BIOTECHNOLOGY: PHARMACEUTICAL ASPECTS

Prodrugs: Challenges and Rewards

Part 1

Edited by
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Preface

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The astute reader will recognize that this concept is as applicable now as it was in the 1950s. At present we use terms such as "drugability" or the need for "drug-like" properties to describe the requirement to incorporate these properties so that the complex synthetic molecules of today can be clinically tested and potentially commercialized. In the mid-1980s, with the advent of molecular biology and the availability of pure protein targets, many new chemical entities were found to be effective at the molecular/receptor level but ineffective as molecules of commercial value. Maximizing binding often led to molecules so chemically constrained that drug-like properties could no longer be built in without significantly compromising activity. Some have referred to this as the "high affinity trap." Of necessity, this began to change. The papers and talks given by C.A. Lipinski and his collaborators made medicinal chemists aware that high throughput screening for drug-like properties should be performed in parallel with molecular/receptor screens so that leads and, ultimately, drug candidates had a better chance of clinical success.

Therefore, in the two parts of this book, we attempt to present the current status of the prodrug concept and its many applications and to highlight its many successes in overcoming the formulation and delivery of problematic drugs. Dictated by the quantity of material, this book is divided into two parts.

The first part is composed of chapters that address the ability of prodrugs to overcome biopharmaceutical challenges resulting from poor permeability of polar drug entities, poor aqueous solubility of oral and parenteral drugs, inadequate targeting of the drug to the brain or particular diseased tissues, etc. This part was specifically directed to teams responsible for the design and delivery of problematic drugs in which the flaw in the parent drug was clearly identified and a prodrug solution sought. Approved and marketed prodrug examples as well as experimental and research concepts are presented.

The second part begins with a series of chapters describing a functional group approach to prodrugs designed more for synthetic medicinal chemists. What type of prodrug can be used to modify functional groups x, y and z? Some functional groups are not covered because they have had little attention paid to them, or because we, as editors, had to draw the line somewhere. The next series of chapters is directed to organizational groups responsible for issues such as toxicology and product development, namely, preclinical and clinical considerations. Finally, the last series of chapters consists of 25 case studies of marketed prodrugs. These represent some of the more successful examples of the prodrug approach from which one might learn and apply to future strategic endeavors in the development of prodrug candidates.

We had intended to supply a list of prodrugs that are currently in clinical trials, but found that in many cases the structures were not available or companies were reluctant to provide publishable information. A search of the topic "prodrugs in clinical trials" in one of the major search engines resulted in 92,000 hits. Of course, these include many redundancies, and many of the older citations are no longer relevant. Nevertheless, one would conclude from this cursory search that prodrugs continue to be a fertile area of research. This is also borne out by the large number of patents on the subject. There are a number of small pharmaceutical/biotech companies dedicated to using prodrugs for the delivery of older but problematic drugs as well as to developing broad-based prodrug technologies that can be applied to new and future drugs. At least one major pharma company has started a prodrug group within their R&D structure. As further evidence of the interest in prodrugs, the American Association of Pharmaceutical Scientists (AAPS) recently started a prodrug focus group. There is no question that the use of prodrugs will be considered earlier and with greater frequency in solving challenges to the delivery of problematic drugs.

Our goal, therefore, in editing and contributing to this book is to provide sufficient examples and supporting literature to introduce this topic to the novice as well as to help the professional in the design of prodrugs. We hope our enthusiasm for this topic is obvious and infective with a full recognition of the challenges and rewards that prodrugs can bring.

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For the editors

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Part 2

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For the editors

Contents

Part I

1

A Case for Prodrugs

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List of Abbreviations

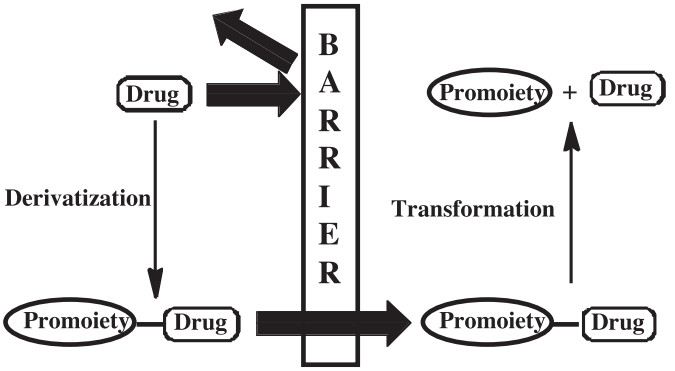
ADEPT .....Antibody-directed enzyme prodrug therapy
BBB.....Blood-brain barrier
GDEPT .....Gene-directed enzyme prodrug therapy
GIT .....Gastrointestinal tract
HTS.....High throughput screening
IM .....Intramuscular
IV.....Intravenous
NCE .....New chemical entity
PEG 400 .....Polyethylene glycol 400
PK.....Pharmacokinetic
SGC.....Soft gelatin capsules
TPGS.....d-alpha Tocopheryl polyethylene glycol

Key words

Prodrugs, History, Rationale, Patents, Latentiation, Suicide inhibitors, Teamwork, Bioreversible derivatives, ADEPT, Promoiety, Paradigm shifts

What are Prodrugs?

A prodrug is a bioreversible derivative of a molecule that has some barrier to its utility as an effective drug. The prodrug concept is illustrated by Scheme 1.



Scheme 1. An illustration of the prodrug concept.

The design and use of a prodrug can best be considered a problem-solving technique. As such, it is not new. The terms "pro-drug" and "pro-agent" were first used by Albert in 1958; the concept was expanded upon in various editions of his book *Selective Toxicity*, including the final edition published in 1985, which was subtitled *The Physico-Chemical Basis of Therapy*. Others such as Harper (1959, 1962) also promoted the concept, but used the term drug latentiation. A prodrug is designed to overcome the barrier or barriers to utility through a chemical approach rather than a formulation approach. Thus, it is an alternative to the redesign of the drug molecule or what is commonly called an analog approach.

Making a Case

The promoiety illustrated in Scheme 1 might refer to an appendage to the molecule that changes the physicochemical properties or bioproperties of the parent drug molecule in such a way as to enhance its deliverability. A good example of a property barrier might be poor water-solubility when an intravenous (IV) injectable form of the drug is desired. The physicochemical properties and bioproperties of drugs that allow their formulation and delivery have recently been defined by the terms "drugable" or "drug-like" properties. Therefore, if a drug candidate does not have drugable properties while an appropriate prodrug of that molecule does, use of this prodrug can result in effective, safe, and efficient delivery of the parent drug to the systemic circulation and to the desired site of action.

The most common barriers to drug delivery include:

1. Poor aqueous solubility preventing, for example, safe parenteral, mainly IV, administration or leading to dissolution rate-limited oral availability of the drug.
2. Poor lipid solubility resulting in poor membrane permeation across various biological barriers including the gastrointestinal tract (GIT), the blood brain barrier (BBB), the skin, etc.
3. Fast elimination from the body resulting from high clearance values and high presystemic metabolism. For drugs with short biological half-lives, controlled release dosage forms are desired.
4. Lack of site specificity, resulting in undesirable effects.
5. Economic barriers caused by limited or no patent protection for the parent drug and resulting in too high a risk for commercialization.

The purpose of this book is to review the literature on prodrugs, updating the concept from earlier books, book chapters, and major reviews (Harper, 1959, 1962; Higuchi and Stella, 1975; Sinkula, 1975; Sinkula and Yalkowsky, 1975; Roche, 1977; Bundgaard, 1985, 1989; Sloan, 1992; Stella, 1996a; Wermuth, 1996; Testa and Mayer, 2003; Testa, 2004). Also, reviews more specific to classes of compounds or therapeutic areas such as the value of prodrugs in cancer chemotherapy (Denny, 2001, 2003; Seddon *et al.*, 2004) are covered. In addition, it will provide examples of both clinical and commercial successes while highlighting industrial and academic research that could lead to greater utilization of this technique in the future.

That the technique has utility is unquestioned. For example, it was noted that, of the 43 new drugs approved worldwide in 1993, five were clearly prodrugs (12%) and two or three probably exerted their therapeutic benefits through being metabolized to parent active drugs (Stella, 1996b). Recently, Bernardelli *et al.* (2002) and Doherty (2003) reported on new approved drugs for 2001 and 2002, respectively. Of the total of 49 new chemical entities approved over the two-year period, seven were clearly prodrugs (14%), an additional three were possibly acting as prodrugs, and one was a soft drug, raising the total to 20–22% (Stella, 2004). I recently analyzed a listing of all drugs approved worldwide and estimated that 5–7% were in fact prodrugs, with the 5% representing the obvious cases and the 7% including those drugs not claimed to be prodrugs. However, in this latter category, the additional 2% were probably acting as prodrugs based on their structural elements or supportive literature. I think that most readers would find these numbers surprisingly high.

The role of prodrugs in modern drug discovery programs can also be gleaned from the recent patent literature. An opinion paper entitled "Prodrugs as Therapeutics" analyzed the prodrug patent literature over the last 10 years (Stella, 2004). In preparing that paper, a search of the patent literature identified 1,396 patents with the keywords prodrug, drug latentiation (an older term used to

describe prodrugs), bioreversible derivatives, and ADEPT, an acronym for Antibody-Directed Enzyme Prodrug Therapy. Figure 1 is a plot of the number of patents per year collected from the search. Clearly, the number of prodrug patents per year has increased significantly over the last 10 years. A separate search at the U.S. Patent Office site for 1976 to the present using just the word prodrug(s) resulted in about 6,500 hits.

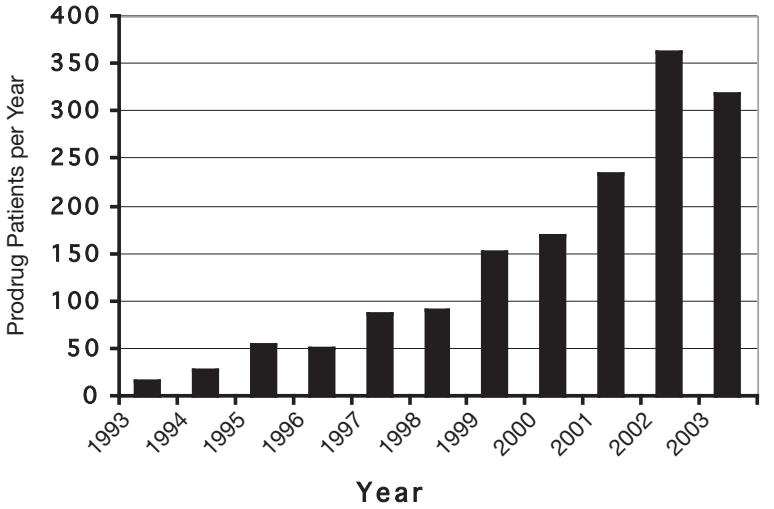
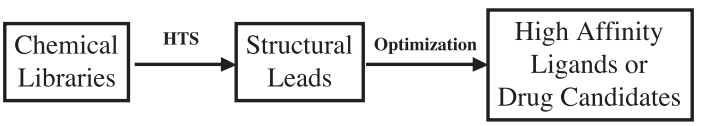


Figure 1. Plot of the number of patents per year over the 10 year period 1993-2003 a search using the terms prodrugs, drug latentiation, bioreversible derivatives, and ADEPT (Stella, 2004). The number of patents for 2003 was estimated from the numbers collected over approximately the first half of the year.

Similar global searches for publications in PubMed and SciFinder with the keyword prodrug(s) resulted in high hit rates and a similar trend of increasing numbers of papers per year.

Rationale for Prodrugs

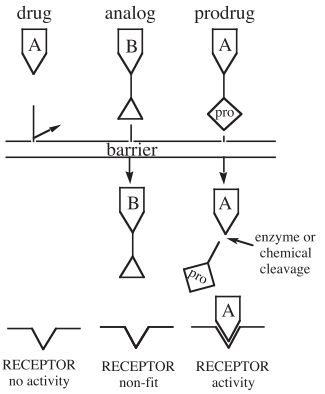
During the drug discovery process, new chemical entities (NCEs) are discovered or identified through a plethora of techniques including the screening of natural products, rational design of agents based on a receptor model, combinatorial chemistry approaches, and pure chance screening of vast chemical libraries. During the 1990s the paradigm used to identify "structural lead" compounds often used high throughput screening (HTS). Subsequent further



Scheme 2. Paradigm for drug discovery in the early 1990s.

chemical manipulation combined with HTS led to the identification of entities labeled drug "candidates." This process is illustrated by Scheme 2.

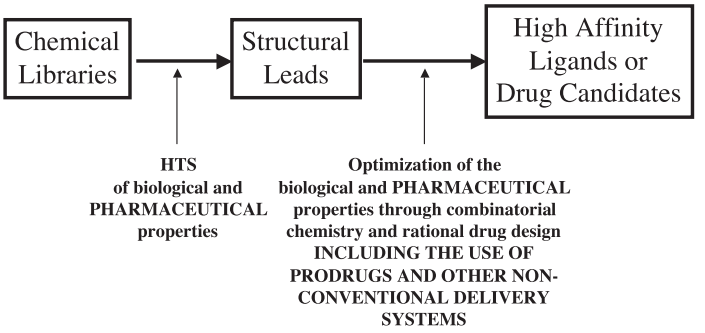
Although these NCEs now had drug candidate status, these agents often performed poorly on further *in vitro* tests for permeability and formulatability and *in vivo* testing in experimental animals. Some have referred to this as the "high affinity trap." Basically, the molecular elements were so constrained that "drugable" or "drug-like" properties could no longer be incorporated without unduly compromising receptor activity. A cartoon representation of this idea was suggested earlier by Ferres (1983).



Scheme 3. Illustration used by Ferres (1983) to show the potential differences between analog and a prodrug approaches to drug receptor activity. Reproduced with permission (Ferres, 1983).

A paradigm shift occurred in the mid-1990s whereby HTS of pharmaceutical properties was combined with HTS of receptor-based activity, resulting in "lead" compounds and, ultimately, drug candidates with higher probabilities of success. This paradigm shift is illustrated by Scheme 4.

Why was this paradigm shift necessary? Part of this may be attributed to the enthusiastic embrace in the early 1990s of combinatorial chemistry, a very useful but somewhat flawed tool now better integrated into the drug discovery paradigm, and the advent of HTS. The hits came, but drugs did not. Lipinski and colleagues (Lipinski *et al.*, 1997; Lipinski, 2000; Horspool and Lipinski, 2003) also



Scheme 4. Drug discovery paradigm at the beginning of the 21st century.

noted the rise in complexity and size of the average drug molecule, resulting in non-deliverable agents, while Veber *et al.* (2002) observed that the number of rotatable bonds was also important. With increased size, greater numbers of hydrogen bond donor and acceptor groups, high log P values, and greater numbers of rotatable bonds came lower water solubility, greater propensity to be efflux candidates, higher protein binding, and higher probability of being rapidly cleared metabolically or via biliary excretion.

Even though the use of HTS of pharmaceutical properties allowed the identification of better drug candidates, often the properties of the "candidate" molecules were not adequate. This is where prodrugs appear to have moved to the front. That is, through prodrugs the temporary modification of the properties can result in drug-like properties (see the Ferres cartoon). It appears that the pharmaceutical industry and academics have rediscovered prodrugs and that this technique has become an integral part of the drug discovery paradigm. The strongest evidence for this conclusion is the large percentage of recently approved drugs that are prodrugs and similar trends in the patent literature.

Trends in Prodrug Patents

Of the 1,396 patents identified in the search described earlier, 605 or 43% of the patents appear to be what might be called defensive patents (Stella, 2004). What does the term defensive patent mean? Beginning in the early to mid-1990s, more and more patents that mentioned prodrugs were actually patents for the parent active drug where terms such as "and prodrugs thereof" were included and where patent claims included specific prodrug examples. That is, the drug and its prodrugs were claimed in the same patent and its extensions in much the same way as "and physiologically acceptable salts thereof" are claimed. In the past, researchers in both industry (the innovator or a competitor) and academia would perform prodrug studies when it was obvious that a drug suffered from some shortcoming. A novel prodrug solution by someone other than the innovator often proved embarrassing but also raised some economic impact issues. By claiming "and prodrugs thereof," companies appear to be attempting to be proactive to this potential challenge. When the chemical structures of the parent drugs in many of these patents were analyzed by this author, it was not always obvious what prodrugs could be made of those compounds and what was the potential barrier being addressed. It did seem that the claim of prodrugs thereof was, in many instances, a legal defensive maneuver.

Table 1 shows the range of patents per year, expressed as a percentage, issued to companies from various regions of the world. Not surprising, the assignees of the majority of patents claiming prodrugs were North American-based companies, especially the multinationals.

The most aggressive company is Pfizer, especially when patents attributed to Pharmacia/Upjohn, Parke-Davis, Warner-Lambert, Searle, and Agouron are included. However, many of these patents were of the defensive type mentioned above. Smaller companies were also aggressive. Examples include Gilead

Assignee	as % of total by year										
	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003
Companies Americas[1]	44	52	39	42	39	39	54	31	41	46	30
Asia[2]	0	4	26	10	6	2	7	33	24	24	28
Europe[3]	19	26	13	22	19	26	19	15	19	20	28
Research institutes[4]	19	19	19	22	32	27	15	15	13	9	13
Individuals	6	0	4	2	1	4	1	3	3	1	1
Unknown	12	0	0	424	4	1	3	2	1	1	0

Table 1. The percent distribution of patents claiming prodrugs broken down by assignee type and by year, 1993–2003. Elements of this table have appeared earlier (Stella, 2004)

[1] Includes companies from North and South America with the majority being large multinational companies based in the USA.

[2] Includes companies from India, Australia and New Zealand. The majority are medium-to-large sized Japanese companies.

[3] Includes western and eastern European, Middle Eastern and African companies, with the majority being large multinational companies based in Western Europe.

[4] Includes universities and foundations.

Sciences, Metabasis, Xenoport, and Nobex. The trends for European, Middle Eastern and African-based companies, as a percentage of total patents, remain within a fairly narrow range. One interesting trend was the percentages seen for Asian (mainly Japanese) companies. Between 1993 and 1999, Asian companies accounted for only 0–10% of all prodrug patents except for an outlier of 26% in 1995. Beginning in 1999, however, the numbers jumped from 11 patents in 1999 to 56 (33%), 55 (24%), and 83 (24%) for 2000 thorough 2002, respectively. In the limited data for 2003, 28% were issued to Asian companies. Many of these patents were of the defensive type. Two companies that appeared very active were Shionogi and Takeda.

When analyzed by therapeutic area, the numbers seemed to follow the current trends seen with new drug discovery efforts in general. These numbers are summarized in Table 2. Note that the percentages add up to greater than 100%; this is because therapeutic benefits in more than one area are often claimed in a patent.

Not surprising is the high percentage of prodrug patents that claim the treatment of cancer and related diseases. The targeting category was included in this table for comparison purposes. The majority of patents mentioning drug

Therapeutic Area	% of prodrug patents (1993–2003)
Cancer	36.8
targeting[2]	14.9[3]
Immunology	10.3
Inflammation	17.6
Antimicrobials (excluding antivirals)	10.0
Antivirals	12.6
Central nervous system (CNS)	18.7
Cardiovascular	25.6
Hormones	11.1
Ophthalmic/dermal/nasal	1
Other	4.6

Table 2. Percentage[1] of prodrug patents (1993–2003) broken down by therapeutic areas (included is a percentage for patents claiming targeting—not a therapeutic area). Elements of this table have appeared earlier (Stella, 2004)

[1] Percentage adds up to >100% since more than one therapeutic area is often claimed.

[2] Technically not a therapeutic area, but included here to show the relatively high percentage of patents claiming the ability to target sites to improve therapy.

[3] Most of the patents that claim targeting are directed toward cancer treatment.

targeting are those for drugs used in the treatment of cancer. Many of these patents are for ADEPT, Gene–Directed Enzyme Prodrug Therapy (GDEPT), and their variants. The anthracycline glycosides seemed to be the most popular family of drugs chosen for targeting.

The barrier that was overcome was not easily identified in all patents, especially the 605 out of 1,396 mentioned earlier as defensive-style patents. Of the rest, the apparent barriers that were overcome could be identified. The numbers are listed in Table 3. Again, the numbers add up to greater than the 1,396 because one or more barriers were often noted in the patents. Other than improved targeting, the largest numbers can be seen for improved solubility for either oral or parenteral delivery and prodrugs for increased permeability, mainly from the GIT. Many in this latter category are for newer as well as older polar antivirals and antibiotics. There were around 23 patents that identified paclitaxel as the drug candidate of choice for solubility manipulation. Patents addressing the poor solubility of camptothecins were also popular.

Barrier Overcome	% of Total Prodrug Patents (1,396)	% of Prodrug Patents with Identifiable Barrier (791)[2]
Oral delivery or formulation		
solubility	5.1	9.0
permeability	14.1	24.9
bioavailability	5.4	9.6
formulation (stability etc)[3]	2.7	5.7
sustained release	1.9	3.4
safety and toxicity	3.0	5.3
targeted absorption	3.3	5.8
presystemic metabolism	0.2	0.4
Parenteral (injectable)		
solubility	6.7	11.8
targeting	14.9	26.3
formulation (stability etc)[3]	0.2	0.4
sustained release	2.1	3.8
Transdermal/ophthalmic/nasal	2.3	4.0
Improved activity	0.6	1.1
Methods patents	1.1	1.9
CNS delivery	0.4	0.8
Barrier not identifiable *(labeled in text as defensive)*[4]	43.3	—

Table 3. Percentage of prodrugs patents categorized by barrier overcome. Elements of this table have appeared earlier (Stella, 2004)

[1] Percentage adds up to >100% since more than one barrier is often claimed. For example, improved permeation often manifests improved bioavailability.

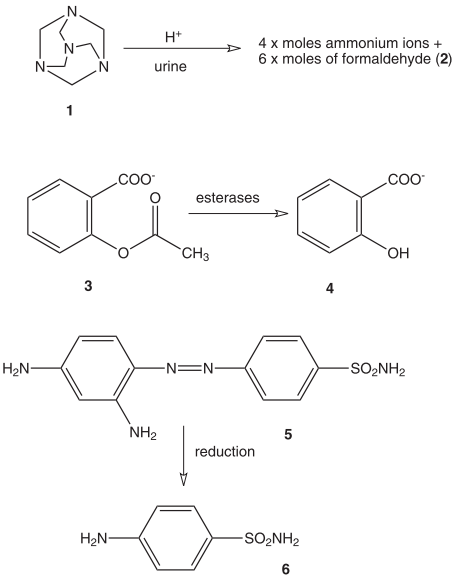
[2] Number used to estimate percentage (1.396–605).

[3] Quite a few of these patents are prodrugs for the delivery of gaseous agents such as NO, CO and CS_2, and drugs such as butyric acid.

[4] In many patents, language such as "and prodrugs thereof" is used. In the text I have referred to these as defensive patents. In the majority of these patents it was not possible to identify why a prodrug claim was made.

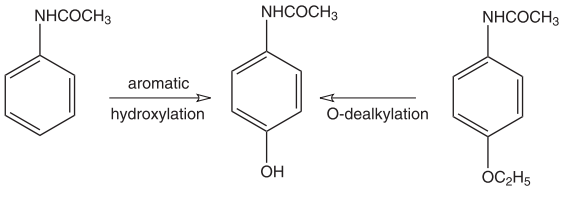
Early History of Prodrug Research

Albert (1958) in his article in *Nature* defined the term pro-drug (Albert also used the word pro-agent in some later papers), but the concept predated this period. Earlier examples of prodrugs include methenamine (**1**), which was introduced in 1899 by Schering, a Berlin-based company (Albert, 1985), as a urinary tract prodrug that delivers the antibacterial formaldehyde (**2**); aspirin or acetylsalicylic acid (**3**), which was introduced in 1899 as a less irritating form of sodium salicylate (**4**), an anti-inflammatory agent; and prontosil (**5**), which was the first sulfa drug and a prodrug of sulfanilamide (**6**).



Structures 1-6.

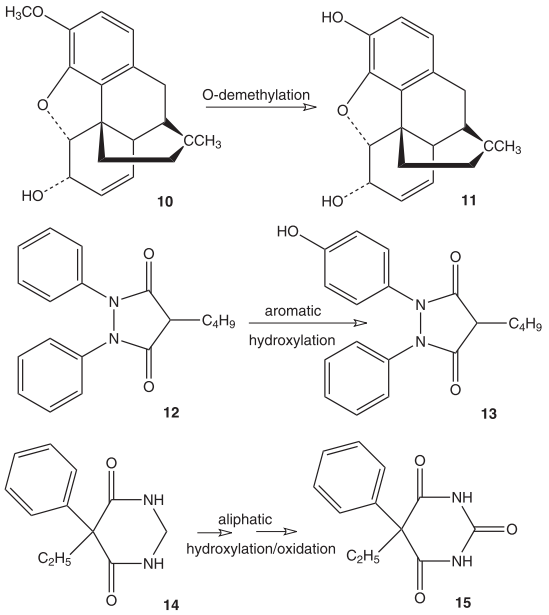
Acetanilide (**7**) was used as early as 1886 as a pain reliever, but its activity was subsequently traced to its metabolism to acetaminophen (**8**, also called paracetamol outside of the U.S.). Phenacetin (**9**), which was removed from the market due to renal toxicity, also exhibited its activity due to O-dealkylation to acetaminophen. Acetanilide and phenacetin were not designed as prodrugs, but their prodrug nature was determined in hindsight.



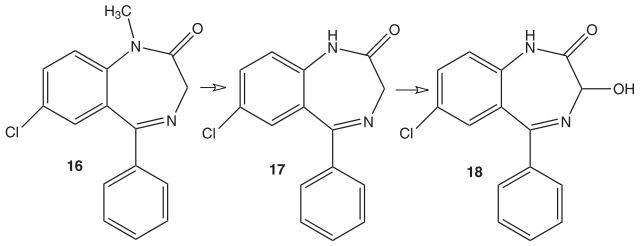
Structures 7-9.

Other hindsight examples include codeine (**10**) being partially metabolized to morphine (**11**), phenylbutazone (**12**) to oxyphenylbutazone (**13**), primadone (**14**)

to phenobarbitone (**15**), and diazepam (**16**) to desmethyldiazepam (**17**) and oxazepam (**18**). In these examples, the prodrugs are active drugs in their own right as are their metabolites.



Structures 10-15.

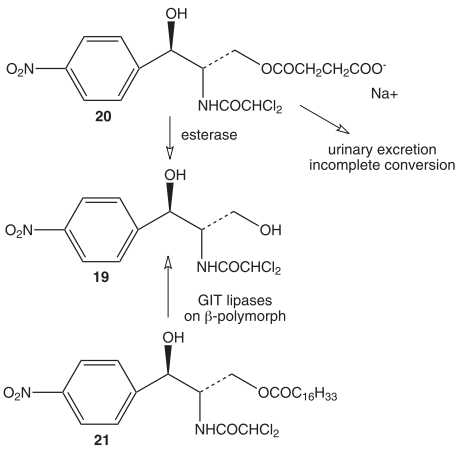


Structures 16-18.

Albert, in various editions of his book, *Selective Toxicity: The Physico–Chemical Basis of Therapy*, provided some of the best early rationale for the use of prodrugs. Albert essentially gave the concept legitimacy as a tool to be used by drug discovery teams to solve issues with problematic drugs. The first edition of *Selective Toxicity* was published in 1951 and the 7th and last in 1981, with a paperback version available in 1985. An interesting footnote in the 1985 edition (p. 97) states, "I apologize for having invented the term, now too widely used to alter, for literary purists tell me they would have preferred 'pre-drug'."

One of the first systematic examples of the application of a prodrug solution to a problematic drug was the work performed at Parke-Davis in the 1950s as applied to the antibiotic chloramphenicol (**19**). Chloramphenicol is sparingly water-soluble (2.5–4 mg/mL) and bitter to the taste. Parke-Davis developed,

shortly after launching chloramphenicol (also called chloromycetin), two prodrugs, chloramphenicol hemisuccinate (**20**), sodium salt, for IV, IM, and ophthalmic administration and chloramphenicol palmitate (**21**) as a suspension for pediatric oral use. Although the water-soluble hemisuccinate ester was a successful product, it led to incomplete *in vivo* conversion to chloramphenicol due to incomplete metabolism and simultaneous renal excretion of the unchanged prodrug (Glazko *et al.*, 1957–1958; Nahata and Powel 1981; Burke *et al.*, 1982). The palmitate ester was designed to be a tasteless form of chloramphenicol for pediatric use (Glazko *et al.*, 1952), and the problems with the polymorphic behavior of this prodrug are legend (Andersgaard *et al.*, 1974; Aguiar *et al.*, 1967; Glazko *et al.*, 1958). Only the β-polymorph led to significant plasma levels of chloramphenicol after oral administration (Aguiar *et al.*, 1967). Nevertheless, in the early-to-mid-1950s, drug discovery researchers in major drug companies and their pharmacokinetic (PK) colleagues clearly recognized the value of bioreversible derivatives as drug delivery forms even though they did not initially refer to them as prodrugs. Glazko and his colleagues at Parke-Davis should be recognized for their pioneering efforts in prodrugs.

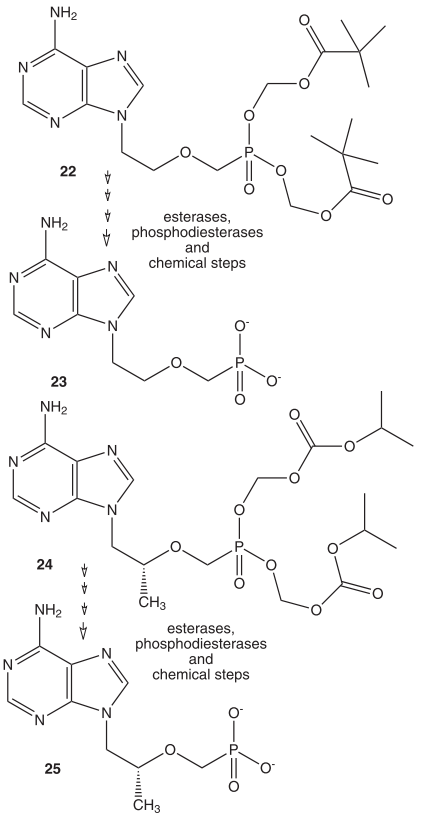


Structures 19-21.

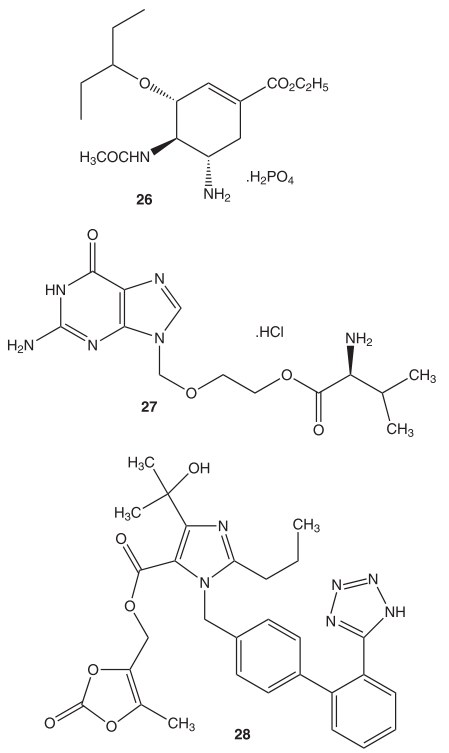
Paul Ehrlich, the 1908 Nobel Laureate, coined the term "magic bullet" to describe drugs or therapies that selectively acted at their site of action with minimal exposure to the rest of the body (Ehrlich, 1906, 1908). He appeared to be one of the first to talk about a "receptor" in molecular terms. In addition, he also studied the role of drug metabolism in activating drugs in his seminal work on arsenicals (see Albert, 1985). Effectively, Ehrlich's magic bullet concept and his work on arsenicals were the precursor to today's ADEPT and GDEPT and prodrugs in general.

What Have You Done For Me Recently?

Examples of very recent commercial successes include the antivirals Hepsera® (**22**), a prodrug of adefovir (**23**) used to treat hepatitis B, and Viread® (**24**), a prodrug of tenofovir (**25**) used to treat HIV infections. Both Hepsera® and Viread® were developed at Gilead Sciences. Gilead was also responsible for the development of the anti-influenza drug Tamiflu® (**26**) or oseltamivir, an ethyl ester prodrug. Roche Holding AG introduced another antiviral prodrug Valcyte™ (**27**) while Sankyo/Forest introduced Benicar® (**28**), an antihypertensive prodrug. Prodrugs **22**, **24**, **26**, **27**, and **28** all show superior oral bioavailability compared to their active forms. Pfizer introduced Dynastat® (**29**), or parecoxib sodium, a water-soluble injectable form of its new COX-2 inhibitor valdecoxib (**30**).

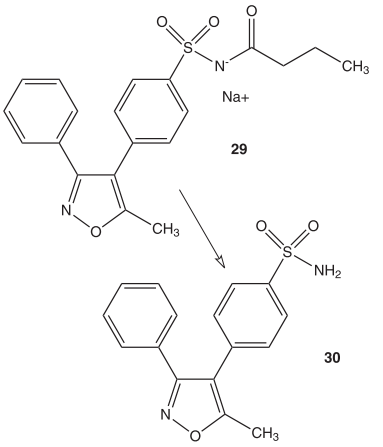


Structures 22-25.

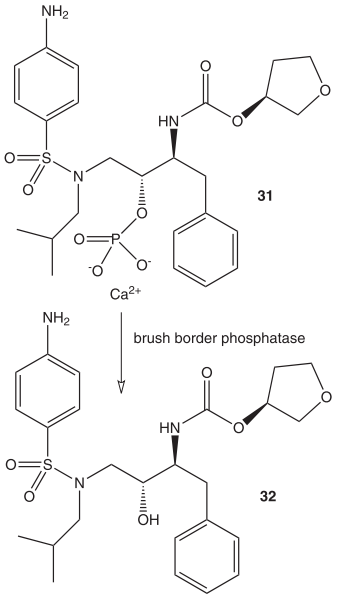


Structures 26-28.

Another very recent approval is Lexiva® (**31**), or fosamprenavir, a water-soluble phosphate ester, calcium salt, of amprenavir (**32**), which is used in the treatment of HIV infections (Anon, 2003). Jointly developed by Vertex and GlaxoSmithKline, fosamprenavir offers interesting clinical and economic advantages over amprenavir (Agenerase®), the parent drug. Amprenavir has an aqueous solubility of only 0.04 mg/mL and is formulated for adult use in soft gelatin capsules (SGC) containing 150 mg of drug along with *d*-alpha tocopheryl



Structures 29-30.



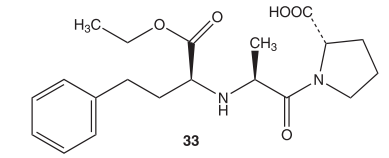
Structures 31-32.

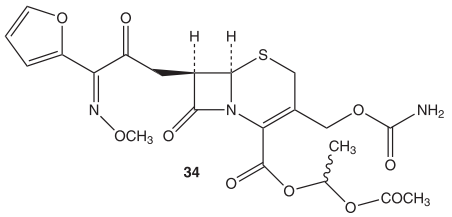
polyethylene glycol (TPGS), polyethylene glycol 400 (PEG 400), and propylene glycol for a final weight of about 1 g. Because the daily dose of amprenavir is 1,200 mg twice a day, this requires patients to take eight capsules twice daily, which is clearly inconvenient. Although amprenavir is well absorbed from this dosage form, patient convenience and compliance is a problem. The aqueous solubility of fosamprenavir is said to be >0.3 mg/mL; it is supplied as a tablet containing 700 mg of the prodrug, equivalent to 600 mg of amprenavir. Thus, dosing becomes more convenient at two tablets twice a day, and blood levels of amprenavir comparable to those from the less convenient amprenavir SGC formulation are seen. The greater drug loading in the tablet is possible because of the higher solubility of fosamprenavir. For those unfamiliar with the use of

polar phosphate esters for oral dosing, reading the work of Heimbach *et al.* (2003) is suggested. Although phosphate esters should show poor permeation from the GIT, the presence of high alkaline phosphatase levels on the surface of the enterocytes (the cells lining the small intestines) allows bioconversion of fosamprenavir to amprenavir at the brush-border lining followed by sequential amprenavir absorption. The "coupling" of metabolism and absorption is used by the body for the absorption of molecules such as folic acid (Rosenberg, 1981). The application of this coupling concept to prodrugs has been summarized previously by Fleisher and co-workers (1985) and more recently confirmed by the work of Heimbach *et al.* (2003) as applied to a number of phosphate ester prodrugs.

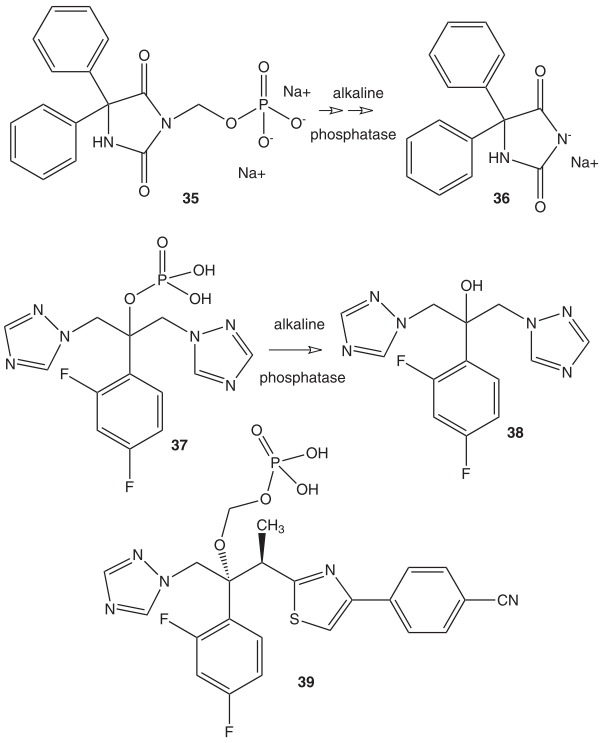
Fosamprenavir, as a prodrug of amprenavir, offers an additional advantage over amprenavir; it has restarted the patent clock. Amprenavir was patented earlier (Tung *et al.*, 1996), whereas the fosamprenavir patent was issued in 2002 (Hale *et al.*, 2002). Thus, while the protection by the composition of matter patent for amprenavir would expire around 2013, protection of the fosamprenavir patent would continue to at least 2019. While the development of fosamprenavir as a prodrug of amprenavir was probably costly, introducing fosamprenavir and encouraging physicians to prescribe fosamprenavir in place of amprenavir prior to patent expiration of amprenavir, those additional costs should be recouped by the longer exclusivity. That is, the additional costs to develop fosamprenavir would presumably be leveraged against future gains created by the extended patent life. Similar advantages were seen when Parke-Davis/Warner-Lambert introduced fosphenytoin or Cerebyx® as a safer injectable form of sodium phenytoin (Stella, 1996c), thus recapturing a market position lost earlier when sodium phenytoin injectable became generic.

Many of the ACE inhibitor antihypertensive drugs in the recent past are prodrugs, the first one being enalapril (**33**), the ethyl ester enalaprilate also known as MK-422 (Ulm, 1983). Poor oral bioavailability of the active species due to poor GIT permeation was the need addressed by these prodrugs. Similar improvements after oral dosing were seen with various ester prodrugs of third generation, non-amino side chain cephalosporins such as cefuroxime when administered as cefuroxime axetil (**34**) (Dellamonica, 1994).





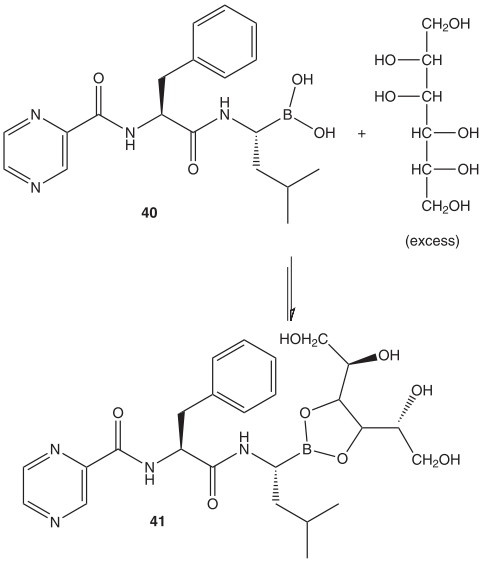
Structures 33-34.



Structures 35-39.

In 1996, Warner-Lambert, now part of Pfizer, launched Cerebyx® (**35**), or fosphenytoin, as a water-soluble, safer form of sodium phenytoin (**36**) for the treatment of seizures (Stella, 1996c). Unlike some of the examples above, fosphenytoin is a prodrug of an older off-patent established product. The advantages of fosphenytoin over sodium phenytoin were such that commercialization of the prodrug could be justified. A similar scenario could be painted for Procif®, or fosfluconazole (**37**) (Bentley *et al.*, 2002), a water-soluble injectable phosphate prodrug of Pfizer's very successful antifungal agent fluconazole (**38**). Fosfluconazole was approved in October 2003 in Japan. A water-soluble prodrug of ravuconazole (Bristol-Myers Squibb/Eisai), referred to as BMS-379224 (**39**), is in early clinical trials (Ueda *et al.*, 2003).

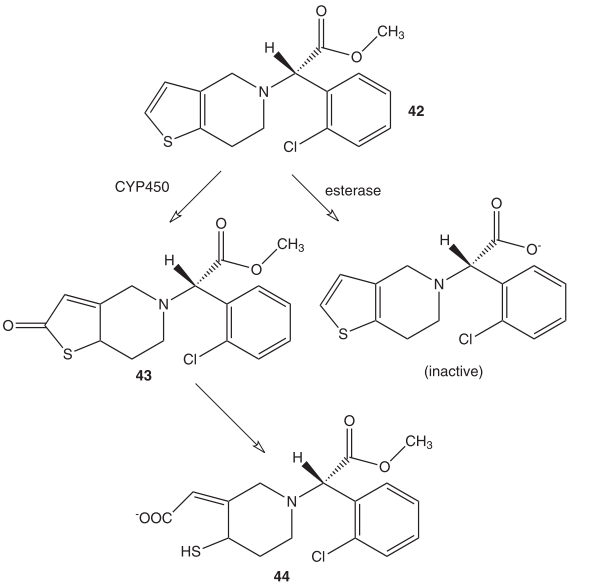
An interesting example of a recently approved drug not recognized as a prodrug is Velcade® (**40**), or bortezomib. The aqueous solubility of bortezomib is quite limited (0.6 mg/mL, observations in our laboratory), but the parenteral freeze-dried form consists of 3.5 mg of drug and 35 mg of mannitol, which is reconstituted with 3.5 mL of normal saline to 1 mg/mL just prior to injection. Bortezomib in the formulation is present as a boronic acid ester with the mannitol (**41**). On reconstitution, the solution shows a solubility of >1 mg/mL because **40** exists in equilibrium with bortezomib (see Scheme 5) and the excess mannitol (Plamondon *et al.*, 2004). Further dilution results in complete dissociation, which is presumably what happens upon IV administration of the drug.



Scheme 5. Scheme showing the proposed reaction of bortezomib (40) with mannitol to form a boronic acid ester with superior water solubility (Plamondon *et al.*, 2002).

Another set of drugs not always recognized as prodrugs are so-called "suicide" inhibitor molecules. These are prodrugs that are activated to reactive species at or near their site of action. Upon metabolic activation, the reactive intermediate chemically reacts with a critical receptor components inactivating the receptor. Consider the approved drug Plavix® (**42**), or clopidogrel bisulfate, a platelet-aggregation inhibitor. Only a small portion of clopidogrel, a methyl ester, is metabolized by a complex P450-dependent pathway to **43** (2-oxoclopidogrel) and then to a thiol **44** (Pereillo *et al.*, 2002), the stereospecific active metabolite. Cleavage of the methyl ester of clopidogrel leads to the inactive corresponding carboxylic acid. **44** is thought by Savi *et al.* (2001) to form a disulfide bond with the $P2Y_{12}$ ADP-receptor on platelets, thus preventing ADP binding, a critical step in the platelet aggregation pathway. Disulfide formation with **44** may also contribute to the CYP2B6 inhibition by clopidogrel (Richter *et al.*, 2004). The metabolic pathway for clopidogrel is shown in Scheme 6.

Earlier examples of suicide inhibitors as potential drugs led Bey (1978) and his colleagues at Merrell Research in Strasbourg to suggest that these inhibitors should be considered prodrugs. He reproduced one of the best cartoons in science to describe these suicide or K_{cat} inhibitors (Scheme 7). Here the prodrug (hand grenade) binds to the enzyme active site (the receptor), undergoes a chemical/biochemical event (pulling the pin of the hand grenade), producing a reactive species (the actual active species), which then destroys the enzyme/receptor by reacting with an essential functionality at the site (BOOM). The cartoon can be attributed to Professor Robert Rando of Harvard, one of the earlier researchers in this field. Numerous prodrug examples were discussed in the review by Bey (1978), including β-fluro D-alanine (**45**), a suicide substrate

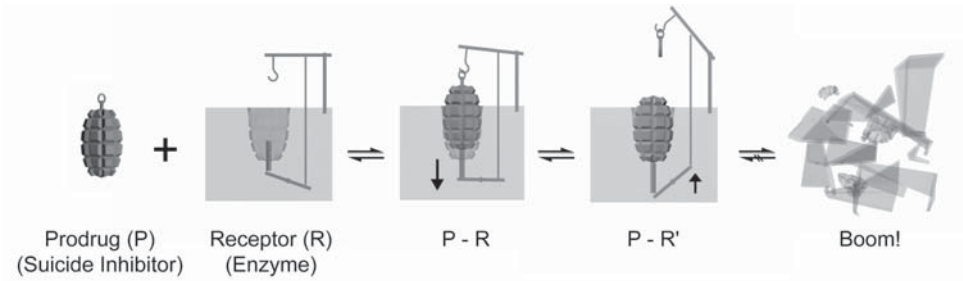


42 43 44

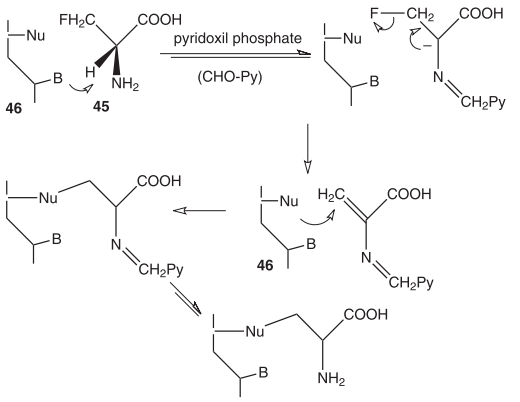
Scheme 6. Scheme showing the metabolism of clopidogrel (42) (Pereillo *et al.*, 2002; Richter *et al.*, 2004).

prodrug for the enzyme alanine racemase (**46**), and various irreversible inhibitors of GABA-transaminase. The proposed mode of action of **45** is shown in Scheme 8. Other workers such as Professors Baker, Rando, Abeles, and Wermuth had all contributed earlier to this area of research.

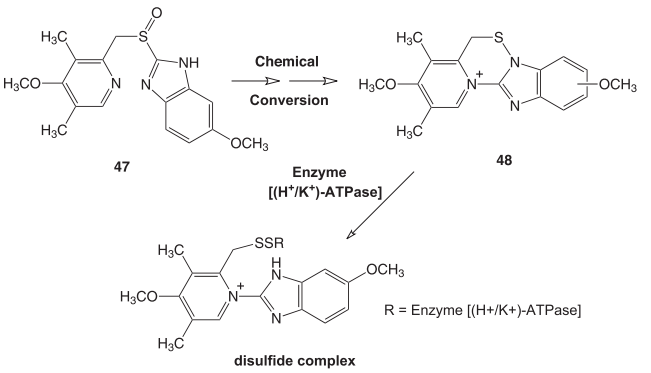
Drugs such as omeprazole (Prilosec®) and lansoprazole, which are used for the treatment of high stomach acid output, are site-activated prodrugs. Omeprazole is a specific inhibitor of gastric (H^+-K^+)-ATPase. Im *et al.* (1985) showed that, under acidic conditions, omeprazole (**47**) is chemically degraded to a reactive intermediate capable of trapping a sulfhydryl group on (H^+-K^+)-ATPase. The initial structure proposed by Im *et al.* was later challenged, and intermediate **48** was proposed by Lindberg *et al.* (1986). These reactions are illustrated in Scheme 9.



Scheme 7. Cartoon reference by Bey (1978) but attributed to Professor Robert Rando of Harvard illustrating how suicide substrates, also called K_{CAT} inhibitors, can inactivate an enzyme active receptor site. Here the prodrug (hand grenade) binds to the enzyme active site (the receptor), undergoes a chemical/biochemical event (pulling the pin of the hand grenade), producing a reactive species (the actual active species), which then destroys the enzyme/receptor by reacting with an essential functionality at the site (BOOM).



Scheme 8. Scheme showing the inactivation of alanine racemase (46) by β-fluro D-alanine (45) a suicide substrate (Bey, 1978).



Scheme 9. Scheme showing the activation of omeprazole, a specific inhibitor of gastric (H^+-K^+)-ATPase, to a reactive intermediate capable of trapping a sulfhydryl group on (H^+-K^+)-ATPase (Im *et al.*, 1985).

Antiviral nucleoside and nucleoside phosphonate drugs have played a significant role in improving the quality of life for many virally infected patients in recent years. Not always thought of as prodrugs themselves, molecules such as acyclovir (parent compound of the prodrug valcyclovir, **27**) and tenofovir (**25**) are, in fact, prodrugs (De Clercq, 1998) in their own right. Upon entering virally infected cells, these nucleosides and nucleoside phosphonates are phosphorylated to their active triphosphates or diphosphates, respectively, by viral nucleoside kinases. These phosphorylated species act as competitive inhibitors of reverse transcriptase and as chain terminators, thus preventing viral replication. These active polar-phosphate metabolites have prolonged intracellular half-lives (Balzarini *et al.*, 1989). In the case of very polar nucleoside phosphonates such as tenofovir, which is very effective at reducing HIV RNA (Deeks *et al.*, 1998) after IV administration, it is interesting to speculate how little material must enter virally infected cells to be effective. This is especially the case because cellular uptake is likely to be very permeation limited. Imagine how much more potent tenofovir

might be if it could enter virally infected cells more efficiently. Is there a place for prodrugs to effect such selectivity?

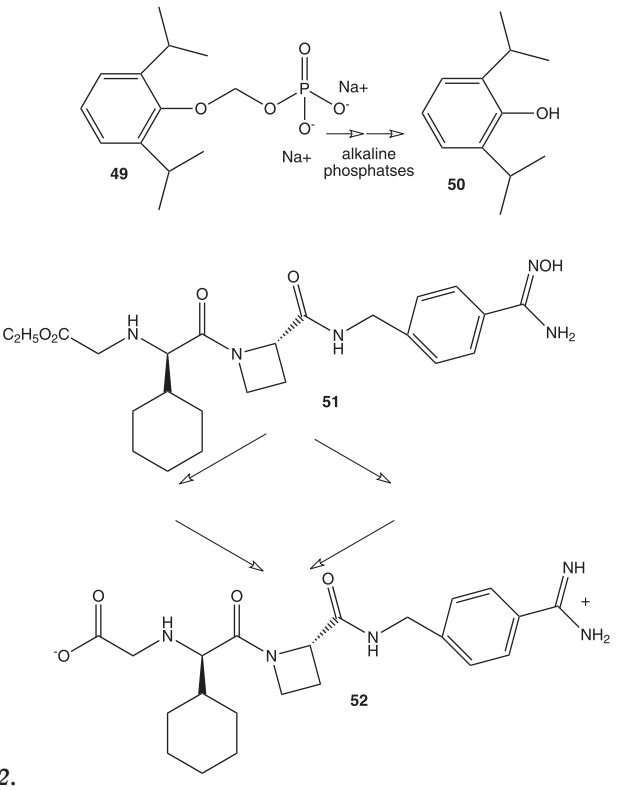
Derivatives of acyclovir, such as valcyclovir (**27**), and of tenofovir, such as tenofovir disoproxil (**24**) (Shaw *et al.*, 1997), are readily recognized as prodrugs with improved oral availability. Therefore, valcyclovir and tenofovir disoproxil are prodrugs of prodrugs of prodrugs, etc.

These illustrative examples of recent prodrugs and some drugs not generally recognized as prodrugs are presented to show that prodrugs play a much larger role in the development of NCEs than is generally perceived around the pharmaceutical industry.

Prodrugs in Clinical Trials.

There are currently a number of prodrugs in clinical trials, some approaching regulatory filing. Two examples are Aquavan™ (**49**) (Stella *et al.*, 2001), a water-soluble prodrug of the anesthetic propofol (**50**) being developed by MGI Pharma and licensed from ProQuest Pharmaceuticals and the University of Kansas, and ximelagatran (**51**) and BIBR 1048 (dabigatran etexilate), two anticoagulant drugs being evaluated/developed by AstraZeneca (Gustafsson, 2003).

Aquavan™ has just successfully completed Phase II clinical trials in the U.S., but the first clinical studies in humans were performed in Germany (Fechner *et al.*, 2003; Schywalsky *et al.*, 2003). The drug is now in extensive phase III clinical



Structures 49-52.

trials. Propofol is currently formulated as an oil in water (o/w) 10% emulsion with the propofol dissolved in the oil phase of the emulsion. Although a very effective anesthetic, propofol in its emulsion formulation causes significant pain on injection, and the emulsion is prone to bacterial contamination. One of the major current clinical applications of propofol is to maintain patients in a coma after head injuries. Because this requires high doses of propofol over an extended time period, it can result in hyperlipidemia from the lipid emulsion dosage form (Fechner *et al.*, 2003; Schywalsky *et al.*, 2003). Aquavan™, being formulated as a purely aqueous solution, avoids the lipid load and bacterial growth issues and causes no pain on injection. The lack of brachial pain on injection is due to the venous tissue not seeing propofol but largely the inactive and non-pain-producing Aquavan™. Similar observations were seen with fosphenytoin as a water-soluble prodrug of sodium phenytoin, a drug that required dosing from a caustic vehicle (Stella, 1996c).

Ximelagatran, sold as Exanta™, which has received approval overseas and has been filed for approval in the U.S. by AstraZeneca, is a prodrug of a new class of anticoagulant drugs. Following absorption, it is broken down to melagatran (**52**) (Gustafsson, 2003), whose oral bioavailability is limited by high polarity (Gustafsson *et al.*, 2001). The bioavailability of melagatran is only 3–7%, while that of melagatran from ximelagatran is about 20%. Gustafsson attributed this to the 80-fold differences in cellular permeation of the prodrug compared to the parent drug. The high polarity of melagatran comes from having both an ionizable carboxylic acid and a protonated benzamidine group in its structure. In the case of the ACE inhibitors, only one polar functional group required prodrug modification; however, in the case of melagatran both ends of the molecule required alterations. Gustafsson (2003) has stated that two intermediates are seen in plasma after oral dosing, ethyl-melagatran (formed by reduction of the -NH-OH to an NH_2) and melagatran-OH (formed by the action of esterases on the ethyl ester group), but the plasma levels of both are low.

What Does It Take To Have A Successful Prodrug Program?

In the past 1–2 years I have been asked this question more than in my previous 30 years as a consultant to the pharmaceutical industry. The personnel and skills needed are no different from those for analog development—it takes a team. The ideal drug is one that is active, easy to formulate, well absorbed after oral dosing, has an acceptable PK profile, and is both renally cleared and metabolized to 1–2 non-toxic metabolites that are rapidly excreted after being formed. If a prodrug intervention is necessary, obviously this ideal scenario is not met. The ideal prodrug, therefore, is one that readily achieves its desired goal, is non-toxic, and breaks down efficiently and quantitatively to the drug and to known and safe byproducts. Like the drug discovery process, this goal is not often met.

Does a prodrug solution to the problem add greatly to the development time and costs? The answer is yes and no. If the need for a prodrug intervention is

recognized early, then it simply becomes an integral part of the drug discovery paradigm (see Scheme 4). In what way might a prodrug add complications? Some potential complications are additional synthesis effort, more complex analytical profiling, metabolism and PK studies requiring the analysis of both the prodrug and drug, and concerns about the toxicity of not only the prodrug and drug but also the released promoieties or byproducts. Some prodrugs, by nature, are chemically more unstable, and this can require additional effort by formulators to solve.

How might it be less complicated? The most obvious reason is to consider the consequences if a prodrug intervention is not initiated early. What if a drug candidate or a class of drug candidates are found, in hindsight, to have poor drugable properties? This is not recognized until 6–12 months into the discovery/development program. The program is evaluated and the decision is made that the only logical solution is a prodrug solution. It then takes 3–6 months to solve the problem, including assembling a new team. This team may have to be taken off of other programs. In all, 12–18 months are lost. Which was more costly, the early prodrug intervention and the complications caused by the additional studies or the 18 months of lost revenue and the disruption to other programs? I believe it will be the latter.

Should pharmaceutical companies assemble a prodrug team? I tend to believe that a prodrug group may be unnecessary unless the types of molecules being developed within a company show the propensity for prodrug intervention on a routine basis. Many companies such as Pfizer and Bristol-Myers Squibb have been successful at implementing prodrug solutions to problems within the normal drug discovery paradigm. For example, Ueda *et al.* (2003) recently published a paper on a prodrug strategy for one of their antifungal agents. A perusal of the authors of that publication showed contributions from numerous departments. Were these authors a separate "team" set apart from the normal design/development team? To my knowledge that was not the case. Ueda and his colleagues at Bristol-Myers Squibb have produced some creative prodrug solutions to problematic drugs over the last 10 years. So it appears that prodrug solutions for problematic drugs is simply on their "radar" screen.

Are prodrug strategies difficult to implement within the normal drug discovery paradigm if there is no previous history of success within a company? If not part of the normal *modus operandi* of the company, the answer is usually yes. I found this particularly the case in small companies without a strong pharmaceutical development leader or where drug discovery scientists have a poor relationship with their development colleagues. I have also seen this problem within companies where potential teams are geographically separated. The resistance to a try prodrug strategy can disappear quickly after a success or when the medicinal chemistry members of a team "buy in" to the concept. This "buy in" in my experience, is critical since no prodrug program can move forward without contributions from competent and committed synthetic chemists with the support of research management.

Today, drug discovery teams in pharmaceutical companies, big and small, are usually multidisciplinary and, because of this, the idea of implementing a

potential prodrug solution to a problematic drug is more likely to be put into action early. It is one of the reasons that I am so confident that the science of prodrugs will continue to grow and evolve.

What Are the Unmet Needs?

Two major barriers to effective drug development that have not received a great deal of attention from prodrug researchers, perhaps because of limited success in the past, are the prevention of presystemic drug metabolism and the bypassing of efflux-limited drug absorption.

Preventing presystemic drug metabolism will be discussed in greater detail in a later section of this book. Johnson (1980) and Svensson and Tunek (1988) detailed the role of presystemic metabolism in preventing drug delivery and the potential use of prodrugs. Anecdotally, the number of drug candidates that do not pass muster due to presystemic drug metabolism is quite high in the opinion of this author. The incidence is probably higher than generally thought because often drug candidates are discarded due to having short biological half-lives and poor PK properties. Such drugs invariably have high clearance values that also often translate to their being good candidates for presystemic clearance. A reasonable half-life does not mean that a drug does not have high clearance because the half-life of a drug is a function of both its clearance (Cl) and distribution properties (as indicated by its volume of distribution, Vd). This is discussed by Jusko (1986) and can seen by examining Eq. 1.

$$t_{1/2} = 0.693\ Vd/Cl$$

Equation 1.

A drug can display high clearance but still have a reasonably long half-life if it has a large Vd. Poor solubility and/or poor permeability properties may also mask high presystemic clearance. If presystemic clearance of a drug candidate could be prevented via the design of a prodrug that allows the drug to reach systemic circulation largely intact, more drug candidates could be moved forward to the clinic. This would especially be the case for drugs undergoing presystemic clearance due to metabolism in the enterocytes (intestinal epithelial cells) during the act of absorption. An example is the work of Elger *et al.* (1995, 1998) on estrogen sulfamate prodrugs as a new approach to hormone therapy. A few patents covering this and similar work have appeared. Nevertheless, the design of novel and creative prodrugs for the prevention of presystemic metabolism would be welcomed and may be a fruitful if challenging area of research.

The role of efflux transporters slowing cellular uptake from the GIT and into organs like the brain has recently been reviewed by Lin and Yamazaki (2003a,b). As pointed out by others (Lipinski *et al.*, 1997; Lipinski, 2000; Horspool and Lipinski, 2003; Veber *et al.*, 2002), the increase in molecular weight, rotatable bonds, etc. seen with newer drug molecules contributes to their increasing problematic drug delivery properties. Size and complexity appear to play a role

in more of these molecules being efflux candidates. Could a prodrug strategy facilitate the delivery of drugs that are efflux candidates? This question has not been systematically addressed, and is one that begs attention. First, efflux transporters are a family of membrane-embedded molecules that have wide specificity. So, predicting whether a molecule will be a substrate for an efflux transporter does not appear to be possible at this time. Therefore, chemically modifying a molecule with the hope of preventing efflux transporter candidacy is somewhat hit and miss. This is also true if the chemical modification involves a prodrug strategy. A prodrug of an efflux candidate might not itself be an efflux candidate and, therefore, prove useful. A drug with low water solubility may also be an efflux candidate and, therefore, efficiently effluxed. Increasing the water solubility of the drug through a prodrug strategy could also facilitate absorption even if both the drug and prodrug are efflux candidates. However, presenting high prodrug concentration to the transporter might permit increased passage if the transporter is one that is readily saturated.

Achieving true targeting via a prodrug strategy is not fully realized, although good examples of successes (and failures) will be discussed later. Historically, one of the first prodrugs (methenamine (**1**)) represented a targeted system. The relatively selective breakdown of methenamine to formaldehyde in acidified urine (urine pH is usually on the acidic side) meant that the antibacterial formaldehyde was released in the urine and not in plasma or other tissues at normal physiological pH of 7.4. A similar concept has been applied to the hypoxic nature of tumor cells in an attempt to effect targeting of anti-tumor prodrugs (Seddon *et al.*, 2004).

These represent the perceived current unmet needs that I would call obvious. There are also unmet needs for specific drugs and classes of drugs. For example, consider the oral delivery of non-amino side chain β-lactam cephalosporins, phosphonate antivirals, and the bisphosphonates used to prevent or treat osteoporosis. All are poorly permeable across biomembranes due to polarity and, although prodrug strategies have achieved some significant successes for the antibiotic cephalosporins and the phosphonate antivirals, the oral bioavailability of the parent drugs from their prodrugs is <50%. For the antibiotics, this results in GIT side effects such as an increased incidence of diarrhea that can be dangerous, especially in young and geriatric patients. The majority of prodrug strategies for masking the charge on a carboxylic acid or phosphonate group seem to focus on ester formation. Yet esters are often prematurely cleaved in the lumen of the intestines or efficiently cleaved in the epithelial cells lining the small intestine, resulting in less than complete absorption of the parent drug. What if a prodrug strategy could be designed whereby the prodrug survives the small intestine and passage though the epithelial cells and is cleaved only after reaching systemic circulation? This should result in 100% delivery of the parent drug. Is this possible? Sure, but it is unlikely to occur with an ester-based prodrug strategy.

Conclusion

I hope that I have made a case for continued prodrug research. Clearly, there are unmet needs that require addressing, and it is unlikely that we will be able to solve drug design and delivery problems without occasionally resorting to the use of prodrugs. Based on what I have seen and heard in my capacity as a consultant to the pharmaceutical industry, especially over the last 3–5 years, the interest in the use of prodrugs is growing as molecules become more complex and their delivery more problematic. The prodrug strategy is becoming an integral part of the drug design and delivery paradigm, a conclusion that is reinforced by the significant percentage of NCEs that are prodrugs.

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Part II

Problems Addressable by Prodrugs

2.1.1

Prodrug Approaches to Enhancing the Oral Delivery of Poorly Permeable Drugs

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Table of Contents

List of Abbreviations

ACE .....Angiotensin-converting enzyme
AE2 .....Anion antiporter
ARB .....Angiotensin receptor blockers
Caco-2 .....Human colon carcinoma cell line
CHO .....Chinese hamster ovary cells
CNS .....Central nervous system
D .....Diffusion coefficient
DADLE .....An opioid peptide made up of ($^{+}H_3N$-Tyr-D-Ala-Gly-Phe-D-Leu-COO^{-}
EPA .....Environmental protection agency
GIT .....Gastrointestinal tract
HIV .....Human immunodeficiency virus
hPepT-1 .....Human peptide transporter
Log P .....Logarithm of the partition coefficient
MCT .....Monocarboxylic acid transporter
OCT .....Organic cation transporter
pK_a .....Negative logarithm of the dissociation constant (Ka) of an acid
P .....Partition coefficient
P_{app} .....Permeability coefficient
P_w .....Wall permability
Pept-1 .....A peptide transporter
SMVT .....Sodium-dependent multivitamin transporter

Key words

Passive diffusion, Transcellular transport, Transporters, Paracellular transport, Prodrugs, Oral delivery, Efflux transporters

Introduction

It is appropriate that the improved oral delivery of problematic drugs via prodrugs is treated early in this book. This chapter provides the rationale for the use of prodrugs along with selective examples of prodrugs used to effect improved oral delivery of poorly permeable drugs. Oral drug delivery is the preferred route of dosing in the USA and much of the rest of the world. Marketing forces therefore dictate that every attempt be made to provide an oral form of a drug that shows promise. Major exceptions are drugs used to treat emergencies where fast-acting injectables or inhalables are desired or drugs used to treat cancer where traditionally injectables have been well accepted and many older anticancer drugs are irritating to the gastrointestinal tract (GIT).

The major barriers to oral delivery of drugs are:

- The drug does not dissolve in the contents of the GIT
- The drug is too polar and does not readily permeate the cells lining the GIT (enterocytes)
- The drug is a substrate for an efflux pump present in enterocytes
- The drug undergoes presystemic metabolism in the luminal contents, the brush-border of the enterocytes, the enterocyctes and/or the liver before reaching systemic circulation.

This chapter focuses on the second of these causes with some additional comments on the third.

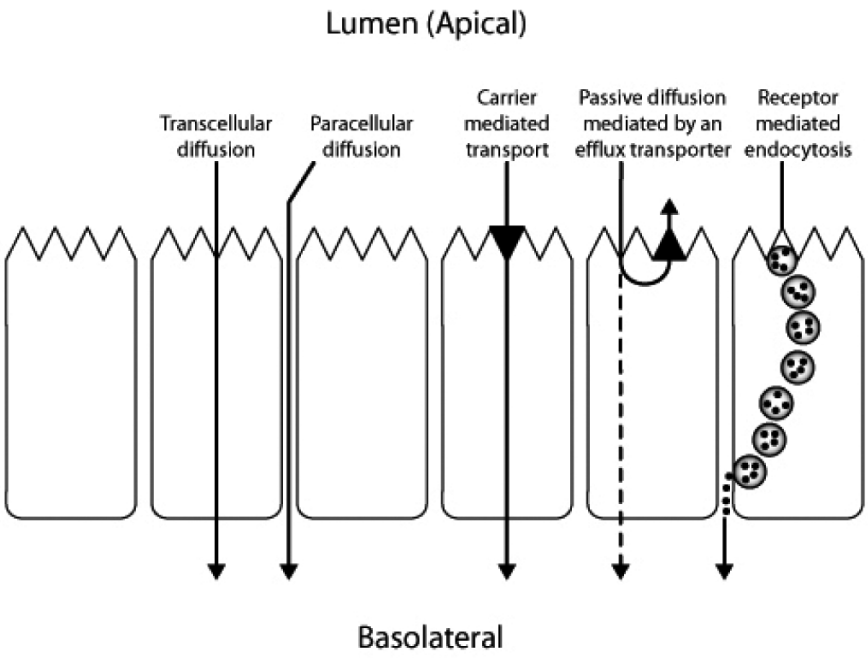
How Are Drugs Absorbed From The Gastrointestinal Tract?

Orally administered drug can physically permeate across a membrane by any one, or a combination of the following mechanisms:

- passive transcellular diffusion
- passive transcellular diffusion modified by efflux transporters
- passive paracellular diffusion
- carrier-mediated transport
- receptor-mediated endocytosis

Scheme 1 illustrates these pathways. The fifth pathway will not be addressed here. Apparent permeation across a membrane is the summation of permeation occurring by each of these routes.

Transcellular passive diffusion is, in general, the preferred route for permeation across a membrane. This is mainly due to the high surface area



Scheme 1. Illustration showing the five major routes for drug transport from the gastrointestinal tract.

available for passive diffusion and lower constraints on size than for paracellular diffusion (Ho *et al.*, 1999). Compared to carrier-mediated transport and receptor-mediated endocytosis, transcellular passive diffusion is also preferred due to lower constraints on structural elements needed for recognition by transporters or receptors, as well as not being capacity limited. This section will focus on transcellular passive diffusion and how prodrug strategies can be used to target this permeation pathway.

Mathematically, the passive diffusion of compounds through a simple membrane can be described by applying Fick's law:

$$\frac{dQ}{dt} = \frac{DP}{h} \bullet A \bullet (C_D - C_A) = P_{app} \bullet A \bullet (C_D - C_A)$$

Equation 1.

where P_{app} can be defined as:

$$P_{app} = \frac{DP}{h}$$

Equation 2.

In these equations, dQ/dt indicates the flux or mass of material of material crossing the membrane, D is the diffusion coefficient across the membrane (cm^2/s), P is the partition coefficient between the membrane and the donor phase, h is the

thickness of the membrane (cm), A is the membrane surface area (cm^2), and C_D and C_A are the initial concentrations of the compound in the donor and acceptor compartment, respectively. P_{app} (cm/s) can then be defined as the apparent permeability coefficient of a compound across a particular membrane.

These equations provide the bases for how the passive diffusion of a molecule across a biological membrane can be improved by altering its physicochemical properties. As Eq.1 illustrates, the quantity of drug that can cross the intestinal membrane is a product of the ability of the drug to permeate a membrane and the concentration of the drug presented to the membrane. Permeation can be improved by increasing the diffusivity (D) and/or increasing the partition coefficient between the membrane and the donor phase. For oral drugs this is the content of the intestinal lumen. The concentration of the compound presented to the membrane can be improved by increasing the solubility of the drug in the intestinal contents in the vicinity of the membrane.

Diffusivity is determined by the size and molecular flexibility of the molecule. Most prodrug strategies will increase the size and, potentially, the flexibility of a molecule. However, because diffusivity is usually related to the square or cube root of molecular weight or size, unless these changes are excessive in going from drug to prodrug, diffusivity is usually not adversely affected by prodrug design strategies. The exception is small molecules coupled to a macromolecule.

The partition coefficient of compounds is controlled by the ability of the drug/prodrug to dissolve in the membrane components relative to their affinity for the intestinal contents. Therefore, parameters such as lipophilicity (hydrophobicity and hydrogen-bonding potential) and the charge properties of the compounds are all important physicochemical properties that affect passive diffusion (Artursson and Matsson, 2004).

Clearly, in effecting change in one parameter by design, an analog or a prodrug can lead to other barriers becoming rate-limiting. This could be due to altered metabolism, lowered solubility and hence dissolution, or the prodrug becoming a substrate for one or more of the apically polarized efflux systems known to hinder oral absorption. This last point will be discussed later.

To be able to permeate the intestinal mucosa via the passive transcellular pathway, it is crucial for the molecule to be able to partition into the cell membrane. Optimal lipophilicity has, therefore, traditionally been considered one of the most important physicochemical parameters when targeting transcellular passive diffusion.

The relationship between permeability and some relative measure of lipophilicity is well established in the literature (Artursson and Karlsson, 1991; Ho *et al.*, 1977; Kansy *et al.*, 2004). The most common parameter quoted is log P or the logarithm of the partition coefficient between some organic solvent (often octanol) and water. More sophisticated solvents and solvent combinations have been proposed and validated for peptide drugs (Conradi *et al.*, 1992; Godwin *et al.*, 1999). Use of *in silico* calculations has also become popular (Borchardt *et al.*, 2004). Log P appears to be made up of a volume- or size-related term reflecting the energy needed to create a cavity in the solvent and an interactive term for

polarity that reflects the solute-solvent interaction. Many have validated some relationship between cellular membrane permeability and log P (variably defined). The relationship usually takes the form illustrated in Figure 1.

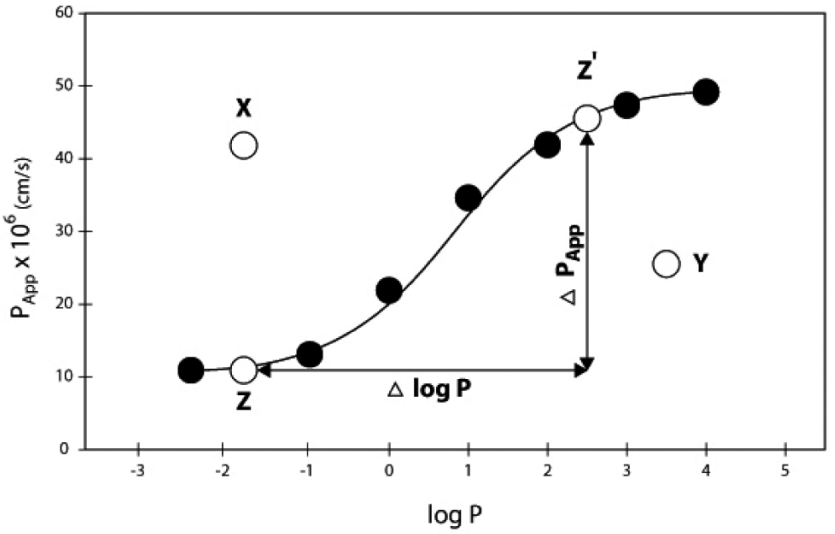


Figure 1. Hypothetical plot of intestinal mucosa P_{APP} versus log P for a series of molecules.

The sigmoidal-shaped curve illustrated in this figure has been used by Borchardt and others in numerous oral and written presentations to account for normal and abnormal permeation behavior of some drug and prodrug molecules. For example, the higher than expected permeation of compound X suggests that this enhancement is due either to its small size, which permits paracellular passive transport, or that the molecule is absorbed via carrier-mediated transport. The poor permeation of compound Y is probably due to its being a substrate for one of the apically polarized efflux systems (Hockman *et al.*, 2002; Lin *et al.*, 2003a,b; Polli and Serabjit-Singh, 2004). Compound Y is said to undergo passive transcellular diffusion modified by efflux transporters with metabolism also possibly playing a role (see Scheme 1).

For this chapter, molecule Z is of interest. Molecule Z shows poor permeation due to its high polarity i.e., it sits on the low end of the sigmoidal curve. Typically, partition coefficients on the order of log P = 1–3 are needed for efficient passive transcellular diffusion, although molecules with slightly lower log P values are often well absorbed if they also show good solubility. It should also be noted that some workers have seen a decline in absorption for extremely lipophilic compounds (Ho *et al.*, 1977; Kansy *et al.*, 2004).

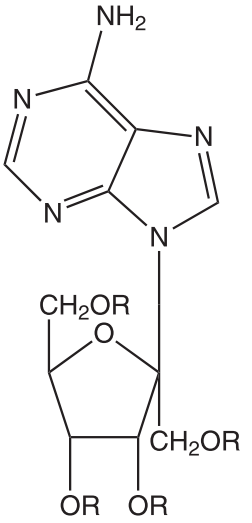
For Z to be able to permeate, its lipophilicity can, for example, be increased through the conjugation of a lipophilic promoiety group to a functional group within Z that contributes to its polarity to produce prodrug Z', i.e., change the log

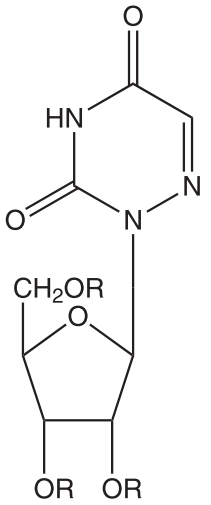
P from a low to a higher value (log P + Δlog P), as illustrated in Fig.1. Later, an alternative strategy for increasing log P will be discussed, i.e., rather than increase log P, design a prodrug that is a substrate for a carrier-mediated transporter.

Rationale For More Lipophilic Prodrugs Of Polar Drugs

Alter Polarity By Masking Polar Non-Ionized Functional Groups

Molecules show low lipophilicity due to the presence of polar functional groups that are not adequately balanced by the presence of adequate numbers of non-polar groups. Consider an older example such as the nucleoside drug psicofuranine (**1**), which has an octanol/water log P value of –1.95 (Ho *et al.*, 1977). It is poorly absorbed after oral dosing presumably because of its low lipophilicity. Its tetraacetate prodrug (**2**) on the other hand is well absorbed (Hoeksema *et al.*, 1961). Ho *et al.* (1977) showed that acetylation of a single alcohol group results in a Δlog P of 0.82. Figure 2 shows the serum level versus time profile of **1** from oral administration of **1** and the much higher levels of **1** from **2**. Similarly, another nucleoside, 6-azauridine (**3**), has a log P of –2.14 and is poorly absorbed after oral dosing while its triacetate (**4**) is efficiently absorbed (Welch, 1961). For both **2** and **4**, cleavage of the acetyl groups to produce the parent drug presumably occurs via the action of esterases





1 R = -H
2 R = -$COCH_3$

3 R = -H
4 R = -$COCH_3$

Structures 1-4.

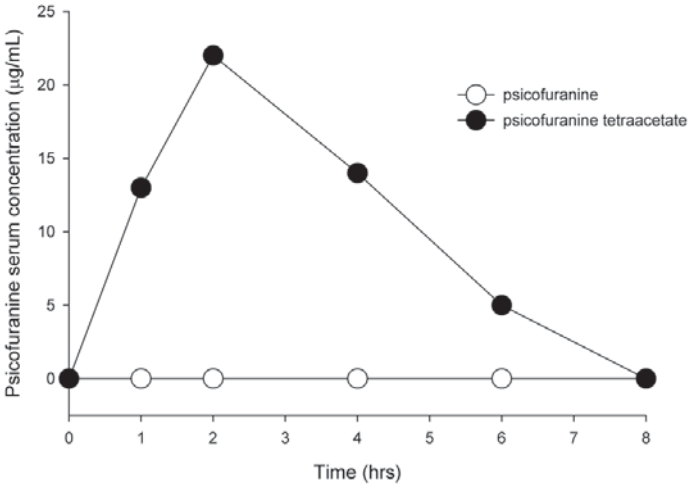
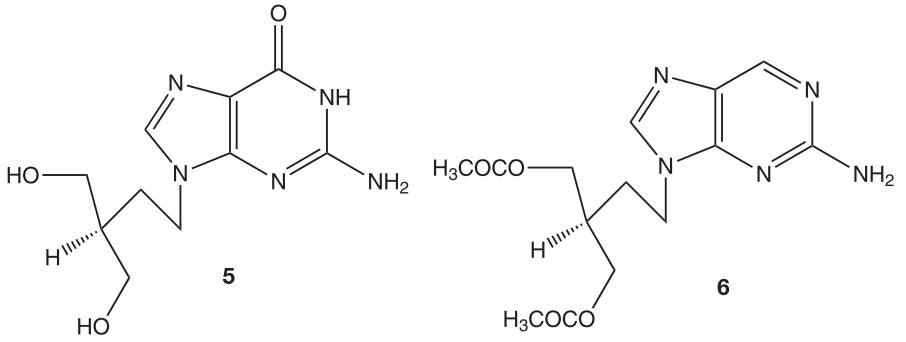


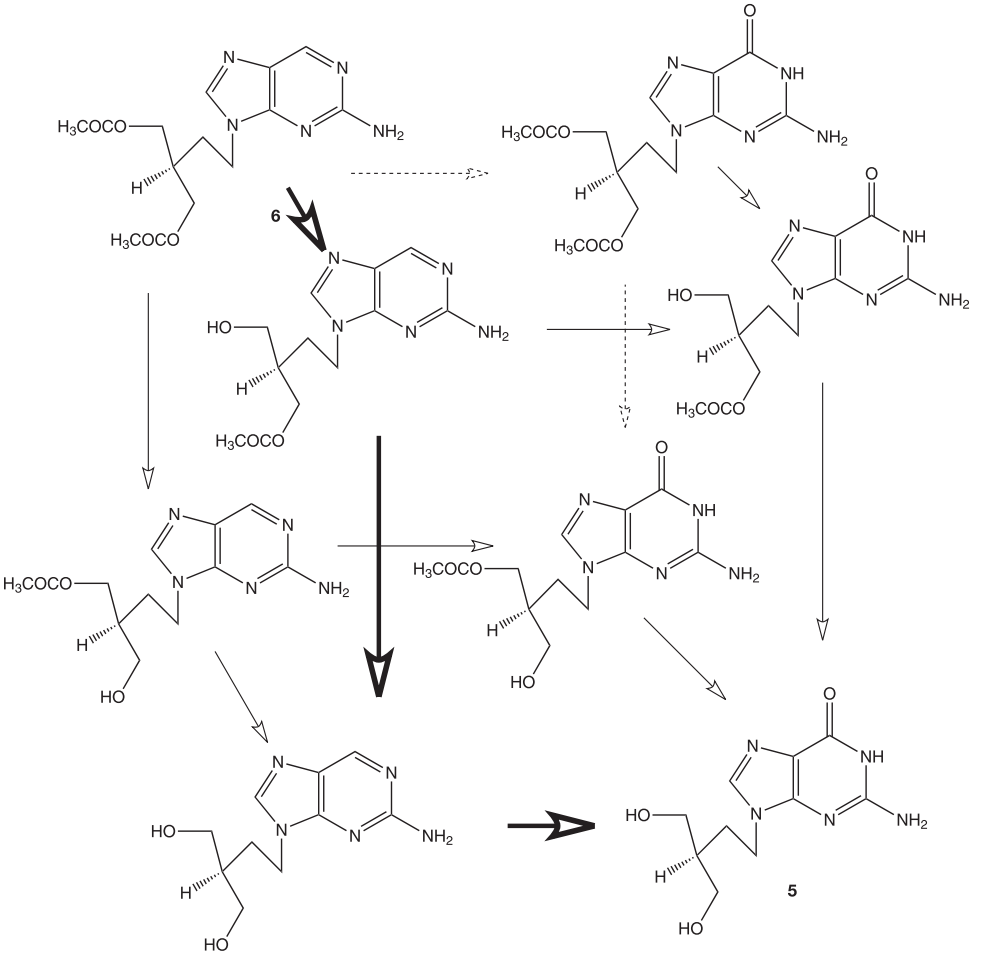
Figure 2. Psicofuranine serum levels in humans after a 1.5 g oral dose of psicofuranine (1, ○) compared to an equimolar dose of its tetraacetate prodrug (2, ●). From Hoeksema et al. (1961).

A more recent example is famciclovir (**6**), a deoxydiacetate prodrug of penciclovir (**5**). In this case, not only were two primary hydroxl groups acetylated but additional lipophilicity was gained by removal of the 6-oxy functional group. Bioconversion of **5** required two deacetylation steps and an oxidation event (probably xanthine oxidase). Initial bioavailability studies for famciclovir were performed in rodents (Vere Hodge *et al.*, 1989; Jarvest *et al.*, 1998). In rats the oral bioavailability of penciclovir was only 1–2%. When 6-deoxy penciclovir was dosed, the bioavailability of penciclovir was increased to 9%. However, when the diacetate ester of 6-deoxy penciclovir, famciclovir, was dosed, the oral bioavailability of penciclovir increased to 41% (Vere Hodge *et al.*, 1989; Jarvest *et al.*, 1998). When penciclovir was dosed orally to human volunteers, its bioavailability was about 4%, which is similar to what had previously been observed in rats. However, when the prodrug famciclovir was dosed orally, the bioavailability of penciclovir was significantly improved to 75% (Pue and Benet, 1993; Jarvest, 1994; Pue *et al.*, 1994; Gill and Wood, 1996; Jarvest *et al.*, 1998). Vere Hodge *et al.* (1989) showed that even though there were multiple possible intermediates and competing pathways (see Scheme 2) the major pathway was sequential cleavage of the two acetyl groups followed by oxidation at the 6-carbon. The metabolic pathways and their relative importance proposed by Vere Hodge *et al.* (1989) are illustrated in Scheme 2 while Figure 3 shows the blood concentration time profiles of **5** from various prodrugs including famciclovir (**6**).

A precursor to the use of an oxidative event as in famciclovir was an earlier study by Krenitsky *et al.* (1984) who looked at a 6-deoxy prodrug (**8**) of acyclovir (**7**). Oxidation at the 6-position by xanthine oxidase proved to be a very successful strategy for increasing the lipophilicity and thus the absorption of acyclovir. Unfortunately, oxidation at the 8-position on 6-deoxyacyclovir produced a toxic



Structures 5-6.



Scheme 2. Metabolic pathways for famciclovir (from Vere Hodge et al., 1989).

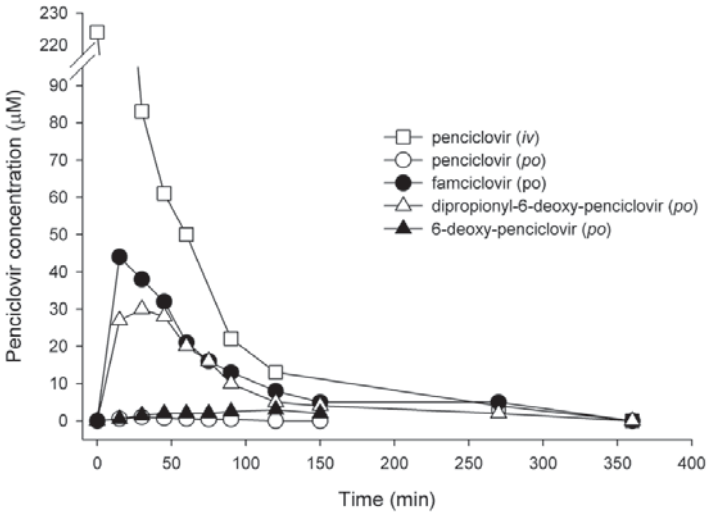
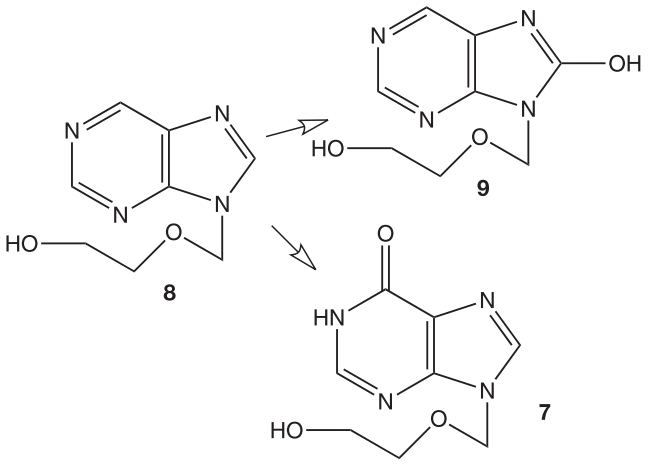


Figure 3. Mean concentrations of penciclovir in blood after administration of penciclovir (□. IV; ○, po) and various penciclovir prodrugs (●, △, and ▲), including famciclovir (●), to rats. From Vere Hodge *et al.*, 1989.

metabolite (**9**), limiting the development of **8**. Nevertheless, this was one of only a few examples available in the prodrug literature of non-ester or non-ether prodrugs of nucleosides and obviously played a role in the identification of famciclovir. For a history of the identification, assessment, and development of acyclovir prodrugs, the reader is directed to the excellent review by Beauchamp and Krenitsky (1993).

The development of valaciclovir as a successful prodrug of acyclovir (Beauchamp and Krenitsky, 1993) will be discussed later in this chapter as an example of a prodrug that utilized a carrier-mediated transporter. However, it has been readily admitted that this was a serendipitous finding and not one that was "by design."



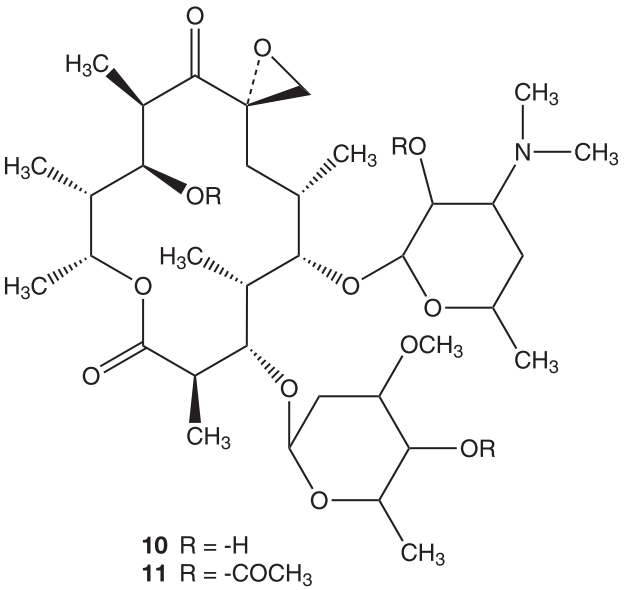
Structures 7-9.

Another good historical example can be seen with the polar macrolide antibiotic oleandomycin (**10**), whose oral availability was significantly improved when administered as it triacetate (**11**) or troleandomycin (Shubin *et al.*, 1957–1958).

Alter Polarity By Masking Polar Ionizable Functional Groups

The high polarity of many drug candidates is due to the presence of one or more ionizable functional groups, mainly groups such as a phosphonic acid, a low pK_a carboxylic acid, a sulfonic acid, or a strongly basic amine group. Often more than one of these groups are present. Because the range of pH of the GIT tract spans the range 1–8, many of these drugs are in their fully ionized state in a large portion of the GIT and so are unable to cross biological membranes efficiently. If these molecules have a small molecular weight, some can be reasonably well absorbed via the passive paracellular route (Ho *et al.*, 1999). However, for drugs having one or more ionizable groups within their structure, hydrophilic/lipophilic balance and, consequently, permeation from the GIT will depend on the pH of the solution. Most prodrug strategies used to date to improve permeation of such drugs are designed to mask the charge of these functional groups or altering the ionization state of the molecules to render them neutral under pH conditions in a significant portion of the GIT. Note, however, that increasing size will decrease the rate of diffusion through membranes, so prodrug designs should also attempt to minimize the molecular size increase as much as possible.

Some of the better historical examples of lipophilic prodrugs of polar ionized drugs can be seen with those drugs that have a carboxylic functional group as at least one major source of their high polarity. There are many drugs with a carboxylic acid functional group present in their structure that are perfectly well

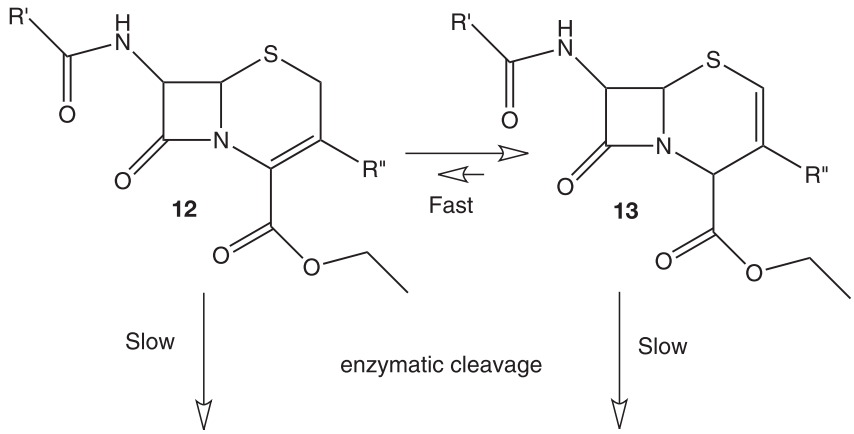


10 R = -H
11 R = -$COCH_3$

Structures 10-11.

absorbed. This is because the rest of the molecule is either relatively lipophilic and/or the pK_a of the carboxylic acid group is >4. Some polar carboxylic drugs are absorbed either via paracellular transport or are substrates for a carrier-mediated transporter (see later discussion). Various prodrug strategies for modifying carboxylic acids are extensively discussed in a later chapter and a number of successful examples are discussed in the Case Studies section of this book.

For illustrative purposes, a number of examples will be discussed here. One class of polar carboxylic acid drugs is the β-lactam antibiotics such as various penicillins and cephalosporins. Penicillin G, for example, although a life-saving orally effective antibiotic discovered in the 1930s, is erratically orally bioavailable both because of its inherent chemical instability when exposed to acidic pH values seen in the stomach and its high polarity due to having a carboxylic acid with a pKa of only 2.6–2.9 in its structure. Various prodrugs of penicIllin G in which the carboxylic acid group was esterified were attempted, but the prodrugs either failed or met with very limited commercial success. Among many, two early broad reviews of prodrugs of β-lactams, one by Sinkula (1975) and one by Ferres (1983), should be consulted.



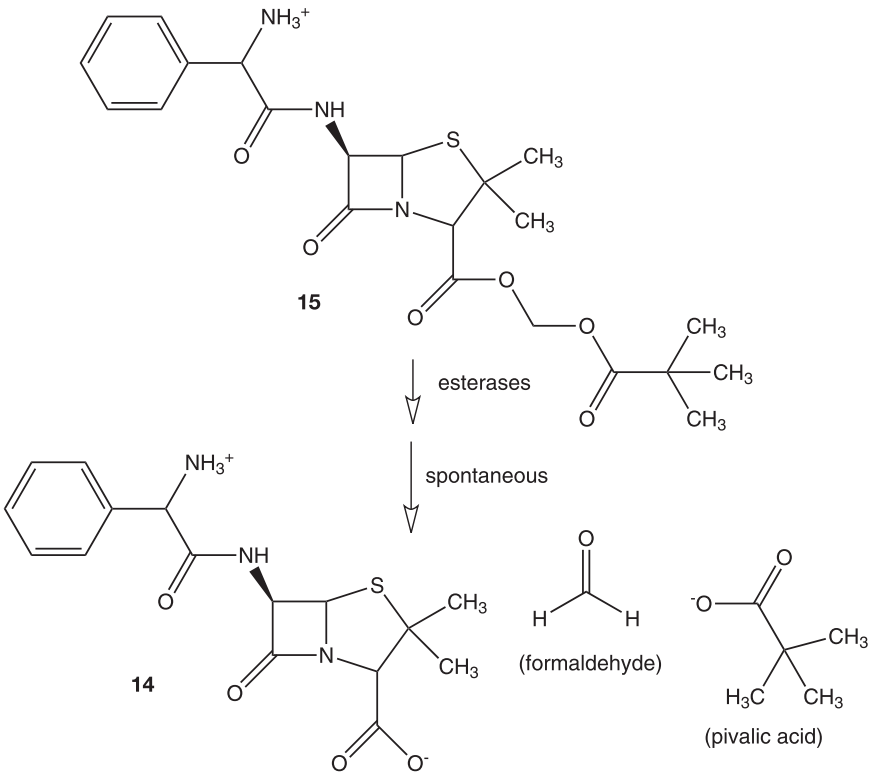
Scheme 3. The reaction showing the Δ3 to Δ2 double bond shift in the case of stable cephalosporin esters (Richter et al., 1990).

In the case of both the penicillins and cephalosporins, direct esterification of the carboxylic groups with simple primary alcohols led to potential prodrugs that were incompletely converted to drug on absorption or adversely affected the chemical stability of either the β-lactam group or other parts of the molecule (Nielsen and Bundgaard, 1988). For example, consider the basic cephalosporin nucleus (**12**) shown in Scheme 3. If the ester is the ethyl ester, such a potential prodrug is poorly cleaved even in the presence of procine liver esterases both because the carbonyl group of the ester has high π-bond overlap with the 3–4 unsaturated double bond, making the carbonyl poorly electrophilic, and because the β-lactam group hydrolysis competes with the de-esterification reaction.

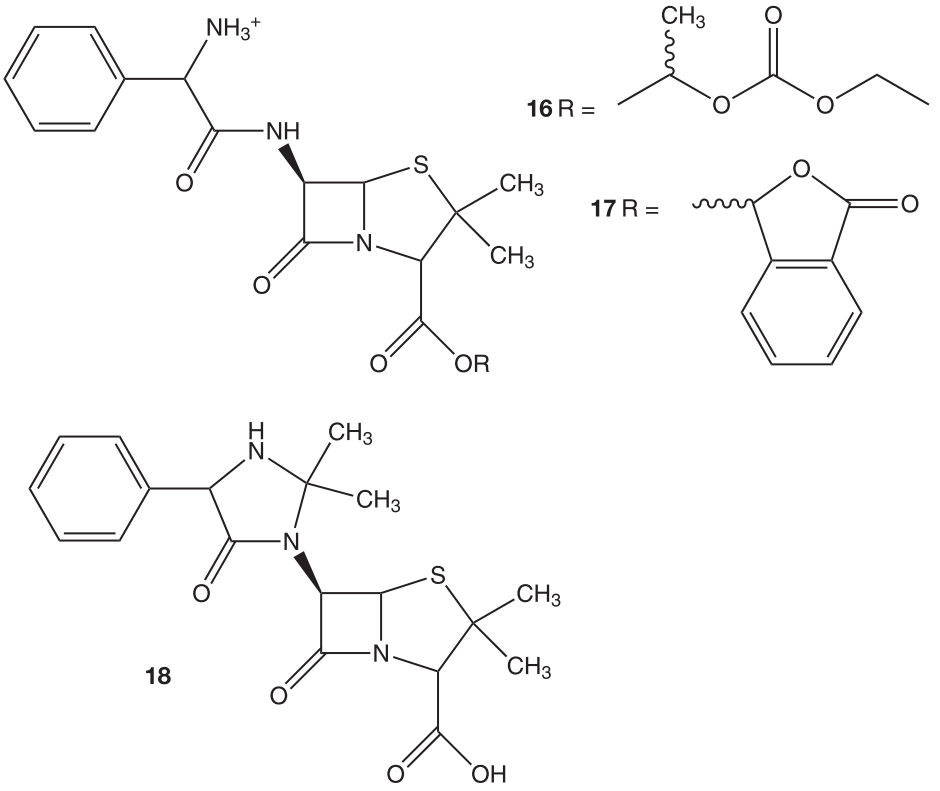
Esterification promotes a facile Δ3 to Δ2 (**13**) shift in the double bond in the ring (Richter at al., 1990; Stoeckel *et al.*, 1998).

Therefore, most prodrugs of penicillins and cephalosporins have been focused on the use of acyloxyalkyl esters where cleavage does not involve an attack of a nucleophile at the β-lactam carboxylic acid ester group but at the ester group a few atoms removed. This is followed by a cascade reaction releasing the drug and an aldehyde spacer group. A good historical example (von Daehne *et al.*, 1970a,b) is the pivaloyloxymethyl ester (pivampicillin, **15**) of ampicillin (**14**), see Scheme 4. Other ampicillin prodrugs are **16–18**. The serum levels of ampicillin from pivampicillin and an equimolar dose of ampicillin can be seen in Figure 4. The bioavailability of ampicillin from pivampicillin is approximately double that from ampicillin. Chanteux *et al.* (2005) recently showed that when pivampicillin was transported across Caco-2 cells, ampicillin accumulated in the cells, i.e., the pivampicillin was effectively converted to ampicillin in the Caco-2 cells, but the ampicillin formed was only slowly released.

Ampicillin is reasonably well absorbed despite its polar zwitterionic nature and is available commercially as such. Therefore, the intent of the prodrug is to improve the delivery. The pivaloyloxymethyl ester strategy used in pivampicillin generates a mole of pivalic acid and formaldehyde. The toxicity from carnitine



Scheme 4. The metabolic/chemical pathway for the conversion of pivampicillin (15) to ampicillin (14).



Structures 16-18.

depletion caused by pivalic acid generation is discussed in a later chapter of this book and an earlier review by Brass (2002).

The generation of formaldehyde is always raised as a possible toxicity problem; however, it is important to keep this issue in perspective. According to the EPA, we are exposed, on average, to significant quantities of methanol per day from the atmosphere, 90% of which is metabolized to formaldehyde and then to formic acid. Two glasses of red wine are said to expose one to 40–50 mg of methanol and, as a result, formaldehyde. In addition, any oxidative O-demethylation metabolic step in the body generates formaldehyde. Formaldehyde is immediately metabolized to formate with a half-life of 1–2 minutes (Bardana and Montanaro, 1991). Therefore, the additional danger from formaldehyde exposure from drugs such as pivampicillin is minimal. In a very recent study of Aquavan, a prodrug that is converted to one mole each of propofol, phosphate, and formaldehyde, circulating formate levels were not raised even after a 30 mg/kg dose of Aquavan to human subjects (Gibiansky *et al.*, 2005). A 30 mg/kg dose of Aquavan generates about 4 mg/kg or 250–300 mg of formaldehyde; however, endogenous formate levels were unaffected.

To avoid the formaldehyde question, some workers have designed acyl- and carbonate-oxyalkyl derivatives that generate inert alcohols, carbon dioxide, and acetaldehyde. An example is bacampicillin (**16**). The only issue with the

acetaldehyde spacer is the generation of an additional chiral center in the molecule.

Why is a prodrug strategy with improved delivery important for ampicillin and many other penicillins and cephalosporins important? A significant side effect of oral antibiotic therapy is diarrhea and subsequent fluid loss caused by bacterial imbalance in the large intestine. Complete or more complete absorption of the antibiotic should cut down the incidence of this side effect (Cullmann, 1995).

Figure 4 shows the plasma level-time profile of ampicillin from pivampicillin compared to that of ampicillin. More than double the plasma levels from an equimolar dose can be seen.

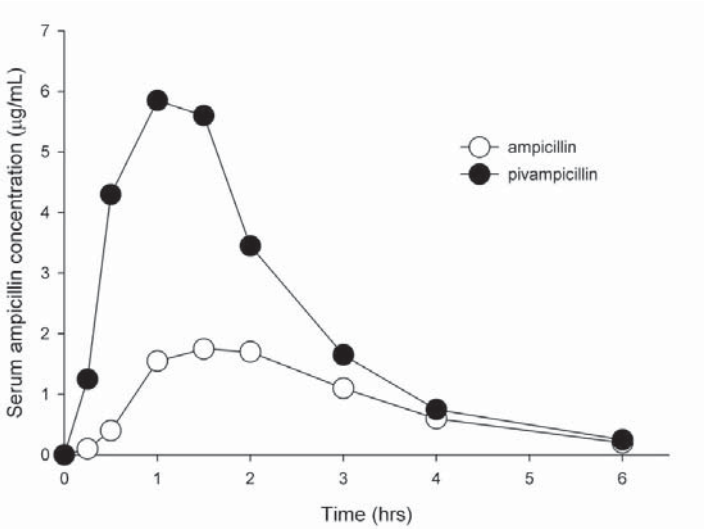
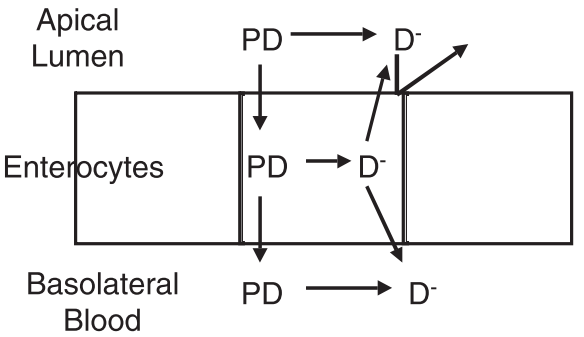


Figure 4. Mean serum concentrations of ampicillin from oral ampicillin (○) and equimolar oral pivampicillin (●) in volunteers (von Daehne et al., 1970b).

A number of penicillins and cephalosporins are absorbed orally without the need for prodrug intervention because they are substrates for a carrier-mediated transporter. Many others, however, are not candidates for a transporter. Most broad-spectrum cephalosporins fit this latter class. Cephalosporins that are not substrates for carrier-mediate transport are incapable of passive transcellular transport because the pK_a of the carboxylic group in cephalosporins is less than that seen with penicillins. The literature is replete with papers from various research groups regarding both novel and rather pedestrian studies into prodrugs of cephalosporins.

From the late 1970s to the mid 1980s this author was involved in one such study in collaboration with a Japanese company. At that time a criterion for success was >40% oral availability of the parent cephalosporin. Although our group tried both tested and novel strategies, we consistently hit a ceiling of

30–40% oral availability. Why this ceiling? Although one might argue that premature cleavage in the GIT or lack of systemic cleavage can account for this ceiling, often overlooked is cleavage in the enterocytes. This is illustrated in Scheme 5. The ideal prodrug is one that is stable to chemical and enzymatic cleavage in the GIT and the enterocytes but is quantitatively cleaved upon reaching the systemic circulation. Is this possible through the use of ester type prodrugs? Probably not, because the contents of both the lumen and the enterocytes have enhanced esterase activity due to the their role in the food digestion process. The prodrugs are simply viewed as a "food source" or an unwelcome xenobiotic. If cleavage occurs in the enterocytes as illustrated in Scheme 4, efflux, both passive and/or carrier-mediated, of the polar drug back into the lumen is possible and, in fact, highly probable since there is just as much driving force for the drug to go in that direction as there is for systemic delivery. An *in vitro* simulation of this process in Caco-2 cells resulted in Caco-2 cell accumulation of a penicillin prodrug (Chanteux *et al.*, 2005).

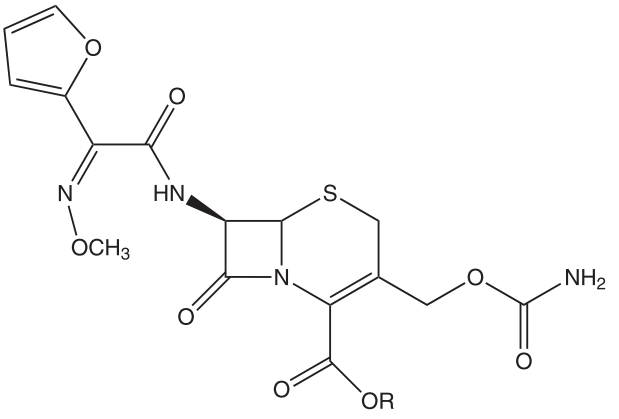


Scheme 5. Illustration showing that a non-polar prodrug (PD) of a polar drug (D^-) may show less than complete oral availability due to premature cleavage in the GIT or the enterocytes.

Despite the ceiling and a reasonable incidence of diarrhea, GlaxoSmithKline (GSK) did get a prodrug of cefuroxime (**19**), cefuroxime axetil (**20**), approved; this was followed by introduction of a number of similar prodrugs of other cephalosporins both in the USA and in other countries (Dellamonica, 1994). These include cefpodoxime proxetil, cefetamet pivoxil, cefditoren pivoxil, cefotiam hexetil, cefteram pivoxil, and cefcapene pivoxil. For a review of many ester prodrugs of marketed drugs including the cephalosporins, see Beaumont *et al.* (2003).

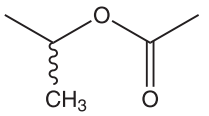
The less than complete absorption seen with ester prodrugs of the penicillins and cephalosporins, as well as others to be discussed later, points out an unmet need for continued research to identify novel prodrug strategies that result in minimal-to-no cleavage in the lumen and enterocytes but complete cleavage only upon reaching the systemic circulation.

A recent example of another carboxylic acid prodrug is oseltamivir (**22**), Tamiflu®, an ethyl ester prodrug of the polar antiviral oseltamivir carboxylate (**21**).



19 R = -H

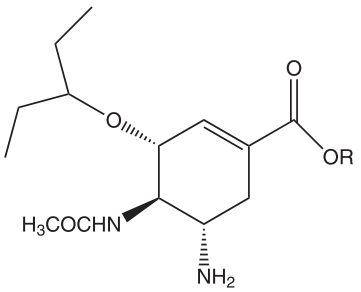
20 R =



Structures 19-20.

Oseltamivir carboxylate (**21**) showed very poor oral availability (4.3 ± 1.6%) in rats, consistent with what was seen with a competitor neuraminidase inhibitor, zanamivir, sold as Relenza®. Zanamivir is available only as an inhaled product. A competitive marketing advantage was gained with the oral availability of oseltamivir carboxylate from oseltamivir. Table 1 summarizes the data of Li *et al.* (1998) showing that the oral availability of **21** from **22** varied from 11% in the ferret (an antiviral animal model) to 73% in the dog, with the rat showing an availability of 35%, a sevenfold greater bioavailability compared to **21** dosing. Human availability is very good and is consistent with the animal data.

Esters other the ethyl ester were tried but not reported (see case study in Section 5 of this book). The ethyl ester has the significant advantage of generating on conversion only the drug and ethanol, an alcohol with known



21 R = H

22 R = -CH_2CH_3

Structures 21-22.

Animal species	Compound	Oseltamivir carboxylate % bioavailability
Mouse	Oseltamivir ethyl ester	30
Rat	Oseltamivir ethyl ester	35
Ferret	Oseltamivir ethyl ester	11
Dog	Oseltamivir ethyl ester	73
Human	Oseltamivir carboxylate	4.3
Human	Oseltamivir ethyl ester	35

Table 1. Bioavailability of oral oseltamivir carboxylate, and oseltamivir carboxylate from oral oseltamivir ethyl ester in various animal species (Li *et al.*, 1998)

minimal side effects in the quantity generated from a 75 mg dose of **22**. Keeping the prodrug strategy as simple as possible, such as in the use of the ethyl ester, has its advantages. On the other hand, for some drugs the ethyl ester may not be the best.

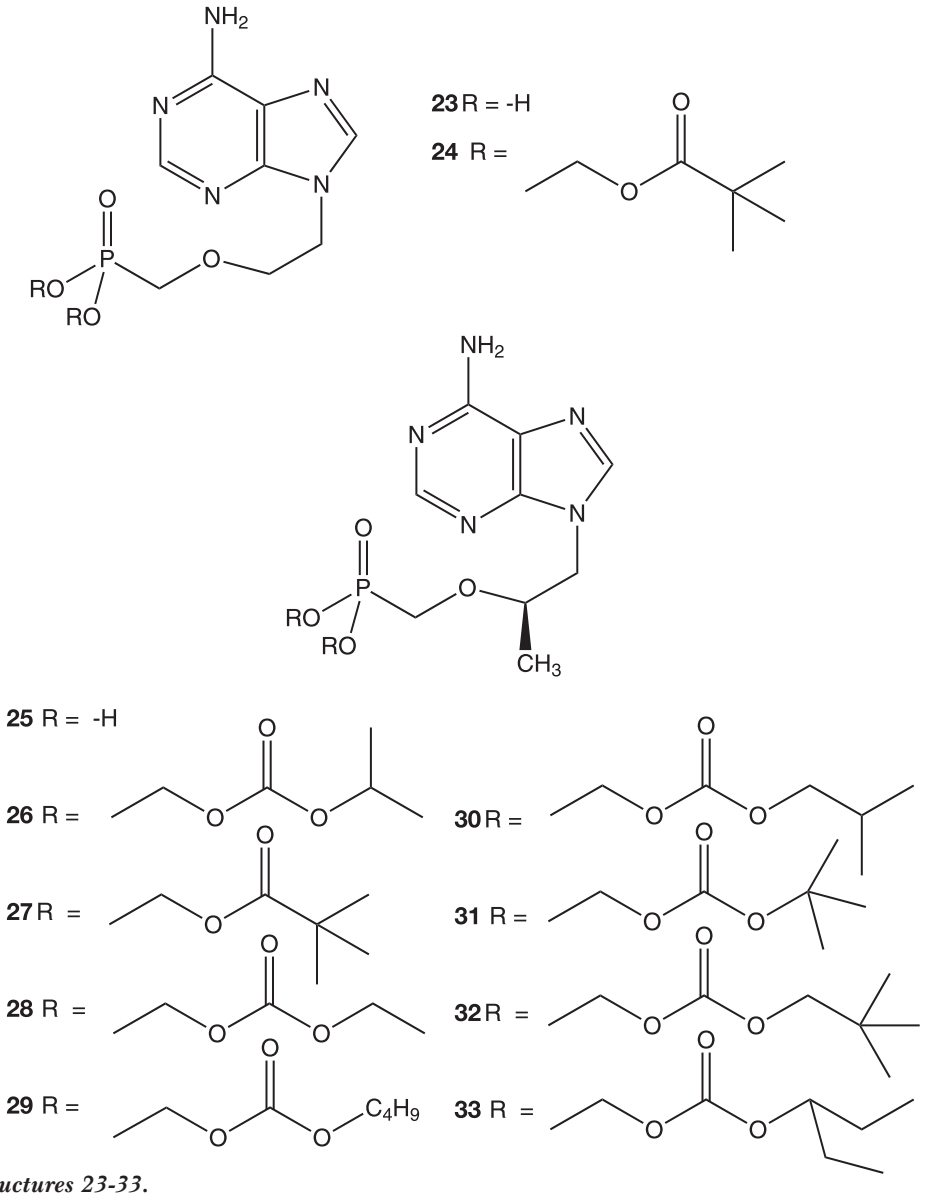
The reader is directed to some of the case studies in the final section of this book for other prodrugs of carboxylic acids as well as Chapter 3.1 for ways in which others have attempted to modify the properties of various carboxylic acids.

Phosphonates, phosphinates, and bisphosphonates all demonstrate high polarity due to their low pK_a values and the intrinsic polarity that they impart. Thus, they show poor oral availability. Some of the bisphosphonates used to treat osteoporosis are sold despite having a low bioavailability and are presumably absorbed via passive paracellular transport. Prodrugs of phosphonates, phosphinates, and bisphosphonates are discussed in Chapter 3.6, and there are three case studies in the last section of this book. For completeness, some selective examples will be briefly discussed here.

Recent commercial successes of phosphonate prodrugs include the antivirals adefovir dipivoxil (Hepsera®, **24**), a prodrug of adefovir (**23**) used to treat hepatitis B, and tenofovir disoproxil (Viread®, **26**), a prodrug of tenofovir (**25**) used to treat HIV infections. Adefovir dipivoxil, like pivampicillin discussed earlier, generates pivalic acid and formaldehyde (in this case two moles of each). When adefovir dipivoxil was first tested as an antiviral for HIV infections, concerns about carnitine depletion were raised (Brass, 2002, and his chapter in this book). After adefovir dipivoxil was found to be effective in the treatment of hepatitis B at a lower dose, those concerns became less serious. When prodrugs of tenofovir were first evaluated, the dipivoxil prodrug was considered (Shaw *et al.*, 1997); however, due to various toxic issues including carnitine depletion, a series of carbonate bis-esters were considered with the tenofovir disoproxil, fumarate salt, being chosen for clinical trials (Kearney *et al.*, 2004). This prodrug is now approved and sold

under the name Viread®. The choice of the disoproxil group highlights the decision-making process when considering the choice of final promoiety (Shaw *et al.*, 1997). In addition to the disoproxil derivative, derivatives **27–33** were extensively evaluated; **27** is the dipivoxil derivative and **28–33** are other biscarbonate prodrugs.

Properties tested were chemical stability at pH 7.4 and 37°C, log P values, stability in dog intestinal, plasma and liver homogenates, and absolute oral bioavailability in dogs by comparing AUC values to those of IV **25** (Shaw *et al.*, 1997). Table 2 is a compilation of the critical data. Although the dipivoxil derivative (**27**) showed the best oral bioavailability, it was discarded because of GIT



Structures 23-33.

side effects, apparently due to the pivalic acid. Of the prodrugs remaining, **26**, **31** and **33** showed equivalent oral bioavailability. **31** was discarded because of inadequate chemical stability (see Table 2) that would have made its formulation even as a solid a nightmare and **33** was discarded because of the unknown toxicity of 3-pentanol, one of the breakdown compounds. The disoproxil releases carbon dioxide, formaldehyde (at total of 35 mg from a 300 mg dose of tenofovir disoproxil) and two moles of isopropyl alcohol, an alcohol known to be safe. Figure 5 shows a plot of tenofovir oral bioavailability versus the half-life for these prodrugs in dog intestinal homogenates. This confirms that a critical criterion for availability is the ability of the prodrugs to survive premature cleavage in the lumen and possibly the enzymes embedded in the brush border of the intestinal cells (enterocytes). The apparent ceiling of about 30% bioavailability in this animal model, the beagle dog, is consistent with the model proposed earlier in Scheme 5.

Compound number	Log P[1]	$t_{1/2}$ (hr) pH 7.4, 37°C	$t_{1/2}$ (min) dog intestinal homogenates[3]	$t_{1/2}$ (min) dog plasma[3]	$t_{1/2}$ (min) dog liver homogenates[3]	% F in dogs[4]
26	1.3	9.2	52.6	20.5	<5	30.1
27	2.1	14	10.4	35.5	<5	37.8
28	0.6	7.0	23.3	16.6	<5	24.5
29	2.7	6.0	<5	<5	<5	18.0
30	2.0	9.0	15	<5	<5	20.8
31	1.9	0.4	26.6	21.2	14.9	30.7
32	>3.0	6.0	<5	<5	<5	16.0
33	>3.9	8.0	30	15	<5	28.8

Table 2. Various physical and biological properties of a series of tenfovir prodrugs, 26–33, and the oral bioavailability of tenofovir from the prodrugs (Shaw et al., 1997)

[1] pH 6.5 and 25°C [2] phosphate buffer

[3] performed at 37°C [4] beagle dogs

Other workers have suggested chemistries other than the use of acyloxyalkyl esters to modify phosphonates and bisphosphonates (Bidanset *et al.*, 2004; Hartline *et al.*, 2005; Erion *et al.*, 2005; Vepsalainen, 2002; Peyrottes *et al.*, 2004). Some of these prodrugs have entered preclinical and early clinical trials.

As previously discussed, the ability to cross a biological membrane by passive diffusion depends on the partition coefficient between the membrane and the aqueous environment. The pH partition theory suggests that partitioning is

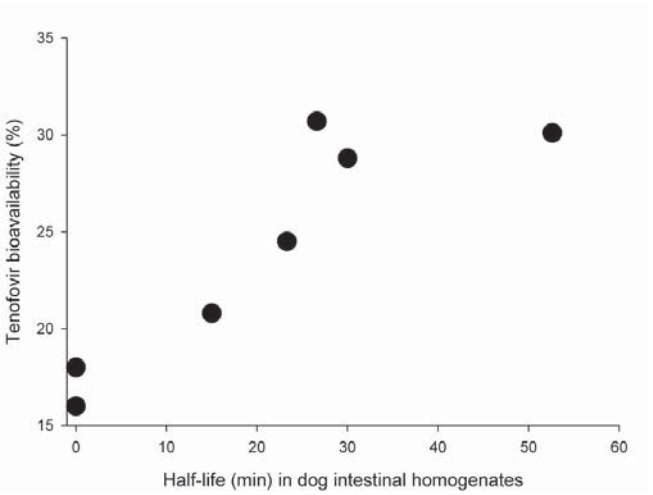


Figure 5. Relationship between oral availability of tenofovir from various alkoxycarbonyloxymethyl ester prodrugs in dogs and the stability of the prodrugs in dog intestinal homogenate (from Shaw *et al.*, 1997).

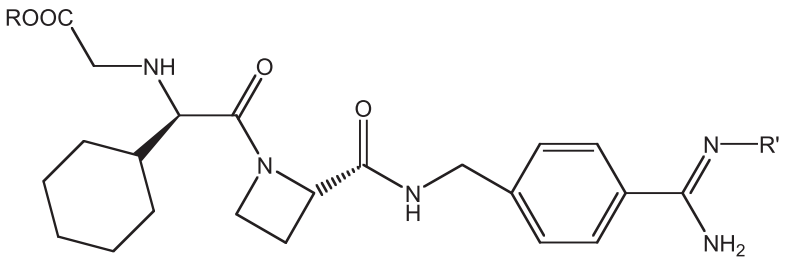
dependent not only on the intrinsic partition coefficient of the uncharged compound (log P) but also on the degree of ionization. Therefore, the interplay between pH in the GIT and the pK_a of a compound affects its passive diffusion. In general, it is the un-ionized form of the compound that permeates across the membrane, due to its higher partition coefficients, even though some evidence shows that ionized species can also permeate but to a lesser extent (through passive paracellular transport and possible carrier-mediated transport). To optimize passive transcellular transport across a membrane, it is important to have a significant fraction of the drug in its un-ionized state at the site of absorption. In some cases a change in the pK_a of a compound is sufficient to render a significant fraction of the molecule in its neutral state under the physiological conditions in the GIT and thus improve the oral absorption of the compound.

An example of this is the modification of the strongly basic amidine and benzamidine functional groups. The reader is directed to Chapter 3.5 of this book for a more extensive discussion of prodrugs of benzamidines. Unsubstituted benzamidines have a pK_a of 11.6, causing them to be fully protonated at all pH values seen in the GIT. Substitutions on the benzene ring can slightly lower the pK_a value but not enough to significantly alter the ionization state of these molecules in the GIT. Permeation of molecules containing this functional group is therefore generally poor. Prodrugs of the amidine group have been designed with a view to altering their pK_a values. Examples of this include preparation of hydroxyamidines and alkoxycarbonyl derivatives. When this approach is used, the pK_a of the benzamidine drops from the previously mentioned 11.6 to values as low as 5.

A recent example of the use of this approach is the thrombin inhibitor ximelagatran (**35**). The parent molecule, melagatran (**34**), contains a strongly basic benzamidine group with a pK_a of 11.5 that is protonated at the pH of the intestinal

tract and hinders intestinal absorption. Furthermore, melagatran also contains an acidic carboxylic group with a pK_a of 2.0 that is ionized at most physiological pH values and a secondary amine with a pK_a of 7.0. Therefore, at pH 7, melagatran is positively charged overall and above 7 it exists in a zwitterionic state. This is illustrated in Scheme 6 where the ionic state of melagatran is shown to exist in multiple forms with only a very small fraction able to exist as an uncharged species (**000** in Scheme 6). The effect of the charges on the oral absorption of melagatran was observed when the compound was initially dosed orally and low and variable bioavailability of 3–7% was observed (Gustafsson *et al.*, 2001). The double prodrug ximelagatran was developed to try to improve the oral absorption of melagatran. Ximelagatran has an ethyl ester group in place of the carboxylic acid and an N-hydroxyamidine group in place of the amidine. By introduction of the hydroxyl group into the amidine functional group, the basicity is lowered to a pK_a of 5.2. The ethyl ester of the carboxylic acid not only eliminates its charge but also reduces the pK_a of the secondary amine to 4.5. These two protecting groups significantly change the apparent physicochemical properties of the molecule, such that greater than 90% of the ximelagatran molecules are neutral at pH values above 6.2. This is also illustrated in Scheme 6. This change in the physico-chemical properties of the compound results in an approximately 80-fold increase in permeation of ximelagatran compared to melagatran across human colon epithelial cell (Caco-2 cell) monolayers (Gustafsson *et al.*, 2001; Gustafsson, 2003). When ximelagatran was dosed orally, the bioavailability of melagatran was observed to be in the range of 18–24% (Gustafsson *et al.*, 2001).

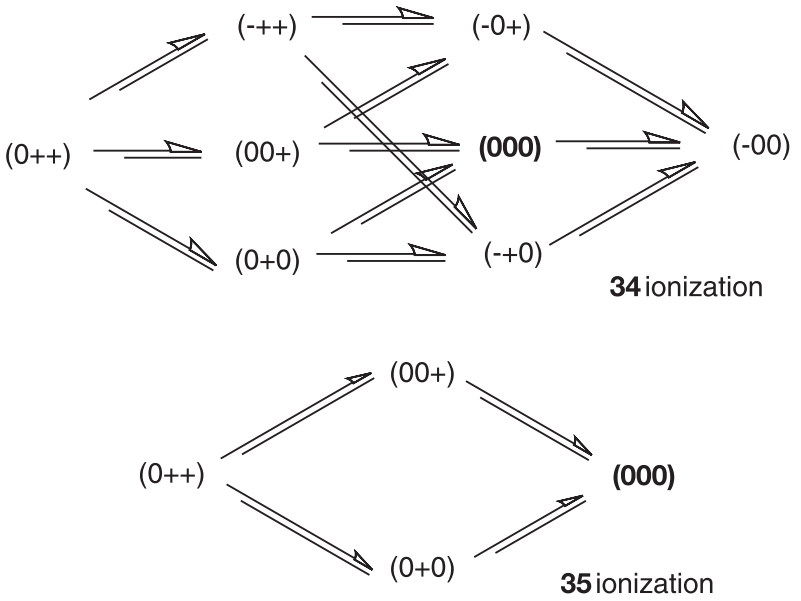
Poly- or zwitterionic compounds present a challenge. Like the example of melagantran discussed above in relation to the changes in the pK_a value of a benzamidine group, other poly-charged molecules are difficult to absorb from the GIT unless the molecule, by chance or design, is a substrate for carrier-mediated transport. Angiotensin-converting enzyme (ACE) inhibitors revolutionized the treatment of hypertension. The first of these, captopril, did not require prodrug intervention for oral delivery, but many subsequent examples did. The first of these was enalapril (**37**), the ethyl ester prodrug of enaliprilat (**36**), which was developed at Merck (Ulm, 1983). As can be seen from its structure, enaliprilat has



34 R = -H R" = -H pKa_1(COOH) = 2.0 pKa_2 (2° amine) = 7.0 pKa_3 (benzamidine) = 11.5

35 R = $-C_2H_5$ R" = -OH pKa_1 (2° amine) = 4.5 pKa_2 (hydroxybenzamidine) = 5.2

Structures 34-35.

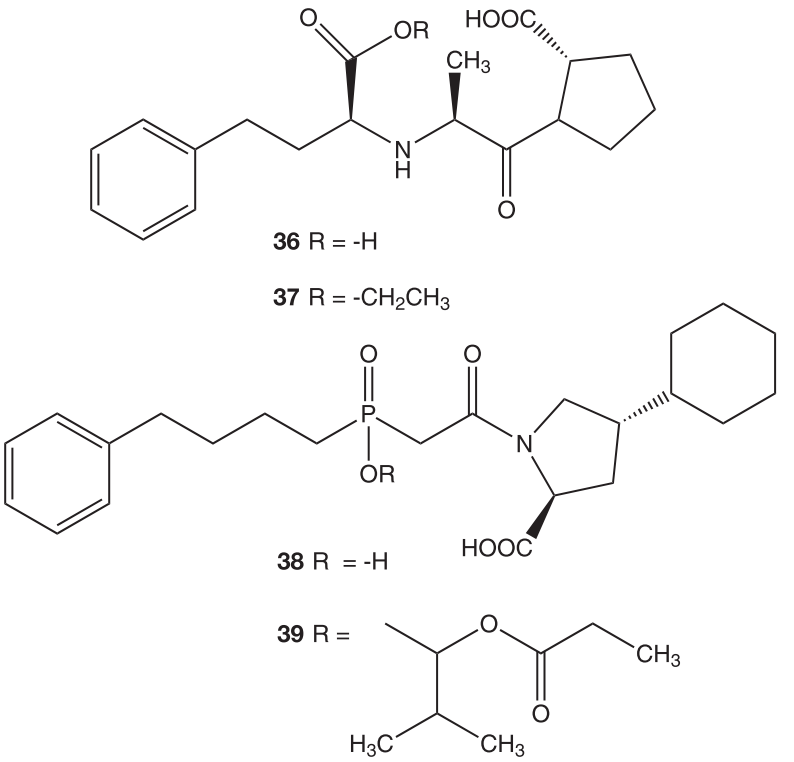


Scheme 6. The complex ionization schemes for melagatran (34) having three apparent pK_a values corresponding to a carboxylic acid group (can exist as the neutral acid (0) or as an anion (–), and two amino groups that can be exist in their charged form (+) or neutral form (0)) and its prodrug ximelagatran (35) where only the amino groups are ionizable. The bolded species, the neutral forms, are those most likely to be passively absorbed after oral dosing.

two carboxyl groups and an amine function such that at all pH values it exists as a charged species. Upon esterification of one of the carboxyl groups, it still exists in various charged forms; however, one of these is a zwitterionic species that is in equilibrium with a neutral form (see Scheme 6 for a similar ionization scheme). As will be discussed later, there is solid evidence that enalapril is both actively transported from a selective region of the GIT and passively absorbed, presumably due to the small fraction of the molecule present in its neutral form. Enalapril has significant di- and tripeptide character that can account for the active component of the transport form the GIT (Schoenmakers *et al.*, 1999; Friedman and Amidon, 1989; Swaan *et al.*, 1995).

Subsequent to enalapril, numerous other ACE inhibitors were identified, most of which required prodrug intervention to effect adequate oral delivery. Examples include benazepril, quinapril, ramipril, moexipril, peridopril, trandolapril, and fosinopril. Many of these have been shown to have both passive and active components to their oral absorption.

One ACE inhibitor that was a little different was fosinoprilat (**38**), the active form of the prodrug fosinopril (**39**). Here the parent drug was too polar due to the presence of both a carboxyl group and a phosphinic acid functionality. Although not documented in the literature, ester prodrug efforts to modify the carboxyl group for this compound were unsuccessful because the much lower pK_a

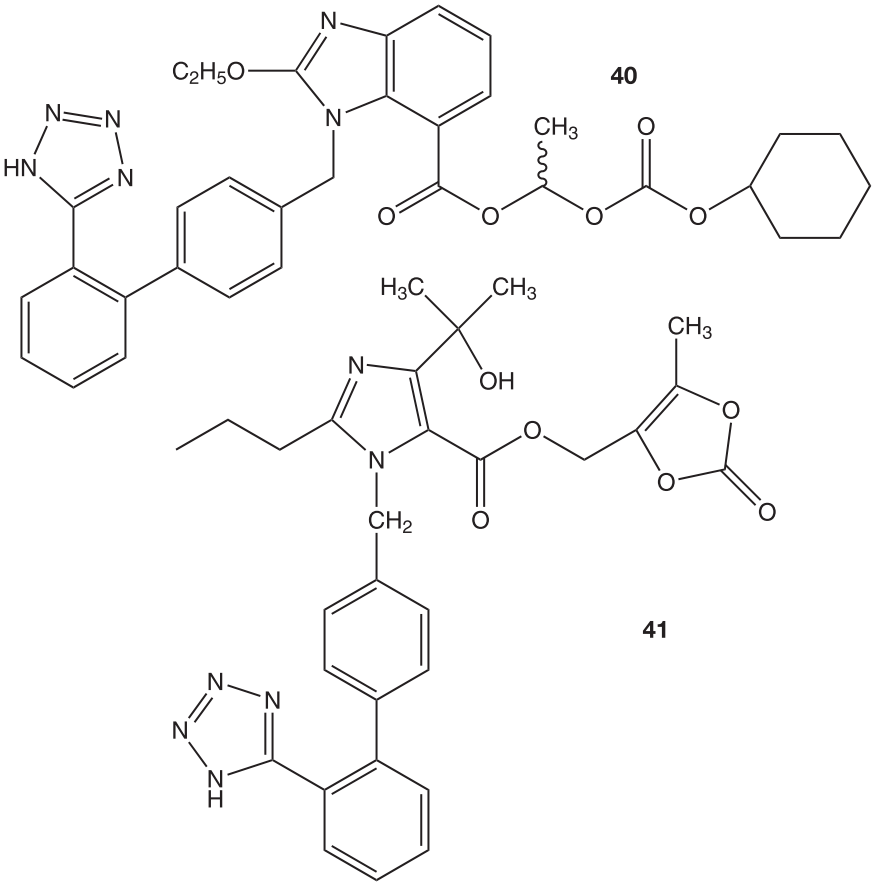


Structures 36-39.

of the phosphinic acid group meant that the molecule was still effectively ionized throughout the pH values seen in the GIT. Blocking the charge on the phosphinic acid group did prove successful (see case study in Section 5 of this book) with the desired prodrug being an acyloxyalkyl ester of the type discussed earlier for phosphonic acid derivatives. A difference was that the acyl function was a proprionyl group and, in place of the formaldehyde, isobutryaldehyde was used. Such a double ester is presumably enzymatically cleaved at the acyl function, releasing the proprionic acid, the isobutryaldehyde, and fosinoprilat while adequate chemical stability is gained from its sterically hindered nature.

Like the ACE inhibitors, some angiotensin receptor II blockers (ARB) also required prodrug intervention to effect oral delivery because of their polar nature. Examples include candesartan cilexetil (**40**) and olmesartan medoxomil (**41**). Both of these prodrugs have promoieties that have received minimal attention.

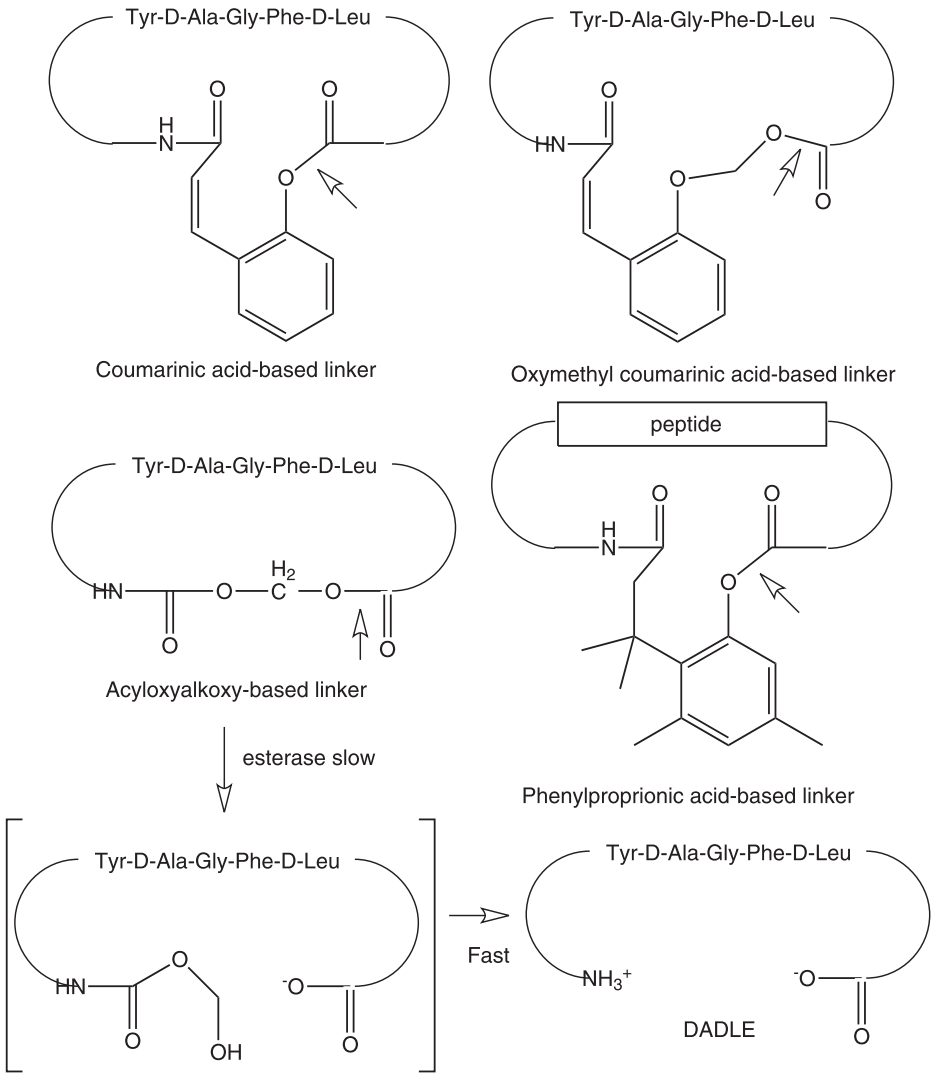
Linear peptides and peptide mimetics have always presented a challenge to efficient oral drug delivery because of both their propensity to be metabolized by peptidases and their poor permeation. This is especially the case for peptidic drugs with both an N-terminal free amine and a C-terminal free carboxyl group. Other workers had observed that some cyclic peptides such as cyclosporine A are metabolically stable and reasonably well absorbed. Borchardt and coworkers (Borchardt and Wang, 2000 and references therein) therefore attempted to capitalize on this observation by tying the two ends of a linear peptide together



Structures 40-41.

through a biodegradable linker to form a cyclic prodrug. This is illustrated in Scheme 7 for the peptide DADLE ($^{+}H_3N$-Tyr-D-Ala-Gly-Phe-D-Leu-COO^{-}) with three examples of linkers explored by this group, and an additional linker attempted earlier in their studies. To release the parent peptide, all three linkers were expected to be initially cleaved by esterases (see arrows in Scheme 7) followed by a second chemically driven event. Although quite promising, this prodrug concept proved ineffective for an unexpected reason. All of the cyclic prodrugs proved to be excellent substrates for efflux pumps. Thus, while promising markers such as higher log P values, smaller molecular radius, less rotatable bonds, lower molecular flexibility, and improved metabolic stability to peptidases were observed for the cyclic prodrugs, effective oral (and CNS) delivery was not observed in a number of cases (Ouyang *et al.*, 2002a,b; Tang and Borchardt, 2002a,b; Liederer and Borchardt, 2005). An interesting question is whether these observations can be generalized to all linear peptides or peptide mimetics. Intuitively, this would seem unlikely.

Most amino acids or amino acid mimetics are also polyionic or zwitterionic in nature. The natural L-amino acids are well absorbed from the GIT by carrier-mediated transport (Ganapathy *et al.*, 1994). D-amino acids and amino acid

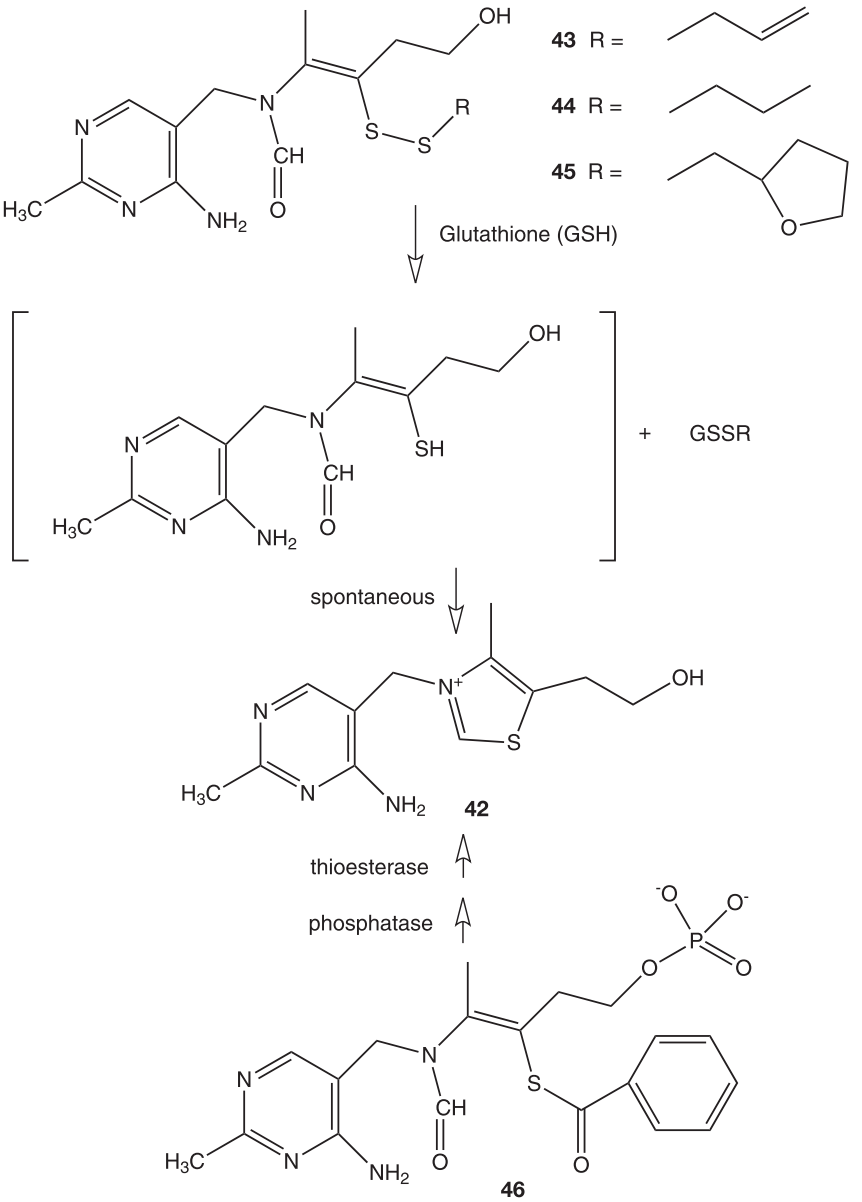


Scheme 7. Various strategies used to convert linear peptides with both a free N-terminus amino group and a C-terminus carboxyl group to cyclic peptides. Also shown is the metabolic pathway for conversion of an acyloxyalkoxy-based prodrug of DADLE (Tyr-D-Ala-Gly-Phe-D-Leu) to DADLE

mimetics may be well absorbed, depending on the degree of paracellular transport or their ability to be recognized by a transporter. It is also possible to deliver these polar drugs orally if they are converted to less polar prodrugs by modifying either the carboxy or the amino terminus.

Like the amino acids, many nutrients and essential vitamins are absorbed via transporter-mediated mechanisms. However, these transporters can be inhibited and, occasionally, it may be best not to rely on the transporter. An interesting example is the absorption of thiamine or vitamin B_1 (**42**). Although thiamine, a

quaternary ammonium compound, is actively transported from the GIT, its transport is depressed on acute alcohol ingestion (Thomson *et al.*, 1971; Thomson and Leevy 1972; Thomson and Majumdar, 1981). Consequently, chronic alcoholics, due to this inhibition and poor diet, often demonstrate symptoms consistent with Wernicke's disease and the accompanying bilateral and ocular palsy resulting from thiamine deficiency (Baker *et al.*, 1974). In Mediterranean populations, where garlic is frequently used in cooking, these symptoms are seen



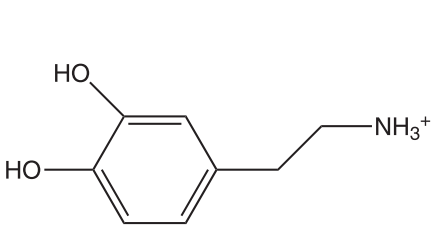
Scheme 8. Two metabolic schemes showing the conversion of thiamine disulfide prodrugs and benfotiamine to thiamine

less frequently. This has been traced to the presence in garlic of a lipid soluble form of thiamine, allithiamine (**43**), that acts as a lipid soluble, passively transported prodrug (Fujiwara, 1976). Japanese researchers extensively studied thiamine deficiency that was observed due to the extensive use of polished rice where rice hull removal eliminated a major thiamine source from the diet (Kawai *et al.*, 1980). A number of prodrugs underwent extensive trials (**43–45**); Loew (1996) reported on the pharmacokinetics of benfotiamine (**46**). Their structures and modes of reversion are shown in Scheme 8.

Facilitated Permeability By Utilizing A Gastrointestinal Tract Transporter:

It is now generally well recognized that active and/or facilitated transport plays an important role in oral absorption of many nutrients and some drugs (Yang et al, 2001; Majumdar *et al.*, 2004; Oh *et al.*, 1999; Tsuji and Tamai, 1999). Various transporters, including those needed for amino acids (Ganapathy *et al.*, 1994), oligopeptides (Smith *et al.*, 1993; Ganapathy *et al.*, 1994), monosaccharides, inorganic phosphate, monocarboxylic acids (Enerson and Drewes, 2003), bile salts (Wilson, 1981), and nucleosides (Baldwin *et al.*, 1999) have been identified. For a comprehensive general discussion of these transporters in drug absorption, the reader is directed to the following references (Oh *et al.*, 1999; Tsuji and Tamai, 1999; Yang *et al.*, 2001; Majumdar *et al.*, 2004). If the structural elements of a drug result in its being recognized by one of the GIT transporters, then its oral availability will be better than predicted based on its physicochemical properties. Therefore, the intestinal absorption of certain drugs can be significantly enhanced by chemically converting them to a prodrug mimicking the natural substrates for the active transporter(s) (Oh *et al.*, 1999; Tsuji and Tamai, 1999; Yang *et al.*, 2001; Majumdar *et al.*, 2004).

The best historical example of this concept is L-dopa (**48**), a prodrug of dopamine (**47**), a natural neurotransmitter whose CNS deficiency as has been linked to Parkinson's disease. Dopamine can be replaced by administering L-dopa, which in the CNS and peripherally is readily decarboxylated by dopa decarboxylase. L-dopa appears to be a substrate for the L-aromatic amino acid transporter (Shindo *et al.*, 1973; Wade *et al.*, 1973; Tsuji, 1999;) both in the intestine and at the blood-brain barrier (Shindo *et al.*, 1971).



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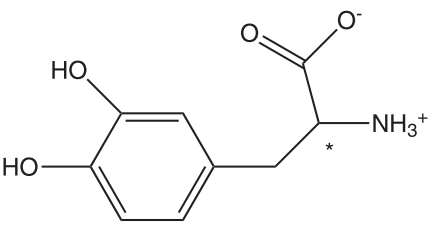


48

Structures 47-48.

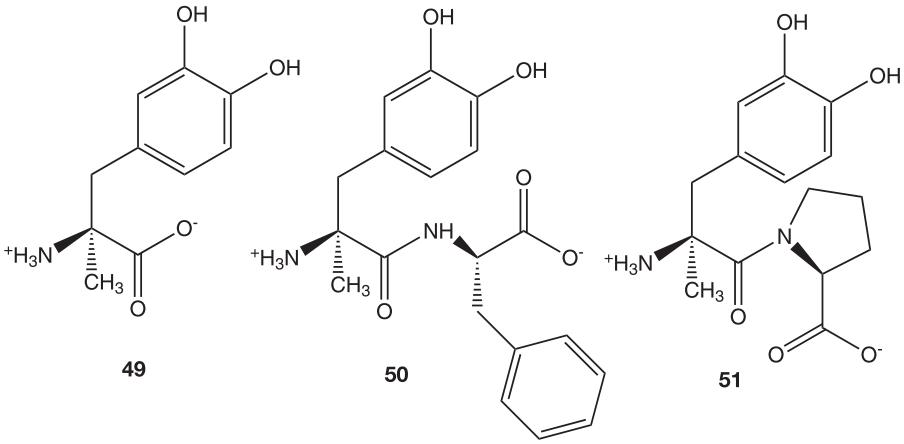
The intestinal peptide transport system has been a key target for prodrug approaches—some by design, some by serendipity. For example, the intestinal peptide transport system has broad substrate specificities and is involved in the transport of natural di- and tripeptides. The most cited transporter is PepT-1 (Liang et al, 1995; Tsuji and Tamai, 1996; Adibi, 1997; Yang *et al.*, 1999). This transport system also absorbs various orally administered peptidomimetic drugs, including some β-lactam antibiotics and angiotensin-converting enzyme inhibitors. A number of prodrugs have been engineered to recognize the high capacity oligopeptide transporters.

Hu *et al.* (1989) prepared a dipeptidyl prodrug of the antihypertensive agent L-α-methyldopa (**50**), L-α-methyldopa-L-phenylalanine (**49**). L-α-methyldopa is itself a relatively poor substrate for L-aromatic amino acid transporter (Hu and Borchardt, 1990; Amidon *et al.*, 1986). The prodrug, 49, displayed up to 20 times higher intestinal permeation (Hu *et al.*, 1989) and its uptake was inhibited in the presence of dipeptides and cephradine (Tsuji *et al.*, 1990), a drug known to be transported by the peptide transporter (Table 3). Of interest were the relative capacities for the carrier to the amino acid coupled to the L-α-methyldopa on the amino or carboxy terminus (Hu *et al.*, 1989). The same group also evaluated L-α-methyldopa-L-proline (**51**) as a prodrug of L-α-methyldopa (Bai *et al.*, 1991, 1992) in rats. A similar effort was made to deliver L-dopa as the tripeptide, L-pyroglumate-L-dopa-L-proline (Bai, 1995). These results demonstrate the feasibility of using the peptide transport system to improve the intestinal absorption of L-α-methyldopa and L-dopa.

Various nucleoside antiviral agents show poor oral bioavailability (see earlier discussion) due to their polarity. Initial prodrug strategies tended to focus on the preparation and evaluation of more lipophilic prodrugs (see earlier discussion) until the observation that the L-valine ester prodrug of acyclovir (**52**), valacyclovir (**53**), showed excellent availability after oral dosing. One could make a reasonable argument that this superior performance was due to a change in polarity and the presence of an ionizable amino group in the structure that could facilitate

Compound	P_W ± SE
L-α-methyldopa (49)	0.4 ± 0.22
Gly-L-α-methyldopa	4.34 ± 0.27
Pro-L-α-methyldopa	1.68 ± 0.23
L-α-methyldopa-pro (51)	5.41± 0.55
Phe-L-α-methyldopa	5.29 ± 1.57
L-α-methyldopa-Phe (50)	10.22 ± 0.45

Table 3. Wall permeability (P_W) of various L-α-methyldopa prodrugs (0.1 mM) using a rat small intestine perfusion study (Hu et al., 1989)



Structures 49-51.

solubility/dissolution. Subsequently, the permeation of valacyclovir across Caco-2 monolayers was found to be about sevenfold higher than that of the parent molecule (Han *et al.*, 1998). Others have explored this phenomenon (Balimane *et al.*, 1998; Ganapathy *et al.*, 1998; Sinko and Balimane, 1998; Guo *et al.*, 1999). There was also a strong stereospecific preference for the L-valine ester compared to the D-valine ester. For example, the urinary recovery of acyclovir from acyclovir itself given IV is 96% and given orally is 19%; however, its recover from valacyclovir was 63%. The D-valinyl ester resulted in a urinary recovery of only 7%. The only other prodrugs that showed some promise were the L-alanyl ester (42% urinary recovery), the L-isoleucyl ester (43% urinary recovery), and the (S)-2-aminobutryl ester (50% urinary recovery) (Beauchamp *et al.*, 1992; Beauchamp and Krenitsky, 1993). Subsequent human studies confirmed the superior behavior of valacyclovir (Beauchamp and Krenitsky, 1993; Weller *et al.*, 1993).

The carrier-mediated transport of the valacyclovir was further confirmed by competitive uptake studies in the stably transferred Chinese Hamster Ovary (CHO) cells overexpressing the hPEPT1 transporter (Balimane and Sinko, 2000)

Compound and Addition	Apical to Basolateral Permeability
Acyclovir (52)	0.0013 ± 0.0002
Valacyclovir (53)	0.0197 ± 0.0028
D-Valine-acyclovir	0.0024 ± 0.0006
Valacyclovir (53) + L-valine	0.0224 ± 0.0029
Valacyclovir (53) + Gly-Sar	0.0086 ± 0.011

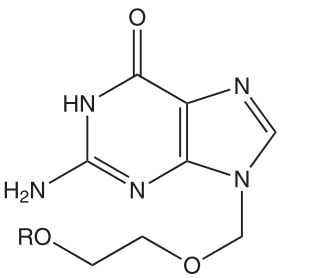
Table 4. Apical to basolateral permeability of acyclovir, the L-valine (valacyclovir) and D-valine esters of acyclovir, and valacyclovir in the presence of L-valine and Gly-Sar across Caco-2 cells (cm/hr) (de Vrueh *et al.*, 1998)

and the selective permeation through Caco-2 cell monolayers of the L-isomer in comparison to the D-isomer (Han *et al.*, 1998; de Vrueh *et al.*, 1998). The lack of transport inhibition by the amino acid L-valine and the significant inhibition by the dipeptide Gly-Sar can be seen in the results presented in Table 4. These findings confirm that valacyclovir owes its superior performance on oral dosing to its capacity to be transported from the GIT by the peptide transporter.

Like acyclovir, ganciclovir (**52**) was poorly absorbed after oral dosing. The L-valine ester prodrugs of ganciclovir (valganciclovir, **53**) and NM107 (NM283) have also been developed (see case study section of this book). The oral bioavailability of ganciclovir from valganciclovir is significantly higher than the parent drug. The absolute oral bioavailability of ganciclovir from valganciclovir following administration with food is approximately 60% in human subjects while the absolute bioavailability of ganciclovir itself is less than 10% (Brown *et al.*, 1999). Sugawara *et al.* (2000) showed that valganciclovir transport through Caco-2 cell monolayers, like that of valacyclovir, was inhibited by a dipeptide, in this case Gly-Phe. Ganciclovir was poorly able to permeate, as expected, and its transport was not affected by the presence of Gly-Phe. The bis-L-valine ester of ganciclovir was better transported than ganciclovir but not as efficiently as the monoester.

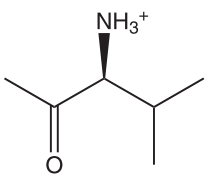
The mono-L-valine ester may have the optimal combination of structural elements that results in reasonable metabolic stability in the lumen, adequate solubility and, thus, dissolution, carrier recognition etc., for good oral availability. For a more complete history of the identification of the mono-L-valine ester as the prodrug of choice for ganciclovir, the reader is directed to the Case Study section of this book where the innovators recall its development. With the successes seen with valacyclovir and valganciclovir, others have used this idea to alter the properties of similar drugs. Examples are valtocitabine used to treat hepatitis B infections and NM 283 used to treat hepatitis C infections. Both of these experimental drugs are undergoing clinical trials.

At this point one is tempted to ask the question, *a priori*, "based on the structural elements of both valacyclovir and valganciclovir, would one have



52 R = -H

53 R =



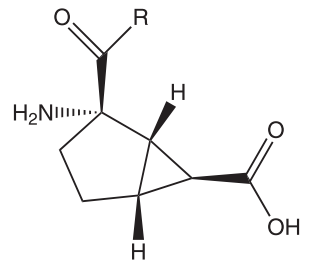
Structures 52-53.

expected them to be candidates for a peptide transporter?" The legitimate answer is no. It was a surprise.

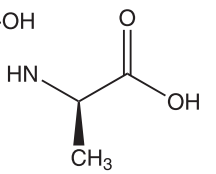
The peptide transporters have also been implicated in the absorption of enalapril. Enalapril is an oral prodrug of enalaprilat, an angiotensin-converting enzyme (ACE) inhibitor (Friedman and Amidon, 1989). As discussed earlier, enalaprilat is poorly absorbed from the gastrointestinal tract because of the presence of three ionizable groups within its structure; however, enalapril is well absorbed. The permeation of enalapril was shown to be concentration dependent, consistent with a saturable non-first-order process, and decreased in the presence of the dipeptide Tyr-Gly and the dipeptide mimetic cephradine, indicating a non-passive absorption mechanism consistent with a peptide carrier-mediated transport system (Friedman and Amidon, 1989; Oh *et al.*, 1999). Similar results have been observed for other ACE inhibitor prodrugs (Oh *et al.*, 1999; Shu *et al.*, 2001). In the case of the ACE inhibitor prodrugs, it is much easier to see the structural elements that make them substrates for the peptide carrier system. They look like peptides!

An L-alanylamide prodrug of LY354740 (**54**), LY544344 or talaglumetad (**55**), shows improved oral bioavailability facilitated by PepT-1 transport (Bueno *et al.*, 2005). A number of dipeptide prodrugs showed good oral availability. An additional example of a prodrug targeting the peptide transporter is alafosalin (Grappel *et al.*, 1985). Bisphosphonates, pamidronate and alendronate, have also been modified by the addition of a Pro-Phe to give Pro-Phe-pamidronate and Pro-Phe-alendronate, respectively (Ezra *et al.*, 2000). These were designed to target the hPepT-1 transporter. Two- to threefold increases in oral bioavailability over that of the parent drugs were seen.

Monocarboxylic acid transport systems have been favorably viewed for drug delivery purposes. Included are monocarboxylic acid-proton contraporter, MCT1 (Price *et al.*, 1998), and an anion antiporter, AE2 (Yabuuchi *et al.*, 1998). Cundy *et al.* (2004a,b) prepared a novel prodrug of gabapentin (**56**), XP13512 (**57**), recognized by MCT1 and the sodium-dependent multivitamin transporter



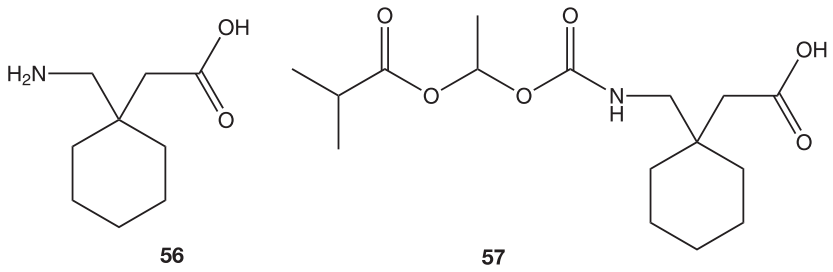
54 R = -OH

55 R = 

Structures 54-55.

(SMVT). These two high capacity nutrient transporters are broadly distributed in the intestinal tract of humans. The MCT1 is responsible for the absorption of small chain fatty acids derived from the diet or produced by microflora (Enerson and Drewes, 2003; Halestrap and Price, 1999). MCT1 has a high transport capacity and is expressed along the length of the intestine. The SMVT is also expressed along the small and large intestine and is responsible for absorption of the essential co-factors biotin, lipoate, and pantothenate. XP13512 was designed to mask the amine group of gabapentin with an acyloxyalkylcarbamate promoiety, resulting in an anionic compound with the potential to be a substrate for the carboxylate transporters. The bioconversion of XP13512 to gabapentin leads to the formation of isobutyric acid, acetaldehyde, and carbon dioxide.

In rats and monkeys, XP13512 showed improved gabapentin bioavailability and increased dose proportionality compared to oral gabapentin (Cundy *et al.*, 2004b). Thus, XP13512 was efficiently absorbed and rapidly converted to gabapentin after oral dosing. In monkeys, the oral bioavailability of gabapentin from XP13512 was about 84% compared to 25% after similar oral administration of gabapentin.



56 **57**

Structures 56-57.

An additional candidate for MCT1 is the prodrug carindacillin, an ester prodrug of carbenicillin. Carbencillin is a dicarboxylic acid that shows poor passive permeation through Caco-2 cell monolayers while carindacillin is transported by a carrier-mediate process. In studies in brush border vesicles and Caco-2 cells, carindacillin was transported via a monocarboxylic acid transporter and not a peptide transporter (Li *et al.*, 1999a,b). Carindacillin shows significantly higher oral availability than carbenicillin (Tanigawara *et al.*, 1982).

Organic cation transporters (OCTs) are expressed in the intestines, and it is possible that a number of cationic drugs are also absorbed via this route. More often than not these act as efflux transporters. To our knowledge these transporters have not been utilized for the enhanced oral delivery via the design of appropriate prodrugs.

Many have tried with little success to utilize the monosaccharide, bile acid, nucleoside, and phosphate transports to effect improved oral delivery of drugs. The utilization of receptor-mediated endocytosis systems for the transport of some

essential vitamins has been attempted with some success. However, these latter systems have low levels of expression and, therefore, low capacities (Yang *et al.*, 2001).

Can Prodrugs Be Used To Overcome The Limited Permeation Of Drugs That Are Efflux Candidates?

One can make a case for efflux candidacy being a major cause for the poor permeation characteristics of many new drug candidates. Lipinski's guidelines (Lipinski *et al.*, 1997, 2000; Horspool and Lipinski, 2003) related to size, lipophilicity and numbers of hydrogen-bond acceptors and donors and the observations of Veber *et al.* (2002) on rotatable bonds in many new molecules speak to this issue to some extent. The relatively promiscuous nature of many efflux systems makes them particularly difficult to engineer around. Could one design a prodrug that is not a substrate? Yes, but what are the rules? If a drug is a substrate for an apical efflux pump, one could in theory design a prodrug with sufficiently different properties and structural elements for the prodrug to be absorbed and then converted to the drug *in vivo*. The prodrug could be a poorer efflux substrate and/or perhaps because of adequate solubility and dissolution saturate the efflux transporter.

There no obvious examples in the literature, to our knowledge, in which prodrugs of efflux transporter permeation-limited molecules have been proven to be successful.

Why Do Prodrug Strategies For Some Drugs Fail? Can Efflux Transporters And/Or Metabolic Enzymes Be Used To Explain Some Failures With Oral Prodrugs?

When researchers meet with limited success using a prodrug approach in an attempt to deliver a polar drug candidate, a great deal of skepticism within their organization often results. Being "burned" never makes one want to try a second time. In part, prodrug failures often result from incomplete knowledge of the total cause of the poor performance of the parent drug in the first place. While the parent drug may have had a low log P value and, therefore, was expected to have limited permeation, the drug may also have suffered from other shortcomings. Its poor permeation may have had an efflux component, the molecule may have been subject to presystemic metabolism, or the drug may have undesirable pharmacokinetic properties such as being rapidly sequestered by the liver and rapidly biliary excreted, resulting in rapid and efficient clearance. That is, permeation was not the only problem, and the wrong candidate or strategy was chosen for prodrug intervention. As discussed in Chapter 1, it takes a "team" to develop a good prodrug program. That team may not exist in some environments, and so the causality may not be properly identified.

Today we are better able to identify the etiology of the poor performance of drug candidates and, therefore, decide logically if prodrug intervention has a

reasonable probability for success. A drug with poor intrinsic pharmacokinetic properties will most often not benefit from a prodrug strategy.

Assuming that the poor delivery of the parent drug was in fact due its high polarity, a prodrug strategy could still fail. The vast majority of the failures are probably due to a poor choice of prodrug strategy, premature metabolism of the prodrug in the contents of the GIT prior to reaching the absorptive surface, or the fact that the prodrug strategy resulted in incomplete conversion of the prodrug to drug on reaching systemic circulation.

It is our belief that additional considerations have been overlooked. Consider the example discussed earlier, namely, the conversion of linear peptides to cyclic prodrugs. The prodrugs were great substrates for apical efflux systems. Ten years ago, this would not have been routinely recognized. So, while the prodrugs had higher log P values, smaller radii, slower metabolism etc., they continued to poorly permeate biomembranes but for a different reason. Therefore, as one looks back on many failed examples in the literature, a case could be made for two major etiologies; the first and most obvious of these is that the prodrugs were prematurely cleaved in the contents of the GIT. The second is that the prodrugs were efflux candidates. While it is hard to document what fraction of failures were due to this latter cause (one does not always publish failures), it could account for many of the anomalies.

What Are The Unmet Needs?

Prodrug research is viewed by many as simply the application of old and tried techniques to newly identified problematic drugs. Rarely does one see truly innovative ideas being put forward. This is because many organizations prefer to utilize chemistries with known biological and toxicological endpoints that have historical precedence. Few major organizations can muster the support for new creative prodrug studies in either chemistry groups or groups needed for their biological evaluation. That appears to be changing as smaller "biotech" companies become involved and the oral delivery of the newer generation of drug candidates in major pharmaceutical companies continues to be problematic. Clearly, research is needed to assess whether the oral delivery of drugs poorly permeable due to efflux candidacy can be positively affected by prodrug intervention.

For those drugs containing one or more low pK_a carboxylic acid or phosphonic acid groups, could their delivery be better if one were not to rely solely on ester type prodrugs where luminal and enterocyte cleavage can lead to incomplete parent drug delivery? Ditto for polar basic drugs. The answer is probably yes. Let's be creative.

Could our understanding at the molecular level of the structural requirements for carrier-mediated transporters help us design better substrates for these carriers either in the form of new drugs or prodrugs? Clearly, the answer is yes. Companies such as Xenoport in California are attempting this approach. However, it would seem that the application of this technology would, by nature, be more limited because of structural constraints.

One could argue that as an industry we have been drawn to molecules that are likely to be absorbable while avoiding those with structural elements that lead to high polarity. Therefore, have we missed a chemical space because of delivery concerns when those concerns could be overcome through the use of prodrugs?

Conclusion

Successful examples of prodrug intervention to improve the oral availability of polar drugs have been well documented. One needs only to look at many illustrated in this chapter and the commercial success of drugs like valacyclovir. However, despite its many successes, the technique is still met with skepticism (it is more work to develop a prodrug than an analog) in many organizations. There are still unmet needs that require addressing, and this will take some creativity by scientists venturing into this area of research. We hope that the selected examples shown here and the references provided will encourage future researchers to venture on.

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2.1.2

Topical Delivery Using Prodrugs

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Table of Contents

List of Abbreviations

C_v .....concentration in vehicle
IPM .....isopropyl myristate
J .....flux
J_{AQ} .....flux from water
J_{IPM} .....flux from IPM
J_{MO}.....flux from mineral oil
$J_{4.0}$.....flux from pH 4.0 buffer
$J_{5.0}$.....flux from pH 5.0 buffer
$J_{7.0}$.....flux from pH 7.0 buffer
$J_{7.4}$.....flux from pH 7.4 buffer
K .....partition coefficient
$K_{IPM:4.0}$.....partition coefficient between IPM and pH 4.0 buffer
$K_{OCT:AQ}$.....partition coefficient between octanol and water
$K_{OCT:4.0}$ .....partition coefficient between octanol and pH 4.0 buffer
$K_{OCT:5.0}$ .....partition coefficient between octanol and pH 5.0 buffer
$K_{OCT:7.4}$ .....partition coefficient between octanol and pH 7.4 buffer
MO.....mineral oil

MW.....molecular weight
P.....permeability coefficient
PG.....propylene glycol
SC.....stratum corneum
S.....solubility
S_{AQ}.....solubility in water
S_{IPM}.....solubility in isopropyl myristate
S_{LIPID}.....solubility in lipid
S_{MO}.....solubility in mineral oil
S_{OCT}.....solubility in octanol
$eS_{4.0}$.....estimated solubility in pH 4.0 buffer
$S_{4.0}$.....solubility in pH 4.0 buffer
$S_{5.0}$.....solubility in pH 5.0 buffer
$S_{7.0}$.....solubility in pH 7.0 buffer
$S_{7.4}$.....solubility in pH 7.4 buffer
AA.....dialkylaminoalkyl
AAC.....alkylaminocarbonyl
AACOA.....dialkylaminoalkylcarbonyloxymethyl
AAM.....dialkylaminomethyl
AC.....alkylcarbonyl
ACAA.....alkylcarbonylaminoalkyl
ACOM.....alkylcarbonyloxymethyl
AOC.....alkyloxycarbonyl
AOCAM.....alkyloxycarbonylaminomethyl
AOCOM.....alkyloxycarbonyloxymethyl
DAAC.....dialkylaminoalkylcarbonyl
DOHOC.....dihydroxylalkyloxycarbonyl
POE.....polyoxyethylene
APAP.....acetaminophen
5-FU.....5-fluorouracil
MOR.....morphine
6-MP.....6-mercaptopurine
NSAID.....non-steroidal anti-inflammatory drug
NTX.....naltrexone
PL.....propranolol
Th.....theophylline

Introduction

The objective of any prodrug approach is to transiently mask a particular functional group in a drug and thus change its physicochemical properties (solubility profile, degree of ionization), protect it from premature chemical or biological metabolism, or facilitate its active or passive transport across a biological barrier, to name only a few of the more obvious applications. In the example of the use of a prodrug approach to enhance topical delivery, a change in the physicochemical properties of the drug is the objective. That change is designed to facilitate the passive absorption across the stratum corneum (SC)—wherein lies the major barrier to permeation—into the viable epidermis and beyond, if necessary. More complete descriptions of the skin can be found in other references (Barry, 1983). Here, we use the term "topical delivery" to include dermal delivery (delivery into the skin) and transdermal delivery (delivery through the skin into systemic circulation).

Although the possible applications of prodrug technology to solve topical delivery problems are quite broad in scope, the commercial use of prodrugs to enhance topical delivery has been limited. Topical formulations of 21-esters of glucocorticoid steroids comprise the best known example. For example, the 21-esters of betamethasone are well established as functioning as prodrugs because they bind less tightly to the glucocorticoid receptor (Ponec *et al.*, 1986) yet are up to 150 times more effective in the vasoconstriction assay than betamethasone (McKenzie and Atkinson, 1964). One possible reason for the lack of other commercial successes is that for some time it was believed that for successful enhancing of topical delivery with a prodrug approach it was necessary only that the prodrug permeant exhibit increased lipophilicity compared to the parent drug and, of course, revert to the parent drug *in vivo*. Numerous models and their corresponding equations are available for predicting flux (J, the amount of material permeating the skin per unit area and time) or normalized J (J/(concentration in vehicle, C_v) = permeability coefficient, P) from direct relationships with lipid solubility (S_{LIPID}) or its surrogate partition coefficient ($K_{LIPID:V}$), and from molecular volume or weight (MW) of the permeant. A well-known example of this sort of relationship is found in the Potts-Guy (1992) equation: $\log P = -6.3 + 0.71 \log K_{OCT:AQ} - 0.0061$ MW, where $K_{OCT:AQ}$ is the partition coefficient between octanol and water. Any statements about the importance of water solubility were in reference to the solubility of the permeant in the standard water vehicle and not in a water phase of the barrier to permeation.

However, even a cursory examination of the literature on the topical delivery of homologous series of molecules shows that water solubility of the permeant is important as well, regardless of whether the vehicle is water or a lipid. As an example of a non-prodrug homologous series, early data from Scheuplein and Blank (1973) showed that fluxes of a homologous series of aliphatic alcohols through human skin *in vitro* from the application of the pure alcohols were greatest for the smaller, more water-soluble members of the series and that normalized fluxes, P, were also greatest for the more water-soluble members. The rank order of these results was confirmed later in *in vitro* diffusion cell experiments

using hairless mice (Sloan *et al.*, 1997). From the application of the alcohols in water, fluxes from saturated solutions were again greatest for the smaller, more water-soluble members. Only the values for P from water increased as the alkyl chain length increased and the alcohols become more lipophilic. In this regard, it is important to remember that P has units of distance per time and no units of amount permeated as found in J. Thus, J is the clinically relevant measurement, not P.

Although it was noted earlier (Sloan *et al.*, 1984) that water solubility was an important criterion in designing prodrugs to optimize topical delivery, the first complete series of homologous prodrugs exhibiting a relatively wide range of solubilities to be characterized and analyzed for the existence of such relationships were the two series of alkylcarbonyloxymethyl (ACOM) derivatives of 6-mercaptopurine (6-MP) (Waranis and Sloan, 1987, 1988). In both the mono- and bisalkylated series, although a balance between lipid and aqueous solubilities was obviously important, the more water-soluble members of each series and not the more lipid-soluble members gave the greatest delivery of 6-MP through hairless mouse skin *in vitro* from saturated isopropyl myristate (IPM) vehicles. In those experiments there was no stagnant water layer at the interface between the vehicle and the skin upon which to blame the poor performance of the more lipophilic members since a lipid, IPM, in which the prodrugs were reasonably soluble, and not water was the vehicle. Also, since these more lipophilic prodrugs were almost completely hydrolyzed to the much more water-soluble parent drug during their permeation, the aqueous dermis was not functioning as a rate-limiting barrier to their permeation. Moreover, the same rank order of the flux values among the members of the bisalkyl prodrugs series was obtained when they were applied in water or propylene glycol (PG) vehicles; as predicted, the more water-soluble members and not the more lipid-soluble members gave the greatest flux values regardless of the vehicle used (Sloan *et al.*, 1984). Finally, it should be noted that values for P for delivery from the IPM vehicle decreased while P for delivery from water increased as the alkyl chain length increased and the prodrugs became more lipophilic. Thus, trends in P for prodrugs as well as those for non-prodrug alcohols are an artifact of their calculation.

The structure of the barrier to topical delivery, the stratum corneum (SC), is the reason that increased aqueous as well as increased lipid solubilities are important physicochemical objectives to meet if optimization of topical delivery is to be realized. It is generally agreed that diffusion through this barrier takes place through the intercellular compartment in which the cells of the SC—the corneocytes—are embedded (Hadgraft and Pugh, 1998). The intercellular compartment consists of lamellar double bilayers. The lipid component of the bilayer comprises ceramides, cholesterol, and fatty acids, in order of abundance. The polar head groups of these lipid components have a water layer associated with them. Thus, a permeant has to pass across alternating lipid and aqueous phases to diffuse through the intercellular barrier, and a balance of solubility in both phases should be essential for optimization of permeation (Sloan *et al.*, 1984). In addition, due to lateral hydrophobic mismatches among the lipid components

of the bilayer, domain mosaics or lipid microdomains can form (Lehtonen *et al.*, 1996). Subsequently, this can lead to lipid-poor (water phases) and lipid-rich phases within the bilayer (van Hal *et al.*, 1996). These water phases can appear as hydrophilic, electron-rich fenestrations that bridge the broad hydrophobic lipid layers between adjacent hydrophilic bands upon ruthenium tetroxide fixing of normal skin (Hou *et al.*, 1991). Thus, these aqueous defects in the intramembrane transbilayer barrier to permeation can lead to shortcuts through the mostly lipoidal-in-nature barrier that would best accommodate permeants exhibiting better aqueous solubility as well as good lipid solubility.

Just as the structure of the stratum corneum is responsible for the need to increase aqueous as well as lipid solubilities in the design of prodrugs to optimize topical delivery, so too is the structure of the parent drug responsible for the need to design a prodrug in the first place. In many drug molecules there are functional groups of the form drug-X-Y-H where Y is O, N or S, and X frequently contains a carbonyl group directly attached to Y. The electron-withdrawing effect of the carbonyl group causes the Y-H bond to further polarize so that it forms strong intermolecular hydrogen bonds within the crystal lattice and causes drug-X-Y-H to exhibit high melting points and low general solubilities. Examples of this type of drug molecule are heterocycles such as 5-fluorouracil (5-FU, mp 284°C), 6-mercaptopurine (6-MP, mp 320°C), theophylline (Th, mp 274°C) (O=C-NH and S=C-NH) and phenols such as narcotic analgetics: morphine (MOR, mp 256°C) (Aryl-OH). In other examples, the functional group is so highly polarized that it becomes significantly ionized at physiological pH, and ionized molecules do not permeate the lipid phase of the alternating lipid and aqueous phase barrier well. Examples of this type of highly ionized drug molecule are ones that contain carboxylic acid and phosphate functional groups such as in non-steroidal anti-inflammatory drugs and nucleotide drugs, respectively. Another example of a highly ionized functional group that is found in drug molecules is the basic amine group. However, drug molecules that contain basic amine functional groups may actually permeate skin better than expected, and basic amine groups incorporated into a prodrug may enhance its skin permeation (Sloan *et al.*, 1984).

For both types of these highly polar and relatively lipid insoluble parent drugs, increased lipid solubility remains as the primary design focus for improving topical delivery. But, since most of these molecules are not very soluble in water as well, the optimum members of any series of prodrugs will again be those that are also more water soluble (Sloan, 1989, 1992). Fortunately, masking one of these highly polar functional groups usually leads to substantial increases in lipid solubilities and one or more members that are more water soluble as well. On the other hand, if the parent is already highly lipophilic, then increased aqueous solubility, S_{AQ}, becomes the primary design focus. Regardless of the solubility of the parent drug, the effect of increasing both lipid and aqueous solubilities of the prodrug can be dramatic. For example, 5-FU is 1700 times less soluble in IPM (S_{IPM}) than in pH 4.0 buffer ($S_{4.0}$) (Table 1). By increasing S_{IPM} and $S_{4.0}$ by 450 and 1.4 times, respectively, using the 1-acetyl-5-FU prodrug (**47**), the flux of 5-FU from an IPM vehicle through hairless mouse skin *in vitro* (J_{IPM}) was increased by 39-fold.

Compounds[a]	mp[b]	MW	S_{IPM}[c]	$S_{4.0}$[c]	log K	J_{IPM}[d]	$eS_{4.0}$[c]
7-ACOM-Th							
1, C1	165	252	2.75	12.4	–0.85	0.58	19.5
2, C2	147	266	2.93	5.00	–0.20	0.31	4.62
3, C3	105	280	25.4	9.96	0.38	1.06	10.5
4, C4	87	294	44.0	4.29	0.93	0.59	5.22
5, C5	60	308	77.8	1.72	1.45	0.47	2.77
6, $C(CH_3)_3$	110	294	37.1	--	0.75	--	6.55
1-ACOM-5-FU							
7, C1	124	202	3.29	204	–1.74	2.88	183
8, C2	102	216	9.83	151	–1.23	3.82	167
9, C3	91	230	14.4	52.3	–0.47	2.57	42.4
10, C4	88	244	14.8	14.3	0.08	1.29	12.3
11, C5	91	258	14.7	3.37	0.82	0.56	2.23
12, C7	108	286	9.99	0.16	1.77	0.12	0.17
13, C9	115	314	4.28	0.0031	--	0.015	--
14, $C(CH_3)_3$	165	244	7.78	9.95	–0.01	0.30	7.85
3-ACOM-5-FU[e]							
15, C1	161	202	1.22	63.1	–1.62	0.60	51.3
16, C2	96	216	15.9	178	–1.03	2.18	171
17, C3	76	230	26.4	85.1	–0.45	2.87	74.1
18, C4	94	244	29.8	20.9	0.13	1.32	21.9
19, C5	71	258	42.8	8.32	0.74	1.01	7.74
20, C7	70	286	40.2	0.56	1.90	0.17	0.51
1-AAC-5-FU							
35, C1	212	187	0.30	3.48	–1.09	0.208	3.69
36, C2	180	201	2.79	7.71	–0.44	0.600	7.76
37, C3	139	215	12.4	9.44	0.14	0.746	8.98
38, C4	133	229	24.6	4.80	0.68	0.515	5.11
39, C6	113	257	44.9	0.36	2.09	--	0.36
40, C8	91	285	46.9	0.030	3.21	0.060	0.030

Table 1. Melting points (mp), molecular weights (MW), solubilities in IPM (S_{IPM}), solubilities in pH 4.0 buffer ($S_{4.0}$), log partition coefficients between IPM and pH 4.0 buffer (log K), fluxes from IPM suspensions through hairless mouse skin in vitro (J_{IPM}), solubilities in pH 4.0 buffer estimated from S_{IPM}/K ($eS_{4.0}$).

Compounds[a]	mp[b]	MW	S_{IPM}[c]	$S_{4.0}$[c]	log K	J_{IPM}[d]	$eS_{4.0}$[c]
1-AOC-5-FU							
41, C1	160	188	2.13	120	–1.72	2.62	112
42, C2	128	202	13.1	263	–1.12	5.92	175
43, C3	126	216	15.2	55.4	–0.44	2.31	42.2
44, C4	98	230	33.8	29.2	0.15	2.23	24.1
45, C6	67	258	153	5.38	1.49	1.54	4.94
46, C8	98	286	36.2	0.13	2.45	0.29	0.13
1-AC-5-FU							
47, C1	130	172	22.1	--	–0.73	9.3	120
48, C2	131	186	36.4	--	–0.12	4.3	47.6
49, C3	146	200	17.4	--	0.43	1.3	6.50
50, C4	121	214	39.2	--	1.05	1.0	3.48
51, C5	102	228	112.7	--	1.58	1.1	2.94
52, C7	84	256	110.7	--	2.88	0.60	0.15
3-AC-5-FU							
53, C1	117	172	4.3	166	–1.39	4.4	105
54, C2	103	186	14.0	198	–0.97	5.2	135
55, C3	112	200	22.0	53	–0.01	2.2	23.0
56, C4	111	214	9.2	5.0	0.22	0.55	5.50
bis-1,3-AC-5-FU							
57, C1	113	214	26.0	--	0.32	2.2	12.4
58, C2	101	242	72.0	--	1.35	0.69	3.2
6-ACOM-6-MP [e,f]							
59, C1	194.5	224	1.05	7.17	–0.83	0.203	--
60, C2	165	238	2.30	4.08	–0.25	0.214	--
61, C3	154	252	3.29	2.04	0.21	0.262	--
62, C4	148.5	266	4.21	0.79	0.73	0.220	--
63, C5	151	280	3.68	0.24	1.19	0.055	--
64, C7	148	308	4.14	0.024	2.24	0.013	--

Table 1 (continued).

Compounds[a]	mp[b]	MW	S_{IPM}[c]	$S_{4.0}$[c]	log K	J_{IPM}[d]	$eS_{4.0}$[c]
bis-6,9-ACOM-6-MP[e,f]							
65, C1	122.5	296	5.27	2.88	0.26	0.227	--
66, C2	77	324	33.6	1.67	1.30	0.232	--
67, C3	69.5	352	90.9	0.20	2.66	0.141	--
68, C4	57.5	380	174	0.047	3.57	0.102	--
69, C5	72	408	49.8	0.0011	4.67	0.012	--
4-AOC-APAP[e]							
82, C1	115	209	11.9	20.6	–0.16	0.998	17.1
83, C2	122	223	9.29	3.78	0.32	0.174	4.50
84, C3	106	237	23.7	2.67	0.90	0.355	3.00
85, C4	120	251	13.9	0.42	1.50	0.097	0.44
86, C6	110	279	16.6	0.047	2.71	0.032	0.033
87, MOC2[g]	81	253	10.3	34.4	–0.30	0.776	20.6
88, MOC3i[h]	123	267	3.38	3.28	0.13	0.087	2.43
Parent Drugs							
Th[e,f]	274	180	0.34	46.1	–2.13	0.479	--
5-FU[f]	284	130	0.049	85.4	–3.24	0.240	--
6-MP[e,f]	320	152	0.022	1.12	–1.71	0.0038	--
APAP[e,f]	170	151	1.90	100.1	–1.72	0.509	--

[a]C1, C2, etc refer to the number of carbons in alkyl chain. [b]Units of [o]C. [c]Units of mM. [d]Units of μmol cm^{-2} h^{-1}. [e]Solubilities in water (S_{AQ}) not pH 4.0 buffer ($S_{4.0}$). [f]Log.solubility ratio between water (S_{AQ} or $S_{4.0}$) and IPM ($SR_{IPM:AQ}$ or $SR_{IPM:4.0}$) not log K. [g] $CH_3OCH_2CH_2$- [h]CH_3OCH_2 $CH(CH_3)$-.

Instead of delivering 0.03 mg cm^{-2} h^{-1} of 5-FU, the prodrug delivered 1.2 mg cm^{-2} h^{-1}. This degree of improvement would be more than sufficient to allow the use of topical 5-FU to treat psoriasis (Tsuji and Sugai, 1972) instead of only actinic keratoses of more permeable areas of the body such as the scalp (Dillaha *et al.*, 1965).

Many different types of functional groups can be used successfully to mask drug-X-Y-H in the parent drug and create various types of promoieties. The combination of masking functional group and drug-X-Y-H is referred to as an enabling functional group because it enables the prodrug to revert to the parent drug. H is replaced by the masking functional group to which is attached alkyl chains of varying length to create a homologous series within a type of promoiety.

A reasonably complete list of possible promoieties is given elsewhere (Sloan, 1992). They are generally of two types: acyl and soft alkyl promoieties. These two types of promoieties and mechanisms whereby they release the parent drugs have been discussed in detail elsewhere (Sloan, 1989; 1992; Sloan and Wasdo, 2003), but almost universally a carbonyl group is the target for a nucleophilic attack that either releases the parent drug immediately (acyl) or after the intermediate release of a methylene=Y (e.g., $H_2C=O$) (soft alkyl).

There does not seem to be any optimum enabling functional group that imparts maximum increases in lipid and aqueous solubilities and, hence, optimized increased flux, regardless of the parent drug. Generally, the largest differences in increased flux values are realized within the members of the homologous series and not from series to series. In other words, for homologous series the length of the alkyl chain group and not the enabling functional group in the promoieties, with a few exceptions, determines how effective the prodrug is at enhancing topical delivery; and the shorter chain, more water-soluble members are the most effective. However, several examples are given below where promoieties containing basic amino or dihydroxyalkyl groups are especially effective, probably because of their unique abilities to increase S_{AQ} without decreasing S_{LIPID}.

Model Development

In order to reflect the obvious dependence of flux on aqueous as well as lipid solubility properties of the intercellular barrier, Roberts and Sloan (1999) developed a transformation of the Potts-Guy equation (Roberts-Sloan equation) which could accommodate lipid vehicles and which contained parameters for solubilities in IPM ($\log S_{IPM}$) and pH 4.0 buffer ($\log S_{AQ}$): $\log J = x + y \log S_{IPM} + (1 - y) \log S_{AQ} - z\,MW$. When this equation was fit to data from seven series of homologous prodrugs of 6-MP, 5-FU and Th (**1–14**, **35–52** and **59–69**; see Table 1), a reasonably good fit was obtained where x, y, z and r^2 were –0.211, 0.534, 0.00364 and 0.937, respectively, for n = 42. Thus, aqueous solubility ($1 - y \log S_{AQ} = 0.464 \log S_{AQ}$) was almost as important as lipid solubility in *in vitro* diffusion cell experiments using hairless mice skin with IPM as the vehicle. The same equation was subsequently fit (Roberts and Sloan, 2001) to data obtained from the application of ten unrelated non-steroidal anti-inflammatory drugs in mineral oil to the skin of human volunteers (Wenkers and Lippold, 1999); x, y, z and r^2 were –1.459, 0.722, 0.00013 and 0.934, respectively. The dependence on aqueous solubility was not nearly as great for flux through human skin *in vivo* as for flux through hairless mouse skin *in vitro*; this was expected for the thicker and less highly hydrated human skin *in vivo*, but the dependence was still significant and substantial ($1 - y \log S_{AQ} = 0.278 \log S_{AQ}$). These latter results showed that, although the barrier to permeation is predominantly lipophilic, hydrophilic contributions to the barrier are important and aqueous solubility needs to be included as a design consideration, especially for optimization of a particular prodrug approach.

Although the Roberts-Sloan equation is a logical extension of the Potts-Guy equation, conceptually it marks a dramatic departure from previous models for permeation that assumed that the barrier to permeation could be modeled by a lipid only. In the Roberts-Sloan model, the barrier to permeation contains aqueous as well as lipid phases, which makes it necessary to design a balance between solubilities in those two phases into the characteristics of the prodrug in order to optimize delivery of the parent drug across such a heterogeneous barrier. However, the concept that the barrier itself comprises aqueous as well as lipid microbarriers is not inconsistent with the lamellar double bilayer structure of the intercellular matrix in the stratum corneum, as described earlier.

To better define permeation through the heterogeneous barrier, a model was developed that allowed for the existence of three parallel paths for permeation of the stratum corneum: a lipid-only, an aqueous-only, and an alternating lipid and aqueous (series) path (Roberts and Sloan, 2000). In this series/parallel model, the permeation through any one path or combination of paths depended on the solubilities of the solute in each phase that comprised the path and the diffusivities of the solute in each phase as well. The solubilities were considered to be analogous to conductivities in electric circuits such that the greater the solubility of a solute in the phase(s) that constituted a path, the greater the carrying capacity of that path and the greater the flux through that path. Similar to electric circuits, the equation for flux that resulted gave:

$$J = 1/(1/a\ S_{LIPID}\ D_{LIPID} + 1/b\ S_{AQ}\ D_{AQ}) + c\ S_{LIPID}\ D_{LIPID} + d\ S_{AQ}\ D_{AQ}$$

where the first term on the right hand side of the equation represents flux through the lipid-aqueous series path, followed by the term for flux through the lipid-only path and, subsequently, the term for flux through the aqueous-only path. The coefficients a, b, c and d are the areas divided by the thicknesses of each phase or path. Substituting S_{IPM} for S_{LIPID} and assuming that diffusivity in the aqueous phase, (D_{AQ}) is inversely proportional to $MW^{1/2}$ while diffusivity in the lipid phase (D_{LIPID}) is proportional to the product of some negative parameter, Ø, and MW gave the complete equation for the series/parallel model.

$$\log J = \log \{1/[1/(a\ S_{IPM}\ 10^{ØMW}) + 1/(b\ S_{AQ}/MW^{1/2})] + c\ S_{AQ}\ 10^{ØMW} + d\ S_{AQ}/MW^{1/2}\}$$

Using the same database as had been used for the Roberts-Sloan model, regression of the data on the equation for the series/parallel model showed that the aqueous-only path was not necessary to explain the results and that the alternating lipid and aqueous (series) path was the high capacity path for permeation of the skin. The lipid-only path became a major contribution to permeation only for the more lipophilic permeants, but it was of low capacity. This result explains why increasing only lipophilicity generally results in only modest increases in flux, but the greatest increases in flux are obtained from the shorter alkyl chain, more water-soluble members of almost any series.

Application to Functional Groups

There are four functional groups found in drug molecules for which the greatest number of promoieties have been designed, synthesized, characterized (solubility, stability, purity), and evaluated in diffusion cell experiments. These four functional groups are the amide or imide (O=C-NH), the thioamide (S=C-NH), the hydroxy group (OH) substituted on aliphatic or aromatic carbons, and the carboxylic acid (O=C-OH) mentioned above. For each functional group there are two general types of promoieties possible; these are the acyl and soft alkyl types also mentioned above and described in detail elsewhere (Sloan, 1989, 1992, Sloan and Wasdo, 2003). Since a recent review of various combinations of functional group and promoiety is available, we will limit our discussion to those prodrugs that have played a role in developing general synthetic approaches to modifying the physicochemical properties of the parent drug or that contribute to databases (Table 1) from which models for topical permeation have been derived that form the basis for the design of better prodrugs.

Drug (O=C)-NH

Parent drugs that contain an amide- or imide-like functional group provide good opportunities for a prodrug approach to significantly change their physicochemical properties by masking the polarized N-H group. These types of parent drugs are generally characterized as exhibiting high melting points and poor lipid and aqueous solubilities. Thus, masking the polarized N-H group by replacing the H with a non-polarized promoiety can dramatically increase the lipid and aqueous solubilities and, hence, potentially increase the topical delivery of the parent drug.

Alkylcarbonyloxymethyl (ACOM)

The first report of the use of ACOM prodrugs to enhance the topical delivery of a parent drug containing an amide- or imide-like functional group was for 7-ACOM derivatives to deliver theophylline (Th) (Bodor and Sloan, 1977). The evaluation of only two members of the complete series (**3** and **6**) was reported initially (Sloan and Bodor, 1982). The characterization and evaluation of the complete series (**1** to **6**) was reported later (Table 1) (Kerr *et al.*, 1998). The results were typical of what has been observed for most other ACOM derivatives of heterocyclic parent drugs containing amide- or imide-like functional group. All of the prodrugs were much more lipid soluble (increased S_{IPM}) than Th, varying from 8 times for **1** to 75 times for **3** to 230 times for **5**. Although in many series at least one member was more soluble in pH 4.0 buffer (increased $S_{4.0}$) than the parent, in this series the best $S_{4.0}$ values were 0.27 and 0.22 times that of Th for **1** and **3**, respectively. The trend in solubilities followed the trend in melting points (mp): **2** exhibited a higher than expected mp and lower than expected S_{IPM} and $S_{4.0}$. **3**, which exhibited almost the same $S_{4.0}$ value as **1** (and over twice the $S_{4.0}$ values of

the other members) and a S_{IPM} value 9 times that of **1**, gave the best flux value from IPM as would be predicted (Sloan, 1989, 1992); **3** exhibited the best balance of $S_{4.0}$ and S_{IPM}. **4** and **5**, which exhibited the greatest S_{IPM} values, did not give the greatest fluxes. The percent of intact prodrug measured in the receptor phases from the diffusion cell experiments varied from 47% for **1** to 7% for **4** and 2% for **5** (data not shown). This result was also typical for ACOM derivatives. The longer chain members of the series underwent more complete conversion to the parent drug even if the total amount permeated was about the same as in **1** versus **4**.

The initial report of 7-ACOM-Th derivatives (Sloan and Bodor, 1982) also described the synthesis of ACOM derivatives that contained other types of functional groups substituted on or into the alkyl chain that were designed to further enhance topical delivery or enhance stability. Examples in which a dialkylamino group or a dialkylaminocarbonyl group were substituted on the alkyl chain were described. Both substituents were designed to increase both lipid and aqueous solubility, but were never evaluated in diffusion cell experiments. Another example where an oxygen was substituted into the alkyl chain to give an alkyloxycarbonyoxymethyl derivative (AOCOM) was described, but was never evaluated. Because the ACOM derivatives of the O=C-NH functional group are not always effective at delivering the parent drug through skin, evaluation of an even more stable carbonate analogue (AOCOM) is probably not warranted unless transdermal sustained systemic delivery is desired.

The second report of the use of ACOM type promoieties to deliver a parent drug containing an amide- or imide-like functional group topically was for 1-ACOM to deliver 5-FU (Mollgaard *et al.*, 1982). Again, only two members (**9** and **14**) were evaluated initially, and propylene glycol (PG) was used as vehicle instead of IPM. The characterization and evaluation of the complete series (**7** to **14**) was published later (Table 1) (Taylor and Sloan, 1998). Although all of the prodrugs were more lipid soluble than 5-FU, because 5-FU was much less lipid soluble than Th (0.14 times S_{IPM}) the increases in S_{IPM} values were relatively much greater for the ACOM derivatives of 5-FU than for Th, especially for the first few members of the series: 70-, 200- and 300 times that of 5-FU for **7**, **8** and **9**, respectively. However, among these, only the C2 member **8** was substantially more lipid soluble than the corresponding C2 member **2** of the 7-ACOM-Th. On the other hand, the aqueous solubility ($S_{4.0}$) of 5-FU was about twice that of Th and the C1 (**7**) and C2 (**8**) members were 2.4 and 1.8 times, respectively, more soluble in water than 5-FU. So the 1-ACOM-5-FU members were much more water soluble than the corresponding 7-ACOM-Th derivatives: 16 times for **7**, 30 times for **8**, and 5 times for **9** compared to **1**, **2** and **3**, respectively. The balance between increased $S_{4.0}$ and S_{IPM} values was better for the 5-FU ACOM derivatives. The result was that the flux values for the 5-FU derivatives from IPM were also greater than those for the Th derivatives: 5 times for **7**, 12 times for **8**, and 2.5 times for **9** compared to **1**, **2** and **3**, respectively. Within the 1-ACOM-5-FU series, **8** exhibited the best balance between $S_{4.0}$ and S_{IPM} values (1.8 times greater $S_{4.0}$ and 200 times greater S_{IPM} than 5-FU) and, hence, exhibited the greatest increase in flux values compared to 5 FU, as would be predicted (Sloan, 1989, 1992), 16 times. The percent of intact

prodrug measured in the receptor phase from diffusion cell experiments varied from 55% for C1 (**7**), 50% for C2 (**8**), and 29% for C4 (**10**) to 12% for C7 (**12**). Thus, the most effective prodrugs were delivering only about 50% of the parent drug.

The 3-ACOM-5-FU prodrugs (**15** to **20**) were designed (Roberts and Sloan, 2003) not only to be more soluble in lipids and water than 5-FU but also to deliver more 5-FU topically than the 1-ACOM prodrugs; this was based on reports that the C1 member of the 3-ACOM series, **15**, was 5 times more labile enzymatically than the corresponding 1-ACOM member, **7**. Complete characterization of the members of the 3-ACOM series (Table 1) found that they were also more lipid soluble and water soluble than the members of the 1-ACOM series except for the C1 member (**15** versus **7**). Based on those solubilities, the results that the C1 member of the 3-ACOM series gave a much lower flux value from IPM than did the C1 member of the 1-ACOM series and that the C3 to C7 members of the 3-ACOM series gave higher flux values than the corresponding member of the 1-ACOM series were expected (Sloan, 1989, 1992). However, the result that the C2 member of the 3-ACOM series (**16**), which was more soluble in lipid and water than the C2 member of the 1-ACOM series (**8**), gave only 0.60 times the flux of **8** was unexpected and could not be explained. It could have been argued that **16** also exhibited the best balance of lipid and aqueous solubilities in the 3-ACOM series, yet **17**, which was almost twice as soluble in IPM as **16** but only 0.50 times as soluble in water, gave the greatest flux value for the series. As expected, the 3-ACOM series was more effective at delivering more parent drug (5-FU) than the 1-ACOM series. Intact prodrug varied from 20% for C1 (**15**), 31% for C2 (**16**), 22% for C3 (**17**) and about 6% for **18** through **20**. Thus, the 3-ACOM-5-FU series, except for **16**, behaved as expected based on their measured solubilities and reported hydrolyses.

Since Th itself is 7 times more soluble in IPM and only half as soluble in water as 5-FU, Th gave about twice the flux as 5-FU from IPM. On the other hand, although S_{IPM} values for the 7-ACOM-Th prodrugs were comparable to those of the corresponding ACOM-5-FU prodrugs, their $S_{4.0}$ values were much lower. The balance between S_{IPM} and $S_{4.0}$, which was better for Th, was worse for its ACOM prodrugs compared to those for 5-FU. Thus, it would be predicted (Sloan, 1989, 1992) that not only would the increase in flux for the 7-ACOM-Th derivatives compared to Th be less than the increase in flux for the ACOM-5-FU derivatives compared to 5-FU, but the absolute values for the 7-ACOM-Th derivatives would be much less than for the ACOM-5-FU derivatives; their absolute $S_{4.0}$ values were less.

Dialkylaminomethyl (AAM)

The dialkylaminomethyl (AAM) or N-Mannich base derivatives represent another type of soft alkyl derivative which is complementary to the ACOM derivatives. While the ACOM derivatives generally undergo enzymatic hydrolysis to the parent drug, the AAM derivatives are chemically labile; the mechanism for

their hydrolysis is described in detail elsewhere (Sloan, 1992). Generally, the AAM derivatives are more soluble in lipids than the parent drug because the promoiety masks the highly polarized O=C-NH group. The facile chemical hydrolysis of the AAM derivatives precludes a realistic, accurate measurement of their aqueous solubilities. However, aqueous solubilities for some N-Mannich bases have been reported under acidic conditions where they are more stable (Johansen and Bundgaard, 1980). Based on those results, which show that the N-Mannich bases are much more soluble in water than the parent amide or imide, it was anticipated that the AAM derivatives would also generally be more soluble in water at physiological pH. Although N-Mannich bases have the potential to increase both aqueous and lipid solubility and hence flux (especially from a nonprotic vehicle such as IPM in which they would be stable), they are not sufficiently stable (even in IPM) to give a reasonable shelf-life and, upon hydrolysis, they liberate a secondary amine that may be irritating to the skin. Thus, their commercial use may be limited to applications where the functional group to be masked is a higher pKa amide for which the promoiety would be more stable and where the secondary amine is a proline derivative which, as an amino acid, should be less toxic.

N-Mannich base derivatives of Th (Sloan *et al.*, 1984, 1988), 5-FU (Sloan *et al.*, 1984, 1988), 6-MP (Siver and Sloan, 1988), 5-fluorocytosine (Koch and Sloan, 1987a) and 5 iodocytidine (Koch and Sloan, 1987b) have been synthesized, characterized and evaluated in diffusion cell experiments. In all examples, the derivatives exhibited higher solubility in IPM than did the parent drugs, as expected, and enhanced delivery of the parent drug through hairless mouse skin from suspensions in IPM. Data for Th and 6-MP, which are typical, are given in Table 2. It is interesting that the most effective N-Mannich base derivatives of Th (**22** and **23**) were over 300 to 1000 times more soluble in IPM than Th and up to

	Th			**6-MP**		
R_2		S_{IPM}[a]	J_{IPM}[b]		S_{IPM}[a]	J_{IPM}[b]
$(CH_3)_2$	**21**	48.4	1.17	**29**	0.038	0.151
$(C_2H_5)_2$	**22**	121	2.06	**30**	0.276	0.790
$(C_3H_7)_2$	**23**	431	2.00	**31**	0.507	0.039
$(C_4H_9)_2$	**24**	478	1.68	**32**	0.118	0.022
$(CH_2)_5$	**25**	63.4	0.89	**33**	0.033	0.014
$(CH_2CH_2)_2O$	**26**	5.83	0.61	**--**	--	--
$(CH_2CH_2)_2NCH_3$	**27**	15.8	0.50	**--**	--	--
$(CH_2)_4$	**28**	47.1	1.28	**34**	0.016	0.027

Table 2. Solubilities in IPM (S_{IPM}) and fluxes from suspensions of N-Mannich bases (AAM prodrugs) of theophylline (Th) and 6-mercaptopurine (6-MP) in IPM through hairless mouse skin (J_{IPM}) *in vitro*.

[a]Units of mM. [b]Units of μmol cm^{-2} h^{-1}.

5 times (for **23**) more than the 7-ACOM-Th derivatives (Table 1, **1** to **6**), yet the increase in delivery of Th from IPM was only 4 times that of Th. On the other hand, the best Mannich base of 6-MP (**30**) was only about 10 times more soluble in IPM than 6-MP yet it gave a 200-fold increase in delivery of 6-MP from IPM. The result was that, although Th flux from IPM was 126 times that of 6-MP and the best Th N-Mannich base **22** was over 400 times more soluble in IPM than the best N-Mannich base of 6-MP (**30**), the absolute value for the rate of delivery of Th by **22** was only about 2.6 times that of 6-MP by **30** from IPM. Large increases in lipid solubility are not effective at causing large increases in flux.

N-acyl

Generally, N-acyl derivatives of amines are considered to be very stable toward hydrolysis because of the poor leaving-group properties of the amine anion (the pKa of the conjugate acid is about 40) in the addition-elimination mechanism for their hydrolyses. However, in the cases of N-acyl derivatives of amides and especially imides, where the typical anion is much more stable (the pKa values of the conjugate acids are about 15 and 8, respectively), hydrolyses are much more facile. Detailed descriptions of the mechanisms for hydrolyses are reported elsewhere (Sloan and Wasdo, 2003). For this type of promoiety, most of the reported examples are for derivatives of 5-FU. Here we would like to show the effect of variations in the heteroatom substituted into the alkyl chain and the effect of the position of substitution of the promoiety onto the parent drug on the solubilities, stabilities and abilities to deliver 5-FU from suspensions in IPM in diffusion cell experiments by the N-acyl prodrugs of 5-FU.

Alkylaminocarbonyl (AAC)

The first type of N-acyl derivative that was reported to enhance the topical delivery of a drug containing an amide or imide functional group was the AAC-type derivative of 5-FU (Sasaki *et al.*, 1990). Initially, only the longer alkyl chain members (**38** to **40**) were evaluated for their abilities to enhance the flux of 5-FU from suspensions in IPM through rat skin. The shortest alkyl chain member evaluated (**38**), which was the least soluble in IPM, gave the greatest increase in flux. Three years later all the shorter alkyl chain members, **35** through **38**, and **40** were evaluated using an IPM vehicle but with hairless mouse instead of rat skin (Table 1) (Sloan *et al.*, 1993). In this series the C3 member, **37**, was the most soluble in water (estimated $S_{4.0}$ from $S_{IPM}/K_{IPM:4.0} = eS_{4.0}$), albeit only 0.11 times more soluble than 5-FU, and over 250 times more soluble in IPM; it gave the greatest increase in flux, as would be predicted (Sloan, 1989, 1992), albeit only 3-fold greater. Regardless of the large increase in S_{IPM} values, the $eS_{4.0}$ values were actually lower, so the flux was not increased greatly. The low $eS_{4.0}$ values were attributed to the fact that one polarized O=C-NH group in 5-FU was exchanged for another polarized O=C-NH group in the promoiety; the potential for intermolecular hydrogen bonding in the crystal lattice was not significantly

decreased. Despite these shortcomings, this type of N-acyl derivative was effective in delivering 5-FU and not intact prodrugs: for **35**, **36** and **37**, only 10, 10 and 6% intact prodrug, respectively, was measured in the receptor phases of diffusion cell experiments.

Alkyloxycarbonyl (AOC)

The second type of N-acyl derivatives that was reported to enhance the topical delivery of 5-FU was the AOC type (Table 1) (Beall *et al.*, 1994). Although all of the AOC derivatives were more soluble in the IPM than was 5-FU (up to 3000 times for **45**), they were not substantially more soluble in IPM as a series than the members of the 1-AAC-5-FU series. However, C1 (**41**) was about 7 times, C2 (**42**) was about 5 times and C6 (**45**) was about 3 times more soluble in IPM than the corresponding members of the AAC series, **35**, **36** and **39**, respectively. The big difference was in the $eS_{4.0}$ values for the shorter alkyl chain members of the AOC series where **41**, **42**, and **43** were 30-, 22- and 4.7-fold more soluble in water ($eS_{4.0}$) than their AAC counterparts **35**, **36** and **37**, respectively. Since the AOC prodrugs were more soluble in IPM and water, it would be predicted (Sloan, 1989, 1992) that **41**, **42** and **43** would outperform their AAC counterparts (12.5, 9.8 and 3 times, respectively) at increasing the delivery of total 5-FU species through hairless mouse skin. **41** and **42** were also more soluble in water than 5-FU (1.3 and 2.0 times, respectively, $S_{4.0}$ versus $eS_{4.0}$), and, since they were also much more soluble in IPM, it would be predicted (Sloan, 1989, 1992) that they would give the greatest increases in flux of total species containing 5-FU (11 and 25 times, respectively). Based on their mechanism of hydrolysis and previous literature data, the AOC derivatives were expected to be more stable than the AAC derivatives. Unfortunately, this increased stability translated into the result that mostly intact prodrug was delivered through the hairless mouse skin in the diffusion cell studies: 42% C1, 90% C2, 78% C3, 73% C4, and 74% of intact C6 as percentages of the total 5-FU species in the receptor phases.

Alkylcarbonyl (AC)

In order to overcome the result that the 1-AOC derivatives were too stable to deliver the parent drug 5-FU in the diffusion cell experiments, alkylcarbonyl derivatives of 5-FU, which were known to be much more labile, were synthesized, characterized and evaluated (Beall and Sloan, 1996). As examples of the application of this type of promoiety, three series of AC-5-FU prodrugs were developed: 1-, 3- and bis-1,3-AC-5-FU prodrugs (Table 1). No series of 3-isomers were developed for the AAC- or AOC-type promoieties because they could not be synthesized (AAC) or had been reported to be even more resistant to hydrolysis than the 1-isomer, which had already been shown to deliver mostly intact prodrug through skin (AOC).

All of the members of the three AC series were much more soluble in IPM than 5-FU. However, direct comparisons between members of the 1-AC-5-FU

series and the 1-AOC-5-FU (or 1-AAC-5-FU) series with the same alkyl chain length in the promoiety can be misleading. For example, the C1 member of the 1-AOC series also has an oxygen inserted between the carbonyl and the C1 alkyl so it is more comparable to a C2 in the 1-AC series in total chain length. Thus, the OC1 member of the 1-AOC series will be compared with the C2 member of the 1-AC series, the OC2 with the C3, the OC3 with the C4, the OC4 with the C5 and the OC6 with the C7. Using these interseries comparisons, all of the members of the 1-AOC series are substantially (8 to 30 times) more soluble in water ($eS_{4.0}$) than the members of the corresponding 1-AC series, except for OC1 (**41**), which is only twofold more soluble than C2 (**48**). In addition, the S_{IPM} values of the 1-AOC series do not differ substantially from those of the 1-AC series, except that OC1 (**41**) is 17 times less soluble in IPM than C2 (**48**). Thus, it would be predicted (Sloan, 1989, 1992) that all of the 1-AOC-5-FU prodrugs are more effective at delivering total 5-FU species than the corresponding 1-AC-5-FU prodrugs, except for **41**, which is over twice as soluble in water but 17 times less soluble in IPM than **48**. **48** exhibits a better balance of S_{IPM} and $eS_{4.0}$ and gives 1.6 times greater flux than **41**, but generally the series or member of a series that exhibits the higher $eS_{4.0}$ values gives the higher flux values (Sloan, 1989, 1992). For example, in the 1-AC series, **47** is 2.5 times more soluble in water ($eS_{4.0}$) than **48** (the next most water-soluble member of the series) and is 1.6 times less soluble in IPM, but gives 2.1 times greater flux than **48**. Also, as predicted from reports on the stability of the 1-AC-5-FU prodrugs, only 5-FU was observed in the receptor phases in the diffusion cell experiments.

In the comparison between the 1-AAC-5-FU series and the 1-AC-5-FU series the NC1 member **35** should be compared with the C2 member **48**, NC2 with C3, etc., as in the comparison with the 1-AOC-5-FU series. The members of the 1-AC-5-FU series are 2.5 to over 100 times more soluble in IPM than the corresponding members of the 1-AAC series. However the eS4.0 solubilities of the 1-AAC-5-FU series are greater than or comparable to those of the 1-AC series except for the NCl member, which is 13 times less soluble than the C2 member. Thus, the flux generated by the C2 member **48** is 20 times greater than that produced by the NCl member **35**. The NC2 member **36** exhibits a comparable $eS_{4.0}$ value, but is 6 times less soluble in IPM than the C3 member **49** and gives half the flux. The NC3 member **37** exhibits a 2.6 times greater $eS_{4.0}$ value, but is 3 times less soluble in IPM than the C4 member and gives a comparable flux value. Again, a balance of S_{IPM} and $eS_{4.0}$ is important.

In comparisons between the 3-AC-5-FU series (Beall and Sloan, 2001) and the 1-AOC-5-FU series, the same trends were seen as in the comparisons between the 1-AC-5-FU series and the 1-AOC-5-FU series. The more water-soluble members gave the greater flux values, which resulted in the OC2 and OC3 members, **42** and **43**, giving 2.7 and 4.2 times greater flux values than the C3 and C4 members **55** and **56**, respectively, of the 3-AC-5-FU series, and C2, **54**, giving twofold greater flux than OCl, **41**. In comparison between the 1-AC and 3-AC-5-FU series, the more water-soluble members gave the greater flux values, which resulted in the C2 and C3 in the 3-AC series and the C1 member in the 1-AC series giving greater

flux values than the corresponding members in the other series. In comparisons between the C4 members, the 3-AC member **56** is only slightly (1.6 times) more soluble in water ($eS_{4.0}$) but substantially (4.2 times) less soluble in IPM than the 1-AC member **50**, so that **50** gives the greater increase in the topical delivery of total 5-FU species, 1.8 times.

The members of the 3-AC series are much more stable toward hydrolysis than the members of the 1-AC series because they must rearrange in a slow first step to a 2-AC intermediate which then hydrolyzes (Beall *et al.*, 1993). A side reaction of the intermediate also leads to the formation of 1- and 3-ACOM-5-FU during hydrolysis in the presence of formaldehyde in the receptor phase; the formaldehyde is used as a preservative in the diffusion cells (Sloan *et al.*, 1991). Thus, with the formation of so many different products and so few available authentic standards for them at the time the experiments were run, it was difficult to determine how much intact prodrug was delivered. An estimate of 35% intact prodrug was given for the C1 member of the 3-AC-5-FU series.

In the bis-1,3-AC-5-FU series (Beall and Sloan, 2002) only the first two members of the series were amenable to estimation of their $eS_{4.0}$ values from $S_{IPM}/K_{IPM:4.0}$. Since S_{AQ} ($S_{4.0}$ or $eS_{4.0}$) has been shown to be such an important criterion for predicting flux (Roberts and Sloan, 1999), only results for those two members are reproduced here for inclusion in the database for model development (Table 1). Comparing members of the bis-1,3-AC series with members of the 1-AC series containing the same total number of carbons in the promoiety shows that the C1 bis-1,3-AC member, **57** (4 total carbons in the promoieties), is more soluble in IPM (1.5 times) and water ($eS_{4.0}$) (1.9 times) than the C3 1-AC member and gives the greater flux value, while the C5 1-AC member is more soluble in IPM (1.5 times) and only slightly less soluble in water (0.92 times) than the C2 bis-1,3-AC member, **58**, and gives the greater flux value. Only one comparison with the 3-AC series is possible. The C1 bis-1,3-AC member, **57**, is more soluble in IPM but less soluble in water than the C3 3-AC member, **54**, so that the balance in solubilities is about the same and the flux values are identical. In the bis-1,3-AC series, the 1-AC group hydrolyzes very quickly ($t_{1/2}$ = 1 to 3 min) to give the corresponding 3-AC series, which then hydrolyze to give the same complex mixture of products observed in the receptor phases from the diffusion cell experiments run with the 3-AC-5-FU series.

Drug (S=C)-NH

There are very few examples of thioamide or thioimide functional groups in drug molecules. Drugs containing this functional group exhibit the same physicochemical properties that amides and imides exhibit: high mp and low lipid and water solubilities. The one example in the literature is 6-mercaptopurine (6-MP). 6-MP exhibits an even higher mp, even lower solubilities in IPM and water, and even lower flux from IPM than the amide and imide parent drugs already described. On the other hand, there is an even greater potential for increasing solubilities and flux values with a prodrug approach.

Alkylcarbonyloxymethyl (ACOM)

The members of the 6-ACOM series of prodrugs of 6-MP (**59** to **64**) (Table 1) are all much more soluble than 6-MP in IPM (50 to 200 times), but there are not large differences among the members, probably because the melting points do not vary much either (Waranis and Sloan, 1988). In addition, the absolute S_{IPM} value for the best 6-ACOM-6-MP prodrug for increasing the topical delivery of 6-MP (**61**) is much less than the S_{IPM} values for the best 7-ACOM-Th (**3**) and 1- (**8**) and 3-ACOM-5-FU prodrug (**17**): 8, 3 and 8 times less, respectively. Similarly, although the S_{AQ} for **61** is twice that of 6-MP and that increase was greater or comparable to the increase in $S_{4.0}$ or S_{AQ} values for the best 7-ACOM-Th and 1- and 3-ACOM-5-FU prodrugs compared to Th and 5-FU (0.2, 1.8 and 1 times, respectively), the absolute S_{AQ} value for **61** is much less than the $S_{4.0}$ or S_{AQ} values for the best ACOM prodrugs in the other series (4.9, 74 and 41 times less, respectively). Thus, although the best 6-ACOM-6-MP prodrug delivers 6-MP through hairless mouse skin from IPM 69 times better than 6-MP, the absolute value for its flux is much less than those for the best 7-ACOM-Th and 1- and 3-ACOM-5-FU prodrugs (4, 14.5 and 11 times less, respectively) at delivering their parent drugs through hairless mouse skin. The absolute values for S_{IPM} or S_{AQ} predict the absolute values for flux (Roberts and Sloan, 1999) and not, for example, the partition coefficients $K_{IPM:4.0}$ (or solubility ratios, SR). The log $K_{IPM:4.0}$ values are negative for the best 1- and 3-ACOM-5-FU prodrugs and slightly positive (greater lipophilicity) for the best 7-ACOM-Th and 6-ACOM-6-MP prodrugs (SR), yet the flux values for the latter are from 2.7 to 14.5 times less, respectively, than for the former.

Although the mechanism for the hydrolysis of the 6-ACOM-6-MP prodrugs has not been studied, by analogy to other ACOM prodrugs it is probably a typical addition-elimination mechanism followed by rapid loss of formaldehyde (Sloan, 1989, 1992; Sloan and Wasdo, 2003). What is known is that in diffusion cell experiments the 6-ACOM-6-MP prodrugs hydrolyze completely to 6-MP during their permeation through hairless mouse skin from IPM. The second 6-MP ACOM series, the bis-6,9-ACOM-6-MP prodrugs, also underwent complete hydrolysis to 6-MP during permeation except for the first two members of the series (**65** and **66**); 12 and 1%, respectively, of intact prodrug was found upon analysis of total 6-MP species in the receptor phases (Waranis and Sloan, 1987). Here, as in most other homologous series (see below), the shorter alkyl chain members are less stable in buffer but more stable in biological media (Kawaguchi *et al.*, 1988) than the intermediate chain-length members.

The best bis-6,9-ACOM-6-MP prodrugs gave flux values that are comparable to those by the best 6-ACOM-6-MP prodrugs (Table 1). However, a more accurate comparison of the two series and the effect of dialkylation on the performance of the prodrugs can be obtained if the total number of added carbons and heteroatoms in the promoieties are normalized as in the comparison of the bis-1,3-AC-5-FU with the 1- and 3-AC-5-FU series, for example. Thus, the C1 member **65** of the bis-6,9-ACOM series should be compared with the C5 member **63** of the 6-ACOM series and the C2 member **66** with the C7 member **64**. In these

comparisons, **65** exhibited a similar S_{IPM} value but a much greater S_{AQ} value (10 times) than **63**; **66** was 8 times more soluble in IPM and 70 times more soluble in water than **64**. Thus, **65** and **66** gave 4 and 18 times greater flux values than the corresponding members (**63** and **64**, respectively) in the 6-ACOM series. This result may be due to the incorporation of two enabling groups into the bis-ACOM series and not merely additional methylene groups. However, there is no advantage gained by masking two polarized amide-like functional groups unless there are concomitant increases in S_{AQ} and S_{IPM}.

N-Alkyl-N-alkyloxycarbonyl)aminomethyl (AOCAM)

The AOCAM type promoiety was developed as an alternate to the ACOM type, which it was thought would exhibit greater S_{AQ} values because of the substitution of an amide group for an ester group in the promoiety. The 6-AOCAM-6-MP prodrugs were similar to the 6-ACOM-6-MP prodrugs in that their melting points and S_{IPM} values did not vary much within the series with the exception of the N-butyl-N-butyloxycarbonyl member (Siver and Sloan, 1990). The absolute S_{IPM} values in the two series were also comparable. However, the AOCAM prodrugs were much less stable in water than anticipated (half-lives of 80–100 minutes at pH 7.1 and 32ºC) for an amide analogue of an ester, and no accurate S_{AQ} values were determined. Rough estimates of S_{AQ} were reported for several prodrugs that were also evaluated in diffusion cell experiments. The best member of the series that was evaluated, the S-(N-methyl-N-methyloxycarbonyl) aminomethyl prodrug, was also estimated to be more soluble in water than was 6-MP. It increased the delivery of 6-MP through hairless mouse skin from IPM sixfold. The S_{IPM} values for the 6-AOCAM prodrugs were comparable to those for the 6-ACOM prodrugs, but their S_{AQ} values were probably less and the flux values were almost 10 times less. Despite their shortcomings the AOCAM prodrugs show that heteroatoms other than oxygen can be incorporated into the soft alkyl type promoiety, and the resulting prodrugs can revert to the parent drug in a timely manner.

Drug Aryl-OH

A hydroxy group (OH) attached to an aromatic ring (phenol) in a drug molecule is an attractive target for a prodrug approach. When delivered orally, drugs containing a phenol group suffer from premature glucuronidation and sulfation of the aryl hydroxy group during the absorption process. This leads to poor oral bioavailability of the drug. One approach to improve bioavailability is to design a prodrug to transiently mask the aryl hydroxy group during oral absorption; the other is to change the route of administration to a topical one. However, as is often the case, the parent phenol lacks the requisite balance of lipid and aqueous solubilities necessary to ensure good topical delivery, and a prodrug approach is also required to enhance those physicochemical properties transiently to increase delivery from a topical route of administration.

O-Acyl

Because of the modest amounts of drug that need to be delivered, a particularly attractive class of drugs for prodrug modification is the narcotic agonists and antagonists, which usually contain a phenolic functional group. There are several reports of the use of O-acyl-type promoieties to enhance the topical delivery of narcotic drugs such as morphine (Drustrup *et al.*, 1991), ketobemidone (Hansen *et al.*, 1992), buprenophine (Stinchcomb *et al.*, 1996), naltrexone (Stinchcomb *et al.*, 2002), and nalbuphine (Sung *et al.*, 2000). However, only two of these reports provide sufficient solubility and flux data with which to analyze their performances and provide any predictive value.

Alkylcarbonyl (AC)

The first report of the use of O-AC derivatives to enhance the topical delivery of a narcotic agonist involves morphine (MOR) (Drustrup *et al.*, 1991). Examples

bis-3,6- and 3-AC-MOR

	S_{IPM}[a]	$S_{7.0}$[a]	J_{IPM}[b]	$J_{7.0}$[b]
70, R=R^1=H; MOR	0.081	6.3	<0.035	<0.035
71, R=R^1= (C=O) C_2H_5	103	9.07	30.5	8.77
72, R=H, R^1= (C=O) C_2H_5	232	61.6	--	--
73, R=R^1 = (C=O) C_5H_{11}	>415	0.04	41.1	5.96
74, R=H, R^1 = (C=O) C_5H_{11}	>392	6.79	125	88.8

3-AC-NTX[c]

	mp[d]	MW	S_{MO}[a]	$S_{7.4}$[a]	J_{MO}[b,e]	J_{MO}[b,f]
75, NTX	169	341	0.26	15.2	2.5	3.26
76, C1	116	383	2.04	1.93	15.6	15.5
77, C2	149	397	4.41	1.25	11.1	11.2
78, C3	108	411	4.16	1.05	5.6	5.7
79, C4	85	425	9.62	0.59	6.7	4.79
80, C5	64	439	6.95	0.28	8.6	8.69
81, C6	60	453	7.62	0.18	7.1	7.87

Table 3. Melting points (mp), molecular weights (MW), solubilities in IPM (S_{IPM}), mineral oil (S_{MO}) and pH 7.0 and 7.4 buffers ($S_{7.0}$ and $S_{7.4}$), and fluxes from suspensions of morphine (MOR) and naltrexone (NTX) prodrugs in IPM (J_{IPM}), pH 7.0 buffer ($J_{7.0}$) and mineral oil (J_{MO}) through human skin *in vitro*.

[a]Units of mM. [b]Units of nmol cm^{-2} h^{-1}. [c]C1, C2, etc. refer to number of carbons in alkyl chain. [d]Units of ^{o}C. [e]Data from a combination of fresh and previously frozen skin. [f]Data from previously frozen skin.

from two series (bis-3,6- and 6-AC-MOR) were synthesized, characterized and evaluated in diffusion cells using human skin and two different vehicles (IPM and pH 7.0 buffer). The solubilities (S_{IPM} and $S_{7.0}$) and flux data (J_{IPM} and $J_{7.0}$) are given in Table 3. All of the prodrugs that were reported except for the bis-3,6-hexanoyl prodrug **73** were more soluble not only in IPM but also in pH 7.0 buffer. The result is that all of the prodrugs increased the delivery of MOR (or total MOR species for **71**) through human skin *in vitro* from suspensions or solutions in IPM by at least 800 times and from suspensions in pH 7.0 buffer by at least 170-fold. The trend was the same from either vehicle. Regardless of the assumed higher S_{IPM} value for **73**, **74** was 170 times more soluble in pH 7.0 buffer than **73** and gave a 3 times greater flux from an IPM solution and 15 times greater flux from pH 7.0 buffer. This confirms the previous results reported for bis-6,9-ACOM-6-MP prodrugs delivered from IPM and water (Waranis and Sloan, 1987); the more water-soluble members of a more lipophilic series of prodrugs give the greater fluxes regardless of the vehicle (also see discussion below). Unfortunately, **72**, which was 10 times more soluble in water and 3000 times more soluble in IPM than MOR and, hence, exhibited the best balance of S_{IPM} and $S_{7.0}$ solubilities among the reported prodrugs, was not evaluated.

Although both of the hexanoyl prodrugs (**73** and **74**) delivered only MOR, the propionyl prodrug **71** delivered 50:50 intact prodrug:MOR. This is consistent with other reports that the medium alkyl chain length homolgues in an ester series are more completely hydrolyzed to the parent drug than are the shorter or longer alkyl chain homologues (Kawaguchi *et al.*, 1988).

The second report concerns the use of 3-AC prodrugs of the narcotic antagonist naltrexone (3-AC-NTX) to deliver NTX (Stinchcomb *et al.*, 2002). The solubilities in mineral oil (S_{MO}) and pH 7.4 buffer ($S_{7.4}$) and the fluxes through human skin *in vitro* using mineral oil as the vehicle are given in Table 3. Although all of the prodrugs were at least 7 times more soluble in MO than NTX, NTX was at least 8 times more soluble in pH 7.4 buffer than was the best prodrug. By comparison, the MOR prodrugs were at least 11 times more soluble in a non-polar lipid and 3.5 times more soluble in a buffer (except for **73**) than were the NTX prodrugs. Thus, the 3-AC-NTX prodrugs were not as effective at enhancing the topical delivery of NTX as were similar prodrugs of MOR—only 4.8 times NTX flux for the C1 (**76**) prodrug from MO compared to 3500 times MOR flux for the C5 (**74**) prodrug from IPM. The prodrug that was the most soluble in MO, **79**, gave the lowest flux value, while **76**, the prodrug that was the least soluble in MO, but still more soluble in MO than NTX, and the most soluble in water, gave the greatest flux value, as predicted (Sloan, 1989, 1992). The authors also reported that the prodrugs completely reverted to NTX during their permeation of human skin *in vitro*.

An attempt was made by the authors to show that the flux data for the 3-AC-NTX series did not depend on $S_{7.4}$. Their fit of the solubility and flux data to the Roberts-Sloan equation (Roberts and Sloan, 1999) gave y and 1–y coefficients for the S_{MO} and $S_{7.4}$ parameters of 0.97 and 0.03, respectively, which at face value seemed to support their conclusion. However, careful examination of their fit

shows that their coefficients are not statistically different from zero and that a more rigorously determined r^2 value for the fit (1–residual sum of squares/corrected sum of squares) is only 0.388. In addition, the database is not homogeneous. Using a leave-one-out analysis of the database shows that the data for NTX itself, which is the only example at the extreme of high aqueous solubility and low flux, is unduly affecting the fit. If the data for NTX are left out, y becomes 0.18 instead of 0.97. Finally, using only flux data from previously frozen skin shows that there is a subset of the database (**76** to **79**) that does give a good fit to the Roberts-Sloan equation (y log S_{MO} = 0.65 log S_{MO}, 1–y log $S_{7.4}$ = 0.35 log $S_{7.4}$, and r^2 = 0.993) and does fit the obvious trends in prodrug solubilities versus flux in the database.

Alkyloxycarbonyl (AOC)

In addition to the use of O-AC prodrugs of phenolic parent drugs there is also a report of the use of the hydrolytically more stable O-alkyloxycarbonyl prodrugs of 4-hydroxyacetanilide (4-AOC-APAP) to enhance its topical delivery (Wasdo and Sloan, 2004). Compared to the other parent drugs (5-FU, Th and 6-MP) in Table I, APAP is much more soluble in IPM and at least as soluble in water. APAP is 5.6 times more soluble in IPM and twice as soluble in water as Th; 39 times more soluble in IPM and as soluble in water as 5-FU; and 86 times more soluble in IPM and in water than 6-MP. Thus, it may be more difficult to dramatically increase the S_{IPM} (and S_{AQ}) values of APAP with a simple prodrug approach because APAP is already relatively more soluble in IPM than other parent drugs evaluated.

The solubilities in IPM (S_{IPM}) and water (S_{AQ}) or estimated solubilities in pH 4.0 buffer ($eS_{4.0}$) are given in Table 1 together with the flux values for the delivery of total APAP species from IPM (J_{IPM}) through hairless mouse skin by the 4-AOC prodrugs. The S_{IPM} values for the 4-AOC-APAP prodrugs varied from 2 to 12 times greater than those of APAP, while the two members that were substantially more soluble in water than the other members, **82** and **87**, were each less soluble in water than APAP, 0.2 and 0.3 times, respectively. Thus, it is not surprising that **82** and **87**, which are the more water-soluble members of a more lipophilic series of prodrugs, give the greatest flux values for the series (Sloan, 1989, 1992), albeit only 2.0 and 1.5 times, respectively, that of APAP because they are less soluble in water than APAP. Because the O-AOC type prodrugs (carbonates) are inherently more stable than the O-AC type prodrugs (esters), significant amounts of intact prodrugs were measured in the receptor phases of the diffusion cell experiments: for **82** to **88**, 64, 14, 25, 0.0, 51 and 0%, respectively, of intact prodrug. The greater the flux value, the greater the percent of intact prodrug that permeated, which is the opposite of what would result if fast hydrolosis of the shorter chain members of the series to the more water soluble parent drug was responsible for the greater flux values abtained from the shorter chain members.

Drug Alkyl-OH

Although an aliphatic hydroxy (OH) is a polar group and can be the cause of low solubilities (lipid and aqueous) and, hence, low topical delivery, simply masking the OH group with a simple alkylcarbonyl promoiety (O-AC) usually does not lead to substantial increases in topical delivery. There are numerous examples where series of O-AC type prodrugs have been synthesized and evaluated (Sloan, 1992). Among the members of those series, several that will enhance the topical delivery of the parent drug by several-fold can usually be identified. However, here we will focus on two types of prodrugs that incorporate potential penetration-enhancing functional groups (i.e., leading to increased water solubilities) into the promoiety and have led to substantial increases in the delivery of the parent drug.

The first type of prodrug contains a dihydroxyalkyl group as part of a carbonate promoiety (DOHOC) (Friend *et al.*, 1988). The original OH group in the parent drug has been replaced with two OH groups in the promoiety to increase the water solubility of the parent drug. In this example, the parent drug is levonorgestrel, **89**, and the solubilities, partition coefficients and fluxes for two DOHOC type prodrugs, **92** and **93**, are reproduced in Table 4 together with the corresponding data for two simple O-AC prodrugs, **90** and **91**, for comparison.

R	mp °C	C_v[a]	log K[b]	J[c]
89, H	240	19.2(100)	3.70	0.00019
90, $(C=O)C_5H_{11}$	84-86	604(95)		
		12.9 (62)		0.00058
91, $(C=O)C_4H_9$	170	28.3 (95)		0.00026
92, $(C=O)OCH_2CH(OH)CH_2OH$	147–148	30.2 (40)	3.22	0.0063
93, $(C=O)O(CH_2)_4CH(OH)CH_2OH$	49–53	396 (40)	3.75	0.0030

Table 4. Melting points (mp °C), solubilities in mixtures of ethanol: water (C_v), partition coefficients (K) and fluxes of total species from suspensions in ethanol: water through rat skin (J) *in vitro*.

[a]Solubilities in mixtures of ethanol:water in units of mM where the value in parentheses is % ethanol in mixture. [b]Octanol: water at 24°C. [c]Flux in units of μmol cm^{-2} h^{-1} from suspensions in mixtures of ethanol:water given in parentheses in C_v column.

The two simple O-AC prodrugs of **89** (**90** and **91**) give results that are typical for this type of prodrug. **90** and **91** are more soluble in 95% ethanol than **89** is in 100% ethanol. Extrapolation of their solubilities in ethanol to solubilities in the homologous alcohol octanol would suggest that **90** and **91** are more soluble in octanol than **89** and, hence, are considered more lipophilic. In addition, partition coefficients could not be determined for **90** and **91** because no **90**, **91**, or **89** could be detected in the aqueous phases; thus, they are definitely less soluble in water. The flux of **89** from various ethanol-water mixtures (40–100%) did not vary much (0.00019 to 0.00022 μmol cm^{-2} h^{-1}) so the delivery of total species containing **89**

from suspensions of **90** and **91** in the various ethanol mixtures can be easily compared to fluxes of the parent drug. The O-AC prodrugs **90** and **91** were more soluble in lipids but less soluble in water than **89**, and gave 3 and 1.3 times, respectively, greater delivery of total species containing **89** than did **89**. Both O-AC prodrugs delivered only **89**; they reverted completely during permeation.

On the other hand, the two DOHOC prodrugs **92** and **93** are more soluble in ethanol-water mixtures that are primarily aqueous in nature (40% ethanol) than **89** is in 100% ethanol. Thus, **92** and **93** are more soluble in water than is **89**. In addition, **92** and **93** exhibit log $K_{OCT:7.4}$ values that are comparable to that of **89** which suggests that they are also more soluble in octanol. Thus, **92** and **93** exhibit increased aqueous as well as lipid solubilities compared to **89** and give 30 and 15 times greater delivery of total species containing **89**, respectively, which would be predicted (Sloan, 1989, 1992). Because of the greater stability of the carbonates (**92** and **93**) compared to the esters (**90** and **91**), **92** and **93** delivered mostly intact prodrug, 80 and 96%, respectively.

The rationale for the synthesis, characterization and evaluation of these more hydrophilic prodrugs was to overcome a presumed rate-limiting, hydrophilic, viable epidermis barrier to permeation of the skin. However, the success of these more hydrophilic prodrugs of an already highly lipophilic parent drug also supports a model for permeation where the high capacity path for permeation is through a series of alternating lipid and aqueous phases in the intercellular barrier to permeation (Roberts and Sloan, 2001) and where a balance between increased lipid and aqueous solubilities is essential for increased permeation of such series (Sloan, 1989, 1992). In addition, although the DOHOC prodrugs were not good prodrugs because they did not revert to levonorgestrel, their performance certainly emphasizes the importance of designing increased aqueous as well as increased lipid solubility into the promoiety to optimize flux.

The second type of prodrug contains a dialkylamino group as part of an ester promoiety (DAAC). The delivery of testosterone by its 17-(4'-dimethylaminobutyrate) ester, **94**, from a 10% solution in pH 7.4 buffer was 60 times greater than the delivery of testosterone from a suspension in the pH 7.4 buffer (Milosovich *et al.*, 1993). In addition, delivery of testosterone from 50 mg ml^{-1} solutions of **94** and testosterone in propylene glycol led to a 35 times greater flux from **94**. No solubility data were reported, but the fact that a 10% aqueous solution of **94** was evaluated suggests that **94** was substantially more soluble in water than was testosterone, 0.040 mg/ml or 0.004%.

Drug (O=C)-OH

Drugs that contain a carboxylic acid, (O=C)-OH, functional group exhibit the same problems that drugs containing other polar, ionized functional groups have—high melting point and low solubilities in lipids and water. The highly ionized (O=C)-OH group tends to slow diffusion into and through the skin by the drugs so that transient masking of the ionized OH with an unionizable promoiety, or one that contains an amino group (see above), tends to improve their topical

permeation. Among the various classes of drugs containing a (O=C)-OH group, the non-steroidal anti-inflammatory drugs (NSAID) have perhaps the most potential to be used topically. Conditions such as psoriasis, erythema, atopic dermatitis, topical cancers (such as basal cell carcinoma) and, of course, local inflammation could all benefit from improved topical delivery of NSAIDs. As for the Drug Aliphatic-OH class, there are numerous examples where series of simple alkyl (A) esters of the (O=C)-OH group, (O=C)-OA, have been synthesized and evaluated; among the members of those series, several that enhance the topical

	S_{AQ}[a]	S_{IPM}[a]	J_{AQ}[b]		log K	S_{AQ}[a,c]	J[b,d]
95	0.011	7.82	0.23	**95**	3.10	2.22	0.35
96, m=1	0.0096	6.00	0.80	**105**, n=1	4.52	0.067	0.21
97, m=2	0.016	7.34	0.96	**106**, n=2	4.39	0.092	0.37
98, m=3	0.012	19.0	0.77	**107**, n=3	4.15	0.17	0.45
99, m=4	0.0074	27.5	0.19	**108**, n=4	3.99	0.25	1.90
				109, n=5	3.81	0.39	2.27
100	0.045	23.5	5.1	**100**	3.0	2.85	2.25
101, m=1	0.355	21.1	13.8	**110**, n=1	3.90	0.31	1.46
102, m=2	0.249	18.8	8.9	**111**, n=2	3.70	0.51	2.17
103, m=3	0.032	16.7	4.00	**112**, n=3	3.50	0.83	1.83
104, m=4	0.011	7.64	2.79	**113**, n=4	3.20	1.74	4.46
				114, n=5	2.90	3.65	5.13
				115	2.8	4.68	5.29
				116, n=1	3.6	0.65	2.71
				117, n=2	3.4	1.06	4.62
				118, n=3	3.3	1.36	4.04
				119, n=4	3.1	2.23	14.3
				120, n=5	2.6	7.67	7.92
				121	3.2	1.74	3.33
				122, n=1	4.1	0.19	2.58
				123, n=2	3.9	0.31	4.04
				124, n=3	3.5	0.83	4.88
				125, n=4	3.2	1.74	5.83
				126, n=5	2.6	7.67	8.04

Table 5. Solubilities in water (S_{AQ}) and IPM (S_{IPM}), log partition coefficients between octanol and water (log K) and fluxes for the delivery of total species from suspensions in water (J_{AQ}) or from ethanol deposited compounds (J) through human skin *in vitro*.

[a]Units of mM. [b]Units of nmol cm^{-2} h^{-1}. [c]Calculated from log S_{AQ} = –1.072 log K + 0.672. [d]Calculated from Q_{24} (in μmol cm^{-2})/24 h from ethanol-deposited compounds.

delivery of the parent drug several-fold can be identified. These series have been described elsewhere (Sloan, 1992). Here we will focus on several examples of more complex esters that incorporate potential penetration-enhancing functional groups into the promoiety.

The first example of these is alkylcarbonylaminoalkyl (ACAA) esters of indomethacin (**95**) (Bonina *et al.*, 1991) and naproxen (**100**) (Bonina *et al.*, 1993). Since alkylazacycloalkan-2-ones have been used as penetration enhancers, it was anticipated that the incorporation of the structurally similar ACAA (see Table 5) promoiety into the prodrugs would give the prodrugs properties like the penetration enhancers and that they would penetrate the skin better. Table 5 presents the S_{IPM}, S_{AQ}, and J_{AQ} values through human skin for the ACAA prodrugs of **95** and **100** that have been evaluated. In the indomethacin series, (**96** to **99**), **97** is the only member that is more soluble in water than **95**; it gives the largest increase in delivery of total indomethacin-containing species (4.2 times), regardless of the fact that it is much less soluble in IPM than **98** and **99**. In the naproxen series (**101** to **104**), **101** is not only more soluble in water than **100** and the other members of the series, but it is also more soluble in IPM than the other members of the series. Thus, it is not surprising that **101** gives the largest increase in delivery of total naproxen-containing species (2.7 times). It is interesting to note that neither **97** or **101** are more lipid soluble than their parent compounds **95** and **100**, respectively, yet, as predicted (Sloan, 1989, 1992), they gave the greatest increase in flux in the series because they were more soluble in water. The drawback to this approach is that, at best, only 10–12% of either parent drug was delivered in the diffusion cell experiments. Interestingly, more hydrolytically labile prodrugs were synthesized in both series where n = 1, but they were never evaluated because they were considered to be too labile. Had the hydrolytically more labile prodrugs been evaluated from an IPM vehicle, in which they would presumably been more stable, they may have been equally effective at increasing delivery of the parent drug as the n = 2 series had been at increasing delivery of the intact prodrug.

The second example is the use of polyoxyethylene (POE) esters of indomethacin (**95**) (Bonina *et al.*, 1995), naproxen (**100**), ketoprofen (**115**), and diclofenac (**121**) (Bonina *et al.*, 2001) to enhance their topical delivery. The rationale for the synthesis and evaluation of the POE esters was that they would possess inherent skin penetration-enhancing properties because they were hydrophilic and would increase the S_{AQ} value for the parent drug in addition to increasing its S_{LIPID} value. Although these results were reported by the same research group that had reported on the ACAA esters, the experimental procedures used to evaluate these POE esters were quite different. First, no experimental S_{AQ} values were measured. Instead, $K_{OCT:AQ}$ values (Table 5) were determined, and the S_{AQ} values in Table 5 were calculated from $\log S_{AQ} = -1.072 \log K_{OCT:AQ} + 0.672$. Second, the flux values were single time point measurements 24 h from the application of the POE esters in ethanol. The ethanol was allowed to evaporate to give a film of the ester as an oil on the skin surface. Regardless of the fact that the S_{AQ} values were only calculated, the $K_{OCT:AQ}$ values decreased as

more oxyethylene units were added as expected if S_{AQ} values increased with added oxyethylene units. Also as expected, the flux values generally increased as $K_{OCT:AQ}$ (an indicator of lipophilicity) decreased and calculated S_{AQ} increased. All of the POE prodrugs were more lipophilic than their parents on the basis of $K_{OCT:AQ}$ except for the n = 5 members of most of the series, but only the more water-soluble members gave higher flux values, as would be predicted (Sloan, 1989, 1992). Only intact prodrug was observed upon analysis of the receptor phases in the diffusion cell experiments, but the authors attributed that to experimental conditions: heat separation of the stratum corneum plus epidermis from the rest of the skin may have damaged it, and a 50% ethanol receptor phase may have leached the necessary enzymes out of the skin.

The third example is the use of dialkylaminoalkyl (AA) esters and amino- or dialkylaminoalkylcarbonyoxyalkyl (AACOA) esters of carboxylic acids to enhance their topical delivery. The rationale for incorporating a basic amino group into the promoiety is the same as for incorporating the POE group: increased S_{AQ} as well as S_{LIPID} values. The 2-diethylaminoethyl ester of indomethacin (**127**) is representative of the AA type (Jona *et al.*, 1995). **127** was 3.7 times more soluble in pH 7.4 buffer, and its $K_{OCT:7.4}$ was 6.2 times greater so it was also 23 times more soluble in octanol than indomethacin. Thus, **127** gave 4.3 times greater delivery of total indomethacin-containing species on a molar basis from a 10% suspension in pH 7.4 buffer through human skin than a similar suspension of indomethacin, as would be predicted (Sloan, 1989, 1992).

	$S_{5.0}$[a]	log $K_{OCT:5.0}$	S_{OCT}[a]	$J_{5.0}$[b]
100	0.40	2.38	95	1.8
128, n=2, R=H	10.38	0.67	48	4.0
129, n=2, R=CH(C_2H_5) CH_3	1.69	2.13	227	5.1
130, n=3, R=H	5.87	0.99	57	1.5
131, n=4, R=CH(C_2H_5) CH_3	1.07	2.72	561	1.8
	$S_{7.4}$[a]	log $K_{OCT:7.4}$	S_{OCT}[a]	$J_{7.4}$[b]
100	102	0.30	204	6.5
132, n=2, m=1, X=0	0.07	2.14	9.77	0.7
133, n=2, m=1, X=NCH_3	4.1	1.16	59	24.6
134, n=4, m=1, X=NCH_3	8.8	1.30	174	7.2
135, n=4, m=2, X=NCH_3	432	3.04	47.9[c]	7.7
136, n=4, m=3, X=NCH_3	50	2.69	2.5[c]	13.2

Table 6. Solubilities in pH 5.0 ($S_{5.0}$) or 7.4 ($S_{7.4}$) buffer, partition coefficients between octanol and pH 5.0 ($K_{OCT:5.0}$) or 7.4 buffer ($K_{OCT:7.4}$), solubilities in octanol calculated from ($S_{5.0}$) ($K_{OCT:5.0}$) or ($S_{7.4}$) (KOCT:7.4), and fluxes for the delivery of total species from pH 5.0 ($J_{5.0}$) or 7.4 buffer ($J_{7.4}$) by suspensions of the compounds through human skin *in vitro*.
[a]Units of mM. [b]Units of nmol cm^{-2} h^{-1}. [c]Units of mM × 10^4.

Members of several series of AACOA-type esters of naproxen have been reported to increase delivery of total naproxen-containing species through human skin from suspensions in pH 5.0 or 7.4 buffer. Solubilities and flux data for representative esters are give in Table 6. For the first series (**128** to **131**) (Rautio *et al.*, 1999), changes to longer alkyl chains connecting the two carboxylic acid groups (one in the drug, the other in the promoiety) led to decreased $S_{5.0}$ (compare **128** to **130** and **129** to **131**), increased S_{OCT} and decreased $J_{5.0}$ (2.7 and 2.8 times less, respectively), as would be predicted (Sloan, 1989, 1992). On the other hand, increasing S_{OCT} by adding an alkyl group R to the aminoalkylcarbonyl (compare **128** to **129** and **130** to **131**) led to decreased $S_{5.0}$, increased S_{OCT} and slightly increased $J_{5.0}$ values (1.3 and 1.2 times, respectively). All of the prodrugs were significantly more soluble in pH 5.0 buffer, and all of them delivered a mixture of intact prodrug and naproxen of an unspecified composition. The two best prodrugs (**128** and **129**) were 2.2 and 2.8 times, respectively, more effective at delivering total naproxen-containing species than naproxen.

For the second series (**132** to **136**) (Rautio *et al.*, 2000), only one member, **135**, was more soluble in pH 7.4 buffer than naproxen and it was also over a 1000 times more soluble in octanol as well; however, it was only slightly more effective (1.2 times) than naproxen at delivering total naproxen-containing species through human skin from suspensions in pH 7.4 buffer. The best ester was **133**, which was 100 times less soluble in pH 7.4 buffer and 8,000 times less soluble in octanol than **135**, yet gave a 3.2 times greater $J_{7.4}$ value than **135** and a 3.8 times greater $J_{7.4}$ value than naproxen. **133** was 25 times less soluble in pH 7.4 buffer and 3 times less soluble in octanol than naproxen, so its enhanced performance versus naproxen (**100**) or **135** cannot be rationalized. Much larger increases in flux by this series relative to naproxen (3 to 100 times) would result if the value of $J_{7.4}$ for naproxen from two previous papers by the same authors (0.23 nmol cm^{-2} h^{-1}) had been used, but it would not change the relative performance within the series.

Effect of Structure of Prodrugs on Reversibility

The importance of the ability of a prodrug to revert to its parent drug has been covered explicitly in the characterization of each type of prodrug discussed. For example, the trends in stability that depend upon chain length, which switches from shorter chain members of a homologous series being less stable under chemical hydrolysis to being more stable under enzymatic hydrolysis has been noted for the bis-6,9-ACOM-6-MP and 3-AC-MOR prodrugs. However, there are two other aspects of the rate of reversion of prodrugs to their parent during topical application that need to be discussed separately.

The first of these aspects is the effect of rate of hydrolysis on dermal (as opposed to transdermal) delivery. It was anticipated that during permeation a more stable prodrug would retain those improved physicochemical properties of the prodrug that enabled it to better partition into the skin so that the prodrug would also partition through the skin better (improved transdermal delivery at the expense of dermal delivery). For convenience in Table 7 we have repeated the flux

	J_{IPM}[a]	C_s[b]		J_{IPM}[a]	C_s[b]
5-FU	0.24	3.7		0.24	3.7
1-AOC-5-FU			**1-AC-5-FU**		
41,C1	2.6	8.3	**47**, C1	9.3	68
42,C2	5.9	18.0	**48**, C2	4.3	69
43, C3	2.3	5.0	**49**, C3	1.3	8.2
44, C4	2.2	4.2	**50**, C4	1.0	16
45, C6	1.5	11.0	**51**, C5	1.1	11
46, C8	0.30	3.2	**52**, C7	0.60	12

Table 7. Values for flux from IPM suspensions through hairless mouse skins *in vitro* (J_{IPM}) and amounts of total 5-FU containing species leached from the skins 24 hours after the donor phases had been removed (C_s).
[a]Units of μmol cm^{-2} h^{-1}. [b]Units of μmol.

values for two series of acyl prodrugs of 5-FU from Table 1 along with the amounts of total 5-FU-containing species leached from hairless mouse skin *in vitro* 24 h after the donor phases had been removed (C_s) (Beall and Sloan, 1996). These C_s values have been reported to be representative of relative dermal delivery by these two series. The 1-AOC-5-FU prodrugs **41–46** are reasonably stable in pH 7.4 buffer (t1/2 190–550 min) and are readily reversible in 80% human plasma (t1/2 2–3 min) (Buur and Bundgaard, 1986), but they all delivered significant amounts of intact prodrug (40–90%); the 1-AC-5-FU prodrugs **47-52** are much less stable even in pH 7.1 buffer ($t_{1/2}$ 3–5 min) and delivered only 5-FU. Thus, it is not surprising that the 1-AC prodrugs generally are much better at increasing C_s values than are the 1-AOC prodrugs, in spite of the fact that in most comparisons their J_{IPM} values are lower. A fast rate of reversion of prodrug to parent drug is necessary to optimize dermal delivery.

The second aspect is the effect of the rate of hydrolysis on transdermal delivery. It was anticipated by some (Stinchcomb *et al.*, 2002) that the transdermal delivery of a more stable, more lipophilic, longer alkyl chain prodrug would be limited by the aqueous dermis barrier. A representative study of this effect is the evaluation of the alkylcarbonyl prodrugs of the R and S isomers of propranolol (PL, **137**), **138–142** (Ahmed *et al.*, 1996). The experimental solubilities in pH 4.0 buffer ($S_{4.0}$), partition coefficients between octanol and pH 4.0 buffer ($K_{OCT:4.0}$), and flux values for each isomer as total intact prodrug and propranolol ($J_{4.0}$) are given in Table 8 along with their calculated solubilities in octanol (S_{OCT}). The most effective prodrug was not the most labile member but the most water-soluble and most lipid-soluble member of the series **140** R and **140** S as would be predicted (Sloan, 1989, 1992), but it was less soluble in water (0.4 times) and only 6 times more soluble in OCT than PL so it only gave a twofold increase in flux. Regardless of the fact that the R isomer hydrolyzed about 5 times faster than the S isomer to the more water-soluble parent drug whose transdermal delivery would not be

Compound[a]	$S_{4.0}$[b]	log K[c]	S_{OCT}[b,d]	$J_{4.0}$[e]	%[f]
137, PL R	392	0.38	938	30.6	
S				30.2	
138, C1 R	33.7	0.62	140	24.2	7
S				24.5	2
139, C2 R	31.2	1.05	355	19.5	37
S				22.4	2
140, C3 R	163	1.54	5630	57.0	65
S				52.2	13
141, C4 R	17.2	1.99	1671	31.5	90
S				26.0	33
142, C5 R	6.4	2.29	1261	26.1	97
S				18.9	70

Table 8. Values for solubilities in pH 4.0 buffer ($S_{4.0}$) and octanol (S_{OCT}), log partition coefficients between octanol and pH 4.0 buffer (log K) fluxes of acyl prodrugs of R and S isomers of propranolol ($J_{4.0}$) and percent of prodrugs hydrolyzed during permeation (%).
[a]C1, C2 etc. indicate the number carbons in the alkyl chain. [b]Units of mM. The values for the solubilities of racemic compounds are twice the listed value. R and S isomers have identical values. [c]Log partition coefficient between octanol and pH 4.0 buffer. [d]Calculated from (K) ($S_{4.0}$). [e]Units of nmol cm^{-2} h^{-1}. [f]Percent of prodrug hydrolyzd during permeation.

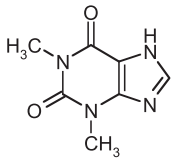
limited by the aqueous dermis, the flux of the R isomer was only 9% greater. Thus, the rate of hydrolysis may have an effect on transdermal delivery, but in this example it was not sufficiently substantial to cause a change in the relative order of effectiveness of the prodrugs in the R series, instead solubility factors dominated the result just as they did with the 4-AOC-APAP prodrugs.

Conclusions

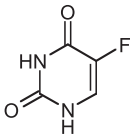
The focus of this chapter has been to describe and analyze various prodrug approaches toward enhancing topical delivery and to define design features to incorporate into prodrug approaches to accomplish that goal. Two general types of prodrug approaches have been described: the use of acyl and soft alkyl promoieties. The results have been analyzed in terms of the effect of lipid and aqueous solubilities on delivery of the parent drug (and/or intact prodrug) and of the effect of the enabling group in the promoieties on reversion of the prodrug to the parent drug during its permeation of skin. Based on these analyses, it becomes obvious that to optimize the delivery of the parent drug (and/or intact prodrug) it is essential to pursue a design process that can result in a prodrug that will exhibit increased aqueous as well as increased lipid solubility. Examples of

design processes that have identified prodrugs exhibiting increased aqueous and lipid solubility include synthesizing and evaluating (a) the shorter alkyl chain and more water-soluble members of homologous series of prodrugs instead of only the longer chain and more lipid-soluble ones and (b) prodrugs containing water-solubilizing functional groups such as tertiary amine, tertiary amide and polyoxyethylene groups in the promoiety.

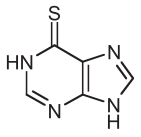
Besides a qualitative approach based on identifying "the more water-soluble members of a more lipophilic series of prodrugs" (Sloan, 1989, 1992) to optimize flux, quantitative analyses of the available data provide a good basis for predicting flux from different vehicles. The Roberts-Sloan equation derived from a rather large (now n = 63, Table 1) database shows that flux from suspensions of prodrugs and parents from IPM can be predicted from $\log J_{IPM} = -0.502 + 0.517 \log S_{IPM} + 0.483 \log S_{4.0} - 0.00266$ MW. In addition, although the database is small (n = 18), the flux of prodrugs and parents from suspensions in water can be predicted from the analogous equation: $\log J_{AQ} = -1.497 + 0.66 \log S_{IPM} + 0.34 \log S_{AQ} - 0.00469$ MW (Sloan *et al.*, 2003). Equally important, the rank order of flux values within each series was the same regardless of the vehicle. Properties that enhance delivery from IPM enhance delivery from water as well. Finally, the reason for the importance of both aqueous and lipid solubilities lies in the structure of the skin and the pathways for its permeation. Analysis of the original database for the Roberts-Sloan equation, but fitting the data to the series-parallel equations instead, showed that the high capacity pathway for permeation of the skin is the series pathway (Roberts and Sloan, 2000). Diffusion across alternating lipid and aqueous phases in the intercellular barrier to permeation demands solubility in both phases.



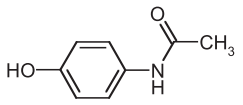
Theophylline
(Th)



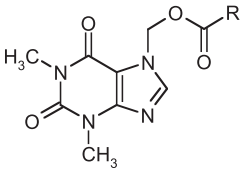
5-Flurouracil
(5-FU)



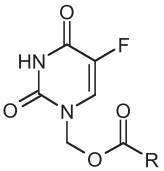
6-Mercaptopurine
(6-MP)



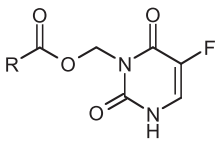
Hydroxyacetanilide
(APAP)



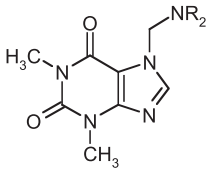
7-ACOM-Th
1 - 6



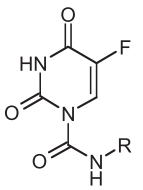
1-ACOM-5-FU
7 - 14



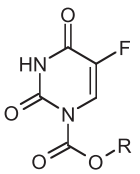
3-ACOM-5-FU
15 - 20



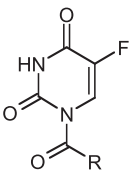
7-AAM-Th
21 -28



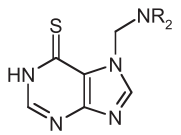
1-AAC-5-FU
35 - 40



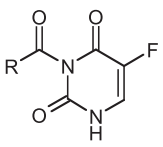
1-AOC-5-FU
41 - 46



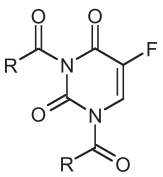
1-AC-5-FU
47 -52



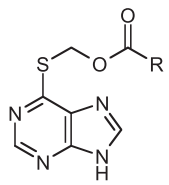
7-AAM-6-MP
29 -34



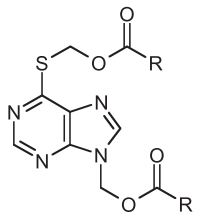
3-AC-5-FU
53 - 56



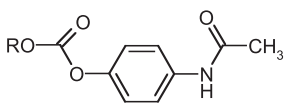
bis-1,3-AC-5-FU
57 - 58



6-ACOM-6-MP
59 - 64

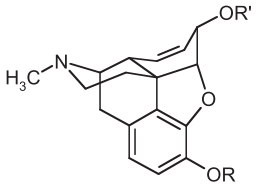


bis-6,9-ACOM-6-MP
65 - 69



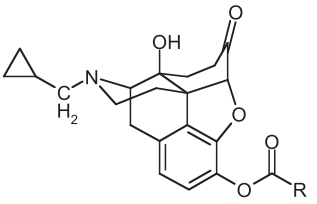
4-AOC-APAP
82 - 88

Representative Structures for compounds 1-69 and 82-88.



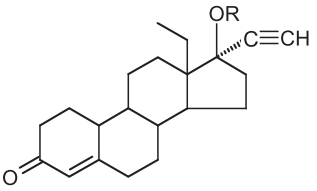
bis-3,6 and 3-AC-MOR

70 - 74

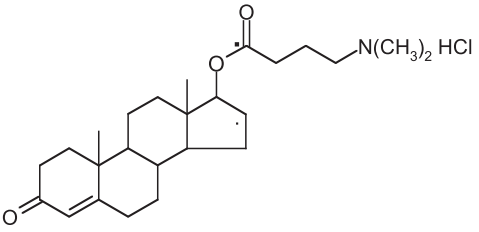


3-AC-NTX

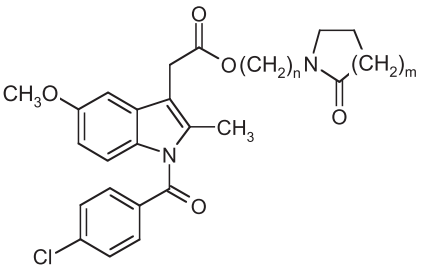
76 - 81



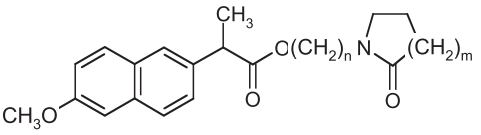
89 - 93



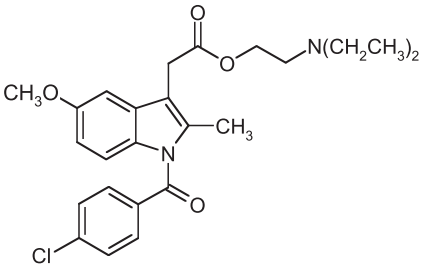
94



96 - 99 (n = 2)

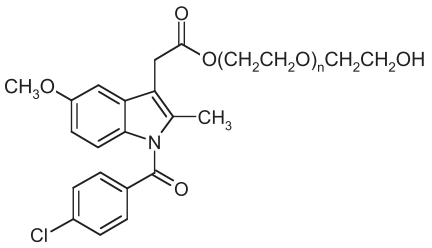


101 - 104 (n = 2)



127

Representative Structures for compounds 70-74, 76-81, 89-93, 94, 96-99, 101-104 and 127.



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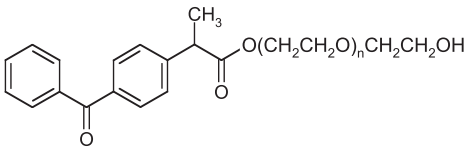

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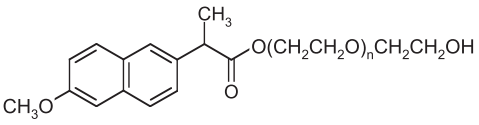

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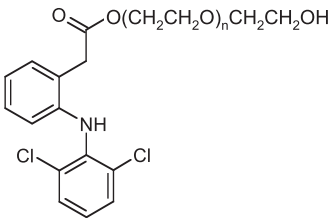

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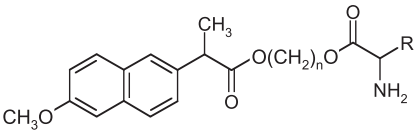

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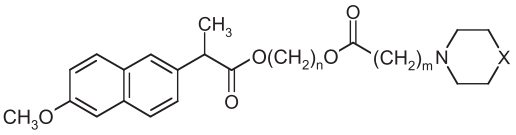

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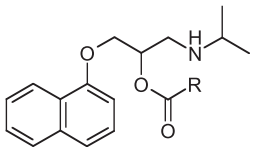

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Representative Structures for compounds 105-109, 110-114, 116-120, 122-126, 128-131, 132-136 and 138-142.

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2.1.3

Prodrug approaches to ophthalmic drug delivery

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Table of Contents

List of Abbreviations

AUC .....area under the curve
CAI .....carbonic anhydrase inhibitor
CD .....cyclodextrin
D .....distribution coefficient
HP .....hydroxypropyl
HSV .....herpes simplex virus
IOP .....intraocular pressure
P_{app} .....apparent partition coefficient
PG .....prostaglandin
PVA .....polyvinyl alcohol
SBE .....sulfobutyl ether

Introduction

Ophthalmic medications are administered either systematically or locally to the eye. In order to treat ocular disorders, the topical delivery of eye drops into the lower cul-de-sac of the eye is preferred since the drug effects are localized and less drug enters into the systemic circulation (i.e., systemic side effects are minimized). In addition, adequate ocular drug concentrations may be difficult to achieve with systemic administration, as the blood-ocular barrier effectively restricts the access of drugs from systemic circulation into intra-ocular targets.

Unfortunately, even after instillation of an eyedrop, typically less than 5% of the applied dose reaches the intraocular tissues. The main reasons for this low ocular drug availability are poor drug permeation across the corneal barrier and a rapid loss of the instilled solution from the precorneal area (Lee and Robinson, 1979). Various drug delivery approaches have been investigated to increase the ocular bioavailability of topically administered drugs (Table 1). Traditional methods of improving ocular bioavailability increase the ocular contact time of the drug (viscous solutions, suspensions, ointments, gels, thermosetting gels, polymeric inserts, micro- and nanoparticles, and liposomes). Chemical approaches, such as prodrugs, aim to enhance the ocular bioavailability by improving physicochemical properties of a drug molecule, with the goal of improving drug permeation across the cornea.

OPHTHALMIC DRUG DELIVERY STRATEGIES		
Chemical technologies	**Formulation strategies**	**Physical approaches**
Prodrug technology Cyclodextrin technology Soft drugs	Penetration enhancers Emulsifiers / liposomes Suspensions Micro- and nanoparticles Bioadhesive hydrogels Ocular inserts	Iontophoresis Phonophoresis

Table 1. Various strategies that are used to increase bioavailability of the ophthalmic drugs (Järvinen and Järvinen, 1996; Bourlais *et al.*, 1998; Loftsson and Järvinen, 1999; Sasaki *et al.*, 1999; Bodor and Buchwald, 2000; Duvvuri, 2003).

The concept of prodrugs was first introduced to ophthalmology about 30 years ago with the introduction of dipivefrin (Hussain and Truelove, 1976). Since then, numerous other prodrugs have been designed to improve the ophthalmic bioavailability of various drug molecules, prolong duration of action, improve formulation properties or reduce systemic side-effects.

Specific Considerations for Ocular Prodrug Design

Topical delivery of eyedrops into the lower cul-de-sac is the most common method of drug treatment in ocular diseases. In topical administration, the aqueous eyedrop solution mixes with tear fluid and is dispersed over the eye surface. However, various precorneal factors, such as drainage of the instilled solution, non-corneal absorption, and induced lacrimation limit ocular absorption by shortening the cornea contact time of the applied drug. These factors, and the corneal barrier itself, limit permeation of topically administered ophthalmic drugs. As a result, only a few percent of the applied drug dose is actually delivered into the intraocular tissues, and the major part (50–99%) is absorbed into the systemic circulation (Järvinen *et al.*, 1995), which can cause various side effects.

Corneal Barrier

The cornea is generally considered to be a major, but not exclusive, pathway for ocular permeation of a topically applied drug (Doane *et al.*, 1978). Compared to many other epithelial tissues (e.g., bronchial, intestinal, nasal, tracheal), the corneal epithelium is relatively impermeable but less so than the stratum corneum of the skin (Rojanasakul *et al.*, 1992). The conjunctiva, which is mainly responsible for the non-corneal absorption of drugs, is significantly more permeable than the cornea (Ahmed *et al.*, 1987; Wang *et al.*, 1991), but blood circulation removes the drug from this superficial tissue before it can enter the deeper ocular tissues.

Although the cornea is composed of five layers, the outer epithelium and stroma are the most significant for drug delivery (Huang *et al.*, 1983). The lipophilic epithelium is the primary barrier for corneal permeation of highly hydrophilic drugs, whereas the partitioning from the epithelium to the hydrophilic stroma is a rate-limiting barrier for highly lipophilic compounds.

Physicochemical Properties

Lipophilic drugs permeate the corneal epithelium *via* the transcellular pathway while hydrophilic molecules utilize the paracellular route; the latter process involves passive or altered diffusion through intercellular spaces. Passive diffusion along a concentration gradient, which is largely influenced by various physicochemical properties, is the main corneal permeation mechanism for most topically applied drugs.

Drug lipophilicity is the most important property for corneal permeation, and both parabolic (Schoenwald and Ward, 1978; Chien *et al.*, 1991) and sigmoidal (Wang *et al.*, 1991) curves have been used to describe their relationship. The optimum apparent partition coefficient (P_{app}; octanol/pH 7.4 buffer) for corneal drug absorption is in the range of 100–1000 (log P_{app} 2–3) (Schoenwald and Ward, 1978; Schoenwald and Huang, 1983), indicating that the absorption of moderately lipophilic compounds is favoured. In the case of prodrugs, it is more complicated to estimate the optimal P_{app} value for corneal permeability because their corneal permeability depends on the lipophilicity of both the prodrug and

parent drug and also on the conversion rate of prodrug to the parent drug *in vivo*.

Aqueous solubility is another important physicochemical property for efficacious ophthalmic delivery. The surface of the eye is constantly being cleaned and moistened by the aqueous tear fluid. Thus, it is difficult for drug molecules to be absorbed by the corneal epithelium without being soluble in the tear film. In addition, the water solubility of the drug must also be sufficient to enable the formulation of aqueous eyedrops. The dilemma here is that an ideal ophthalmic drug should simultaneously be both water-soluble and lipid soluble, but only a few molecules are known to fulfill these criteria.

In addition to the lipophilicity and aqueous solubility of a drug, molecular size (Liaw and Robinson, 1992), charge (Liaw *et al.*, 1992), and degree of ionization (Maren and Jankowska, 1985; Brechue and Maren, 1993) also affect corneal penetration. Tear fluid has a limited buffering capacity (Carney and Hill, 1979). Thus, pH and buffering capacity of the instilled eyedrops affect the pH of tear fluid and, consequently, drug ionization in the precorneal area. The non-ionized form of a drug usually permeates the cornea more easily than does the ionized form, so both the pH and buffering capacity of instilled eyedrops can have a significant effect on ophthalmic drug absorption.

Drugs developed for ophthalmic use should have good chemical stability to allow formulation into topical ready-to-use aqueous eyedrops, which is the most practical and commonly used administration route for treatment of ophthalmic diseases. This is often challenging in the development of ophthalmic prodrugs that are intended to be rapidly converted to active drug after absorption. Therefore, only those prodrugs that show good chemical stability combined with high enough enzymatic lability can be easily developed without resorting to multivial reconstitutable products.

Criteria for an Ideal Ophthalmic Prodrug

The major goal in designing prodrugs is to overcome various physico-chemical, biopharmaceutical, and/or pharmacokinetic problems that may be associated with the parent drug molecules, which would otherwise be of limited clinical use. The most common barriers in ophthalmic drug formulation and delivery that may be overcome by a prodrug are:

1) Low aqueous solubility
   - which prevents the development of aqueous eye-drops
2) Low lipid solubility
   - which results in low corneal permeation and low ophthalmic bioavailability
3) Short duration of action (due to rapid drug elimination from the site of action)
   - which necessitates frequent administration of a drug and often leads to poor patient compliance
4) Systemic side effects (due to low corneal and high systemic absorption)
   - which may lead to safety concerns and also poor patient compliance

A successful ophthalmic prodrug strategy requires that the reasons for developing a prodrug are clearly defined and the limitations for a clinically acceptable prodrug are understood. In general, ophthalmic prodrugs should fulfill at least most of the following criteria:

1) Adequate chemical stability
   - providing sufficient shelf life in aqueous eye-drops
2) Adequate aqueous solubility
   - allowing preparation of aqueous eye-drops
3) Optimal lipophilicity
   - confirming efficient corneal absorption before precorneal drug removal
   - possessing a log P_{app} value of 2–3 for optimal corneal permeation
4) The prodrug must release the parent drug within the eye at a reasonable rate
   - the bioconversion rate of the prodrug should be fast enough to minimize the elimination of intact prodrug from the site of action
   - the bioconversion rate of the prodrug should be slow enough if prolonged duration of action is the goal
5) Neither the prodrug nor the released promoiety should be toxic or irritating.

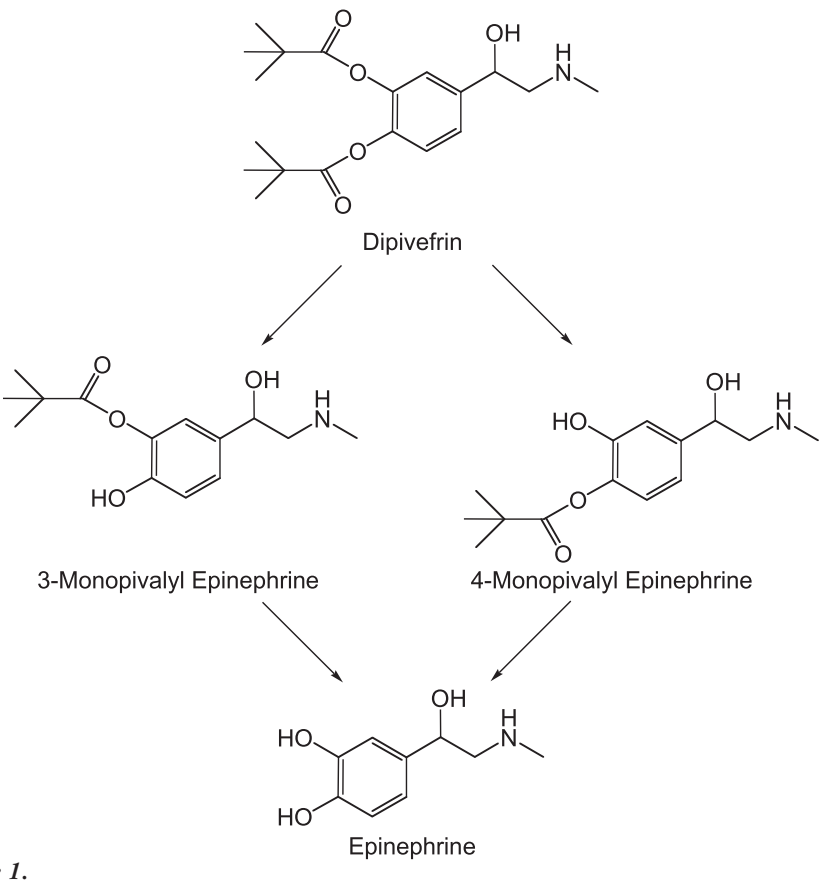
In summary, the design of ophthalmic prodrugs not only includes the optimization of physicochemical properties (e.g., lipid and aqueous solubilities, chemical stability) but should also consider the evaluation of enzymatic degradation properties within the cornea and intraocular tissues. When local treatment is the aim, the active parent drug should be released during permeation or soon afterward.

Selected Case Studies of Ophthalmic Prodrugs

Prodrugs of Adrenergic Agonists

Dipivefrin

Prodrugs were introduced to ophthalmology nearly 30 years ago, when the corneal permeation of epinephrine was improved by using the prodrug approach (Hussain and Truelove, 1976). Dipivefrin, a pivalic acid diester of epinephrine, is 600 times more lipophilic (at pH 7.2) than epinephrine (Wei *et al.*, 1978), and is able to permeate the human cornea 17 times faster than epinephrine (Mandell and Stentz, 1978). After absorption, dipivefrin is hydrolyzed to epinephrine by esterase activity (Scheme 1), and the major site for this hydrolysis is the cornea. Most of dipivefrin appears as epinephrine and metabolites of epinephrine within 15 minutes after topical administration (Hussain and Truelove, 1976; Anderson *et al.*, 1980; Redell *et al.*, 1983). As a consequence of enhanced ocular absorption, a smaller topical dose of dipivefrin is required to achieve a therapeutic effect comparable to that of epinephrine in the eye (Kaback *et al.*, 1976). Since the



Scheme 1.

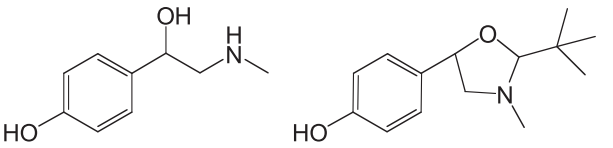
fraction of the dose of both epinephrine and dipivefrin that is absorbed systemically after ocular administration is within a similar range (55% and 65%, respectively) (Anderson, 1980), the smaller prodrug dose results in lower systemic levels of epinephrine. A 0.1% dipivefrin eyedrop results in only a slightly less effective intraocular pressure (IOP) lowering effect compared to the conventional 2% epinephrine hydrochloride eyedrop, while its systemic side effects are greatly reduced compared to those of epinephrine (Kohn *et al.*, 1979).

Phenylephrine Prodrugs

Phenylephrine (**1**) is an α-adrenergic agent that is used clinically for pupil dilation either in eye examinations or in ocular surgery. It has a low ocular bioavailability due to its hydrophilic nature (log $D_{oct}^{7.4} = -1.89$) (Schoenwald *et al.*, 1987) and, therefore, concentrated eyedrops (up to 10%) are required to produce mydriasis. The large topical dose of phenylephrine may cause adverse systemic side effects, such as severe hypertension, ventricular arrhythmia, and possible myocardial infarction (Miller-Meeks *et al.*, 1991 and references cited therein).

Phenylephrine oxazolidine (**2**) is a lipophilic (log $D_{oct}^{7.4} = 1.38$) phenylephrine prodrug (Chien and Schoenwald, 1986), which is converted to phenylephrine in

aqueous solution at pH values between 1 and 7.4 ($t_{1/2}$ = 6–13 min). Thus, phenylephrine oxazolidine eyedrops must be formulated in a non-aqueous solution (sesame oil). Compared to 10% phenylephrine eyedrops, 10% oxazolidine prodrug eyedrops increased phenylephrine levels in aqueous humor by 6–8-fold and improved mydriatic activity 4-fold in rabbits (Schoenwald and Chien, 1987; Chien and Schoenwald, 1990). In monkeys, 1% phenylephrine oxazolidine increased mydriatic response and decreased the plasma levels of phenylephrine 3.5-fold compared to 10% phenylephrine (Schoenwald *et al.*, 1987). The lower plasma levels would be expected to result in fewer systemic side effects. In a clinical trial with healthy subjects, 1% phenylephrine oxazolidine produced greater pupillary dilation than 10% phenylephrine (Miller-Meeks *et al.*, 1991). Despite these encouraging results, phenylephrine oxazolidine was not advanced to additional clinical trials.



phenylephrine, **1** phenylephrine oxazolidine, **2**

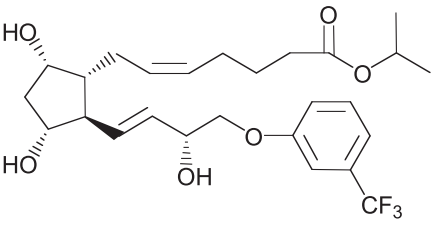
Structures 1-2.

The phenylephrine pivalate prodrug showed 15 times higher mydriatic activity in rabbits than phenylephrine itself (Yuan and Bodor, 1976). However, the pivalate prodrug also has an intrinsic activity about half that of phenylephrine, which has raised the question of whether its action is due to its intrinsic activity or if it is actually behaving as a prodrug (Mindel *et al.*, 1980).

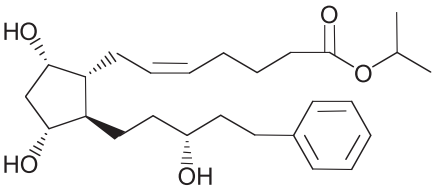
Prostaglandins

The most recent achievements for prodrug technologies in ophthalmology have been with prostaglandin prodrugs for the reduction of IOP in patients with open angle glaucoma or ocular hypertension. The first prostaglandin prodrug, latanoprost (**3**) (Xalatan®), was approved by the FDA in 1996, followed by bimatoprost (**4**) (Lumigan®), travoprost (**5**) (Travatan®) and unoprostone isopropyl (**6**) (Rescula®) in the early 2000s. Although some of these compounds are reviewed in case histories at the end of this book, a brief introduction is also given in this chapter.

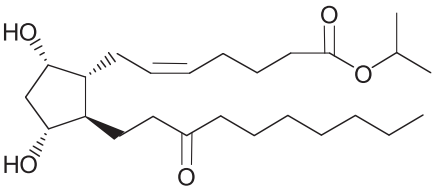
The starting point of the development of prostaglandin (PG) analogs goes back to mid 1970s, when it was noticed that low topical doses of PGs decreased IOP, which was contradictory to the previous findings that high doses of PGs increased IOP (Bito *et al.*, 1983 and references cited therein; Lee *et al.*, 1984). Although many PGs decreased IOP, major attention was focused on $PGF_{2\alpha}$ and its analogs. $PGF_{2\alpha}$ (**7**) is an effective ocular hypotensive agent in humans (Giuffre, 1985; Lee *et al.*, 1988), but it is not clinically acceptable due to its local adverse



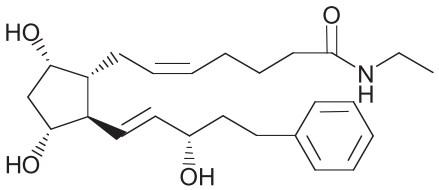
travoprost, **3**



latanoprost, **4**



unoprostone isopropyl, **5**



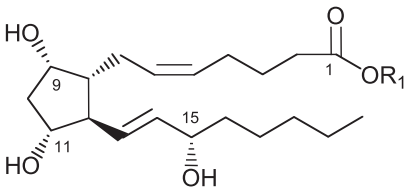
bimatoprost, **6**

Structures 3-6.

effects, i.e., conjunctival hyperemia and irritation (Bito, 1987 and references cited therein). Thus, the main objective of $PGF_{2\alpha}$ research became an effort to design a non-irritating ophthalmic drug delivery system. To achieve this, two approaches were adopted (Woodward *et al.*, 1994). One approach was to increase the ocular absorption of $PGF_{2\alpha}$ by making a prodrug and thereby reduce the exposure of the drug on the ocular surface. The other approach was based on the design of more selective prostaglandin analogs for the prostaglandin $F_{2\alpha}$ receptor.

The first $PGF_{2\alpha}$ prodrugs involved esterification of the 1-carboxylic acid group. $PGF_{2\alpha}$-1-methyl (**9**) and 1-benzyl (**10**) esters were 2–3 orders of magnitude more lipophilic than $PGF_{2\alpha}$ and enhanced the corneal permeability of $PGF_{2\alpha}$ 25–40-fold *in vitro*. $PGF_{2\alpha}$-esters were hydrolyzed upon passage through the cornea, and subsequent hydrolysis studies in corneal homogenates have indicated

that the main site of hydrolysis is the corneal epithelium (Camber *et al.*, 1986). Compared to the ocular administration of $PGF_{2\alpha}$ itself, $PGF_{2\alpha}$-1-esters increased the concentration of $PGF_{2\alpha}$ in intraocular tissues (e.g., aqueous humor, ciliary body, iris) and improved its ocular hypotensive potency (Bito and Baroody, 1987; Villumsen *et al.*, 1989). Despite the fact that $PGF_{2\alpha}$-1-esters improved bioavailability of $PGF_{2\alpha}$, they did not appear to result in a clinically acceptable ophthalmic drug due to ocular side effects (Camras *et al.*, 1989).



7 $PGF_{2\alpha}$; R= H
8 $PGF_{2\alpha}$-1-isopropylester; R= $CH(CH_3)_2$
9 $PGF_{2\alpha}$-1-methylester; R= CH_3
10 $PGF_{2\alpha}$-1-benzylester; R= CH_2Ph

Structures 7-10.

A series of $PGF_{2\alpha}$-esters and -diesters having acyl group(s) at the 9, 11 and 15 positions represents an alternative strategy that has been explored to circumvent the ocular surface side effects of $PGF_{2\alpha}$ (Cheng-Bennett *et al.*, 1994; Woodward *et al.*, 1994) . The $PGF_{2\alpha}$-9-acyl esters did not prove to be prodrugs as they were not able to release $PGF_{2\alpha}$. $PGF_{2\alpha}$-15-pivaloyl ester hydrolyzed to $PGF_{2\alpha}$ about one order of magnitude faster than $PGF_{2\alpha}$-11-pivaloyl ester in all ocular tissues. Interestingly, the $PGF_{2\alpha}$-1-isopropyl ester (**8**) was rapidly hydrolyzed in corneal epithelium, compared to 11- and 15-pivaloyl esters. All the prodrugs that converted to $PGF_{2\alpha}$ decreased IOP in rabbits. $PGF_{2\alpha}$-11-acyl-, $PGF_{2\alpha}$-15-acyl- and $PGF_{2\alpha}$-11,15-diacyl esters seemed promising, as they lowered IOP efficiently with mild ocular side effects. These compounds had corneal permeabilities about 20-fold higher than $PGF_{2\alpha}$ but still 4-fold lower than the $PGF_{2\alpha}$-1-isopropyl ester (Chien *et al.*, 1997). The cited investigators reasoned that results from the 11- and 15-acyl esters indicated a way to separate the hypotensive and ocular surface hyperaemic effects of $PGF_{2\alpha}$ due to their more favourable enzymatic hydrolysis profiles (i.e., slower conversion rates in epithelium and higher conversion rates in anterior segment tissues).

Internal esters of $PGF_{2\alpha}$ have also been investigated (Woodward and Chan, 1992; Cheng-Bennett *et al.*, 1994; Woodward *et al.*, 1994; Chien *et al.*, 1997). $PGF_{2\alpha}$-1,11-lactone and $PGF_{2\alpha}$-1,15-lactone both decreased IOP in rabbits, but only $PGF_{2\alpha}$-1,11-lactone lowered IOP without substantial ocular side effects. Although the corneal permeabilities of lactones are relatively high, their slow bioconversion seems to result in poor bioavailabilities of $PGF_{2\alpha}$.

The prodrug approach alone was not able to provide a satisfactory solution for increasing the therapeutic index of $PGF_{2\alpha}$. Consequently, several synthetic approaches were adopted to modify the selectivity of the $PGF_{2\alpha}$ backbone (e.g.,

Resul *et al.*, 1993). As a result of these approaches, none of the currently available prostaglandin prodrugs has the exact $PGF_{2\alpha}$ backbone. Although these prostaglandin analogs have differences in their selectivities toward PG-receptor subtypes (Sharif *et al.*, 2003), they all have similar kinds of side effects and are closely comparable in their ability to reduce IOP (Kaufman, 2003; Parrish *et al.*, 2003). The design of new prostaglandin analogs having different selectivities continues (e.g. Liljebris *et al.*, 1995; Woodward *et al.*, 2000; Hellberg *et al.*, 2002).

Three of the current prostaglandin analogs that are approved (latanoprost, travoprost, unoprostone isopropyl) are clearly prodrugs, as they are isopropyl esters of their parent acids. Bimatoprost is an N-ethyl amide of bimatoprost acid, and it has been reported to be pharmacologically unique in that it has no meaningful activity on prostanoid receptors (Woodward *et al.*, 2001, 2003). Therefore, it has been postulated to act on yet uncharacterized prostamide receptors. However, several studies have shown that bimatoprost is hydrolyzed in ocular tissues to bimatoprost acid (Maxey *et al.*, 2002; Hellberg *et al.*, 2003), which has been reported to be a prostanoid FP receptor agonist (Sharif *et al.*, 2003). Therefore, in the light of the current knowledge, bimatoprost is also a prodrug.

Prodrugs of β-Adrenergic Antagonists

β-Adrenergic antagonists (β-blockers) are effective ocular hypotensive agents that act by decreasing the formation of aqueous humor (Sugrue, 1989). Timolol, a nonselective β-adrenergic antagonist, was introduced in 1978 for the treatment of glaucoma. Today, timolol is one of the most frequently prescribed drugs for this disease. In addition to timolol, betaxolol, carteolol, levobunolol and metipranolol are currently in clinical use. Additionally, various other β-blockers, such as atenolol, labetalol, metoprolol, nadolol, pindolol, and propranolol, have been evaluated for their topical ocular hypotensive activities.

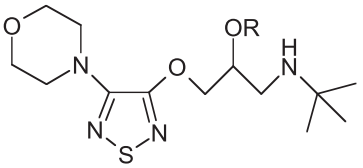
The clinical acceptance of ophthalmic β-blocker therapy, especially with a nonselective β-blocker, is usually limited by systemic side effects. For example, timolol eyedrops have caused serious cardiovascular, respiratory, and central nervous system side effects (Nelson *et al.*, 1986). β-Blockers are only moderately lipophilic. Thus, more lipophilic prodrugs have been studied to enhance their ocular absorption. Enhanced ocular absorption would allow the use of smaller topical doses, which would decrease systemic absorption and thus the risk of systemic side effects.

Timolol Prodrugs

Timolol is a base with a pK_a value of 9.2 (Schoenwald and Huang, 1983). At physiological pH, 98% of timolol is protonated, and it therefore shows low lipophilicity with a $\log D^{7.4}_{oct}$ of –0.04 (Bundgaard *et al.*, 1988a). Due to its low lipophilicity, less than 5% of the instilled timolol dose gains access to internal eye structures (Urtti *et al.*, 1990).

Various alkyl, cycloalkyl, and aryl esters and a carbamate ester have been synthesized by esterifying the hydroxyl group of timolol (Table 2) (Bundgaard *et al.*, 1986a,1988a,b). All studied prodrugs were more lipophilic than timolol, and a parabolic dependence of corneal drug permeation on drug lipophilicity was observed (Chang *et al.*, 1987; Chien *et al.*, 1991). These prodrugs converted to timolol within the eye. Compared to an equivalent timolol dose, *O*-butyryl timolol increased the corneal absorption of timolol in rabbits by four to six times, but did not affect systemic absorption of timolol *via* the nasal mucosa and conjunctiva of the eye (Chang *et al.*, 1988a,b). These results are consistent with a study showing that corneal drug permeation is more sensitive to changes in drug lipophilicity than is conjunctival permeation (Wang *et al.*, 1991). Compared to a 15.0 μM timolol solution, 3.75 μM O-butyryl timolol increased timolol concentration in aqueous humor and decreased systemic absorption of timolol by 10-fold. Thus, *O*-butyryl timolol improved the therapeutic index of timolol by 15-fold, as assessed by the ratio of aqueous humor to plasma concentrations. *O*-butyryl timolol also showed a prolonged duration of action in rabbits (Potter *et al.*, 1988).

Alkyl, cycloalkyl, and aryl ester-prodrugs of timolol were hydrolyzed at about equal rates in plasma and phosphate buffer, making it difficult to design a



Compound	R	Log $D_{oct}^{7.4}$ [a]	P_{app} (10^5 cm s^{-1})[b]
Timolol	-H	–0.04	1.13 ± 0.19
O-Acetyl timolol	$-COCH_3$	1.12	2.31 ± 0.48
O-Propionyl timolol	$-CO(CH_2)_2CH_3$	2.08	3.23 ± 0.45
O-Pivaloyl timolol	$-COC(CH_3)_3$	2.68	1.36 ± 0.61
O-Octanoyl timolol	$-CO(CH_2)_6CH_3$	4.66	0.89 ± 0.19
O-Cyclopropanoyl timolol	—CO—	1.74	4.15 ± 0.31
O-Cyclohexanoyl timolol	—CO—	3.30	0.68±0.10
O-Benzoyl timolol	—CO—	2.55	1.68±0.25

Table 2. Structures, lipophilicities and corneal permeability coefficients of timolol and its ester prodrugs.

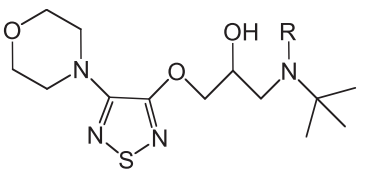
[a]Bundgaard *et al.*, 1988a

[b]Corneal permeability coefficient (mean ± SE, n = 4–6) (Chien *et al.*, 1991)

prodrug that quickly converts to an active drug *in vivo* and has a sufficient shelf life. Thus, the poor aqueous stability of these prodrugs limits their clinical usefulness, although they show good biopharmaceutical properties (Bundgaard *et al.*, 1988a).

A series of amphiphilic timolol ester prodrugs with long aliphatic chains (C8-C16) have been tested in rabbits to antagonize isoprotenol-induced hypotension (Pech *et al.*, 1993). *O*-palmitoyl (C16) timolol was the most amphiphilic/lipophilic compound in the series and showed the highest activity. This prodrug was hydrolyzed completely in the cornea *in vitro*. Interestingly, the corneal permeability coefficient of *O*-palmitoyl timolol (0.42×10^{-6} cm/s) was lower than that of timolol (2.2×10^{-6} cm/s). Based on its high lipophilicity, the corneal uptake of this prodrug can be assumed to be effective. Therefore, the low ability to permeate (measured by the appearance of timolol in the receiver compartment) may reflect slow enzymatic hydrolysis of the prodrug in the cornea. In hypertensive rabbits, IOP reduction with *O*-palmitoyl timolol was significantly greater and of longer duration than that produced by timolol alone. According to Pech *et al.* (1993), trans-scleral absorption of *O*-palmitoyl timolol might explain its greater IOP lowering effect, and the prolonged action is probably due to accumulation of the prodrug in the corneal epithelium.

(Acyloxyalkyl)carbamates represent an alternative approach to modify the physicochemical properties of timolol, where prodrug derivatization has been accomplished on the secondary amine group of timolol (Alexander *et al.*, 1988). The *N*-(acetoxymethoxy)carbonyl prodrug of timolol (**11**) hydrolyzed 100–500 times faster in plasma than in a buffer solution (pH 7.4) at 37°C. The shelf life in aqueous solutions (4°C, pH 4) was estimated to be 3–5 years. The esterase-mediated hydrolysis of this prodrug leads to a hemiacetal, which spontaneously decomposes to regenerate the parent timolol in an aqueous environment through the intermediacy of carbamic acid. The side-products in this activation are acetic acid, formaldehyde, and carbon dioxide. A fivefold enhancement in the *in vitro* corneal permeation of timolol was reported for this prodrug. However, the likely disadvantage to this approach is that the hydrolysis in phosphate buffer led to the formation of oxazolidine derivative, which was found to be resistant to enzymatic hydrolysis in separate experiments.



11

N-(Acetoxymethoxy)carbonyl timolol
R=-CO-O-CH_2-O-CO-CH_3

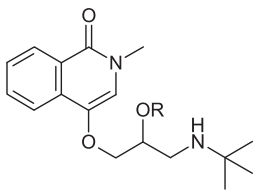
Structure 11.

Tilisolol Prodrugs

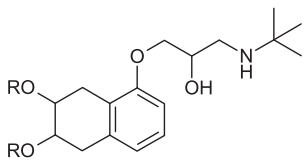
Tilisolol (**12**) is a nonselective, hydrophilic (log $D_{oct}^{7.4}$ = –0.27) β-blocker. Like most other β-blocker prodrugs, tilisolol prodrugs are esters that are more lipophilic than the active drug itself. The first reports of tilisolol prodrugs described short carbon-chain ($C_{2\text{-}5}$) esters (**13–16**) (Sasaki *et al.*, 1993). The corneal permeation of these prodrugs *in vitro* was 3–6-fold higher than that of tilisolol. Prodrug derivatization did not affect the conjunctival and scleral permeation of tilisolol. A linear relationship was found between the aqueous humor concentrations of tilisolol after prodrug instillation and the corneal permeability coefficients of the prodrugs *in vitro*, which suggests that the tilisolol prodrugs are absorbed into the eye *via* the corneal route.

The *O*-palmitoyl ester (C_{16}) of tilisolol (**17**) is an amphiphilic prodrug that delivers tilisolol into the iris-ciliary body, which is the target tissue for β-blockers to treat glaucoma at significantly higher concentrations compared to tilisolol itself (Kawakami *et al.*, 2001a). It is apparent that the palmitoyl ester has enhanced affinity for hydrophobic ocular tissues (such as cornea), which causes a retardation of drug action. The *in situ* absorption experiments suggested that the enhanced ocular bioavailability of this prodrug was due to increased retention in the precorneal area. In addition, the blood concentrations of palmitoyl tilisolol and tilisolol after prodrug instillation were greatly reduced compared to tilisolol instillation.

The *O*-butyryl and *O*-palmitoyl esters of tilisolol have also been evaluated for their ability to deliver tilisolol into the eye from ophthalmic inserts (Kawakami *et al.*, 2001b). The *O*-butyryl prodrug incorporated into inserts performed better in this application, providing a ratio of the AUC in aqueous humor to the AUC in plasma 3.1-fold higher than tilisolol inserts.



Tilisol **12** R = H
Tilisol prodrugs: **13** R = -$COCH_3$
14 -$COCH_2CH_3H$
15 -$CO(CH_2)_2CH_3$
16 -$CO(CH_2)_3CH_3$
17 -$CO(CH_2)_{14}CH_3H$



Nadolol **18** R = H
Nadolol prodrug **19** R = -$COCH_3$

Structures 12-19.

Nadolol Prodrugs

Nadolol (**18**) is a hydrophilic (log $D_{oct}^{7.4}$ = –0.82, pK_a 9.39), nonselective β-blocker (Schoenwald and Huang, 1983). The low corneal permeation of nadolol is probably the main reason for its poor clinical efficacy. Diacetyl nadolol (**19**) is about 20 times more lipophilic than nadolol, and this prodrug enhanced the *in*

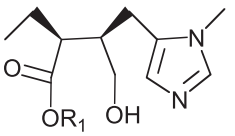
vivo ocular absorption of nadolol in rabbits by about 10-fold compared to nadolol (Duzman *et al.*, 1982). Despite the improved ocular absorption of nadolol with a prodrug, both diacetyl nadolol and nadolol (equal doses) decreased human IOP to a similar extent in a single-dose study. However, in a 3-month clinical study, diacetyl nadolol maintained its IOP reducing effect, while nadolol had no long-term ocular hypotensive effect (Duzman *et al.*, 1983).

Pilocarpine Prodrugs

Pilocarpine is a direct-acting cholinergic agonist that is used to control the elevated IOP associated with glaucoma. Pilocarpine shows a low ocular bioavailability (1–3% of instilled dose) due to poor absorption into the cornea coupled with a short duration of action. Because of these drawbacks, pilocarpine is generally instilled 3–4 times per day, which results in undesirable side effects and poor patient compliance. The ocular absorption of pilocarpine is mainly limited by its low lipophilicity (log P_{app} = –0.15) (Bundgaard *et al.*, 1986b). Thus lipophilic prodrugs have been studied to improve both the ocular absorption and delivery of pilocarpine.

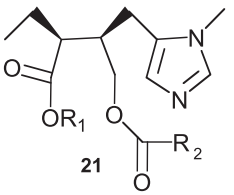
Pilocarpic Acid Mono- and Diesters

Bundgaard and co-workers (1985,1986b,c) developed a series of mono- and diester prodrugs from pilocarpic acid. Pilocarpic acid monoesters (**20**) underwent spontaneous lactonization to pilocarpine in aqueous solution. Monoesters were more lipophilic than pilocarpine and increased the ocular bioavailability of pilocarpine using miosis as a bioassay indicator (Mosher *et al.*, 1987). Several monoesters prolonged the duration of action by 1.5-fold compared to pilocarpine. The usefulness of the monoesters is, however, limited by their poor aqueous stability.



20

Pilocarpic acid monoester



21

Pilocarpic acid diester

Structures 20-21.

The stability problem of pilocarpic acid monoesters was overcome by producing the pilocarpic acid diesters (**21**) by esterification of the remaining pilocarpic acid monoester hydroxyl group (Bundgaard *et al.*, 1986c). The resulting diesters were highly stable in aqueous solution because the first step in metabolic degradation of the diester is *via* enzymatic hydrolysis. The shelf-lives ($t_{90\%}$) for these diesters in aqueous solutions were more than 5 years (pH 5–6, 25°C) (Bundgaard *et al.*, 1985). The half-times for the enzymatic conversion of various

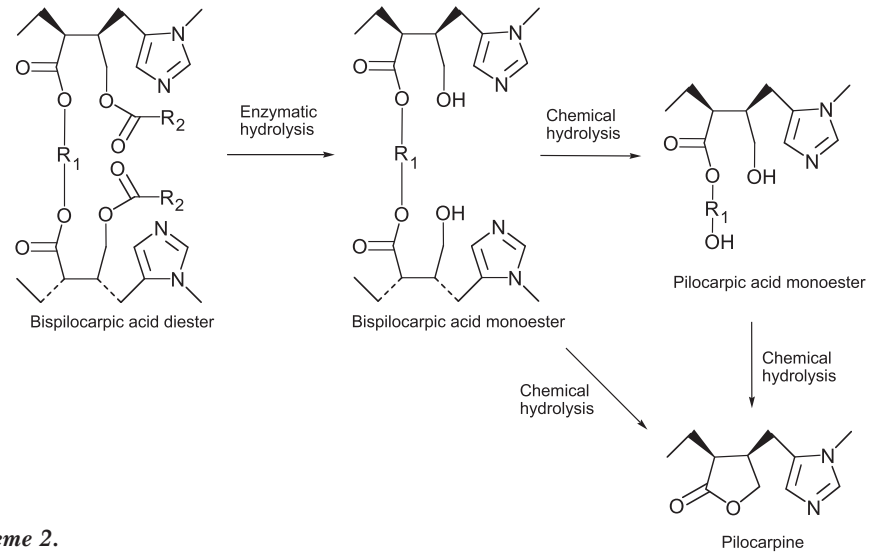
diesters to their corresponding monoesters varied from 3 min to 330 min in 75% human plasma solutions (pH 7.4) at 37ºC (Bundgaard *et al.*, 1986c). Compared to pilocarpine solution (0.5%), adequately lipophilic diesters (eq. to 0.25% pilocarpine) that had a favourable rate of conversion from the diester to the monoester and, subsequently, to pilocarpine also had a prolonged (2.25-fold) duration of miotic action in rabbits (Mosher *et al.*, 1987). The disadvantages of these effective pilocarpine diester derivatives are significant ocular irritation and a low aqueous solubility at pH values higher than 4.5 (Mosher, 1986). However, O-benzoyl pilocarpic acid methyl ester (pilocarpic acid diester) decreased IOP significantly in both normotensive and glaucomatous monkeys without significant eye irritation (Weinkam *et al.*, 1990).

Bispilocarpic Acid Mono- and Diesters

Bispilocarpic acid mono- and diesters are dimeric pilocarpine prodrugs with two pilocarpine units attached *via* a carbon chain, which minimizes the molecular weight contribution of the promoieties (Järvinen *et al.*, 1991a,b,1992a).

Bispilocarpic acid monoesters were prepared by esterifying pilocarpic acid with appropriate di-halogenated alkyls/aryls (Järvinen *et al.*, 1992a). Bispilocarpic acid monoesters spontaneously cyclized to pilocarpine in aqueous solution (Scheme 2). Unfortunately, the shelf-lives of these bispilocarpic acid monoesters at pH 4.5 and 4ºC were, at best, only a few months.

Bispilocarpic acid diesters were synthesized by esterification of the two alcoholic hydroxyl groups of bispilocarpic acid monoester (Järvinen *et al.*, 1992b). These diesters enzymatically hydrolyzed to provide bispilocarpic acid monoesters, which cyclized to the active pilocarpine in quantitative amounts, i.e., 1 mol of diester released 2 mol of active pilocarpine (Scheme 2) (Järvinen *et al.*, 1991b). These bispilocarpic acid diesters were more water-soluble than pilocarpic acid diesters, with corresponding lipophilicities (Järvinen *et al.*, 1991b,1992c). This



Scheme 2.

useful property may due to a favourable conformation of the bis-structure, where the hydrophobic areas are surrounded by hydrophilic portions of the molecule (Konschin and Ekholm, 1991). Due to their higher lipophilicity, the bispilocarpic acid diesters improved corneal penetration of pilocarpine and increased corneal uptake with increasing lipophilicity (Suhonen *et al.*, 1991b). However, a parabolic relationship between lipophilicity and corneal permeability was achieved for these prodrugs.

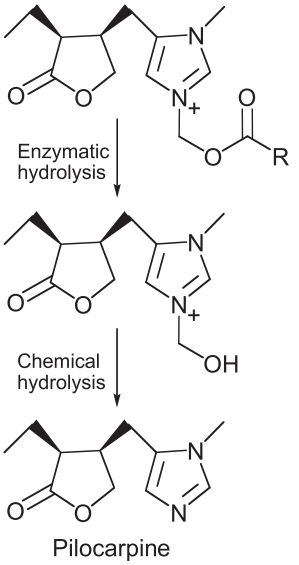
The ocular delivery of pilocarpic acid diesters was studied in rabbits using miosis as a bioassay (Järvinen *et al.*, 1995; Suhonen *et al.*, 1995,1996). Compared to 1% pilocarpine, these pilocarpine prodrugs (equivalent to 0.25% or 0.5% pilocarpine solution) extended the duration of miosis from 3 h (pilocarpine) to 4–5 h, and decreased the peak miotic response (Suhonen *et al.*, 1996). Unfortunately, these pilocarpine prodrugs also caused significant eye irritation immediately after eyedrop administration, which increased with increasing lipophilicity of the prodrug. This reaction was thought to be due to the rapid absorption of lipophilic prodrug into the lipophilic corneal epithelium and/or precipitation of prodrug molecules in the precorneal area. However, the eye irritation was substantially decreased by co-administered cyclodextrins (CDs), such as hydroxypropyl-β-cyclodextrin (HP-β-CD) and sulfobutyl ether β-cyclodextrins (SBE-β-CD) (Järvinen *et al.*, 1995; Suhonen *et al.*, 1995; Jarho *et al.*, 1996).

Inclusion complex formation between prodrug and CD probably slows the corneal absorption of prodrug as well as increasing prodrug solubility in the precorneal area. Co-administered CDs did not affect the miotic response of bispilocarpic acid diester solutions when the molar ratio of CD to prodrug was low (Järvinen *et al.*, 1995). However, increasing the molar ratio of CD to prodrug decreased the ocular absorption of prodrug due to substantial complexation of prodrug and CD in the precorneal compartment. CD/drug-complexes are not thought to permeate biological membranes and, thus, the CD/drug complex must dissociate before drug absorption (Nakanishi *et al.*, 1989; Frijlink *et al.*, 1990). The rate of dissociation of drug/CD complexes in the precorneal area should be faster than the rate of their clearance from the precorneal area. There is a very short time that is available for inclusion complexes to release a drug on the precorneal area, as aqueous eyedrops are removed from the precorneal area within couple of minutes after eyedrop administration. The use of a viscous vehicle is a simple method to increase the precorneal residence time of a drug/CD complex. When the viscosity of a bispilocarpic acid diester eyedrop containing SBE-β-CD was increased with polyvinyl alcohol (PVA), the ocular absorption was improved without inducing eye irritation (Jarho *et al.*, 1996). CD technology, combined with a viscous vehicle, is a feasible approach to develop clinically acceptable ophthalmic formulations from lipophilic prodrugs possessing solubility, stability, and/or irritation problems (Loftsson and Järvinen, 1999).

Soft Quaternary Salts of Pilocarpine

Soft quaternary ammonium salts of pilocarpine are water-soluble prodrugs that release pilocarpine *via* enzymatic and spontaneous chemical hydrolysis

(Scheme 3) (Bodor, 1977; Druzgala *et al.*, 1992). As quaternary salts, their aqueous solubility should be high, which could limit their corneal permeation. The *in vivo* studies, however, have shown that their bioavailability is better than that of pilocarpine. Compared to a 2% pilocarpine solution, 0.5% *N*-hexadecanoyloxymethyl pilocarpinium chloride solution showed equivalent miotic effect. The same prodrug derivative decreased IOP in glaucomatous beagle dogs with greater potency and longer duration of action than pilocarpine (Druzgala *et al.*, 1992).



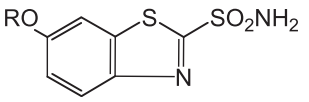
Scheme 3.

Carbonic Anhydrase Inhibitor Prodrugs

Oral carbonic anhydrase inhibitors (CAIs) have already been in clinical use for 50 years in the treatment of increased IOP (Sugrue, 1989). The currently used oral CAIs include acetazolamide and methazolamide. However, patient compliance with systemic CAI medication is poor, due to side effects such as hypokalemia, fatigue, depression, gastrointestinal disturbances, and anorexia (Epstein and Grant, 1977; Lichter *et al.*, 1978). Because the topical administration of CAIs was clinically ineffective, researchers started to explore other methods of drug delivery, including the prodrug approach (Sugrue *et al.*, 1985; Grove *et al.*, 1988; Woltersdorf *et al.*, 1989) and cyclodextrin-technology (Javitt *et al.*, 1994; Loftsson *et al.*, 1994) to increase the topical activity of CAIs. The prodrug approaches, described below, were undertaken before the advent of topical dorzolamide solution and brinzolamide suspension in the 1990s (Sugrue, 2000). These compounds are CAI analogs, however, and not prodrugs.

Prodrugs of CAI derivatives have been designed either to increase low aqueous solubility or to increase lipophilicity as strategies to enhance corneal permeation. A series of *O*-acyl derivatives of 6-hydroxybenzothiazole-2-

sulphonamide (L-643,799) were evaluated as topically active CAIs (Woltersdorf *et al.*, 1989). L-643,799 (**23**) is an analog of ethoxzolamide (**22**), which has been used in oral CAI medication. Prodrugs were converted to active CAI (L-643,799) in cornea homogenate and improved the corneal permeation of L-643,799 *in vitro*. The pivaloyl ester of 6-hydroxybenzothiazole-2-sulphonamide (L-645,151) (**24**) was the most interesting prodrug of the series; it significantly lowered IOP in rabbits (Sugrue *et al.*, 1985). However, clinical development of this prodrug was discontinued due to problems of sensitization (Grove *et al.*, 1988). Schoenwald and Barfknecht (1991) have subsequently described water-soluble prodrugs of 2-benzothiazolesulphonamide, hydroxymethazolamide, and dichlorphen-amide.



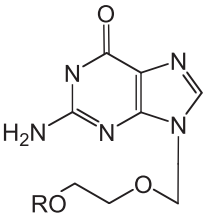
Compound		R
Ethoxzolamide	**22**	$-CH_2CH_3$
L-643,799	**23**	-H
L-645,151	**24**	$-COC(CH_3)_3$

Structures 22-24.

Acyclovir Prodrugs

Acyclovir (**25**) is a potent and selective anti-herpes drug (Schaeffer *et al.*, 1978) that could offer a means for topical treatment of herpes simplex virus (HSV) keratitis. However, the delivery characteristics of acyclovir are far from optimal, which can be attributed to its limited aqueous solubility (1.4 mg/mL) and moderate lipophilicity (logP –1.47) (Bundgaard *et al.*, 1991). Thus, acyclovir cannot be given as eye drops, which would be the most practical formulation for the treatment of ocular infection. The efficacy of 3% acyclovir eye ointment has been reported in the treatment of epithelial keratitis (Richards *et al.*, 1983). Due to various side effects associated with the use of ointments in the eye, however, acyclovir has not been approved for the treatment of HSV keratitis in the United States. In addition, acyclovir ointment is not effective against stromal keratitis or when deeper tissues are involved (Sanitato *et al.*, 1984). Prodrug strategies have been applied to improve delivery properties of acyclovir by increasing aqueous solubility or by enhancing membrane permeability, either by increasing lipophilicity or targeting the oligopeptide transporter on the cornea.

Several water-soluble ester derivatives of acyclovir (**26–31**), including glycyl (**26**), alanyl (**28**), and succinate (**31**) esters, have been prepared and evaluated for their anti-viral activity (Colla *et al.*, 1983). These prodrugs were as active as or only slightly less active than acyclovir itself against HSV-1 and HSV-2 in primary rabbit kidney cell cultures. Chromatographic analyses of the incubation media confirmed the ester hydrolysis to acyclovir. The solubility of these esters allowed formulation of 6% solutions at pH 7.4 (30-fold increase compared to acyclovir).



R=H; Acyclovir
25

Water-soluble ester prodrugs		Lipophilic ester prodrugs		Dipeptide prodrugs	
	R		R		R
26	$-COCH_2NH_2$	**32**	$-COCH_2CH_3$	**38**	-Val-Val
27	$-COCH(CH_3)NH_2$	**33**	$-CO(CH_2)_2CH_3$	**39**	-Val-Gly
28	$-CO(CH_2)_2NH_2$	**34**	$-COCH(CH_3)_2$	**40**	-Val-Tyr
29	$-COCH(CH_3)NHCOOCH_2C_6H_5$	**35**	$-CO(CH_2)_3CH_3$	**41**	-Tyr-Val
30	$-CO(CH_2)_2NHCOOCH_2C_6H_5$	**36**	$-COC(CH_3)_3$		
31	$-CO(CH_2)_2COOH$	**37**	$-CO(CH_2)_4CH_3$		

Structures 26-41.

When administered as 1% eye drops, glycyl ester effectively suppressed the development of both epithelial and stromal keratitis in rabbits (Maudgal *et al.*, 1984).

The hydrophilic nature of acyclovir contributes to its low ocular bioavailability. Thus, lipophilic aliphatic 2'-esters of acyclovir have been synthesized (**32–37**) (Hughes *et al.*, 1993). As expected, these esters were more lipophilic (up to 140-fold) and showed lower aqueous solubility than acyclovir. The esters hydrolyzed to acyclovir in ocular tissue homogenates and exhibited an increase in corneal permeation (Hughes and Mitra, 1993).

Recently, dipeptide prodrugs of acyclovir (**38–41**) have been targeted to the oligopeptide transporter with the aim of improving the ocular bioavailability of acyclovir (Anand *et al.*, 2003a). The existence of an oligopeptide transporter on the cornea was supported by the results obtained in permeability studies of valacyclovir using excised rabbit cornea. Valacyclovir transport was found to be saturable, and energy- and pH-dependent, and was strongly inhibited by dipeptides (Gly-Sar, Gly-Pro), ACE inhibitors (enalapril, captopril), and β-lactam antibiotics (cephalexin, cephazolin) (Anand and Mitra, 2002). The aqueous solubility of these dipeptide prodrugs ranged from 15–30 mg/mL, and they all hydrolyzed to yield acyclovir in ocular tissue homogenates. The permeabilities of dipeptide prodrugs through rabbit cornea were 1.7–2.9-fold higher compared to acyclovir. Valacyclovir transported through the cornea was inhibited by dipeptide prodrugs, which suggests that dipeptide prodrugs are recognized by the oligopeptide transporter and are possibly transported actively (Anand *et al.*, 2003a). This is further supported by Caco-2 permeation experiments, where the transport of Gly-Val-acyclovir was inhibited in the presence of glycylsarcosine (Anand *et al.*, 2003b). Val-Val-acyclovir has been demonstrated to be effective against HSV-1 in the rabbit epithelial and stromal keratitis models *in vivo* (Anand *et al.*, 2003c).

Steroids

Steroids applied to the lower conjunctival sac (e.g., dexamethasone, fluorometholone, hydrocortisone, prednisolone) are used clinically to treat inflammations of the eye. Acetate ester prodrugs have been prepared in order to increase the lipophilicity and the corneal absorption of steroids (Kupferman *et al.*, 1974,1982; Leibowitz *et al.*, 1977; Schoenwald and Ward, 1978). Phosphate esters of steroids have been developed to increase the aqueous solubility of steroids and allow formulation of aqueous eyedrop solutions (Cox *et al.*, 1972; Musson *et al.*, 1989).

Prednisolone acetate hydrolyzes completely to prednisolone *in vitro* and *in vivo*, and enzymatic conversion is assumed to occur in the cornea (Musson *et al.*, 1989,1991). Unlike prednisolone acetate, prednisolone phosphate was not hydrolyzed completely to prednisolone *in vitro* or *in vivo*, and the presence of both intact prodrug and released prednisolone was observed. The phosphate ester is most probably hydrolyzed by the action of phosphatase enzymes in the eye (Lojda *et al.*, 1976; Schive and Volden, 1982).

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2.2.1

Overcoming Poor Aqueous Solubility of Drugs for Oral Delivery

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† *"Sadly, while this book chapter was in preparation our mentor and research advisor Dr. David Fleisher passed away. At the College of Pharmacy at the University of Michigan, in Ann Arbor, Dr. Fleisher mentored countless undergraduate, graduate and post-graduate students and fellows who now carry on his teachings and strive to propagate his gentle and wise manner of nurturing young minds that need some direction. Dr. Fleisher was a remarkable intellect and an outstanding researcher/contributor in the fundamentals of oral drug delivery, food effects as well as prodrugs and his pioneering efforts have stood the test of time and veracity. Dr. Fleisher's ability to instinctively empathize with those that needed solace and comfort and perhaps a gentle steering hand to walk the maze of life, particularly in difficult times, endeared him to all that were fortunate to have known him. Dr. Fleisher is missed dearly and will always be in our hearts. He was 61."*

Table of Contents

List of Abbreviations

2F-ara-A.....Fludarabine
ADMET..Absorption, Distribution, Metabolism, Elimination and Toxicity
AP.....Alkaline Phosphatase
ara-A 9-β-D-arabinofuranosyladenine
BCS.....Biopharmaceutical Classification System
CLL .....β-Cell Chronic Lymphocytic Leukemia
ClogP .....Calculated log of the partition coefficient
COMT .....Catechol-O-Methyl Transferase
CSA .....Cyclosporine A
DDC.....Dopa Decarboxylase
DKP.....Diketopiperazine
DMSO.....Dimethyl Sulfoxide
Do.....Dose Number
EMP .....Estramustine Phosphate
EPR .....Enhance Permeability and Retention Effect
GI .....Gastrointestinal
HIV-1 PR .....Human Immunodeficiency Virus type -1 Protease
i.v. .....Intravenous
m(PEG) .....Methoxy Polyethylene Glycol
MAP .....Microtubule Associated Proteins
MFP.....Mono-Fluoro-Phosphate
MRD1 .....Multi-Drug Resistance 1
NCE .....New Chemical Entities
PD.....Parkinson's Disease
PEG .....Polyethylene Glycol
P-gp .....P-glycoprotein
TAT-59 .....Miproxifene Phosphate

Introduction

It is estimated that 40% of active new chemical entities (NCEs) identified in combinatorial screening programs employed by many pharmaceutical companies are poorly water soluble, i.e., these compounds have an aqueous solubility less than 10 μM (5 μg/mL for a compound with a molecular weight of 500) (Lipinski, 2002, 2004). When these poorly soluble NCEs are further advanced in discovery and ultimately brought into development they are often plagued by incomplete absorption and low, erratic bioavailability. There are a limited number of options available to drug discovery scientists to enhance the solubility of a compound by conventional formulation approaches. These include the identification and selection of stable pharmaceutical salts (Stahl, 2003). However, salt formation requires an ionizable group and, therefore, this is not a viable option for neutral compounds or those with ionization constants that do not fall within the physiological range. Other common approaches are reducing solid particle size by micronization, such as milling or the formation of nanosuspensions (Müller *et al.*, 2001), the use of complexation agents such as cyclodextrins (Rao and Stella, 2003), or the use of solubilizing excipients (Strickley, 2004). While these solubilization techniques often lead to significant improvement in systemic exposure when availability is solubility- or dissolution-rate limited, conventional formulation approaches are not always successful and an alternate strategy is required.

One effective strategy for overcoming the low aqueous solubility is to convert the water-insoluble parent drug into a soluble prodrug. Broad objectives of a prodrug design often include enhanced bioavailability, fewer side effects, and improved patient acceptance and compliance. In some cases, a solubility-enhancing prodrug can successfully achieve all these goals.

Oral prodrugs are often designed to overcome an absorption limitation or related problem of their parent drugs (Notari, 1981; Stella *et al.*, 1985; Neau, 2000; Ettmayer *et al.*, 2004), such as poor solubility and/or poor permeability. Prodrugs can increase absorption relative to their parent drugs *via* several mechanisms, including (a) targeting carrier-mediated transporters such as hPepT1 with e.g., valacyclovir, which has enhanced permeability and solubility compared to acyclovir (Bai *et al.*, 1992; Smiley *et al.*, 1996; Sinko and Balimane, 1998; Anand *et al.*, 2004; Sun *et al.*, 2004), or XP13512, a prodrug of gabapentin that targets a nutrient transporter (Cundy *et al.*, 2004); (b) masking a polar/charged functional group that prevents a molecule from penetrating lipophilic membranes, e.g., adefovir dipivoxil, (Annaert *et al.*, 2000; Niemi *et al.*, 2000; Van Gelder *et al.*, 2000) and ester prodrugs of carboxylic acids (Beaumont *et al.*, 2003); (c) perturbing crystal lattice packing by decreasing hydrogen bonding, yielding to lower melting point parent drugs with enhanced solubility in simulated gastric fluids (Stella *et al.*, 1998); and (d) improving dissolution rate and/or solubility of a lipophilic drug, such as amprenavir and buparvaquone (Fleisher *et al.*, 1996; Furfine *et al.*, 2004; Mäntylä *et al.*, 2004a,b).

While there are many "oral" prodrugs (Stella, 2004), including lipophilic carboxylic ester prodrugs that overcome poor cellular permeability and result in

low systemic exposure of the polar carboxylic acid parent drug (Testa and Mayer, 2001; Beaumont *et al.*, 2003), relatively few examples of marketed water-soluble oral prodrugs have been described (Ettmayer *et al.*, 2004). In addition to standard modification such as the addition of ionizable groups, e.g., phosphates, or covalently linking polar neutral molecules, e.g., polyethylenglycols, some novel prodrug activation approaches have been described recently (Hamada *et al.*, 2003).

Prodrug activation can occur enzymatically or non-enzymatically, i.e., by chemical hydrolysis such as intramolecular cyclization-elimination (Tamamura *et al.*, 1998; Matsumoto *et al.*, 2000,2001; Sohma *et al.*, 2003; Hamel *et al.*, 2004) or by O→N intramolecular acyl migration reactions (Kiso *et al.*, 1999a; Matsumoto *et al.*, 2001; Hamada *et al.*, 2002, 2003, 2004; Hayashi *et al.*, 2003a; Skwarczynski *et al.*, 2003; Sohma *et al.*, 2003). There is current interest in non-enzymatic hydrolysis or intramolecular catalysis of prodrugs, presumably due to their advantage in reducing biological variability.

While a number of prodrug design strategies have proven therapeutically successful (Ettmayer *et al.*, 2004; Stella, 2004), there are only a few examples in which solubility-enhancing prodrugs have been of demonstrable clinical utility. This chapter describes prodrug strategies that discovery scientists can pursue in cases where conventional approaches to enhance solubility have either failed or are impractical (Tong, 2000). Some of the more commonly used water-soluble prodrugs for oral delivery that will be discussed include a) phosphate ester prodrugs where the phosphate promoiety is often attached directly to a functional group of a NCE (e.g., a hydroxyl) or, less commonly, *via* a small spacer group (Kearney, 1990; Stella, 1996) and b) polyethyleneglycol-conjugated parent drugs using a labile linker or spacer.

Phosphate Ester Prodrugs

Phosphate prodrugs offer several advantages for formulation and development of poorly water-soluble compounds. Phosphate prodrugs are chemically stable, their synthesis is usually straightforward in the presence of a hydroxyl moiety (Kearney and Stella, 1993), and the increases in solubility imparted by the dianionic phosphate group are often several orders of magnitude (Stella, 1996; Rodriguez *et al.*, 1999; Zhu *et al.*, 2000; Heimbach, 2003; Furfine *et al.*, 2004). For oral dosage forms, the reduction or elimination of solubilizing excipients can lead to cost reduction through greater drug loading capacity (Sorbera *et al.*, 2001; Becker and Thornton, 2004), providing a more convenient dosing regimen, especially for high dose compounds. Moreover, phosphate ester prodrugs are readily cleaved by endogenous phosphatases to rapidly release the pharmacologically active parent drug (McComb *et al.*, 1979).

There are many examples of successful phosphate prodrugs for parenteral administration, including fosfluconazole (Bentley *et al.*, 2002; Sobue *et al.*, 2004), etoposide-phosphate (Greco and Hainsworth, 1996; Schacter, 1996), clindamycin-phosphate, (Soejima and Saito, 1994), hydrocortisone-phosphate (Arky, 2000),

and fludarabine-phosphate (Grever *et al.*, 1990; Rossi *et al.*, 2004). Several phosphate prodrugs that have been at various stages in drug discovery or development include the antifungal SCH 59884 (Kim *et al.*, 2002), PA2808 (Baker *et al.*, 2004), ZD-6061 (Soltau and Drevs, 2004), camptothecin-phosphate (Hanson *et al.*, 2003), and antitumor prodrugs of various combretastatins, including combretastatin-A4-phosphate, which is currently in Phase I/II clinical trials (Grosios *et al.*, 1999; Hill *et al.*, 2002; Pettit *et al.*, 2002; NIH, 2004; Young and Chaplin, 2004). Aquavan® is a phosphate ester prodrug of the analgesic drug propofol (Banaszczyk *et al.*, 2002) that employs a phosphonooxymethyl spacer approach that has been successfully employed for fosphenytoin and camptothecin (Stella, 1996; Hanson *et al.*, 2003) to enhance enzymatic hydrolysis rates. Derivatization of tertiary amine-containing drugs such as loxapine resulted in quaternary amine phosphate prodrugs with greatly enhanced aqueous solubility (Krise *et al.*, 1999a,b,c).

Very few phosphate prodrugs have been marketed exclusively for oral administration. Estramustine phosphate has been used since the mid 1970s for the management of prostate cancer (Nilsson and Jonsson, 1975; Perry and McTavish, 1995). Fosfosal, a phosphate prodrug of salicylic acid, has reduced gastrointestinal (GI) side effects compared to the parent compound (Ramis *et al.*, 1988). Inorganic disodium monofluorophosphate (MFP) is on the market as an alternative to NaF for the oral treatment of osteoporosis. This unusual prodrug eliminates the gastric irritation by the reaction of gastric HCl with NaF (Rigalli *et al.*, 1994; van Asten *et al.*, 1996). In addition, the fluoride bioavailability of the fluoride-containing prodrug is nearly double that of NaF since precipitation of calcium fluoride is prevented. Pediapred® (Medeva Pharmaceuticals, Inc.) is a liquid formulation of prednisolone phosphate used to overcome the poor palatability of prednisolone tablets to children. Recently, fludarabine was marketed as an oral phosphate prodrug (Boogaerts *et al.*, 2001) by Berlex-Schering. For fludarabine, marketing as the oral prodrug may have been a consequence of prior approval of its parenteral formulation, fludarabine-phosphate (Fludara®) and, thus, may not have been a deliberate phosphate prodrug strategy based on a biopharmaceutical advantage. Fosamprenavir, a novel oral phosphate prodrug of the antiviral drug amprenavir (Agenerase®) that allows a more simplified dosage regimen was recently approved in Europe and the US (Becker and Thornton, 2004); it is discussed later in this chapter as well as in another chapter in this book.

Why is it that very few phosphate prodrugs seem to survive beyond the discovery stage? One reason is that not all phosphate derivatives offer significant biopharmaceutical advantages compared to their parent drugs, and there are no clear guidelines to identify drug candidates for which this can be achieved. Criteria for suitable oral phosphate candidates were discussed by Fleisher (Fleisher *et al.*, 1996). Oral phosphate prodrugs are especially promising for insoluble parent drugs that must be administered at high doses since, despite low solubility, "low-dose" compounds will be completely dissolved and absorbed within normal GI residence times. Thus, a phosphate prodrug strategy is most likely to

succeed for drug candidates where absorption is dissolution-rate limited due to low solubility and high projected dose.

Dissolution rate limits to drug absorption can be evaluated by the familiar Noyes-Whitney equation (Eq. 1) (Noyes and Whitney 1897):

$$\frac{dM}{dt} = \frac{DS}{h}(C_s - C_b)$$

Equation 1.

This equation describes the dissolution rate for a solid solute into a non-reactive medium in units of mass/time. *D* is the diffusion coefficient of the drug or prodrug in the medium of dissolution, h is the diffusion layer thickness around the drug particles, S is the exposed surface area of the solid, C_s is the solubility of the drug, and C_b is the concentration of the drug in the bulk medium. *S* and C_s lend themselves to easy pharmaceutical manipulation to increase dissolution rates (Kearney, 1990).

Phosphate prodrugs markedly increase a drug's aqueous solubility, C_s, and consequently increase its dissolution rate (Amidon, 1981). Mass transport rates across the GI barriers are increased by soluble prodrugs due to their higher concentration gradients across the intestinal mucosa (Amidon, 1981; Fleisher *et al.*, 1986, 1996; Stewart, 1986), as long as there are no offsetting decreases in partitioning into the membrane. Even when the latter is the case, absorption may still be increased if bioconversion maintains a higher parent drug concentration at the cell membrane-lumen interface. The maximum flux, or amount of parent drug passively absorbed per time per area can be expressed as Eq. 2 (Amidon *et al.*, 1980):

$$J_m = P_{eff}(C_s - C_b)$$

Equation 2.

In this simplified equation, J_m is the mass flux in units of g/cm^{2}s and P_{eff}, the effective permeability coefficient, has the units of a velocity e.g., cm/s. C_b and C_s are as defined for Eq. 1 and are in units of g/cm^3. Under sink conditions, when $C_b \cong 0$, the maximal flux is defined by Eq. 3 (Amidon *et al.*, 1980,1985):

$$J_m = P_{eff} C_s$$

Equation 3.

Phosphate prodrug bioconversion at the intestinal brush border by membrane bound enzymes, such as alkaline phosphatase, is depicted in Figure 1 (Fleisher *et al.*, 1985; Stewart, 1986). The concentration of the parent drug is elevated in the vicinity of the mucosal membrane due to enzymatic conversion of the prodrug, which can lead to local supersaturation of the parent drug (Heimbach *et al.*, 2003a), resulting in a larger concentration driving force and absorptive flux. In

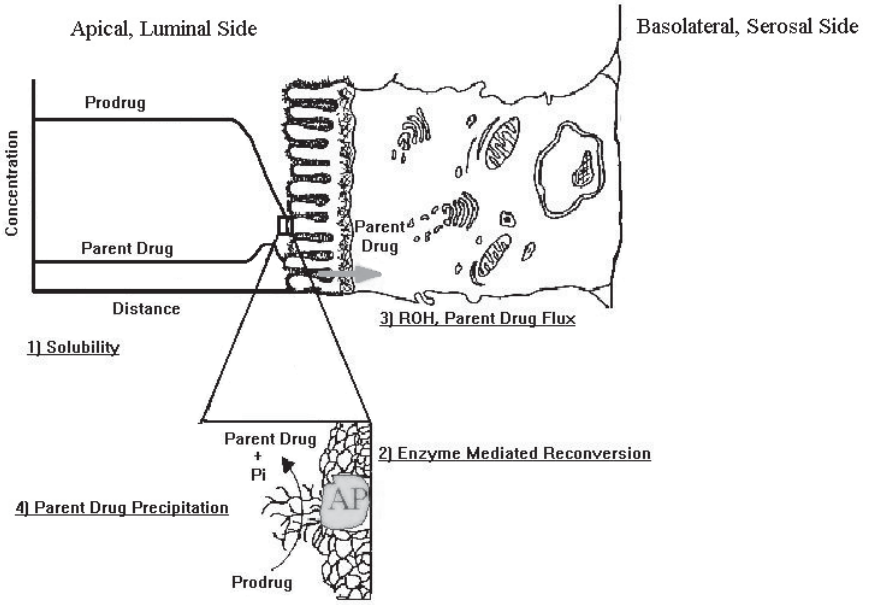


Figure 1. Absorption model with potential rate lmits for water-soluble phosphate prodrugs. Adapted from the literature (Stewart, 1986; Fleisher *et al.*, 1996). The soluble (phosphate) prodrug provides a greater concentration driving force across the intestinal lumen. The cleavage of the phosphate group by membrane-bound alkaline phosphatases releases the usually lipophilic parent drug in the vicinity of the mucosal membrane. Potentially rate-limiting steps include: Dissolution/Solubility (not common); Enzymatic Bioconversion, i.e., enzyme-mediated parent drug generation; and Permeability/Transport of the parent drug. Enzyme-mediated supersaturation leading to precipitation of the parent drug after prodrug hydrolysis can negatively impact the prodrug strategy. Notably, in some cases, the prodrug can increase the solution concentration of the parent drug through supersaturation or solubilization from a prodrug surfactant effect (Heimbach *et al.*, 2003a).

some cases the solubility of the parent drug is also enhanced in the presence of the surface-active phosphate prodrug (Heimbach *et al.*, 2003a).

Many lipophilic drug substances are substrates of the multi-drug resistance 1 (MDR1) gene product P-glycoprotein (P-gp), which can limit systemic drug exposure after oral dosing (Lown *et al.*, 1997; Ekins *et al.*, 2002). Few, if any studies have been published in which a solubility-enhancing prodrug was demonstrated to saturate intestinal efflux or metabolism due to an increased solution concentration (C_s), leading to enhanced transepithelial flux and increased systemic exposure. A recent study described the use of a soluble dendrimer prodrug of propranolol to bypass efflux transporters and enhance oral bioavailability (D'Emanuele *et al.*, 2004). However, propranolol is a highly permeable compound, where efflux is unlikely to be rate-limiting to absorption. For most drugs, efflux at the intestine is not clinically significant (Lin, 2003), except for a few low dose compounds generating intestinal drug concentrations that are too low to saturate the efflux pumps, i.e., at concentrations below the Michaelis constant (K_m) (Lin, 2003).

However, the possibility that supersaturated drug solutions resulting from hydrolysis of soluble prodrugs in the vicinity of these pumps might saturate capacity-limited efflux should be considered as a strategy to increase intestinal absorption.

Phosphate Prodrugs that Enhance Drug Absorption

Oral phosphate prodrugs may result in higher plasma levels (C_{max}) and increased bioavailability compared to the parent drug form. This very outcome has been demonstrated for oxyphenbutazone phosphate (Hook *et al.*, 1975) as well as fosphenytoin in dogs (Varia and Stella, 1984; Lai *et al.*, 1987). Fosphenytoin also yielded lower t_{max} and higher C_{max} in rats (Burstein *et al.*, 1999) as shown in Figure 2. Plasma levels of a stachyflin analog were also greatly increased in rats after phosphate prodrug dosing, as shown in Figure 3 (Yagi *et al.*, 1999; Yoshimoto *et al.*, 2000). However, these drugs can show considerable variability in their pharmacokinetic profiles after oral prodrug dosing even though the aqueous solubilities of the prodrugs are significantly higher than those of their parent forms. While variability due to poor dissolution of the parent is eliminated, an additional level of variability appears to be introduced through variable enzymatic conversion kinetics (Fleisher *et al.*, 1986; Ruiz-Balaguer *et al.*, 1997), or by precipitation of the parent after rapid bioconversion at high levels of supersaturation (Heimbach *et al.*, 2003a). For example, the variability in absorption after oral administration of etoposide phosphate was of the same order as that seen for etoposide itself, leading to questions of the value of the phosphate prodrug approach (de Jong *et al.*, 1997).

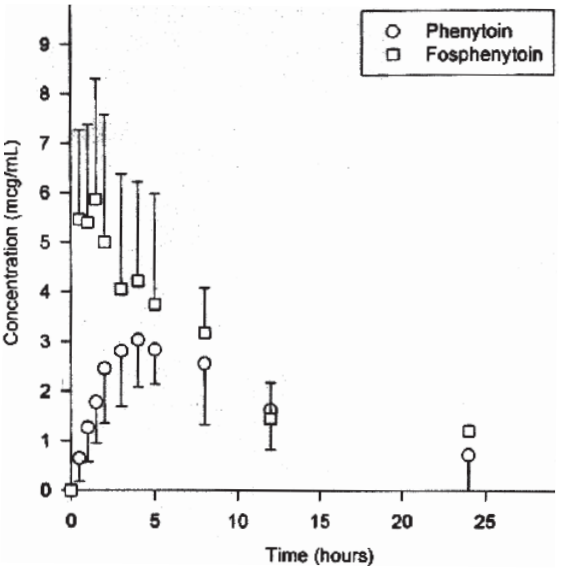


Figure 2. Plasma levels of phenytoin in rats following oral administration of phenytoin and its phosphate prodrug, fosphenytoin. Figure was modified from Burstein et al., 1999. Plasma levels of phenytoin in rats following a single oral dose of (○) phenytoin suspension (n = 6), (□) fosphenytoin (n = 7); phenytoin equivalent doses were 30 mg/kg.

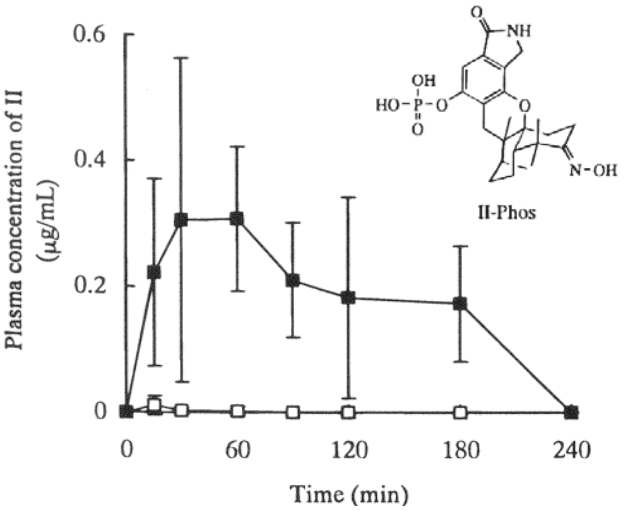


Figure 3. Plasma levels of stachyflin II in rats following oral administration of stachyflin II-phosphate. Figure was adapted from Yagi *et al.*, 1999. The prodrug stachyflin phosphate (■) was dosed as a solution and the parent (□) was dosed as a suspension at 20 mg/4 mL/kg. The aqueous solubility of the prodrug was >20 mg/mL vs. 0.001 mg/mL (water, 25°C) of the parent drug.

Phosphate Prodrugs that Fail to Enhance Drug Absorption

Failure of phosphate prodrugs to increase drug absorption can be attributed to several potential rate-limiting factors in the drug absorption process (Figure 1). After oral administration, phosphate ester prodrugs are dephosphorylated in the GI tract by membrane-bound alkaline phosphatase (AP). In theory, lipophilic parent drugs generated at the membrane surface should be well absorbed compared to their ionic polar phosphate counterparts, and there appears to be little luminal metabolism from biliary or pancreatic input or from shed enterocytes in the intestinal fluid (Fleisher *et al.*, 1986). Exceptions to this scenario may occur if phosphate prodrugs are administered with a fat-containing meal that stimulates the intestinal secretion of surfactant vesicles containing alkaline phosphatase (Engle *et al.*, 2001). Given this mechanism and fate, phosphate ester prodrugs may encounter at least three potential rate limits to oral absorption (Figure 1): a) inadequate dissolution/solubility, b) unsuitably slow enzymatic bioconversion and/or c) low permeation of the parent drug. While dissolution is not usually rate-limiting to absorption, poor permeation and/or drug efflux of the parent drug can lead to prodrug failures.

Etoposide phosphate failed in clinical trials due to its "lack of offering a clinically relevant benefit" over oral etoposide (de Jong *et al.*, 1997). Other drugs have failed because they did not improve the rate and/or extent of absorption of the parent drug in animal models. LY307853 is an example of such a drug (Zornes *et al.*, 1993). Still others, such as phosphate esters of taxol (Ueda *et al.*, 1993) and PD 0154075 (Zhu *et al.*, 2000) have failed due to poor bioconversion.

Challenges with Phosphate Esters Prodrugs

There are several limiting attributes of phosphate prodrugs with respect to their oral delivery. Phosphate groups impart negative charges to drug compounds. For that reason they are functionally extremely polar and thus are thermodynamically unlikely to passively cross the GI-membrane (Krise and Stella, 1996). Passively absorbed drugs are thought to be absorbed primarily in their neutral, non-ionized form (Winne, 1977). This principle is captured in the so-called "pH-partition hypothesis" (Shore *et al.*, 1957). Because of their ionic charge, phosphate prodrugs have octanol-water partition coefficients that are significantly below the often quoted "reasonable" log P of 2 to project good absorption (Ho *et al.*, 1977). For example, the reported log D of stachyflin phosphate (Figure 3) at pH 7.4 is –2.5 (Yagi *et al.*, 1999). For fludarabine phosphate, the reported log D is less than –3 (Kusui *et al.*, 2000). Each value is indicative of the high polarity imparted by the phosphate group, which in these two cases exists mainly as a dianion at neutral pH. For small ionic and non-ionic molecules, however, passive absorption through aqueous pathways has to be considered as an alternative means of uptake (Ho *et al.*, 1977). This hydrophilic, small molecule pathway explains the absorption of MFP as a prodrug for fluoride (van Asten *et al.*, 1996).

Another limitation of oral phosphate prodrugs appears to be their tendency to exhibit variability in the fraction of drug absorbed. In general, there are hosts of factors that can lead to variable absorption; many of them are compound dependent. Variability can be caused by poor permeability of the parent compound (Lee and Chiou, 1983; Obermeier *et al.*, 1996). This mechanism apparently explains the equivocal uptake observed for the phosphate prodrug ethiofos (Fleckenstein *et al.*, 1988). Low lipophilicity, as reported for antibiotics, can also be a contributing factor (Ho *et al.*, 1977; Kakeya *et al.*, 1985). Variability can also result from variable enzymatic bioconversion (Ruiz-Balaguer *et al.*, 1997), a phenomenon seen, for example, with succinate esters (Fleisher *et al.*, 1986) or from instability at gastric pH (Shah *et al.*, 1989), also reported for ethiofos (Fleckenstein *et al.*, 1988). The stochastic process of precipitation due to formation of insoluble calcium salts (van Asten *et al.*, 1996) can contribute to absorption variability as has been reported for estramustine phosphate (Gunnarsson *et al.*, 1990). Variable gut-wall (Lee and Chiou, 1983; Selen, 1991) and hepatic metabolism (Yuasa *et al.*, 1996) provides an additional variability contribution. Significant variability may result when drugs or prodrugs are administered with food. This can result from drug solubilization by bile salts (Humberstone *et al.*, 1996) or from luminal enzyme hydrolysis of phosphate prodrugs as generated by AP-containing vesicle secretion with lipid meals (Engle *et al.*, 2001). This could also contribute to the negative meal effect observed for estramustine phosphate (Gunnarsson *et al.*, 1990).

It is important to recognize that not all drugs can be converted into phosphate ester prodrugs. The parent drug should possess one hydroxyl moiety as a "synthetic handle" so that a phosphate monoester can be synthesized readily onto the drug molecule (Kearney, 1990; Krise and Stella, 1996). Therefore, a large

number of insoluble compounds do not readily lend themselves to chemical modification with a phosphate moiety.

Phosphate Prodrugs Case Examples

TAT-59, Miproxifene Phosphate

TAT-59 (Miproxifene Phosphate) is a triphenylethylene analog of tamoxifen that was under development and in Phase II–III clinical trials (Nomura *et al.*, 1998b; Monograph, 1999) by the Taiho Pharmaceutical Company (Tokushima, Japan) when its development was discontinued. TAT-59 is the phosphate ester prodrug of the practically insoluble parent drug DP-TAT-59 (Figure 4), which has a solubility of 58 ng/mL at pH 7.4 (Heimbach, 2003). Formulation strategies to identify a crystalline pharmaceutical salt of DP-TAT-59 to increase its solubility and dissolution rates, as was done successfully with tamoxifen citrate, apparently failed.

Prodrug chemical stability was verified to confirm the viability of a phosphate prodrug strategy. TAT-59 powder was determined to be chemically stable at neutral pH over GI residence times, and formulated tablets were shown to be chemically stable in long-term stability studies. In these studies, parent drug formation was minimal (Matsunaga *et al.*, 1993, 1994; Matsunaga *et al.*, 1996; Heimbach, 2003), a result that is typical for most phosphate esters under ambient conditions. The degradation rate of TAT-59 was slightly higher at or near pH 3-4 due to the less stable monoanionic phosphate form that exists predominantly at that pH range,; this has also been observed for many phosphate esters (Flynn and Lamb, 1970; Kearney and Stella, 1993; Heimbach, 2003).

It is expected that a phosphate prodrug would exhibit a higher solubility and dissolution rate compared to a poorly soluble parent drug. Indeed, the TAT-59 prodrug increased the parent drug's equilibrium solubility by three orders of magnitude at pH 7.4, from 58 ng/mL (0.1 μM) for DP-TAT-59 to 52 μg/mL (~100

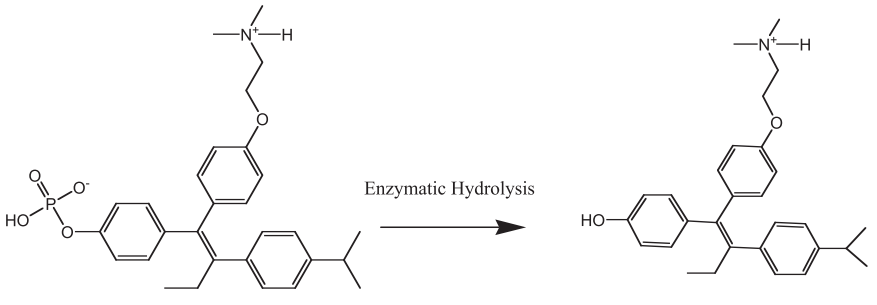


Figure 4. Structures of TAT-59, shown in its neutral, zwitterionic form, and its precursor DP-TAT-59.

μM). However, unlike other phosphate esters, TAT-59 exhibits unusually low solubility (Table 1), even though solubility and dissolution rate are increased with increasing pH. The higher solubility is promoted as the second phosphoric acid hydrogen undergoes ionization followed by deprotonation of the basic amine (Heimbach, 2003). Similarly, apparent solubility is increased from 8 μg/mL (pH 6.5) to 52 μg/mL (pH 7.4) and 830 μg/mL (pH 8.0) (Heimbach, 2003). Despite TAT-59's unusually low solubility, the prodrug did show a phosphate prodrug solubility advantage by having nearly 1000-fold higher phosphate prodrug solubility compared to that of DP-TAT-59 (Table 1), near pH 7.4. However, at pH 6.0, the prodrug did not significantly improve the solubility over that of the parent drug, and both are insoluble at 0.0037 μg/mL and 0.0040 μg/mL, respectively (Heimbach, 2003).

The low solubility of TAT-59 is surprising since most phosphate esters are freely soluble in water. In a similar case, the prodrug metronidazole phosphate had a minimal solubility at pH 2 that was attributed to zwitterion formation (Cho *et al.*, 1982). The low aqueous solubility of TAT-59 is most likely linked to the formation of a poorly soluble zwitterion, resulting in an overall neutral molecule in much of the pH range of the GI tract. TAT-59 has a melting point of over 210°C, a relatively high value, especially when compared to that of tamoxifen citrate, at 140–142°C. High levels of crystallinity as reflected in high melting points will also adversely affect solubility since a high melting point reflects poor solubility in all solvents (Amidon, 1981).

The prodrug approach was successful because, despite the relatively low solubility of TAT-59, its solubility and dissolution rate were significantly higher than those of the parent drug (see also Table 2). This was demonstrated in a study showing that 20 mg TAT-59 tablets were completely dissolved within 30 min at pH 7.4; these results could not be achieved with the parent drug (Heimbach, 2003).

The prodrug approach was also successful from a drug delivery point of view. In Caco-2 cells, at 100 μM dosing TAT-59 increased the absorptive flux of DP-TAT-59 nearly 10-fold, which is in accordance with the increased solubility achieved by the prodrug approach (Heimbach *et al.*, 2003b). *In vivo* pharmacokinetic studies showed that TAT-59 is rapidly dephosphorylated. When given as a single oral dose of 0.3 mg/kg to rats with induced carcinoma, no intact TAT-59 was detected in plasma (Toko *et al.*, 1990), but significant levels of the parent drug were detected. The bioavailability of DP-TAT-59 after oral TAT-59 dosing was 28.8% in rats and 23.8% in dogs, and no intact prodrug was detected in the plasma of either species (Masuda *et al.*, 1998). For humans, no bioavailability data have been published, but the t_{max} of DP-TAT-59 after prodrug dosing ranged from 5 to 7.3 h; and its C_{max} and AUC were dose-dependent (Nomura *et al.*, 1998a). There were no reported food effects with TAT-59 from the Phase I studies (Nomura *et al.*, 1998a). A report from early Phase II trials recommended a daily low dose of 20 mg (Aoyama *et al.*, 1998), and in late Phase II studies TAT-59 was found to show efficacy and safety comparable to that of tamoxifen (Nomura *et al.*, 1998b). The development of TAT-59 was discontinued in 1999, possibly due to lack of differentiation from tamoxifen.

Phosphate Ester Prodrug	Aqueous Solubility Prodrug (mg/mL)[a]	Aqueous Solubility Parent Drug $C_{s\ min}$, (mg/mL)[a]	Targeted Oral Dose (mg)[b]	Dose Number (Do)[c] based on Parent drug solubility
Acetaminophen phosphate	372[d]	16.6	200–650	0.05–0.16
Prednisolone phosphate	>10	0.3	5	0.1
Fludarabine phosphate	28[f]	0.33	10–50	0.1–0.6
Entacapone phosphate	30[e]	1.75[e]	200	0.5
Hydrocortisone phosphate	>10	0.28	50–240	0.7–3.4
Etoposide Phosphate	>20	0.10	260–900	10–36
Fosphenytoin	140[g]	0.019	100	21
Fosamprenavir-Ca Fosamprenavir-Na	0.3[h] >100	0.04[h]	700–1200[h]	70–120
LY307853 (LY303366-phosphate)	50i	<0.005	350[j]	>280
Estramustine phosphate	0.2	<0.001	400–1000	>1600–4000
TAT-59	0.05	0.008 (pH 6.5) 0.00005 (pH 7.4)	20–80	1600–6400
Stachyflin phosphate	>20[k]	0.001[k]	1000[l]	4000
Buparvaquone phosphate	>3.5	0.00003[m]	500–750[n]	67000–100000

Table 1. Oral Dose, Aqueous Solubility, and Dose Number of Selected Prodrugs and Their Parent Drugs.

[a]The aqueous solubilities were determined in pH 6.5 phosphate buffer or were taken from the literature. [b]Targeted oral doses were taken from the literature, and the Physicians Desk Reference. [c]Dose Number (Do) for prodrug evaluation, which is based on the parent drug solubility = targeted oral Dose/250 mL/C_{smin}, where C_{smin} is the lowest solubility of the parent at intestinal pH. The Do is calculated analogous to the BCS dose number (Kasim *et al.*, 2004). [d]from Taniguchi (Taniguchi and Nakano, 1981). [e]from Savolainen (Savolainen *et al.*, 2000b), at pH 7.4, [f]from reference (McEvoy, 2000), at pH 4. [g]from Stella (Stella, 1996) at pH near 9 in water. [h]: from Sorbera (Sorbera *et al.*, 2001), solubility determined at pH 3–4. [i]from Zornes (Zornes *et al.*, 1993). [k]from Yagi (Yagi *et al.*, 1999). [j,l]The oral dose was estimated based on doses given in animal studies. [m]from Mättylä (Mäntylä *et al.*, 2004a) at pH 7.4.

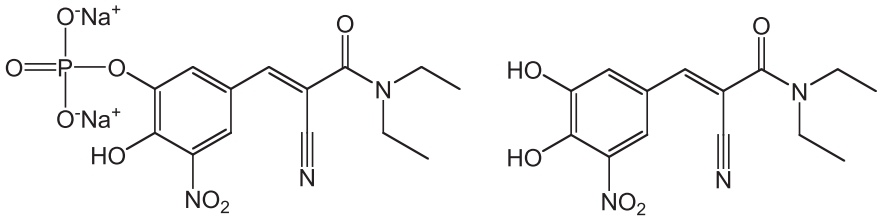
Parent Drug[a]	Parent T_{Diss}/T_{Si}[b]	Prodrug T_{Diss}/T_{Si}[b]	Parent Drug BCS Class[c]	Parent Drug Rate Limits[d]	Prodrug Rate Limit[e]	Prodrug Performance
Acetaminophen	0.002	0.0001	I	high solubility/high permeability	Parent Permeability	not published
Amprenavir	1.1	0.14 (Ca-salt)	III	low solubility/high permeability	Parent Permeability	Bioequivalent, compact dosage form
Buparvaquone	1240	<0.011	NA	low solubility	NA	not published
DP-TAT-59	11-890[f]	24 (pH 5.5) 12 (pH 6.0) 5.5 (pH 6.5) 2.6 (pH 6.8) 1.7 (pH 7.0) 0.9 (pH 7.4) 0.05 (pH 8)	III	low solubility/high permeability	Dissolution rate = Parent Permeability	Parent Drug Flux higher across Caco-2, Development Discontinued
Entacapone	0.016	0.001	III, IV[f]	high solubility/low permeability	Permeability	Prodrug failed in rats
Estramustine	41.2	0.020	III	low solubility/high permeability	Parent Permeability	Prodrug marketed
Etoposide	0.3	0.002	IV	low solubility/efflux	Parent Permeability	Bioequivalent in Humans
Fludarabine	0.08	0.001	NA	NA	NA	Prodrug marketed
Hydrocortisone	0.13	0.0037	I, II	high solubility/high permeability	Parent Permeability	Prodrug marketed for i.v. only
LY303366	>14.5	0.001	IV	high solubility/low permeability	Parent Permeability	Prodrug failed in rats
Phenytoin	1.5	0.0002	II	low solubility/high permeability	Parent Permeability	Bioequivalent in dogs, marketed for i.v. only
Prednisolone	0.1	0.004	I	high solubility/high permeability	Parent Permeability	Bioequivalent in Humans
Stachyflin	37.7	0.002	NA	Low solubility	NA	Plasma levels higher (mice)

Table 2. Calculated Dissolution Ratios and Rate Limits of Parent Drugs and Prodrugs.

[a]Calculations are based on earlier work by Yu (Yu, 1999; Zhang and Yu, 2004); [b]The dissolution ratios were calculated by dividing the dissolution time (T_{Diss}), by the mean small intestinal residence time, T_{Si}, 199 min; [c]Based on targeted oral dose. BCS Class I: high solubility, high permeability; BCS Class II: low solubility, high permeability; BCS Class III: high solubility, low permeability; BCS Class IV: low solubility, low permeability (Amidon *et al.*, 1995), high solubility is defined as the Dose/C_s <250mL; [d]Based on high membrane permeability in Caco-2 cells, when no human data is available; [e]Rate limits assume that enzymatic bioreversion by alkaline phosphatase is not rate limiting; this non-specific enzyme hydrolyzes nearly all phosphate esters rapidly (McComb *et al.*, 1979). [f]Entacapone has a strongly pH-dependent solubility; the drug is insoluble at pH 3 (near 17 μg/mL), but is soluble at pH 7.4 (near 1.75 mg/mL), thus the drug can be considered to possess an "intermediate" solubility. NA: Not available, since permeability is unknown.

Entacapone Phosphate

Entacapone phosphate is an investigational phosphate ester prodrug of entacapone (Figure 5), a drug marketed in 1999 as Comtan® by Novartis and Orion Pharma for the treatment of Parkinson's disease (PD) which results from low levels of dopamine in the brain. Common treatment of PD involves oral administration of levodopa, a precursor of dopamine that can cross the blood-brain barrier, combined with an inhibitor of dopa decarboxylase (DDC), such as carbidopa, to inhibit decarboxylation of levodopa in the periphery. Entacapone is clinically used as an adjunct to the levodopa/carbidopa (Sinemet®) therapy (Chong and Mersfelder, 2000; Forsberg *et al.*, 2002). It acts peripherally as a selective and reversible inhibitor of catechol-O-methyltransferase (COMT), the main enzyme responsible for metabolic loss of levodopa after blockage of DDC (Chong and Mersfelder, 2000; Henchcliffe and Waters, 2002).



Entacapone Phosphate Entacapone

Figure 5. Structures of the investigational prodrug entacapone phosphate and its precursor, entacapone.

Entacapone exhibits linear pharmacokinetics over the dose range of 5 mg to 800 mg. While it is absorbed rapidly, within 1 h of administration, its oral bioavailability (~25–35%) is low (Heikkinen *et al.*, 2001). The factors contributing to this poor bioavailability have not been resolved. Savolainen *et al.* (2000b) suggested that it may be caused by entacapone's low aqueous solubility and slow dissolution in the pH range of the stomach and upper small intestine. To improve the aqueous solubility and dissolution rate of entacapone, Leppänen and coworkers (Leppänen *et al.*, 2000a,b; Heimbach *et al.*, 2003b) have synthesized a prodrug by splicing a phosphate functional group onto entacapone using phosphorus oxychloride as a phosphorylation agent. The resulting prodrug had an aqueous solubility of over 30 mg/mL at pH 7.4 while that of entacapone was 1.75 mg/mL at the same pH value. The prodrug was enzymatically labile, yet highly stable toward chemical hydrolysis (Leppänen *et al.*, 2000b). This phosphate prodrug strategy did not succeed, as the prodrug did not yield higher entacapone plasma levels in rats (Heimbach *et al.*, 2003b), which may be the result of entacapone's low intestinal permeability. Other attempts to increase entacapone's bioavailability included the synthesis of lipophilic prodrugs (Savolainen *et al.*, 2000a) to enhance its permeability.

The main barrier to achieving high bioavailability for entacapone, however, may lie in its high systemic clearance (Heikkinen *et al.*, 2001), which may not be saturated by optimizing presystemic drug absorption through a prodrug approach. In general, metabolic liability is unlikely to be addressed *via* a solubility-enhancing prodrug strategy, and ideal parent drugs are those with low-to-medium clearance.

Fludarabine Phosphate

Fludarabine phosphate (Fludara®), is a fluorinated nucleotide analog of the antiviral agent vidarabine, 9-β-D-arabinofuranosyladenine(ara-A), which differs only by the presence of a fluorine atom at position 2 of the purine moiety and a phosphate group at position 5 of the arabinose moiety (Plunkett *et al.*, 1993), shown in Figure 6. These structural modifications result in increased aqueous solubility and resistance to enzymatic degradation by adenosine deaminases compared to vidarabine (Brockman *et al.*, 1977; Plunkett *et al.*, 1990). Fludarabine phosphate is indicated for the treatment of patients with B-cell chronic lymphocytic leukemia (CLL) who have not responded to or whose disease has progressed during treatment with at least one standard alkylating agent containing regimen (Boogaerts *et al.*, 2001; Rossi *et al.*, 2004). The parent drug, fludarabine, is sparingly soluble in water (Walsh, 2000; Heimbach, 2003), see also Table 2. However, the phosphate prodrug is soluble in water at approximately 9 mg/mL and in aqueous buffers at 28 mg/mL at pH 4 and 57 mg/mL at pH 9 (McEvoy, 2000). Until recently, fludarabine phosphate was available only as a liquid formulation for intravenous administration. Now, a solid formulation 10 mg tablet has been developed for oral administration (Foran *et al.*, 1999; Oscier *et al.*, 2001). A dose-dependent increase in systemic exposure of 2F-ara-A (fludarabine) was observed after oral administration of fludarabine phosphate, with an AUC (0–24 h) of fludarabine similar to that obtained *via* intravenous administration (Foran *et al.*, 1997, 1999). Dose-independent and predictable bioavailability with low intraindividual variation (Foran *et al.* 1997, 1999; Rossi *et al.*, 2004) and no food effects were observed (Oscier *et al.*, 2001). Based on the relatively low dose, combined with moderate parent drug solubility, this successful oral prodrug was marketed as the phosphate probably because it already existed as a commercial parenteral product.

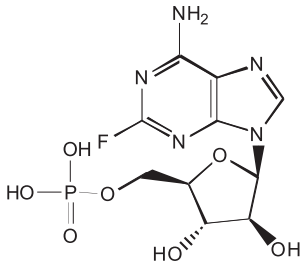


Figure 6. Structure of the marketed oral prodrug Fludara® (fludarabine phosphate), a phosphate ester prodrug of fludarabine.

Estramustine Phosphate

Estramustine phosphate (EMP) is the phosphate ester prodrug of the practically insoluble, non-ionizable parent drug estramustine, shown in Figure 7 (Wadsten and Lindberg, 1989). Emcyt® (Pharmacia, Pfizer) is a cytotoxic drug that has long been used in the treatment of advanced prostatic carcinoma and is available in both injectable and oral formulations (Bergenheim and Henriksson, 1998). The oral prodrug is a "high-dose" compound with suggested dosages from 140 mg to 1400 mg/day divided in 2–3 doses (Perry and McTavish, 1995). The cytotoxic mechanism of action is complex and not completely understood. However, EMP is thought to bind to microtubule-associated proteins (MAPs) and/or to tubulin, which results in the arrest of cell division in the G_2/M phase of the cell cycle.

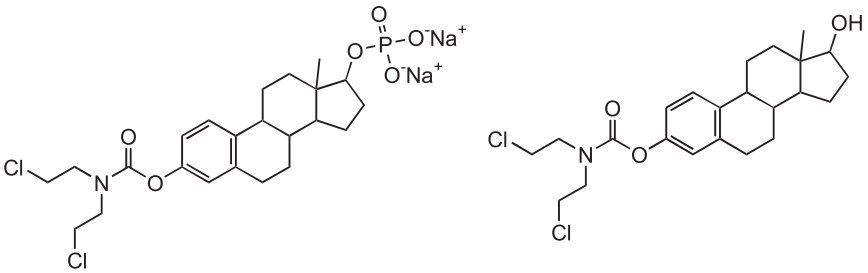


Figure 7. Structures of the estramustine phosphate disodium salt and its parent drug, estramustine.

After oral administration, EMP is rapidly dephosphorylated (Tritsch *et al.*, 1974) and reaches the blood circulation as the active estramustine drug or as its oxidized isomer, estromustine. Its metabolite concentrations are variable and the probable cause might be the substantial first-pass metabolism by phosphatases from various tissues including small intestine (Bergenheim and Henriksson, 1998). Pharmacokinetic studies showed that EMP has low and variable bioavailability, ranging from 44 to 75%, which indicated that absorption might be incomplete. Its bioavailability is also reduced in the presence of milk, food, or free calcium (Gunnarsson *et al.*, 1990). In contrast, no studies have been published on oral dosing of estramustine, likely due to challenges in identifying a suitable conventional formulation for this parent drug.

Reasons for the success in the prodrug strategy with EMP may lie in the biopharmaceutical properties of estramustine. Estramustine has a low solubility compared to the high-targeted dose, and this lipophilic compound, which lacks ionizable groups but possesses a primary alcohol group, lends itself to phosphorylation. Moreover, unlike some other phosphate esters, the prodrug is also known to significantly enhance the solubility of estramustine through a surfactant effect (Heimbach, 2003; Heimbach *et al.*, 2003a).

Fosamprenavir

Fosamprenavir (Telzir®, Lexiva™) is the phosphate ester prodrug of the protease inhibitor (PI) amprenavir (Agenerase®), which was co-discovered by Vertex Pharmaceutical (VX-175) and GlaxoSmithKline (GW433908). This novel prodrug addresses several challenges. It shows improved solubility and equivalent or higher bioavailability compared to the parent drug, and permits more convenient once- or twice-daily tablet dosing instead of multiple capsules (Corbett and Kashuba, 2002; Falcoz *et al.*, 2002; Becker and Thornton, 2004; Ellis *et al.*, 2004).

A prodrug screening strategy in the preclinical stage that systematically identified amprenavir's pharmacokinetic liabilities was successfully employed for the identification of the now launched phosphate prodrug (Furfine *et al.*, 1999, 2004). Amprenavir is a high-dose parent drug (1200 mg bid, or 8 capsules) with low aqueous solubility (0.04 mg/mL) and high permeability in the Caco-2 cell model (Polli *et al.*, 1999), identifying amprenavir as a typical BCS Class II drug (Amidon *et al.*, 1995) with a dose number near 100 (see also Tables 1 and 2). Poor dissolution/solubility as well as its high lipophilicity (clogP = 3.3) are thought to contribute to the variable bioavailability of amprenavir, which ranges from 30 to 90% (Polli *et al.*, 1999; Noble and Goa, 2000).

Conventional formulation strategies to enhance amprenavir's dissolution rate utilized a large percentage of solubilizing excipients in the marketed Agenerase® formulation. For that reason, amprenavir's Agenerase® capsules have a relatively low drug content, which results in extensive "capsule-burden." This cumbersome dosage regimen can cause poor patient compliance, especially when patients are on combination drug therapies with other PIs such as ritonavir (Anderson, 2004; Chapman *et al.*, 2004).

Like other phosphate esters, fosamprenavir is rapidly and extensively hydrolyzed by alkaline phosphatase in the GI tract to yield amprenavir during absorption with minimal fosamprenavir reaching the systemic circulation (Furfine *et al.*, 2004; Wood *et al.*, 2004). Despite the high solubility of the sodium salt (>100 mg/mL) (Furfine *et al.*, 2004) the prodrug is marketed as the mono-calcium salt (Figure 8). In this form, the solubility of the prodrug is only ~10-fold higher at GI pH compared to that of the parent drug (0.31 mg/mL vs. 0.04 mg/mL)

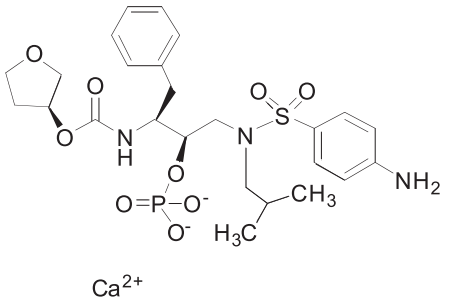


Figure 8. Structure of the oral prodrug fosamprenavir (Lexiva™ ,Telzir®), the calcium phosphate ester salt of amprenavir (Agenerase®).

(Sorbera *et al.*, 2001; Furfine *et al.*, 2004). Selection of the calcium salt of fosamprenavir produced a crystalline form favorable to dosage formulation and product development as compared to the hygroscopic sodium salt (Furfine *et al.*, 2004). Fosamprenavir, as the stable calcium salt, has allowed the development of a compact tablet dosage form, permitting more convenient and flexible dosing schedules compared to amprenavir capsules. The FDA recently approved a once-daily fosamprenavir/ritonavir dosage regimen that consists of only 4 tablets/capsules compared to 10 capsules of amprenavir that are required (Anderson, 2004; Chapman *et al.*, 2004; Furfine *et al.*, 2004).

In summary, a phosphate prodrug strategy led to a marketed product because amprenavir is a high-dose parent drug that exhibits high permeation and low aqueous solubility, making it an ideal candidate for an oral solubility prodrug strategy.

Dose, Solubility and Dissolution Rate Considerations

The Dose Number as a Tool in Selection of Parent Drugs

The dimensionless dose number (Do) as described by Amidon and colleagues (Amidon *et al.*, 1995; Kasim *et al.*, 2004; Sun *et al.*, 2004) can be used as a tool to evaluate the practicality of a solubility enhancing prodrug approach. The Do was calculated as the targeted oral parent equivalent dose/250 mL/C_{smin}, where C_{smin} is the lowest parent drug solubility at relevant intestinal pH and 250 mL represents the 8 oz volume of fluid routinely recommended with oral drug administration. The targeted doses were taken from the literature or scaled from animal experiments based on equivalent mg/kg body-weight dosing (Table 1). A high solubility drug is conservatively defined as a drug in which the highest prescribed dose is soluble in 250 mL of fluid, or where Do is ≤1 (Kasim *et al.*, 2004).

The straightforward dose number concept can be extended to aid in the identification and selection of ideal parent drugs for a solubility prodrug approach. A prodrug strategy can be considered for poorly soluble parent drugs with high-targeted doses (relative to their solubility) when formulation approaches prove futile. A recent list of 123 drugs with marketed immediate release dosage forms on the WHO Essential Medicines List contained only six drugs with Do >100, suggesting that drugs with high Do (Do >100) are challenging to develop (Kasim *et al.*, 2004). To assess the potential advantage of a solubility prodrug approach, Do were calculated for selected parent drugs (Table 1). The case will be made that parent drugs with a Do >100 and high intestinal permeability are best suited for a solubility prodrug approach.

Solubility and Dose Numbers for Parent Drugs and Prodrugs

Phosphate prodrugs had solubilities higher than that of the corresponding parent drugs by 10–100000-fold (Table 1), as expected. Most prodrugs are "highly soluble" compounds, as defined in the Biopharmaceutical Classification System

(BCS), and their Do is ≤1 (data not shown) (Kasim *et al.*, 2004). For example, fosphenytoin has a Do of 0.003 for a 100 mg dose, which compares to a Do of 21 for phenytoin, the parent drug (Table 1).

Parent drugs were classified based on their measured aqueous solubility at intestinal pH and their membrane permeability (Heimbach *et al.*, 2003b). From Table 2, it can be seen that the soluble prodrugs nearly always lead to a change in the solubility classification in the biopharmaceutical classification systems (BCS) (Amidon *et al.*, 1995; Sun *et al.*, 2004). BCS Class II drugs are converted to BCS Class I prodrugs (phenytoin/fosphenytoin), while BCS Class IV drugs are converted to BCS Class III prodrugs (LY303366/LY307853). Prodrug approaches for BCS Class I parent drugs are rare since the prodrug would remain a Class I drug (low dose hydrocortisone/hydrocortisone-phosphate) and the prodrug would not likely enhance drug absorption. TAT-59 is an unusual case as the prodrug and DP-TAT-59 are both BCS Class II drugs (Table 2).

Low Do Drugs (Do<1) with High Permeability:

Hydrocortisone-phosphate (Do = 1) is available only as the prodrug for parenteral use. For oral administration, the parent drug hydrocortisone is marketed. Hydrocortisone for oral delivery is a low dose (5–20 mg) compound, and this polar steroid has a relatively high solubility near 0.3 mg/mL at intestinal pH (Pedersen, 2000). Combined with its high P_{eff} in rats (Fleisher *et al.*, 1986) and its low-dose bioavailability of 96%, hydrocortisone can be classified as a Class I compound according to the BCS (Amidon *et al.*, 1995; Kasim *et al.*, 2004; Sun *et al.*, 2004) (Table 2). Phosphate prodrug strategies for Class I parent drugs are likely to yield bioequivalent results, with no direct biopharmaceutical advantage. Consistent with this projection, both hydrocortisone and hydrocortisone-phosphate were well absorbed and had similar high absorptive fluxes in Caco-2 experiments (Heimbach *et al.*, 2003b). Another example is the Class I drug prednisolone (Olivsei, 1985) (Tables 1, 2) where prodrug dosing was found to be bioequivalent to parent drug dosing in humans and no significant increase in plasma levels was seen at the low dose of 5 mg. These steroid drugs might be regarded as Class II compounds at the very high oral doses sometimes employed with chemotherapy (prednisone 100 mg). However, even at high doses the Do value for similar steroids is low (Table 2) and suggests no oral delivery advantage for the prodrug. Fludarabine is another example of a BCS Class I parent drug with a marketed oral phosphate prodrug. As discussed, marketing as an oral prodrug may have been a consequence of prior approval of the parenteral formulation fludarabine-phosphate (Fludara®) (Boogaerts *et al.*, 2001) and not a consequence of an oral prodrug strategy, as illustrated by its low Do.

Low Do Drugs (Do<1) with Low Permeability:

For low dose parent drugs with moderate solubility and low permeability, such as entacapone, an oral phosphate prodrug approach may fail while the strategy may be viable for parenteral delivery. As discussed, entacapone is a compound

with high solubility at neutral pH, low solubility at gastric pH, and low cellular permeability. One of the reasons for the low bioavailability (Savolainen *et al.*, 2000a) was thought to be entacapone's pH dependent solubility, which is that of a typical weak acid. However, the prodrug failed in rat studies, and entacapone plasma levels were actually slightly higher after dosing an entacapone solution at pH 7.4 than after dosing the phosphate prodrug (Heimbach *et al.*, 2003b). The fact that entacapone flux was not significantly increased in Caco-2 cells is not surprising since the parent drug has sufficient solubility and its absorption is likely permeability rate-limited (Tables 1, 2).

Medium Do Drugs (1<Do<100) with High Permeability:

Fosphenytoin (Parent Do 21, Dose 100 mg) is a successful parenteral prodrug of phenytoin (Dilantin®) with rapid dissolution and increased solubility of 140 mg/mL (Stella, 1996) compared to the poor solubility of phenytoin, near 19 μg/mL. Phenytoin is a BCS Class II drug whose absorption is nearly complete in humans when dosed as the soluble sodium salt, but whose absorption is erratic (Neuvonen, 1979) at doses of 100 mg (Sietsema, 1989). For that reason, the prodrug would offer only a flux advantage in humans if very high doses of phenytoin were administered. Consistent with this projection, oral dosing of fosphenytoin has been found to yield a lower t_{max} and higher C_{max} in rats (Burstein *et al.*, 1999) and dogs (Varia and Stella, 1984) and resulted in an increased relative bioavailability in dogs (Lai *et al.*, 1987).

High Do (Do>100) Drugs with Low Permeability:

While these drugs are not likely to be suitable candidates for oral delivery, they can be ideal prodrug candidates for the parenteral route. An example of such a BCS Class IV parent drug is the echinocandin B analog LY303366 targeted for systemic fungal infections. LY303366 has poor intestinal permeability as measured in the Caco-2 model (Li *et al.*, 2001) (Table 1), in spite of a favorable log octanol/water partition coefficient of 2.0 and a solubility less then 0.005 mg/mL. The low permeability is likely a function of moderate molecular size and high hydrogen bonding potential. The phosphate prodrug, LY307853, failed to improve (Zornes *et al.*, 1993) LY3030366 plasma levels from oral dosing in dogs, and the overall bioavailability was less then 5%. Thus, high Do alone is not a sufficient criterion for this oral delivery strategy as highly soluble prodrugs can still fail to improve absorption of poorly permeable parent drugs.

High Do (Do>100) Drugs with High Permeability:

These are "ideal" solubility prodrug candidates, as the rate-limiting step for absorption is addressed and absorption is enhanced by the solubility prodrug approach. The targeted doses of these compounds are high with respect to parent drug solubility, and conventional formulation approaches often fail to improve

oral delivery. Estramustine (Do = 1600–4000) and TAT-59 (Do = 4000–6400) are examples of successful prodrugs in this class (see also Table 1). Parent drugs such as phenytoin may fall into this category if very high oral doses are required.

Estimation of Dissolution Rates and Ratios

To evaluate or rank-order solubility enhancing prodrugs, it is useful to calculate the estimated dissolution times of prodrugs and parent drugs relative to the GI residence time. Here, the dissolution times (TDiss) for selected (phosphate) prodrugs were calculated based on Eq. 4 published by others (Yu, 1999; Zhang and Yu, 2004).

Equation 4.

$$T_{Diss} = \rho h r_0 / 3DC_s$$

ρ = drug density (which is generally near 1200 mg/cm^3), h = aqueous diffusion layer thickness set to 30 μm, r_0 = initial particle size set to 25 μm, D = diffusion coefficient in cm^2/s calculated by the method of Hayduk and Laudie (Reid and Prausnitz, 1977; Fleisher *et al.*, 1986), and C_s is the estimated aqueous solubility in the GI lumen. Dissolution rates were estimated as $k_{\mathrm{Diss}} = T_{\mathrm{Diss}}^{-1}$. For compounds that were not available for analysis, solubility data from the literature values were utilized. The dissolution ratios (Table 2) were calculated by dividing the dissolution time, T_{Diss}, by the mean small intestinal residence time, T_{Si}, = 199 min (Yu, 1999). High solubility was defined here as a parent dose/solubility ratio <250 mL, as discussed by others (Hoerter and Dressman, 1997). High permeability drugs, such as phenytoin, exhibit a fraction absorbed >90%. However, for some phosphate esters listed, human permeability data are not available. In those cases, "high permeability" is consistent with "high permeability" as measured in the Caco-2 model (Heimbach, 2003; Heimbach *et al.*, 2003b).

When T_{Diss}/T_{Si}, is near unity, dissolution may be slow yet complete within the intestinal residence time, as is the case with phenytoin (Table 2). For T_{Diss}/T_{Si}, $<<1$, dissolution is rapid and complete, and absorption is permeability rate-limited; examples of this are entacapone and fludarabine. For T_{Diss}/T_{Si}, $>>1$, dissolution is incomplete within GI residence times. Estramustine and DP-TAT-59 fall into this category. Not surprisingly, for all prodrugs with the exception of TAT-59 T_{Diss}/T_{Si}, was $<<1$, indicating rapid dissolution (Table 2, Figure 9). For fosamprenavir (GW433908), the calculated ratio was 0.14, which may be due to the relatively low solubility of this calcium salt of this phosphate ester prodrug (Furfine *et al.*, 2004).

For the unusual, zwitterionic TAT-59, calculated dissolution times below pH 7 were long compared to the GI residence time. As shown in Table 2, the T_{Diss}/T_{Si}, ratios for TAT-59 were pH-dependent and ranged from 24 (pH 5.5) to near unity (pH 7.4), suggesting that dissolution is slow compared to other prodrugs. At pH 6, T_{Diss}/T_{Si}, = 11 for DP-TAT-59 and 12 for TAT-59, indicating that the prodrug actually has a slower dissolution rate than the parent drug at this pH (Table 2). This is the result of zwitterion formation from a basic group within the TAT-59

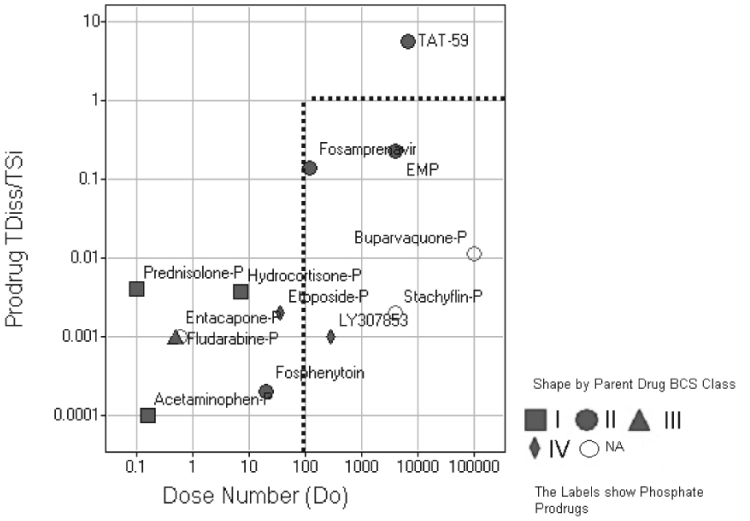


Figure 9. Plot of phosphate ester prodrug dissolution ratios against their Parent Drug Dose number (Do). The enclosed rectangular area represents medium-to-high dose numbers for parent drugs targeted for a phosphate prodrug strategy with surveyed prodrugs that yield the highest solubility advantage over the parent drug. For compounds with low Do, conventional formulations of the parent drug may be successful for oral delivery, such as for acetaminophen and fludarabine. Dissolution is not rate-limiting absorption with the prodrugs, (i.e., $T_{Diss}/T_{Si} > 1$) (Yu, 1999; Zhang and Yu, 2004), except for TAT-59, a zwiterionic prodrug (Heimbach, 2003).

molecule. In general, weakly basic amines on an NCE negate a soluble prodrug approach, as they can partially render a phosphate prodrug strategy ineffective through zwitterion formation. Therefore, if parent drugs contain basic centers, their ionization constant (i.e., pKa) should be determined prior to embarking on a phosphate prodrug strategy.

Figure 9 shows the Do of various prodrugs compared to their calculated prodrug dissolution ratios. Prodrugs of parent drugs with Do in the range of 0.1–10 (Table 1), such as prednisolone phosphate, fludarabine phosphate and acetaminophen phosphate, are shown in the lower left corner. Compounds with high Do characteristic of high dose parent drugs with low solubility, such as estramustine phosphate and TAT-59 (Table 1), are shown on the left side of Figure 9. For prodrugs with high Do (i.e., >100) and $T_{Diss}/T_{Si} \leq 1$, the highest dissolution-solubility advantage over the parent drug is gained (enclosed area in Figure 9).

Summary on Solubility and Dose

To summarize, parent drugs with a high targeted oral dose and low solubility (high Do, >100), such as DP-TAT-59, are well suited for a phosphate prodrug strategy. For successful oral delivery, high intestinal permeability of parent drugs is required, i.e., they are BCS Class II drugs.

A phosphate prodrug strategy has been utilized on occasion for BCS Class I drugs (low Do, i.e., Do <1), but no biopharmaceutical advantage is achieved. For BCS Class III and IV (low permeability) parent drugs with high Do, such as entacapone and LY307853, a phosphate prodrug strategy may fail to improve delivery for orally targeted drugs, but will likely succeed for parenteral administration of the drugs.

Other Solubility Prodrugs

From a chemical perspective, it may be convenient to distinguish between carrier-linked prodrugs, i.e., drugs linked to a carrier moiety *via* a labile bridge, and bioprecursors, which do not contain a carrier group that are activated by the creation of a functional group (Wermuth, 1984). Sulindac (Clinoril®), a non-steroidal anti-inflammatory indene derivative, is a bioprecursor prodrug in which the marketed sulindac sulfoxide is a water-soluble precursor of sulindac sulfide (Figure 10). Following absorption, sulindac sulfoxide undergoes two major biotransformations—one reversible reduction to its active metabolite sulindac sulfide and another irreversible oxidation to its inactive sulfone metabolite (Figure 10). The sulfide is excreted in the bile and reabsorbed from the intestine after rapid re-oxidation to the sulfoxide. This reversible oxidation is thought to maintain constant blood levels and may explain the long half-life of the sulfide metabolite (Duggan, 1981). Sulindac sulfoxide, the prodrug, is nearly completely absorbed from the GI tract (Shen and Winter, 1977) and is 100 times more water-soluble (3.3 mg/ml) than the sulfide metabolite (0.03 mg/ml) at pH 7.4. It is this greater solubility in addition to its ideal lipophilicity (log P of 1.52 at pH 7.4) that results in great intestinal absorption of this successful drug (Davies and Watson, 1997).

For carrier-linked prodrugs, the carrier moiety is conveniently linked to a hydroxy, amino, or carboxy group. Derivatization of the latter is particularly rewarding in terms of lipophilicity, since a highly polar carboxylate group becomes masked inside an ester group whose properties can be broadly modulated (Testa and Mayer, 2001).

Poly(ethylene glycol) Ester Prodrugs

Polyethylene glycol (PEG) is a polyether diol manufactured by the aqueous anionic polymerization of ethylene oxide. Polymerization is initiated using anhydrous alkanols such as methanol or derivatives including methoxyethoxy ethanol, which result in a mono alkyl, capped poly(ethylene glycol) such as methoxy PEG (mPEG). mPEG is acceptable for drug modification because of its low potential reactivity. The polymerization can be controlled and a variety of linear or branched neutral polymers possessing broad molecular weight range (1000–50,000) can be obtained (Hooftman *et al.*, 1996; Greenwald *et al.*, 2003). These polymers are amphiphilic and dissolve in organic solvents as well as water. PEGs are non-toxic and their hepatic and renal clearance makes them ideal to use

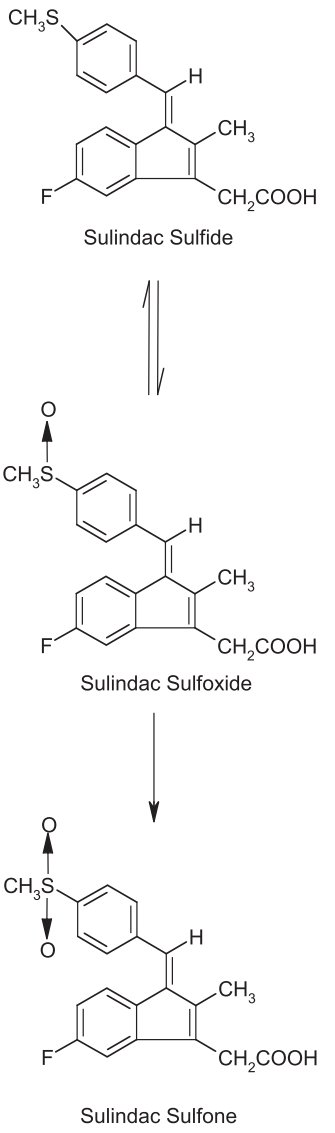


Figure 10. Structures of Sulindac®, also known as sulindac sulfoxide, and its metabolites.

in pharmaceutical applications. Thus, PEG has been approved by the FDA for human intravenous (i.v.), oral, and dermal applications. These characteristics allow PEG to be used as carrier system through a prodrug approach either to enhance aqueous solubility of a poorly soluble parent drug or for other drug delivery purposes. The general scheme of the PEG-prodrug approach is illustrated in Figure 11. PEG is covalently conjugated to the parent drug through a linker.

PEG prodrugs can be designed to undergo either a) chemical cleavage, with linkers including carbamate or carbonate moieties (Greenwald *et al.*, 1995; Cho and Chung, 2004); 1,6-elimination systems (Greenwald *et al.*, 1999), trimethyl

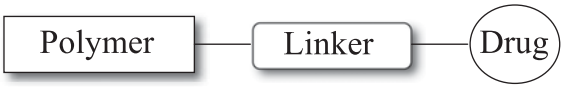


Figure 11. General scheme of polymeric prodrugs layout.

lactonization (Greenwald *et al.*, 2000), or b) enzymatic cleavage where the linker or spacer is sensitive to specific enzymes (Kopecek and Duncan, 1987; Dubowchik and Firestone, 1998; Caiolfa *et al.*, 2000).

Anti-cancer agents for parenteral administration have particularly benefited from the PEG prodrug approach. In addition to increasing solubility, PEG prodrugs enhance permeability and retention (EPR) effect (Maeda and Matsumura, 1989), where the prodrug is able to extravasate through the large fenestrations of the tumor vessels concentrating in the tumor matrix and the linked parent drug is released either in the extracellular compartment or inside the tumor cell. In this approach the pharmacokinetics of the parent drug are modified by increasing the circulating half-life of the prodrug, leading to enhanced systemic exposure (Greenwald *et al.*, 2003, 2004; Guiotto *et al.*, 2004).

Several applications of this approach for enhancing the solubility of a parent drug to increase oral bioavailability have been investigated. Cyclosporine A (CSA) is a highly lipophilic compound with poor aqueous solubility. Its absorption from the GI tract is incomplete with oral bioavailability ranging between 10 and 89% (Novartis Rev 7/97, Rec 2/98). To overcome its low and variable bioavailability, CSA has been administered in an excessive amount; however, this strategy was accompanied by serious side effects, including nephrotoxicity, hypertension, and hepatotoxicity in some patients. In order to increase its bioavailability, Cho and co-workers (Cho and Chung, 2004) developed a soluble mPEG-CSA prodrug through ester formation. One alcohol group of the PEG polymer was used to covalently link the PEG polymer to a chemical linker. The authors examined variable linkers such as succinate, propionic acid, methylsuccinate and thiodidlycolic acid (Figure 12). Esters with PEG as an electron-withdrawing substituent (alkoxy) in the α-position proved to be an effective linking group since they aid in the rapid hydrolysis of the ester carbonyl bond, releasing CSA (alcohol). The hydrolysis of the investigated prodrugs, expressed as half-life, was variable and depended on the linker moiety. In rat whole blood, the prodrugs showed half-lives for hydrolysis in the range 1.6–65 min, compared to 2.8–28 h in phosphate buffer at pH 7.4. The PEG-CSA conjugate, using succinate as a linker (KI-306, Figure 12) showed about 1.5-fold increase in C_{max} and AUC compared to equivalent dose of unmodified CSA after oral administration to rats. This type of prodrug may enhance the solubility of the parent drug and increase its bioavailability. The advantage is mainly related to its ability to reduce the administered dose, which may contribute to reduction in CSA side effects. Although the bioavailability of CSA was enhanced, the impact of this prodrug approach on high variability accompanying CSA dosage was not reported.

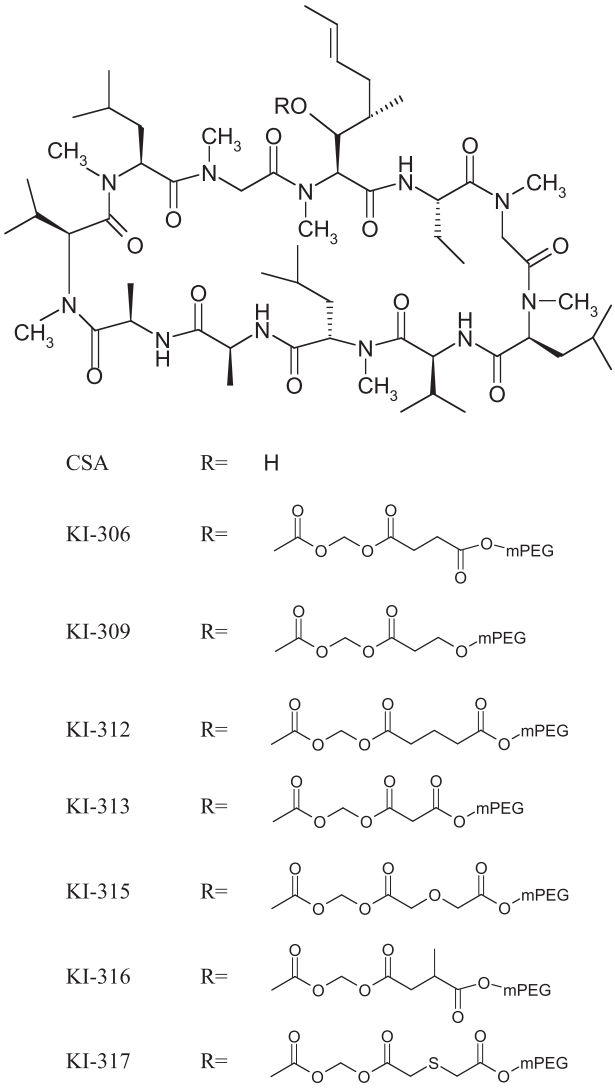


Figure 12. Structures of CSA and prodrugs adapted from the literature (Cho and Chung, 2004).

The insoluble nature of paclitaxel, one of the most potent chemotherapeutic agents used in the treatment of breast and ovarian cancers, has led to the formulation of this drug as a 1:1 ethanol:cremophore mixture, which is diluted prior to lengthy infusions. Various hypersensitive reactions have been found in patients undergoing paclitaxel treatments, mainly due to histamine release mediated by cremophore. Accordingly, paclitaxel was considered as a candidate for a soluble prodrug strategy and, specifically, conjugation with PEG. A series of water-soluble paclitaxel prodrugs was synthesized by attaching paclitaxel to mPEG either through amino acids (Pendri *et al.*, 1998; Feng *et al.*, 2002), succinate (Li *et al.*, 1996; Choi *et al.*, 2004) or methoxyacetate (Greenwald *et al.*, 1996) as linkers.

In most of these studies, the enhancement of solubility was demonstrated for i.v. administration to overcome the side effects accompanying the use of solubilizing agents.

When paclitaxel is orally administered, it is poorly absorbed for two reasons. In addition to its low solubility, paclitaxel is a substrate for the multidrug efflux transporter P-gp in the GI tract (Terwogt *et al.*, 1999). Therefore, this drug is mainly used intravenously. However, in a study utilized by Choi and Jo (2004), a PEG prodrug of paclitaxel was synthesized specifically for oral delivery. The authors introduced a water-soluble PEG-paclitaxel prodrug with succinyloxy-methyloxy carbonyl as a new self-immolating linker that is spontaneously decomposed into paclitaxel by an esterase enzyme. In animal studies, the absolute oral bioavailability of paclitaxel released from the hydrolysis of the PEG prodrug increased to 6.3% compared to 1.6% for unmodified paclitaxel administered as a suspension in a water-Tween-80® co-solvent. C_{max} was increased as well, while t_{max} and half-life were unchanged. The increased bioavailability of paclitaxel by PEG might have resulted from a reduced effect of intestinal P-gp-mediated drug efflux as a result of the higher solubility of the prodrug.

Although CSA and paclitaxel pegylated prodrugs have not been tested in clinical trials, *in vivo* results obtained from animal experiments were successful. However, when this prodrug strategy was applied to the HIV protease inhibitors saquinavir, indinavir, and nalfinavir by conjugation to PEG2000, *in vitro* results obtained utilizing Caco-2 cells were disappointing. The conjugation of HIV protease inhibitors to PEG2000 significantly increased their hydrophilicity, decreasing their membrane permeation and passive diffusion across the cell membrane monolayer. This prodrug approach may not be applicable for increasing oral exposure for such compounds (Rouquayrol *et al.*, 2002).

Amino Acid Ester Prodrugs

Additional strategies to enhance drug solubility include amino acid ester prodrugs. In general, amino acid pro-groups introduce a cation or anion to the parent drug. At physiological pH, the prodrugs are ionized and have greater water solubility than the parent. In the literature, a few investigations have been reported with only a limited number of prodrugs reaching clinical stages. Examples of amino acid ester prodrugs are listed in Table 3, and their structures are shown in Figure 13.

CAM 4451 is a non-peptide, α-methyl tryptophan derivative used as a selective high-affinity NK1 neurokinin receptor antagonist. It is a lipophilic compound with Clog P of 4.4, which demonstrates very low aqueous solubility. Consequently, data obtained from oral administration of this compound to rats showed poor oral bioavailability. The introduction of amino acids such as glycine and leucine increased the solubility of CAM 4451 from less than 2 μg/mL to 3 and 0.1 mg/mL for dimethyl glycine (CAM 4562) and leucine (CAM 4580) ester prodrugs, respectively (Chan *et al.*, 1998). These prodrugs were designed for hydrolysis to the parent drug by aminopeptidase enzymes in the brush border

Solubilizing Pro-group	Drug	Reference
Dimethyl glycine	CAM 4451	(Chan *et al.*, 1998)
Leucine	CAM 4451	(Chan *et al.*, 1998)
N,N-dimethyl glycine	CEP-5214	(Gingrich *et al.*, 2003) (Ruggeri *et al.*, 2003)
Glycine	Quercetin	(Mulholland *et al.*, 2001)

Table 3. Examples of Reported Amino Acid Ester Prodrugs

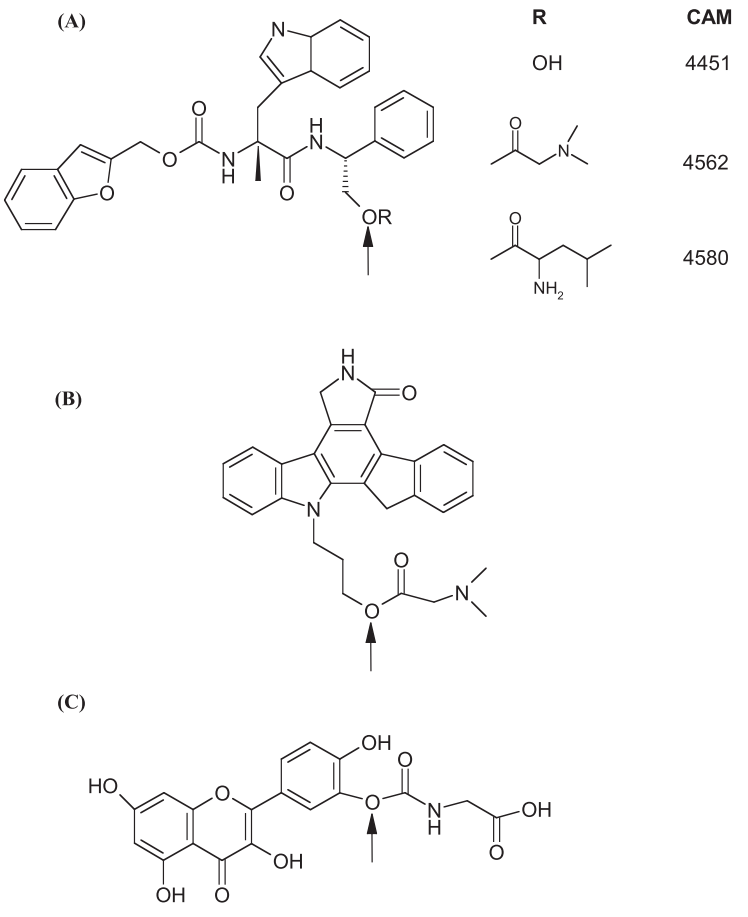


Figure 13. Structures of amino acid ester prodrugs of (A) CAM 4451, (B) CEP-5214, and (C) quercetin. These prodrugs are hydrolysed in vivo at the arrowed oxygen to release the parent drugs and their corresponding amino acids.

membrane of the GI tract. The time of conversion to the parent drug is important to the success of this approach since rapid hydrolysis could result in parent compound precipitation in the intestine. To be successful, the prodrug should escape hydrolysis in the lumen and rapidly hydrolyze at the brush border membrane. In this study, the dimethyl glycine ester of CAM 4451 was more selective for hydrolysis at the brush border compared to its leucine ester, which had short reconversion time; this suggested that this prodrug hydrolyzed before reaching the brush border membrane, thereby providing the potential for precipitation. *In vivo* experiments indicated an increase in the bioavailability of CAM 4451 from 3.6 to 39% following the administration of the dimethyl glycine ester prodrug.

Among the successful examples for amino acid ester prodrugs is N,N-dimethyl glycine ester of CEP-5214 (CEP-7055). CEP-5214 is a potent low nanomolar pan-inhibitor of human VEGF-R tyrosine kinases yet demonstrates significant *in vivo* antitumor activity. CEP-5214 has a very low aqueous solubility (10 μg/mL), which is believed to be responsible for its low bioavailability. Accordingly, to enhance its solubility, the N,N-dimethyl glycine ester was synthesized. CEP-7055 as the HCl salt yielded high aqueous solubility (40 mg/mL) and, when administered to animals as the prodrug, increased plasma levels of the parent drug CEP-5214 with bioavailability ranging from 15–20%. The prodrug has now been advanced to phase I clinical trials (Gingrich *et al.*, 2003; Ruggeri *et al.*, 2003).

Quercetin is a naturally occurring flavonoid with many biological activities, including inhibition of a number of tyrosine kinases. Clinical trials exploring different schedules of administration of quercetin have been held back by its extreme water insolubility, requiring formulation in dimethylsulfoxide (DMSO) where 150 mg quercetin is soluble in 1 mL of DMSO (Ferry *et al.*, 1996). There are concerns about using high doses of DMSO as it causes dose-dependent haemolysis (Muther and Bennett, 1980; Santos *et al.*, 2003). To overcome these limitations, QC12, a water-soluble glycine ester prodrug of quercetin, was synthesized (Mulholland *et al.*, 2001) and evaluated in a clinical study to investigate its pharmacokinetics following oral and i.v. administrations to cancer patients. *In vitro* hydrolysis of QC12 to quercetin at 37°C in water showed the prodrug to be stable with a half-life of 16.9 h while the half-life in whole blood was only 0.39 h. However, following oral administration neither QC12 nor quercetin were detected in the serum, demonstrating that QC12, like quercetin is not orally bioavailable (Gugler *et al.*, 1975). After i.v. administration, both compounds were detected in the serum. It was not clear why both compounds were not bioavailable after oral administration; however, the authors considered the possibility that the rates governing distribution and metabolism of QC12 could be more rapid than the hydrolysis rate generating quercetin, diminishing the utility of the prodrug for oral dosage. The relative availability of quercetin obtained following i.v. dosing of QC12 was estimated to be in the range of 20–25%. The advantage presented by QC12 is its high aqueous solubility compared to that of quercetin, eliminating the need for formulation in DMSO. However, its use is limited to i.v. administration.

Miscellaneous Ester Prodrugs

To improve the poor aqueous solubility of the aforementioned COMT inhibitor drug entacapone, investigators evaluated various alkyl carbamate esters of entacapone as potential prodrugs to enhance its oral bioavailability (Savolainen *et al.*, 2000a; Leppanen *et al.*, 2001; Forsberg *et al.*, 2002). In these studies, the objective was to increase the oral bioavailability of entacapone (reported in the range of 25–35%) by increasing its aqueous solubility at acidic pH and to increase its lipophilicity at neutral pH. Entacapone is a weak acid (pKa 4.5) and, therefore, it is ionized at the pH of the small intestine, hindering its absorption. Its solubility, which is pH-dependent, is 77 μg/mL at pH 5.0 and increases to 1752 μg/mL at pH 7.4. Various carbamate derivatives (Figure 14) of entacapone were synthesized and evaluated *in vitro* and *in vivo* (Savolainen *et al.*, 2000a; Leppanen *et al.*, 2001; Forsberg *et al.*, 2002). Although these authors were successful in increasing entacapone aqueous solubility at acidic pH and lipophilicity as indicated by the log P values for some prodrug candidates, the bioavailability was not increased in spite of enhanced solubility. Relatively lower bioavailabilities of entacapone released from the carbamate ester prodrugs were observed compared to unmodified entacapone after oral administration. The prodrugs were further examined for their pharmacodynamic response in rats and, in line with pharmacokinetic observations, were less effective than entacapone. This result is consistent with those obtained with a phosphate prodrug of entacapone (Heimbach *et al.*, 2003b), discussed earlier in this chapter, where entacapone absorption is controlled by permeability.

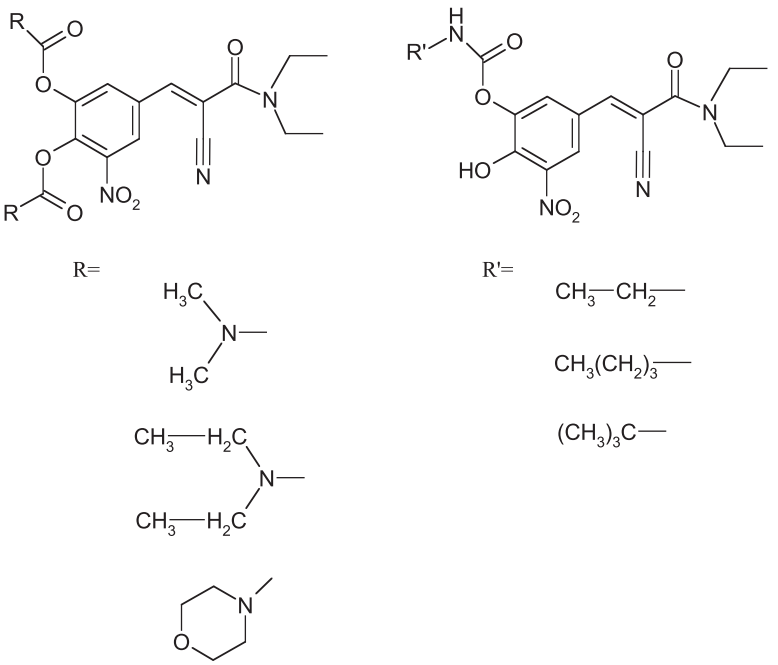


Figure 14. N-alkyl and N,N-dialkyl carbamate esters of entacapone (adapted from Savolainen *et al.*, 2000b).

O→N Acyl Migration Reaction

An acyl migration reaction is observed between adjacent amino and hydroxyl groups, leading to the formation of O-acylpeptides when peptides containing β-hydroxy-α-amino acids such as serine and threonine residues are exposed to strong acids (Wakamiya *et al.*, 1974). The solubility of these O-acylpeptides in aqueous media is generally increased by the newly produced amino group, and reconversion back to the peptides can be achieved by a pH shift to a weakly basic condition in aqueous media (Oliyai and Stella, 1995). This effective strategy to enhance the solubility of parent compounds was extensively investigated by Kiso and his colleagues (Kiso *et al.*, 1999b; Matsumoto *et al.*, 2001; Hamada *et al.*, 2002, 2003, 2004; Hayashi *et al.*, 2003a,b; Skwarczynski *et al.*, 2003). The authors applied the O→N intramolecular acyl migration reaction to potent small-sized peptide human immunodeficiency virus type-1 protease (HIV-1 PR) inhibitors that showed low water solubility and low GI absorption (Hamada *et al.*, 2002, 2003, 2004). These included the dipeptide-type HIV-1 PR inhibitor KNI-727 (Hamada *et al.*, 2004) and the tripeptides-type KNI-272 and KNI-279 (Hamada *et al.*, 2002). These anti-HIV drugs consist of an allophenylnorstatine-thiazolidine-4-carboxylic acid core structure with highly selective and superpotent HIV-1 PR as demonstrated by potent *in vitro* and *in vivo* antiviral activities with low cytotoxicity. The O→N acyl migration reactions for these drugs are outlined in Figure 15.

The prodrugs of these compounds contained an O-acyl peptidomimetic structure with an ionized amino group that produced an increase in water solubility and were designed to regenerate the corresponding parent drugs based on O→N acyl migration reaction *via* a five-membered ring intermediate at the α-hydroxy-β-amino acid residue. The prodrug of KNI-727 increased water solubility

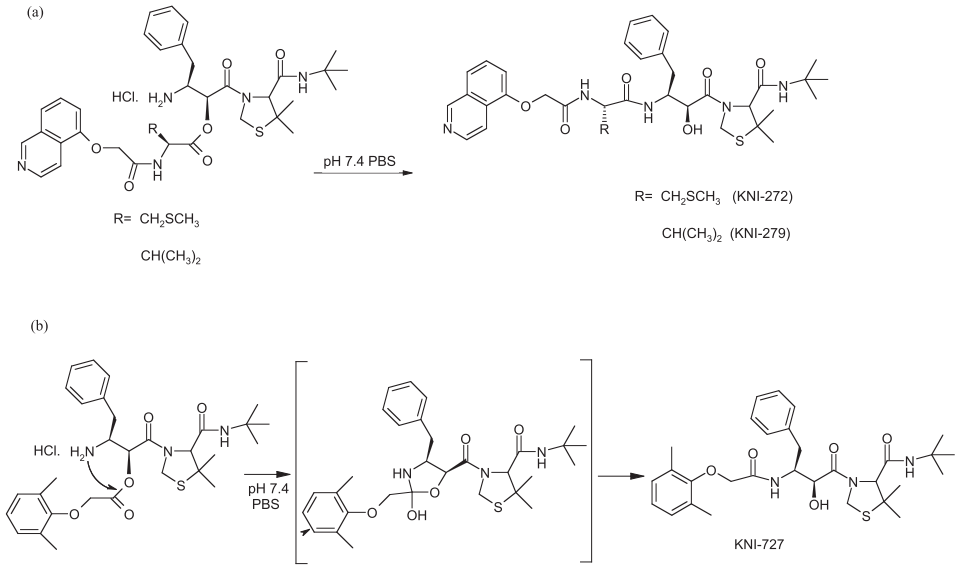


Figure 15. Prodrugs based on O→N intramolecular acyl migration reaction of: (a) KNI-272 and KNI-279; and (b) prodrug of KNI-727. Adapted from Hamada *et al.*, 2004.

more than 800-fold compared to the parent compound (Hamada *et al.*, 2004), while KNI-272 and KNI-279 prodrugs improved solubility more than 4000-fold (Hamada *et al.*, 2002). These prodrugs have the unique advantage of generating the parent compounds without the formation of additional by-products; this is in contrast to other prodrugs, which contain spontaneously or enzymatically cleavable moieties or linkers.

However, to achieve a successful prodrug, the migration rate to the parent drug should be fast enough (within GI residence time) to ensure higher GI absorption. For example, when this strategy was applied to the HIV-1 PR inhibitor ritonavir, which has a valine residue, the resulting prodrug was very slowly converted to ritonavir with a half-life value of 32 h at pH 7.4 (Hamada *et al.*, 2002). This very slow migration could be the result of the fact that this ritonavir prodrug forms an energetically unfavorable six-membered ring intermediate in the migration step.

The migration rate is significantly affected by the steric effect of the O-acyl groups, as is the case for the migration rates of the prodrugs of the HIV-1 PR inhibitors mentioned above. Their migration rates were ranked in the order KNI-727>KNI-272>>KNI-279. The KNI-279 prodrug, which showed the slowest migration, has a bulky isopropyl group. The KNI-272 prodrug has a methylthiomethyl group as an α-substituent at the O-acyl group while the KNI-727 prodrug has a non-branched α-substituent. The data indicate a significant effect of the O-acyl group in modulating the migration rate where less hindered side chains more effectively increase the migration rate. In addition, for a constant steric effect, the migration rate of the prodrugs depends on the electrostatic effect. In this regard, the introduction of an electron-withdrawing group, such as a nitro group or a chlorine atom, to the phenoxyacetyl group of the KNI-727 prodrug accelerates the migration rate, while the introduction of an electron-donating group, such as methyl or methoxy groups, slows the migration rate. Thus, controlling the migration rate is a key issue in utilizing the O→N acyl migration reaction for soluble prodrug development.

Furthermore, the same workers applied this strategy for synthesizing water-soluble prodrugs of taxoides, including paclitaxel, docetaxel, and canadensol (Hayashi *et al.*, 2003a; Skwarczynski *et al.*, 2003). The successful results obtained from applying O→N acyl migration to paclitaxel and canadensol suggest that this strategy can be applied to 3'-N-acyl taxoids, i.e., paclitaxel and canadenasol, but not to docetaxel, which possesses a *t*-butyloxycarbonyl group at the 3'-position (Hayashi *et al.*, 2003a; Skwarczynski *et al.*, 2003). In the case of docetaxel, although O→N migration occurs, the undesired hydrolysis of the *t*-butyloxycarbonyl group occurs as well.

Intramolecular Cyclization-Elimination Reactions

In this design, the prodrug has a water-soluble moiety attached, which contains two covalently and tandem-linked units through the hydroxyl group of the parent drug. These units, composed of a self-cleavable spacer and a

hydrophilic solubilizing moiety with an amino group that is ionized at physiological pH, maintain the prodrug in a soluble form in GI fluids and then gradually revert to the lipophilic parent drug. Conversion to the parent drug involves a chemical cleavage at the self-cleavable spacer through an intramolecular cyclization-elimination reaction *via* imide formation under physiological conditions (Figure 16) (Bodanszky and Kwei, 1978; Tamamura *et al.*, 1998; Matsumoto *et al.*, 2000). Although high water solubility is required, the conversion time for the parent drug to be completely released is again a significant consideration. The conversion time is under the control of 1) chemical modification of the solubilizing moiety, 2) the bond length of the spacer, and 3) the pH of the medium.

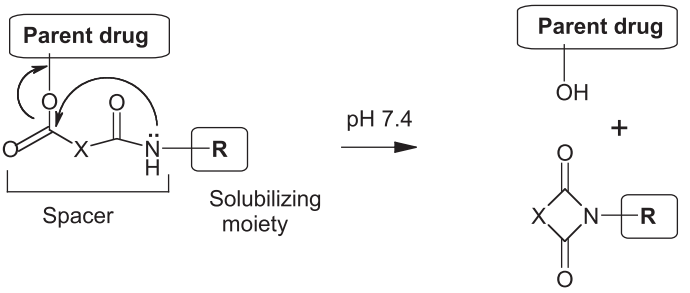


Figure 16. General structure of a water-soluble prodrug and its intramolecular cyclization-elimination reaction releasing the parent drug and an imide fragment (adapted from Matsumoto *et al.*, 2000).

Recent *in vivo* and *in vitro* studies detailed the generation of the parent drug KNI-727, an HIV-1 PR inhibitor that is sparingly soluble, from designed prodrugs with variable solubilizing moieties and spacer lengths. These studies suggest that a prodrug with a conversion time around 35 min was required for significant improvement in the GI absorption following intraduodenal administration (Matsumoto *et al.*, 2001; Sohma *et al.*, 2003). On the basis of the structure-conversion time relationship, a series of water-soluble prodrugs with different auxiliary units designed for KNI-727 were developed with solubilities ranging from less than 5.5 μg/mL to 93.8 mg/mL (Sohma *et al.*, 2003). However, few candidates yielded constant drug conversion time, which significantly increased the GI absorption of the parent drug *in vivo* and improved its systemic bioavailability (1.5–1.9-fold higher). Other prodrugs showed similar or decreased bioavailability values compared to that of the parent drug, KNI-727, suggesting that, despite the higher water solubilities for the designed prodrugs, there was no correlation between the water solubility and bioavailability values. These results indicate that enhancing water solubility alone is insufficient for improving GI absorption.

Hamel *et al.* (2004) applied this prodrug strategy to the cyclic undecapeptide drug CSA. However, they did not utilize the spacer and solubilizing moiety approach involving imide formation attempted by Kiso and co-workers. Instead,

they employed the well-established intramolecular cyclization reaction of dipeptide ester attachment to the parent drug (Figure 17) forming diketopiperazine (DKP) in aqueous solution (Goolcharran and Borchardt, 1998; Fischer 2003). This approach showed delivery benefits for ara-C (Wipf *et al.*, 1991, 1996) and CSA (Rothbard *et al.*, 2000) for topical use. The solubilities of CSA dipeptide ester prodrugs were improved 1000–5000-fold compared to those of the parent drug, and the conversion rate of CSA-prodrugs to release CSA and DKP ranged from 2 min to >5 days under physiological conditions. Such large differences illustrate the potential of this approach for precisely tailoring the release of the parent compound at physiological conditions providing versatile lead compounds for further optimization.

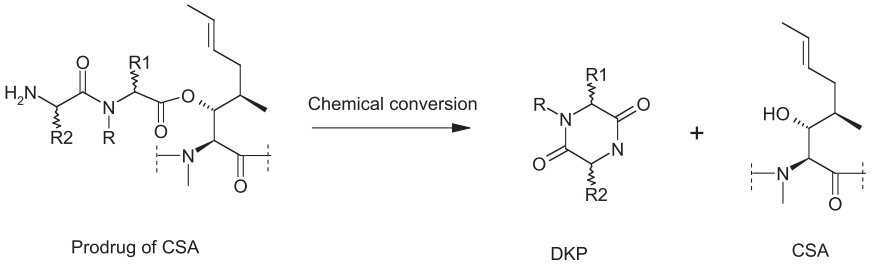


Figure 17. General structure of the prodrug and conversion to DKP and CSA at physiological pH, where R = Me, Et, Pr; R_1 and R_2 are amino acid side chains, adapted from Hamel *et al.*, 2004.

Strategic Considerations for Orally Administered Solubility Prodrugs

Phosphate Prodrug Screening Strategy

The successful identification of ideal candidates for an oral phosphate prodrug strategy requires a careful analysis of the properties of the parent drug and prodrug. These include ionization, drug and prodrug solubility/dissolution, enzyme-mediated parent drug generation, transport and efflux, targeted dose, and the ratio of targeted dose relative to parent drug solubility as well as the potential for precipitation. Based on these considerations, a flowchart detailing a decision-making process to aid in identifying useful NCEs as candidate phosphate prodrugs in drug discovery is depicted in Figure 18. The flowchart provides guidance in selecting drug candidates for phosphate and other water-soluble prodrugs that release the parent drug presystemically. The flowchart is not to be construed as all-inclusive, and the following assumptions and limitations apply:

1) The parent drug possesses no major ADMET (absorption, distribution, metabolism, elimination, and toxicity) liabilities. Liabilities of particular concern include poor metabolic stability,

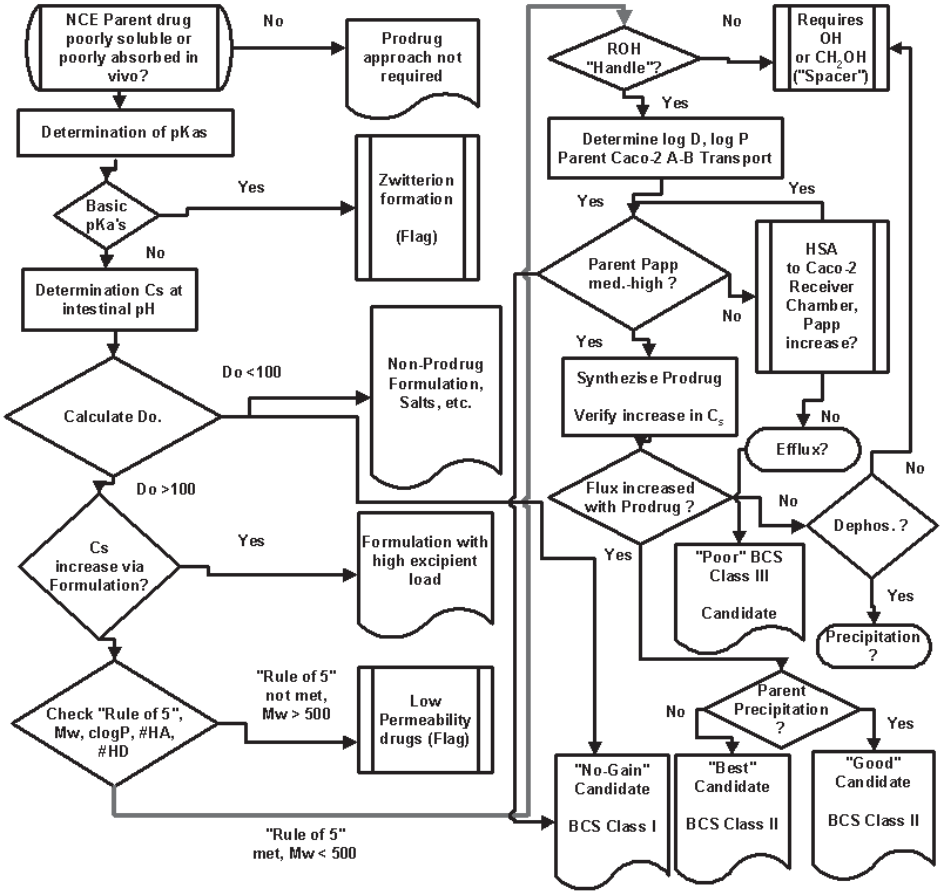


Figure 18. Identification of suitable parent drugs for an oral (phosphate) prodrug strategy. Cs, Aqueous solubility; Do, Dose Number; HSA, Human Serum Albumin; Papp, Apparent Caco-2 Permeability; #HA, Number of Hydrogen Bond Acceptors; #HD, Number of Hydrogen Bond Donors; BCS, Biopharmaceutical Classification Scheme.

drug-drug interaction potential, or toxicity. A phosphate prodrug is not expected to alter the distribution, systemic elimination, or side effects of a drug, since the parent drug is liberated at or near the apical membrane in the GI lumen. Thus, a parent drug with high metabolic clearance or extraction ratio is often a poor choice for a soluble prodrug. Even with a fraction absorbed (F_{abs}) near 1, a drug can still be poorly bioavailable due to significant first-pass elimination because the maximum achievable bioavailability (F_{max}) is a function of clearance (CL) and liver blood flow (Q), according to Eq. 5 (Kwon, 2001; Ward *et al.*, 2001).

$$F_{max} = F_{abs} * (1\text{-}CL/Q)$$

Equation 5.

2) A soluble prodrug approach is generally considered for the purpose of enhancing oral drug delivery by increasing passive drug absorption. In some cases, this increase could serve to saturate first-pass elimination. However, alternate motivations for developing a soluble prodrug may include non-biopharmaceutical considerations such as extending patent life or overcoming toxicity (e.g., monofluorophosphate).
3) The prodrug is chemically stable and shows no appreciable chemical or luminal enzymatic degradation while in the GI tract. As discussed, most phosphate esters do exhibit excellent chemical stability.

The flowchart in Figure 18 depicts the sequential decisions required in evaluating the various scenarios that may develop with a poorly water-soluble or poorly absorbed NCE, for which a prodrug approach is under consideration. In accordance with the decision tree, the following issues must be reviewed for successful development of an oral phosphate prodrug candidate:

a) To select parent drugs with desirable properties it is important to understand their ionization behavior. The determination of ionization constants, i.e., pK_as, is critical. Phosphate prodrugs containing basic amines may result in poorly soluble zwitterions at intestinal pH and can significantly compromise the solubility advantage typically offered by phosphate prodrugs.
b) To emphasize the importance of solubility relative to the targeted dose, a dimensionless dose number (Do) based on the targeted parent drug dose and parent drug solubility should be calculated. If the targeted dose has not yet been determined, a dose of 500 mg can be used as a conservative estimate. Parent drugs with low solubility and a high-targeted dose yield high Do, while parent drugs with low dose and moderate to high solubility yield low Do. Compounds with Do >100 are the best candidates.
c) The octanol/water partition coefficient (log P) can be used as a surrogate parameter, if a cell-based permeability model, such as Caco-2 monolayers, is not available. However, log P can be a poor predictor of membrane transport. As an example, the parent drug LY303366 of phosphate prodrug LY307853 was assessed to have a favorable membrane partition coefficient (log P near 2.0), and the prodrug increased the aqueous solubility by over three orders in magnitude. Yet, as alluded to earlier, the prodrug failed to increase bioavailability in rats and dogs (Zornes *et al.*, 1993). Transport studies in Caco-2 revealed that the parent drug LY303366 has poor intestinal permeability (Li *et al.*, 2001), probably due to its large molecular weight (MW >1100) and hydrogen-bonding potential.
d) The success of a soluble prodrug strategy is greatly influenced by the magnitude of parent drug permeation across intestinal

membranes. Poor membrane permeation and/or parent drug efflux can lead to poor prodrug performance as was shown for entacapone phosphate. Ideal candidates, such as DP-TAT-59, possess a permeability that is in the range of that for high permeability marker compounds such as metoprolol (Heimbach, 2003).

e) The enzymatic hydrolysis rate is often fast compared to the membrane permeation rate as is the case for TAT-59, fosphenytoin, and entacapone phosphate (Heimbach *et al.*, 2003a). However, parent drug absorption from sterically hindered phosphate prodrugs may lead to prodrug failure. As an example, slow enzymatic bioreversion has been reported for phosphate prodrugs of taxol (Mamber *et al.*, 1995). In some cases a "spacer" can be employed to render the phosphate group more accessible for faster enzymatic hydrolysis, as was done for fosphenytoin (Stella, 1996). To verify the parent drug release from a phosphate prodrug a kit, e.g., Enzcheck® (www.molecularprobes.com) can be employed.

f) As for any enzymatic process, the release of parent drug from a phosphate prodrug by intestinal alkaline phosphatases may depend on animal species, regional enzyme distribution and intestinal cell type, and differences in isozyme activities. This could yield problems in scaling from *in vitro* or animal studies to systemic parent drug pharmacokinetics from oral administration of a phosphate prodrug in a clinical study. For example, it has been reported that the activity of intestinal phosphatases to cleave alkyl versus aryl phosphates is a variable function of intestinal region and animal species (McComb *et al.*, 1979). However, it has also been reported that alkaline phosphatase activity in Caco-2 cells is similar to that observed in the small intestine of other species (Hidalgo *et al.*, 1989).

g) It is recognized that the potential for *in vivo* precipitation of parent drugs is a complex process. However, simplified *in vitro* assays can be used to screen for enzyme-mediated precipitation in aqueous buffered solutions (Heimbach *et al.*, 2003a). In some cases, the surface activity of a phosphate prodrug may aid in solubilizing the parent drug and preventing parent drug nucleation from prodrug solutions. Thus, the solubility of the parent drug should be determined in the presence and absence of relevant prodrug solution concentrations.

Flags shown in Figure 18 indicate challenges for an oral phosphate prodrug approach that can lead to failure to improve parent drug absorption. These include slow enzymatic prodrug bioreversion and undesirable parent drug properties as determined in a drug discovery setting. Lipinski's "rule of 5" predicts that poor absorption or permeation is more likely when there are more

than 5 H-bond donors, 10 H-bond acceptors, drug candidate molecular weight is >500, and the calculated log P (ClogP) is >5 (Lipinski, 2004; Lipinski *et al.*, 1997, 2001).

Rewards Provided by Orally Administered Solubility Enhancing Prodrugs

- Utilization of a soluble prodrug may overcome oral bioavailability limitations for a poorly water-soluble drug candidate, resulting in higher drug plasma levels to improve therapy.
- The consequences of the increased oral bioavailability may favorably alter both drug dosage regimen and toxicity. The capacity to achieve higher plasma concentrations with a soluble prodrug may permit a reduced drug load in a solid formulation (e.g., amprenavir) to improve the dosage regimen. In addition, decreased drug dosing with a solubility prodrug may reduce the toxicity and side effect potential accompanying drugs requiring high dosage as parent compounds (e.g., cyclosporine).
- Ionic prodrugs of poorly soluble lipophilic drugs such as phosphate prodrugs may show surfactant activity. This property can help to prevent the parent drug from precipitating subsequent to reconversion.
- Water-soluble prodrugs may smooth the development process for both parenteral and oral dosage formulations of a poorly water-soluble drug candidate.

Screening Tools for Successful Prodrugs

- Prodrug solubility can be determined as a function of pH by either potentiometric methods or equilibrium shake-flask experiments followed by HPLC, UV, or LCMS analysis. Prodrug solubilities can then be compared with parent drug solubility over the range of GI pH.
- Chemical stability must be verified for prodrugs targeted for enzymatic hydrolysis while aqueous stability as a function of pH must be characterized for prodrugs designed for chemical hydrolysis. In addition, solid-state stability of prodrugs must be tested.
- Physicochemical properties including lipophilicity as indicated by log P and log D, polarity (charge, hydrogen bonding), and molecular volume should be predictive of passive permeation. Permeability can be predicted through in silico approaches or through artificial model membranes.
- *In vitro* conversion time to the parent drug in both aqueous media and biological fluids such as plasma or blood should be examined.

- The potential for the parent drug to precipitate following its release from the prodrug must be evaluated to assess the role of supersaturated parent drug solutions to increase absorptive flux.
- The toxic potential of metabolite intermediates, of the carrier moiety or of a prodrug fragment should also be considered and explored.
- The massive increase in drug development costs forces early careful selection of prodrug candidates that display the greatest likelihood of success. Thus, the development of new experimental strategies and methods using high-throughput measurements for solubility profiles to rapidly screen and select the most promising compounds becomes a real necessity.

Subsequent to the above screening considerations, prodrug candidates can be considered for *in vivo* animal studies to investigate oral and i.v. parent drug and, in some cases, prodrug pharmacokinetics.

Concluding Remarks

- In the process of the developing a prodrug candidate, one or more synthetic steps are needed. This implies that additional resources are required, in addition to increased production cost. Thus, a solubilizing prodrug strategy should only be considered after confirmation that poor solubility and dissolution are the major pharmacokinetic liabilities. The soluble prodrug strategy should be weighed against the potential for dosage formulation to solve these problems.
- Careful prodrug design is required to minimize the number of proposed candidates and maximize the explored space of candidate physicochemical and pharmacokinetic properties. The ability to predict target properties such as drug solubility, extent of absorption, and rate of prodrug activation is critical.
- The rate of activation of a prodrug candidate represents another challenge in a prodrug strategy. The diversity of enzyme reconversion sites and interspecies variation make rational optimization of the rate of activation a difficult task. Chemical activation of prodrugs offers less variability than enzymatic activation, but this approach has received little attention.
- When selecting poorly soluble drugs to be candidates for a soluble prodrug strategy, parent drugs with a high dose and low solubility (Do>100), which belong to BCS class II, are the best candidates.
- Prodrugs described here target solubility/dissolution limitations that must be confirmed as a rate-limiting step in drug absorption. Thus, parent drugs with low permeability or high first-pass metabolism (high clearance) are likely to be poor choices for a solubility prodrug approach and provide little or no increase in systemic exposure.

- Lastly, it is important to realize that the successful drug discovery implementation of an oral prodrug strategy often requires a multidisciplinary teamwork approach toward drug design, ADMET, and formulation considerations.

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2.2.2

Prodrugs and Parenteral Drug Delivery

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List of Abbreviations

AUC .....area under the curve
CPT.....camptothecin
D5W .....dextrose 5% in water
EPR.....enhanced permeability and retention
IM.....intramuscular
IP .....intraperitoneal
IV .....intravenous
HAS .....human serum albumin
MW.....molecular weight
NS.....normal saline solution
PAF .....platelet activating factor
PBS .....phosphate buffered saline
PEG .....polyethylene glycol
PG .....poly-(L-glutamic acid)
PK.....pharmacokinetics
POM .....phosphonooxymethyl
SC.....subcutaneous
$t_{1/2}$ .....half-life
t_{90}.....shelf-life (time for 10% loss)
w/v.....weight per volume

Parenteral Dosing

Parenteral or injectable drug dosing is the desired route of drug administration under a number of clinical circumstances. In some countries, such as Italy, patients often prefer this route to oral dosing, while in countries like the USA the parenteral drug market is more limited. The most obvious reasons for a parenteral dosage form are when a drug cannot be taken orally due to oral absorption limitations, e.g., insulin for the treatment of diabetes, or when the patient cannot swallow oral medication, e.g., a comatose patient. Additionally, if the drug is orally toxic, as are some anticancer drugs, or immediate action is required, for example, acute life-saving treatment in an emergency room for conditions like a heart attack or an anaphylactic reaction, then injectable dosage forms are essential. For some drugs, a single sustained release intramuscular (IM) injectable dosage resulting in once a month dosing might be desired for patient convenience or for those patients known to be non-compliant.

For a drug to be given by intravenous (IV) injection, the most common parenteral route, the drug should be in solution and free of particulates of a size and number that can cause lung emboli or other blockages. Particulate specifications set by the regulatory authorities also need to be met. Not all drugs for which an IV dosage form is desirable will have the desired aqueous solubility. IV solutions ideally should be made up under isotonic conditions similar to normal saline solution (NS), dextrose 5% in water (D5W), with a pH value as close to physiological pH of 7.4 as possible. Various formulation strategies such as the use of aqueous co-solvents, surfactants, solubilizers such as the parenterally safe cyclodextrins, or pH adjustments well removed from pH 7.4 have been used to overcome aqueous solubility limitations. However, some of these techniques can contribute significantly to toxicity. The major adverse effects associated with IV administration resulting from formulation issues are hemolysis, injection site precipitation, lung entrapment, phlebitis and pain (Yalkowsky *et al.*, 1998). Additionally, other formulation techniques may still not be able to reach the desired goal of a stable formulation at an effective drug concentration.

Some drugs are also administered by subcutaneous (SC), intramuscular (IM), or other parenteral routes. For these routes, clear solutions are not always needed, depending on the desired temporal pattern of release. That is, for rapid action and release, solutions are best, but if a sustained release pattern is desired, suspensions of drug may work to advantage.

Sometimes, the barrier overcome is not inadequate solubility but some other limitation. These might include poor chemical stability, pain, or tissue damage on injection.

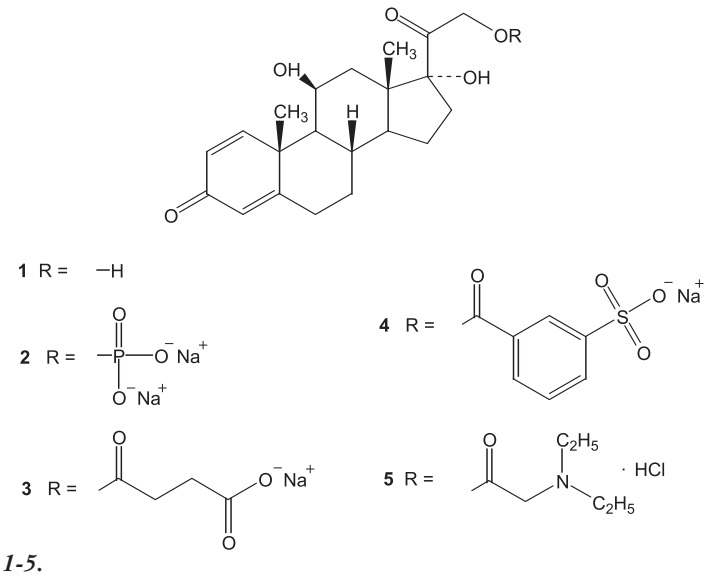
When formulation techniques prove toxic or inadequate, changing the physico-chemical properties of the drug via the design of a prodrug can result in a more desirable product. Some of the best examples of commercialized prodrugs are parenteral products. The literature is replete with examples of prodrugs for parenteral applications. The following is not meant to be comprehensive but, rather, a collection of examples to show the application of prodrug strategies to parenteral formulation of problematic drugs.

Prodrug Strategies to Increase Solubility for Parenteral Formulations

Historical Perspective

The most widely cited examples of parenteral prodrugs are water-soluble prodrugs of steroids. Adrenal corticosteroids such as prednisolone, methylprednisolone, hydrocortisone, betamethasone, and dexamethasone exhibit poor aqueous solubility. They are all commercially available as water-soluble sodium hemisuccinate esters and/or sodium phosphate esters. These water-soluble prodrugs are used in the emergency treatment of bronchial asthma, acute adrenal cortical insufficiency, and allergic drug reactions and are given intraarticularly or intrasynovially in the treatment of joint inflammation. These prodrugs regenerate the parent steroid by enzymatic cleavage of the hemisuccinate or phosphate ester *in vivo* (Melby and St. Cyr, 1961).

Prednisolone (**1**) is commercially available as several water-soluble derivatives such as prednisolone sodium succinate (**2**), and prednisolone sodium phosphate (**3**). These prodrugs are parenterally bioavailable forms of prednisolone (Melby and St. Cyr, 1961). Similarly, methylprednisolone sodium succinate (Derendorf *et al.*, 1985; Mollmann *et al.*, 1988), hydrocortisone sodium succinate (Melby and St. Cyr, 1961; Kawamura et al, 1971a), dexamethasone sodium phosphate (Melby and St. Cyr, 1961; Rohdewald *et al.*, 1987), and betamethasone sodium phosphate (Lederer, 1984) are also commercially available water-soluble forms of their parent steroids. There are many other historic examples of effective parenteral prodrug strategies for steroids in the literature. Prednisolone sodium 21-m-sulfobenzoate (**4**) (Kawamura *et al.*, 1971c), prednisolone 21-diethylaminoacetate (**5**) (Morita *et al.*, 1963), methylprednisolone sodium phosphate (Mollmann *et al.*, 1988), methylprednisolone 21-diethylaminoacetate (Schoettler and Krisch, 1974),



Structures 1-5.

hydrocortisone sodium phosphate (Melby and St. Cyr, 1961), hydrocortisone 21-m-sulfobenzoate and hydrocortisone 21-sulfate (Kawamura *et al.*, 1971b), and dexamethasone sodium succinate (Laboratoires Francais de Chimiotherapie, 1961) have all been explored. These prodrugs have all been synthesized and found to be water soluble, parenterally bioavailable forms of the parent steroid.

The hemisuccinate esters of these various steroids are useful, but undergo quite rapid hydrolytic degradation in aqueous solution and, as a result, are insufficiently stable to afford ready-to-use injectable formulations (Anderson and Taphouse, 1981). Therefore, they must be supplied as sterile powders for reconstitution. Furthermore, the hemisuccinate esters do not convert rapidly and completely *in vivo*. In a study of the pharmacokinetics of methylprednisolone hemisuccinate, Derendorf *et al.* (1985) found that 10% of the dose was excreted as the unchanged prodrug in the urine after IV administration.

In contrast to hemisuccinate esters, phosphate esters of steroids are found to be very stable in aqueous solution, allowing in some cases the formulation of ready-to-use injectable solutions with practical shelf lives (Flynn and Lamb, 1970; Kreienbaum, 1986). Phosphate prodrugs of corticosteroids are also rapidly converted to their parent steroid *in vivo* (Melby and St. Cyr, 1961). Moellmann *et al.* (1988) found that the peak human plasma levels of methylprednisolone from the sodium phosphate ester were 3–4-fold higher after IV administration than peak levels from the corresponding hemisuccinate ester. Significantly higher plasma AUC values for methylprednisolone from the phosphate ester, which also shows lower mean residence times than the hemisuccinate ester, were found. As much as 14.7 % of the hemisuccinate dose was excreted unchanged, while only 1.7% of the phosphate prodrug was excreted unchanged in the urine when given at molar equivalent doses.

The stability of steroid hemisuccinate esters is decreased by intramolecular carboxylate-catalyzed ester hydrolysis. This intramolecular catalytic hydrolysis, along with the 21⇄17 acyl migration of methylprednisolone 21-succinate, was examined in depth by Anderson *et al.* (1984). Figure 1 shows the pH-rate profile for the hydrolysis of methylprednisolone 21-succinate. The rate of hydrolysis deviates from the usual V-shaped profile due to intramolecular catalysis by the terminal carboxylate group. It is predicted from reactions of similar esters such as methylprednisolone 21-acetate that if it were not for the intramolecular carboxylate catalytic hydrolysis, methylprednisolone 21-succinate would degrade less than 10% in two years at 25°C (Anderson *et al.*, 1985a).

Another problem with the aqueous parenteral formulation of succinate esters, as well as other dicarboxylic acid esters, is their limited solubility in the pH range of optimal ester stability. The optimal pH for stability of methylprednisolone 21-succinate is in the range of 3–4. However, in this range, the solubility is poor, and a pH of 7–8 is required to achieve the necessary solubility for parenteral formulations (Anderson *et al.*, 1985a).

Anderson *et al.* (1985a) synthesized a number of derivatives of methylprednisolone 21-esters with sulfonate and tertiary amino groups as promoieties, which are much more soluble in the pH range of 3–5. Sulfonate and amino groups are

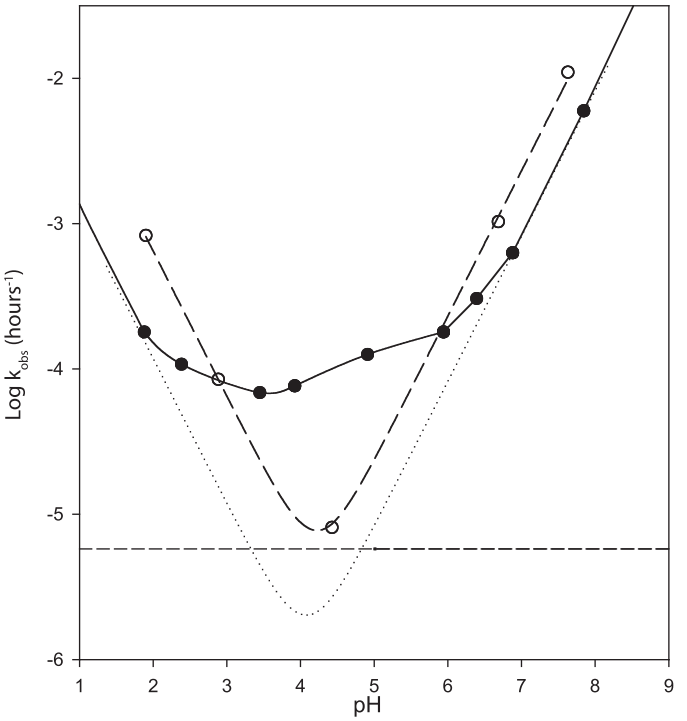
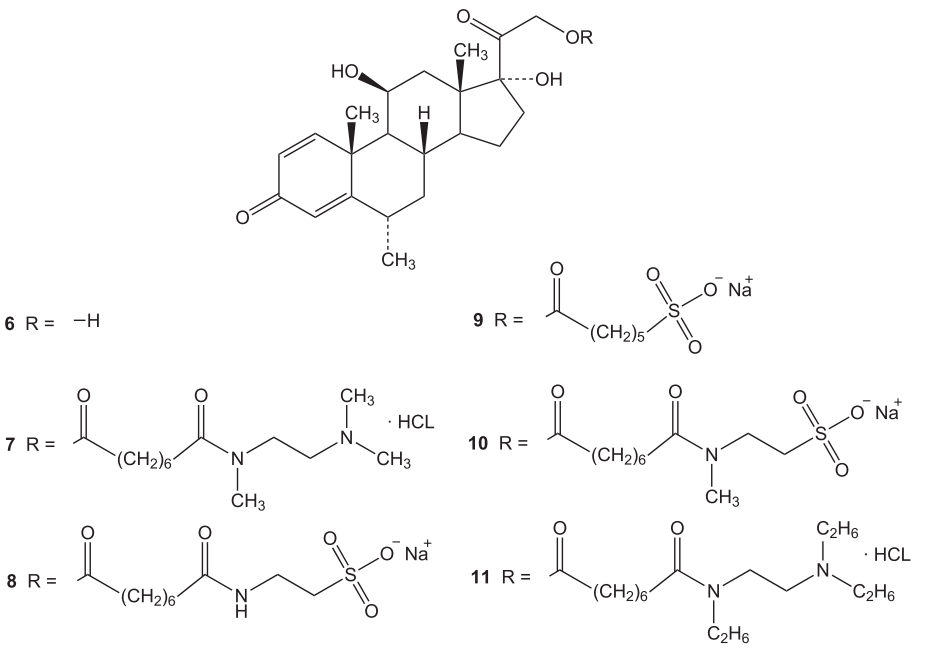


Figure 1. pH rate profile for hydrolysis of methylprednisolone 21-succinate (●), methylprednisolone 21-acetate (○). The horizontal dashed line (– – –) represents the rate constant necessary for 10% degradation over a 2-year shelf-life (Anderson *et al.*, 1995a).

both electron withdrawing, so the derivatives included additional methylene spacer groups in between the ionizeable group and the ester function. Compounds (**7–11**) are examples of sulfonate and amino prodrugs of methylprednisolone (**6**) that have predicted shelf lives of greater than 2 years at 25°C in dilute solution (Anderson *et al.*, 1995a). Compounds **10** and **11** were investigated in concentrated solutions and found to self-associate and form micelles at higher concentrations (Anderson *et al.*, 1985b). These compounds were shown to exhibit a greater chemical stability at high concentrations, with t_{90} values exceeding 2 years at 30°C, the upper limit of what is considered controlled room temperature. Furthermore, in concentrations equivalent to 100 mg/mL of methylprednisolone, these micellar prodrug solutions show approximately a 40-fold increase in the solubility of methylprednisolone. This significantly increases the potential shelf life of ready-to-use parenteral formulations of methylprednisolone prodrug by solubilizing the parent drug formed from prodrug degradation that would otherwise precipitate (Anderson *et al.*, 1995b).

Clinical studies of methylprednisolone suleptanate (Promedrol®, **10**) have shown it to be bioequivalent to methylprednisolone succinate, and it has been deemed a pharmaceutically acceptable substitute (Daley-Yates *et al.*, 1997; Parker *et al.*, 1997). Promedrol® (**10**) was approved in several countries for the treatment of asthma, but its current status is unknown to this author.

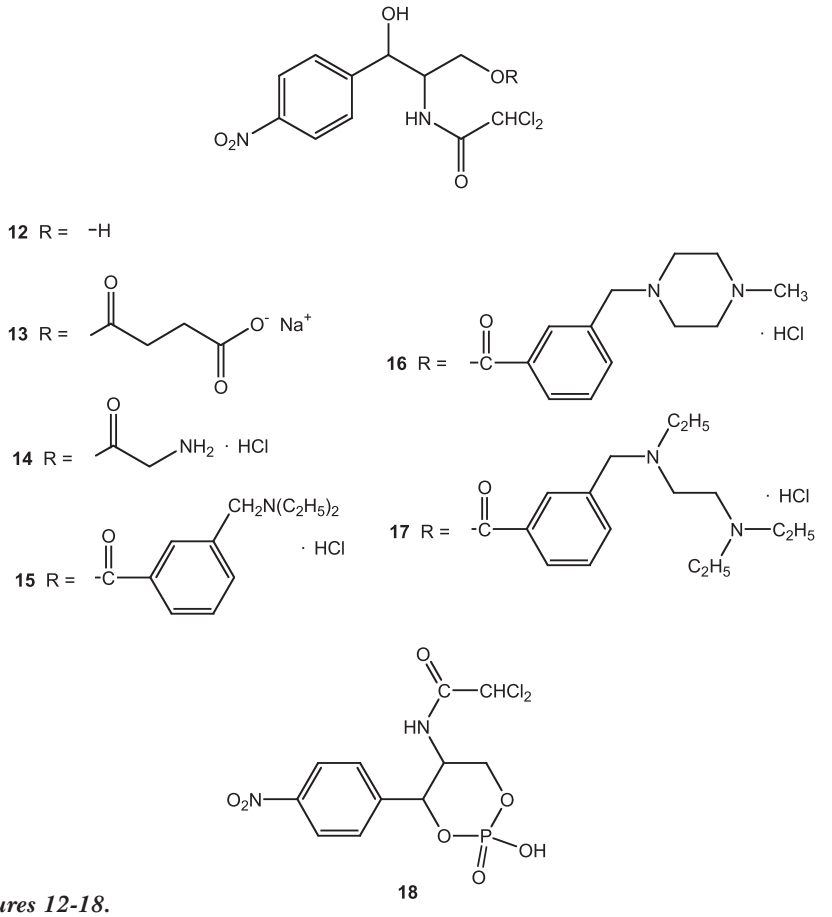


Structures 6-11.

Another classic historical example of a sparingly water-soluble drug that has had several prodrug strategies implemented to produce an injectable formulation is the antibiotic chloramphenicol. Chloramphenicol (**12**) is an older antibiotic that has gained more recent interest because of its effectiveness in the treatment of meningitis and pneumonia caused by ampicillin-resistant Haemophilus influenzae (Ambrose, 1984). **12** is a neutral compound that is only slightly soluble in water, but it is commercially available as a sodium monosuccinate ester (**13**) for parenteral administration (Glazko *et al.*, 1958).

Chloramphenicol monosuccinate (**13**) is inactive in its intact form and must be hydrolyzed to **12** for activity. The pharmacokinetics of **13** have been studied in children (Kauffman *et al.*, 1981) and adults (Burke *et al.*, 1982). The bioavailability of **12** from intravenous administration of **13** is about 70%, but the range is quite variable as a result of the variable and incomplete hydrolysis of the ester *in vivo* (Ambrose, 1984). Kauffman *et al.* (1981) found that from 6 to 80% of the administered dose was recovered as unchanged prodrug in the urine following IV administration of **13** to infants and young children (ages 2 weeks to 7 years). Similar results have been found in older children and adults. Nahata and Powell (1981) found that in children and young adults ages 2.5 months to 20 years 8 to 43% of administered dose was excreted unchanged, and Burke *et al.* (1982) found that 13 to 26% was excreted unchanged in adults ages 19 to 64 years.

Phosphorylation of chloramphenicol has been investigated by Mosher *et al.* (1953) as an alternative to the hemisuccinate. A cyclic phosphoric acid ester of chloramphenicol (**18**) was synthesized. This compound is soluble in water and very stable to acid hydrolysis. Unfortunately, **18** shows no antibiotic activity and is not enzymatically hydrolyzed to form chloramphenicol *in vivo*. When



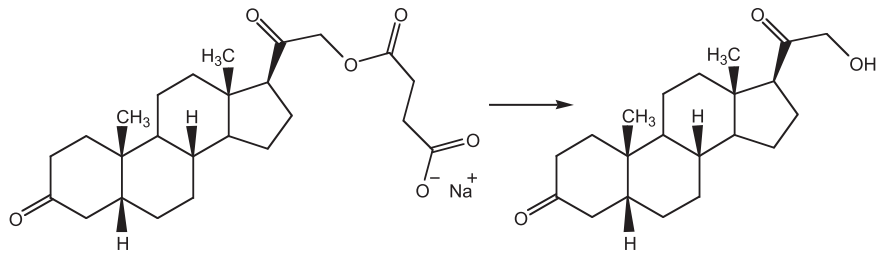
Structures 12-18.

administered parenterally to rats, **18** is rapidly excreted unchanged in the urine (Mosher *et al.*, 1953).

Amino acid prodrugs of chloramphenicol such as chloramphenicol glycinate (**14**) have been evaluated. **14** shows a faster rate of enzymatic hydrolysis than **13** (Trivellato *et al.*, 1958; Serembe and Ospedale, 1960). However, **14** shows poor aqueous stability (Concilio *et al.*, 1958). Amino acid esters typically exhibit poor aqueous stability in the pH range of 3–7 as a result of the catalytic effect of the protonated amino group (Bundgaard *et al.*, 1984b; Johnson *et al.*, 1985).

Bundgaard *et al.* (1991) demonstrated a novel prodrug strategy to overcome the stability problems associated with chloramphenicol glycinate. A phenyl group was incorporated between the ester and the amino group. A number of N-substituted 3-aminomethylbenzoate esters of chloramphenicol such as **15** were reported. This prodrug is actually more stable than **12** itself in the pH range 3–5 at 80°C and is converted to **12** in 80% human plasma at 37°C with a half-life of 8.0 min. The water solubility of **15** is only 1 mg/mL, but other derivatives that had solubilities greater than 15% w/v and plasma conversion half-lives between 0.9 and 5.0 minutes (**16** and **17**) were reported (Bundgaard *et al.*, 1991).

Two additional historical examples of sodium succinate prodrugs of interest are the succinate esters of hydroxydione and oxazepam. Hydroxydione (**20**) is a basal anesthetic that is insoluble in water but may be given IV as a sodium succinate ester (**19**) (Laubach *et al.*, 1955). It showed anesthetic effects in laboratory animals with a wide margin of safety (Murphy *et al.*, 1955). Oxazepam (**22**) is a metabolite of diazepam and is itself a mild short-acting tranquilizer with anticonvulsant activity. It is sparingly soluble in water and has been solubilized as a sodium succinate ester (**21**) for parenteral use. Oxazepam sodium succinate appears to act by conversion to **22** *in vivo* but, similar to other sodium succinate esters, part of the prodrug dose is excreted unchanged in the urine of dogs and pigs following IV administration (Walkenstein *et al.*, 1964). **21** and **22** have a chiral center and can be resolved into optically active isomers. Mussini *et al.* (1972) found that the (+) isomer of **21** gives much higher plasma levels of **22** and shows a threefold higher anticonvulsant activity than does the (–) isomer when administered IV to rats and mice. The different rates of *in vitro* hydrolysis of these optical isomers of oxazepam succinate by stereospecific soluble esterase from rats, mice and guinea pigs appears to be animal species-dependent (Salmona *et al.*, 1974).



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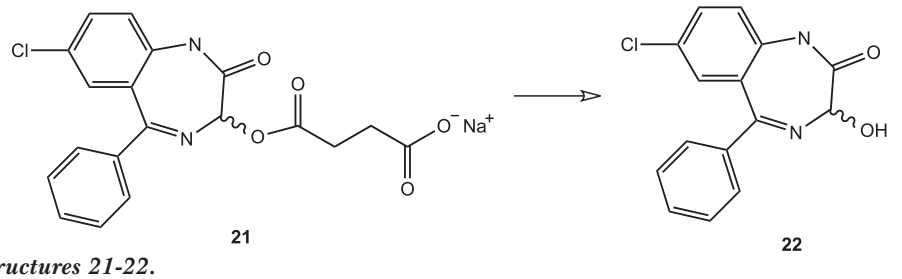
Structures 19-20.



21 22

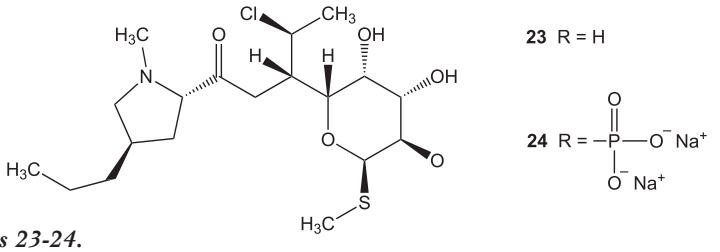
Structures 21-22.

Further Development of Parenteral Prodrug Strategies

Phosphate Prodrugs

The use of phosphate esters as prodrugs had emerged as a viable strategy to increase the aqueous solubility of poorly soluble drugs for parenteral applications.

Their advantages over succinate esters were demonstrated in their application to steroids, and this strategy appeared to have great potential for future parenteral applications. Phosphate esters are readily ionizable and have significantly higher aques solubility than the parent drug. They are often sufficiently stable in the solid state and in aqueous solution to allow development of parenteral applications, and they are rapidly cleaved *in vivo* by alkaline phosphatases to release the parent drug as well as inorganic phosphate, a non-toxic naturally occurring entity.



Structures 23-24.

Clindamycin (**23**) is an antibiotic used in the treatment of gram-positive bacterial infections. Clindamycin hydrochloride salt can be administered IM or IV, but injections of this drug are found to be irritating. This irritation is thought to be a result of precipitation of the free base and/or rapid partitioning of drug into tissue at the injection site (Novak *et al.*, 1970). Clindamycin phosphate (**24**) is a prodrug of clindamycin used for parenteral administration. It is supplied as a powder for reconstitution prior to injection. **24** has approximately threefold higher IV and IP LD50 values in mice than does clindamycin HCl (Gray *et al.*, 1974). IM testing of **24** shows it to be a bioreversible form that does not seem to exhibit the injection site irritation associated with the parent drug (Edmondson, 1973).

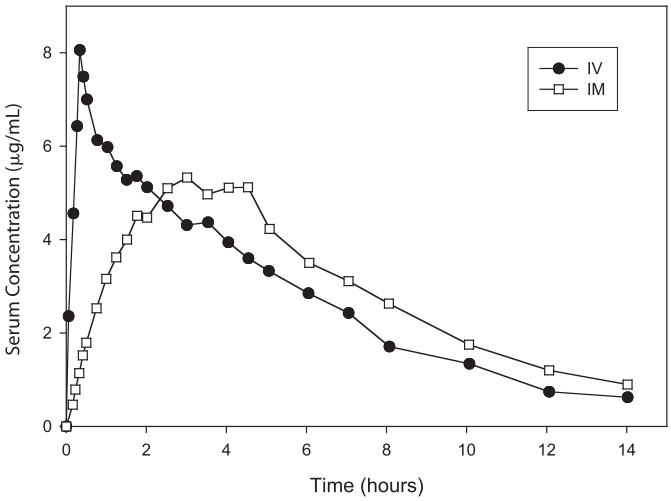
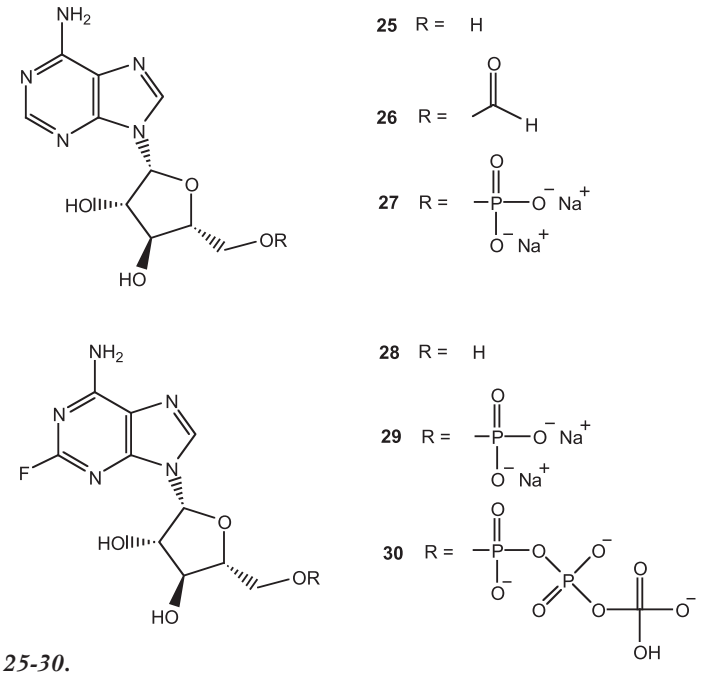


Figure 2. Human serum concentration of clindamycin (23) following 600 mg IV (●) and 600 mg IM (□) administration of clindamycin phosphate (24) in same subject (DeHaan *et al.*, 1973).

The phosphate ester itself is inactive against bacteria *in vitro*, but is rapidly converted to **23** *in vivo* (DeHaan *et al.*, 1973) after which the parent drug is rapidly distributed to other fluids and tissues. Figure 2 illustrates the human serum concentration-time profile of **23** following IV and IM dosing of **24**. The hydrolysis of the phosphate ester to form **23** occurs more rapidly in the case of IV administration, and IM administration exhibits slower absorption and a lower peak concentration of **23**. In multiple dose regimes, equilibrium is reached by the third dose, after which serum levels of **23** are readily predictable (DeHaan *et al.*, 1973).

Vidarabine (**25**) is an antiviral cytotoxic agent with poor aqueous solubility. The 5'-formate ester (**26**), which was synthesized by Repta *et al.* (1975), has a 60-fold higher aqueous solubility than the parent cytotoxic nucleoside and is rapidly and completely hydrolyzed to form the parent drug in the presence of endogenous enzymes in human whole blood. The 5'-phosphate ester (**27**) was also examined by LePage and Hersh (1972) for use as a small volume IV formulation. Its solution stability was examined and found to be excellent near physiological pH (Hong and Szulczewski, 1984). **27** is water-soluble and converts to **25** *in vivo*, but **25** undergoes deamidation rapidly enough to limit its usefulness (LePage and Hersh, 1972; Hanessian, 1973).



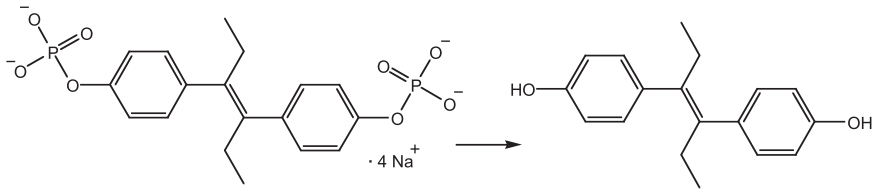
Structures 25-30.

The problem of rapid *in vivo* deamidation associated with vidarabine-5'-phosphate (**27**) was overcome by the development of a synthetic analogue that does not undergo deamidation as readily. Montgomery and Hewson (1969) synthesized the fluorine derivative 9-β-D-arabinosyl-2-fluoroadenine (**28**), which is resistant to adenosine deaminase. The 5'-phosphate derivative with enhanced

water solubility, fludarabine (**29**, sometimes referred to as fludarabine phosphate), was reported by Montgomery and Shortnacy (1982). Fludarabine is used in the treatment of lymphoid malignancies including chronic lymphocytic leukemia and non-Hodgkin's lymphoma (Keating, 1990; Hiddemann and Pott-Hoeck, 1994). For a review of the pharmacology of **29**, see Gandhi and Plunkett (2002) and references therein.

29 is supplied as a powder for reconstitution prior to injection. It is converted to the parent drug **28** rapidly and quantitatively after an IV bolus dose (Malspeis *et al.*, 1990), rapid infusion (Hersh *et al.*, 1986; Danhauser *et al.*, 1987), or continuous infusion (Avramis *et al.*, 1990). **29** is transported into cells by nucleoside transport systems (Barrueco *et al.*, 1987). Intracellular **29** is rephosphorylated and accumulates as the active cytotoxic species fludarabine-5'-triphosphate **30** (Avramis and Plunkett, 1982).

The synthetic non-steroidal estrogen-based compound diethylstilbesterol (**32**) is practically insoluble in water. It is available commercially as a ready-to-use solution of its diphosphate ester (**31**) for IV administration in the treatment of prostatic and postmenopausal breast carcinoma. The solution is stable for >2 years at room temperature (Mueller and Wollmann, 1986), and is hydrolyzed to form diethylstilbesterol in rat, dog, and man (Abramson and Miller, 1982).

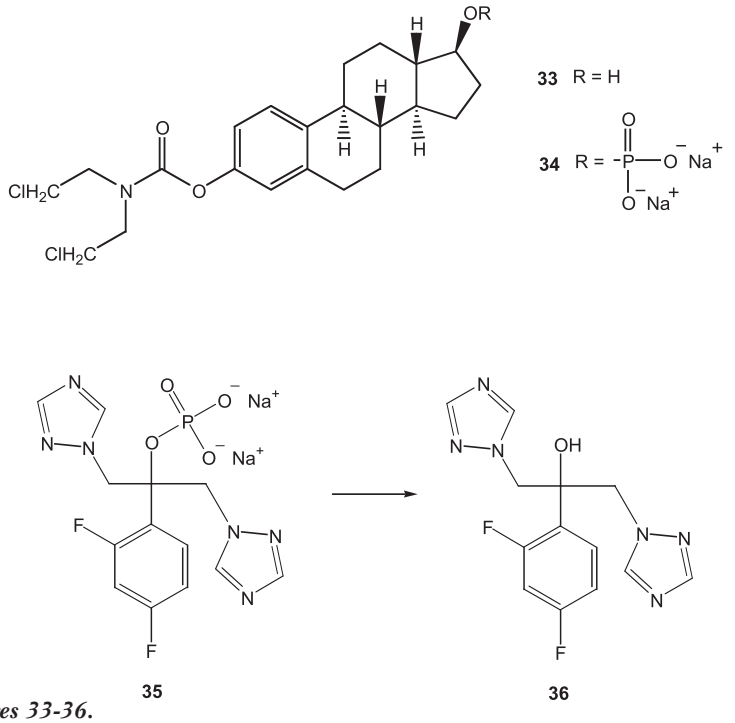


31 **32**

Structures 31-32.

Estramustine phosphate (**34**) is a prodrug of the cytotoxic agent estramustine (**33**). It has been used in the treatment of advanced prostatic carcinoma for some time, and has more recently gained interest as a radiosensitizer. For a review, see Bergenheim and Henriksson (1998). It is typically administered orally, but can be given in high doses intravenously (Hudes *et al.*, 2002). **34** is stable in human blood, but rapidly dephosphorylates to estamustine by liver, intestine, and prostatic enzymes (Gunnarsson *et al.*, 1983). It is being investigated as a combination therapy with paclitaxel and carboplatin for patients with advanced prostate cancer (Kelly *et al.*, 2003; Simpson and Wagstaff, 2003).

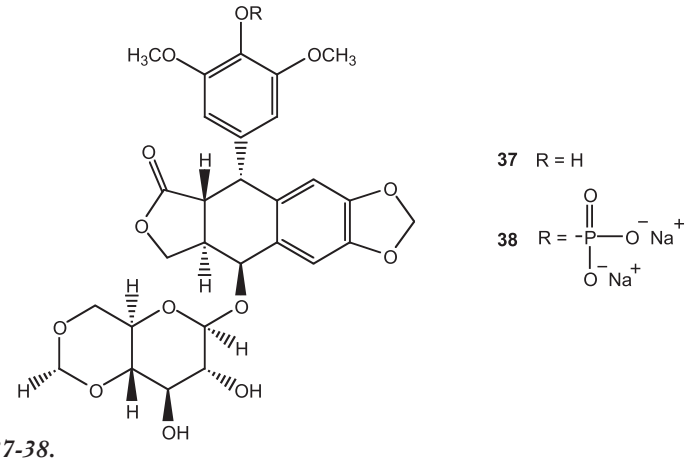
Fluconazole (**36**) is a broad-spectrum antifungal agent that is active by both oral and intravenous administration for the treatment of superficial and systemic infections. However, the IV formulation available is a 2 mg/mL dilute solution in saline for infusion. The prodrug fosfluconazole (**35**) was developed to increase the solubility of the parent drug for production of a low volume parenteral formulation, which would allow access to bolus administration as well as higher IV doses. Fosfluconazole is a unique example of a phosphate prodrug in that it results from direct phosphorylation of a tertiary alcohol (Murtiashaw *et al.*, 1997).



Structures 33-36.

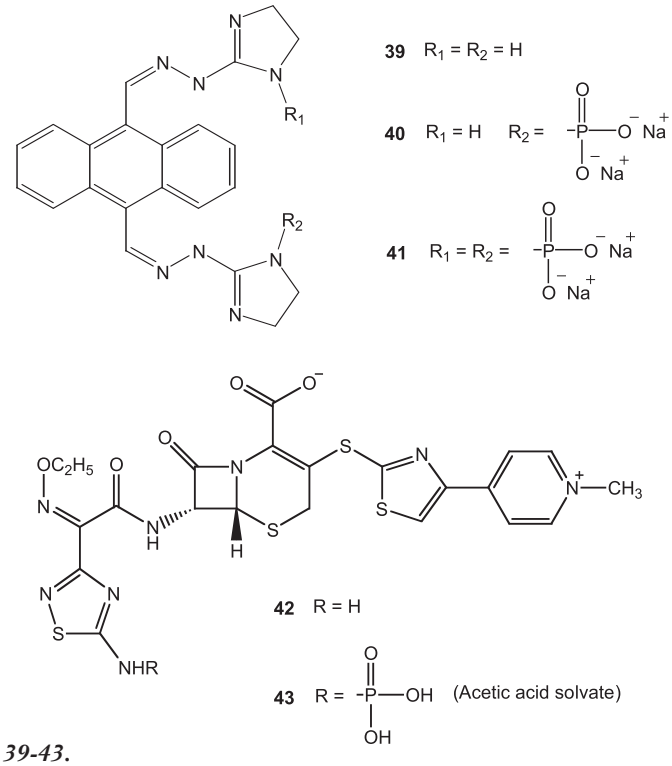
Fosfluconazole (**35**) has an aqueous solubility of >300 mg/mL as its disodium salt, and is sufficiently stable for IV formulation (Bently *et al.*, 2002). The prodrug is converted to **36** in humans following IV bolus injection of up to 2000 mg, with less than 4% of the dose excreted unchanged in the urine (Sobue *et al.*, 2004). In this pharmacokinetic (PK) study, **35** had a volume of distribution of 0.2 L/kg at higher doses, was highly plasma protein bound (98% at 10 μg/mL), and was eliminated with a terminal half-life of 1.5 to 2.5 hours depending on dose. The PK parameters of **36** produced from **35** in this study were similar to those of equivalent doses of **36** reported in the literature. Fosfluconazole was approved in October 2003 for marketing in Japan.

Another recent example of a successful phosphate prodrug is etoposide phosphate (**37**). Etoposide (**38**) is a semi-synthetic derivative of podophyllotoxin that is useful in the treatment of a variety of malignancies (Slevin, 1991). The equilibrium aqueous solubility of etoposide is about 0.16 mg/mL, making use of organic solvents and large volumes of saline necessary for IV administration (Shah *et al.*, 1989). Etoposide phosphate is water-soluble (20 mg/mL) and is supplied as a sterile powder for reconstitution prior to injection. **38** converts rapidly and completely to **37** following IV administration to humans, and the PK profile of **37** from **38** is nearly identical to that of **37** *per se* (Schacter 1996 and references there in). The therapeutic activity and toxicity are also similar (Fields *et al.*, 1995, 1996; Kreis *et al.*, 1996). Etoposide phosphate was approved by the FDA in 1996 for the treatment of testicular tumors and small cell lung carcinoma, and is being investigated for its safety and efficacy in the treatment of AIDS-related non-Hodgkin's lymphoma.



Structures 37-38.

All of the examples of phosphate prodrugs given so far have consisted of phosphate esters of drug alcohol groups. N-phosphono type prodrugs have also been investigated to improve delivery properties of drugs for parenteral applications. Murdock *et al.* (1993) reported N-phosphono prodrugs of bisantrene (**39**), an antitumor agent with poor solubility at physiological pH that causes phlebitis due to precipitation at the injection site (Powis and Kovach, 1983). The mono- and di-substituted N-phosphate compounds (**40**,**41**) showed greatly improved aqueous solubility at pH 7.4 and show activity comparable to that of **39** against P-388 leukemia and B-16 melanoma with no evidence of precipitation on



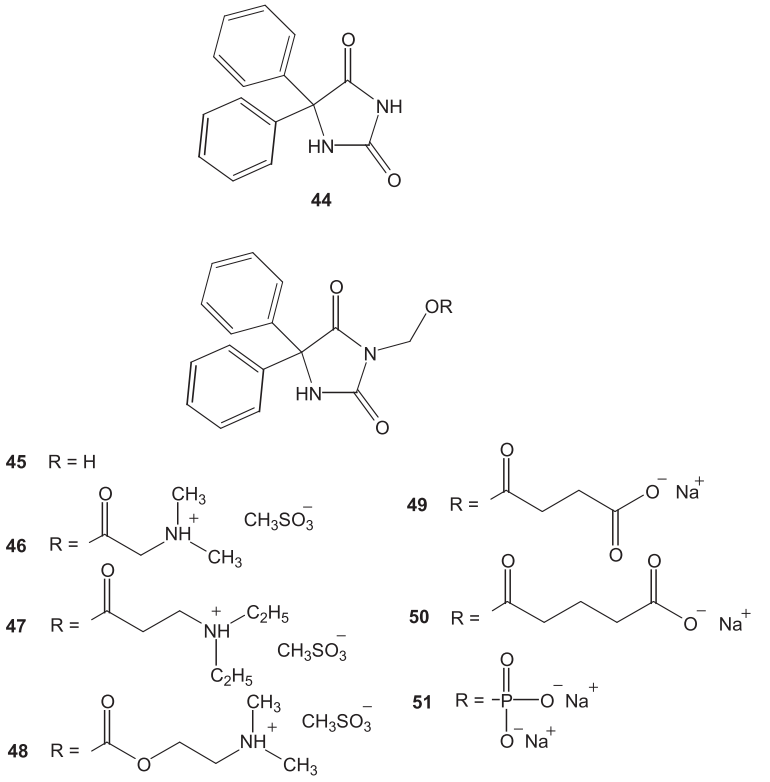
Structures 39-43.

injection. **40** and **41** are inactive *in vitro* against tumor cells, indicating that their *in vivo* activity is a result of their ability to function as prodrugs.

Ishikawa *et al.* (2001) used a similar approach to improve the water solubility of several antibacterial agents. The cephalosporin derivative T-91825 (**42**) exhibits potent antibacterial activity against methicillin-resistant *Staphylococcus aureus*, but the zwitterionic compound shows limited aqueous solubility (2.3 mg/mL). The N-phosphono derivative TAK-599 (**43**) shows aqueous solubility of >100 mg/mL at pH 7 near physiological pH (Ishikawa *et al.*, 2003). TAK-599 was crystallized from aqueous acetic acid in the form of an acetic acid solvate. It was found to be 98% stable in pH 7 aqueous solution for 8 hours. **43** was shown to rapidly convert to **42** following IV administration to rats and monkeys, and showed *in vivo* activity superior to that of vancomycin in systemic bacterial infection in mice (Ishikawa *et al.*, 2003).

Phosphonooxymethyl Prodrugs

An extension of the successful phosphate prodrug strategies is the development of phosphonooxymethyl (POM) prodrugs. With this type of prodrug, the phosphate group is not directly attached to the drug but, rather, is linked to the drug through a methoxy spacer. This prodrug strategy was first applied to the antiepileptic drug phenytoin (**44**). Phenytoin is a sparingly water-soluble (25 μg/mL), weakly



Structures 44-51.

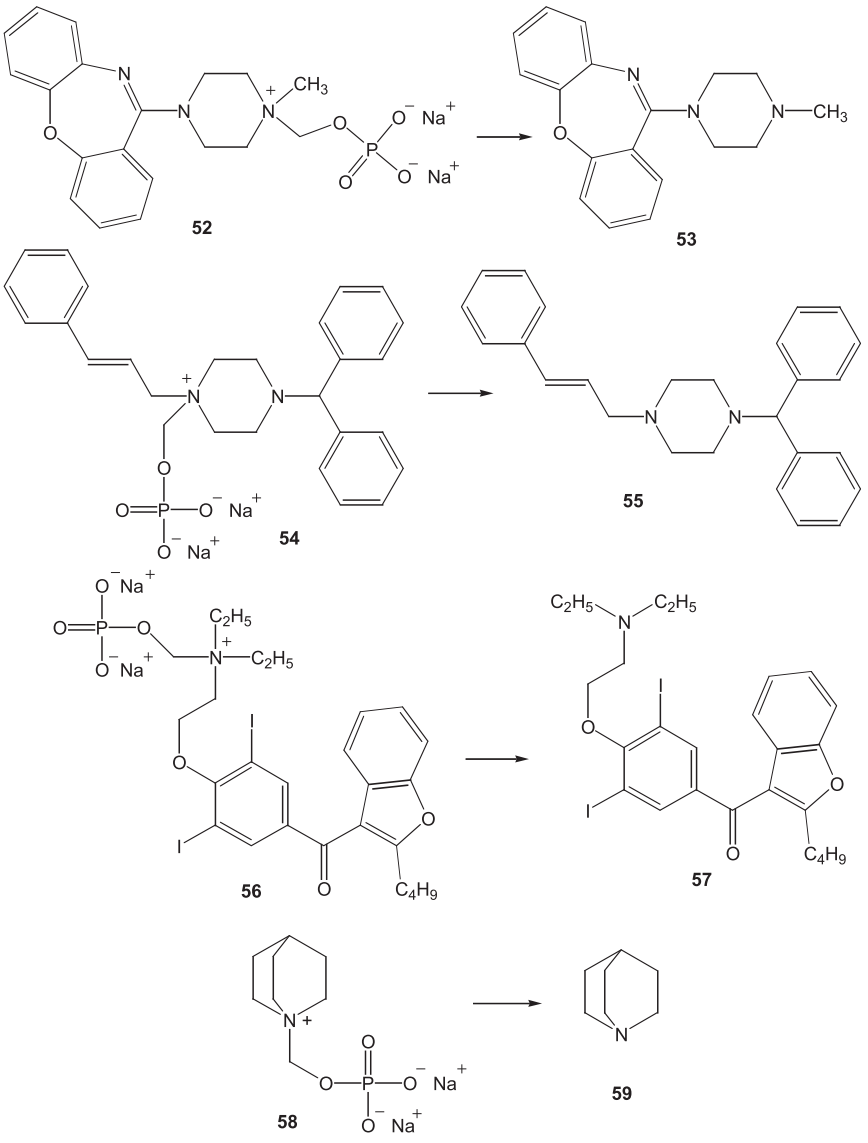
acidic anticonvulsant (Newton and Kluza, 1980). There are a number of seizure-related indications for which a rapid onset is required and a parenteral formulation of phenytoin is necessary. A concentrated solution (46 mg/mL) of phenytoin for parenteral application was achieved by using the sodium salt of phenytoin in an aqueous solution of 10% ethanol and 40% propylene glycol adjusted to pH 12. Intravenous administration of this alkaline solution is known to cause pain, burning, and phlebitis (Earnest *et al.*, 1983; Spengler *et al.*, 1988). It appears that phenytoin may also precipitate at the injection site (Gatti *et al.*, 1977).

A number of prodrugs of phenytoin (**46–51**) were synthesized and examined by Varia *et al.* (1984a). The prodrugs consisted of a number of water-solubilizing functional groups attached to 3-(hydroxymethyl)phenytoin (**45**), of which the disodium phosphate ester (**51**) was the most promising. **51**, now called fosphenytoin, is commercially marketed as Cerebyx®, a ready-to-use injectable form of phenytoin; it has been the subject of many reviews (Bebin and Bleck, 1994; Boucher, 1996; Browne *et al.*, 1996; Ramsay and DeToledo, 1996; Stella, 1996; Uthman *et al.*, 1996; Browne, 1997; Luer, 1998; DeToledo and Ramsay, 2000; Fischer *et al.*, 2003). Fosphenytoin is an example of a successful parenteral prodrug strategy and appears in the case studies section of this book.

Fosphenytoin (**51**) has a solubility of 142 mg/mL equivalent to 88.2 mg/mL phenytoin near physiological pH (Varia *et al.*, 1984a). Its maximum stability is in the pH range of 7.5 to 8. Fosphenytoin is 90% stable in water at pH 7.4 for 2.8 years at 25°C (Varia *et al.*, 1984b). However, a shelf-life limiting condition of parenteral formations of **51** is the precipitation of phenytoin produced from the degradation of **51** in solution. Fischer *et al.* (1997) demonstrated that aqueous formulations of **51** including 0.9% NaCl and dextrose 5% do not precipitate phenytoin for at least 30 days at 25°C, 4°C and –25°C. Parenteral formulations of **51** with increased shelf life are exhibited in the pH range of 8.3–9.4 in which the major degradant diphenylglycinamide is more water soluble than is phenytoin (Herbranson *et al.*, 1990). However, refrigeration is still required at these pH values. Parenteral formulations of **51** with extended shelf life, projected as high as 9 years at 25°C, involve the use of the cyclodextrin $(SBE)_{7m}$-β-CD to help solubilize phenytoin produced at a pH value 7.4 (Narisawa and Stella, 1998).

Fosphenytoin bioreverts to phenytoin via a two-step process. The phosphate group is enzymatically cleaved to give 3-(hydroxymethyl)phenytoin (**45**), which spontaneously hydrolyzes to release formaldehyde and phenytoin. **51** converts rapidly and completely to **44** *in vivo* after IV and IM administration in rats, dogs, and humans (Gerber *et al.*, 1988; Varia and Stella, 1994a,b). **51** has a conversion half-life of about 8 min in humans receiving IV doses of 150–1200 mg phenytoin equivalent (Gerber *et al.*, 1988). The absolute bioavailability of **44** from **51** was determined to be >99% by comparing the area-under-the-curve (AUC) values of **44** from equivalent doses of **51** and sodium phenytoin infused over the same time period in a crossover study with 12 healthy male volunteers (Jamerson *et al.*, 1990). There have been many studies in humans that have yielded similar results (Boucher *et al.*, 1989; Jamerson *et al.*, 1990; Leppik *et al.*, 1990; Ramsay *et al.* 1997; Pryor *et al.*, 2001).

In addition to the utility of POM prodrugs used with phenytoin, the POM prodrug approach has also been applied to tertiary amines. Krise *et al.* (1999a) developed POM prodrugs of loxapine (**53**), cinnarizine (**55**), amiodarone (**57**) along with a model compound; quinuclidine (**59**). **53** is a antipsychotic agent (Heel *et al.*, 1978; Menuck and Voineskos, 1981), **55** is a calcium channel blocker (Godfraind *et al.*, 1982), and **57** is an antiarrhythmic agent (Chow, 1996). These drugs are all tertiary amines with poor pH-dependent aqueous solubility. The POM prodrug strategy was applied to these drugs (**52**, **54**, **56**, **58**) to overcome the barrier of their poor aqueous solubility to improve parenteral and oral delivery of these drugs.



Structures 52-59.

The loxapine POM prodrug (**52**) is approximately 15,000 times more soluble at pH 7.4 than **53**, and is very stable in aqueous solution. However, precipitation of parent drug from concentrated prodrug solutions is expected to be the limiting factor in shelf-life. The solubility of **52** at pH 7.4 is only 30 μg/mL, which would only allow for 0.06% degradation before **53** would theoretically precipitate, resulting in a shelf-life limited to about 6.7 days. However, the intrinsic solubility of **53** is increased to 2.7 mg/mL in an aqueous solution of 72 mg/mL of **52** at pH 11.8. This projects a shelf life of 1.7 years, and the solubility of **53** would be greater at pH 7.4 than pH 11.8, so that the actual shelf life of a ready-to-use parenteral dosage form of **52** formulated at pH 7.4 may very well exceed 2 years (Krise *et al.*, 1999b).

The bioreversion of **52** and **54** has been evaluated in rats and dogs by Krise *et al.* (1999c). Prodrugs were administered IV and IM to rats and dogs. Plasma levels of the prodrugs drop rapidly with a half-life of approximately 1 min and fall below limits of detection 5 min after infusion in rats and dogs. **52** was not observed in detectable levels in the plasma following IM administration to rats. The mean AUC values of **53** and **55** following IV and IM dosing of the parent drugs are not statistically different than AUC values of **53** and **55** produced from equivalent doses of their respective prodrugs. An example of the PK profile of **55** following IV administration of **55** as a cyclodextrin solution and its prodrug **54** to a beagle dog can be seen in Figure 3. The plasma concentration profile of **55** from **54** is nearly identical to that from equivalent dose of **55**. These results are consistent with the rapid and complete conversion of the prodrug to parent drug following IV and IM administration to rats and dogs. Enzyme saturation of enzymes responsible for the bioreversion is not observed when the IV dose of **54** is tripled from 6.2 μmol/kg to 18.6 μmol/kg in rats (Krise *et al.*, 1999c).

These prodrugs are shown to be substrates for alkaline phosphatase, which facilitates the release of parent drug from their respective prodrugs (Krise *et al.*,

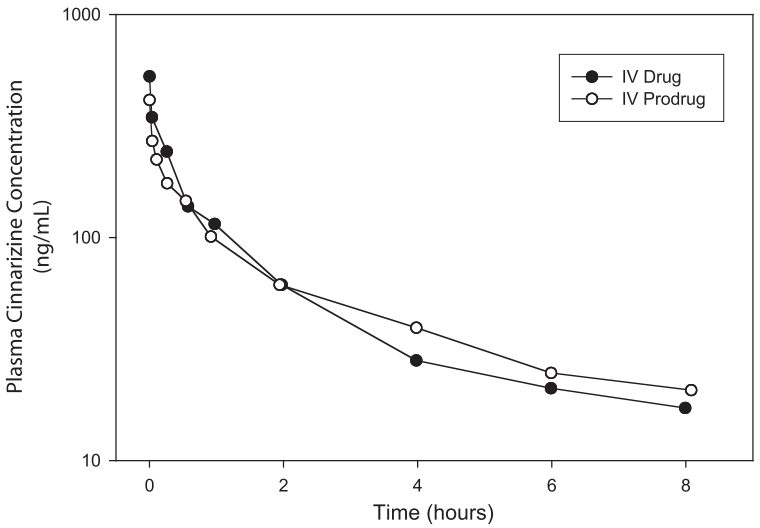
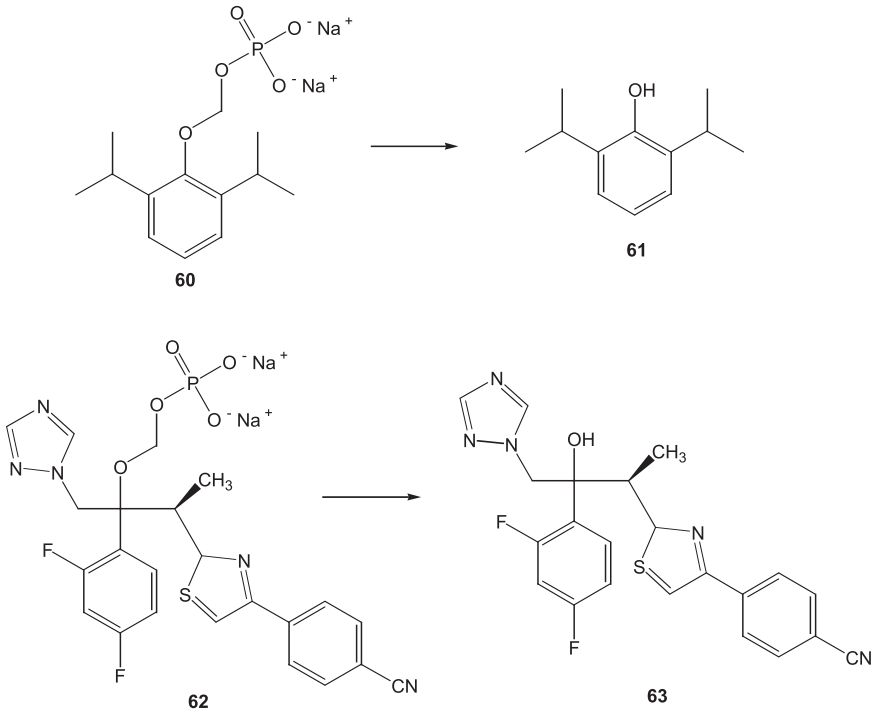


Figure 3. Plasma concentration of cinnarizine (55) following IV administration of equimolar (3 μmol/kg) doses of 54 (○) and 55 (●) to beagle dog (Krise *et al.*, 1999c).

1999b). The *in vivo* conversion of these prodrugs is extremely rapid compared to the relatively slow *in vitro* hydrolysis in the presence of alkaline phosphatase. It is likely that a significant fraction of the enzymes responsible for the rapid *in vivo* conversion are arterial and venous membrane-associated (Krise *et al.*, 1999c).

The success of the POM prodrug strategy with fosphenytoin and other drugs has led to its utilization in more recent applications. Propofol (Diprivan®, **61**) is an IV sedative-hypnotic agent widely used for anesthesia and sedation (Bryson *et al.*, 1995). **61** is an oil, and is only sparingly soluble in water. It is currently formulated as an oil-in-water emulsion, which has several disadvantages including poor physical stability, potential for emulsion-induced embolism, and a risk of infection due to bacterial contamination (Prankerd and Stella, 1990; Bennett *et al.*, 1995). Pain at the site of injection is also a major adverse effect (Nakane and Iwama, 1999).



Structures 60-63.

GPI 15715 (Aquavan®) (**60**), is a water-soluble phosphonooxymethyl ester prodrug of propofol. It is reported to have an aqueous solubility of approximately 500 mg/mL and is converted to **61** by alkaline phosphatase *in vitro* (Stella *et al.*, 2000). Furthermore, **60** is nontoxic in rats and produces anesthesia in dogs similar to **61**. In rats, **61** from **60** showed a slightly delayed onset (as expected), longer apparent half-life, increased volume of distribution, greater potency, and sustained duration of action compared to that of known formulations of **61** (Schywalsky *et al.*, 2003).

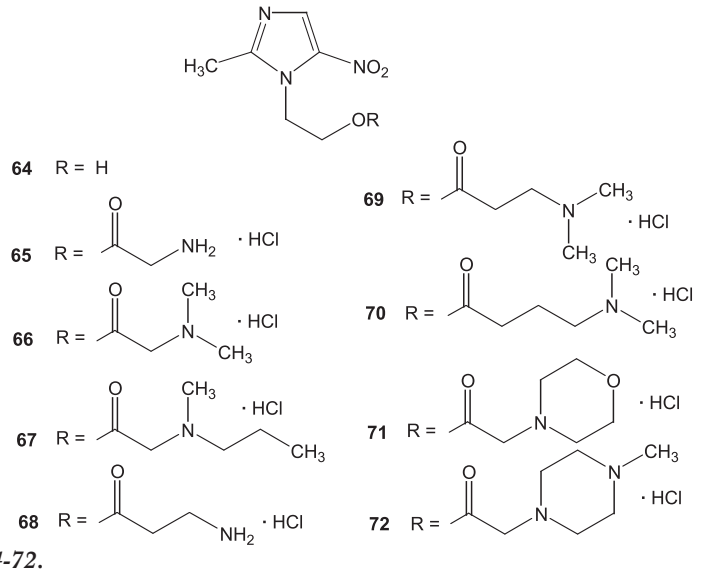
In the first study in humans by Fechner *et al.* (2003), **60** was well tolerated with no signs of pain on injection. Two of the nine subjects reported a transient unpleasant tingling or burning sensation in the groin area at the start of infusion. The pharmacokinetics of **60** were best described by a two-compartment model, and the hydrolysis half-life was 7.2 ± 1.1 min (central volume of distribution, 0.07 L/kg; clearance, 7 mL · kg^{-1} min^{-1}; terminal half-life, 46 min). The PK parameters of **61** produced from **60** were somewhat different than parameters reported in the literature for lipid emulsion formulations of **61**. Due to the conversion process of **60** to **61**, the time to achieve peak concentration of **61** after bolus injection of 60 was longer than that from **61**, and the time for elimination of **61** after infusion of **60** was longer than for **61** from the lipid emulsion formulation.

Ueda *et al.* (2003) have reported the synthesis and biological evaluation of a new POM prodrug of ravuconazole, BMS-379224 (**62**). Ravuconazole (**63**) is a potent broad-spectrum antifungal agent, which exhibits excellent activity against fungal pathogens such as *Candida albicans* and *Cryptococcus neformans* and, especially, against *Aspergillus* species (Arikan and Rex, 2002). Bristol-Myers Squibb is developing **63** as an oral agent, but its overall usefulness is limited by its poor aqueous solubility (0.6 μg/mL). An IV formulation would be of considerable benefit for the treatment of serious systemic fungal infections. **62** has an aqueous solubility of >30 mg/mL, and is relatively stable in solution and very stable in its solid form. **62** was rapidly converted to **63** *in vivo* following IV administration to rats, dogs, and monkeys, and shows efficacy comparable to that oral **63** against systemic *C. albicans* infection in mice. BMS-379224 appears to be in late stage clinical trials.

Amino Acid Prodrugs

Amino acids and amine-containing derivatives have been used for parenteral prodrug strategies, such as the previously mentioned steroid and chloramphenicol examples. They have been explored further for their use as promoieties for parenteral prodrugs, and metronidazole provides several good examples of amino acid ester type prodrug strategies. Metronidazole (**64**) is a drug used for the treatment and prevention of infections caused by anaerobic bacteria (Tally and Sullivan, 1981; Edwards, 1983). It is usually administered orally, but can also be administered by IV infusion for rapid onset; however, because of its relatively poor solubility, administration typically requires the infusion of 100 mL of a 5 mg/mL solution (Bundgaard *et al.*, 1984a). There appears to be some interest in developing a prodrug of **64** for parenteral administration. Bundgaard *et al.* (1984a) synthesized and evaluated a number of amino acid esters of metronidazole (**65–72**), among them, metronidazole N,N-dimethylglycinate (**66**) appeared to be the most promising.

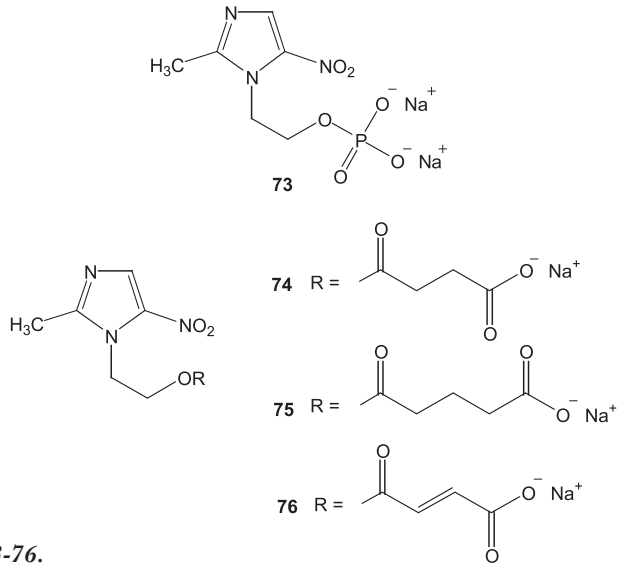
The hydrochloride salt of metronidazole N,N-dimethylglycinate (**66**) has an aqueous solubility of >50% w/v, and is rapidly cleaved ($t_{1/2}$, 12 min) to give **64** in human plasma at 37°C (Bundgaard *et al.*, 1984a). This prodrug is apparently rapidly and quantitatively hydrolyzed to **64** following IV administration to beagle dogs (Bundgaard *et al.*, 1984b). The plasma-concentration time curve of **64**



Structures 64-72.

produced from **66** following IV administration is very similar to that from **64**. While **66** does not exhibit sufficient aqueous stability to formulate as a ready-to-use solution, it has adequate stability to prepare a freeze-dried dosage form that could be reconstituted up to several hours prior to use.

Several other prodrug strategies have been applied to **64**. The phosphate ester of metronidazole (**73**) was evaluated by Cho *et al.* (1982). The solubility of **73** is >730 mg/mL at pH 7 and 25°C, more than 50 times the solubility of the parent drug. Subcutaneous administration of the **73** to rats produces blood levels of **64**, are only slightly less than that from an equivalent **64** dose (0.88:1.00 AUC ratio). It is possible that some of the prodrug may be excreted unchanged prior to enzymatic conversion. Maleic acid, succinic acid, and glutaric acid hemiesters (**74–76**) of metronidazole have also been examined to establish the kinetics of

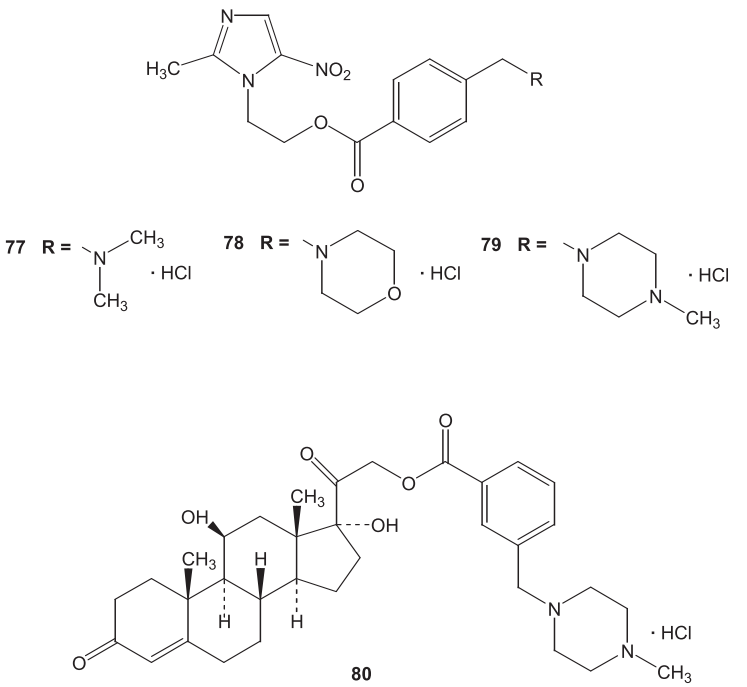


Structures 73-76.

regeneration of **64** in buffered solutions, 80% human plasma, and pig liver homogenate (Larsen *et al.*, 1988). This study was performed as part of an investigation of the potential of using dextrans as carriers for drugs, which is discussed in the macromolecular prodrug chapter of this book.

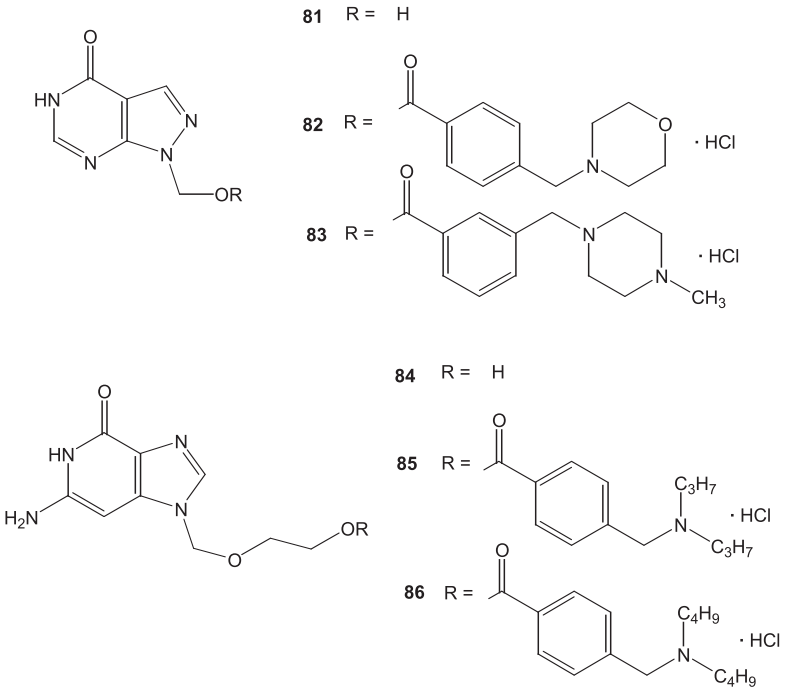
The reason for the unfavorable high instability of amino acid ester prodrugs such as **66** even at low pH values where the prodrug is most soluble and stable is due to the proximity of the amino group to the ester linkage. The neighboring amino group can participate in intramolecular catalysis or assistance of ester hydrolysis, and the strong electron-withdrawing effects of the protonated amino group activate the ester bond linkage toward hydrolysis (Bundgaard *et al.*, 1984b, and references there in). In an effort to overcome this problem, Bundgaard *et al.* (1989) synthesized a number of aminomethylbenzoate esters of metronidazole (**77–79**) and other drugs in which a phenyl group is placed between the ester bond linkage and the solubilizing amino group to distance the ester bond from the electron-withdrawing and catalytic effects associated with the promoiety. Elevated temperature stability studies predict a shelf life (t_{90}) of 12 to 14 years for compounds **77–79** with consideration given to precipitation of formed drug (Jensen *et al.*, 1990). These prodrugs are very soluble at low pH and show excellent solution stability. Furthermore, these prodrugs are rapidly cleaved in 80% human plasma with $t_{1/2}$ values of 0.4 and 0.6 min for prodrugs **78** and **79**, respectively.

This aminomethylbenzoate ester prodrug approach has been applied to a number of other drugs that suffer from poor aqueous solubility with similar success. N-substituted 3- or 4-(amino¬methyl)benzoate 21-esters of hydrocor-



Structures 77-80.

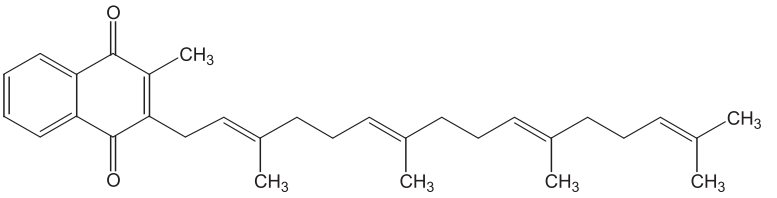
tisone, prednisolone, and methylprednisolone were synthesized and evaluated as water-soluble prodrugs (Jensen and Bundgaard, 1992). Temperature-accelerated stability studies of 3-[(4-methylpiperazin-1-yl)methyl)]benzoate ester of hydrocortisone (**80**) in pH 4 aqueous solution predict a t_{90} of 6 years at 25°C. Aminomethyl-benzoate esters of allopurinol such as **82** and **83** show reasonably high chemical stability with predicted t_{90} values of 2.9 and 14.7 years, respectively, when stored at 5°C (Bundgaard *et al.*, 1990). **82** and **83** show rapid enzymatic hydrolysis in plasma, and are rapidly and completely converted into allopurinol (**81**) following IV administration to rabbits. Aminomethylbenzoate esters of the anti-viral agent acyclovir (**85**,**86**) also show good solubility (~20% w/v) in the pH range of 4–5. They are extremely stable in solution (t_{90}, 26 years) and rapidly produce acyclovir (**84**) in 80% human plasma with half-lives on the order of minutes (Bundgaard *et al.*, 1991).



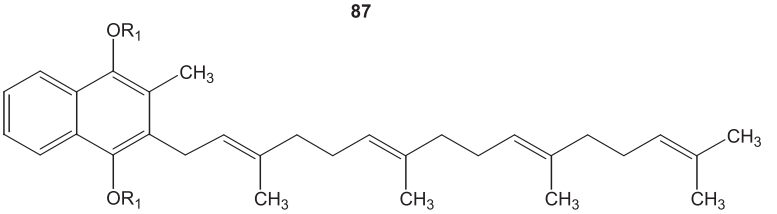
Structures 81-86.

There are several other examples of amino acid ester prodrug strategies applied to poorly water-soluble drugs in attempts to create parenteral prodrugs. Mono- and bis-N,N-dimethylglycine esters of menahydroquinone-4 (**89–91**) have been synthesized and tested (Takata *et al.*, 1995a). Menahydroquinone-4 (**88**) is the reduced/active form of vitamin K derivative menaquinone-4 (**87**). These water-soluble compounds show relatively good stability in pH 7.4 buffer and rapidly regenerate **88** in the presence of rat plasma or rat liver homogenate. *In vivo*, **88** was released in a site-specific manor following the IV administration of **89** to vitamin K cycle inhibited rats. The liver-selective uptake of **88** from **89** was 5.7-fold higher compared to an equivalent dose of **87**, and coagulation activity was

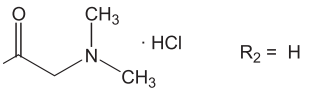
extended 1.9-fold in warfarin-poisoned rats (Takata *et al.*, 1995b). Similar prodrugs of phylloquinone (vitamin K_1) have been investigated (Takata *et al.*, 1999). These types of amino acid ester vitamin K prodrugs have the distinct advantage that they do not require a bioreductive activation process to release the active agent, so they could be particularly useful in the treatment of patients with induced hypoprothrombinaemia undergoing coumarin and cephalosporin therapies with inhibited vitamin K quinone bioreductive activation pathways (Takata *et al.*, 1999).



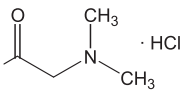
87



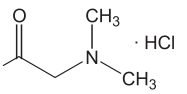
88 $R_1 = R_2 = H$



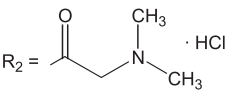
89 $R_1 =$ $R_2 = H$



90 $R_1 = H$ $R_2 =$



91 $R_1 =$



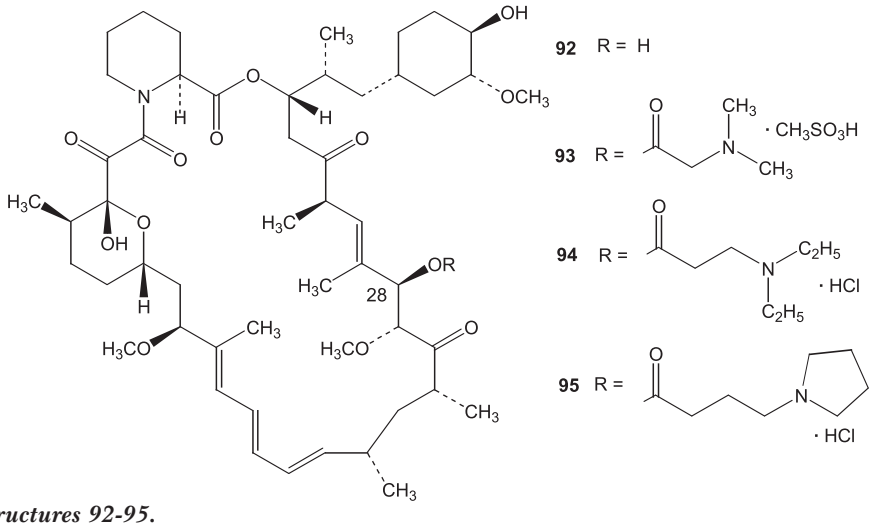
$R_2 =$

Structures 87-91.

Further examples of amino acid ester prodrug strategies are found applied to the drug rapamycin. Rapamycin (**92**) is a macrocyclic triene with immunosuppressive properties (Martel *et al.*, 1977). **92** was also found to have significant antineoplastic activity against a broad spectrum of ascites and transplanted solid tumors in mice (Douros and Stuffness, 1981a,b), even when administered away from the tumor implant site (Eng *et al.*, 1984). Its poor aqueous solubility and poor oral bioavailability led to the investigation of water-soluble derivatives as potential prodrugs for parenteral administration. Ester prodrugs of rapamycin have been synthesized by Stella and Kennedy (1987). Glycine derivatives such as N,N-dimethylglycine and amine-containing acids were used as solubilizing promoieties to form ester prodrugs of rapamycin (**93–95**). **93** and **94** show aqueous solubilities >50 mg/mL. While the prodrugs do not exhibit sufficient

stability to allow for ready-to-use solution formulations, they may be stored in solid form and reconstituted prior to injection.

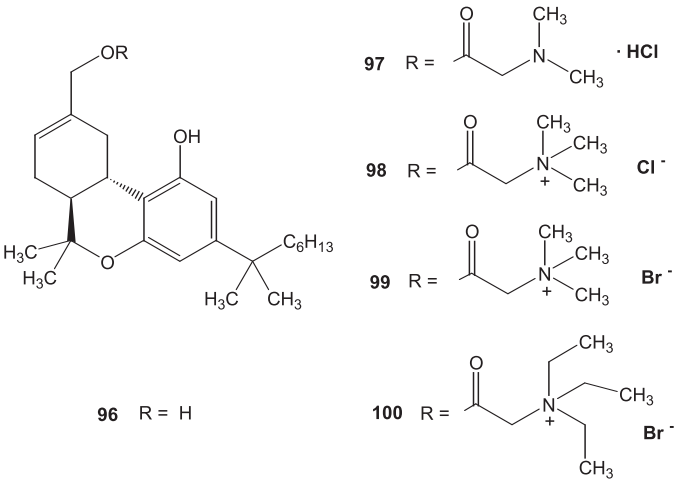
The pharmacokinetics of rapamycin-28-N,N-dimethylglycinate (**93**) were determined after IV administration of 10–100 mg/kg to mice (Supko and Malspeis, 1994). The prodrug remained in the mice for 5–12 hours after administration, and exhibited an atypical dose-dependency that appears to originate from saturable binding of the prodrug to plasma proteins, while tissue binding remains linear. Plasma levels of **92** were rapidly achieved, surpassing the prodrug plasma concentration within 30 min following administration. Plasma concentrations of **92** were sustained near peak levels for approximately 8 h (4.95–8.12 μg/mL), and remained above 1 μg/mL for 48 h. Surprisingly, this prodrug strategy appears to provide an effective slow-release IV delivery system for rapamycin.



Structures 92-95.

Dexanabinol (**96**) is a synthetic, nonpsychotropic cannabinoid (Mechoulam *et al.*, 1990), which is a noncompetitive N-methyl-D-aspartate receptor antagonist (Feigenbaum *et al.*, 1989) that was evaluated for the treatment of severe traumatic brain injury. The lipophilic nature of **96** allows it to pass through the blood brain barrier and access the central nervous system. However, the lipophilicity of the drug is also associated with very poor aqueous solubility, making the development of an injectable formulation extremely difficult.

Pop *et al.* (1996) synthesized a number of glycinate esters of dexanabinol for use as potential water-soluble prodrugs. Of the compounds synthesized and tested, quaternary alkyl ammonium salts of N-substituted glycine esters (**97–100**) seemed to be the most promising. The aqueous solubility of these prodrugs is approximately 50 mg/mL (53.4, 52.2, 47 mg/mL for **98**, **99**, **100** respectively) (Pop *et al.*, 1999). These prodrugs are reasonably stable in solution with half-lives between 3.64 and 14.64 days at pH 7.4 and 21°C. Like many other amino acid ester prodrugs, **97–100** have greater stability at lower pH values. Prodrugs **98** and **100** exhibit stability half-lives of 26–28 days at pH 5.5 and just over 50 days at pH 3.0, both at 21°C. This stability would not permit a ready-to-use formulation, but



Structures 96-100.

a sterile solid for reconstitution prior to administration may be possible. These prodrugs are rapidly hydrolyzed to give **96** in human plasma with concentration-dependent half-lives on the order of minutes. Figure 4 shows the plasma and brain concentration versus time profile of **96** following IV administration of **98** to rats. Peak concentrations of drug in blood and brain were achieved in less than 5 min. These data suggest that **98** is rapidly enzymatically hydrolyzed in the blood to **96**, which then passes through the blood-brain barrier to the central nervous system of the rat.

Amino acids have been used predominantly to form ester prodrugs, but there are a few examples where amino acids have been used as promoieties linked to amine drugs via an amide bond. Pochopin *et al.* (1995) synthesized several D- and

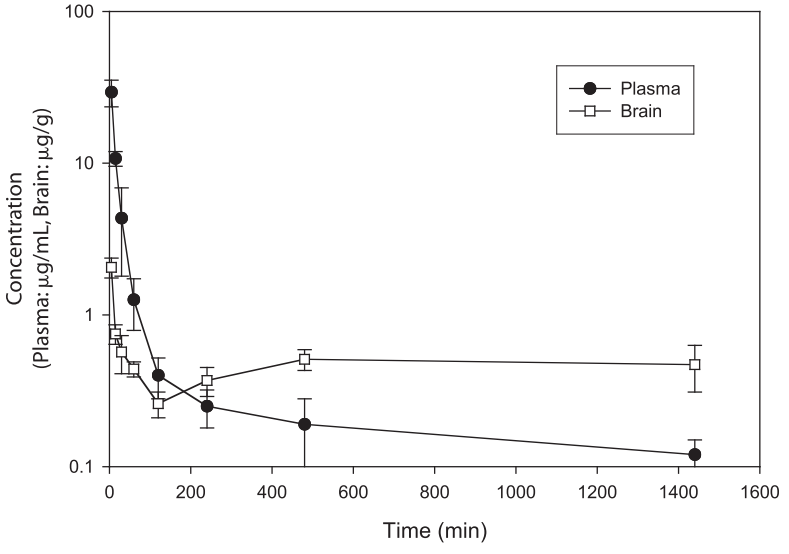
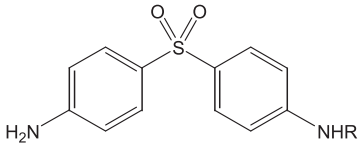
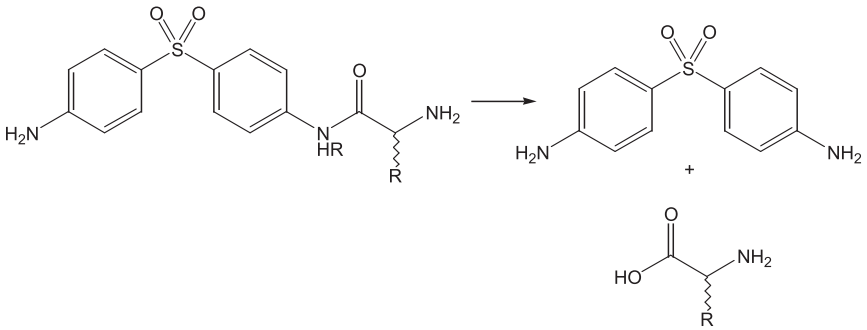


Figure 4. Concentration of dexanabinol (96) in rat plasma (●) and rat brain (□) following IV administration of 98 (Pop *et al.*, 1999).

L-amino acid amide prodrugs of the bacteriostatic and antileprosy drug dapsone. Dapsone (**101**) is a synthetic sulfone anti-infective, containing primary aromatic amine groups with an aqueous solubility of 0.16 mg/mL. It was chosen as a model to examine the feasibility of using amino acid amides as prodrugs to improve the aqueous solubility of amine-containing drugs. These prodrugs were expected to undergo enzymatic cleavage of the amide bond to release parent drug *in vivo*. Dapsone is a symmetric drug with two identical aromatic amines, but only the mono-substituted amino acid amides were deemed desirable (**102–110**).



101	R = H	**106**	R = -D- leucine
102	R = glycine	**107**	R = -L- phenylalanine
103	R = -L- alanine	**108**	R = -D- phenylalanine
104	R = -D- alanine	**109**	R = -L- lysine
105	R = -L- leucine	**110**	R = -D- lysine



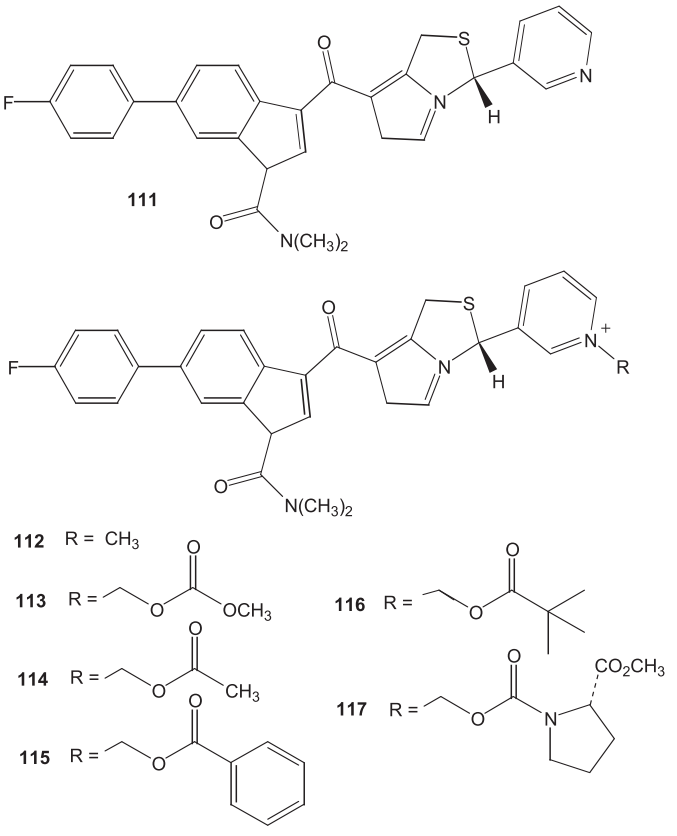
Structures 101-110.

With the exception of **107**, the HCl salts of the various L-amino acid amide and glycine prodrugs of dapsone are very soluble in water. L-lysine-dapsone (**109**) had an apparent solubility of >65 mg/mL even at pH 7.4. Elevated temperature studies indicate that the predicted t_{90} values for the amino acid amide prodrugs of dapsone are >2 years at pH 4 (Pochopin *et al.*, 1995). The stability of the L-amino acid amides and the glycine prodrugs in human blood at 37°C was found to be good, with $t_{1/2}$ values in the range of 9 to 20 min except for L-leucine-dapsone (**106**), which was 1.7 min. The L-amino acid amide (including glycine) prodrugs were rapidly hydrolyzed after being administered IV to rabbits (Pochopin *et al.*, 1994). The L-amino acid and glycine prodrugs were quantitatively converted to **101** within 2 min following administration, giving a blood concentration profile indistinguishable from that of a bolus dose of **101** *per se*. Interestingly, the D-

amino acid amide prodrugs of dapsone were also quantitatively converted to **101** *in vivo*, but with $t_{1/2}$ values of 32–56 min compared to <2 min for the L-amino acid prodrugs. These results are consistent with the *in vivo* conversion of the prodrugs by stereospecific peptidase enzymes.

Miscellaneous Parenteral Prodrug Strategies

As detailed above, Stella and Krise used the POM technology to successfully modify the delivery properties of tertiary amines. Their work included the synthesis of several bioreversible quaternary ammonium derivatives of tertiary amines. Their work was partially inspired by Davidsen *et al.* (1994), who synthesized a number of N-(acyloxyalkyl)pyridinium salts of the tertiary amine pyrrolothiazole (**112–117**) in an effort to create water-soluble prodrugs. Pyrrolothiazole (**111**) is a potent platelet-activating factor (PAF) antagonist. PAF is an important mediator in life-threatening conditions such as septic shock (Sanchez-Crespo, 1993), and the PAF antagonist **111** has shown activity in models of inflammation and septic shock (Davidsen *et al.*, 1994). **111**, however, has an aqueous solubility of less than 1 μg/mL, making intravenous administration of the drug impossible. These N-(acyloxyalkyl)pyridinium salts present an interesting prodrug strategy for pyridine containing drugs. The pyridine nitrogen is

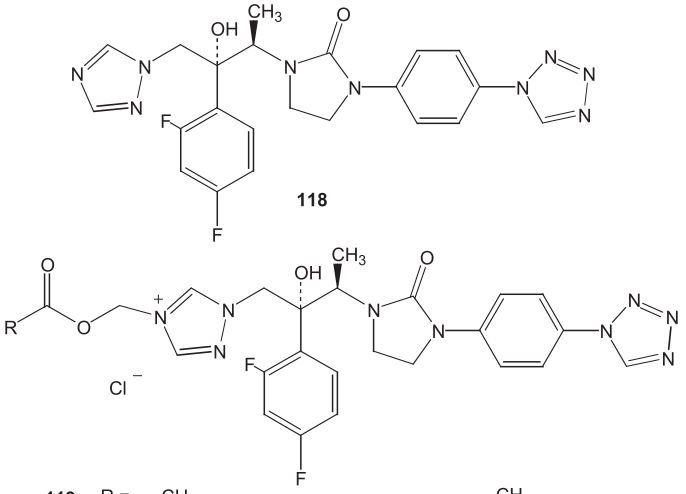


Structures 111-117.

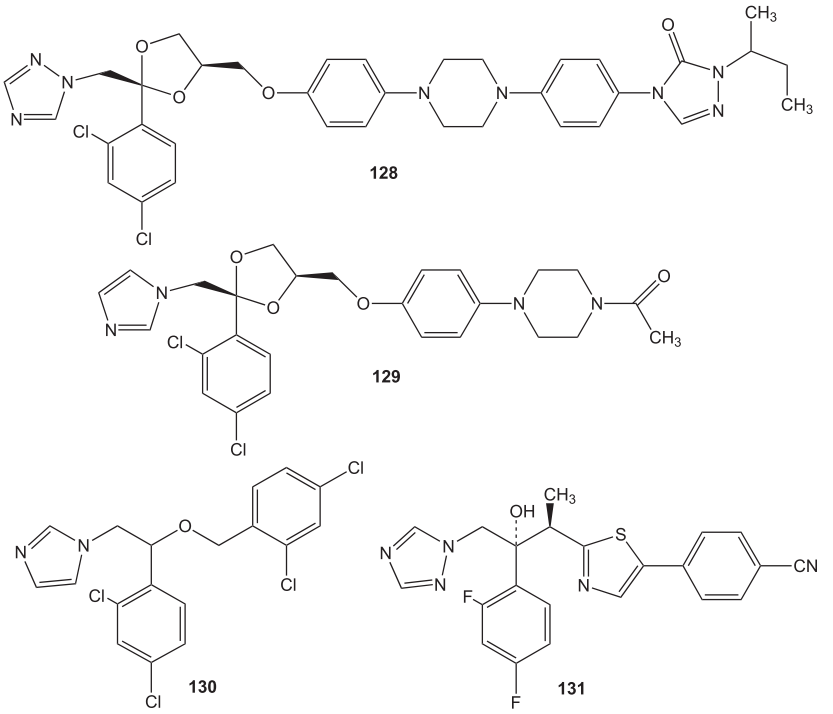
alkylated with an acyloxyalkyl promoiety creating charged pyridinium compounds with solubilities of 20 mg/mL or greater.

The buffer and plasma stabilities of this type of compound vary with the composition of the promoiety; ABT-299 (**114**), the acetyl-substituted analog, was selected for further investigation. **114** has as conversion $t_{1/2}$ of 510 min in pH 7 buffered solution at 25°C and a $t_{1/2}$ of 2.6 minutes in human plasma adjusted to pH 7 at 37°C. The prodrug conversion occurs by enzymatic cleavage of the ester bond followed by spontaneous release of formaldehyde to give the parent drug. PK studies also show that this conversion occurs *in vivo* in a number of animal models, and **114** is effective in the treatment of endotoxic shock in animal models (Davidsen *et al.*, 1994; Summers *et al.*, 1995; Albert *et al.*, 1996a).

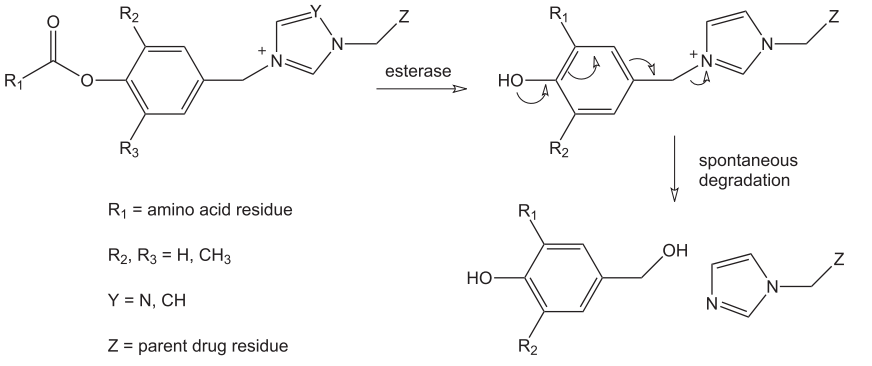
The results of administration of **114** to human subjects show the rapid conversion of the prodrug to **111**. Blood samples taken from subjects following the administration of **114** were challenged with PAF *ex vivo* and showed inhibited β-thromboglobulin release, a measure of platelet degranulation, for at least 12 h. This indicates that administration of **114** to humans results in a pronounced inhibition of platelet activation coupled to the platelet PAF receptor, which supports the potential therapeutic benefits of this prodrug in the treatment of PAF-mediated diseases (Albert *et al.*, 1996b; 1997).



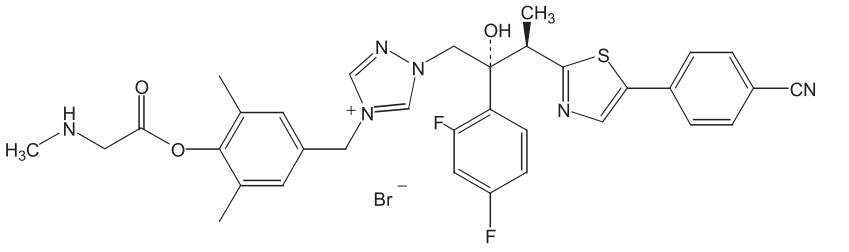
Structures 118-127.



Structure 128-131.



Scheme 1.



Structure 132.

A somewhat similar new approach has been applied to lipophilic azoles containing antifungal agents. Ichikawa *et al.* (2001) prepared several derivatives of TAK-456 (**118**), a poorly water-soluble (5 μg/mL) triazole containing antifungal agent, in attempt to create an injectable form of the drug for the treatment of systemic fungal infections. The prodrug strategy involves the addition of a variety of acyloxyalkyl groups to the nitrogen of the triazole ring forming charged triazolium salts (**119–127**).

These compounds were designed to undergo enzymatic cleavage of the ester bond followed by spontaneous release of formaldehyde to give the parent drug *in vivo*. Of the compounds synthesized, TAK-457 (**119**) was selected as the most promising compound for clinical evaluation. The solubility of **119** is 10 mg/mL (4 mg/mL for the hydrate) and is 98% stable for 24 h in a 5% aqueous glucose solution. **119** rapidly converts to **118** in mouse, rat, and human plasma with $t_{1/2}$ values of 5–6 min. Rapid *in vivo* conversion was also observed in rats (Ichikawa *et al.*, 2001). Furthermore, **119** shows activity in mouse models of invasive pulmonary aspergillosis fungal infection (Hayashi *et al.*, 2002).

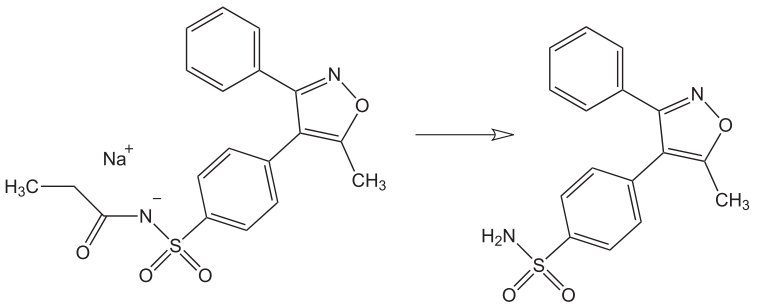
Ohwada *et al.* (2002) investigated the feasibility of a similar prodrug approach in attempts to create water-soluble prodrugs of itraconazole (**128**), ketoconazole (**129**), miconazole (**130**), and BMS-207147 (**131**). This approach consists of attaching various 4-hydroxybenzyl self-cleavable linkers with amino acid ester solubilizing moieties to the azole (triazole or imidazole) groups of the parent drugs, forming water-soluble benzylazonium salts. The ester bond of the promoiety can be enzymatically hydrolyzed to generate a phenolic intermediate, which undergoes spontaneous degradation to release the parent drug and a benzylalcohol derivative (scheme 1).

Of the compounds examined, **132** appears to be the most promising. It has an aqueous solubility of 49 mg/mL, and is 97% stable after 7 days in 0.1% buffer at pH 3 and 25°C. The conversion rate for **132** in human plasma was extremely rapid, with a $t_{1/2}$ value of less than 2 min at 37°C. IV administration of **132** was found to be very effective against systemic candidasis and systemic and pulmonary aspergillosis in rat models of fungal infection (Ohwada *et al.*, 2002).

Selective cyclooxygenase-2 (COX-2) inhibitors such as celecoxib (Penning *et al.*, 1997) and rofecoxib (Prasit *et al.*, 1999) are currently marketed for the treatment of inflammatory conditions without the side effects of non-selective inhibitors, which inhibit both COX-1 and COX-2. These new selective inhibitors reduce the risk for gastrointestinal ulceration associated with COX-1 inhibitors. COX-2 inhibitors such as celecoxib and rofecoxib exhibit modest aqueous solubility, which limits their therapeutic usefulness by limiting their dosing options (Talley *et al.*, 2000). Parecoxib (**133**) is a prodrug of the COX-2 inhibitor valdecoxib (**134**), which had greatly improved solubility for parenteral application. Parecoxib is an excellent and unusual example of a successful prodrug strategy; it also appears in the case studies section of this book.

Parecoxib is formed by the acylation of the sulfonamide group of **134**. The nitrogen of the acylated sulfonamide can be deprotonated with base to give parecoxib sodium salt. Parecoxib sodium has a solubility of 22 mg/mL in phosphate buffered saline at 25°C. The conversion of **133** to **134** is mediated by

hepatic esterases with a K_m value greater than 470 μM and can be completely inhibited by esterase or amidase inhibitors. The *in vivo* conversion of **133** to **134** was rapid and complete following IV administration to rats, dogs and cynomolgus monkeys. Furthermore, **133** showed potent efficacy and rapid onset in a therapeutic model of pain, with results similar to those of the potent non-selective COX-1 and COX-2 inhibitor ketorolac (Talley *et al.*, 2000).



Structures 133-134.

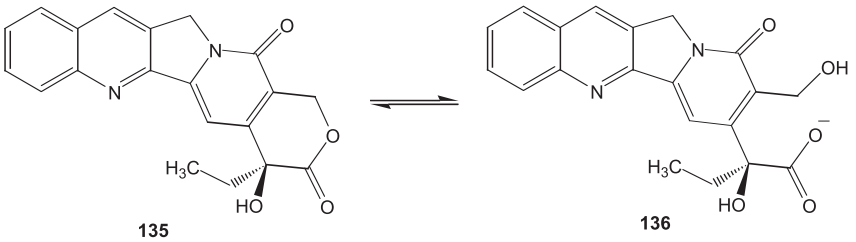
Parecoxib (**133**), commercially available under the trade name Dynastat®, is supplied as a lyophilized powder for reconstitution with saline prior to injection. Pharmacokinetic studies show that **133** is rapidly converted to **134** following IM or IV injection in humans, and in clinical trials **133** compares favorably with ketorolac (Jain, 2000; Cheer and Goa, 2001; Daniels *et al.*, 2001; Karim *et al.*, 2001).

Recent Parenteral Prodrug Strategies for Important Cancer Drugs

Prodrugs of Camptothecin

Camptothecin (CPT, **135**) is a naturally occurring antineoplastic agent that targets topisomerase I, which is an essential enzyme in DNA replication and RNA transcription (Vanhoefer *et al.*, 2001). **135** is sparingly water soluble (2–3 μg/mL) (Wall *et al.*, 1966), and exists in a pH-dependant equilibrium between its lactone and carboxylate (**136**) forms. Clinical trials in the 1970s using the sodium carboxylate form of CPT were unsuccessful because of its low activity and severe toxicity (Muggia *et al.*, 1972). Intact **135** (lactone) was later found to be approximately 10 times more potent than **136** (carboxylate) (Wani *et al.*, 1980). **136** has a high affinity for human serum albumin (HSA); thus, when **135** comes in contact with human blood, it is quickly converted to **136** (Mi and Burke, 1994).

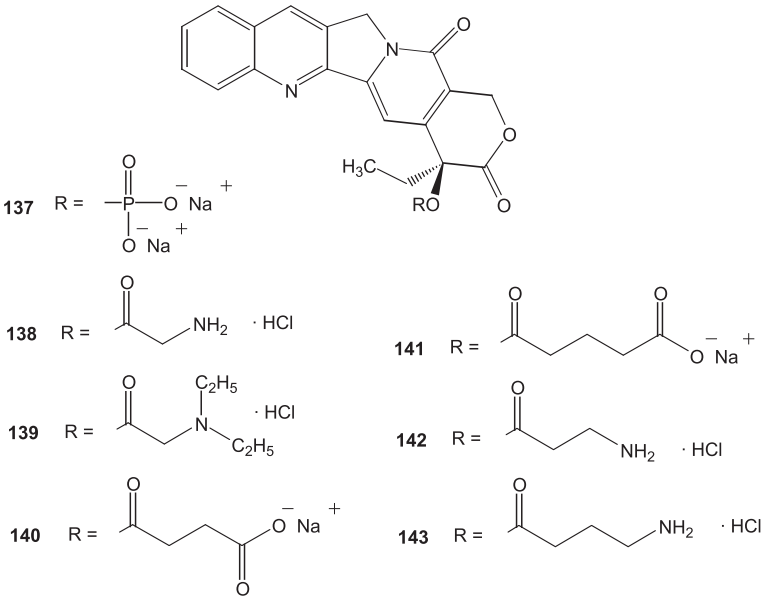
A number of CPT derivatives were reported by Vishnvvajjala and Garzon-Aburbey (1990) in a U.S. patent application for their use as potential parenteral prodrugs. 20-ester derivatives such as the phosphate, glycinate, N,N-diethylgly-



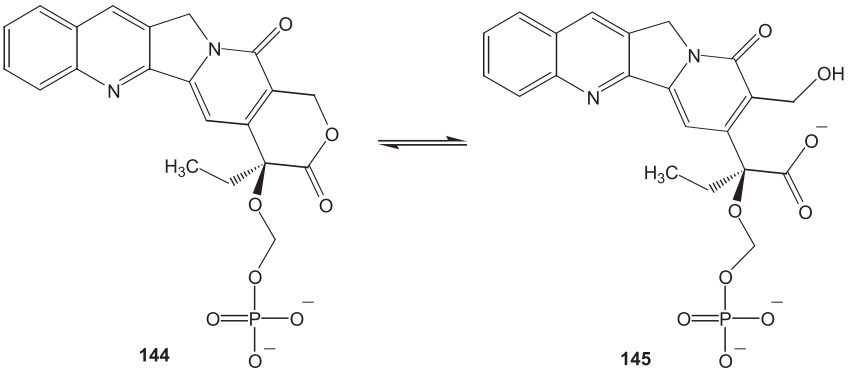
Structures 135-136.

cinate, succinate, and glutarate, as well as other similar derivatives (**137–143**), were synthesized. The glycinate ester (**138**) and longer chained amine-containing acid esters **142** and **143** were later investigated for their use in liposomal core-loaded drug formulations (Liu *et al.*, 2002). The water-soluble prodrugs were found to be relatively stable at lower pH values and break down rapidly at physiological pH. The glycine derivative **138** generated some unusual decomposition products, and it was also noted that increased length of alkyl chain between amine and the ester slowed the rate of hydrolysis.

Another interesting prodrug of camptothecin is the 20-phosphoryloxymethyl derivative (**144**). The solubilizing POM promoiety is attached to the alcohol group adjacent to the ketone of the lactone ring. Hanson *et al.* (2003) found that the POM promoiety apparently catalyzes the opening of the lactone ring. The opened form of the POM-CPT lactone ring (**145**) is favored above pH 4.5 at equilibrium. The bioconversion of **144** *in vivo* results predominantly in the release of the open ring form (**136**) following IV administration to rats, but the equilibrium between the lactone and carboxylate is then rapidly established (Hanson, 2002).

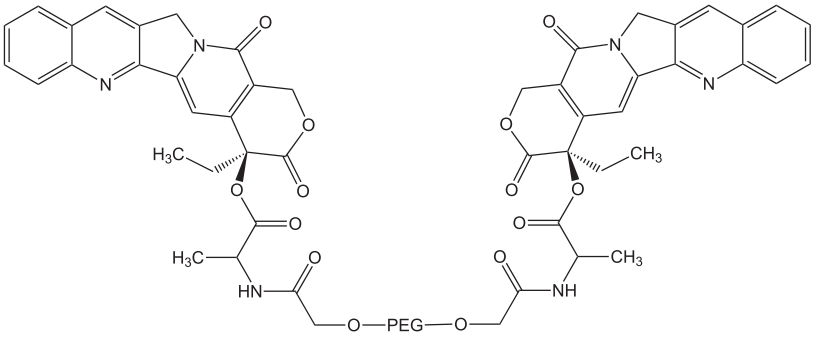


Structures 137-143.



144 145

Structures 144-145.



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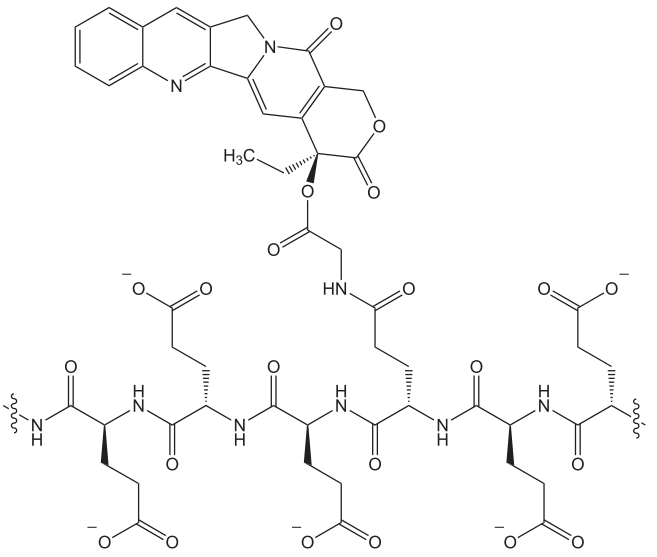


147

Structures 146-147.

Polymer bound CPT prodrugs have been investigated for their use in parenteral applications. Water-soluble polymers not only increase the solubility of poorly soluble drugs, but polymer-bound drugs see a particular enhancement in tumor targeting as well. This enhanced permeability and retention (EPR) effect is a result of increased permeability of tumor vasculature to macromolecules and lack of lymphatic vessels in tumors to return the macromolecules to systemic circulation (Maeda *et al.*, 2000; Maeda, 2001).

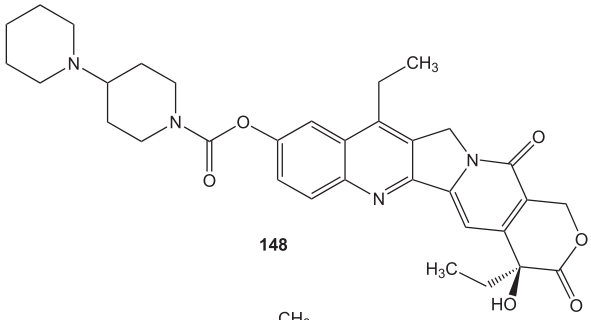
Polyethylene glycol (PEG) prodrugs of CPT have been extensively studied. A variety of spacer groups have been employed to achieve the most desirable stability and enzymatic release (Greenwald *et al.*, 1998) profiles. Clinical trials for the PEG-CPT prodrug pegamotecan (**146**) have been reported (Rowinsky *et al.*, 2003; Scott *et al.*, 2004). Peak levels of **135** following IV infusion were observed after about 24 h, and patients responded well with low incidence of toxicities. More details on this and other PEG prodrugs can be found in the PEG prodrug chapter of this book.

Another interesting water-soluble polymer prodrug of CPT is poly-(L-glutamic acid)-Gly-camptothecin (**147**). Poly-(L-glutamic acid) (PG) is a biodegradable peptide homo-polymer with carboxylic acid side chains that carry multiple anionic charges; CPT binds to the polymer at multiple sites. Several different linkages for the CPT-PG conjugates have been investigated (Bhatt *et al.*, 2003). CPT-Gly-PG conjugate **147** has loaded up to 50% w/w ratio of CPT on the polymer (Singer *et al.*, 2001). As in the PEG prodrugs, increasing the MW of the PG polymer increases the plasma residence time by decreasing renal clearance. The increase in MW of PG from 33 to 50 kDa resulted in greater anti-tumor activity with no significant change in maximum tolerated dose. **147** showed enhanced efficacy compared to **135** in athymic mice bearing human colon and lung tumor models (Singer *et al.*, 2000; Bhatt *et al.*, 2003).

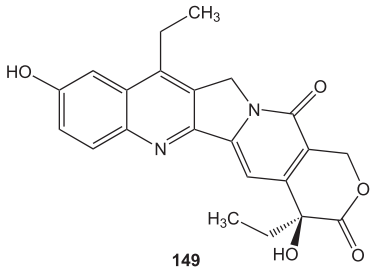
Irinotecan (CPT-11, **148**) is a water-soluble prodrug of the CPT derivative SN-38 (**149**) that is used for IV administration. For a review see Mathijssen *et al.* (2002). The solubilizing pro-moiety of **148** is a cyclic tertiary amine linked by a carbamate ester to the phenol group of **149**, and **148** is converted into **149** predominantly by hepatic carboxylesterases in humans (Creemers, *et al.*, 1994). Like CPT, **148** and **149** exist in open carboxylate and closed lactone ring forms; only the lactone forms of these compounds are active. **135** exists predominantly in its carboxylate form, but **149** is present predominantly in its lactone form at equilibrium in human plasma.

148 and **149** display linear PK profiles in humans over a wide range of doses (Slatter *et al.*, 2000). Peak plasma levels of **148** are reached at the end of infusion and decay rapidly with distribution and elimination; **149** is produced rapidly, with peak plasma concentrations occurring at the end of infusion and up to 4 h after. The terminal half-life of 148 is 14.6 h, while **149** has a terminal half-life of 47 h. **148** is the main circulating component in plasma following IV administration and is the major excretion product in urine, bile and feces. The pharmacokinetics of **148** and **149** are extremely complex and have been extensively investigated. For further review see Mathijssen *et al.* (2001). **148** has a broad spectrum of activity in

solid tumors (Rougier *et al.*, 1998; Bleiberg, 1999; Sandler and van Oosterom, 1999), and was approved by the FDA in 2000 as a first-line therapy in combination with 5-fluorouracil and leucovorin for the treatment of metastatic colorectal cancer.



148

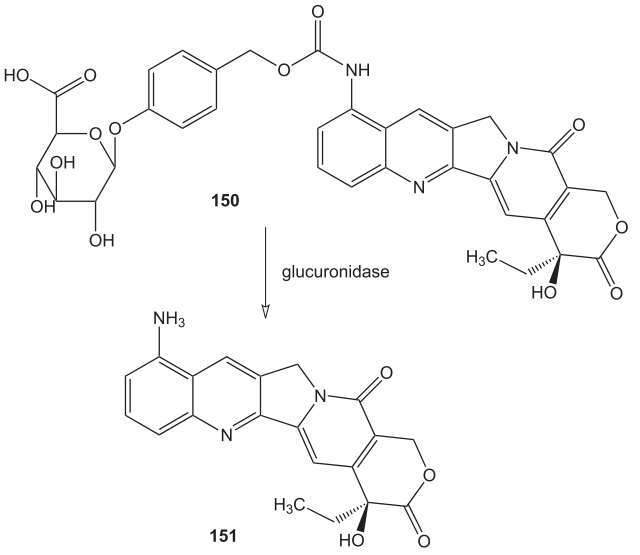


149

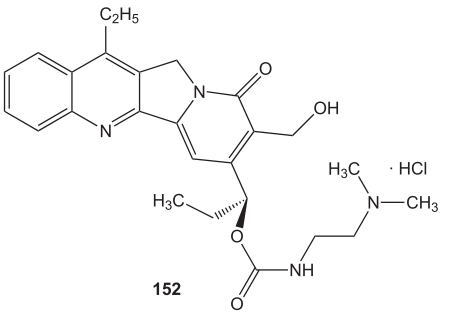
Structures 148-149.

Leu *et al.* (1999) reported the synthesis and testing of a water-soluble glucuronide derivative (**150**) of 9-aminocamptothecin (**151**). The glucuronide promoiety was selected not only for its solubility properties but also because of its ability to be preferentially activated at tumor cells targeted with β-glucuronidase-antibody conjugates (Haisma *et al.*, 1992; 1994; Wang *et al.*, 1992). **150** had a solubility of 25 mg/mL at pH 4 where the lactone form is dominant compared to 0.006 mg/mL for **151** at the same pH value; **150** is very soluble at physiological pH (109 mg/mL at pH 7), preventing precipitation at the injection site (Leu *et al.*, 1999). **150** was stable in PBS at pH 7 and 37°C and in 90% human plasma at the same pH and temperature for at least 48 h. A 25 μM concentration of **150** converted nearly quantitatively to **151** in 2 h in the presence of 0.05 μg/mL β-glucuronidase. Human serum albumin (HSA) did not affect the equilibrium of lactone versus carboxylate forms of **150** compared to **150** in PBS alone, while **135** and **151** are both shifted to the inactive carboxylate form in the presence of HSA (Prijiovich *et al.*, 2003). Similar results were also seen in human serum and whole blood.

150 was 25–60 times less toxic to 5 human cancer cell lines than was **151**, but **150** exhibited activity similar to that of **151** in the presence of β-glucuronidase (Prijovich *et al.*, 2002). *In vivo*, **150** exhibited efficacy against CL1-5 human lung cancer xenografts similar to or greater than that of **151**, CPT-11, and topotecan. Also worth mentioning are a series of aminoethylamide derivatives of 7-



Structures 150-151.

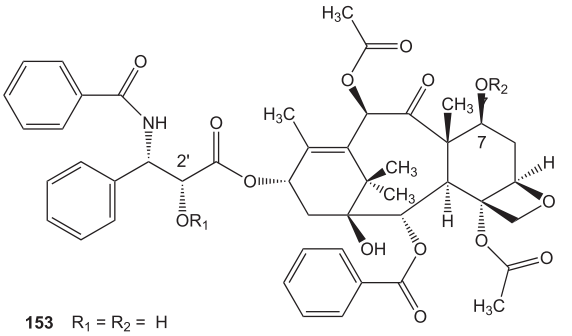


Structures 150-151.

ethylcamptothecin such as **152** reported by Sawada *et al.* (1988). Compounds of this nature are potential prodrugs of the carboxylate form of 7-ethylcamptothecin.

Prodrugs of Paclitaxel

Paclitaxel (Taxol®, **153**) is a well-known cytotoxic agent that is currently used to treat breast, ovarian, and non-small cell lung cancers (Donehower and Rowinsky, 1993; Rowinsky and Donehower 1993). **153** is a unique anti-tumor agent; however, its poor aqueous solubility (0.25 μg/mL) is a significant disadvantage to its therapeutic usefulness (Adams *et al.*, 1993). Clinical formulations of Taxol® contain polyoxyethylated castor oil (Cremophor EL®) and ethanol. Cremophor EL® is considered to be the source of hypersensitivity reactions observed with the infusion of this formulation (Weiss *et al.*, 1990; Brown *et al.*, 1991). There have been numerous publications on the subject of water-soluble prodrugs of paclitaxel and its semisynthetic analogs. This section will outline a few examples of prodrug strategies that have been applied to this drug in attempts to create a safe injectable formulation utilizing these prodrugs.



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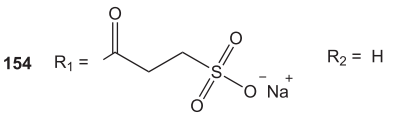

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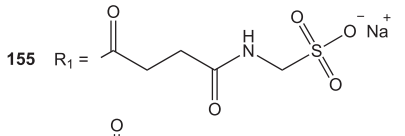

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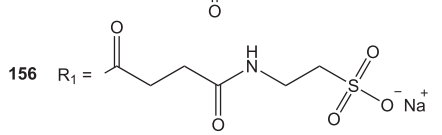

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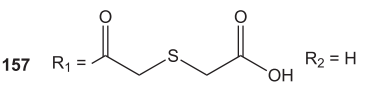

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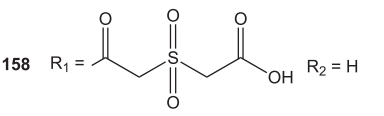

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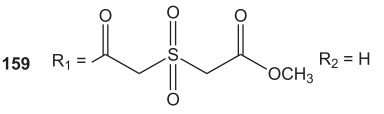

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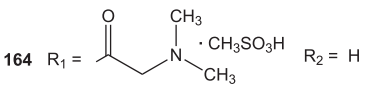

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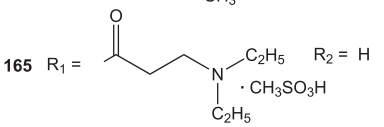

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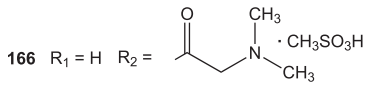

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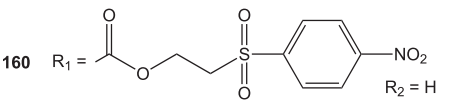

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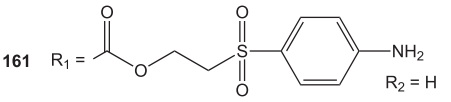

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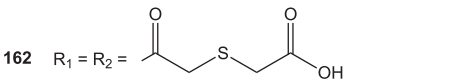

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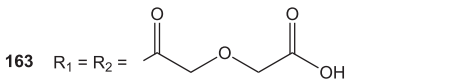
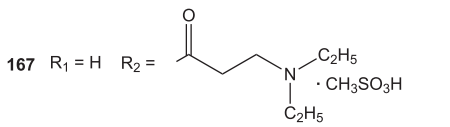
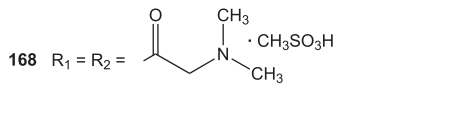


163 $R_1 = R_2 =$



167 $R_1 = H$ $R_2 =$



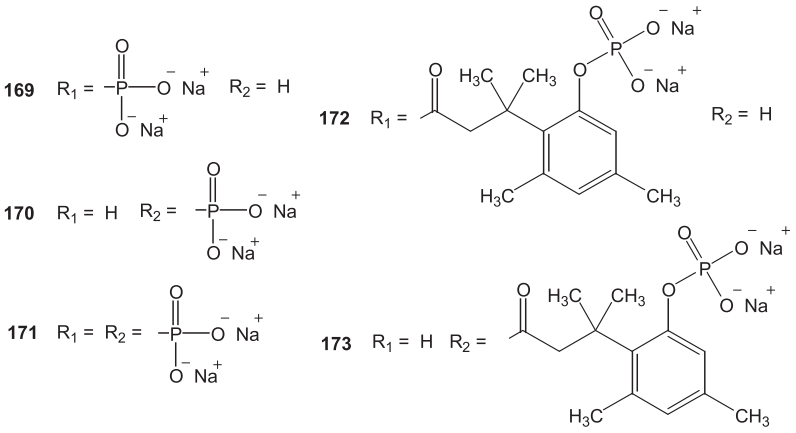
168 $R_1 = R_2 =$

Structures 153-168.

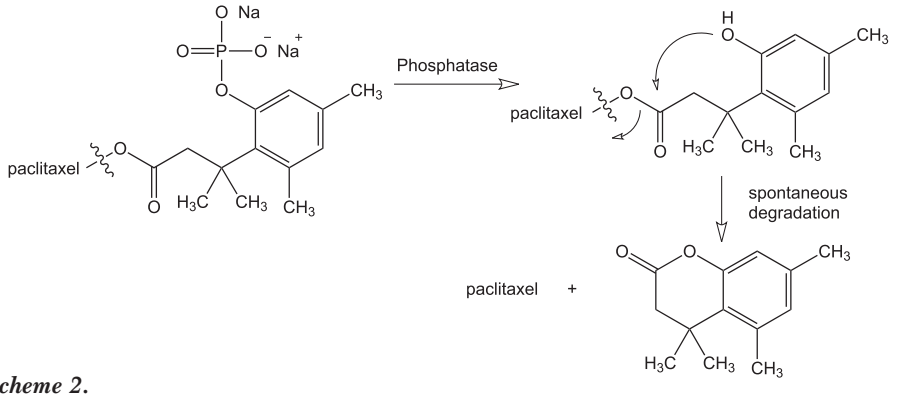
The majority of paclitaxel prodrug strategies involve the esterification of the alcohol groups at the C-2' or C-7 position of the parent compound. 2'-ester derivatives of **153** with sulfonate containing promoieties (**154–156**) were prepared by Zhao *et al.* (1991). Compounds **154**, **155**, and **156** showed somewhat reduced activity compared to **153** when evaluated using four different cell lines *in vitro* and P-388 lymphocytic leukemia *in vivo*. Nicolaou *et al.* (1993) synthesized a number of compounds designed to increase aqueous solubility and permit paclitaxel release under physiological conditions (157–163). **159** has a solubility of 1.2 mg/mL and a $t_{1/2}$ of >8.3 h at pH 7.5 and 37°C. The $t_{1/2}$ for the release of **153** from **159** decreases to 1.6 h in the presence of human plasma. These compounds display cytotoxicity similar to that of **153** in various cell lines.

Mathew *et al.* (1992) prepared and evaluated some 2'- and 7-amino acid esters of paclitaxel (**164–168**) as potential prodrugs. The methanesulfonic acid salts of these derivatives showed much improved solubility. **165** and **168** had water solubilities of > 10 mg/mL. The 7-derivatives were effective in promoting microtubule assembly *in vitro*, suggesting intrinsic activity, while the 2'-derivatives showed little activity. The 7-derivatives appeared to be more chemically and enzymatically stable than the 2'-derivatives. The 2'-derivatives **164** and **165** inhibited the *in vitro* proliferation of B16 melanoma cells to an extent similar to that of **153**, and **165** showed the greatest antitumor activity in mammary tumor xenografts in mice. The cytotoxic and antitumor activities of the 2'-derivatives are likely due to their release of **153**.

Early attempts by Vyas *et al.* (1993) included the synthesis and evaluation of water-soluble (> 10 mg/mL) 2'- and 7-phosphate paclitaxel and docetaxel derivatives (**169–171**). Unfortunately, these derivatives showed no significant *in vitro* enzymatic conversion to **153** and no *in vivo* anti-tumor efficacy. This was likely a result of steric hindrance around the phosphate promoiety, so new derivatives, which utilized a methylated 2'-hydroxyphenylpropionic acid spacer group between the phosphate promoiety and paclitaxel, were introduced (**172**, **173**) (Ueda *et al.*, 1993). This approach made the phosphate promoiety more accessible to phosphatase enzymes and, following dephosphorylation, the spacer group can rapidly lactonize to generate paclitaxel and hydrocoumarin (Scheme 2).



Structures 169-173.



Scheme 2.

172 and **173** are water soluble (>10 mg/mL) and relatively stable in solution. No decomposition was observed in pure water at room temperature for 24 h, while >90% was cleaved to generate **153** in 25 min in the presence of alkaline phosphatase at 37°C. These compounds did not convert significantly in rat, dog, or human plasma, presumably because of high plasma protein binding and low *in vitro* phosphatase activity. **172** was found to be marginally active in the M109 tumor model, where **173** was surprisingly found to be as active as paclitaxel (Ueda *et al.*, 1993). **172** and **173** were active in tubulin polymerization assays only in the presence of alkaline phosphatase (Mamber *et al.*, 1995). The t_{90} values for **172** and **173** were later reported to be 20 h and >100 h, respectively, in 50 mM Tris buffer at 37°C and pH 7.4 (Vyas *et al.*, 1995).

2'- and 7-malic acid esters of paclitaxel were prepared by Damen *et al.* (2000) (**174–176**). The apparent solubilities of **174**, **175**, and **176** are 0.6, 0.3, and 0.5 mg/mL, respectively, at pH 7.4. These compounds did not significantly degrade for 48 h in water at 37°C buffered to pH 7.4, but **174** did release paclitaxel with a $t_{1/2}$ of 4 h in 80% human plasma under the same conditions (**175** and **176** did not). **174** was also found to be more active than 153 *in vivo* in the P-388 tumor model.

A 2'-(N-methylpyridinium acetate) prodrug (**177**) of paclitaxel was reported in a communication by Nicolaou *et al.* (1994). **177** exhibits a solubility of 1.5 mM (1.5 mg/mL) in various buffer systems between pH 6.2 and 7.4, and its solubility in pure water appears to be limited only by gel formations above concentrations of 20 mM (20 mg/mL). **177** was found to form various assemblies, including micelles and helices, depending on the type of media present. **177** is stable for at least 21 days in phosphate-buffered saline (PBS) of pH 6.0 to 7.3, but shows rapid conversion to **153** in human plasma at 37°C ($t_{1/2}$ = 2.4 min). **177** showed no activity *per se* in tubulin polymerization assays, while **153** released from **177** in human plasma did exhibit the expected stabilization of microtubules, indicating that **177** acts as a prodrug of paclitaxel. The release of **153** from **177** is presumed to occur by a neucleophilic attack at the 2 position of the pyridinium moiety (Scheme 3).

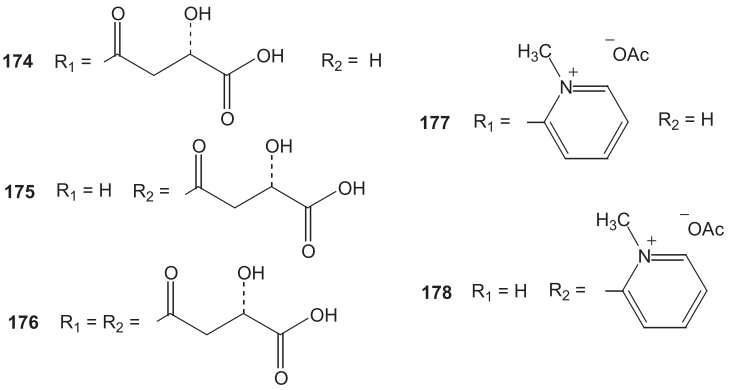
The cytotoxicity of **177** was tested against a series of cell lines that included leukemia, ovarian, lung, breast carcinoma, and melanoma cells, and was found to be very similar to that of **153** itself. Furthermore, **177** was found to inhibit tumor

growth in nude mice with human prostate carcinoma xenografts to the same extent of equal molar concentrations of **153**. Also, **177** showed no toxicity at the maximum tolerated dose of **153**, where **153** showed significant toxicity; four of eight animals died before the end of the study (Nicolaou *et al.*, 1994).

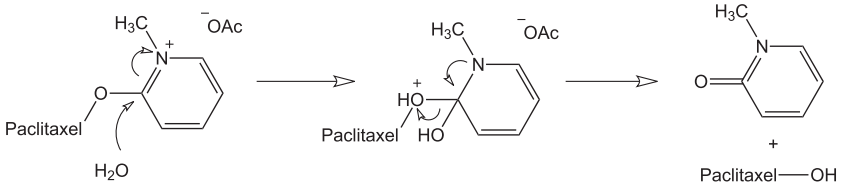
The 7-(N-methylpyridinium acetate) salt of paclitaxel (**178**) was also prepared and evaluated (Wrasidlo *et al.* (2002). **178** has a much longer half-life than **177** in human plasma at 37°C (180 min compared to 2.4 min for **177**), and did not show antitumor effects *in vivo*.

Deutsch *et al.* (1989) synthesized and evaluated some 2'- and 7-succinate, glutarate and amine derivatives of paclitaxel as potential prodrugs (**179–183**). The aqueous solubilities of the sodium salts of **179** and **180** were about 0.1% and about 0.3% for the disuccinate **181**. The alternative triethanol ammonium salts of **179** and **180** showed solubilities of about 1%. The mono substituted 2'-succinate (**179**) showed the best antitumor activity. The 2'-monoglutarate (**182**) showed improved properties compared to the succinates, and the amino amide hydrochloride salt derivative (**183**) exhibited aqueous solubility of about 1% and was extremely potent and active.

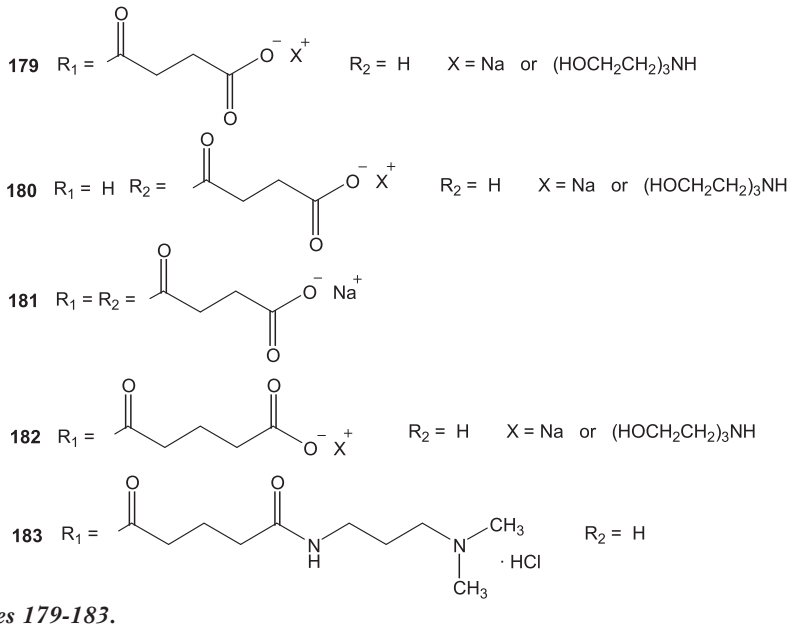
Another example is found in the double prodrug strategy of coupling an adenine-containing triphosphono-gamma-(Z)-ethylidene-2,3-dimethoxbutenolide (**184**) to paclitaxel. The promoiety itself shows notable anticancer activity and possesses high water solubility. The paclitaxel esters of this adenine-containing triphosphate, **185** and **186**, show aqueous solubilities of 26 and 29 mM (22 and 25 mg/mL **153** equivalent), respectively. Solutions of **185** and **186** (3 mg/mL) remained clear and precipitate-free for >15 h. **186** is rapidly hydrolyzed to



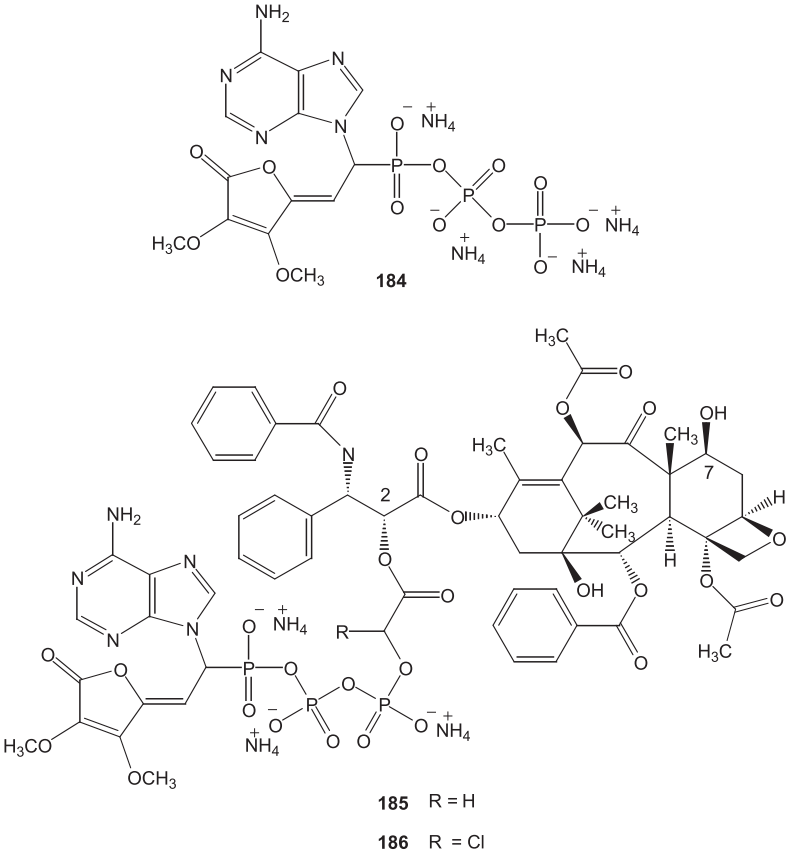
Structures 174-178.



Scheme 3.



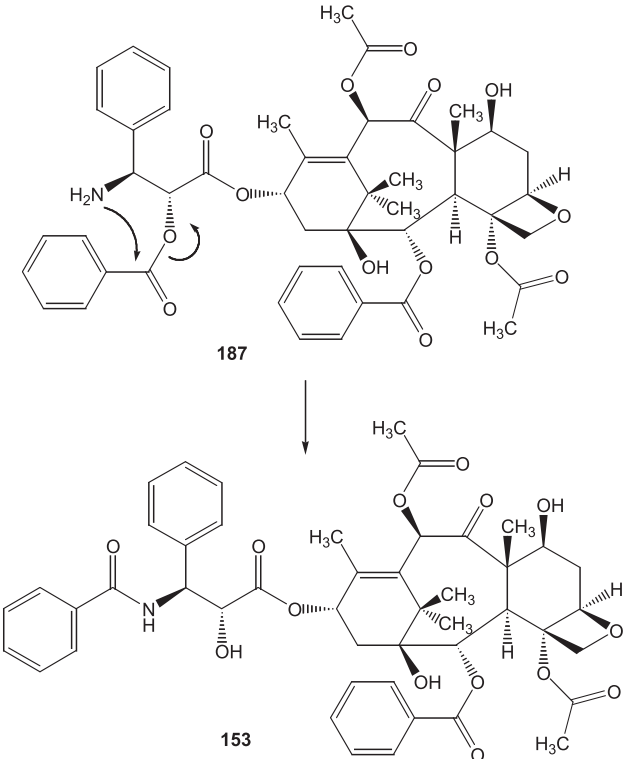
Structures 179-183.



Structures 184-186.

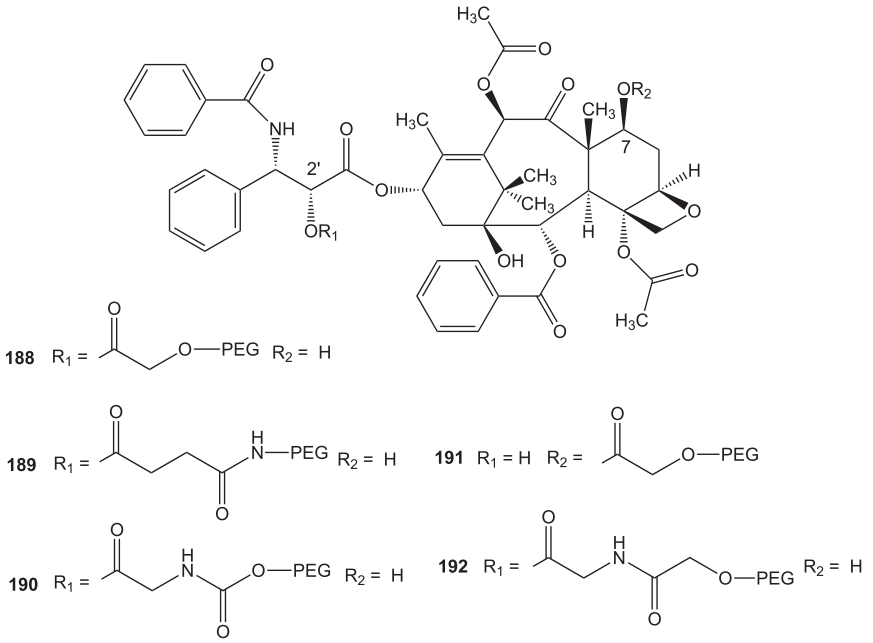
paclitaxel and the triphosphonate **184** under physiological conditions, while the conversion of **185** to **184** and **153** is much slower.

A recent somewhat unique example of a paclitaxel prodrug with increased solubility has been reported by Hayashi *et al.* (2003). This prodrug (**187**) is a structural isomer of paclitaxel, which has a free amine group. The water solubility of **187** as its HCl salt is 0.45 mg/mL and it is relatively stable in pH 4 citrate buffer at room temperature (<3% release of 153 after 3 h). Solid-state stability was maintained for one month at 4°C with no decomposition. **153** is released with a $t_{1/2}$ value of 15 min in PBS at pH 7.4 and 37°C. **153** is formed through an intramolecular pH-dependent chemical mechanism via O–N acyl migration. No promoiety or functional auxiliary is released in the conversion.



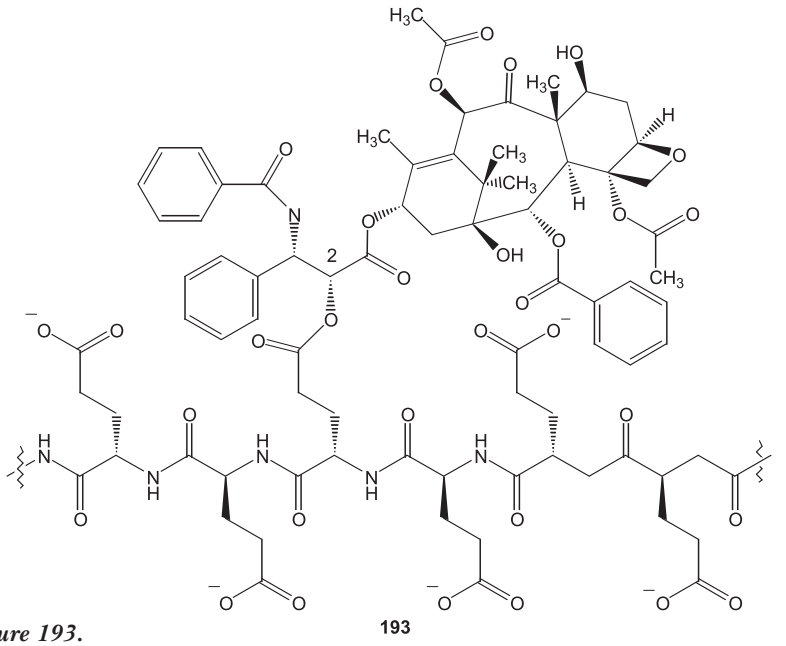
Structure 187.

There have also been many attempts to solve the solubility problem of paclitaxel by the use of high molecular weight prodrugs. 2'- and 7-polyethylene glycol (PEG) esters of paclitaxel (**188–192**) have been prepared and found to be quite water-soluble (Greenwald *et al.*, 1994). The aqueous solubility of **188** was estimated to be ≥ 666 mg/mL at room temperature, and it released paclitaxel with a $t_{1/2}$ of 5.5 h at 37° and pH 7.4. The $t_{1/2}$ of **189** was 3.0 h under the same conditions, but in the presence of rat plasma, a $t_{1/2}$ of 1.1 h was observed. The *in vitro* activity of the 2'-esters was similar to that of **153** *per se*, but the activity of the 7-ester (**191**) showed a reduced activity. PEG 5000 was used for these studies; larger MW PEG derivatives have also been examined (Greenwald *et al.*, 1996) and



Structures 188-192.

found to increase life expectancy in the P-388 leukemia mouse model. Other types of spacer groups between paclitaxel and PEG such as the paclitaxel-2'-glycinate-PEG conjugate (**192**) have been prepared and evaluated as well (Pendri *et al.*, 1998).

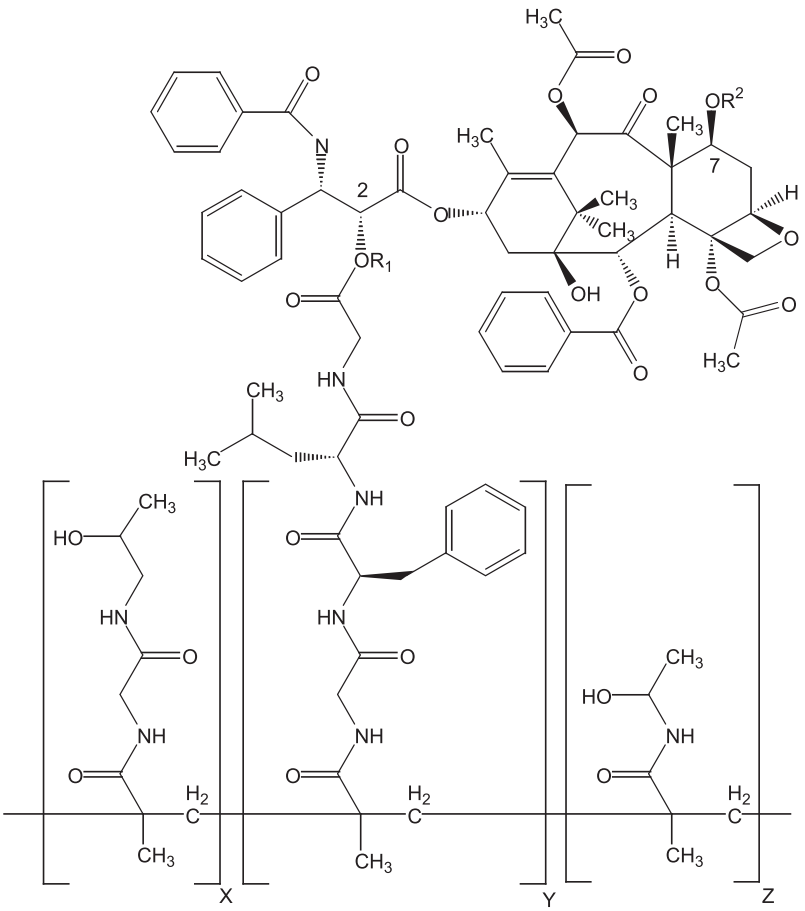


193

Structure 193.

Poly-(L-glutamic acid) (PG)-paclitaxel conjugates have been investigated for their use as prodrugs. PG is a water-soluble biodegradable polymer with carboxylic acid side chains to which paclitaxel can be attached. CT-2103, Xytotax® (**193**) is a PG-paclitaxel conjugate (PG-TAX) weighing approximately 80 kD that binds approximately one molecule of paclitaxel per 10.4 glutamic acid monomers (Singer *et al.*, 2003). Compared to free **153**, **193** has an increased tumor uptake and retention of **153**, and shows greater efficacy than **153** in a number of human cancer models (Li *et al.*, 1998; Auzenne *et al.*, 2002; Singer *et al.*, 2003). **193** releases very little **153** into systemic circulation. **193** appears to be taken up by tumor tissue and releases **153** following cleavage of the PG backbone by cathepsin B (Singer *et al.*, 2003).

CT-2103 (**193**) is in Phase II clinical trials as a single use agent in patients with relapsed ovarian cancer, relapsed colon cancer, and primary non-small-cell lung cancer and in combination with platinates in ovarian and lung cancer (Singer *et al.*, 2003). **193** appears to be well tolerated by short-term infusion with reduced toxicity compared to **153**, and shows activity, even in patients with failed prior taxane therapy. The reduced toxicity and potential enhancement of efficacy are



194

Structure 194.

thought to be due to reduced exposure to normal tissue in systemic circulation and enhanced permeation and retention (EPR) in tumor tissue.

There are other examples of macromolecular prodrugs of paclitaxel. Carboxymethyldextran paclitaxel prodrugs with amino acid linkers are reported by Sugahara *et al.* (2002). PNU166945 (**194**), a polymer-conjugated water-soluble prodrug of paclitaxel, reached Phase I clinical trials (Terwogt *et al.*, 2001). **194** consists of a hydroxypropyl-methacrylamide polymer linked through an amino acid chain to the 2'-position of paclitaxel. 194 showed high antitumor activity and low toxicity in preclinical studies (Pesenti *et al.*, 1995). **194** was well tolerated in humans, with no dose limiting toxicities up to 196 mg/m^2. **194** produced active levels of **153** with linear pharmacokinetics for both **194** and **153**. Unfortunately, the study was ended prematurely because of severe neurotoxicity observed in additional rat studies (Terwogt *et al.*, 2001).

Paclitaxel has had many known prodrug strategies and several new imaginative prodrug strategies applied to it in an attempt to create a safe injectable form of the drug. There are dozens of patents filed every year claiming new paclitaxel prodrug entities with improved delivery properties but, at this time, a successful prodrug has yet to be commercialized.

Conclusions

Prodrug technology offers reasonable strategies to improve the parenteral delivery properties of poorly soluble drugs. Different prodrug strategies offer their own advantages and disadvantages. In the early days, sodium hemi-succinate prodrugs were the most common choice for poorly soluble alcohol functional group-containing drugs, but they often suffer from poor aqueous stability and slow and incomplete *in vivo* conversion. The phosphate prodrugs are usually much more stable, and appear to bioconvert rapidly, but there are some applications in which they are unable to be synthesized or do not bioconvert due to steric or other factors. POM prodrugs have demonstrated good stability and rapid *in vivo* conversion, but there are some concerns about the pruritis/paresthesia noted in the clinic for these as well as other phosphate-based prodrugs.

Many of the prodrugs discussed here have little or no toxicity data reported about non-naturally occurring side products formed during bioconversion. This is an issue with any new prodrug strategy—that is, the need to assess the safety of the prodrug and the released promoiety components.

There are some problems that are common to almost all parenteral prodrugs. The precipitation of poorly soluble drugs generated from a concentrated prodrug solution on storage often is the shelf-life limiting factor. We have seen some examples of how such problems have been addressed. For example, some prodrugs have been found to increase the solubility of their respective parent drugs through the formation of self-associated complexes or micelles, and cyclodextrins can be used to solubilize small amounts of insoluble drug formed during storage.

In all, the advantages of prodrug strategies for parenteral applications often outweigh the disadvantages. Use of these prodrug strategies can increase the solubility of poorly water-soluble drugs to levels sufficient to create safe effective parenteral formulations that otherwise would not be possible. Many of these prodrug techniques described here can be applied to future drug entities to achieve delivery characteristics that are suitable for parenteral applications.

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2.3.1

Poly (ethylene glycol) Prodrugs: Altered Pharmacokinetics and Pharmacodynamics

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Table of Contents

List of Abbreviations

6-MP.....6-mercaptopurine
AA.....Amino acid
AE.....Adverse events
ala.....Alanine
ALL.....Acute lymphoblastic leukemia
AML.....Acute mylogenous leukemia
AmB.....Amphotericin
Ara-C.....Cytosine arabinose, 1-(β-D-arabinfuranosyl) cytosine
AUC.....Area under the curve

gly .....Glycine
L.....Leucine
BE .....1, 6-Benzyl elimination
Bicine (Bicin) .....bis-Hydroxyethyl glycine
C_{max} .....The peak or maximum concentration
CDK.....Cyclin-dependent kinase
CL .....Clearance
CML.....Chronic mylogenous leukemia
CPT.....Camptothecin
DNR .....Daunorubicin
DOX .....Doxorubicin
EPR .....Enhanced permeability and retention effect
GE junction.....Gastro-Esophageal junction
FITC.....Fluorescein isothiocyanate
GFP .....Green fluorescent protein
ILS.....Increased life span
i.p. .....Intraperitoneal
i.v. .....Intravenous
mAb .....Monoclonal antibody
MTD .....Maximum tolerated dose
MTX.....Methotrexate
MW .....Molecular weight
ODN .....Oligonucleotide
PD .....Pharmacodynamic(s)
PEG .....Poly (ethylene glycol)
rPEG .....Releasable poly(ethylene glycol)
PK.....Pharmacokinetics
SD.....Stable disease
T/C .....Mean survival time of the treated group/mean survival time of the control group
TML .....Trimethyl lock lactonization
U-PEG .....Branched PEG
Vss.....Volume of distribution at a steady state

Introduction

Pharmacological activity and therapeutic significance are often correlated to the plasma concentration versus profile of the drug. Polymeric prodrugs, as a class, generally achieve areas under the curve (AUCs) of great magnitude, as they slowly release native drug over longer periods of time (Sinko and Kohn, 1993). It has been demonstrated conclusively by Greenwald *et al.* (1996a,b) that substantial increments to the AUC can be achieved by polyethylene glycol (PEG) conjugation to small molecule drugs if the molecular weight (MW) of the PEG exceeds 20,000 Da. This effect is essentially one of increasing the apparent AUC for the drug itself. It is important that the polymeric species demonstrates good blood and tissue compatibility; for this purpose, a neutral or slightly negative electric charge appears to be optimal since polycationic polymers are readily captured by the first-pass effect, and are known to possess various degrees of toxicity (Maeda, 1992; Nishikawa *et al.*, 1996). The focus of this review will be on the design, applications, and current investigations of polymeric prodrugs with the main emphasis on PEG. The exploitation of PEG prodrugs has been especially effective with anticancer agents, but other drugs in the areas of antibacterials and antifungals have also been successfully modified with PEG to produce prodrugs that demonstrate the desired increased AUCs. It would appear that other types of drugs such as antiviral agents can be similarly modified when a continuous controlled release of drug is desired, but only one report using low molecular weight (LMW) PEG has appeared in the literature (Zacchigna *et al.*, 2002).

Prodrug design comprises an area of drug research that is concerned with the optimization of drug delivery. A prodrug is a biologically inactive derivative of a parent drug molecule that usually requires a chemical or enzymatic transformation within the body to release the active drug, and has improved delivery properties over the parent molecule (Stella *et al.*, 1985; Bundgaard, 1989, 1991; Sinhababu and Thakker, 1996). In the case of polymer prodrugs, it is clear that the solubility of the conjugate will almost always exceed that of the original drug, usually overcoming any existing aqueous insolubility and, thus, increasing the possibility of more effective drug delivery. If the conjugate persists in the body for a period of time, either in the compartment of administration or moving from one compartment to another, it can potentially release drug as long as it stays in the body. Of course, the rate of drug release will be dependent on the nature of the polymer-drug linkage. Linkages could theoretically be chosen so that either pH or enzymatic degradation mediates prolonged drug release. The increased circulating half-life resulting from polymer conjugation can also benefit water-soluble drugs where rapid renal elimination, due to low molecular weight (LMW), is often problematic. The host of various polymers that have been employed in prodrug strategies underscores the fervent effort chemists have used in attempting to exploit the unique virtues of the conjugated species. Styrene-maleic anhydride neocarzinostatin (SMANCS) copolymer (Maeda, 1991), hydroxypropyl methacrylamide (HPMA) copolymer (Putnam and Kopecek, 1995), dextran (Bernstein *et al.*, 1978; Danhauser-Ried *et al.*, 1993), polyglutamic acid (Jultani *et al.*, 1997; Bhatt *et al.*, 2003), and polyaspartic acid (Zunino *et al.*, 1982) are but a

few of the other polymeric systems that have been employed to accomplish delivery in analogous ways. PEG conjugation offers the unique advantage of being a telechelic or semitelechelic polymer (activated at one or both the termini). Thus, loading is quite predictable with proteins as well as organic species. From this diverse group of polymers, only one small molecule conjugate employing the SMANCS approach has so far been approved to treat hepatic cancer in Japan (Maeda, 1991). However, this achievement has generated a great deal of enthusiasm and commitment by many groups to succeed with other polymeric prodrug strategies. This is underscored by the recent positive reports encountered in clinical cancer trials of PEG-camptothecin (Rowinsky *et al.*, 2003; Scott *et al.*, 2004) and poly (L-glutamate paclitaxel) (Singer *et al.*, 2003). For the sake of brevity this review will focus mainly on PEG as the polymeric carrier. All of the examples provided in this work underscore the fact that molecular weight (MW) ultimately controls PK. It should also be pointed out that development of permanently bonded PEG-protein drugs has benefited the high molecular weight (HMW) protein by attachment of LMW PEG (2–5 kDa) because multiple attachment sites further increase the effective MW, and elimination of the PEGylated protein is virtually halted, leading to a longer plasma $t_{1/2}$ and enhanced pharmacokinetics (PK).

Circulatory Retention: Molecular Weight Considerations of the Polymer

Yamaoka *et al.* (1994) conducted a detailed study that measured the distribution and tissue uptake of PEG of different molecular weights after i.v. administration to mice. Yamaoka reported that the renal clearance of PEG decreased with an increase in molecular weight, with the most dramatic change occurring at 30,000 Da. The $t_{1/2}$ of PEG circulating in blood also showed a concomitant and dramatic increase. For example, the $t_{1/2}$ for PEG went from approximately 18 min to 16.5 h as the molecular weight increased from 6,000 Da to 50,000 Da. In fact, PEG molecules of 10^4 Da or greater MW demonstrate a significantly higher accumulation in tumors than within normal tissues, irrespective of the tumor site over time (Murakami *et al.*, 1997). Recent studies by DeNardo *et al.* (2003) of PEGylated peptides in lymphoma-bearing mice using different MW PEG derivatives demonstrated that, as molecular size increased, blood and body clearances decreased. The effect of molecular size on blood clearance was not altered by ligand binding specificity; $t_{1/2}$ ranged from 5.4 h (40,000 Da) to 17.7 h (150,000 Da). However, ligand specificity was found to alter body clearance.

It has long been recognized that for comb (branched) polymer drug conjugates, the biodistribution of the polymer, based on MW considerations, will determine the fate of the conjugate as well. Seymour *et al.* (1987) detailed the effect of molecular weight of the comb copolymer HPMA on body distribution and rate of excretion and identified a molecular weight threshold-limiting glomerular

filtration at 45,000 Da; below this limit the $t_{1/2}$ of the polymer was quite short, e.g., $t_{1/2}$ for a 12,000 MW copolymer was reported to be only 3 min.

Low MW prodrugs are generally designed to be cleaved efficiently and rapidly ($t_{1/2}$ < 20 min) in blood by enzymatically mediated processes, resulting in an accelerated rate of conversion of the inert form to the biologically active parent (Stella *et al.*, 1985). Thus, the PK of the parent drug is only minimally affected by prodrug modification. However, prodrug efficacy can be addressed by extending the circulating lifetime of a polymeric water-soluble prodrug through modification. By increasing the circulating life of the prodrug in plasma relative to its rate of hydrolysis, equivalent or greater potency should result with a gradual controlled release of the drug as long as therapeutic levels can be reached and maintained without causing toxicity. One way to accomplish this objective is to prevent rapid renal excretion of the hydrophilic form of the drug by increasing the molecular weight of the solubilizing agent, as was demonstrated for HPMA-doxorubicin (Seymour *et al.*, 1987), PEG-paclitaxel (Greenwald *et al.*,1996a), and PEG-CPT (Greenwald *et al.*,1996b).

The shape of the polymer (linear, starburst, or various branched types) appears to be of lesser importance than the MW of the prodrug in achieving an optimal PK. As will be seen from subsequent examples, PEGs of MW between 35,000 Da and 40,000 Da seem optimal for most applications. Longer residence times can be achieved with polymers of higher MW, but their use is probably restricted to biodegradable polymers with a prescribed time frame to prevent toxic accumulations of the drug conjugate. In most cases, employing polymer of MW < 20,000 Da with a LMW drug produces a rapidly excreted species that can be erroneously interpreted as inactive.

On the other hand, development of PEG-protein permanently bonded conjugates has benefited protein drugs since multiple attachments (usually through the ε-amino of lysine or the α-amino group) generally of MW 5,000–40,000 Da increase the effective MW of the protein, virtually halt renal excretion, and produce a conjugate that is not recognized by the immune system. This leads to a long circulating half-life and enhanced PK of the non-immunogenic PEGylated macromolecule.

The Prolinker

Regardless of the circulating half-life ($t_{1/2}$) of the polymeric conjugate, the rate at which the connecting bond that holds the drug to the polymer is broken becomes equal in importance to the MW of the polymer. Too rapid a breakdown of the prodrug can lead to spiking of the parent drug and possible toxicity, while too slow a release rate will compromise the efficacy of the drug. Thus, the stability of the drug conjugate linkage and its potential for controlled degradation is an important consideration in determining the effectiveness of the prodrug. A general rule is that if the conjugate is designed as a circulatory depot, the drug must be liberated according to a prescribed regime without immediate total dissociation following administration. Similarly, if the conjugate is meant to reach

a particular extracellular or intracellular target, the linkage must be sufficiently stable to maintain its chemical integrity until the destination is reached. At the target site, the active principal should be released by a specific, enzymatic mechanism. Permanently bonded drug-polymer conjugates are generally ineffective for drug release (Greenwald *et al.*, 1995, 2000b). Recently, however, Riebeseel *et al.* (2002) have shown that permanent methotrexate (MTX)-PEG conjugates inhibited dihydrofoate reductase *in vitro* without release of free MTX. Interestingly, *in vivo* testing of the 40,000 Da conjugate produced greater activity than LMW conjugates or native MTX. However, at the present time, a particular prolinker cannot be reliably predicted and must be determined empirically for each particular conjugate.

Ester Bipartate Prodrugs

The prodrugs most often employed generally are based on hydrolyzable or enzymatically cleaveable bonds such as esters, carbonates, aryl carbamates, and hydrazones. Cleavage of the particular bond employed frees the drug and the inert cohort of the combination; this type of prodrug is referred to as bipartate, or consisting of two parts. Of the bipartate prodrugs, esters are the most ubiquitous in the literature, no doubt since they are often the easiest to synthesize. Prodrugs of this sort can be designed from either an alcohol with an acid parent drug, or an alcohol parent drug with an acid. The rate of breakdown of the ester is generally more easily controlled for an alcohol drug by modification of the associated acid structure (Christenson *et al.*, 1964; Bender *et al.*, 1965).

Esters with PEG as an electron-withdrawing substituent (alkoxy) in the α-position proved to be especially effective linking groups in the design of prodrugs since they aid in the rapid hydrolysis of the ester carbonyl bond. Esterification of the anticancer, tubulin-inhibiting drug paclitaxel (containing a 2° alcohol that is required for activity) with a PEG 40,000 Da acid yielded a highly water-soluble 2'-PEG ester of paclitaxel, **1** (Greenwald *et al.*, 1996a). The polymer conjugate that was shown to function as a prodrug, *i.e.*, breakdown occurred in a predictable fashion *in vitro*; the half-life ($t_{1/2}$) in PBS buffer at pH 7.4 was 5.5 h, while in rat plasma a more rapid breakdown was observed, with a $t_{1/2}$ of about 1 h. Cell tissue culture employing P338/0 and L1210 murine leukemia cells with **1** (Table 1) gave IC_{50} values that were comparable to those of Taxol®. It was established that acute toxicity resulted from high doses of **1** (PEG 40,000); the efficacy of the HMW PEG prodrug was determined *in vivo* using a P388/0 mouse leukemic model and was found to be essentially equivalent to that of a Taxol® (cremophor EL®, ethanol, and paclitaxel) formulation (Table 1).

At a dose of 5.25 μmol, Taxol® was profoundly toxic. It was, therefore, not surprising that no acute toxicity was exhibited in mice when treated *i.p.* with **2**, since this prodrug has a MW of 5,000 Da and presumably is rapidly eliminated by the kidneys. Activity equivalent to that of Taxol® was observed *in vitro* where no such elimination is possible. A lack of *in vivo* activity was also observed for **2** when tested *i.p.* in a P388/0 murine leukemia model (Table 1). This example clearly illustrates the necessity for *in vivo* testing to verify *in vitro* cytotoxicity results.

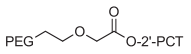
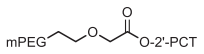
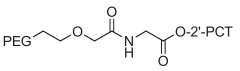
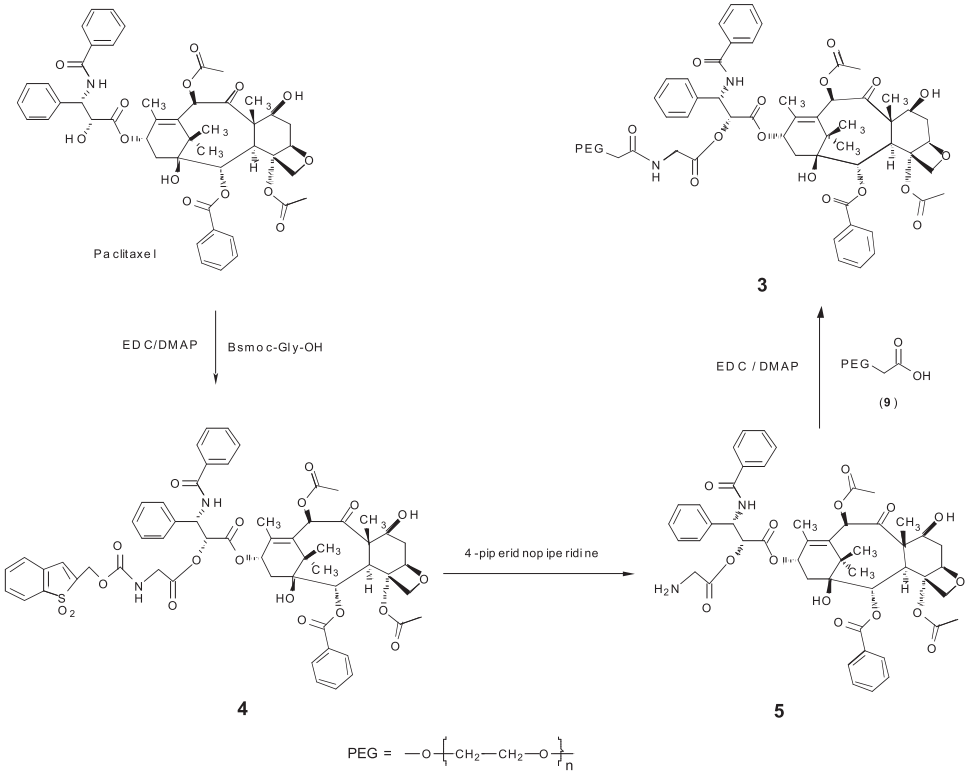
Compound	#	IC_{50} (nM) P388/O	$t_{1/2}$ (h)[b] PBS pH 7.4	$t_{1/2}$ (h)[b] Rat Plasma	P388 *in vivo*[c] Total Dose (mg/kg)	Mean time to Death (days)[d] [cures/group]	% ILS[e]	P values *vs.* Control	P values *vs.* Paclitaxel
Control		—	—	—	—	13.2 ± 1.2 [0/10]	—	—	—
Paclitaxel		6	—	—	75	17.5 ± 1.7 [0/10]	33	P = .0151	—
					100	13.7 ± 1.3 [0/10]	4	P = .7714	—
 PEG MW 40,000	**1**	10	5.5	0.4	75	19.0 ± 1.1 [0/10]	44	P = .0013	P = .3850[f]
 mPEG MW 5,000	**2**	15	5.5	0.5	75	14.1 ± 2.3 [0/10]	13	P < .06	P < .0001
 PEG MW 40,000	**3**	14	7.0	0.4	75	21.8 ± 1.0 [0/10]	65	P < .0001	P = .0151[f]
					100	24.0 ± 8.9 [1/10]	82	P < .0001	P < .0001[g]

Table 1. *In Vitro*[a] and *In Vivo* Results of PEGsPaclitaxel Derivatives

[a]All experiments were done in duplicate: Standard deviation of measurements = ±10%. [b]These results more appropriately, represent the half lives of disappearance of the transport form. [c]*In vivo* efficacy study of the water soluble paclitaxel derivatives using the P388/0 murine leukemia model. Paclitaxel or prodrug derivatives were given, in equivalent dose of paclitaxel, daily [intraperitoneal (i.p.) × 5], 24 h following an injection of P388/0 cells into the abdominal cavity with survival monitored for 40 days. [d]Kaplan-Meier estimates with survivors censored. [e]Increased life span (%ILS) is (T/C–1) × 100. [f]Paclitaxel at 75 mg/kg. [g]Paclitaxel at 100 mg/kg.

Ester Spacers

High molecular weight PEG paclitaxel prodrug strategies were next extended to prodrugs that utilized heterobifunctional spacer groups. Of the various spacers tried, amino acids appeared to be the most useful, reducing toxicity while increasing the efficacy of the paclitaxel conjugate (Pendri *et al.*, 1998). No explanation for this result was given. A synthetic variation of PEG chemistry was applied to paclitaxel in order to avoid the preparation of unstable salts of 2'-amino esters of paclitaxel (Deutsch *et al.*, 1989; Zhao *et al.*, 1991; Mathew *et al.*, 1992). By first preparing the HMW PEG-conjugated amino acid, PEG glycine, condensation with the 2'-OH of paclitaxel resulted directly in a relatively stable PEG amide derivative of paclitaxel-2'-glycinate (**3**) (Pendri *et al.*, 1998). However, a recent method (Greenwald *et al.*, 2003d) to produce the parent small molecule, gly-paclitaxel (**5**) as a pure and relatively stable compound was accomplished by utilizing the Bsmoc protecting group (Carpino *et al.*, 1999) (Scheme 1).



Scheme 1. PEG-gly-paclitaxel

Condensation of **5** with PEG acids gave the desired polymeric prodrug in high yield (Scheme 1). Both prodrugs, with and without a glycine spacer, had a solubility of ~125 mg/mL or 5 mg paclitaxel equivalent per mL. The relative *in vivo* equivalency of paclitaxel and the conjugated forms was assessed by monitoring survival in a P388/0 murine leukemia model (Table 1). The mean time to death for animals treated with unconjugated paclitaxel at a total dose of 75 mg/kg was 17.5 days, resulting in an increased life span (ILS) of 33% with no cures. Similarly, the mean time to death for animals treated with an equivalent dose of PEG-paclitaxel (**1**) was 19 days (ILS = 44%). In contrast, PEG-gly-paclitaxel (**3**) treated animals had a mean time to death of 21.8 days (ILS = 65%), which was significantly ($P < 0.02$) longer than the paclitaxel group. At a total dose of 100 mg/kg, the paclitaxel group's ILS was reduced to 4% due to acute toxicity, while the same active dose of 40,000 PEG-gly-paclitaxel increased the ILS to 82% with one cure. Thus, this screen suggests a greater therapeutic index can be achieved with **3**. In addition, treatments with **3** against HT-29 (colon), A549 (lung) and SKOV3 (ovarian) solid tumor-bearing mice demonstrated significant antitumor activity (Table 2).

The HMW PEG-paclitaxel prodrug conjugates offer improved therapeutic efficacy that likely arises from two outstanding features. First, due to the HMW of the polymer and the relatively slower rate of cleavage of the ester bond, this

Tumor type	Treatment schedule[a]	Total dose (mg/kg)[b]	% T/C[c]
HT-29 (colon)	3 cycles of daily x 5 at intervals of 14 days, i.p.	225	11.3[d]
A549 (lung)	Daily x 5 for 2 weeks, i.p.	150	9.5[d]
SKOV3 (ovarian)	Weekly x 2 for 4 weeks, i.v.	200	8.1[d]

Table 2. Antitumor Activity of PEG-gly-paclitaxel (3) Against s.c. Human Tumor Xenografts in Nude Mice

[a]Treatments initiated when tumor volumes reached ~100 mm^3.

[b]Based on paclitaxel content.

[c]Treatment and control groups were measured when the control group's median tumor volume reached ~800–1100 mm^3.

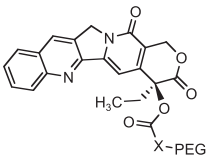
[d]$P<0.01$.

transport form (*i.e.* via HMW PEG prodrug) has an *in vitro* $t_{1/2}$ of 0.5–1 h in rat plasma, thus allowing several complete circulatory passages to be completed and resulting in a modified biodistribution. Secondly, and perhaps most significantly, the HMW polymer-drug conjugate is expected to selectively accumulate in the tumor. This accumulation, known as the enhanced permeability and retention (EPR) effect, or passive tumor targeting, has been postulated by Maeda *et al.* (1992); Nishikawa *et al.* (1996); and Noguchi *et al.* (1998) to occur for HMW polymer conjugate therapeutics. In addition, the reduced toxicity exhibited by these compounds probably results from a more controlled ester bond hydrolysis, possibly from an altered biodistribution, compared with the non-attenuated release of unconjugated paclitaxel. The two properties, passive targeting and continuous release from a depot of the polymeric prodrug, provide what has been termed double targeting (Yokayama and Okano, 1996) and are thought to produce enhanced efficacy.

Another insoluble anticancer drug, camptothecin (CPT), illustrates the general utility of the HMW PEG prodrug approach as well as the use of amino acid spacers. CPT has the unique structural elements of a lactone ring and a 3° alcohol (20-OH), both of which are requirements for activity (topoisomerase I inhibition (Wall *et al.*, 1993; Potmesil, 1994; Bedeschi *et al.*, 1997)). The biggest drawback in the use of this potent drug is that camptothecin is virtually insoluble in water. Amino ester prodrug salts connected to the 20-OH group have been developed as a means of addressing this problem (Vishnuvajjala *et al.*, 1990), but no clinical candidates have been forthcoming using this approach, possibly because of a poor PK profile. Using a PEG prodrug delivery strategy, Greenwald *et al.* (1996b) reported that CPT can be solubilized as a non-ionic α-alkoxyester conjugated to PEG carboxylic acid with a molecular weight of 40,000 Da. CPT's solubility as the 20-camptothecin PEG 40,000 ester prodrug form (**6**) was approximately 2 mg/mL in water and is dramatically greater than that of CPT (0.0025 mg/mL). PEG-CPT has been shown to hydrolyze *in vivo* and gradually release native CPT (Conover *et al.*, 1997). Fortuitously, it was found that modifying CPT at the 20 position as a PEG ester additionally stabilizes the active lactone ring (essential for activity) under physiological conditions (Greenwald *et al.*, 1996b). Stabilization by acylation of the 20-OH group of CPT has been shown to be due to a low degree

of intramolecular H-bonding (Zhao *et al.*, 2000). H-bonding appears to play an important role in the chemistry of CPT. Recently it was shown that PEG conjugation of 10-OH-CPT could be selectively done at the 10 or 20-OH position by choosing an acylating reagent that can affect the degree of intermolecular H-bonding (Greenwald *et al.*, 2003b).

However, while this simple α-alkoxy ester was efficacious (Conover *et al.*, 1997), introduction of various spacer groups between the PEG solubilizing portion



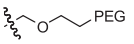
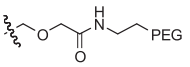
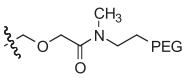
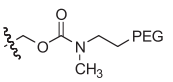
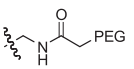
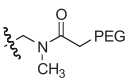
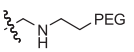
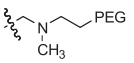
X-PEG	#	IC_{50} (nM) P388/O	$t_{½}$ (h)[b] PBS pH 7.4	$t_{½}$ (h)[b] Rat Plasma	P388 *in vivo*[c] Mean time to Death (days)[d]	P388 *in vivo*[c] %ILS[e]	P388 *in vivo*[c] Survivors on day 40
Control		—	—	—	13.0	—	0/10
Camptothecin		7	—		38.0*	192	7/10
	**6**	15	27	2	38.0*	192	9/10
	**7**	16	5.5	0.8	17.4†	34	4/10
	**8**	21	27	3	31.6*†	143	6/10
	**9**	18	28	5	23.4	80	0/10
	**10**	12	40	6	35.0*	169	8/10
	**11**	15	97	10	19.3*†	48	0/10
	**12**	24	12	3	30.6*	135	0/10
	**13**	42	102	> 24	21.4*†	65	0/10

Table 3. *In Vitro*[a] and *In Vivo* Results of PEG-Camptothecin Derivatives

[a]All experiments were done in duplicate: Standard deviation of measurements = ±10%. [b]These results more appropriately, represent the half lives of disappearance of the transport form. [c]*In vivo* efficacy study of the water soluble camptothecin derivatives using the P388/0 murine leukemia model. Camptothecin or prodrug derivatives were given in equivalent dose of camptothecin (total dose of 16 mg / kg) daily [intraperitoneal (i.p.) × 5], 24 h following an injection of P388/0 cells into the abdominal cavity with survival monitored for 40 days. [d]Kaplan-Meier estimates with survivors censored. [e]Increased life span (%ILS) is (T/C–1) × 100. *Significant ($P < 0.001$) compared to control (untreated). †Significant ($P < 0.001$) compared to camptothecin.

Compound	$t_{1/2a}$ (Distribution)	$t_{1/2b}$ (Elimination)	Mean Residence Time (AUMC / AUC)
PEG-CPT	~4 min	3.4 h	4.9 h
PEG-Gly-CPT	~5 min	5.3 h	7.5 h
PEG-Ala-CPT	~8 min	11.3 h	15.9 h

Table 4. Circulatory Retention of PEG-Camptothecin in Mice

of the molecule and the CPT alcohol again led to significant differences in biological activity, as was the case for paclitaxel (Greenwald *et al.*, 1998; Table 3). Thus, by comparing the rates of breakdown of the PEG ester prodrugs in rat plasma with *in vivo* results, some predictive rules could be winnowed out of the study. It is possible that specific amino acids may result in favorable hydrolysis of the ester bond between the amino acid and CPT via pH or esterase-mediated release (Table 4). Interestingly, two recent papers have reported on the cellular uptake of amino acid ester prodrugs by a peptide transporter, regardless of the fact that no peptide bond is present in their structure (Han *et al.*, 1998; Balimane *et al.*, 1998). Thus, entrance into the cell may be theoretically possible through this type of active transport. Another possibility to consider is that the PEG amide bond undergoes some degree of cleavage (Conover *et al.*, 1998, 1999). It is well documented that certain peptides, quite stable in plasma, can be broken down in the lysosomal compartment by peptidases or cathepsins (Kopecek and Duncan, 1987). In contrast, other enzymes may initially encourage amide bond breakage between PEG and the amino acid by exo-peptidases or proteinases in tumor tissue resulting in an amino acid-CPT ester conjugate that would still possess enhanced efficacy by maintaining the desired lactone form by stabilization through acylation. The ester bond would subsequently be cleaved to release CPT. Early work even demonstrated the lability of PEG-amide conjugates in the presence of chymotrypsin (Ulbrich *et al.*, 1986). Further delineation of such a potential tripartate system would be a worthwhile undertaking. Cross-species differences affecting rates of dissociation of the prodrug also need to be considered when examining the usefulness of any conjugate for application to human therapy.

Interestingly, PEG 40,000 Da ester derivatives of the open lactone form demonstrate efficacy similar to that of the PEG-ala-CPT. This finding strongly suggests that once PEG ester cleavage occurs, the hydroxy acid formed rapidly equilibrates with the more biologically active lactone form to restore activity (Greenwald *et al.*, 2003e).

PEG Ester Prodrugs of Antiviral Agents

Acyclovir and valaciclovir which have limited water solubility were coupled with 1.5 kDa PEG-ala-acid to give an ester prodrug which was water soluble and stable in PBS buffer at pH 5.5. The native drugs were released when treated with human plasma. Because of the LMW of the conjugate *in vivo* testing was not done, but *in vitro* results in human plasma demonstrated a $t_{1/2}$ of about 2 h (Zacchigna *et al.*, 2002).

Enhanced Hydrolysis of Bonds Using Spacers that Provide Anchimeric Assistance

Anchimeric assistance (see Table 3, Greenwald *et al.*, 1998) emerges as a primary mechanism in the hydrolytic process for those structures where NH, NHC=O, and O-NHC=O functionalities are present, and a 3-, 5-, or 6-membered cyclic transition state can be formed with the terminal ester (Bernhard *et al.*, 1962; Fife and DeMark, 1976; Gogate *et al.*, 1987; Saari *et al.*, 1990; Tadayoni *et al.*, 1993).

Even greater rate accelerations for labile functionalities has been demonstrated for CO_2H, and are especially effective for compounds derived from diglycolic anhydride, which has an α-oxygen-withdrawing group (see Greenwald *et al.*, 2003c).

Excretion and Toxicity

CPT derivatives conjugated with PEG 8,000, 20,000 or 40,000 diacid (Martinez and Greenwald, 1997) were administered as single *i.v.* injections to mice. All mice received the same amount of active CPT (25 mg/kg); however, considerable differences in levels of toxicity were demonstrated. Lethality was approximately 50%, 10% and 0% for the PEG-CPT 40,000 Da, 20,000 Da and 8,000 Da constructs, respectively, and is due to continued release of CPT (LD_{50}, 7 nm) over time (Greenwald *et al.*, 2003a). The hydrolysis rates within the PEG CPT drug series were constant; thus, the divergent levels of toxicity were probably due to differences in distribution and circulation, metabolism, and excretion. Urinary excretion studies support this theory. When equimolar amounts of PEG 40,000 Da 20,000 Da and 8,000 Da diacids were administered *i.v.* in rats, an inverse correlation between MW and excretion rate was observed (Figure 1) (Greenwald *et al.*, 2000a,b). All detectable PEG 8,000 Da diacid was excreted within the first 6 h after dosing. In contrast, it took 24 h to recover all of the PEG 20,000 Da diacid

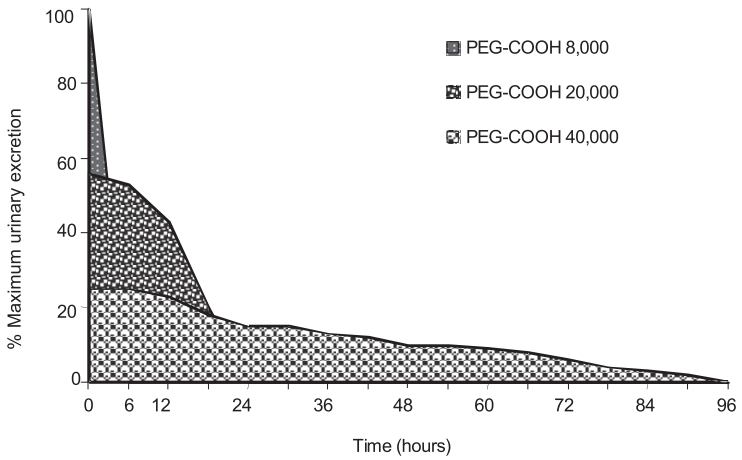


Figure 1. Urinary Elimination of PEG-COOH in Rats Following Intraveous Administration

and over 96 h to excrete the PEG 40,000 Da diacid. Thus, the MW of the conjugate can have a profound impact on its systemic circulation. PEG-organic drug conjugates of MW 5,000 or less synthesized prior to 1994 (Zalipsky *et al.*, 1983) unfortunately were never tested *in vivo*, and lack of drug activity was not detected. Polymer MW, an important feature of drug design, especially where there is only one site available for PEGylation, was not taken into consideration. Ostensibly, employing polymers of MW 5,000 Da to conjugate drugs gave rapidly excreted species that would have little or no effect *in vivo*. Application of PEG to most anticancer prodrugs mandates the use of polymers with a MW of 30,000 Da or greater in order to prevent rapid elimination of the PEGylated species and permit passive tumor accumulation (Knauf *et al.*, 1988; Maeda *et al.*, 1992; Noguchi *et al.*, 1998; Conover *et al.*, 1998).

Clinical Trial Results for PEG Prodrugs

A phase I clinical trial of **3** has been carried out and a human PK study was reported (Beeram *et al.*, 2002) in which it was determined that the MTD (maximum tolerated dose) was in excess of 5,184 mg/m^2, but most treatments were performed at 4,320 mg/m^2 (approximately 235 mg/m^2 of paclitaxel). Similarly to Taxol® neutropenia was the predominant hematological toxicity observed, while others consisted of peripheral neuropathy, vomiting, and diarrhea. Preliminary PK monitoring of free and conjugated paclitaxel demonstrated detectable levels (> 5ng/mL) of free paclitaxel still present after 48 h. A concurrent study of PGA-paclitaxel (also of approximately MW 40,000 Da) has produced similar results and toxicologies (Singer *et al.*, 2003), and it is reported to be entering Phase II trials.

Clinical results have been reported for PEG-camptothecin (Pegamotecan), a conjugate of PEG to CPT using the slightly hindered alanine spacer (Rowinsky *et al.*, 2003), which is a relatively stable spacer in buffer that is hydrolyzed in human serum with a $t_{1/2}$ of about 7 h (*in vitro*, 37 °C). Free CPT levels in plasma were measured in a Phase I study in patients with various types of solid tumors (Ochoa *et al.*, 2000). Maximal levels of free CPT were observed about 24 h after infusion, reflecting the interplay between release of CPT from the PEG conjugate, clearance of the conjugate, and clearance of the released CPT. The maximal concentration of free camptothecin was proportional to the dose of Pegamotecan, reaching about 0.5μg/mL at the MTD of 120 mg/m^2 of CPT (equivalent to 7 g/m^2 of PEG-CPT conjugate). By contrast, patients receiving similar doses of CPT in the form of sodium camptothecinate in earlier clinical trials (Maggia *et al.*, 1972) had maximal plasma concentrations of the more toxic and less active carboxylate form in the range of 30–60 μg/mL. The strikingly high maximal plasma concentration of CPT may have accounted for the poor tolerability of sodium camptothecinate in the earlier clinical trials. Human pharmacokinetic studies of Pegamotecan demonstrated significant levels of free CPT present even after 70 h. Out of the first fourteen patients treated repetitively at 7000 mg/m^2, about 120 mg/m^2 of CPT, five exhibited stable disease states, and one showed a partial response (PR). This initial anti-cancer activity in the form of partial tumor responses and prolonged

stable disease in Phase I prompted initiation of Phase II trials in several solid tumor indications. Single doses of 8,750 mg/m^2 were reached in MTD studies, but neutropenia and leukopenia were observed as major toxicities encountered; therefore, further trials were done at 7,000 mg/m^2. The phase II study was designed to evaluate the activity of Pegamotecan in advanced and metastatic adenocarcinomas of the stomach and GE junction using a multicenter, open label, single arm study with a Fleming 2 stage design (Scott *et al.*, 2004). Fifteen subjects were enrolled in the first stage; one response allowed enrollment of 20 additional subjects. Eligibility criteria included: pathologically confirmed measurable adenocarcinoma of the stomach or GE junction, prior chemotherapy regimens, and no prior treatment with a camptothecin analog. Pegamotecan was administered at 7,000 mg/m^2 every 3 weeks until toxicity occurred or disease progression was detected. The primary endpoint was the response rate. Secondary endpoints included safety, tolerability, survival, and patient-benefit parameters. The median age was 62 years (range 36–79). Among the first 15 patients, PRs were observed in 4 subjects (27%): two responses were noted after 2 cycles, one after 4 cycles, and one after 6 cycles. Two of these subjects remained in the study for 9 and 10 cycles, respectively. Stable disease (SD) was observed in 6 subjects (40%). Five of these subjects remained on study for 4 cycles and one for 6 cycles. The most common adverse events (AE) were nausea (n = 8), anemia (n = 6), vomiting (n = 5), and fatigue (n = 5). Only 1 grade 3 AE (nausea) and 1 grade 4 AE (vomiting) that occurred in the same patient were reported. Two subjects developed grade 2 cystitis, in each of whom dehydration was reported. Another subject who received 10 cycles, in whom aggressive hydration was documented, did not develop cystitis. In conclusion, Pegamotecan is a promising treatment for adenocarcinoma of the stomach and GE junction. It appears to be well tolerated, with a low incidence of grade 3 and 4 toxicities. Grade 2 cystitis, a known toxicity of camptothecin, was observed in 2 subjects and was successfully treated by aggressive hydration.

Bipartate Amides: PEG Prodrugs of the Water-Soluble Small Molecule, AraC

An interesting example of a bipartate prodrug of a water-soluble small molecule is exemplified by an ara-C amide. Ara-C [cytosine arabinose, 1-(β-D-arabinofuranosyl) cytosine] is a pyrimidine nucleoside analog employed for the treatment of acute and chronic human leukemias such as acute lymphoblastic leukemia (ALL), acute mylogenous leukemia (AML), and chronic mylogenous leukemia (CML). Its clinical utility is severely limited by the catabolic action of cytosine nucleoside deaminases, which are widely distributed in both normal and cancerous tissue and which give rise to the inactive metabolite 1-(β-D-arabinofuranosyl) uracil (ara-U). As a consequence, ara-C has a very short plasma $t_{1/2}$, which necessitates continuous infusion to provide maximum therapeutic efficacy (Hadfield and Sartorelli, 1984) but causes some undesirable side effects. To

overcome the shortcomings of ara-C, many prodrug strategies have been explored with varied degrees of success (Wipf and Li, 1994; Fadl *et al.*, 1995).

Recently, site-specific attachment of PEG on the N^4-amino group was reported employing acyl thiazolidine thiones (Choe *et al.*, 2002a). Since ara-C itself shows little or no activity against most solid tumors, passive tumor accumulation of a PEG ara-C prodrug conjugate was expected to provide the means of substantially increasing anticancer activity for this drug. Some of the disubstituted PEG linked ara-C prodrugs are shown in Table 5 with their rates of *in vitro* hydrolysis and results from *in vivo* anti-tumor test. These disubstituted prodrugs were highly soluble (~300 mg/mL in water) and were engineered to vary in their rate of *in vitro* hydrolysis (release of ara-C in plasma) from approximately 1 h to three days: no clear correlation could be observed between hydrolysis rates and *in vitro* cancer cell growth inhibition. A general trend was observed that indicated that those prodrugs, which hydrolyzed either too quickly (< 2 h) or too slowly (> 40 h) showed less anti-tumor activity in the xenograft model examined. Due to the relatively low loading capacity of these disubstituted conjugates (~ 1% ara-C w/w) only 20 mg/kg of active ara-C could safely be given per dose. In contrast, the optimal dose of ara-C is approximately 100 mg/kg/dose in this model. This situation was addressed by the development of new dendritic (branched) PEG linkers thereby allowing higher loading of ara-C on a single PEG strand (Table 6).

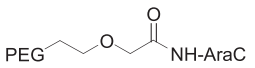
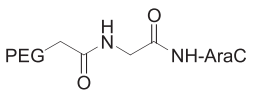
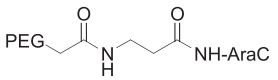
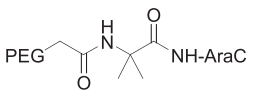
Compound	#	$t_{1/2}$ (h, Rat Plasma)[α]	IC_{50} (nM, P388/O)[α]	%TGI (LX-1)[β]
Ara-C		—	10	26.2
	**14**	3	15	50.5
	**15**	1.7	101	ND
	**16**	16	196	36.9
	**17**	92	297	10.5

Table 5. *In Vitro* and *In Vivo* Results of PEG Ara-C Derivatives

[α]All experiments were done at 37°C in duplicate and $t_{1/2}$ was measured by the disappearance of PEG derivatives. Standard deviation of measurements = ±10%. [β]Subcutaneous injections of LX-1 cells were allowed to reach an average tumor volume of 75 mm^3 prior to treatments (day 1). Ara-C (100 mg/kg/dose, optimal dose) and PEG-Ara-C derivatives (20 mg/kg/dose, volume limitation) were administered i.v. on day 1,4,7 and 10. Percent growth inhibition was calculated from the quotient of the median tumor volume of the treatment group divided by the median tumor volume of the control group {(1–T/C) × 100}. All PEG-Ara-C doses were based on their ara-C content. ND = not determined.

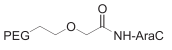
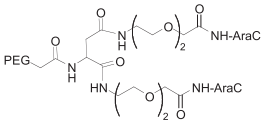
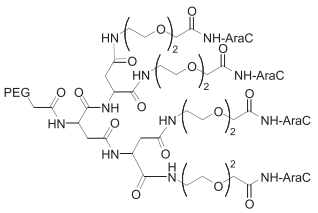
Compound	#	$t_{1/2}$ (h)[a] PBS, pH 7.4	$t_{1/2}$ (h)[a] Human Plasma	Solubility (mg/mL)[β]	Dose (mg/kg)	%TGI[χ] (LX-1) solid tumor
Ara-C					100	26.2
	**14**	32	2.9	~300	20	50.5
	**18**	32	4.4	~400	40	66.3
	**19**	30	4.1	>500	60	78.2

Table 6. Summary of In Vitro and In Vivo Results of PEG Ara-C

[a]All experiments were done at 37°C in duplicate and $t_{1/2}$ was measured by the disappearance of PEG derivatives. Standard deviation of measurements = ±10%. [β]Solubility in acidic formulated buffer. [χ]Percent tumor growth inhibition (%TGI) was calculated from the quotient of the median tumor volume of the treatment group divided by the median tumor volume of the control group [(1-T/C) × 100] when the latter reached 1000.

Bipartate Amides: Branching of the PEG Termini: Extenders

To accomplish multi-loading, branching of the telechelic, or double ended, PEG polymer is required. Branching of the PEG termini could be accomplished in myriad ways; however, it was decided that only components that could biodegrade to innocuous by-products would be utilized. Using a strategy with a scaffolding of polyaspartic or polyglutamic acids linked to a bi-functional adamantine anchor, Ranganathan and Kurur (1997) linked pre-assembled Glu or Asp dendrons to achieve consecutive generations of two-directional peptidic dendrimers consisting of tetramers, octamers, and 16-mers. This type of synthetic approach was well adapted to the substitution of bifunctional PEG as the amphiphilic anchor (Choe *et al.*, 2002b). However, complete conjugation of the dendritic PEG acid (4- or 8-mer) with ara-C was achieved only after certain spatial requirements for the dendrimer-cytarabine conjugation were recognized and addressed. Apparently, the steric bulk of several ara-Cs in proximity to each other precluded the formation of a fully loaded dendrimer compound. This problem was satisfactorily resolved by using an extended spacer prepared from a bifunctional linking moiety, which further separated the branches of the dendron and allowed more complete conjugation to take place. It can be seen from Table 6 that both the tetramer and octamer are more soluble (and less viscous) than the double-loaded derivative, a result which is probably due to the greater number of

polyhydroxy sugars in the conjugates. As was desired, the higher loading of drug provided substantial improvements in an LX-1 solid tumor model when compared to the disubstituted species. The octamer-loaded PEG ara-C conjugates also showed improved efficacy in two other *in vivo* cancer models, PANC-1 and P388/0. In summary, employing a multi-loaded branched PEG amide prodrug of ara-C substantially increased inhibition of tumor growth and there appears to be a threshold value for the amount of ara-C transported to the tumor site for optimal activity, which may be due in part to the presence of metabolic enzymes.

PEG Prodrugs of Indoles

The cyclin-dependent kinases (CDKs) are a group of enzymes that are involved in cell cycle progression regulation (Kunick *et al.*, 2000). The CDKs activate host proteins through phosphorylation on serine or threonine using adenosine triphosphate as a phosphate donor (Sielecki *et al.*, 2000). CDKs have attracted much attention as potential therapeutic targets, especially in treating cancer, because they are key players in the control of cell proliferation. Recently, a novel class of small molecule CDK/cyclin B inhibitors, the paullones, containing a four ring fused system were synthesized, and antitumor activity was demonstrated (Schultz *et al.*, 1999). Structure activity studies yielded 9-nitropaullone (alsterpaullone, **20**, Figure 2) as one of the lead compounds in the series; however due to its poor aqueous solubility, **20** could not be easily formulated, and this precluded *in vivo* testing. Since PEG prodrugs of molecular weight >20,000 can solubilize and release small molecules in a predictable fashion (Greenwald, Conover *et al.*, 2000a,b), methods were sought to find means by which PEG could be used in a similar manner with **20**, which has only indole and amide

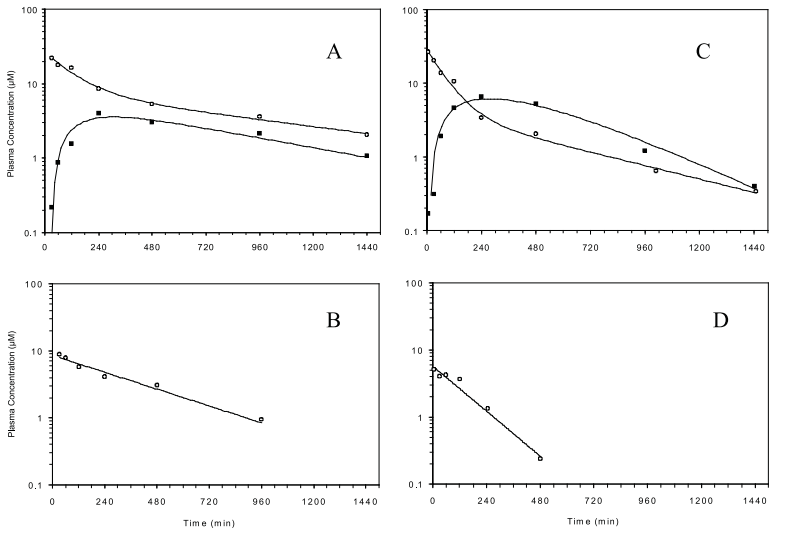
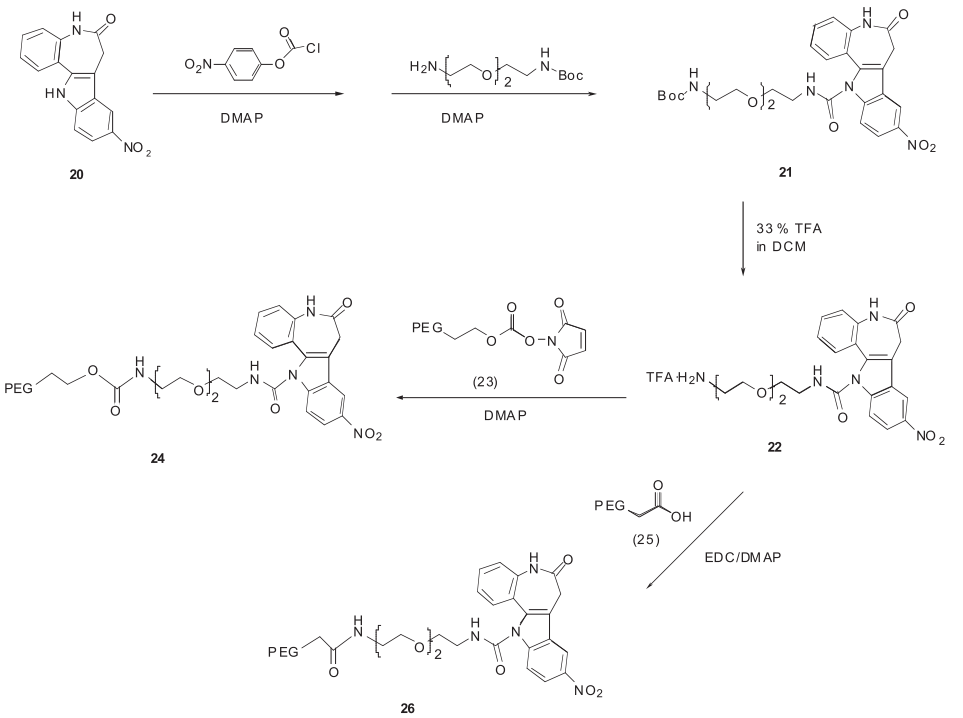


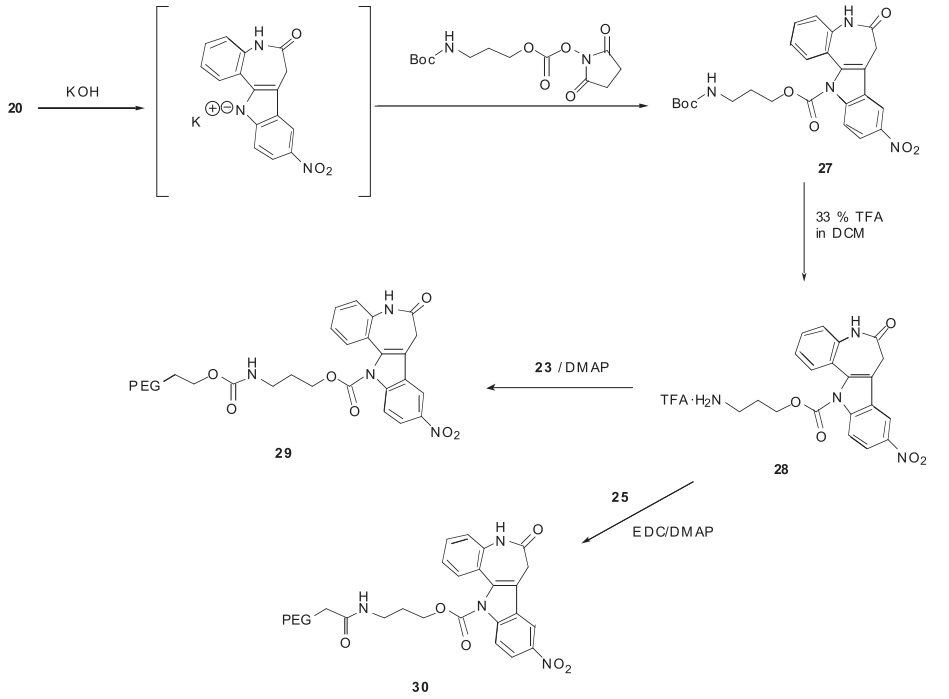
Figure 2. Geometric mean plasma concentrations of 20 and lines of best fit observed following i.v. (○) or i.p. (■) administration of PEG-conjugates to CD2F1 mice at doses equivalent to 14 mg/kg 1 (A, 24, B, 29; C, 26; D, 30).



Scheme 2.

N-H functionalities available for attachment to PEG. A successful conjugation would thus solubilize alsterpaullone and permit intravenous (*i.v.*) or interperatoneal (*i.p.*) administration. To this end, Greenwald *et al.* (2004b) developed two methods based on indole modification that yielded PEG prodrugs and produced usable levels of **20** *in vivo* as evidenced by PK studies.

Two methods were devised that were used to conjugate PEG to alsterpaullone via the N of the indole ring portion of the molecule. In the first approach (Scheme 2), activation of the indole was accomplished by reaction with p-nitrochloroformate to produce a reactive carbamate that was then condensed with a mono blocked diamine followed by deblocking and conjugation to PEG through a urea bond. The second route (Scheme 3) utilized the anion of the indole and produced a carbamate bond. Both compounds were highly water-soluble, stable in buffer, and released alsterpaullone *in vivo*. Studies were conducted in mice to investigate the influence of PEGylation on the plasma pharmacokinetics of alsterpaullone. The total plasma clearance rate was decreased up to 32-fold, and the apparent biological half-life lengthened up to 8-fold when alsterpaullone was injected *i.v.* as a PEG-conjugate when compared to injection of the unconjugated compound. The most pronounced effect on the pharmacokinetic alsterpaullone was produced by a 40 kDa PEG urea-linked conjugate. When the 40 and 20 kDa urea-linked conjugates were administered by *i.p.* injection, high relative bioavailability (46% and 99%, respectively) of alsterpaullone was observed. The PKs of alsterpaullone PEG conjugates are shown in Figure 2, and the *in vitro* measurements are shown in Table 7.



Scheme 3.

Compound	MW	$t_{1/2}$ (saline) 25 °C	$t_{1/2}$ (rat plasma) 37 °C	% Active	Solubility of conjugates (mg/mL)	Solubility of alsterpaullone (mg/mL)
24	40988	> 48 h	23 min	1.44	153	2.20
26	21012	> 48 h	1.0 h	2.80	189	5.30
29	40842	> 48 h	19.9 h	1.44	172	2.48
30	20754	> 48 h	50 h	2.88	186	5.36

Table 7. Properties of PEG Alsterpaullone Conjugates

PEG Prodrugs of Amines

PEG Tripartate Prodrugs: Benzyl Elimination (BE) system

While most amine drugs can be solubilized as acid salts, their rate of renal excretion is high. When converted to neutral small prodrug species, the ability to form salts is lost, and solubility may again become problematic. Not so in the case of PEG-drug conjugates, where PEG confers water solubility on insoluble small organic compounds without the need for forming salts. PEG prodrugs of amino-containing compounds also constitute the basis for solubilization of insoluble drugs while extending the plasma $t_{1/2}$ of the prodrug. Obviously, for PEG prodrug technology to be effective, the compound must have an adequate circulatory

retention to allow ample tumor accumulation (of either the free drug or the conjugate) and if the entire conjugate is taken up it must contain a trigger that is cleaved within either the stromal environment or the neoplastic cells. Successfully designed PEG conjugated specifiers or "triggers" (Denny and Wilson, 1998) were synthesized as part of a double prodrug strategy that relied on enzymatic separation of PEG followed by the classical and rapid 1, 4- or 1, 6-benzyl elimination (BE) reaction, releasing the amine (drug) initially bound (or latentiated) in the form of a carbamate (Figure 3, Wakselman, 1983). This release technology has been developed extensively and is generally referred to as the double prodrug approach (Bundgaard, 1989) since in essence a pro-prodrug has been made. In such systems, the hydrolytic sequence involves a first step which usually is an enzymatic cleavage, followed by a second, faster step, which is a molecular decomposition (Carl *et al.*, 1981). Further refinement of the hydrolytic decomposition can be accomplished by the introduction of steric hindrance through the use of ortho substituents on the benzyl component of the prodrug. This modification leads to a longer plasma $t_{1/2}$ of the final tripartate form. The "ortho" effect also has the beneficial effect of directing nucleophilic attack almost exclusively to the activated benzyl 6-position of the heterobifunctional intermediates. This novel PEG prodrug methodology can be accomplished in a rapid and facile manner. Demonstration of the usefulness of the PEG prodrug strategy to amino-containing anticancer compounds was first accomplished using daunorubicin.

Daunorubicin (DNR)

The efficacy of PEG-DNR conjugates prepared using the BE methodology (Greenwald *et al.*, 1999) was tested within a solid M109 tumor model; their relative activities varied according to route of administration and their rate of dissociation (Table 8), which was determined *in vitro*. When the compounds were dosed *i.p.*, the greatest activity was observed for native DNR, followed by carbamate derivatives (**35–37**). However, when the PEG prodrugs were administered by the more clinically relevant *i.v.* route, those compounds with a rat plasma dissociation $t_{1/2}$ of 2–4 h were predominantly effective in inhibiting solid tumor growth without causing toxicity and displayed a lower% T/C (*i.e.*, greater anti-tumor effect) than an equivalent dose of DNR. The reason behind this phenomenon probably lies in the biodistribution of the PEG-drug conjugates, especially with respect to their ratio of drug elimination versus tumor uptake.

Doxorubicin (DOX)

Analogous conjugates to the DNR series were prepared for doxorubicin (Enzon Pharmaceuticals, Inc. unpublished results), and tested in an MX-1 xenograft mouse model that is very responsive to DOX. Unexpectedly, PEG-conjugated derivatives produced linker profiles (Table 9) different from than that observed for DNR in the M109 mouse model. In the DOX case the aromatic

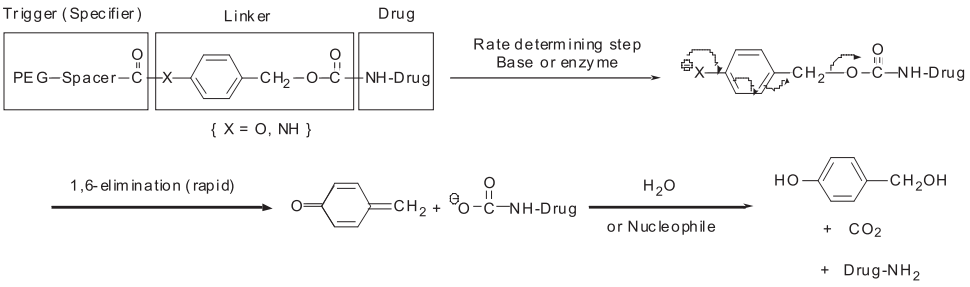


Figure 3. PEG BE prodrug

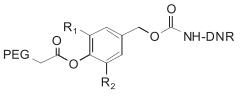
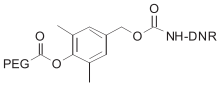
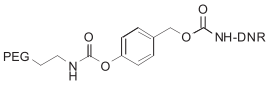
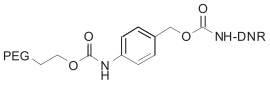
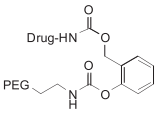
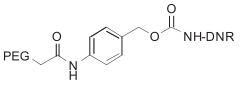
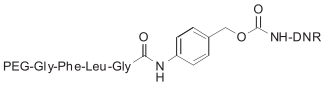
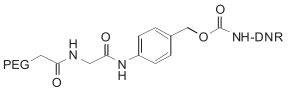
Compound		#	IC_{50} (nM)	$t_{1/2}$ (h)[a]		M109[b]	
			P388/0	PBS (pH 7.4)	Rat Plasma	i.p.%T/C	i.v.%T/C
Daunorubicin·HCl			3			44.8	117.0
Ester							
	$R_1 = R_2 = H$	**31**	8	> 24	0.4	92.8	NA[c]
	$R_1 = R_2 = OCH_3$	**32**	27	> 48	1.0	68.6	48.2
	$R_1 = R_2 = CH_3$	**33**	55	> 48	1.9	90.3	67.9
Carbonate							
		**34**	179	> 48	2.9	90.3	74.4
Carbamate							
		**35**	15	> 48	4	84.1	64.6
		**36**	415	> 8	> 24	75.3	129.0
		**37**	35	> 48	3	91.3	68.7
Amide							
		**38**	457	> 48	> 24	122.7	NA[c]
		**39**	160	> 24	13	87.6	82.6
		**40**	825	> 24	> 24	91.1	204.3

Table 8. *In Vitro* and *In Vivo* Results of PEG-BE-Daunorubicin Prodrugs.

[a]All in vitro experiments were done at 37°C in duplicate. Standard deviation of measurements = ±10%. [b]3 mg/kg/dose of active DNR administered to balb/c mice bearing subcutaneous Madison Lung Carcinoma on 1 & 4 (intraperitoneal) or 3 & 6 (intravenous) days after inoculation. Percent treatment over control (% T/C) median tumor volumes were compared when control groups median tumor volume reached ~ 2000 mm^3. [c]Result not available.

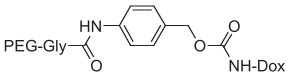
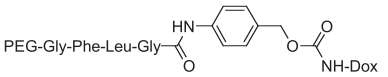
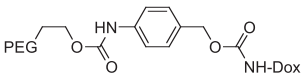
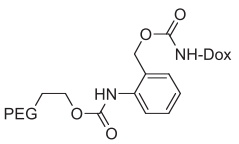
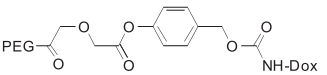
Compound	#	$t_{½}$ (h, rat plasma)	Dose[β] (mg/kg) q7d×3, iv	T/C (%)[χ] 2000 mm^3 (Day 11)
Doxorubicin		—	10	10.2
	**41**	> 24	10	31.3
	**42**	> 24	10	38.2
	**43**	NA	10	85.8
	**44**	NA	10	56.1
	**45**	0.3	10	40.0

Table 9. *In Vitro* and *In Vivo* Results of PEG-BE-Doxorubicin Prodrugs Against MX-1[α]
[α]Mean baseline (initial) tumor volume was approximately 100 mm^3 in this human mammary carcinoma (MX-1) xenograft model. [β]10 mg/kg/dose (DOX content) was given intravenously once a week for three weeks (Qd7 × 3). [χ]Percent treatment over control (%T/C) median tumor volumes were compared when control group's median tumor volume reached 1000 mm^3.

amide derivative **41**, with a $t_{1/2}$ >24 h in rat plasma (analogous to **40** for DNR which had poor activity), provided the most efficacious results in an MX-1 model (Table 9). In fact, after 5 weeks of treatment, **41** was statistically equivalent to DOX in efficacy. This result clearly demonstrates that individual compounds must be subjected to a complete evaluation using different linkers in order to determine the most efficacious combination.

Antifungals: Amphotericin

Drug delivery of insoluble agents using the BE elimination conjugation strategy with PEG was further explored with the antifungal agent amphotericin B (AmB). This fungicide has such broad-spectrum activity that it remains the gold standard agent for many life-threatening fungal infections. However, AmB is virtually insoluble in water and is commercially formulated as a lipid complex (Abelcet®). Prodrugs of AmB were prepared (Conover *et al.*, 2003) using various PEG linkers of MW 40,000. Fortunately, the amino group on the sugar ring of AmB, which is essential for its antifungal activity, provided an ideal site for PEG

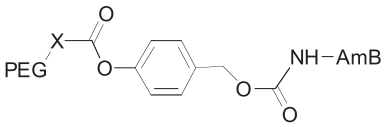
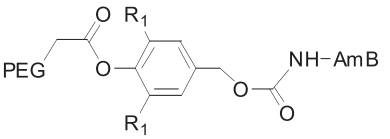
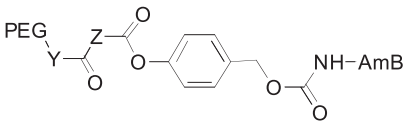
Compound	#	Solubility in saline (mg/mL)[b]	Dissociation $t_{1/2}$ (h) Rat plasma[c]	IC_{50} *S. cerevisiae* (μM)[d]
Amphaotericin B		<0.01	—	0.035
	**46**	49.9 [2.2]	3.0	5.0
X = CH_2CH_2O				
X = CH_2CH_2NH	**47**	66.3 [2.5]	1.3	2.0
	**48**	46.0 [1.4]	1.0	0.3
R_1 = H				
R_1 = CH_3	**49**	60.3 [2.3]	3.0	6.0
	**50**	56.6 [2.5]	1.5	2.0
Y = CH_2, Z = $NHCH_2$				
Y = CH_2CH_2NH, Z = CH_2CH_2NH	**51**	56.3 [2.5]	1.5	4.0

Table 10. *In Vitro* Profile[a] of PEG Conjugated Amphotericin B

[a]All experiments were conducted as a minimum in duplicate. Standard deviation of measurements = ± 10%. [b][ ] solubility of AmB in conjugate (mg/mL). [c]Rates of hydrolysis of the PEG-AmB derivatives were determined in phosphate buffered saline (PBS, pH 7.4) at 25 °C and fresh rat plasma at 37 °C. [d]IC_{50} was measured by an Alamar-blue-based cytotoxicity assay and the broth dilution method, respectively.

attachment as a promoiety. This investigation provided a series of di-substituted PEG-AmB derivatives that had *in vitro* PEG hydrolysis rates in plasma varying between 1 and 3 h (Table 10). Importantly, all PEG conjugates showed solubilities greater than 30 mg/mL in aqueous media with good stability in PBS buffer. Efficacy studies in a Candida albicans infection model showed that conjugate, **46**, when administered *i.v.*, resulted in 100% survival at its MTD and 90% and 80% survival at 1/2 MTD, respectively. Negative and positive controls showed 10% of the vehicle control mice survived compared to 70% of mice treated with 1 mg/kg of AmB. As a major finding, this investigation of AmB demonstrated that its conjugation to PEG could produce conjugates that were significantly (6x) less toxic than AmB-deoxycholate (Figure 4), while maintaining or even improving their *in vivo* antifungal effectiveness. Again, in this tripartate system, the alteration of PEG conjugate pharmacokinetics can be easily achieved by changing the PEG specifier and by adding a spacer and/or introducing steric hindrance. Thus, greater drug efficacy could possibly be accomplished.

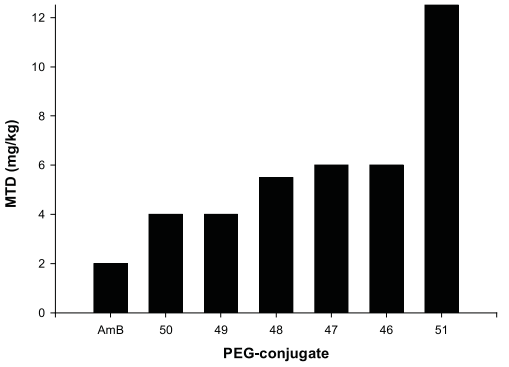


Figure 4. Acute toxicity of formulations. The maximum tolerated single dose (MTD) of selected PEG-conjugated AmB derivatives was estimated by body weight loss in female ICR mice. The MTD was determined following administration ascending i.v. doses of PEG-AmB to mice (n = 4 - 5) at 1 to 2 mg/kg increments. Body weights were measured thrice weekly for two weeks. The highest dose to cause a loss of less than 20 percent of initial weight within the time period was considered the MTD.

Antibacterials: PEG Vancomycin

Vancomycin (Figure 5) is a water-soluble glycopeptide antibiotic that is the drug of choice for the treatment of Gram-positive infections caused by methicillin resistant *Staphylococcus aureus* (Kirby, 1981). It is also used in the treatment of bacterial infections in patients allergic to β-lactam antibiotics (Ingerman, 1987). For the safe and effective use of this drug, quantization of its levels in patient's blood is often required to maintain therapeutic levels (Pryka *et al.*, 1991), and dosing is usually done by infusion every 6 hours for prolonged periods of time (about 10 weeks), depending on the severity of the infection being treated. Several groups have attempted to enhance the performance of vancomycin by continuous infusion (James *et al.*, 1996; Klepser *et al.*, 1998; Wysocki *et al.*, 2001), but it was concluded that no significant enhancement of therapeutic efficacy was realized. However, it was observed (Wysocki *et al.*, 2001) that over a 10-day interval, the cost of treatment per patient could be reduced by 30% using the continuous infusion method.

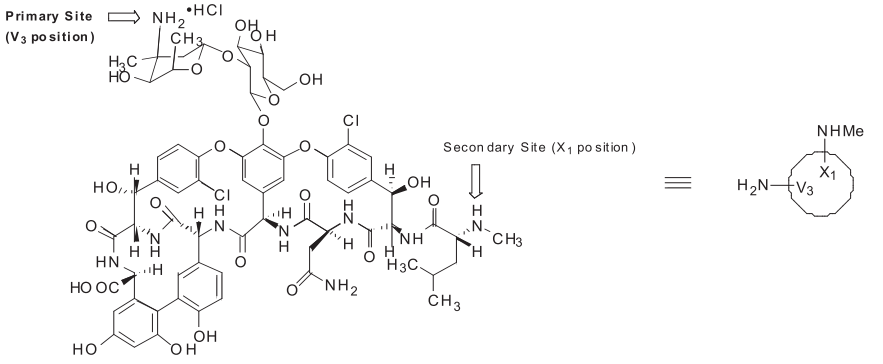
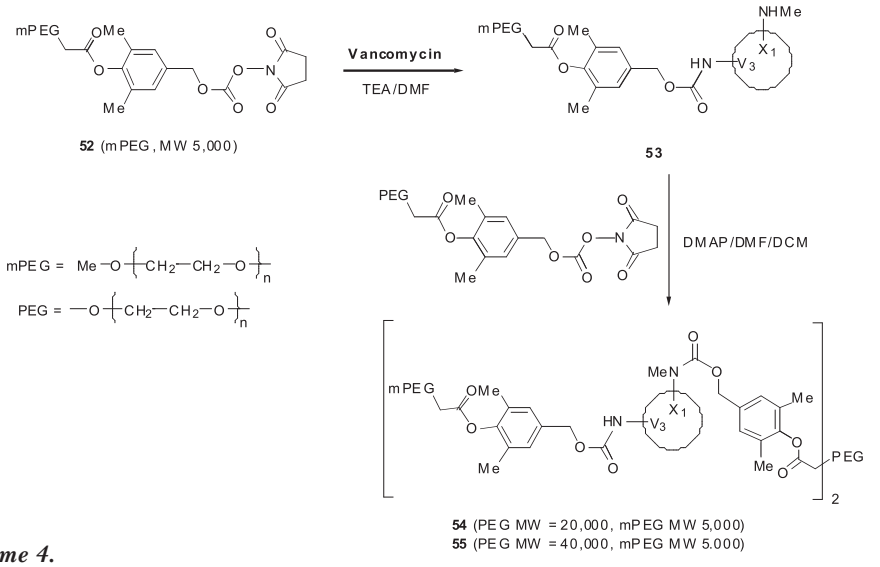


Figure 5. Vancomycin



Scheme 4.

It has now been found that the facile reaction of vancomycin with various PEG linkers, at the V_3 position, can be selectively accomplished by using an excess of base in DMF. Using rPEG (Scheme 4) as a blocking group for V_3 provides crystalline derivatives that can be further PEGylated to give pure V_3-X_1 latentiated species (Greenwald *et al.*, 2003f).

All PEG-Vancomycin transport forms show significant anti-bacterial activity that is of the same order as that of native vancomycin (Table 11). Significant increases in the AUC (Table 12) were observed for all PEG-vancomycin conjugates, thus making them potential single dose therapies per week.

After completion of the synthesis of the various latentiated di-substitued 40,000 MW PEG V_3 derivatives, the physical properties of these derivatives were determined and are listed in Table 11. Very little hydrolysis occurs for all derivatives in saline (pH 7.0), thus enabling stable formulations to be prepared prior to use. However, the rate of decomposition ($t_{1/2}$) in rat plasma for the PEG transport forms varies significantly from 1 h to >24 h; a similar trend is observed in the case of human plasma. However, as shown in Table 11, efficacy for all the PEG conjugates is virtually equipotent to that of vancomycin itself.

It can also be seen from Table 11 that 5–6% by weight of vancomycin is present in the PEG 40,000 conjugates. Substitution of PEG 20,000, which provides higher loading (8.8%, compound **54**), resulted in a very poor PK profile since its clearance was the highest observed in the study (Table 12). This may be the result of more rapid renal and endoreticular clearance, which is known to occur as the MW of PEG changes (Yamaoka *et al.*, 1994). To decrease the volume of formulated drug at a given concentration, resulting in decreased solution viscosity, as previously discussed in the case of ara-C, it was necessary to increase the loading of vancomycin onto the polymer. Thus, tetrameric V_3-PEG transport forms were prepared using the known PEG scaffolding based on aspartic acid as shown in

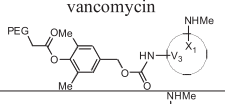
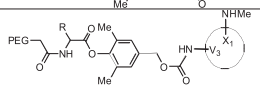
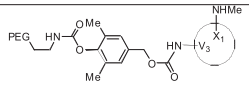
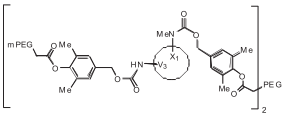
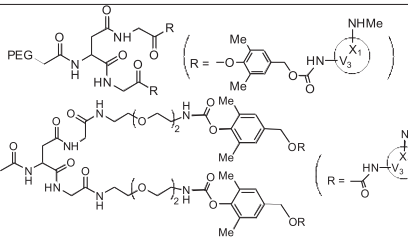
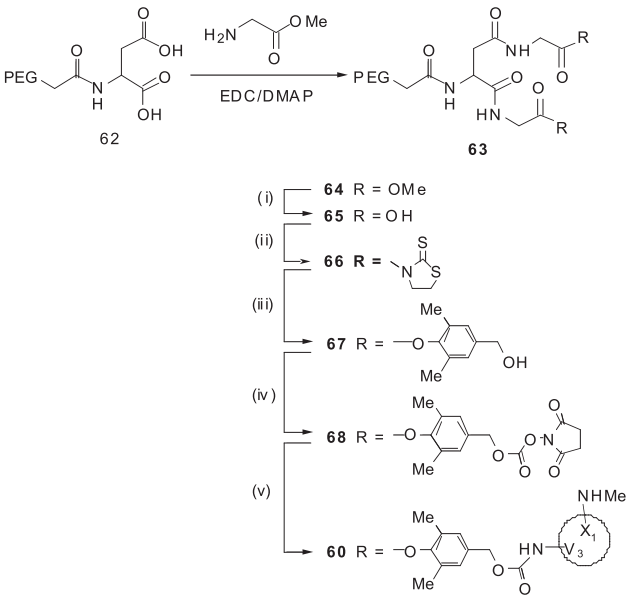
Compound	#	*MW*	% of Vanco[a]	$t_{1/2}$ (rp) (h)	$t_{1/2}$ (hp) (h)	Post[¶] Challenge %Survival
saline		—	—	—	—	10
vancomycin		1449.3	—	—	—	100
	**56**	43332	6.60	2	6.6	100
 R = H	**57**	42669	6.56	18.8	27	100
R = Me	**58**	43457	6.10	16	>24	100
	**59**	43275	5.15	187	277	90
 PEG MW = 20,000, mPEG MW 5,000	**54**	33764	8.80	4.6	32	100
PEG MW = 40,000, mPEG MW = 5,000	**55**	53764	5.39	5.0	22	80
	**60**	47011	10.9	19.9	83.5	100
	61	47483	10.4	36	198	100

Table 11. PEG-Vancomycin Compound Description and Summary of LD90-100 Results [a]% of vancomycin by weight; solubility for all compounds was measured in saline at 25 °C and was in the range of 120–180 mg/mL, and all compounds had <1% decomposes at 25 °C in saline. [¶]Compound given 1h following S. aureus (Smith) challenge, survival after 1 week.

Compound	C_{max} (µg/mL)	Plasma $t_{1/2}$ (hr)	CL (mL/hr/kg)	Vss (mL/kg)	AUC (hr·µg/mL)
vancomycin	162.0 ± 9.0	0.34 ±0.04	642.0 ± 83.7	309.3 ±17.2	78.8 ± 10.8
57	25.5 ± 2.1	10.48 ±0.58	131.1 ± 18.2	2723.3 ±97.3	386.4 ± 51.4
58	47.9 ± 4.3	4.09 ±0.25	178.3 ± 13.4	2640.8 ±23.9	282.2 ±28.5
59	54.6 ± 2.5	9.12 ±0.98	70.0 ± 5.4	1648.3 ±81.5	716.9 ± 56.8
54	53.7 ± 5.4	2.23 ±0.38	293.9 ± 26.1	938.0 ±94.3	171.0 ± 15.2
55	24.8 ± 7.7	21.19 ±6.90	70.9 ± 6.3	2128.6 ±560.1	708.4 ± 61.0
60	29.3 ± 5.1	12.37 ± 2.18	97.8 ± 7.3	3598 ± 462	513 ± 40
61	107.1 ± 16.5	14.2 ± 1.40	23.0 ± 2.1	1341 ± 52.5	2184 ± 189

Table 12. Pharmacokinetics of Intravenous Bolus Administration of 50 mg/kg Vancomycin Equivalents of PEG-vancomycin Conjugates in Rats



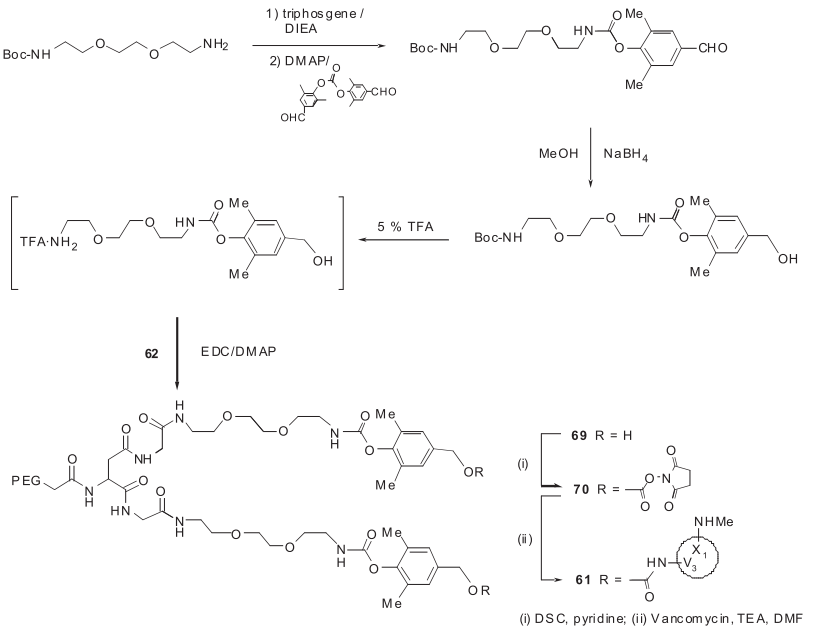
(i) LiOH; (ii) EDC, DMAP, 2-mercaptothiazoline; (iii) **3,5-dimethyl-4-hydroxyl benzol alcohol,** DMAP; (iV) DSC, pyridine; (v) **vancomycin**, TEA, DMF

Scheme 5.

Scheme 5 (Choe *et al.*, 2002b). For instance, conjugation of the activated tetra-acid derivative **63** with 4-hydroxyl-3, 5-dimethyl benzyl alcohol followed by activation of the benzyl alcohol moiety with NHS produced the tetra-substituted 1,6-BE linker, **68**. Reaction of one equivalent of **68** with 4 equivalents of vancomycin, as shown in Scheme 5, gave the desired V_3-substituted tetramer **60** with a loading of 4 vancomycins per PEG as determined by UV analysis (Greenwald *et al.*, 1996a, 1999). In this fashion, the % active of vancomycin was increased to about 10% (theoretical, 12%). Similarly, a tetrameric vancomycin V_3 PEG carbamate derivative, **61**, was also prepared as shown in Scheme 6. For example, conjugation of the activated tetra-acid derivative **70** (Scheme 6) with vancomycin produced the tetra-substituted product **61**.

In the PK study (Table 12), native vancomycin showed a circulatory half-life ($t_{1/2}$) of 0.34 h with a C_{max} of 162 μg/mL and an AUC of 78.8 h·μg/mL. Vancomycin had a clearance (CL) of 642 mL/h/kg and a volume of distribution at a steady state (Vss) of 309 mL/kg in rats.

At the same time, the PEG-vancomycin conjugates uniformly showed a longer sustained, although lower concentration of circulating free vancomycin in rat plasma. All of the PEG-vancomycin conjugates showed a 12- to 64-fold longer $t_{1/2}$ (4.1 h–21.9 h). All of the conjugates achieved C_{max} values that were 16% to 33% (26 μg/mL–54 μg/mL) of those observed for unmodified vancomycin. The AUC for the PEG-vancomycin conjugates were 1.4- to 9.1-fold greater (112 h·μg/mL–717 h·μg/mL) than that observed for vancomycin and had concomitantly slower clearance rates (71 mL/h/kg–178 mL/h/kg) that were 11% to 28% of vancomycins. The Vss of the PEG-vancomycin conjugates were 3–9-fold greater (938 mL/kg–2723 mL/kg) than that observed for vancomycin. The PK profile is summarized in Table 12.



Scheme 6.

PEG Prodrugs of Oligonucleotides

PEG permanently bonded to oligodeoxynucleotides (ODNs or oligos), generally as the phosphate ester, have been synthesized by Bonora (1997), Burchovich *et al.* (1998), Drioli *et al.* (2002), and several other groups in an attempt to stabilize the rapidly metabolized oligo while, hopefully, enhancing-cellular uptake (for reviews on this subject see Bonora, 2002 and Chirila *et al.*, 2002). PEG coupling to the 3' and 5' terminal positions showed more than a 10-fold increase in exonuclease stability while maintaining *in vitro* activity (Jaschke *et al.*, 1994); however, antisense activity *in vivo* has yet to be demonstrated. This may be due in part to the inability of solid-phase methods to produce sufficient quantities of materials to test.

Other cases of oligos with 3' or 5' modifications including dyes, cholesterol, or PEG have shown enhanced antisense binding activities *in vitro* (Letsinger, 1989; Jones *et al.*, 1994; Saison-Behmoaras *et al.*, 1997).

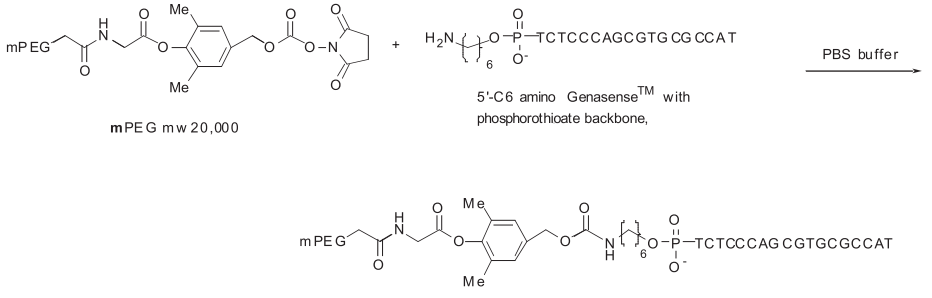
Kawaguchi *et al.* (1995) used a hexylamine linker at the 5' terminal during the automated synthesis of a 15 base pair ODN and then permanently conjugated the basic NH_2 group to a low molecular weight (10,000 Da) branched PEG derivative, 2,4-(O-methoxypolyethylene glycol)-6-chloro-S-triazine. Unfortunately, purity was not defined, and a comparison of half-lives in human plasma in the presence of S1 nuclease demonstrated that the PEGylated mixture had marginally greater stability, while the oligo with a phosphorothioate backbone showed the greatest stability. No PEGylation of the latter was reported.

Construction of a pure, high molecular weight PEG prodrug by using the available NH_2 moiety obtained by introduction of an amino alkyl side chain at

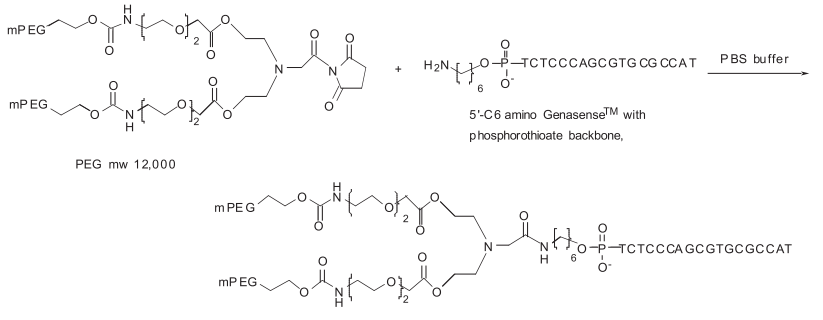
either the 3' or 5' terminal positions should provide a long-lived species and enhance oligo bioavailability using this long circulating prodrug form. PEG conjugation has been shown to enhance cellular uptake *in vitro* (Rapozzi *et al.*, 2002), thus making a PEG prodrug strategy particularly appealing. The use of high molecular weight PEG (>20,000 Da) can control circulating half-life in plasma while the linkage can be designed to release native oligo into the cell. In fact, application of this concept using phosphorothioate based ODNs conceivably can provide constructs with outstanding stability.

Reaction of various mPEG 20,000 Da BE linkers with the model oligo Genasense®, an ODN currently awaiting FDA approval, modified with a 3'- or 5'-aminohexyl functionality as shown in Scheme 7, resulted in 50–80% yield of the desired PEG prodrug, **71**. Unreacted oligo is easily recovered and can be recycled to increase the overall yield. This is in contrast to liquid phase synthesis of a PEGylated mercaptoalkyl ester where the final yield of substituted oligo was 3% (Jones *et al.*, 1994). As was the case with other small molecules, by varying the trigger different rates of release could be obtained (Greenwald *et al.*, 1999). Furthermore, the novel aliphatic bicin prodrug linker was also applied successfully to give prodrug **75** (Scheme 8). In addition to amino alkyl Genasense®, which contains a phosphorothioate backbone, other model oligos (4- and 6-mers) were prepared with a normal phosphate ester backbone modified as the PEG prodrug and, encouragingly, also demonstrated much greater *in vitro* stabilities (Table 13).

The *in vitro* results of the PEG modified oligos (Zhao *et al.*, 2005) are summarized in Table 13 and clearly show a substantial increase in rat plasma half-life and enhanced stability against a variety of nucleases, especially the



Scheme 7.



Scheme 8.

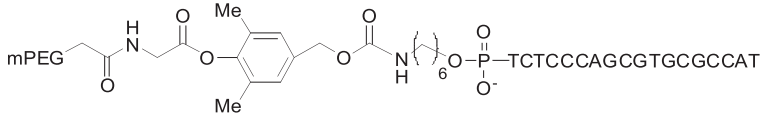
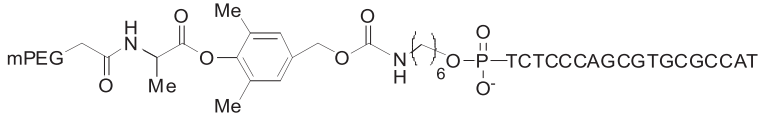
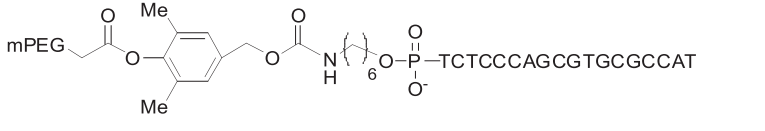
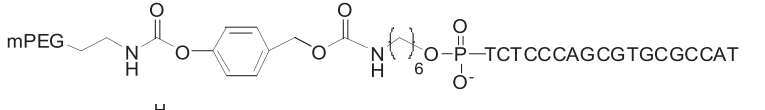
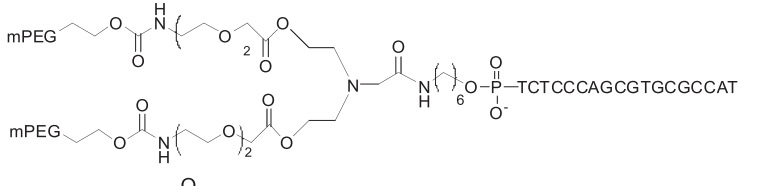
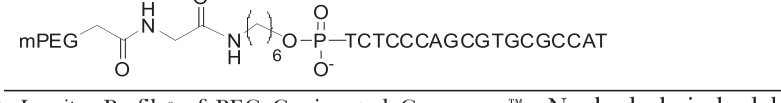
Compound	#	*MW*	%Active	$t_{1/2}$ (rp) h	Stability towards nucleases		
					$t_{1/2}$ (PEI)[b]	$t_{1/2}$ (PEII)[c]	$t_{1/2}$ (nucleaseP1)[d]
	**71**	26168	19.1%	14.7	>24h	38.9 h	0.3 h
	**72**	25994	22.7%		>24h	>48 h	<5 min
	**73**	26114	22.5%	6.5h	>24h	Stable	<5 min
	**74**	26070	21.4%	4.1h	5.6 h	>48h	<5 min
	**75**	30365	17.4%		18.7h	36.8h	
	**76**	25980	22.6%		>24h	>48 h	<5 min

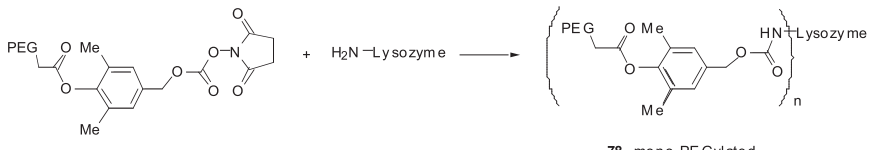
Table 13. *In vitro* Profile[a] of PEG Conjugated Genasense™ No hydrolysis had been found for all conjugates after 24 hrs at 25 °C; [b]PEI = Phosphodiesterase I, 5'-exonuclease; [c]PEII = Phosphodiesterase II, 3'-exonuclease (the dominate nuclease in cell); [d]Nuclease P1 = endonuclease.

predominant nuclease (PEII) in mammals, which is the main source of oligo degradation in cells.

Proprotein Conjugates using Releasable PEG (rPEG)

It is generally observed that protein functions, such as catalysis and receptor binding, are compromised to varying degrees following PEGylation, often resulting in diminished activity; this may be due to the presence of the PEG substituent on or near the active or regulatory sites of the protein. Early attempts to improve this situation led to the use of different activated mPEG linkers, all producing conjugates with hydrolysis-resistant permanent bonds with no one particular linker providing consistently superior results. Therefore, it seems likely that the most effective linker for maintaining activity will vary for different proteins and will have to be determined empirically for each case (Lee *et al.*, 2003). PEG mass and the degree of protein modification have also been explored as a means of preserving activity; the use of fewer PEG strands of higher MW has been reported to produce conjugates with less loss of activity (Knauff *et al.*, 1988; Somack *et al.*, 1991). A novel approach for maintaining maximum activity in a conjugate would be to design a functional mPEG linker that can predictably break down by enzymatic hydrolysis. Such a releasable mPEG (rPEG) would provide PEGylated protein-conjugates that are impermanent and could act as a depot or reservoir, continuously discharging native protein (non-immunogenic) with full, albeit potentially short-acting, activity. BE derivatives based on mPEG 5,000 Da (now termed rPEG) are useful for application to protein and peptide modification using free α- and ε-amino functions, in the same fashion as small amino-containing molecules, and provide predictably unstable conjugates or prodrugs.

Demonstration of this approach to the controlled release of proteins is based on the relatively long $t_{1/2}$ that results from PEG conjugation, and was done using lysozyme as a model protein (Scheme 9) (Lee *et al.*, 2003). Lysozyme, whose structure and function are well understood, also has the advantage that activity is totally lost upon permanent PEGylation, and the presence of even a single PEG on its surface results in complete loss of enzymatic activity. This provides an opportunity to demonstrate unambiguously that PEG can be completely removed by regenerating the fully functional enzyme. Various BE PEG linkers such as the N-hydroxysuccinimidyl-activated carbonates were reacted with the available ε-amino groups of lysine (Table 14). The strategic placement of ortho methyl groups on the aromatic ring served to slow the rate of enzymatic cleavage of the PEG ballast and thus allowed a controlled release of native protein. The rate of



77

78. mono-PEGylated
79. heavily PEGylated

Scheme 9.

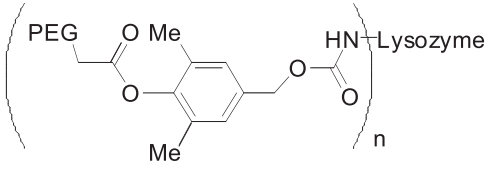
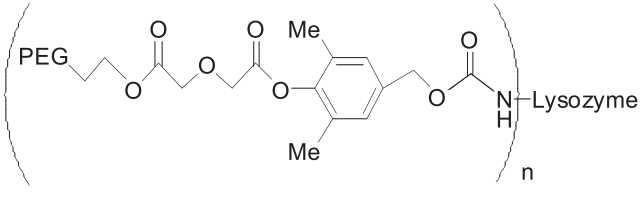
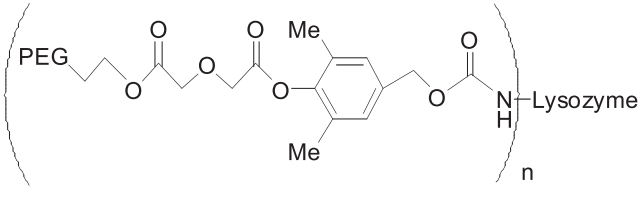
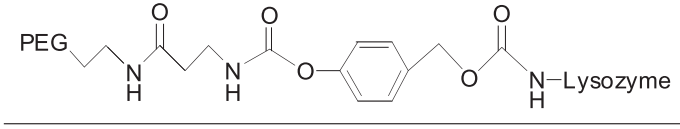
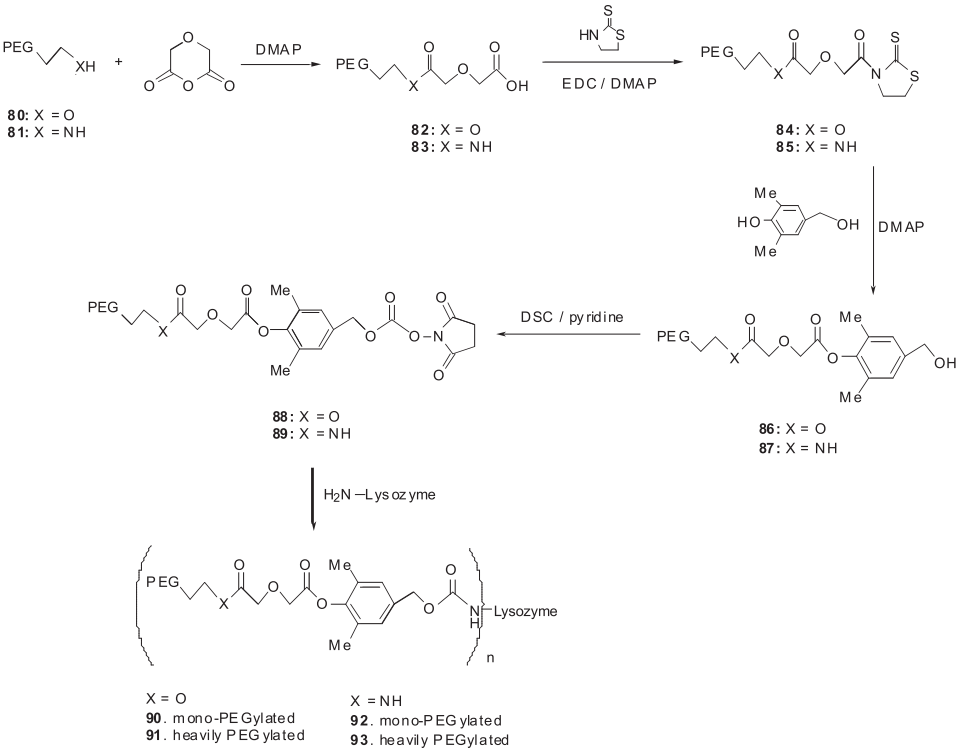
compounds	PEG #	#	Plasma	PBS, 37°C	pH 8.5, 37°C
	n=1	78	17	>168	5.6
	n>1	79	>72	N/R	>120
	n=1	90	4	23	1
	n>1	91	64	>168	22
	n=1	92	4	12	0.6
	n>1	93	21	65	7
		94	4	15	0.8

Table 14. Release Rate of Mono PEGylated Lysozyme in Rat Plasma and in Buffer* *The data are expressed as t1/2 in hours. The release in plasma was monitored for 3 days, the release in pH 8.5 buffer for 5 days, and the release in PBS for 7 days. PBS contains 138 mM NaCl, 2.7 mM KCl, and 10 mM phosphate, pH 7.4. The release of lysozyme was detected by regeneration of lysozyme activity and confirmed by gel electrophoresis. N/R stands for "no release".



Scheme 10.

release was compatible with the circulating $t_{1/2}$ of permanently PEGylated proteins, which typically ranges from a few hours to several days (Witt *et al.*, 2001). For an rPEG protein conjugate to demonstrate effective drug delivery, activity must be regenerated before the protein is eliminated from the body by renal and hepatic clearance pathways. rPEG-lysozyme conjugates were relatively stable in pH 7.4 buffer for over 24 h. However, regeneration of native protein from the rPEG conjugates occurred in a predictable manner during incubation in high pH buffer or rat plasma as demonstrated by enzymatic activity and structural characterization. *In vitro* studies are presented in Table 14. One important feature of these rPEG-conjugates was that regeneration of native lysozyme correlated with PEG number: the native protein was released more rapidly from the monosubstituted conjugate than from the disubstituted species, suggesting possible steric hindrance to the approach of cleaving enzymes. More rapid hydrolysis of the rPEG-lysozyme conjugates was accomplished by employing side chains incorporating diglycolic acid derivatives, which provided anchimeric assistance to hydrolysis of the linker of the tripartate rPEG protein (Greenwald *et al.*, 2003c), as shown in Scheme 10. Interestingly, regeneration of activity was also effected by PEG number in these cases as well, although on a more rapid time scale.

PEG Tripartate Prodrugs: Trimethyl Lock Lactonization (TML)

During the exploration of the limits of the PEG prodrug strategy, it was apparent that the use of the intra-molecular cyclization reaction (lactonization) of a hindered amide (Shan *et al.*, 1997; Testa and Mayer, 1998; Wang *et al.*, 1999) would provide a practical alternative to the BE system. In order to utilize the trimethyl lock system (TML) in a fashion similar to the BE system, it was necessary to first establish various methodologies that allowed the efficient synthesis of different acyl functionalities (triggers) such as esters, carbonates, and carbamates on the phenolic hydroxyl group. The acylating agents were by necessity bifunctional and offered a site for easy PEGylation. Thus, introduction of PEG into the TML system as part of the specifier or trigger resulted in a neutral and highly water-soluble tripartate polymeric prodrug potentially capable of passive tumor targeting (Figure 6). The PEG prodrugs were designed to attain predictable rates of hydrolysis by changing the nature of the trigger/linker bond, by adding steric hindrance on the aromatic ring of the linker, and by the use of spacer groups. This approach resulted in a versatile methodology for easily altering the final design of the prodrug: it enabled a "mix and match" of spacers, triggers, and linkers that were utilized in a meaningful manner and, ultimately, provided optimal plasma $t_{1/2}$ for delivery of different types of drugs (Greenwald *et al.*, 2000b). From the hydrolysis result of PEG TML-DNR conjugates in rat plasma (Table 15), it is evident that adjusting the $t_{1/2}$ of TML linkers is not as simple as was the case for the BE system. A common thread that runs through these derivatives, as well as for **95**, which also possesses quite effective activity, is the incorporation of an amino acid ester spacer. As has been mentioned earlier, amino acid conjugated species may, in some cases, demonstrate enhanced tumor uptake.

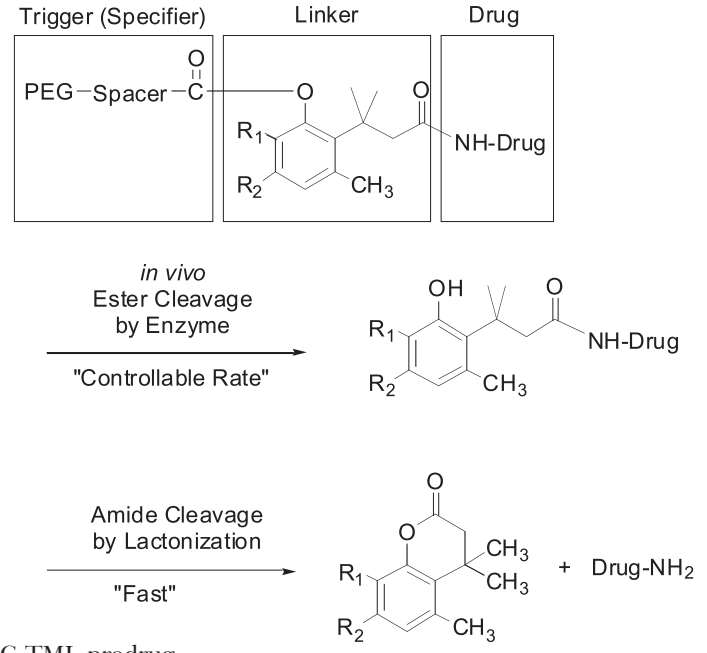


Figure 6. PEG TML prodrug

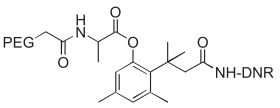
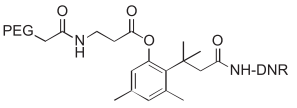
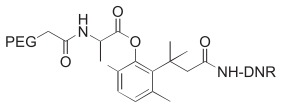
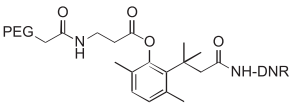
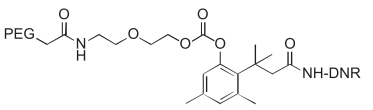
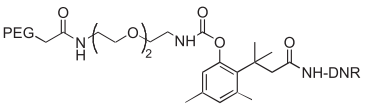
Compound	#	IC_{50} (nM) P388/O	PBS pH 7.4	$t_{1/2}$ (h) Rat Plasma	Cell Media	M109[b] (%T/C) i.p.	M109[b] (%T/C) i.v.
Daunorubicin·HCl	-	3	-	-	-	44.8	117.0
Esters							
	**95**	43	> 24	1.9	14	62.8	92.5
	**96**	203	> 24	0.2	80	101.2	63.7
	**97**	389	> 24	21	36	153.6	72.5
	**98**	411	> 24	8	94	114.8	31.6
Carbonate							
	**99**	142	> 24	1.1	38	57.1	118.4
Carbamate							
	**100**	203	> 24	> 24	53	110.5	93.8

Table 15. *In Vitro*[a] and *In Vivo* Results of PEG TML Daunorubicin Prodrugs.
[a]All in vitro experiments were done at 37°C in duplicate. Standard deviation of measurements = ±10%. [b]3 mg/kg/dose of active DNR administered to balb/c mice bearing subcutaneous Madison Lung Carcinoma on 1 & 4 (intraperitoneal) or 3 & 6 (intravenous) days after inoculation. Percent treatment over control (%T/C) median tumor volumes were compared when control groups median tumor volume reached ~ 2000 mm^3.

A comparable study between TML and BE was also reported. **95** and **35** which appeared to have the closest hydrolysis rate ($t_{1/2}$ = 2–4 h) were evaluated along with a very slow releasing carbamate derivative (**36**) for chemotherapeutic activity against a more established and slower growing solid tumor model, SKOV3 ovarian cancer (~70 mm^3) xenograft using nude mice (Table 16). Both compounds **35** and **95** were quite similar in their ability to significantly inhibit the growth of SKOV3 tumors. In contrast, PEG conjugation did not appear to enhance the activity of DNR against tumor lines that were insensitive to DNR (MX-1, mammary and PC-3, prostate). Thus, the attachment of PEG 40,000 with its

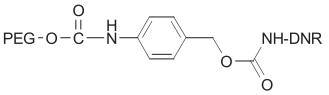
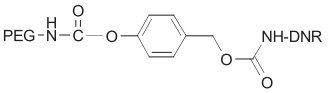
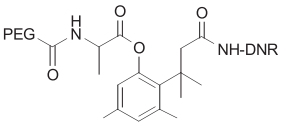
Compound	#	Tumor Vol by week 6 (Mean, mm^3)	%Δ from basal by week 6 (Mean)	T/C (%) by week 5
Control		1557.8	3091.4	—
Daunorubicin HCl		1242.4	3008.1	35.2
	**36**	777.1	1224.8	51.7
	**35**	512.9	699.1	7.6
	**95**	50.0	78.5	4.5

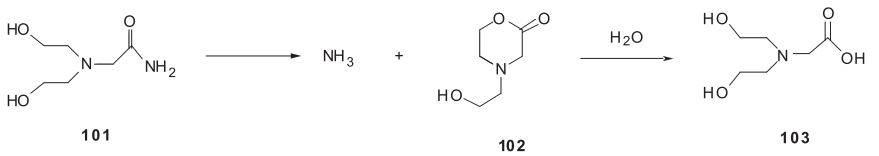
Table 16. Efficacy of PEG-BE & TML-DNR Against s.c. Human Ovarian Tumors (SKOV3) in Nude Mice

ability to accumulate in tumors will not automatically permit drugs to have greater antitumor activity.

PEG Prodrugs Based on bis-Hydroxyethyl Glycine (Bicine or Bicin)

Suggs and Pires (1997) reported the rapid hydrolysis of C-terminal amides of glycine at 25 °C and pH 7 when the N-terminus is N-hydroxyethylated. The $t_{1/2}$ of bis-*N*-2-hydroxyethylglycinamide (**101**) in 0.1 phosphate buffer was determined to be 3 h, which is tremendously faster than the 7 yr reported for the unsubstituted glycinamide. Amide derivatives with a single hydroxyethyl group underwent hydrolysis more slowly than would be predicted by statistical factors alone, suggesting that both hydroxyethyl groups assist in the hydrolysis of the amide and that the mechanism of action is a simple serine protease mimic cyclizing to a morpholinolactone (**102**, detected by NMR analysis), which is then rapidly hydrolyzed by water to the acid **103** (bicine or bicin) as shown in Scheme 11.

Of late, there has been a great deal of interest in prodrug strategies—especially those that can solubilize and release amine-containing bioactive molecules and macromolecules. From inspection of **101**, it was immediately apparent that a PEG prodrug system for amino-containing drugs based on this novel substituted bicin amide hydrolysis could be designed and would, of

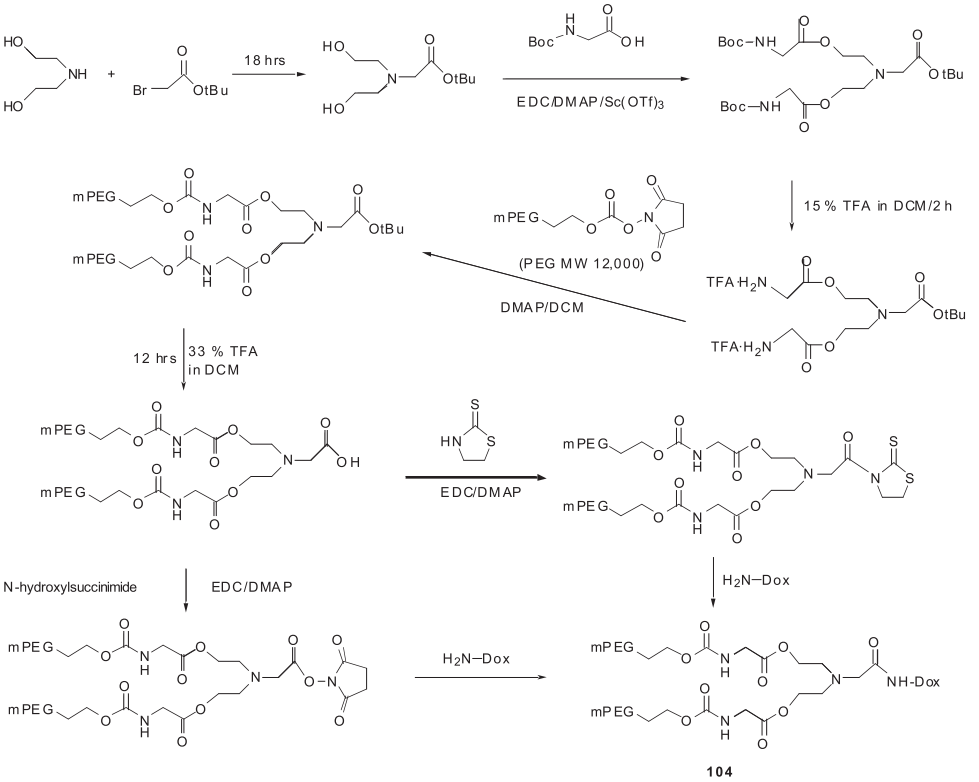


101 102 103

Scheme 11.

necessity, be entirely based on aliphatic chemistries. In the case of polypeptides, this would be especially desirable. Only a very few amino prodrug strategies are practical (Amsberry and Borchardt, 1990; Wang *et al.*, 1996; Greenwald *et al.*, 1999, 2000b) and few of these are aliphatic based (Kondo *et al.*,1986; Alexander *et al.*, 1996; Choe *et al.*, 2002b). Successful development of a bicin-based prodrug would be of major importance to the field of drug delivery, especially if PEG could be incorporated into this system to add solubility and increased circulating half-life ($t_{1/2}$) to the prodrug. The tactical approach used to increase the release rates of the promoiety was, once again, to introduce either an α-heteroatom on the acid portion of the ester or to provide anchimeric assistance when an even more rapid $t_{1/2}$ was desired. Lengthening the $t_{1/2}$ was accomplished by the introduction of steric hindrance and/or by adding α-substituents. This basic strategy worked (Greenwald *et al.*, 2004a) and led to the synthesis of novel branched PEG prodrug derivatives of both proteins and small organic molecules (Scheme 12). As the PEG prodrugs of small molecules underwent self-destruction, a clear demonstration of the effect of PEG MW on *in vivo* activity was again observed (Greenwald *et al.*, 1996a).

The rates of plasma hydrolysis varied from about 3 to 20 h, releasing native DOX with the IC_{50} values shown in Table 17 and thus demonstrating not only that these esters were prodrugs but also that the rates of decomposition could be directed by the usual chemical modifications discussed earlier. For example,



Scheme 12.

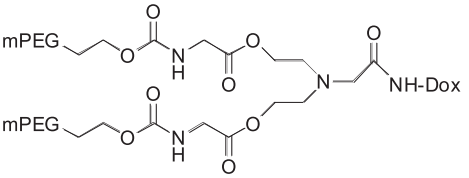
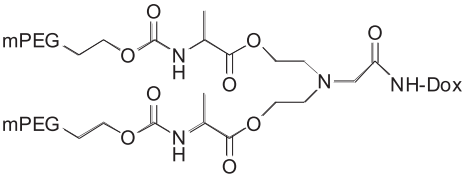
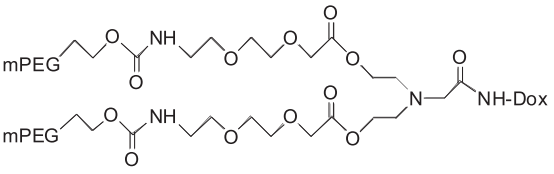
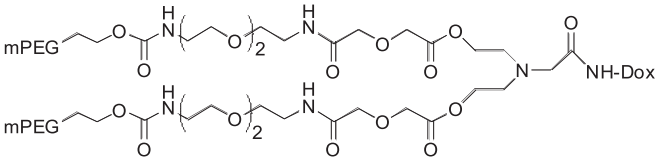
Compound	#	$t_{1/2}$ (h) (rat plasma)	*MW*	IC_{50} (P388) (nM)
Doxorubicin•HCl		—	580	17
	**104**	10	24855	428
	**105**	20	24883	316
	**106**	5.5	25031	1100
	**107**	3.2	25233	455

Table 17. Properties of rU-PEG-DOX Conjugates

diester **104** is a double prodrug, and a proposed mechanism for the release of DOX (which is representative for any amine) is shown in Scheme 13. The first step in the breakdown must, of necessity, be the hydrolysis of one ester bond, producing **108**. Although mono-ester **108** is capable of amide assisted hydrolysis to free amine (DOX) and compound **109**; this reaction is probably on the order of 30–100 times slower than the doubly assisted breakdown of **110** to DOX and **102**, which arises after a second ester hydrolysis (Suggs and Pires, 1997). Although the IC_{50} values indicated that the prodrugs were active *in vitro* (Table 17), unexpectedly, these DOX derivatives, all based on a 24,000 Da molecular weight (MW) U-PEG (two12,000 Da strands), were found to be inactive in a M109 murine tumor model when compared to native DOX. An explanation for this unusual behavior is that in the first step of prodrug breakdown, ester hydrolysis, one PEG 12,000 MW strand is lost leaving a prodrug (compound **108**, Scheme 13) that now can be seen to be a linear PEG prodrug with an MW of only 12,000 Da. It has been clearly demonstrated that LMW PEG (< 20,000 Da) attached to drugs (specifically paclitaxel) undergo rapid renal excretion and show no drug activity (Greenwald *et al.*, 1996a). Therefore, it appears that the lack of *in vivo* activity of

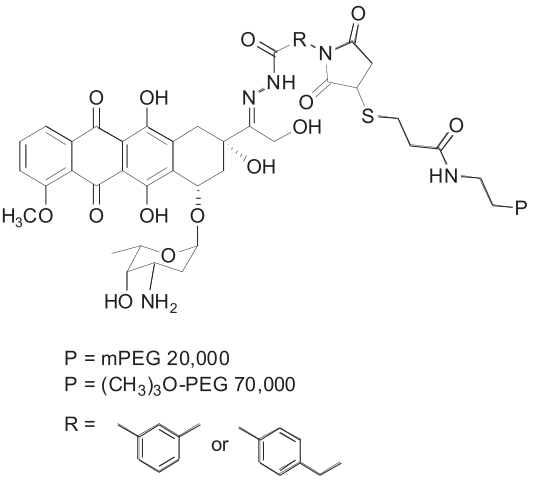
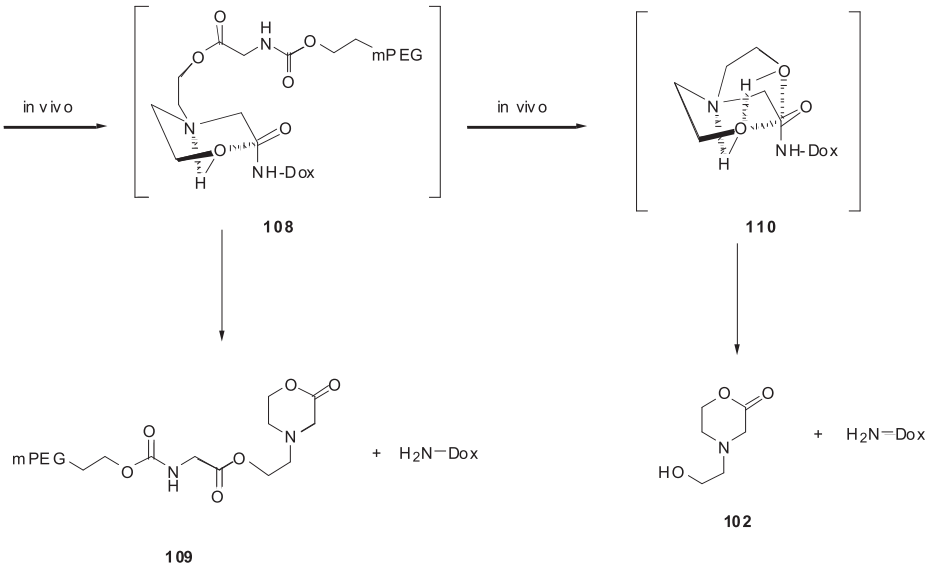


Figure 7. PEG Hydrazone Drug Conjugates.

104 is due to the generation of lower MW polymeric species arising from the initial self-destruction of the diester. In actual fact, the structure of the PEG prodrugs designed in this work presents novel examples of branched PEG, previously described as "umbrella" or U-PEG (Martinez and Greenwald, 1997). The original design incorporates permanent PEG strands (through amide or carbamate linkages) that increase the MW and $t_{1/2}$ of protein conjugates; note that there can be no release of native drug from this type of system. However, in the case of PEG substituted bicins, attachment utilizes ester bonds and, thus, the conjugated PEG strands are labile.



Scheme 13.

Acid-Activated PEG-Drug Conjugates: Hydrazone Derivatives of Doxorubicin

A novel series of HMW PEG conjugates that incorporated acid-sensitive hydrazone linkages have been synthesized using PEG 20,000 Da and 70,000 Da (Rodrigues *et al.*, 1999). Thus, DOX maleimide derivatives containing an acid-sensitive hydrazone linker, and a stable amide linker for comparison, were coupled to PEG using thiopropionic acid spacers as shown in Figure 8. The polymer drug derivatives were designed to release DOX inside a tumor cell by acid-cleavage of the hydrazone bond, after endocytic cellular uptake. The hydrazone conjugates demonstrated activity *in vitro*, albeit the activity was much less than that of DOX itself. In contrast, PEG DOX conjugates containing a stable amide bond at the amino sugar (3'-position) showed no *in vitro* activity at all. Fluorescence microscopy studies revealed that free DOX accumulates in the cell nucleus whereas the acid labile PEG-DOX derivatives are localized primarily in the cytoplasm.

Targeting of PEG Prodrugs

Targeting moieties can be built into PEG using heterobifunctional derivatives. Recent efforts applying biotin as a targeting moiety in combination with LMW PEG-gly-CPT showed higher cytotoxicity and apoptosis-inducing activity than did the simple PEG-CPT conjugates *in vitro*. (Minko *et al.*, 2002). Unfortunately, no *in vivo* data of the 3,400 MW derivative were reported; again, the importance of higher MW and PK becomes of prime importance. Another interesting approach reported by Dharap and coworkers (2003) investigated two types of molecular targets: (1) an extracellular membrane receptor specific to ovarian cancer, LHRH peptide, which is over-expressed in ovarian cancer cells and (2) BH3 peptide, which inhibits anti-apoptotic defense and, therefore, should increase the ability of a PEG (3400)-CPT conjugate to activate caspase-dependent pathways of apoptosis. In both cases, enhanced *in vitro* results were reported but were not accompanied by *in vivo* studies. It is anticipated that many additional combinations of targeting moieties associated with PEG prodrugs will appear in the future.

Novel PEG-immunoconjugates, recently reported by Suzawa *et al.* (2000, 2002) have demonstrated antigen-specific targeting using anticancer agents DU-257 and adriamycin with mAb, KM231 and NL-1, respectively. The enzymatically cleavable linker PEG-L-ala-L-val was coupled with DU-257, a potent anticancer duocarmycin derivative, through an amide bond. Coupling of the PEG-DU-257 conjugate to the KM231 mAb, which is specifically reactive to GD3 antigen, was then carried out. GD3 antigen is expressed on the surface of several malignant tumors such as SW1116, and *in vitro* testing using this cell line demonstrated significant growth inhibition at a concentration of 75μg/mL. Thus, it appears that PEG-dipeptidyl prolinkers may be an effective means with which to prepare novel immunoconjugates.

Labeling Studies of PEG Prodrugs

Fluorescent Dye Labeling

To examine cellular binding, uptake, and trafficking of PEG in cancer cells, FITC-labeled 40 kDa PEG (PEG-FITC) was synthesized employing a stable thiourea bond. This conjugate was shown to be stable under the experimental conditions. Yu *et al.* (2004) examined the cellular uptake and subcellular localization of PEG-FITC in various human cancer cells using FACS and confocal microscope analyses. FACS results revealed that PEG-FITC was taken up by A549 lung cancer cells in a dose- and time-dependent manner. The maximal uptake level was reached after an approximately 16-h exposure. PEG-FITC exhibited significantly higher levels of uptake than native FITC, suggesting that PEGylation may enhance drug transport to cells. The internalized PEG-FITC was retained in the cells at significant levels for over 8 h, although its levels declined slowly over time. Confocal microscopic analysis demonstrated that PEG-FITC was localized predominantly in the perinuclear region of the cytoplasm (Figure 8). Staining of the cellular lysosomes with Lysotracker Red revealed similar distribution, suggesting that the PEG-FITC may reside in lysosomes. This was confirmed by examining the co-localization of PEG-FITC and the lysosome marker. Staining of the nucleus with DAPI showed little or no nuclear localization of PEG-FITC. Significant uptake was also observed in human SKOV3 ovarian, MCF-7 mammary, and 518A2 melanoma cancer cells. The uptake by A549 cells was diminished completely at 4°C, indicating that cellular uptake is an energy-dependent process.

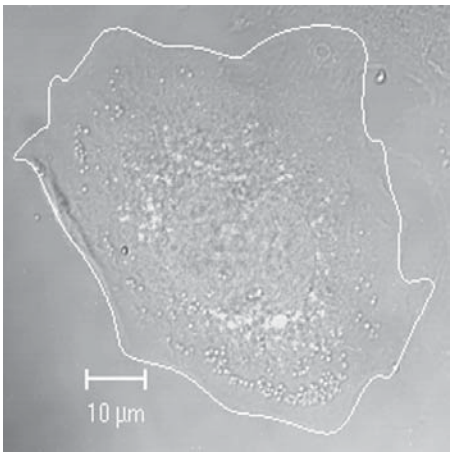


Figure 8. PEG is taken up by cancer cells
Human A549 lung cancer cells were incubated with 25 uM PEG-FITC for 16 h. The cells were washed, stained with DAPI to reveal the nucleus (blue), and analyzed using laser scanning confocal microscope. Shown is superimposition of the confocal image of a cell across the middle of nucleus with its transmitted image. Note that the PEG-FITC is distributed predominantly in the peri-nuclear region of cytoplasm.

These data demonstrate that PEG can be taken up substantially by human cancer cells, presumably via pinocytosis (Yu *et al.*, 2003, 2004).

To test whether a PEGylated compound enters and accumulates in tumor via the EPR effect, a model was established to address this question using GFP with a molecular mass of 27,000 Da (Yu *et al.*, manuscript in preparation). Two PEGylated derivatives of GFP, mono-PEG-GFP and di-PEG-GFP, containing one or two 20 kDa PEG, respectively, on each GFP molecule were synthesized by standard procedures. PEG and GFP were conjugated through a non-biodegradable linker in order to prevent native GFP from being released *in vivo*. Native GFP or the PEGylated forms (100 μg) were administered intravenously to nude mice bearing the LS174T colon cancer. Plasma, tumor and some normal tissues were collected at various time points after the compound injection. Fluorescence levels in the plasma or tissue homogenates were measured based on the GFP fluorescence intensity. In addition, tissues were sectioned, stained with hematoxylin and eosin, and examined for GFP fluorescence under a fluorescence microscope. It was found that both mono- and di-PEGylated GFPs displayed prolonged circulating time in plasma (approximately 5 and 10 h, respectively), as well as a significant and sustained presence in the tumor, whereas native GFP ($t_{1/2}$ < 1 h) revealed little or no signal in plasma and tumor, respectively. Di-PEG-GFP (with a greater MW) showed, as expected, greater tumor accumulation than mono-PEG-GFP. Consistent with this, intense green fluorescence was observed in tissue sections from the PEGylated GFP groups, whereas tumor sections from the native GFP group revealed no signal. In contrast, no or insignificant fluorescence signals were detected in normal tissues, including spleen, kidney, liver, and lung.

Isotopic Labeling

Using ^{3}H-CPT and PEG-gly-^{3}H-CPT, Conover *et al.* (1998) performed a study using HT-29 (colorectal) tumor-bearing mice. The results of the study clearly demonstrated an EPR effect as shown by a tumor accumulation of the PEG that resulted in about a 30-fold greater accumulation of labeled CPT in the tumor than that produced by the native species (Conover *et al.*, 1998). Although there is no doubt that the EPR effect is responsible for tumor accumulation of the PEG-CPT species, it is interesting to note that the focus of greatest effect is not always predictable and must be determined empirically by trained clinicians. The prodrug efficacy is heavily dependent on the type of the linker used and MW of the macromolecule, which could affect the biodistribution of the conjugate. For example, optimum efficacy of a particular polymeric drug may signify some plasma breakdown during circulation before tumor accumulation occurs. This, in fact, may add to the effectiveness of the drug as it is now free to associate with plasma proteins such as serum albumin and enter the tumor via an alternate pathway. Hepatic enzymes may also play a role in cleaving the PEG conjugate, releasing drug to the circulatory system. Acknowledging that the EPR effect provides large tumor accumulations does not appear sufficient by itself to explain the lack of (or lessened) efficacy observed for some PEG prodrugs in several

mouse xenograft models. Even where activity is found, it often cannot be correlated to those same cancer types in humans. Thus, biodistribution of the prodrug in human may differ significantly from that in animals. Polymeric peptide prodrugs designed to be completely stable in plasma and decompose by a cathepsin-mediated hydrolysis after endocytotic capture (Kopeck and Duncan, 1987) also do not appear to have the flexibility to provide this possible assist to the pharmacodynamic (PD) profile.

Concluding Remarks

In a span of about 10 years, PEG prodrug chemistry has been initiated, developed, and applied successfully to anticancer agents as well as several other important classes of medicinal agents such as antibiotics, antivirals, and antifungal agents. The foremost of these to advance to latter stage clinical trials is the PEG 40,000 Da MW ala-ester of CPT, a bipartate prodrug expressing anticancer activity against gastric and esophageal indications. Because of the high MW of PEG required to prevent rapid clearance of the drug conjugate, the native drug employed in the conjugate needs to possess initial high activity (nM or greater) to minimize the amount of PEG conjugate required for dosing. Thus far, no severe toxicities have been observed for PEG 40,000 during systemic administration. One problematic drawback encountered for any additional development of this class of compounds may be a cost of goods (COG) issue. Thus, an important advance in this field has been the terminal branching achieved using aspartic dendrons that permit a payload of up to 16 drug molecules per strand of PEG to be delivered, although a loading of 4 or 8 units appears to be sufficient for most applications. This lessens the total amount of polymer conjugate that needs to be dosed and, thus, the overall cost. Alternatively, the recently commercialized multi-arm PEGs can be used for the same purpose. The utility of the PEG prodrug approach to drug delivery provides a way to solubilize insoluble drugs in a non-ionic matrix and reduce drug clearance while extending the plasma concentration, thus providing a greater apparent AUC. Applications of this concept to water-soluble drugs may also serve to prevent rapid clearance from the body and lead to a greater apparent AUC. In the case of ara-C this passive accumulation led to much greater solid tumor efficacy, thus allowing additional indications to be considered by PEGylation. PEG prodrugs can be used for drugs with amino, hydroxyl, or carboxyl functions, thus giving them great versatility for drug delivery. The chemistry of the PEG linkers seems adequately designed at this point leaving only the choice of small molecule candidate to be determined. However, additional modification of PEG that can further facilitate drug delivery of anticancer agents is exemplified by heterobifunctional derivatives, which can be modified with targeting moieties on one terminus while the drug is attached to the distal terminus. In addition, the role of spacer groups such as amino acid esters (which were mentioned in an earlir section of this chapter) that appear to be recognized by peptide receptors on the cells needs to be further delineated to assist increasing cellular uptake, which will certainly lead to greater drug efficacy.

Inevitably, the next ten years of PEG applications will, no doubt, lead to additional examples of the use of this polymer for drug delivery, which with any luck will provide heightened utilitarian benefits for biologically active agents.

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2.3.2

Prodrugs to Reduce Presystemic Metabolism

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List of Abbreviations

AUC .....Area under the plasma concentration vs. time curve
%CV .....Coefficient of variation (%)
C*max* .....Maximum observed plasma concentration
DOPAC .....3,4-dihydroxyphenylacetic acid
F .....Extent of oral bioavailability
(expressed as a percentage of the administered dose)

Rationale

Why Maximize Oral Bioavailability?

One of the most important pharmacokinetic characteristics of a drug or new drug candidate is its oral bioavailability. The oral route is the preferred means of administration for most drug therapies, particularly those self-administered by the patient on an ongoing basis. Unless a drug is intended to treat a condition of the gastrointestinal tract, its effectiveness after oral administration requires attaining adequate and consistent systemic exposure. The extent of bioavailability determines the levels of exposure as well as the variability in exposure. Hellriegel *et al.* (1996) surveyed 143 literature references reporting absolute oral bioavailability and described the relationship between absolute bioavailability and inter-subject variability (% CV) for 100 drugs studied in those references. It was shown that the variability of systemic exposure after oral dosing was greatest when oral bioavailability was low and, conversely, inter-subject variability was generally low when oral bioavailability was high. Variability of systemic exposure leads to inconsistent and possibly unpredictable pharmacological and toxicological effects of the drug. Therefore, drugs with good oral bioavailability can have a considerable therapeutic advantage over related drugs with poor oral bioavailability.

If a drug is subject to extensive presystemic metabolism, it is likely that there will be high levels of metabolites generated and appearing in the systemic circulation. These metabolites may have pharmacologic effects or unwanted side effects. Another reason to strive for the greatest bioavailability is to reduce the exposure to metabolites and to limit the effects caused by metabolites. For example, oral administration of oxybutynin resulted in plasma AUC of des-ethyl-oxybutynin more than 10-fold greater than the plasma AUC of oxybutynin. Following oxybutynin administration using an alternative delivery method that reduced the extent of presystemic metabolism, the plasma AUC of the metabolite was only 2-fold greater than that of oxybutynin, and fewer systemic side effects resulted (Buyse *et al.*,1998). Similarly, Clarke *et al.* (2003) showed that plasma concentrations of selegiline metabolites were significantly reduced when selegiline was administered using a transmucosal delivery system, which increased selegiline bioavailability relative to oral administration and afforded a potentially safer and more predictable method for treatment of Parkinson's disease.

A third reason to maximize oral bioavailability is related to the efficiency of use of the active drug substance. If oral bioavailability averages 25% (25% of the dose reaching the systemic circulation intact), then 75% of the active drug substance is wasted. This inefficiency of compound usage adds to the cost of the pharmaceutical product and can be an especially important factor for active drug ingredients that are expensive to produce. For these reasons, enormous efforts are made to identify new drug candidates that have reasonably good oral bioavailability in animal models and in humans. Similarly, existing drugs with less than

optimal oral bioavailability represent an opportunity for technologies that afford improved drug delivery.

Incomplete oral bioavailability can be caused by incomplete absorption from the intestinal tract, due to solubility or permeability limitations, or can be related to presystemic metabolism. Presystemic metabolism is typically due to hepatic extraction, although recently the role of intestinal metabolism has become increasingly recognized. Incomplete oral bioavailability due to incomplete absorption can often be addressed with formulation modifications. However, there is no formulation solution to overcome hepatic first-pass metabolism. The chemical approach is required, and often times the prodrug approach has been utilized, especially when chemistry has been optimized for the desired pharmacological properties. This chapter reviews the application of prodrug strategies aimed at improving oral bioavailability by reducing presystemic metabolism.

The Prodrug Approach

Systemic absorption after oral dosing requires the compound to pass through a series of potential sites of metabolism—the intestinal lumen, the intestinal epithelium, and the liver. If the structural position at which presystemic metabolism of a drug occurs is known and if presystemic metabolism is mediated primarily by a single enzymatic reaction at a single site of the molecule, then it may be possible to design prodrugs to block metabolism at that site. The prodrug is therefore intended to pass through the site of metabolism (intestinal membrane or liver) intact and then be hydrolyzed upon reaching the systemic circulation. An illustration of this prodrug strategy is given in Figure 1. One of the challenges in

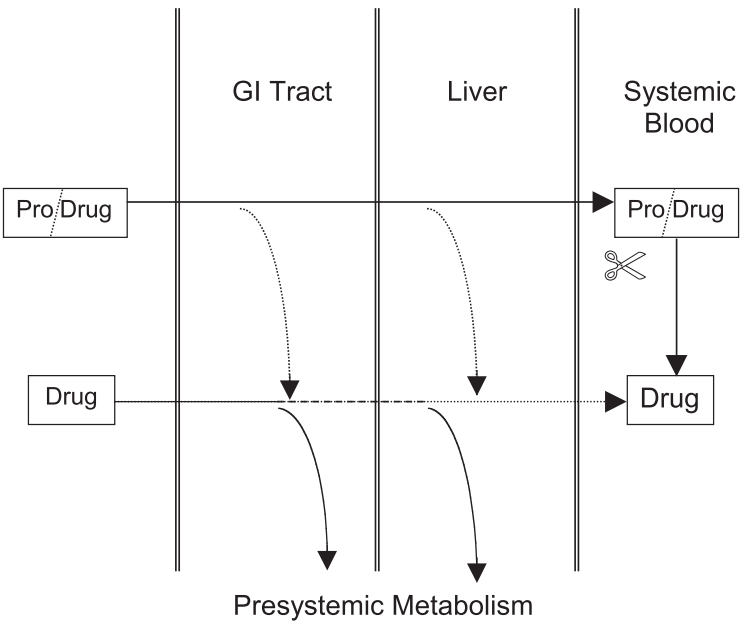

Figure 1. Scheme describing the prodrug approach to reducing presystemic metabolism after oral administration.

oral prodrug delivery is that there are multiple barriers, or potential sites of metabolism, in series. It is not unusual that a prodrug does not completely pass through the intestines and liver intact, because the intestines and liver generally have high levels of activity of the enzymes (e.g., esterases) generally mediating prodrug-to-active drug conversion. These potential pathways of prodrug disposition are also depicted in Figure 1 and will be discussed again later.

In this review, the presystemic metabolism of peptide drugs will also be considered. Peptide drugs have the additional barrier of degradation by digestive proteases and peptidases present in the intestinal lumen as well as on the brush border membrane of the enterocytes. Because of the high levels of these enzymes, transmucosal (*e.g.*, nasal, buccal, rectal, or pulmonary) delivery may be more feasible than oral delivery for some biologically active peptides. However, these absorption sites may also be rich in peptidase enzyme activity. For example, certain peptides are rapidly degraded by aminopeptidases when exposed to the nasal mucosa. The prodrug approach can also be applied to the transmucosal delivery of peptide drugs that are highly metabolized by peptidases at the site of administration.

Examples of the Prodrug Approach

There have been numerous examples of the application of the prodrug approach to improve the oral bioavailability of drugs subject to extensive presystemic metabolism, with varying degrees of success. Some selected examples of prodrugs and the results observed are summarized in Table 1 and are discussed below. Also included in Table 1 are some examples of peptides for which the prodrug approach was utilized to potentially improve oral or transmucosal delivery by reducing metabolic degradation. Structures of some of these prodrugs are shown in Figure 2.

Nalbuphine (**1**) is an opioid analgesic that has incomplete oral bioavailability in animals and humans due to presystemic metabolism. The route of presystemic metabolism is primarily conjugation on the phenolic hydroxyl group. Two prodrugs were identified that markedly improved oral bioavailability in preclinical studies, the acetylsalicylate (**2**) and anthranilate (**3**) esters. These were designed to protect the phenolic position from conjugation. Bioavailability in dogs was approximately 5–7% after oral 1, 17–24% after oral **2**, and approximately 50% after oral **3** (Aungst *et al.*, 1987). Furthermore, the plasma concentrations and AUC of conjugated nalbuphine were reduced in dogs after dosing with the prodrugs, making it clear that first-pass metabolism was reduced. In rats, oral bioavailabilities were 2.7% after **1**, 3.9% after **2**, and 5.1% after **3**, so these prodrugs were significantly less effective in rats than in dogs with regard to absolute oral bioavailability. This same approach was applied to the structurally related opioid antagonist naltrexone, which is similarly subject to presystemic conjugation at the phenolic hydroxyl position. Bioavailability in dogs was increased 45-fold after oral administration of the anthranilate ester prodrug and 28-fold after the acetylsalicylate prodrug, whereas benzoate and pivalate esters

Drug	Presystemic Metabolism	Prodrug	Results	Reference
Nalbuphine (1)	Conjugation at phenolic –OH	Acetylsalicylate (2), anthranilate (3) esters on phenolic -OH	Increased oral F <2-fold in rats, increased oral F 8-fold in dogs, decreased AUC of congugated nalbuphine	Aungst *et al.* (1987)
Naltrexone	Conjugation at phenolic -OH	Anthranilate and other esters on phenolic –OH	Anthranilate ester increased oral F 28–45-fold in dogs	Hussain *et al.* (1987)
Naltrexone	Conjugation at phenolic –OH	Salicylate ester	Increased oral F 30-fold in dogs	Hussain and Shefter (1988)
Nalbuphine, naloxone, naltrexone	See above	N-oxide (4)	Increased oral F several fold in dogs	Boswell and Myers, (1985)
Estradiol (5)	Conjugation at phenolic –OH	Acetylsalicylate, anthranilate esters on phenolic -OH	Increased oral F in dogs 17-fold with acetylsalicylate and 5-fold with anthranilate	Hussain *et al.* (1988)
Estradiol	See above	O-saccharinyl-methyl ester	Increased oral F (bio-activity) 5-fold in rats	Patel *et al.* (1995)
Estrogens	See above	Sulfamates (6)	Increased systemic estrogenic activity	Elger *et al.* (1995)
Salicylamide (7)	Conjugation at phenolic –OH	N-morpholi-nomethyl (N-Mannich base) (8)	Increased oral F 2.3-fold in rabbits	D'Souza *et al.* (1986)
Dopamine (9)	Conjugation of catechol	N-(N-acetyl-L-methionyl)O,O-bis-carbonyl dopamine (10)	Increased oral F 4-fold in dogs, reduced concen-trations of dopamine sulfate and DOPAC	Murata *et al.* (1989)
Methyldopa	Sulfate conjugation of catechol, decarboxylation	Succinimidoethyl (S) and pivaloy-loxyethyl (P) esters	Both (S) and (P) increased oral absorption, (S) may be primarily conjugated before hydrolysis, while (P) is hydrolyzed before conjugation	Vickers *et al.* (1978)
Methyldopa	See above	Pivaloyloxyethyl ester	Increased % of dose excreted as methyldopa, decreased sulfation	Vickers *et al.* (1984)

Table 1. Examples of Prodrugs to Reduce Presystemic Metabolism

Drug	Presystemic Metabolism	Prodrug	Results	Reference
Terbutaline	Conjugation at phenolic –OH	Bis-dimethylcarbamate ester	No significant increase in plasma conc. in humans	Holstein-Rathlou *et al.* (1986)
Propranolol	Glucuronidation on β -OH, hydroxylation	Hemisuccinate on β -OH	Increased plasma AUC 8-fold after oral dosing in dogs	Garceau *et al.* (1978)
Propranolol	See above	Acetate, hemisuccinate on β -OH	Both prodrugs increased propranolol AUC 2.5-fold in rats	Anderson *et al.* (1988)
Propranolol	See above	Alkyl esters on β -OH	Increased oral F 2–4-fold in dogs	Shameem *et al.* (1993)
Di- and tripeptides	Carboxypeptidase	N-α-hydroxyalkylation of the peptide bond	Increased metabolic stability	Bundgaard and Rasmussen (1991)
Various peptides	Chymotrypsin	α-hydroxyglycine	Increased metabolic stability	Kahns and Bundgaard (1991)
Desmopressin	Chymotrypsin	Pivalate ester on tyrosine -OH	Reduced metabolism, increased permeation through Caco-2	Kahns *et al.* (1993)
Tetragastrin	Peptidases	Acetyl, caproyl, lauroyl esters	Increased stability in excised intestinal and liver homogenates, reduced hepatic extraction in rats	Yodoya *et al.* (1994)
Leu-enkephalin, DADLE	Peptidases	Coumarinic acid-based cyclic prodrug	Increased stability to Caco-2 peptidases and increased permeation	Gudmundsson *et al.* (1999a)
Leu-enkephalin, DADLE	Peptidases	Phenylpropionic acid-based cyclic prodrug	Increased stability to Caco-2 peptidases and increased permeation	Gudmundsson *et al.* (1999b)
Calcitonin	Peptidases	PEGylated	Increased stability in rat nasal mucosal homogenate	Na *et al.* (2004)

Table 1 (continued). Examples of Prodrugs to Reduce Presystemic Metabolism

were less effective in improving bioavailability (Hussain *et al.* 1987). A salicylate ester of naltrexone was also equally effective in increasing naltrexone oral bioavailability in dogs (Hussain and Shefter, 1988).

A much different approach to increasing the oral bioavailability of hydroxymorphinans, including nalbuphine and naltrexone, involved derivatization at a position distinct from that involved in presystemic metabolism. Boswell and

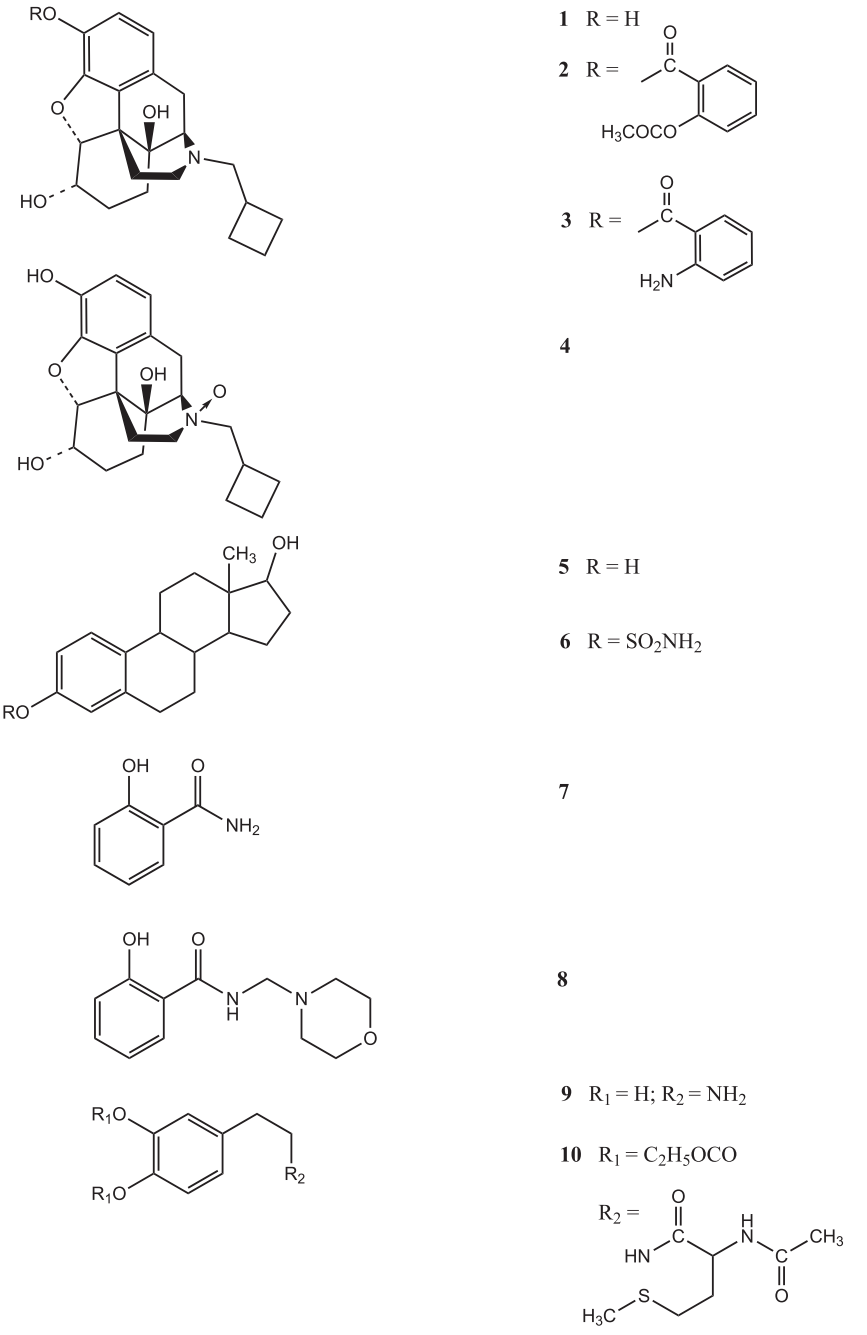

Figure 2. Structures of some of the prodrugs discussed as examples.

Myers (1985) presented results showing that the N-oxides of these compounds were converted to nalbuphine or naltrexone after oral administration to animals, and that oral bioavailability was markedly increased when dosed as the N-oxide. Nalbuphine bioavailability was increased 10-fold in dogs and more than 5-fold in rats when administered as nalbuphine N-oxide (**4**). This indicates that the prodrug approach does not necessarily require direct blocking at the site of metabolism on the molecule, but may only require identifying a prodrug derivative that is less susceptible to presystemic metabolism.

Estrogens, including estradiol (**5**) and ethinyl estradiol, are also subject to conjugation at the phenolic hydroxyl position, which results in incomplete bioavailability after oral dosing. A prodrug approach was tested based on the opioid prodrugs described above, in which the 3-hydroxy group of estradiol was blocked with anthranilate and acetylsalicylate esters. Relative to that after estradiol dosing, oral bioavailability in dogs was improved 17-fold with the anthranilate prodrug and 5-fold with the acetylsalicylate prodrug (Hussain *et al.*, 1988). Another prodrug of estradiol, the saccharinylmethyl ester, was shown to have 9-fold greater oral activity than estradiol in rats and provided approximately 5-fold greater bioavailability than oral estradiol (Patel *et al.*, 1995).

Estrone sulfate is a natural prodrug of estrone that has been used in estrogen replacement therapy. Another group of estrogen derivatives that have been described includes N,N-alkylated and non-alkylated sulfamates of estradiol (**6**), ethinyl estradiol, and estrone. These were shown to convert to the active estrogen after oral dosing in rats and provided potential for a more favorable side effect profile (Elger *et al.*, 1995). These compounds represent examples of a prodrug strategy that is quite different from that of the typical ester prodrugs. In this case, the systemic hydrolysis of the estrogen sulfamate to the parent estrogen was believed to occur efficiently, although the mechanism or tissue location of hydrolysis was not known.

Another phenolic compound that is subject to extensive presystemic metabolism and which has also been considered a candidate for prodrug derivatization is salicylamide (**7**). Rather than directly blocking the phenolic hydroxy position, as in the prodrug strategies described thus far, D'Souza *et al.* (1986) prepared an N-Mannich base, N-morpholinomethyl salicylamide (**8**). In this case, the conversion of the prodrug to the active drug is chemically mediated rather than requiring an enzymatic reaction. Bioavailability in rabbits was increased 2.3-fold, and plasma salicylamide reached C_{max} approximately 1 h after the oral dose. This serves as another example in which derivatization at the position of metabolism was not a requirement for increased oral bioavailability.

Dopamine (**9**) is highly metabolized after oral dosing, both at the amino position and on the catechol hydroxy groups. A group of prodrugs in which the substituents were added onto the amino group, catechol hydroxy groups, or both were prepared and tested (Murata *et al.*, 1989). One prodrug in which both sites of metabolism were protected, N-(N-acetyl-L-methionyl)O,O-biscarbonyl dopamine (**10**), increased dopamine bioavailability in dogs about 4-fold. Plasma concentrations of the metabolites, dopamine sulfate and 3,4-dihydroxyphenylacetic acid (DOPAC), were reduced.

Like dopamine, α-methyldopa is subject to presystemic metabolism by conjugation of the hydroxy groups, as well as metabolism by decarboxylation to methyldopamine. Two prodrugs in which the carboxylate was esterified, the succinimidoethyl and pivaloyloxyethyl esters, were intended to improve absorption in part through increased intestinal permeability (Vickers *et al.*, 1978). However, these could also reduce the extent of presystemic metabolism. Both prodrugs increased oral absorption in rats and humans. In humans it appeared that the succinimidoethyl ester was conjugated prior to the ester being hydrolyzed, whereas the pivaloyloxyethyl ester was hydrolyzed more rapidly than conjugation occurred. The pivaloyloxyethyl ester increased the apparent oral bioavailability of methyldopa in humans, as indicated by the percentage of the dose excreted in urine as methyldopa (Vickers *et al.*, 1984). The amount of methyldopa metabolite (sulfate conjugate) excreted in urine was reduced when the prodrug was administered.

Bambuterol is a bis-dimethylcarbamate prodrug of terbutaline. While this prodrug may reduce first-pass intestinal and hepatic metabolism, plasma concentrations were not significantly increased in humans; however, the time course of plasma concentrations was prolonged after prodrug dosing relative to that after terbutaline dosing (Holstein-Rathlou *et al.*, 1986). This prodrug may have the added advantage of providing a sustained release profile.

Propranolol is a beta-blocker that has had a major role in the treatment of hypertension and other cardiovascular diseases, even though it has relatively low oral bioavailability due to presystemic metabolism. Propranolol undergoes first-pass metabolism by both glucuronide conjugation at the β-hydroxy position and aromatic hydroxylation. In an effort to reduce the extent of first-pass conjugation, Garceau *et al.* (1978) prepared a hemisuccinate ester blocking the site of conjugative metabolism. Oral bioavailability in dogs was increased 8-fold. The hemisuccinate ester disappeared more rapidly from plasma after i.v. dosing in dogs than did propranolol. After oral dosing some prodrug was absorbed intact, and plasma prodrug concentrations were initially much greater than propranolol concentrations. Anderson *et al.* (1988) later prepared both hemisuccinate and acetate esters of propranolol. Both prodrugs afforded increased oral bioavailability of propranolol in rats. Shameem *et al.* (1993) described a series of β-hydroxy ester prodrugs of propranolol, which included straight and branched alkyl, acyloxyalkyl, and cycloalkyl substituents. Hydrolysis rates in buffer, dog plasma, and dog liver homogenate were determined. Oral bioavailability in dogs was increased 2-fold with the acetyl ester, 3-fold with butyryl and cyclopropanoyl esters, and 4-fold with an isovaleryl ester. The authors suggested that the prodrug should be lipophilic and stable in buffer and plasma but susceptible to hydrolysis by the liver, for optimal bioavailability.

Bioactive peptides have long been considered as potential drug candidates, but their poor biopharmaceutical properties (metabolic instability and poor membrane permeability) often make their delivery quite difficult. Various prodrug approaches have been employed to address the metabolic stability problems. These prodrugs could then be candidates for oral delivery or for

delivery via other transmucosal routes that may be more feasible than oral. Kahns and Bundgaard (1991) and Bundgaard and Rasmussen (1991) synthesized various prodrugs of peptides protecting the C-terminal amide group. While some derivatives did not improve metabolic stability, peptidyl-α-hydroxyglycine derivatization increased stability to chymotrypsin 7-fold to 75-fold. Similarly, N-α-hydroxyalkylation increased stability to carboxypeptidase. Kahns *et al.* (1993) also prepared various aliphatic esters and a carbonate ester as prodrugs of desmopressin (dDAVP), derivatizing the tyrosine phenolic group. These prodrugs were converted to dDAVP in human plasma or rabbit liver homogenate. The pivalate ester prodrug had increased stability to chymotrypsin and increased absorption through Caco-2 cells relative to dDAVP.

Another peptide that has been the subject of prodrug studies is tetragastrin. Yodoya *et al.* (1994) prepared acetyl, caproyl, and lauroyl derivatives and evaluated their stability in intestinal and liver homogenates and plasma. The degradation half-lives of these derivatives were significantly greater than that of tetragastrin. Gudmundsson *et al.* (1999a,b) prepared cyclic prodrugs of the peptides leucine enkephalin (Tyr-Gly-Gly-Phe-Leu) and DADLE (Tyr-D-Ala-Gly-Phe-D-Leu). In these cases the C- and N-terminals of the peptides were reversibly cyclized using coumarinic acid or phenylpropionic acid linkers. These prodrugs increased metabolic stability of the peptides to peptidases present in Caco-2 cells and increased the absorption of the peptides through the Caco-2 membranes. Finally, polyethylene glycol (PEG) derivatives of peptides have been considered as prodrugs. A PEGylated salmon calcitonin was shown to afford increased stability in a rat nasal mucosal homogenate, suggesting the potential for its use to improve nasal absorption (Na *et al.* 2004).

Obstacles to Success

Some of the previously described examples appear to be remarkably successful in improving oral bioavailability or having the potential to improve bioavailability. However, the majority of these cited prodrugs studies were preclinical studies and, unfortunately, many of these prodrugs have so far not become products for humans. Undoubtedly, many other prodrug strategies must have been attempted that have not been successful in preclinical studies and have not been described in the literature. Why is it that the prodrug approach for improving the delivery of drugs subject to presystemic metabolism, so simply depicted in Figure 1, has proven difficult to carry through from preclinical success to marketed products for humans? Two major factors would seem to be obstacles for achieving ultimate success with the prodrug approach. First, many prodrugs may be subject to sequential bioactivation to the active drug followed by immediate metabolism or elimination, prior to reaching the systemic circulation. Secondly, there are large inter-species differences in the activities of the enzymes both bioactivating the prodrug and degrading the active drug, making it difficult to develop screening assays that accurately predict the best properties for humans.

Sequential Metabolism

As shown in Figure 1, the intended outcome after oral administration of a prodrug is that the prodrug passes through the intestinal membrane and liver intact and is bioactivated upon reaching the systemic circulation. The other possibilities depicted are that the prodrug can be bioactivated in the intestine or liver and immediately metabolized, or bioactivated in the intestine and metabolized further in the liver. These occurrences would lead to failure of the prodrug approach. Since the intestine and liver have considerable levels of esterase activity, as well as other enzymes that may be involved in prodrug bioactivation, the sequential metabolism of a prodrug and its formed drug could be expected.

In a study of the metabolism of the dopamine prodrug N-(N-acetyl-L-methionyl)O,O-bisethoxycarbonyl dopamine, the relative proportions of prodrug, drug, and metabolite species were evaluated in portal and systemic blood of dogs after oral dosing (Yoshikawa *et al.* 1991). Portal blood C_{max} concentrations were in the order unchanged prodrug > deethoxycarbonylated prodrug > conjugated dopamine > other metabolites. Thus, the intestine performed mainly catechol ester hydrolysis. Systemic blood C_{max} concentrations were in the order conjugated dopamine > deethoxycarbonylated prodrug > homovanillic acid > dopamine. This shows that the liver performed both amide hydrolysis and conjugation reactions. Success of the prodrug depends on the relative rates of these reactions in intestine and liver.

Ximelagatran is a hydroxyamidine and ethyl ester double prodrug of the thrombin inhibitor melagatran, which affords improved oral absorption due to increased permeation. Although ximelagatran is absorbed at least 40–70% in rats, dogs and humans, the bioavailability of melagatran was 5–10% in rats, 10–50% in dogs, and 20% in humans (Ericksson *et al.*, 2003). Partial bioactivation of the prodrug with subsequent biliary excretion of the formed metabolites was suggested to account for the reduced bioavailability of the active compound, melagatran.

In vitro measurement of prodrug bioactivation and prodrug and drug metabolism in tissue preparations and plasma may be useful for predicting *in vivo* disposition, especially if *in vitro* versus *in vivo* correlations can be established. Obermeier *et al.* (1996) described a relationship between hydrolysis rates in rat liver S9 fraction and oral bioavailability in rats for a series of prodrugs of an angiotensin II antagonist. In this case, if hydrolysis was too slow, the prodrug was susceptible to metabolism by routes other than conversion to the active drug. Alternatively, if hydrolysis was too rapid, the prodrug provided little utility relative to the active drug itself. While optimization of hydrolysis rates is desirable, one problem with this strategy is that this prodrug optimization process would be for selection of prodrugs with optimum bioavailability in rats, whereas other species can have markedly different *in vitro* and *in vivo* properties.

Species-Dependence of Bioactivation

One of the most important criteria for evaluating prodrugs is the assessment of how rapidly the prodrugs are converted to the active drug. This is most often evaluated using *in vitro* studies of stability in buffer, plasma, or tissue homogenates or fractions. The goal is to predict bioactivation rates *in vivo* or at least to rank order a series of prodrugs. When prodrugs are converted to the active drug by enzymatic reaction, such as esterase hydrolysis, the conversion can be highly species-dependent. An example of this is given in Table 2, using results adapted from Aungst *et al.* (1987). Hydrolysis rates of these prodrugs had a consistent rank order in that the hydrolysis rate of **2** was always > **3**. However, each preclinical species had hydrolysis rates of both prodrugs that were greater than that in human plasma. For these prodrugs, hydrolysis rates in intestinal and liver homogenates from rats and dogs were greater than those in plasma. This suggests that after oral dosing, the intestine and liver should be the major sites of prodrug bioactivation. Hydrolysis rates of these prodrugs by liver and intestine were also species-dependent, but these were not evaluated using human tissues.

	Plasma Hydrolysis Half-Life (h)	
Prodrug	**2**	**3**
Rat	0.13	1.5
Dog	3.2	15.6
Monkey	0.6	3.3
Human	8.3	55.8

Table 2. Species-Dependence of *In Vitro* Plasma Hydrolysis of Nalbuphine-3-acetylsalicylate (2) and Nalbuphine-3-anthranilate (3) (Adapted from Aungst *et al.*, 1987).

Yoshigae *et al.* (1998a) examined the species differences in intestinal hydrolysis rates of propranolol ester prodrugs. Hydrolyase activity for intestinal microsomes had the rank order man > rat >> Caco-2 > dog. Hydrolyase activity for intestinal cytosol had the rank order rat > Caco-2 = man > dog. Dog showed stereoselective intestinal hydrolase activity, whereas rat and man did not. A separate study (Yoshigae *et al.*, 1998b) examining the species dependence of hydrolysis of these propranolol prodrugs in liver microsomes and cytosol showed that liver activities had a rank order opposite to that of intestine (e.g., dog > rat).

Addressing the Challenges

Inter-species differences in bioactivation rates of prodrugs, as seen in the examples described above, are typical. These differences certainly complicate the identification of prodrugs that are effective in humans. One possible approach to

avoiding this problem is to design prodrugs that react non-enzymatically to form the active drug. For example, an alternative prodrug approach to reducing the presystemic metabolism of phenol functional groups involved phenyl carbamates of N-substituted 2-aminobenzamides (Thomsen and Bundgaard, 1993). Liver, intestinal, or plasma enzymes did not affect conversion of these prodrugs to the active phenol, but prodrugs could be designed with conversion half-lives of 10–60 minutes.

Another approach could be designing prodrugs that are not subject to bioactivation upon passage through the intestinal membrane and liver but are selectively converted to the drug after reaching the systemic circulation. One group of prodrugs appearing to have this property was amino acid carbamates of phenolic drugs, which were not hydrolyzed by intestinal or liver enzymes but were readily cleaved in plasma (Hansen *et al.*, 1992). However, much more work is needed to investigate whether or how this approach could be accomplished.

Finally, it should be pointed out again that directly blocking the position on the molecule at which metabolism occurs is not a requirement for a successful prodrug application. Examples have been presented in which prodrugs were prepared and these prodrugs were apparently no longer susceptible to presystemic metabolism. The N-oxides of hydroxymorphinans increased their oral bioavailabilities even though the phenolic hydroxy group was unprotected. N-Mannich base prodrugs of salicylamide increased its oral bioavailability, but derivatization of the phenolic hydroxy group was not required.

Preclinical studies suggest great potential for the prodrug approach to increase oral bioavailabilities of drugs subject to presystemic metabolism. Much work may be required to develop an understanding of the inter-species differences in prodrug bioactivation rates and in drug metabolism rates. Those workers concerned with prodrug optimization must bear in mind these inter-species differences.

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2.4.1

Controlled Release - Small Molecules

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Table of Contents

List of Abbreviations

PD.....Prodrug
D.....Drug, parent molecule, or an active moiety
CR.....Controlled release
SR.....Sustained release
IV.....Intravenous administration
IM.....Intramuscular administration
SC.....Subcutaneous
k_{con} .....Rate constant for conversion of prodrug to drug in depot, injection site or in plasma (denoted as superscripts).
k_r .....Rate constant for release (1st order) or release rate (Zero order) for prodrug and drug from depot formulation at the injection site.
k_a .....Rate constant for absorption of prodrug and drug from depot at the injection site into plasma, lymphatic and tissue compartment (denoted as superscripts).
k_e .....Elimination rate constant for elimination of prodrug and drug from plasma (denoted as superscripts).
k_{nc} .....Rate constant for conversion of prodrug to other degradants or metabolites in depot, at injection site or in plasma.
Ko/w .....Oil/water partition coefficient
Flu.....Fluphenazine
Fluphenazine-D .....Fluphenazine Decanoate
FLU-D.....Fluphenazine Decanoate (in figures)
PK.....Pharmacokinetics
5-FU.....5-Fluorouracil

PLGA .....Poly-lactic-co-glycolic acid
5-ASA.....5-Aminosalicylic acid

Introduction

A prodrug is generally designed to improve the physical and chemical properties of a parent drug to enable formulation development, improve delivery, and/or achieve targeting. Similarly a prodrug approach can be used to enable development of a controlled release formulation, i.e., to provide a pharmacokinetic profile with sustained plasma levels. In the following discussion the term controlled release (CR) is used to include all prodrug approaches that result in sustained plasma levels over a dosing duration, thus reducing dosing frequency of the parent drug or the active moiety. CR technologies could include osmotic devices, polymeric matrix, implants, and microspheres and suspensions for parenteral depots that result in sustained plasma levels of the drug.

The following scheme (Figure 1) describes the potential means by which a prodrug can enable a controlled release pharmacokinetic profile for a drug molecule. The potential mechanisms that a prodrug could utilize to achieve a CR profile for an active agent can be classified as:

1. Modifying release rate from the dosage form.
2. Altering disposition properties such as absorption and/or tissue distribution to provide controlled release pharmacokinetic (PK) profile.
3. Designing an appropriate rate of conversion from prodrug to drug in plasma or at the site of absorption or target site to provide controlled release pharmacokinetic profile.

Any or all of the above mechanisms can be achieved by modification of the physical and chemical properties of the drug such as aqueous solubility, partition

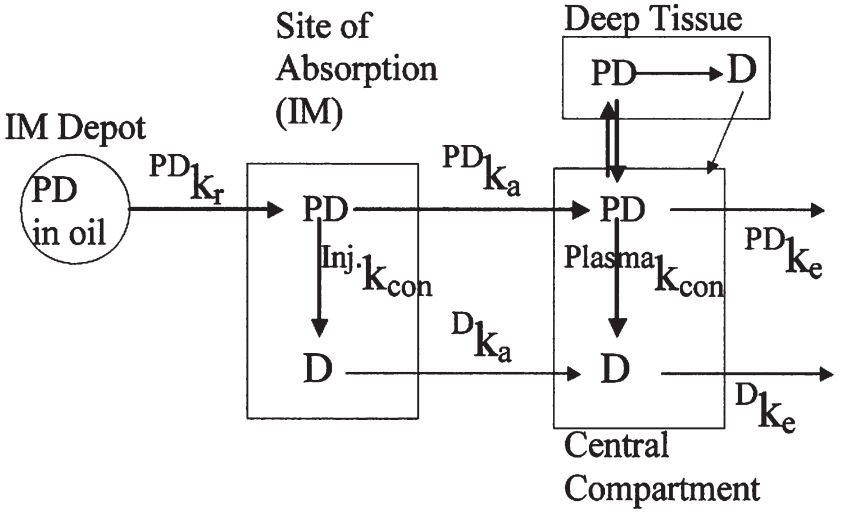

Figure 1. Schematic describing release, conversion, and pharmacokinetic rates that can be altered by a lipophilic prodrug to provide a controlled release profile for a parent drug upon IM administration. Emphasized arrows indicate the rate processes that can be controlled by designing a prodrug to achieve sustained plasma levels for the drug.

coefficient, and dissolution rate; these changes may have either a direct effect on the release of the active moiety or may alter a pharmacokinetic property such as the rate of absorption from the injection site and/or alter the tissue distribution.

Prodrugs for Oral CR

Prodrugs are generally considered new chemical entities and, hence, are infrequently designed to enable a CR formulation of a drug unless all attempts at CR formulations of the parent drug fail. This is particularly true for oral CR formulations since a number of CR technologies such as osmotic, matrix, and multiparticulates have been successfully developed for a variety of drug candidates with different physical and chemical properties. In addition, it is questionable how much benefit a prodrug can provide for CR, considering that the gastrointestinal retention and dosing duration for oral dosage forms cannot exceed 24 h.

Prodrugs for Parenteral SR Formulations

Parenteral sustained release (SR) formulations, conventionally known as depots, in contrast to oral CR formulations are generally designed to maintain plasma levels for more than a few weeks duration, preferably for a month or longer (Chien, 1981; Murdan and Florence, 2000). These formulations are usually administered by either a subcutaneous (SC) or an intramuscular (IM) injection, which provides sustained plasma levels for weeks to months. The parenteral route of administration thus precludes the use of CR technologies such as an osmotic device, which provide an active control over the release characteristics of the dosage form irrespective of the drug properties (Chien, 1981; Murdan and Florence, 2000). Recently, biodegradable polymer-based microspheres and *in situ* gelling depots have been designed to provide CR following IM and SC administration (Chien, 1981; Tipton and Dunn, 2000). Microspheres and gelling depots are limited by the dose, stability, and/or compatibility of the drug in organic solvents and utilize very complex manufacturing processes (Murdan and Florence, 2000, Tipton and Dunn, 2000). Moreover, a large burst is often observed when water-soluble drugs are delivered in these microspheres and gelling depot formulations. Classically, prodrugs have been very successfully used to enable development of parenteral depot products such as haloperidol decanoate and have demonstrated the benefit of the prodrug approach in enabling a monthly depot of a variety of drug compounds as described below (Chien, 1981, Murdan and Florence, 2000).

Lipophilic Prodrugs for IM and SC Depots

In this approach, a drug with an alcohol functional group is esterified with long-chain fatty acids to form the respective ester prodrug derivatives such as the decanoate, cypionate, valerate etc. The resultant prodrug is extremely lipophilic and oil soluble compared to the parent drug, thus enabling high doses of the

prodrug to be administered in relatively small injection volumes (see Table 1). A solution of the prodrug when administered by the IM or SC route as a solution in a vegetable oil (e.g., sesame oil) results in sustained plasma levels of the active drug for over a month (Chien, 1981). This approach has been successfully utilized for a number of drugs and the resultant marketed products with the relevant physical chemical and pharmacokinetic properties are listed in Table 1. As seen from Table 1, a majority of the prodrugs listed are practically insoluble in water; however, they are soluble in various oils, thus enabling formulation concentrations of 50–200 mg/mL. Therefore, dosing durations of 2–8 weeks for sustained plasma levels are possible. This prolonged exposure is possible even for drugs with relatively short biological half-lives. The decanoate ester is the most commonly used prodrug derivative due to its extremely high oil solubility; however, similar results are obtained with other long-chain fatty acid ester derivatives such as enanthate and palmitate. As seen from Table 1, a number of antipsychotics have been developed as IM depots using this prodrug concept since compliance is assured and rate of relapse of an acute psychotic episode is reduced significantly in this patient population (Cario *et al.*, 2003).

Mechanism of CR for Lipophilic Esters for Parenteral Depots

IM Absorption

Water-soluble small molecular weight molecules are absorbed rapidly upon IM administration. Even relatively larger sized carbohydrate molecules are rapidly absorbed after injection in a rabbit hind leg perfusion model (Nara *et al.*, 1992). Similarly, rapid absorption/transport should be expected for lipophilic molecules after IM administration either via the lymphatics or directly into the blood from the interstitial aqueous space in the muscle since it is highly vascularized. In a SC and IM absorption study in pigs utilizing gamma-scintigraphy, although no significant difference in the disappearance rate of various oils was observed, the half-life for disappearance of various oils was greater than 14 days (Larsen *et al.*, 2001). IM absorption of a variety of model compounds from various oils was found to correlate with their oil/water distribution coefficient (Ko/w) independent of the viscosity of the oil, suggesting that release from the oily depot is rate controlling (Tanaka *et al.*, 1974; Hirano *et al.*, 1981). Similarly, the IM absorption of testosterone propionate appeared to be related to the oil/water distribution coefficient; however, urinary elimination half-lives were considerably longer, which suggests a depot formation elsewhere following IM absorption (Al-Hindawi *et al.*, 1981, 1987). The long duration of plasma levels observed for lipophilic prodrugs such as haloperidol decanoate, however, cannot be explained solely by the slow absorption and/or release characteristics from IM dosing.

Drug Product (Proprietary Name) Therapeutic Class	Dosage Form or Delivery System	Formulation Concentration	Depot Dose (max 2 mL injection)	Duration or Dosing Interval	Apparent Elimination $t_{1/2}$ (parent compound)	Aqueous Solubility	References
Estradiol cypionate (Depot-Estradiol®) Class: estrogen	Solution in cottonseed oil	5 mg/mL	1 to 5 mg	3 to 4 weeks		Insoluble (< 0.1 mg/mL)	Parfitt 1999, Gennaro 1995, PDR 2004
Estradiol valerate (Delestrogen®) Class: estrogen	Solution in oil	10 mg/mL 20 mg/mL 40 mg/mL	10 to 20 mg	4 weeks		Insoluble (< 0.1 mg/mL)	Senior 2000, Gennaro 1995, PDR 2004
Fluphenazine decanoate (Prolixin®) Class: antipsychotic	Solution in sesame oil	25 mg/mL	25 mg	3 weeks	33 hours ($t_{1/2}$ of decanoate is14 to 26 days)	Insoluble (< 0.1 mg/mL)	Parfitt 1999, Drug Facts and Comparisons, 2004
Flupentixol decanoate (Depixol®, Depixol® Conc., and Depixol® Low Volume) Class: antipsychotic	Solution in fractionated coconut oil	20 mg/mL, 100 mg/mL, and 200 mg/mL	20 to 40 mg (max 300 mg)	2 to 4 weeks			Murdan and Florence 2000

Table 1. Commercially Available Non-Aqueous Depot Formulations Based on Lipophilic Ester Prodrugs of Active Drug Moiety and their Physical and Chemical Properties and Pharmacokinetic Parameters

Depot-Estradiol (brand of estradiol cypionate injection, USP) Package Insert

Depo®-Testosterone (testosterone cypionate injection, USP) Package Insert

Profile on Norethisterone enanthate. Monograf UBAT 176.

Drug Product (Proprietary Name) Therapeutic Class	Dosage Form or Delivery System	Formulation Concentration	Depot Dose (max 2 mL injection)	Duration or Dosing Interval	Apparent Elimination $t_{1/2}$ (parent compound)	Aqueous Solubility	References
Haloperidol decanoate (Haldol Decanoate 50 and Haldol Decanoate 100®) Class: antipsychotic	Solution in sesame oil	50 mg/mL and 100 mg/mL	Max 100 mg	4 weeks	21 hours ($t_{1/2}$ of decanoate is 21 days)	~0.1 mg/mL	Parfitt 1999, PDR 2004
Hydroxyprogesterone caproate (Hy-Gestrone®) Class: contraceptive	Solution in oil	125 mg/mL 250 mg/mL	375 mg	4 weeks		< 0.1 mg/mL	Senior 2000
Norethisterone enanthate (Noristerat®) Class: contraceptive	Solution in castor oil	200 mg/mL	200 mg	8 weeks	4 to13 hours		Murdan and Florence 2000
Nandrolone decanoate (Deca-Duralolin®) Class: anabolic steroid, erythro-poiesis stimulant	Solution in peanut oil	100 and 200 mg/mL	50 to 200 mg	1 to 3 weeks	6 to 8 hours	< 0.1 mg/mL	Parfitt 1999, PDR 2004
Nandrolone decanoate (Deca-Duralolin®) Class: anabolic steroid, erythro-poiesis stimulant	Solution in peanut oil	100 and 200 mg/mL	50 to 200 mg	1 to 3 weeks	6 to 8 hours	< 0.1 mg/mL	Parfitt 1999, PDR 2004

Drug Product (Proprietary Name) Therapeutic Class	Dosage Form or Delivery System	Formulation Concentration	Depot Dose (max 2 mL injection)	Duration or Dosing Interval	Apparent Elimination $t_{1/2}$ (parent compound)	Aqueous Solubility	References
Pipothiazine palmitate (Piportil Depot®) Class: antipsychotic	Solution in sesame oil	50 mg/mL	Up to 200 mg	1 to 4 weeks	8.8 h		Parfitt 1999, Gennaro 1995
Testosterone cypionate (Depot®-Testosterone) Class: androgen	Solution in cottonseed oil	100 mg/mL 200 mg/mL	Max 400 mg	2 to 4 weeks	~8 days	< 0.1 mg/mL	Parfitt 1999, PDR 2004
Testosterone enanthate (Delatestryl) Class: androgen	Solution in castor oil	100 mg/mL 200 mg/mL	Max 400 mg	2 to 4 weeks		< 0.1 mg/mL	Murdan and Florence 2000, Parfitt 1999
Zuclopenthixol decanoate (Clopixol® Depot) Class: antipsychotic	Fractionated coconut oil	200 mg/mL	100 to 600 mg	2 to 4 weeks	20 h		Parfitt 1999, Murdan and Florence 2000

Table 1 (Continued). Commercially Available Non-Aqueous Depot Formulations Based on Lipophilic Ester Prodrugs of Active Drug Moiety and their Physical and Chemical Properties and Pharmacokinetic Parameters

Depot-Estradiol (brand of estradiol cypionate injection, USP) Package Insert

Depo®-Testosterone (testosterone cypionate injection, USP) Package Insert

Profile on Norethisterone enanthate. Monograf UBAT 176.

Absorption and Disposition of Prodrugs from Oily Depots

One can propose two competing hypotheses for a CR mechanism from oily depots of prodrugs:

1. The sustained plasma levels of the drug is controlled by the slow conversion of the prodrug ester to the active moiety at the depot site or
2. The partitioning of the lipophilic ester and the oil from the depot into the lymphatic and fatty tissue and subsequent slower release of the prodrug into the plasma control the appearance of drug in plasma.

The first hypothesis is difficult to support due to the ubiquitous presence of esterase enzymes, the rapid conversion of various esters observed in plasma, and the highly vascular nature of muscle tissue, which results in fairly rapid conversion at the depot site as well. As described later using the example of fluphenazine (Flu), IM absorption and conversion of the decanoate ester to fluphenazine in plasma is fairly rapid. In contrast, it has been demonstrated that uptake of a series of testosterone esters into lymphatics and fatty tissue and its absorption from muscle is directly proportional to its partition coefficient supporting the second mechanism (Al-Hindawi *et al.*, 1981, 1987). However, until recently there have been very few reports delineating the mechanism by which these prodrug depots provide controlled release, and there are few pharmacokinetic studies that describe the complete PK profile of both the prodrug and the drug after parenteral administration of these depot dosage forms. Although depot haldol (haloperidol) is the most commonly used antipsychotic depot, the following case study with depot fluphenazine decanoate clearly demonstrated the mechanism by which this class of lipophilic ester prodrugs in oily depots provide sustained plasma levels over a long duration.

IV and IM Pharmacokinetics, and Mechanism of CR of Fluphenazine Decanoate Depot

Fluphenazine is an atypical antipsychotic of the phenothiazine class and its decanoate ester (fluphenazine-D) prodrug derivative was designed to develop an IM depot formulation (Table 1). The decanoate ester of fluphenazine at a solution concentration of 25 mg/mL in sesame oil has been approved as a depot formulation. The pharmacokinetics of fluphenazine and fluphenazine-D after IV and IM administration in dogs were evaluated by monitoring both fluphenazine and fluphenazine-D in plasma (Luo *et al.*, 1997). The elimination half-lives of both fluphenazine (6 h) and fluphenazine-D (3.5 h) when administered by the IV route were very rapid as seen in IV PK results in Figure 2; however, the apparent elimination half-life of fluphenazine (42.9 h) derived from IV dosing of fluphenazine-D was prolonged. Therefore, the longer apparent elimination half-life of fluphenazine must be due to slow conversion of decanoate ester to fluphenazine in plasma. However, an apparent elimination half-life of 42.9 h for fluphenazine derived from IV fluphenazine-D cannot totally explain the sustained

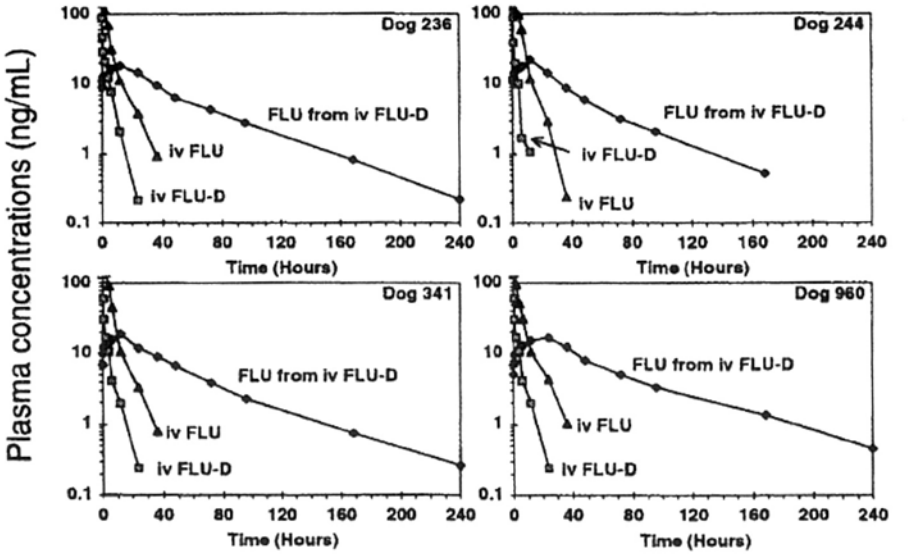

Figure 2. Plasma pharmacokinetic profile in dogs after intravenous administration of (a) fluphenazine HCl (FLU) in water (▲) (b) fluphenazine decanoate (FLU-D) in ethanol (■) and (c) Fluphenazine derived from its decanoate ester by conversion (◆). There was a wash out period of >3 months between the two treatments. (Reproduced with permission from Luo *et al.* 1998)

fluphenazine levels observed in plasma over 40 days after IM administration of fluphenazine-D solution in sesame oil (Figure 3).

In the IM PK study of fluphenazine in dogs, the PK parameters for fluphenazine free base were similar to those after IV administration; thus, fluphenazine base is rapidly absorbed in the muscle into systemic circulation (Figure 3) and absorption of fluphenazine in muscle is not rate limiting. However, levels of both fluphenazine-D and fluphenazine derived from the decanoate ester were sustained in plasma for a long period of time. The apparent elimination half-life of fluphenazine-D was 43-fold that of fluphenazine IV. These results suggested slow release and absorption of fluphenazine-D in the muscle. As seen in Figure 3, fluphenazine levels were sustained with very little fluctuations over a 40-day period, demonstrating the success of the prodrug concept for this IM depot formulation.

The earlier higher depot levels of fluphenazine after IM administration of fluphenazine-D were explained by rapid conversion in the liver in contrast to plasma as both absorption of fluphenazine-D from muscle and its conversion to fluphenazine in plasma are relatively slow processes. The slow disappearance of oils and the dissolved lipophilic drug from muscle has been demonstrated. The absorbed lipophilic drug may be transported by the lymphatics, further protecting the prodrug from plasma esterase-induced conversion and thus contributing to prolonged release of fluphenazine. Lipophilcity of fluphenazine-D was postulated to result in partitioning into the deep tissue compartment. The slower release of the decanoate from the deep compartment along with conversion in systemic

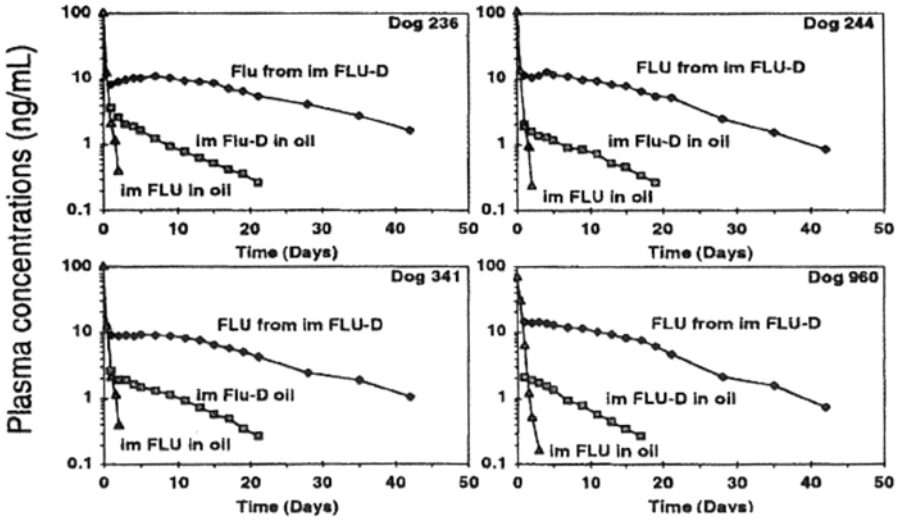

Figure 3. Plasma pharmacokinetic profile in dogs after intramuscular administration of (a) fluphenazine base (FLU) in sesame oil (▲) (b) Fluphenazine decanoate (FLU-D) in sesame oil (■), and (c) fluphenazine derived from its decanoate ester by conversion (◆). There was a washout period of >3 months between the two treatments. (Reproduced with permission from Luo *et al.* 1998)

circulation may contribute to sustained levels of fluphenazine in plasma later in the dosing period.

To determine if the majority of lipophilic ester is indeed absorbed by lymphatics draining the muscle rather than blood and to confirm the mechanism of sustained release from depot, the investigators studied the release and presystemic absorption of fluphenazine-D and fluphenazine administered IM in rats (Luo *et al.*, 1998). Although prodrug and drug both demonstrated exponential decline from the IM injection site, prodrug levels were present at the injection site 28 days post-dose while fluphenazine concentrations were very low, suggesting limited conversion at the injection site. Since fluphenazine base administered IM as a solution in oil was rapidly released and absorbed, fluphenazine must partition quickly from the oil into the interstitial aqueous phase. In contrast, the lipophilic decanoate ester, by slowing the partitioning of the prodrug into interstitial aqueous phase, essentially controlled the release from the depot. The ester prodrug, however, appeared to be absorbed primarily into the iliac and hypogastric lymph nodes closer to the injection site, which appears to be dependent on the slow partitioning out of the oil into the muscle interstitial space and lymphatics. In contrast, when fluphenazine was administered as a solution in oil, levels in proximal lymph nodes were significantly lower; this suggests a smaller extent of partitioning of the hydrophilic free base compared to that of the decanoate ester prodrug. Also, comparatively higher levels of fluphenazine were observed in lymphatics following IM administration of the decanoate ester in comparison to fluphenazine free base. This observation implies that the conversion occurs in the lymphatics and that fluphenazine in plasma is

derived primarily from fluphenazine-D and not from absorption of converted fluphenazine in muscle. Therefore, the rate-controlling steps for appearance of fluphenazine in plasma appears to be slow partitioning of the prodrug from the oily depot at the injection site into the proximal lymph nodes with subsequent hydrolytic activation occurring primarily in the lymph nodes. This is also consistent with the much faster *in vitro* release rate of haloperidol from sesame oil compared to its decanoate ester; an argument can be made that the oil would not control the release of the hydrophilic parent drug for absorption into the systemic circulation (Radd *et al.*, 1985).

The mechanism for release of fluphenazine-D described above was developed using an excellent combination of various PK studies that suggest it is primarily the increased lipophilicity caused by forming the decanoate ester that results in the modification of various release and PK parameters/rates. One can further suppose that this mechanism should be consistent for all lipophilic prodrugs, particularly, decanoate esters, when administered IM irrespective of the physico-chemical properties of the parent molecule. Based upon the above discussion, Figure 4 depicts the primary rate-controlling mechanisms for lipophilic ester prodrugs such as decanoate esters of fluphenazine and haloperidol in providing sustained plasma levels of the parent active moiety when administered as prodrug solutions in oil. The rate-limiting processes appears to be:

1. Partitioning of prodrug from oil at depot injection site into lymphatics.
2. Slow conversion of prodrug to drug in lymphatics.
3. Partitioning of ester prodrug into blood from lymphatics.

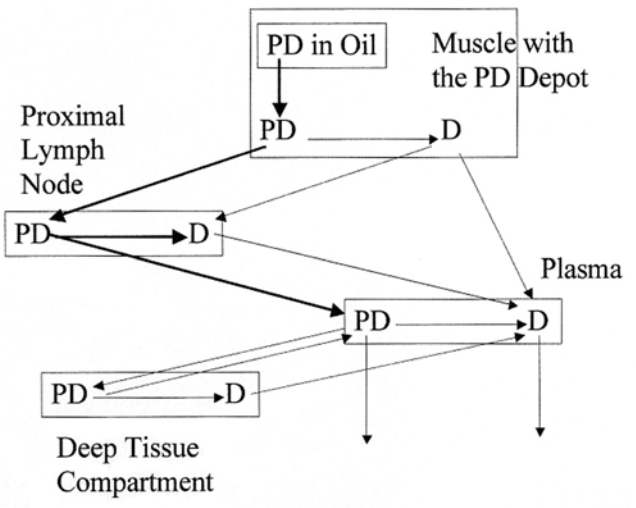

Figure 4. Revised schematic describing the mechanisms by which a prodrug when administered in oil-based IM depot can provide sustained plasma levels of the parent drug by controlling the release, conversion and pharmacokinetic rates. (Bold arrows indicate rates that are known to be rate limiting while dotted arrows indicate rates that have not been confirmed to be rate limiting.)

It is not clear whether the ester prodrug partitions extensively into the deep tissue compartment, thus providing sustained levels; however, when fluphenazine-D was administered by the IV route, fluphenazine levels did decline slowly but not to the same extent as after IM administration. This suggests that fluphenazine-D uptake into the deep tissue compartment does occur; however, it cannot completely account for the prolonged sustained behavior seen after IM dosing.

Dissolution Controlled Depots Based on Prodrugs

The attempt in this type of depot formulation is to control the appearance of drug in systemic circulation by controlling its release from the depot through slowing the dissolution rate of the drug. The Noyes-Whitney equation predicts that dissolution rate is directly proportional to solubility under steady-state conditions. If the solubility of a drug is high at physiological pH, slow release may require the use of sparingly soluble salts and/or poorly soluble complexes of the drug to provide SR from a depot formulation. For example, penicillin depots were developed by forming the penicillin G procaine and penicillin G benzathine salts (Chien *et al.*, 1981; Senior, 2000). Utilizing this same concept, a variety of progestin, metabolic, and anabolic steroids were esterified to form the acetate ester prodrugs, which have very low aqueous solubility and, when administered IM as aqueous suspensions, provide prolonged plasma levels (Table 2). Depo-Provera, an aqueous suspension of medroxyprogesterone acetate, a contraceptive, is a long-acting (3 months) IM depot dosage form utilizing this concept. The apparent elimination half-life of medroxyprogesterone is ~50 h; when it is administered as the acetate ester, it provides even longer duration of sustained plasma levels, potentially due to the low intrinsic dissolution rate of the acetate ester. A partial *in vivo* conversion of medroxyprogesterone acetate to medroxyprogesterone is established, however, systemic preclinical and clinical PK results are not available to demonstrate the complete mechanism of release. Clearly, however, sustained exposure to the medroxyprogesterone ester has been shown in clinical studies (Physician's Desk Reference, 2004).

Parenteral SC or IM SR formulations based on poly-lactic-co-glycolic acid (PLGA) provide long duration of release, often exceeding 3 months for water-soluble drugs (Tipton and Dunn, 2000). However, they are associated with a burst that can be as large as 30% of the total dose (Tipton and Dunn, 2000). To reduce this burst effect with these formulations, the less soluble pamoate salt or esters of the active drug can be used. *In vitro* release of various nalbuphine ester prodrugs with different hydrophilic characteristics from PLGA matrices showed higher release rates for the more hydrophilic prodrugs, suggesting that prodrugs can be used as a tool to achieve the desired release rate even from active matrix-controlled drug delivery systems (Sung *et al.*, 1998). This further demonstrates the utility of prodrugs for parenteral SR formulations even from biodegradable polymer-based depot formulations.

Drug Product (Proprietary Name) Therapeutic Class	Dosage Form or Delivery System	Formulation Concentration	Depot Dose (max 2 mL injection)	Duration or Dosing Interval	Apparent Elimination $t_{1/2}$ (parent compound)	Aqueous Solubility	Reference
Dexamethasone acetate (Decadron-LA®) Class: adrenocortical steroid	Aqueous suspension	8 and 16 mg/mL	8 and 16 mg	1 to 3 weeks 1 to 3 weeks		Insoluble (< 0.1 mg/mL)	Parfitt 1999, Drug Facts and Comparisons 2004
Medroxyprogesterone acetate (Depo-Provera®) Class: contraceptive	Aqueous suspension	150 mg/mL	150 mg	13 weeks	Approx. 50 hours	Insoluble (< 0.1 mg/mL)	Gennaro 1995
Medroxyprogesterone acetate (MPA) and estradiol (E_2) cypionate (Lunelle®) Class: contraceptive	Aqueous suspension	25 mg MPA and 5 mg E_2 per 0.5 mL	MPA: 25 mg E_2: 5 mg	28 to 30 days	MPA: 50 hours E_2: 4 to 5 hours	Both Insoluble (< 0.1 mg/mL)	Gennaro 1995
Methylprednisolone acetate (Depot Medrol®) Class: corticosteroid	Aqueous suspension	20, 40, and 80 mg/mL	40 to 120 mg	1 week	2 to 3 hours	0.7 mg/mL	Parfitt 1999, Gennaro 1995, PDR 2004
Triamcinolone acetonide (Tac®) Class: cortocosteroid	Aqueous suspension	3 mg/mL 10 mg/mL 40 mg/mL	40 to 100 mg	6 weeks	2 to 3 hours	0.1 to 1 mg/mL	Senior 2000, Parfitt 1999
Triamcinolone diacetate (Aristocort Forte®) Class: cortocosteroid	Aqueous suspension	25 mg/mL 40 mg/mL	40 to 80 mg	1 week	2 to 3 hours	< 0.1 mg/mL	Senior 2000, Drug Facts and Comparisons 2004, Parfitt 1999

Table 2. Commercially Available Depot Formulations Based on Aqueous Suspensions of Poorly Water Soluble Prodrugs of Active Drug Moiety and their Physical and Chemical and Pharmacokinetic Properties.

Depot-Medrol® (methylprednisolone acetate injectable suspension, USP) Package Insert

Depo-Provera Contraceptive Injection (medroxyprogesterone acetate injectable suspension, USP) Package Insert

Lunelle Monthly Contraceptive Injection (medroxyprogesterone acetate and estradiol cypionate injectable suspension) Package Insert

Modified Release Formulations Using Prodrugs

Recently there has been increasing interest in modifying the release characteristics of the formulation by designing a prodrug to achieve a specific therapeutic objective or to enhance the efficacy of the drug. Although some of these concepts are still at a preclinical stage, they demonstrate the formulation release characteristics problems that can be addressed by a prodrug approach.

Colonic Delivery

Prodrugs of 5-aminosalicylic acid (5-ASA) such as sulfasalazine and olsalazine containing an azo bond have been designed for colonic delivery in the treatment of ulcerative colitis (Sandborn, 2002). 5-ASA administered orally is absorbed rapidly in the proximal small intestine, thus insufficient 5-ASA reaches the colon to treat the ulcerative colitis. Sulfasalazine releases 5-ASA by selective reduction of azo bond via the action of bacterial azo-reductases in the colonic lumen; this results in a site-specific release of 5-ASA. Cyclodextrin prodrugs of biphenylacetic acid were investigated to provide delayed release of parent drug to the colon (Minami *et al.*, 1998). Cyclodextrin conjugates are hydrophilic and thus not absorbed in the upper gastrointestinal tract; however, the bacterial and endogenous enzymes in the cecum and colon can cleave them to release the parent drug.

Ophthalmic Delivery

Constant removal of foreign matter instilled in the eye by tear secretions generally reduces the effective half-life of drug and exposure of the cornea to the drug, resulting in the need for frequent administration. Analogous to the concept of using lipophilic ester prodrugs for parenteral sustained release, attempts have been made to provide prolonged release in the eye using prodrugs. Butyryl and palmitoyl ester prodrugs of tilisolol were synthesized and incorporated into hydrogel polymer inserts for the treatment of glaucoma (Kawakami *et al.*, 2001). The *in vitro* release of the lipophilic ester was prolonged compared to that of the parent drug with the more lipophilic palmitoyl prodrug releasing only in the presence of serum albumin. The aqueous humor concentrations of tilisolol were prolonged with a lower C_{max} from both prodrug inserts compared to the active drug insert Thus, *in vivo* controlled release of the prodrug from the polymer insert also resulted in higher selectivity with higher ophthalmic/plasma exposure ratios for the butyryl prodrug compared to that of the parent drug and the palmitoyl ester prodrug inserts.

In another ophthalmic condition known as proliferative vitreoretinopathy, intravitreal injection of 5-fluorouracil (5-FU), an antimetabolite, can prevent the rapid uncontrolled growth of ocular cells within the vitreous cavity of the eye. However, 5-FU is rapidly eliminated from the eye (apparent $t_{1/2}$ <3.5 h) since it is extremely hydrophilic and is rapidly dissolved and cleared. Two groups have

investigated the use of lipophilic N_1-alkoxycarbonyl (Steffansen *et al.*, 1996) and N1-alkylcarbonyl (Jolimaitre *et al.*, 2003) prodrugs of 5-FU to increase its solubility in silicone oil, which can be directly administered by intravitreal injection. This concept showed slower release rates of the prodrug from silicone oil with an inverse relationship between surrogate release rates from the oil with log P. The *in vivo* studies should demonstrate if this specific condition and the unique release requirement could be addressed using a prodrug approach.

Site-Directed Delivery

The concept of designing a lipophilic ester prodrug has been applied to a variety of delivery systems and formulations as described above; however, it is unclear if a prodrug can be used to provide targeted delivery. Continuous administration of 17-β-estradiol, a principal estrogen, is required as an estrogen replacement therapy for the treatment of post-menopausal symptoms. Sustained delivery of the estrogen using an intravaginal ring would provide improved patient compliance since it is a relatively non-invasive local delivery device that can be self-administered with minimal systemic side effects. However the intravaginal rings are composed of hydrophobic polydimethylsiloxane in which 17-β-estradiol has poor solubility due to its polarity. A series of hydrophobic ester prodrugs of 17-β-estradiol was designed in which their solubility was significantly improved in the polymer and the release rate was modified (Woolfson *et al.*, 1999). In a clinical study, the 3-acetate ester of 17-β-estradiol delivered from a vaginal ring as estrogen replacement therapy maintained clinically desirable plasma levels for 84 days. This study demonstrated the advantage of using an appropriate prodrug for the delivery system to achieve targeted local delivery.

Prodrugs of mitomycin-C and colchicines have been designed to enhance anti-tumor efficacy either by providing tumor-specific release or by controlling the release duration (Baker *et al.*, 1996; Song *et al.*, 1996). In another interesting application, diclofenac was derivatized with a bisphosphonate moiety in an attempt to increase delivery to the bone (Hirabayashi *et al.*, 2001). The diclofenac prodrug was found to be predominantly distributed to the skeleton with a relatively long half-life after IV administration, thus demonstrating a potential site-specific delivery system to the bone. One of the problematic conditions is prosthetic valve endocarditis, which can be intractable and is sometimes fatal; it is usually treated with systemic antibiotics requiring frequent administration. To address this problem, a lipophilic palmitate derivative of clindamycin was developed as a local implant and shown to provide plasma levels for two weeks following implantation in a preclinical model (Cimbollek *et al.*, 1995).

The above examples demonstrate a wide variety of formulation and drug delivery challenges that may be addressed by prodrugs; however, robust efficacy and improvement of therapy needs to be demonstrated clinically as exemplified by the decanoate ester-based antipsychotic depot products.

In summary, prodrugs of small drug molecules have been successfully used to provide controlled, sustained, and/or modified release characteristics for the

active moiety for a variety of different compounds by various routes of administration. This has involved modifying the physical and chemical properties of the active drug by increasing the lipophilicity and/or reducing aqueous solubility. These prodrugs also have a direct impact on *in vivo* PK disposition, which is primarily responsible for providing controlled or sustained release from parenteral depot products.

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2.4.2

Controlled Release - Macromolecular Prodrugs

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Table of Contents

List of Abbreviations

AUC .....area under plasma concentration-time curve
CNBr .....cyanogen bromide
DMARD.....Disease modifying anti-rheumatic agent
DS .....degree of substitution
5-FUdR.....5-fluoro-2'-deoxyuridine
HA.....hylauronic acid
HPG .....N^5-(3-hydroxypropyl)-L-glutamine
HP(SEC) .....high performance size-exclusion chromatography
i.v.....intra venous
Leu .....leucine
M_n .....number average molecular weight
M_W.....weight average molecular weight
μ .....ionic strength
NCE .....new chemical entity
NSAID.....non-steroidal anti-inflammatory drug
PHEA .....poly[α,β-(N-2-hydroxyethyl)aspartamide]
PHPG .....poly-N^5-(3-hydroxypropyl)-L-glutamine
pHPMA .....poly[N-(2-hydroxypropyl)methacrylamide]
RES .....reticuloendothelial system
SEC .....size-exclusion chromatography
σ.....Hammett substituent parameter

Introduction

The majority of drug candidates have *molecular weights* of about 200–500 Dalton. Bioreversible derivatives obtained by covalent attachment of a promoiety similar in size to drug candidates can be referred to as low molecular weight prodrugs. This approach has been used to improve drug performance by overcoming various barriers to drug delivery. When the barrier is target site access and the target site can only be reached after transport via the systemic circulation, the *in vivo* fate of the drug/prodrug is affected by distribution processes, protein binding, and excretion, not to mention the range of metabolic reactions that prodrugs may undergo. The latter processes are influenced by the physicochemical properties of the prodrug derivative. However, these are less predictable when using the low molecular weight prodrug approach to optimize systemic site-specific drug delivery. The macromolecular prodrug approach, in which the small therapeutic agent is attached to a macromolecular promoiety, has been exploited to provide drug targeting. The basic rationale behind this approach is that the transport properties of the macromolecular prodrug should be dictated predominantly by those of the macromolecular transport vector. Thus, macromolecular conjugates derived from a wide array of macromolecules endowed with intrinsic target receptor affinities, especially of anticancer agents and other therapeutics have been evaluated.

Other important objectives may be achieved by employing high molecular weight compounds devoid of any apparent specificity for discrete cell-surface receptors: (a) stabilization of the therapeutic agent, (b) enhancement/modification of drug solubility, (c) improvement of circulation life time, and (d) extended duration of drug action (Larsen, 1989a). Development of innovative sustained- and controlled-release drug delivery systems is highly warranted, not least in the area of parenteral depot formulations. In an industrial setting the macromolecular prodrug approach has to compete with other potential depot technologies characterized by physical incorporation of the active agent into a polymeric matrix. Initially, cost/risk benefit considerations may advocate for the selection of one or more formulation principles rather than a macromolecular prodrug solution. This is primarily based on the fact that regulatory authorities consider a prodrug of a lead compound as a NCE requiring extensive toxicological qualification (see, for example, Anderson and Langone, 1999). Potential changes in immunological responses resulting from covalent linkage of small ligands to *per se* biocompatible polymeric compounds are one major concern. On the other hand, macromolecule-based chemical drug delivery systems may show superior performance in certain therapeutic modalities and should, in comparison to other depot technologies, be recognized as an alternative avenue rather than a second or third option to solving drug delivery problems.

A wide variety of biodegradable synthetic and natural polymeric drug carriers have been studied (see, for example, Soyez *et al.*, 1996). However, in the present contribution we will focus mainly on the potential utility of high molecular weight bioreversible derivatives derived from relatively simple polymer backbones containing a multitude of hydroxyl (OH)-functional groups ready for derivati-

sation such as (i) polysaccharides including dextrans, (ii) N-hydroxyalkylated polyglutamines and polyaspartamides, and (iii) N-hydroxyalkylated vinyl polymers including polymers of N-(2-hydroxypropyl)methacrylamide (Fig. 1). First, these were chosen because immunogenicity appears to be correlated with the structure and complexity of the macromolecule as discussed by Bennett (1990a). Second, the employment of these more homogenous macromolecules is expected to reduce costs of attachment chemistry and to facilitate the subsequent physicochemical characterization of the macromolecular conjugates (prodrugs). Adequate characterization of these macromolecular prodrugs prior to biological evaluation is of utmost importance since (i) the onset and duration of action of macromolecular prodrugs are dependent on the mode and the rate of release of the active agent from the conjugate, and (ii) the intended pharmacokinetic profile of the conjugate might be altered as a consequence of changes in, for example, molecular weight distribution, drug load/character, and electric charge.

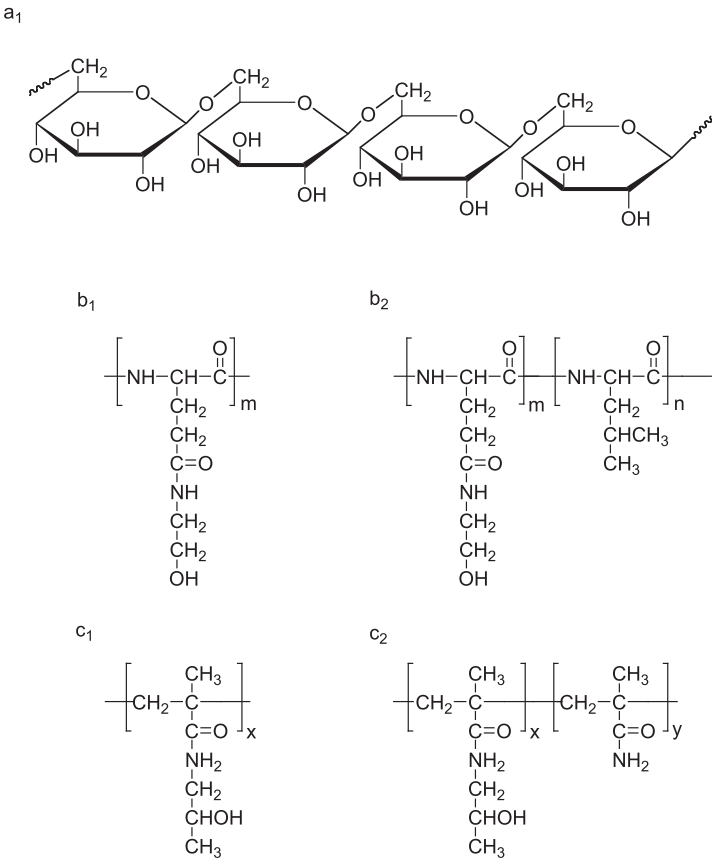


Figure 1. Examples of structures of polymeric transport vectors (macromoles) containing multiple OH-functional groups: dextran (a); poly[N^5-(2-hydroxyethyl)glutamine] (b_1), leucine copolymer (b_2); poly[N-(2-hydroxypropyl)methacrylamide] (c_1), methacrylamide copolymer (c_2) used in the preparation of macromolecular prodrugs.

In principle, macromolecular prodrugs may provide extended duration of drug action after various routes of administration (Table 1). After i.v. injection conjugates exhibiting prolonged residence times in circulation can release the drug in a controlled manner or in the case of passive targeting in the vicinity of the target site. As briefly reviewed, lack of detailed knowledge about mechanisms

1.	Intravenous injection Drug release from long-circulating conjugates into blood or in case of passive targeting in vicinity of target site
2.	Non-intravenous injection Drug release in local compartment (e.g. intra-articularly) or from immobile s.c. instilled conjugate for systemic action
3.	Oral administration Drug release in restricted areas of the gastrointestinal tract for local or systemic action
4.	Pulmonary administration* Drug release from particulate-based macromolecular prodrugs for local or systemic action

Table 1. Examples of how macromolecular prodrugs can result in controlled drug release attainable from different routes of administration.

* Potential applicability discussed by Zeng *et al.* (1995).

and important physicochemical variables that control the *in vivo* disposition, including reticuloendothelial system (RES) uptake, constitutes at present a severe barrier to extensive exploitation of the macromolecular prodrug approach. Alternatively, sustained activity, to be exerted by the locally released drug or after entering the systemic circulation, might be accomplished after local (non-i.v.) administration of such derivatives. To this end we will discuss various aspects of potential relevance for controlled delivery of drugs after oral, subcutaneous, and intra-articular instillation of macromolecular prodrugs. Here, the range of desired release rates may not be governed solely by the hydrolytic lability of the established covalent linkage between the drug and the promoiety but other factors might be used to modify the drug release profile. These include manipulation of aqueous solubility and the use of macromolecular prodrugs as an integrated part of various particulate drug delivery systems, the latter eventually being designed to be formed *in situ* at the administration site (Hatefi and Amsden, 2002). Further, emphasis is placed on different *in vitro* models used for the determination of release profiles from long-acting conjugates.

Structure and Stability of Established Macromolecule-Drug Bond

Water-soluble macromolecular prodrugs can be created using relatively simple chemistries to structures that can be very complex. The rate of drug regeneration from the macromolecular prodrug can be governed by pH-dependent hydrolysis and/or enzyme-mediated bond cleavage (Fig. 2). Prodrug derivatives, in which the drug is linked directly to or near the polymer backbone, may act as a depot releasing the active agent in a predictable manner. Based on the present knowledge, it is to be expected that in most cases regeneration rates of the parent

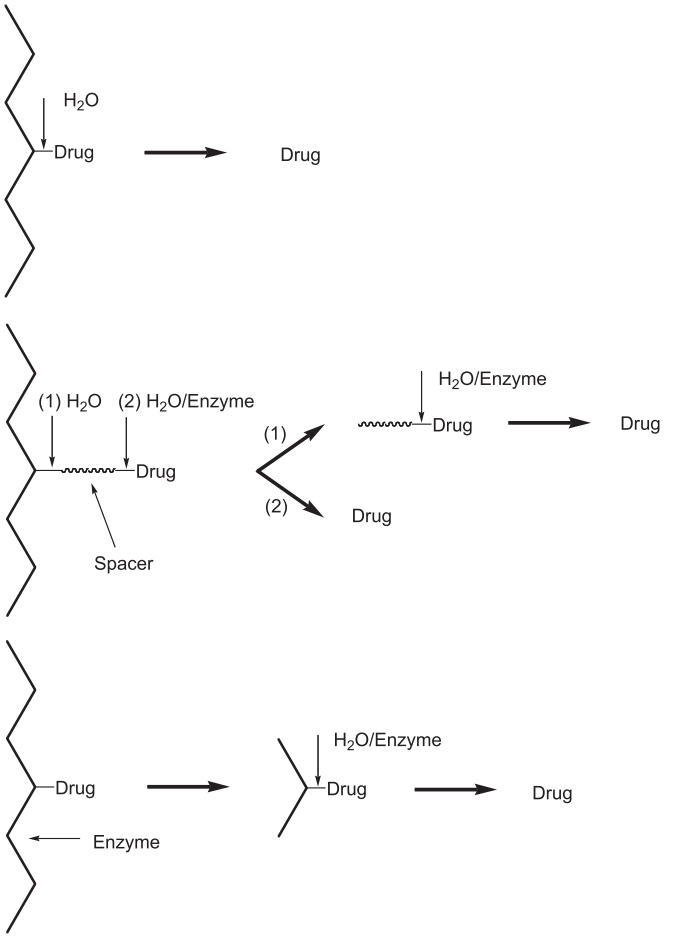


Figure 2. Modes of drug release from macromolecular prodrugs.

drug are confined to pH-dependent hydrolysis since the bulky polymer matrix most often renders the hydrolytic centre inaccessible to enzymatic attack. In particular environments (colon and intracellular lysosomal compartments) concerted enzyme action, however, can lead to drug liberation where one enzyme type is responsible for cleavage of the polymer matrix, allowing smaller drug

containing fragments to be substrates for various hydrolases. Placing a spacer arm between the drug and the carrier may serve three purposes. First, the terminal functional group of the spacer arm can be varied, thus providing a more appropriate covalent attachment of the drug through a variety of chemical bonds to the polymer backbone. Second, enzyme cleavage/release of the drug might be augmented due to the increased distance between the drug and the polymer chain. Third, sequentially labile prodrugs can be constructed in such a way that pH-dependent hydrolysis predominantly liberates the spacer-drug derivative (a corresponding low molecular weight prodrug). In this latter case, after parenteral administration, the macromolecular derivative might therefore act as a local depot, releasing the low molecular weight prodrug that after extravasion or diffusion from the injection site is activated at the target site.

Drug attachment directly to or near the polymer backbone

Covalent linkage of typical drug functional groups (R-COOH, R-OH, R-NH_2) to polymer hydroxyl groups can be established in the form of carboxylic acid, carbonate, and carbamate ester bonds (Fig. 3). Reacting amines with cyanogen bromide activated polysaccharides may further lead to isourea and imidocarbonate structures (see, for example, Larsen, 1990). Whereas several conjugates involving different prodrug bond types have been tested *in vivo*, investigations of the kinetics of hydrolysis of macromolecular prodrugs are primarily limited to studies of the pH-dependent hydrolytic stability of carboxylic acid and carbonate ester derivatives as discussed below. The results obtained indicate that the cleavability of the macromolecule-drug bond is influenced by steric and electronic

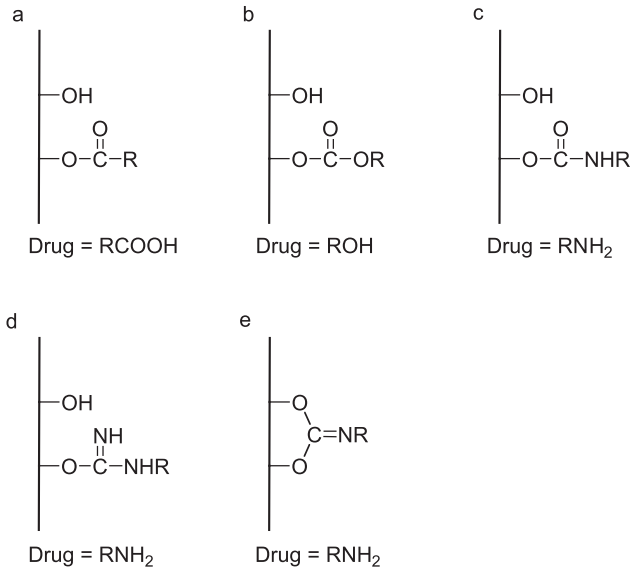


Figure 3. Examples of drug attachment directly or close to polymer backbones containing OH-group functionalities in the form of: a: carboxylic acid ester bond; b: carbonate ester bond; c: carbamate ester bond; d: isourea structure; e: cyclic imidocarbonate structure.

effects in a manner similar to that observed for low molecular weight ester derivatives. The data do not allow for extrapolation to the behaviour of other bond types. However, it is tempting to suggest that both kinetics and mechanism of hydrolysis of macromolecule-drug bonds bear resemblance to those of corresponding low molecular weight prodrug analogues in those cases where the polymer neighbouring group effects on bond cleavage can be neglected.

The basic knowledge of the stability of carboxylic acid esters of polyhydroxy compounds originates from kinetic studies of the hydrolysis of model dextran benzoic acid/*para*-substituted benzoic acid ester derivatives (Larsen and Johansen, 1985; Johansen and Larsen, 1985a; Larsen *et al.*, 1986). Stability studies of the benzoic acid esters of dextran were conducted in aqueous solution over the pH range 3–9.8. U-shaped pH-rate profiles were obtained and the second-order rate constants for specific acid-, water-, and hydroxide ion-catalyzed hydrolysis of the conjugates are shown in Table 2. At pH 7.4, almost identical rates of hydrolysis were found for conjugates with varying molecular weight (10,000–110,000 Dalton) and degree of substitution (3.4–15.8% w/w). In addition, the pH-rate profiles in weak alkaline solution for ethyl and isopropyl benzoate were constructed. The calculated second-order rate constants (k_{OH}) for the latter two compounds are included in Table 2; they show an expected 5-fold increase in the rate of alkaline

	k_H (M^{-1} min^{-1})	k_{H2O} (M^{-1} min^{-1})	k_{OH} (M^{-1} min^{-1})
O-Benzoyl dextran (DS 9.1%)	1.37×10^{-2}	2.3×10^{-9}	232
Ethyl benzoate			15.5
Isopropyl benzoate			3.2

Table 2. Catalytic rate constants for hydrolysis of O-benzoyl dextran, ethyl benzoate, and isopropyl benzoate at 60ºC and $\mu = 0.5$. From Larsen and Johansen (1985)

hydrolysis of ethyl benzoate compared to the isopropyl ester. A comparison between the values for the hydroxide ion catalytic rate constants for hydrolysis of isopropyl benzoate and the dextran benzoic acid esters, esters of secondary alcohols, revealed a 70-fold greater reactivity of the macromolecular ester derivative to specific base-catalyzed hydrolysis. Increased susceptibility of dextran-naproxen ester conjugates towards hydrolytic degradation (pH 6–10) in proportion to naproxen ethyl ester was also observed (Harboe *et al.*, 1988a). The enhanced lability of the dextran esters in alkaline solution might most likely be attributed to the lower pK_a value of the dextran hydroxy groups (11.8 at 37ºC (Larsen, 1989c)) versus those of the aliphatic alcohols since in alkaline ester hydrolysis increasing degradation rates are generally observed with decreasing pK_a of the alcoholic leaving group. This hypothesis is consistent with the

Drug/model drug	Polymer	Bond type	$k_{obs} \times 10^3$ (h^{-1})	References
4-substituted benzoic acids:				
4-H	Dextran	ES	3.8	Larsen and Johansen (1985); Johansen and Larsen (1985a)
4-NO_2	Dextran	ES	86.6[a]	Larsen *et al.* (1986)
4-Cl	Dextran	ES	9.5[a]	Larsen *et al.* (1986)
4-CH_3	Dextran	ES	2.1[a]	Larsen *et al.* (1986)
4-CH_3O	Dextran	ES	1.3[a]	Larsen *et al.* (1986)
Naproxen	Dextran	ES	3.6	Larsen and Johansen (1989)
Ibuprofen	Dextran	ES	4.8	Larsen and Johansen (1989)
Diclofenac	Dextran	ES	17.8	Larsen and Johansen (1989)
Ketoprofen	Dextran	ES	7.4	Larsen and Johansen (1989)
Fenoprofen	Dextran	ES	5.3	Larsen and Johansen (1989)
Naproxen	HES[b]	ES	0.34[a]	Larsen (1989b)
Naproxen	SST[c]	ES	0.81[a]	Larsen (1989b)
Ketoprofen	PHEA	ES	0.10[a]	Jaksic *et al.* (1996)
Fenoprofen	PHEA	ES	0.04[a]	van der Merwe *et al.* (2002)
L-Dopa	PHEA	ES	63.6	Zorc *et al.* (1993)
α-Methyldopa	PHEA	ES	41.5	Zorc *et al.* (1993)
Benzyl alcohol	Dextran	CE	10.8	Weibel *et al.* (1991)

Table 3. Pseudo-first-order rate constants, k_{obs}, for hydrolysis of carboxylic acid ester (ES) and carbonate ester (CE) derivatives derived from polysaccharides and poly[α,β-(N-2-hydroxyethyl)aspartamides], PHEA, in aqueous solution pH 7.4 and 37°C.

[a]From extrapolation assuming specific base catalysis for pH above 7

[b]Hydroxyethyl starch

[c]Soluble starch (Ph. Eur.)

observation that ketoprofen and fenoprofen esters of poly[α,β-(N-2-hydroxyethyl)apartamides] are significantly less labile at pH 7.4 (37°C) than are the corresponding dextran ester derivatives (Table 3). The reactivities of various *para*-substituted benzoic acid ester derivatives of dextran (see Table 3) towards alkaline hydrolysis were assessed from degradation studies performed in borate buffer, pH 8.7 (60°C). Substitution in the 4-position had a significant effect on alkaline hydrolysis rates. The influence was predominantly electronic in nature and the rate data were well correlated by the Hammett equation (Larsen *et al.*, 1986):

$$\log k_{obs} = 1.73\sigma - 2.20 \ (n = 5, r = 0.987) \qquad (1)$$

The calculated Hammett reaction constant of 1.73 (Eq. 1) is of the same order of magnitude as that reported for alkaline hydrolysis of substituted ethyl benzoates (Washkuhn *et al.*, 1971). Practically identical stabilities of the benzoic acid-dextran conjugates were found in aqueous buffer pH 7.4 and in 80% human plasma, strongly suggesting that hydrolysis in plasma proceeds without enzyme catalysis. Similarly, insignificant enzyme-facilitated hydrolysis of various NSAID-dextran conjugates after incubation in different biological media (Table 4) and of ketoprofen-PHEA esters exposed to several enzyme systems (Jaksic *et al.*, 1996) was observed, indicating that in case of drug attachment close to polymer matrix

NSAID	Dextran[a]	DS[b]	5% Liver homogenate		20% Human synovial fluid	80% Plasma			Buffer pH 7.4
			Pig	Rabbit		Pig	Human	Rabbit	
Naproxen	T-70	5.6	n.d.[c]	133	211	178	172	176	175
Naproxen	T-10	9.9	128	126	194	161	134	126	179
Naproxen	T-500	6.6	183	191	217	n.d.	169	n.d.	183
Ketoprofen	T-10	5.2	88	102	120	203	74	89	93
Fenoprofen	T-10	7.4	66	n.d.	n.d.	94	115	98	130
Ibuprofen	T-70	3.9	77	n.d.	n.d.	159	123	n.d.	143
Diclofenac	T-10	1.1	36	n.d.	n.d.	n.d.	29	n.d.	39

Table 4. Regeneration half-lives (hours) of NSAID compounds from their corresponding dextran ester prodrugs in different biological media (pH 7.40 and 37°C). From Larsen and Johansen (1989)

[a]The dextran T-fractions refer to Pharmacia samples

[b]DS, degree of substitution (% w/w)

[c]n.d., not determined

it is to be expected that most often the macromolecular carrier will protect the prodrug bond against enzymatic attack.

Coupling of hydroxyl group containing drugs to polysaccharides in the form of carbonate esters has been reported for the steroids estrone and testosterone (Yolles, 1978; Yolles *et al.*, 1979). A study of release rates of the fertility control agents from hydroxypropylcellulose-based conjugates *in vitro* revealed a release rate of estrone of 2.3 mg/day. The liberation of testosterone proceeded somewhat slower most likely reflecting that the phenolic carbonate ester bond (estrone) is more sensitive to hydrolytic degradation than carbonate esters derived from aliphatic alcohols (testosterone). The U-shaped pH-rate profile obtained from the hydrolysis of model benzyl dextran carbonate ester conjugates with degrees of substitution in the range 2.5–10.4% w/w (Fig. 4) is fairly similar to those of dextran esters of carboxylic acids. In buffer pH 7.4 and in 80% human plasma (37°C) half-lives close to 100 h were calculated (Weibel *et al.*, 1991) which are of the same order of magnitude as those found for the hydrolysis of dextran NSAID ester derivatives (Table 4). Stability data for hydrolysis of trifluorethyl benzyl carbonate ester in alkaline solution are also shown in Fig. 4. The pK_a value for trifluorethanol is about 12.4 and comparable to that of dextran (11.8). Thus, the corresponding

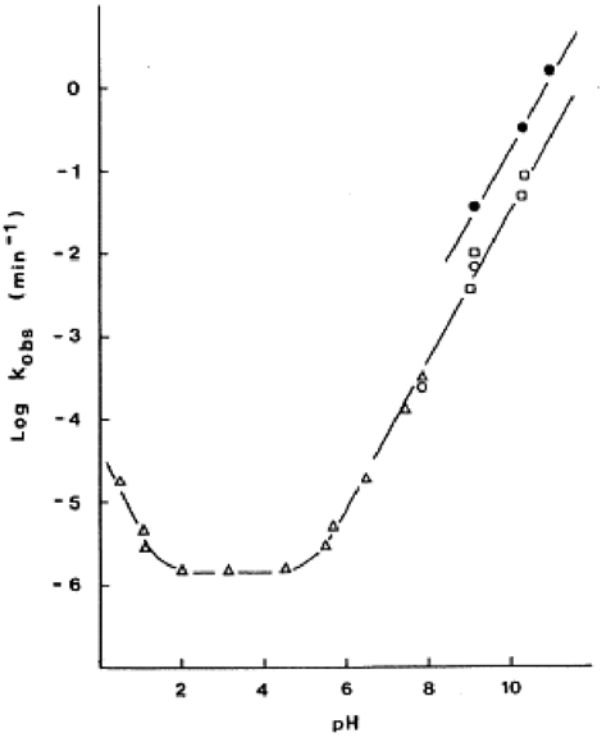
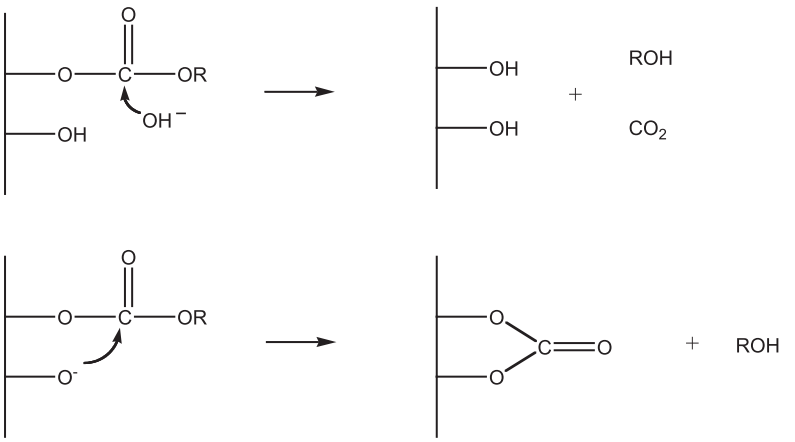


Figure 4. The pH-rate profiles for hydrolysis of benzyl-dextran carbonate ester (DS = 5.7) (DS is the degree of substitution defined as the percentage of mg drug released per mg of the conjugate) and trifluorethyl benzyl carbonate ester (●) at 37°C and μ = 0.5. The symbols (△, ○, □) refer to rate constants derived by various methods. Reprinted from Weibel *et al.* (1991) with permission from Elsevier.

alkoxide ions are expected to exhibit similar leaving abilities. The observed enhanced hydrolytic lability of trifluorethyl benzyl carbonate may indicate that the occurrence of an intramolecularly catalyzed hydrolysis reaction as depicted in Scheme 1 is not favorable for benzyl carbonate ester conjugates. Phenolate anions, in particular 4-nitrophenolate, are much better leaving groups than the benzyl



Scheme 1. Hydroxide ion and intramolecular alkoxide ion catalyzed hydrolysis of dextran carbonate esters.

alkoxide ion. Therefore, in case of 4-nitrophenyl dextran carbonate esters, intramolecular catalysis effected by a neighbouring carbohydrate alkoxide ion to yield a transient cyclic carbonate ester structure may compete with the intermolecular hydroxide ion-catalyzed hydrolysis of the carbonate ester bond (Schacht *et al.*, 1985).

Incorporation of a Spacer Arm

The application of the spacer group between the macromolecule and the drug has hitherto been employed predominantly to allow drug attachment in cases where the functional groups of the active agent and/or the carrier have not allowed direct attachment. In the field of drug targeting, considerable effort has been devoted to the design of conjugates containing oligopeptide spacers that, after pinocytic internalization, release the active principle by action of various lysosomal enzymes (see, for example, Soyez *et al.*, 1996).

Whereas the drug-spacer bond, per definition, has to be cleavable, the spacer-matrix linkage might be stable or cleavable. Examples of spacer arm chemical structures employed in coupling drugs containing various functional groups to dextran are presented in Table 5. Most of these conjugates are probably unable to regenerate parent dextran *in vivo* since the established spacer-dextran bonds appear to be rather stable under physiological conditions where only dextran-

Amine group	|-O-CO-NH-$(CH_2)_5$-COOH[a]	Mitomycin C	Hashida *et al.* (1983)
	|-O-(C=N)-NH-$(CH_2)_n$-COOH[a]	Mitomycin C	Takakura *et al.* (1989)
	|-O-$(CH_2)_5$-COOH	Mitomycin C	Takakura *et al.* (1987)
	|-NH-$(CH_2)_n$-COOH[b]	Procainamide	Schacht *et al.* (1984)
Carbonyl group	|-O-CH_2-CO-NH-NH_2	Daunorubicin	Hurwitz *et al.* (1980)
Hydroxy group	|-NH-$(CH_2)_{10}$-NH-CO-$(CH_2)_6$-COOH	5-FUdR	Onishi *et al.* (1987)
	|-O-CH_2-CO-NH-$(CH_2)_6$-NH-CO-$(CH_2)_2$-COOH	Acyclovir	Rosemeyer and Seela (1984)
	|-O-CO-$(CH_2)_n$-COOH	Metronidazole	Larsen *et al.* (1988)
		Dexamethasone	McLeod *et al.* (1993)
		Methylprednisolone	McLeod *et al.* (1993); Mehvar *et al.* (2000)
		Zidovudine[c]	Giammona *et al.* (1999)
Carboxylic acid	|-NH-$(CH_2)_2$-OH[b]	Naproxen	Azori *et al.* (1986)
	|-O-CO-CH_2-OH	Naproxen	Larsen (1989b)
	|-NH-$(CH_2)_{10}$-NH_2[b]	Methotrexate	Onishi and Nagai (1986)
	|-O-$(CH_2)_2$-NH_2	Methotrexate	Chu and Whiteley (1977)

Table 5. Examples of spacer arm chemical structures employed in coupling of drugs containing various functional groups to dextran.

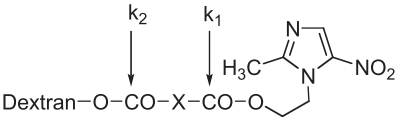
[a]Spacer linked to CNBr-activated dextran

[b]Spacer linked to dialdehyde dextran followed by reductive amination

[c]Linkage to PHEA backbone

spacer ester linkages are with certainty split *in vivo* within a reasonable period of time.

A dicarboxylic acid spacer is often used to attach OH-group-containing drugs to dextrans. For example, succinic and glutaric acid have been used to link metronidazole to dextrans (Larsen and Johansen, 1987; Larsen *et al.*, 1987, 1988). As apparent from Scheme 2, parallel formation of metronidazole and the spacer-drug derivative resulted from the hydrolysis of the dextran conjugates with the apparent first-order degradation rate constant, k_{obs}, given as the sum of the two first-order rate constants k_1 and k_2. From degradation kinetic studies in aqueous



Spacer arm (X):

CH_2CH_2 (succinic acid) (Dextran-MMS)

$CH_2CH_2CH_2$ (glutaric acid) (Dextran-MMG)

Scheme 2. Structures of dextran ester prodrugs of metronidazole. The arrows indicate points of nucleophilic attack associated with the rate constants for the formation of metronidazole (k_1) and the spacer-drug derivative (k_2).

buffer solution, U-shaped pH-rate profiles were obtained. In each case, the conjugates exhibited comparable stability in aqueous buffer and in human plasma (Table 6), indicating that the terminal metronidazole ester linkage due to steric hindrance is not subject to plasma enzyme catalyzed hydrolysis. This suggestion is

	0.05 M phosphate pH 7.40		80% human plasma		5% pig liver homogenate	
	k_{obs} (h^{-1})	$t_{1/2}$ (h)	k_{obs} (h^{-1})	$t_{1/2}$ (h)	k_{obs} (h^{-1})	$t_{1/2}$ (h)
Dextran-MMS	2.16×10^{-2}	32.1	1.2×10^{-2}	57.8	4.8×10^{-2}	14.4
Dextran-MMG	1.37×10^{-2}	50.6	1.3×10^{-2}	53.3	2.4×10^{-2}	28.9

Table 6. Stability of dextran ester conjugates of metronidazole monosuccinate (Dextran-MMS) and metronidazole monoglutarate (Dextran-MMG) in 0.05 M phosphate buffer pH 7.40, 80% human plasma, and 5% pig liver homogenate at 37°C. From Larsen *et al.* (1988)

supported by the fact that the environment in the vicinity of the terminal drug-ester bond appears uncharged at physiological pH. Thus, the chemical structure to be attacked by enzymes may show resemblance to those of the butyrate and valerate esters of metronidazole, which are both extremely good substrates for plasma hydrolases (Johansen and Larsen, 1985b). Compared with plasma, the degradation of the two conjugates proceeded somewhat faster in 5% pig liver homogenate (Table 6). This might simply reflect different enzymes operating in the two biological matrices. Conversely, it has to be taken into consideration that liver dextranases indirectly may accelerate the regeneration of parent metronidazole and the corresponding hemiester derivatives from the conjugates as discussed later.

In Fig. 5, the ratios k_2/k_1 associated with the formation of metronidazole and the hemiester derivatives from the dextran prodrugs are plotted against pH (Larsen *et al.*, 1988). The data clearly demonstrate a reversal of the relative rates of hydrolysis of the dextran-spacer ester linkage compared with the conjugate metronidazole monosuccinate/monoglutarate ester bond going from acid- to base-catalyzed hydrolysis. This behaviour is in accordance with the fact that base-catalyzed ester hydrolysis is influenced by both polar and steric effects whereas steric effects are expected to dominate in acid-catalyzed hydrolysis. It appears that formation of the hemiester derivative accounts for 60–70% of the total degradation of the derivatives at physiological pH. Compared with metronidazole (pK_a about 16), much smaller k_2/k_1 ratios are, a priori, expected for hydrolysis of dextran hemiester conjugates of, for example, steroids such as methylprednisolone and hydrocortisone exhibiting pK_a values of about 11 (Hansen and Bundgaard, 1979) and phenolic drugs (pK_a 9–10). Also, intramolecularly catalyzed hydrolysis of the latter types of dicarboxylic acid hemiesters (Gaetjens and Morawetz, 1960; Thanassi and Bruice, 1966) might contribute to rapid appearance of the active drug *in vivo*. To this end glucocorticoid dextran

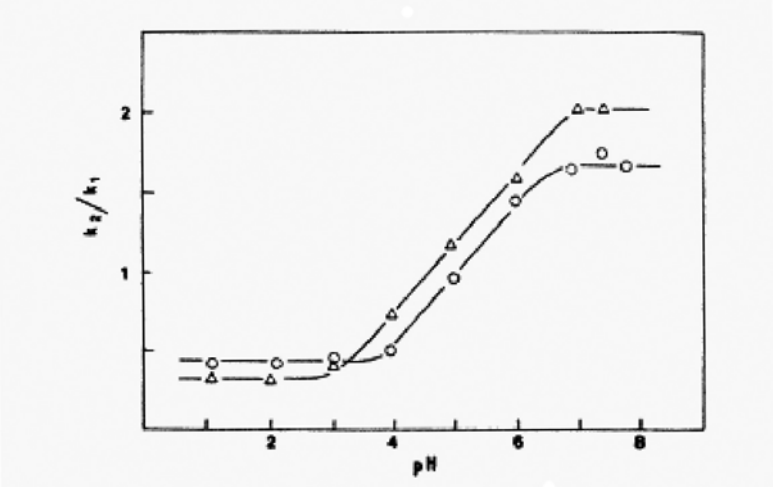
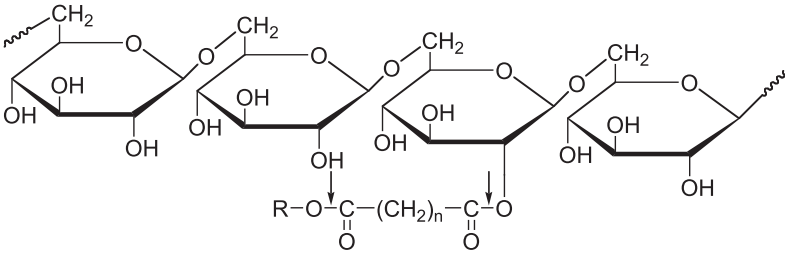


Figure 5. The ratio of the first-order rate constants k_2 and k_1 (see Scheme 2 for definition) as a function of pH (37°C; μ = 0.5). The ratios refer to the dextran ester conjugates of metronidazole monosuccinate (○) and metronidazole hemiglutarate (△). Reprinted from Larsen et al. (1988) with permission from Elsevier.



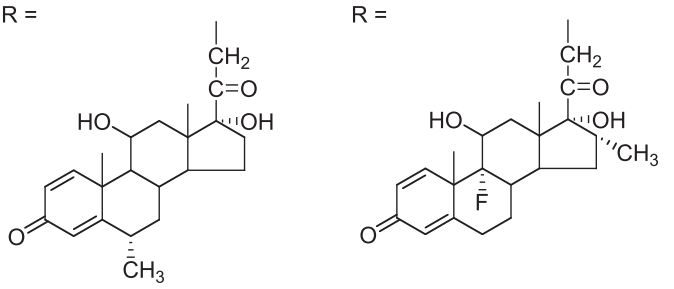


Figure 6. Chemical structures of glucocorticoid-dextran conjugates. Arrows indicate possible sites of ester hydrolysis. Redrawn from McLeod *et al.*, (1993).

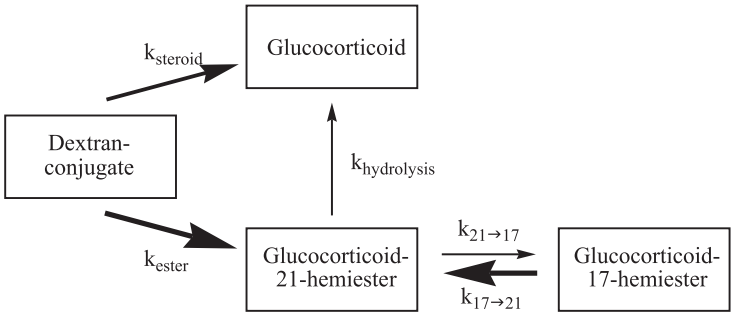


Figure 7. Schematic model showing ester hydrolysis and acyl migration pathways. The thickness of the arrows indicate the relative magnitude of the rate constants. Adopted from McLeod *et al.* (1993).

hemiesters (Fig. 6) have been investigated for varying goals, including possible application to colon site-specific drug delivery (McLeod *et al.*, 1993) and utility in connection with liver transplantation. This was rationalized by the observation that dextran derivatives tend to accumulate in the liver upon i.v. administration (Mehvar *et al.*, 2000). Product analyses from hydrolysis studies of these glucocorticoid prodrugs performed in buffer pH 7.0–7.4 and in rat plasma revealed k_2/k_1 ratios comparable to those reported for the dextran metronidazole hemiester conjugates. Acyl migration equilibria between the steroid C-21- and 17-positions (Fig. 7) may only partly explain the lack of enhanced hydrolytic lability of the terminal spacer-steroid ester bond.

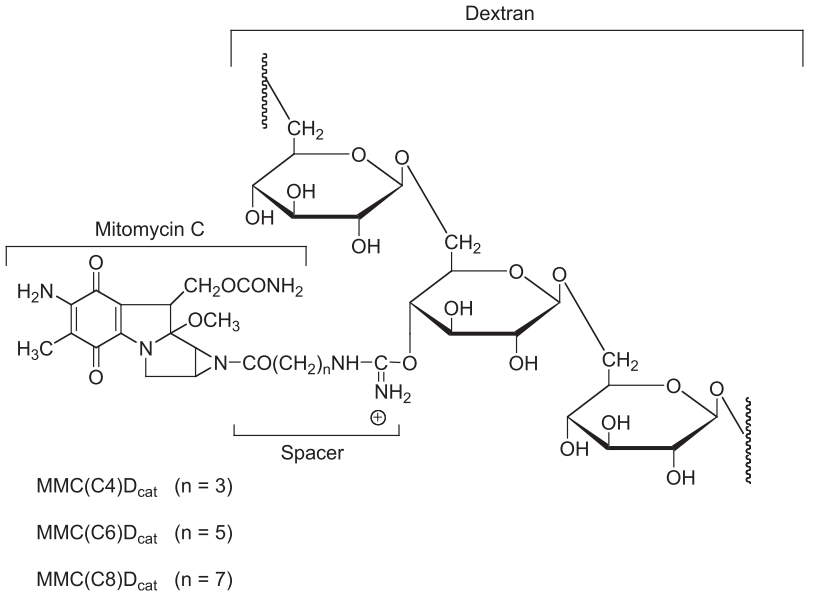


Figure 8. Chemical structures of mitomycin C-dextran conjugates with different spacer arms. Adopted from Takakura *et al.* (1989).

Mitomycin C conjugates of dextran (Fig. 8) have been synthesized employing ω-aminocarboxylic acids (Takakura *et al.*, 1989). The amine function was attached to CNBr-activated dextran and, in addition to the isourea linkage shown, other types of spacer-matrix chemical bonds are presumably present. As with dextran-drug hemiester conjugates, comparable release rates of antitumor mitomycin C release were observed after incubation of the conjugates in aqueous buffer and various tissue homogenates. Surprisingly, the derivatives derived from γ-aminobutyric acid, ε-aminocaproic acid, and ω-aminocaprylic acid differed considerably with respect to rate of amide bond cleavage with half-lives of 11, 24.2, and 42.2 h (pH 7.4, 37oC), respectively. It was suggested that the difference in regeneration rates may involve variable neighbouring group participation in the breakdown of the prodrug amide bond.

Inspection of the spacer arm structures presented in Table 5 reveals that several of the employed spacers should provide sufficient spacing of the drug from the polymer backbone, thus allowing enzyme access to the drug-spacer bond. However, apparently only in the case of a 5-FudR-dextran conjugate (Onishi *et al.*, 1987) and a zidovudine-PHEA conjugate (Giammona *et al.*, 1999) was enzyme-facilitated liberation of the liganded drug possibly operating.

Sequential drug liberation mechanism involving initial enzyme-mediated cleavage of the polymer backbone

Diminished steric protection of the drug-matrix bond towards the action of various hydrolases may result from initial depolymerization of the polymer backbone (Fig. 2). The inspiration to study the fate of dextran prodrugs after oral administration arose from the observation that a starch-nicotinic acid ester prodrug administered orally to rats provided a low but almost constant plasma concentration of the antilipolytic agent for about 12 h (Ferruti, 1981). Also, preliminary studies in the rabbit revealed significant bioavailability of naproxen after oral administration of model naproxen-dextran ester conjugates (Harboe *et al.*, 1988b). Naproxen was attached to dextran fractions in the M_w range 10,000–500,000 and absorption percentages relative to an equimolar oral dose of parent naproxen were observed to be about 60%. Oral bioavailability data obtained in the pig gave an average absorption of 91%, consistent with those found using the rodent animal model (Harboe *et al.*, 1989a). Average naproxen plasma concentration-time curves from 3 pigs after oral administration of a solution of naproxen and an equivalent molar dose of a dextran T-70-naproxen ester prodrug (dextran T-70 referring to a Pharmacia dextran lot possessing a M_w of about 70,000) are depicted in Fig. 9. The animals were fasted for 20 h prior to drug administration and during the experiments. Pure hydrolytic release of naproxen from the conjugates cannot account for the initial phase of systemic drug absorption. The pH-rate profile for hydrolysis of the naproxen conjugate exhibited a U-shape with almost equal degradation rates at pH 1 and 7.4 (k_{obs} of about 4×10^{-3} h^{-1} at 37°C corresponding to a minimum half-life of drug release close to 175 h (Larsen and Johansen, 1989). Taking into account that extensive

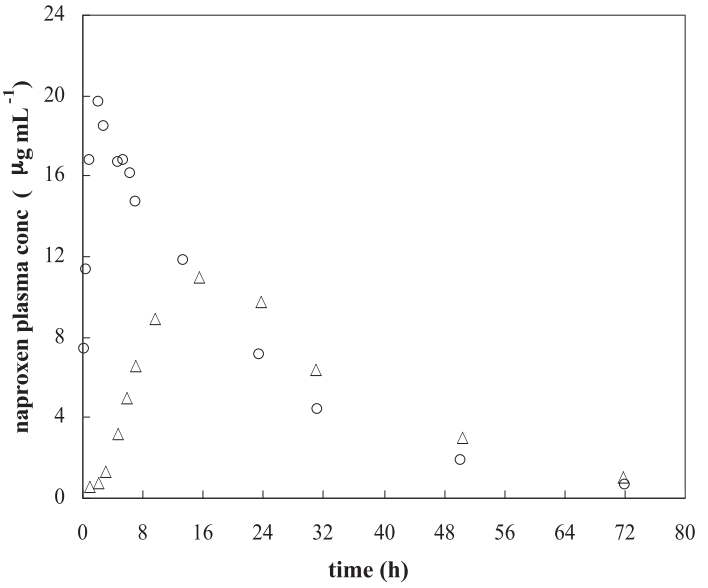


Figure 9. Average naproxen plasma concentration-time curves after oral administration of an aqueous solution of naproxen (3.6 mg per kg body weight) (○) and an equivalent dose of a dextran T-70 conjugate (DS 8.2) (△) in 3 pigs (40–44 kg). Reprinted from Harboe et al. (1989a) with permission from Elsevier.

absorption of intact prodrug was unlikely, it was suggested that naproxen release in the GI tract involved the action of one or more enzyme systems.

Further insight into the mechanism of drug release was obtained by studying drug liberation rates after incubation of dextran-naproxen ester prodrugs in homogenates of various segments of the pig gastrointestinal tract with their contents (Table 7) (Larsen *et al.*, 1989). Naproxen was liberated much faster in caecum and colon homogenates than in stomach and small intestine homogenates. Direct evidence of fragmentation of the dextran backbone was accomplished using high-performance size-exclusion chromatography (HP(SEC)) analysis of the prodrugs in colon homogenates (Larsen *et al.*, 1989). In HP(SEC) polymers are eluted according to their molecular weight with the larger molecules having the shorter retention times. Chromatograms of a dextran T-70 prodrug incubated in a colon homogenate revealed that a reduction in peak height of the conjugate was accompanied by an increase in the peak width and, based on peak area measurements, the degradation kinetics showed indications of zero-order kinetics. The chromatograms provided evidence of breakdown of the dextran chains while naproxen was still attached to the carrier, since only the drug was responsible for the fluorescence detector response. In colon homogenate experiments, the initial rate of dextran depolymerization was observed to decrease as a function of the amount of parent dextran added to the reaction mixtures (Fig. 10).

Based on the above observations and results from more comprehensive bioavailability studies, it appears reasonable to suggest that dextran conjugates are

Homogenate/buffer[a]	Rabbit	Pig		
	v_i (μg ml^{-1}/h)	v_i (μg ml^{-1}/h)	pH (start)	pH (end)
Stomach	-	11.2	7.3	7.5
Duodenum	7.1	7.4	7.4	7.4
Jejunum	6.0	7.1	7.4	7.4
Ileum	6.3	8.1	7.3	7.5
Cecum	65.1	86.5	7.4	7.5
Colon I	32.6	107.0	7.4	7.4
Colon II	-	111.2	7.4	7.4
0.1 M phosphate pH 7.40	6.6	6.6	-	-

Table 7. Initial velocities of naproxen formation, v_i, after incubation of a dextran T-70-naproxen ester prodrug with DS 8.3 in homogenates prepared from various segments of the gastrointestinal tract with their content of rabbits and pigs (37ºC). DS is defined as the percentage of mg drug released per mg of the conjugate. The reaction solutions: 33% homogenate - 0.2 M phosphate buffer pH 7.4 (1:1 v/v). From Larsen *et al.* (1988)

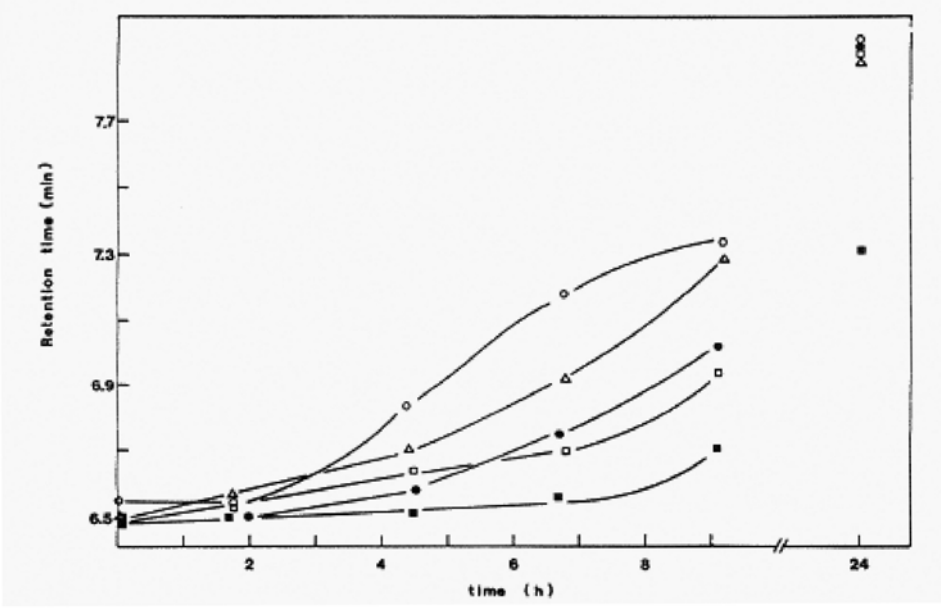


Figure 10. Retention times from HP(SEC) analysis of a dextran T-70-naproxen conjugate (DS 5.6) after incubation in pig colon homogenate (1 mg/mL) at 37ºC. (○): colon homogenate – 0.2 M phosphate buffer pH 7.4 (2:1); (△) do, but contains 5 mg parent dextran T-70; (□): do, but contains 10 mg dextran T-70; (■): do, but contains 50 mg dextran T-70; (●): do, but contains 50 mg glucose. Reproduced from Larsen *et al.* (1989) with permission of the copyright holder.

transported almost intact to the large bowel, where most likely the dextran chains are initially cleaved randomly by dextranases. The progressive increase in fragmentation results in diminished efficiency of steric protection of the dextran-drug ester bond and, consequently, sufficiently small fragments will become substrates for esterases and other hydrolases residing in the colon. In this context, it should be emphasized that Bacteroides species (some of which secrete dextranases) account for approximately 20% of the human fecal flora (Reddy *et al.*, 1984). Such species are also present in patients suffering from ulcerative colitis and Crohn's disease (Drasar and Hill, 1974), diseases treatable with anti-inflammatory drugs. Although extensive release of ketoprofen from dextran ester conjugates (Larsen *et al.*, 1991a) and dexamethasone linked to dextran through a dicarboxylic acid spacer (McLeod *et al.*, 1993; McLeod *et al.*, 1994a) have been observed in caecum and the large bowel, the versatility of the dextran prodrug approach to provide local sustained drug release of the anti-inflammatory drugs to the colon remains to be established. Interestingly, lack of esterase-facilitated hydrolysis of hydrocortisone-hyaluronic acid ester prodrugs was observed even for conjugates that initially had been treated with a hyaluronidase (Rajewski *et al.*, 1992). Negatively charged polymer carboxylate groups formed from hydrolysis of the prodrug bond may potentially render depolymerised fragments poor substrates for esterases.

Modification of Physicochemical Properties to Manipulate Drug Release Rate

Pharmaceutical chemical profiling of a macromolecular prodrug differs to some extent from that routinely performed for small molecule candidates. The characterization has to include polymer solution properties. As required for other drug candidates, basic physicochemical parameters must be incorporated into the drug substance specifications which constitute a part of the quality documentation in a New Drug Application/Common Technical Document. These physicochemical characteristics have to be kept within narrow limits to ensure reproducible drug product manufacture and quality, and, ultimately, constancy of therapeutic action as briefly outlined below. From a drug delivery perspective modification of physicochemical properties of macromolecular constructs may, early in the drug development phase, be used actively as a tool to accomplish a desired drug release profile. A particularly interesting approach to pursue is the manipulation of the hydrophilic/hydrophobic character of the polymer backbone of, intrinsically, insoluble macromolecular prodrugs. Drug liberation from such systems is afforded by complex heterogenous phase hydrolytic reactions where rate of water access, in addition to the nature of the prodrug bond, is a major determinant for the regeneration rate of the active agent (see, for example, Rajewski *et al.*, 2000).

Several physicochemical properties of synthesized macromolecular derivatives (molecular size and shape, flexibility, charge, and hydrophilic/lipophilic balance) are determinants for their pharmacokinetic fate after systemic administration.

Events influencing the pharmacokinetic properties include liver uptake, uptake by RES, macromolecule interactions leading to aggregation, extravasation (and lymphatic transport), residence time locally in tissue/body cavity, and renal clearance.

The intravascular persistence of macromolecular prodrugs is expected to vary considerably with molecular size (Mehvar, 2003). Therefore, the employment of conjugates possessing low polydispersity (M_w/M_n) may minimize undesirable cellular uptake and secure effective glomerular filtration of non-biodegradable polymeric transport groups. The glomerular capillary wall is expected to impede transport of macromolecules with a molecular weight > 45,000, corresponding to that of the largest dextran molecules detected in urine sampled over a 24-hour period (Wallenius, 1954). The weight-average molecular weight, M_w, is easily estimated from light scattering experiments or size-exclusion chromatography (SEC) whereas the number-average molecular weight, M_n, can be calculated from end-group analyses or osmotic pressure measurements. Attachment of drugs to hydrophilic polymers may give rise to conformational changes in an aqueous environment. Thus, the hydrodynamic volume (expressed by the limiting viscosity number) was found to be almost inversely proportional to the degree of substitution (DS, defined as the percentage of mg released drug per mg of the conjugate) of dextran ester conjugates derived from benzoic acid (Johansen and Larsen, 1985a) and naproxen (Harboe *et al.*, 1988a), presumably as a result of intramolecular hydrophobic interactions. Such reductions in hydrodynamic volume of relatively hydrophobic dextran derivatives may enhance the kidney filtration ability in proportion to that of pure dextran of comparable molecular weight. However, by using analytical SEC, the experimentally determined M_w for the dextran-benzoic acid conjugate was only 5% lower than the theoretically calculated value (Johansen and Larsen, 1985a). This apparent discrepancy between the data from viscosity measurements and SEC may most likely be attributed to the different conditions under which the influence of DS on the hydrodynamic volume has been assessed. In the former case, experiments were carried out in rather dilute aqueous solutions, while in SEC intramolecular hydrophobic bonding might be suppressed by interaction of the conjugates with the hydrophilic gel chains. Although the latter hydrophilic environment may mimic *in vivo* conditions, the actual effect of DS on the pharmacokinetic fate of such derivatives has to be established through adequate *in vivo* studies. Mehvar (2003) has reviewed the influence of charge on pharmacokinetic properties of polysaccharide-based prodrugs. The modest number of studies performed does not allow one to draw strong conclusions. However, it does appear that, relative to neutral conjugates, positively and negatively charged derivatives may experience much shorter and slightly extended circulation half-lives, respectively. A further complication concerning the pharmacokinetic fate of macromolecular prodrugs after i.v. administration might be illustrated by the data reported by O'Mullane and Daw (1991) who have studied the *in vivo* disposition of poly[N-(2-hydroxypropyl)methacrylamide] (pHPMA) and the equivalent macromolecule substituted

with 2 mol% cholesterol in mice. As apparent from Table 8, the *in vivo* fate of the substituted macromolecule differed significantly from that of parent pHPMA.

	pHPMA	pHPMA-cholesterol
Blood	48.4 (1.42)	16.3 (1.4)
Skin	16.1 (0.69)	6.7 (0.4)
Liver	1.3 (0.18)	18.4 (0.4)
Gut	3.1 (0.15)	2.9 (0.4)
Muscle	7.8 (0.36)	3.3 (0.2)

Table 8. Comparison of the in vivo distribution, after 24 h, of [^{125}I]pHPMA-cholesterol given as a bolus dose i.v. in mice (n=4). Results expressed as mean (±SD). From O'Mullane and Daw (1991)

The aqueous solubility of macromolecular prodrugs derived from water-soluble transport groups is dependent on the chemical structure of the attached drug and the degree of drug load. Estimation of absolute solubilities might be difficult due to the tendency of many conjugates to gel in concentrated aqueous solution (Van der Merwe *et al.*, 2002). Poorly soluble vinyl-homopolymers are easily prepared by radical polymerization (or solid-state mechanochemical polymerization (Kondo *et al.*, 2002)) of the corresponding vinyl monomer containing the covalently attached drug. Copolymerization using properly selected monomers can provide enormous variability in polymer composition and properties, including enhanced aqueous solubility (Fig. 11).

The use of insoluble macromolecular prodrugs excludes the i.v. route; however, extended duration of drug release might be obtained from these insoluble prodrugs after intra-articular and/or subcutaneous administration. Release kinetics for a soluble polymeric prodrug follow pseudo-first-order in the absence of boundary effects; however, for insoluble macromolecular prodrugs, complex kinetic models are required to describe drug release as a function of time. On contact with the aqueous environment surrounding the administration site, the surface of the solid prodrug has to be hydrated to initiate drug release by heterogenous phase hydrolysis of the drug-macromolecule bond. It is presumed that (i) as the hydrolysis reaction proceeds still new interfaces are created between the water-impermeable solid prodrug core and the swollen drug-depleted region and (ii) the absolute amount of drug liberated per time unit is proportional to the area of this interface (Bennett *et al.*, 1991) implying that different drug release rates result from various formulation geometries of a particular prodrug. Further, the interplay of rates of water permeation (polymer hydration) (k_w), hydrolysis of the prodrug bond (k_{hyd}), polymer matrix degradation, and boundary layer effects adds to the complexity of the overall release mechanism. Mathematical models incorporating the effects of geometry and different k_w/k_{hyd} ratios on drug release have been dealt with in more detail by Bennett (1990b) and Kondo *et al.* (1998).

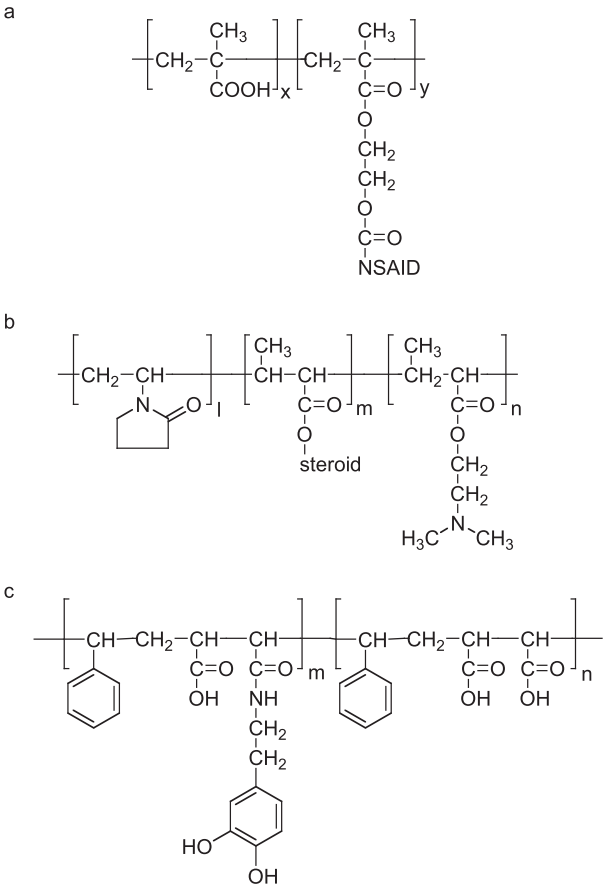


Figure 11. Examples of vinyl-type copolymer conjugates exhibiting significant aqueous solubility at physiological pH. a: conjugate formed by copolymerization of methacrylic acid and NSAID esters of 2-hydroxyethyl methacrylate (Wang and Chang, 1999); b: conjugate formed by copolymerization of 1-vinyl-2-pyrrolidone, dexamethasone 21-crotonate and 2-(diethylamino)ethylcrotonate (Timofeevski *et al.*, 1996); c: conjugate formed by reacting a styrene maleic anhydride copolymer and dopamine (Kalcic *et al.*, 1996).

For a given geometry and prodrug bond type, the magnitude of the k_w term might be manipulated because the rate of water permeation is influenced by the hydrophilic/hydrophobic character ("wettability") of the insoluble macromolecular construct. A means to modify the latter parameter is the design of copolymers comprising the drug-containing monomer and a monomer unit enabling modulation of the overall hydrophilic/lipophilic balance. The narcotic antagonist naltrexone has been linked to poly-N^5-(3-hydroxypropyl)-L-glutamine (PHPG) through a succinic acid spacer arm (Negishi *et al.*, 1987) or by a carbonate ester bond (Bennett *et al.*, 1991) (Fig. 12). *In vitro* release profiles of drug from copolymer conjugates containing leucine in the form of discs and spheres (Fig. 13) demonstrate decreasing naltrexone release rates with increasing proportions of

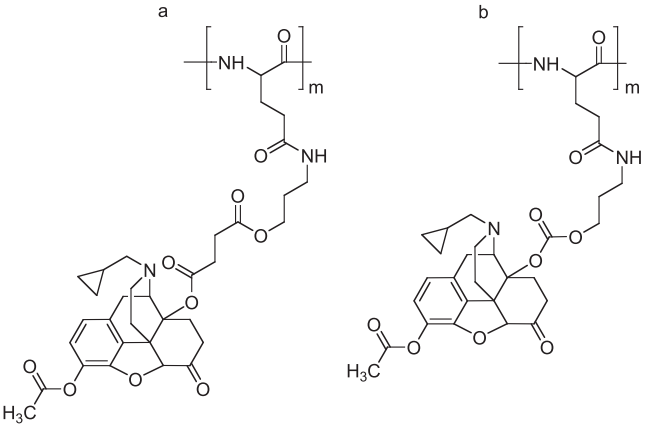


Figure 12. Coupling of naltrexone to PHPG polymer via (a) succinate spacer arm and (b) carbonate ester bond.

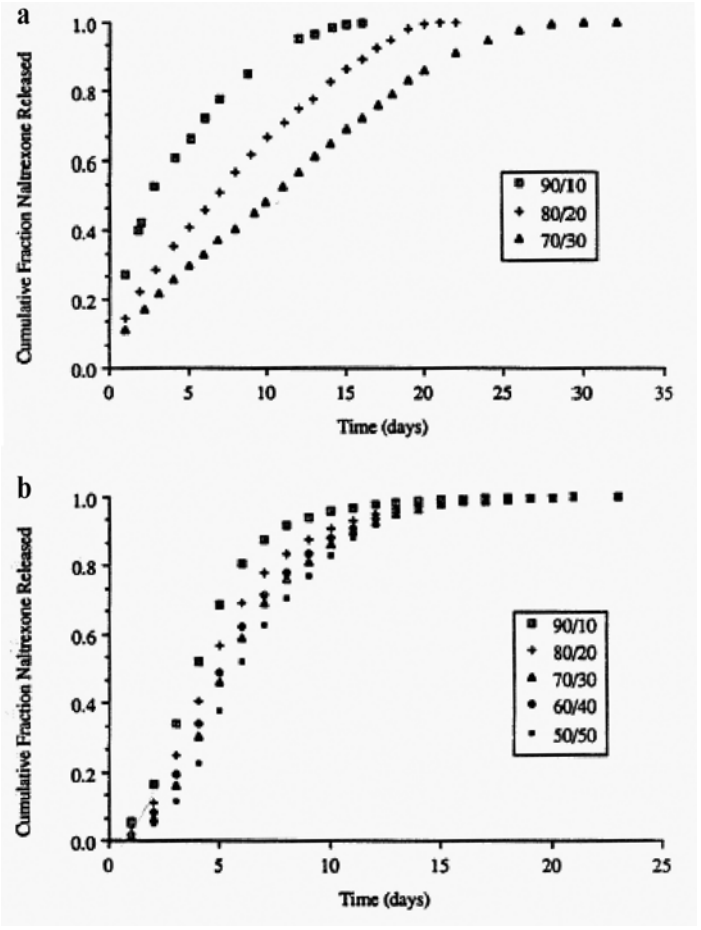


Figure 13. Cumulative fraction of released naproxen from N3A14HS-Copoly(HPG/Leu) conjugate (a) discs (11.0 mm dia.×0.8 mm thick) and (b) particles (150–180 μm). Reproduced from Bennett (1990c) with permission of the author.

hydrophobic leucine residues incorporated into the copolymers. However, direct comparison of the release profiles from the two formulation geometries may not be possible since drug release rates were determined under different experimental conditions. Whereas drug release from the discs was performed under sink conditions, the dialysis bag approach used for the spheres may not be optimal for the study of drug release from sustained release formulations under "true" sink conditions (Washington, 1990). On the other hand, the dialysis bag technique may reasonably simulate drug release from conjugates administered into a small aqueous compartment separated from the sink (the blood) by a sieve-like membrane or other types of diffusional restricted barriers (Parshad *et al.*, 2003). Analogous to the above series of studies, drug release characteristics from vinyl copolymers of various compositions (Kondo *et al.*, 1994) have been assessed utilizing a flow-through-cell apparatus (Kondo *et al.*, 1998).

Route of Administration

Oral Administration

The oral controlled drug delivery concept may embrace dosage forms that release the active agent in a predictable manner over a desired period of time as well as delivery systems designed to liberate the drug locally at specific sites within the gastrointestinal tract. To justify the high costs of developing a macromolecular prodrug for oral drug delivery, the prodrug must provide a superior solution to a drug delivery problem not easily resolved by conventional formulation techniques. Drug delivery to the colon may constitute such a niche. There are various colonic disorders, including ulcerative colitis and Crohn's disease, that warrant delivery of effective amounts of drug compounds such as anti-inflammatory agents selectively to the diseased site. Further, it has been suggested that the large bowel may offer an opportunity for systemic absorption of peptide-like drugs (Saffran *et al.*, 1986; Gruber *et al.*, 1987). Van den Mooter and Kinget (1995) and Sinha and Kumria (2001) have discussed various aspects of colonic drug delivery.

The bioavailability of naproxen, an inflammatory drug, after oral administration of aqueous solutions of various dextran-naproxen ester prodrugs in pigs has been determined employing conjugates ranging in molecular weight from 10,000 to 500,000 (Harboe *et al.*, 1989b). Average naproxen plasma concentration versus time profiles (from three pigs) are shown in Fig. 14, indicating that these dextran prodrugs possessed considerable sustained release capacity. In Table 9 are presented the various average pharmacokinetic parameters observed. The calculated bioavailability of naproxen from all the prodrugs was high relative to both i.v. and p.o. administration of the parent drug. The ratio AUC (parent naproxen p.o)/AUC (naproxen i.v.) was approximately 0.9, which is quite similar to the value found in man (Runkel *et al.*, 1973). Although the experimental design did not allow for statistical analysis, the data suggested that the obtained average naproxen plasma concentration-time profiles differed with respect to time of peak

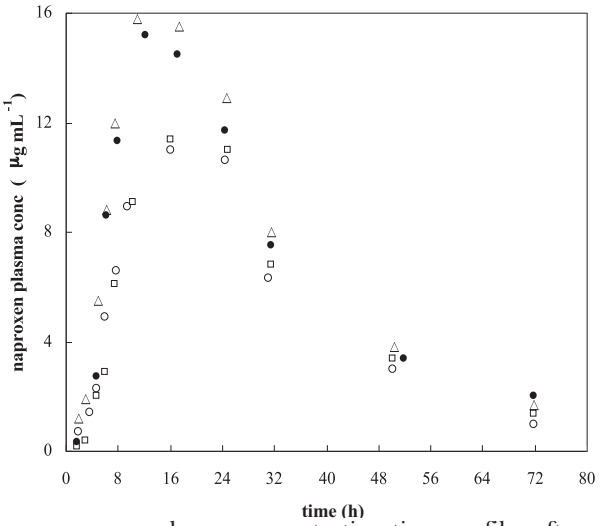


Figure 14. Average naproxen plasma concentration-time profiles after oral administration of solutions of dextran prodrugs varying in molecular size to three pigs (all doses corresponded to 3.6 mg naproxen per kg body weight). (□) M_w 500,000 (DS 6.8); (○) Mw 70,000 (DS 8.2); (●) M_w 40,000 (DS 6.9); (△) M_w 10,000 (DS 7.1). Reproduced from Harboe *et al.* (1989b) with permission of the copyright holder.

Compoundb	C_{max} (µ ml^{-1})	T_{max} (h)	k_e (h^{-1})	AUC (µg ml^{-1} h)	AUC (o.) × 100/AUC (naproxen i.v.)	AUC (conjugate) × 100/AUC (naproxen o.)
Naproxen i.v.	35	-	0.048	479	-	-
Naproxen p.o.	19.7	2	0.049	439	91.6	-
Dextran-T-10 conjugate p.o.	15.6	14	0.052	476	99.4	108.4
Dextran-T-40 conjugate p.o.[c]	15.5	13	0.048	508	106.1	115.7
Dextran-T-70 conjugate p.o.	11.1	16	0.045	398	83.1	90.7
Dextran-T-500 conjugate p.o.	11.6	18	0.044	422	88.1	96.1

Table 9. Bioavailability of naproxen after oral administration of dextran-naproxen ester prodrugs in pigsa (Average pharmacokinetic parameters, determined after administering solutions of dextran prodrugs varying in weight average molecular weight, in comparison to those obtained after oral (p.o.) and i.v. administration of solutions of parent naproxen)

[a]Each conjugate was administered to three pigs ranging in weight from 33 to 45 kg

[b]Equal doses corresponding to 3.6 mg naproxen equivalents per body weight were given

[c]A new group of three pigs was used

From Harboe *et al.* (1989a,b)

level, T_{max}, and maximum plasma concentration, C_{max}. The lower molecular weight conjugates resulted in more rapid appearance of naproxen in the blood and higher plasma levels, as expected from the hypothesis that the smaller prodrugs are depolymerised faster by dextranases than are their high molecular weight counterparts. All the plasma profiles exhibited a characteristic lag time (2–3 h) for naproxen appearance in the systemic circulation. Little is known about the gastric emptying rate in the pig, but preliminary experiments in the pig showed that after 2 h a considerable amount of the administered prodrug dose was still present in the stomach whereas at 4 h the conjugates were detected primarily in the cecum and the colon (Harboe *et al.*, 1989a). Similar bioavailability characteristics have been observed after oral administration of dextran-ketoprofen ester prodrugs (Larsen *et al.*, 1991b). Plasma steady-state concentrations of ketoprofen following multiple dosing (Fig. 15) were between 2 and 4 μg/mL

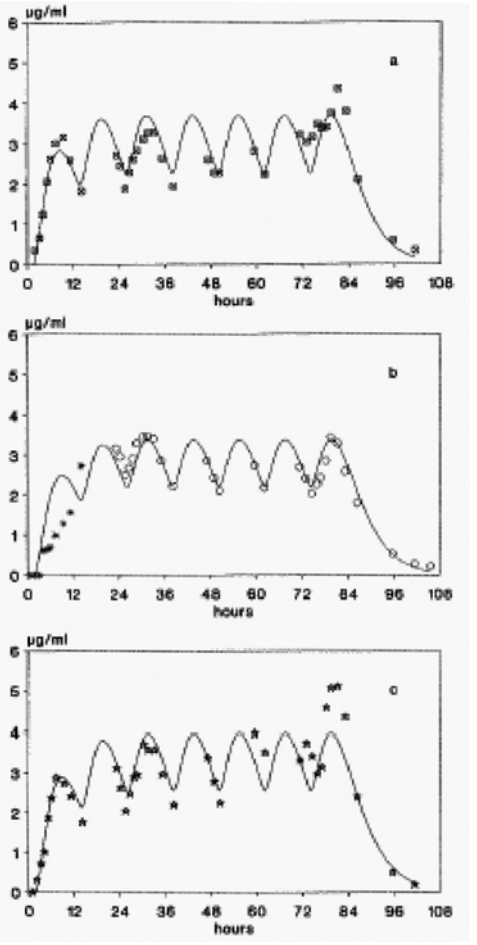


Figure 15. Plasma concentration profiles of ketoprofen following multiple oral administration a ketoprofen-dextran ester prodrug (equivalent to 4 mg/kg ketoprofen) in three pigs (a-c). The curves were obtained by fitting the ketoprofen plasma concentration data to a polyexponential expression. Reproduced from Larsen *et al.* (1992) with permission of the copyright holder.

(Larsen *et al.*, 1992). McLeod *et al.* (1994b) have reported dexamethasone pharmacokinetics after orally administered dextran hemicarboxylic acid ester prodrugs in the rat. Dexamethasone-succinate-dextran and dexamethasone-glutarate-dextran were given to two groups of male Sprague-Dawley rats by intragastric infusion. In two additional groups, disodium dexamethasone phosphate and dexamethasone hemisuccinate were each administered by subcutaneous infusion. In a fifth group, dexamethasone itself was given by intragastric infusion. The hemisuccinate ester macromolecular conjugate resulted in significantly higher concentrations in the cecum and colon than the other modes of dexamethasone administration. The authors suggested that the latter conjugate type had two advantages. First, the conjugate provided significantly higher levels of dexamethasone in the large intestine, which would theoretically lead to enhanced local anti-inflammatory effect. Second, the dextran prodrugs produced significantly lower blood concentrations compared to parenteral and oral administration of dexamethasone, which may reduce systemic side-effects.

Subcutaneous Injection

Some drug candidates intended for chronic use cannot be used orally due to extensive first-pass metabolism or poor biomembrane transport properties. Among alternative routes of administration are depot parenteral formulations, resulting in effective systemic plasma levels and satisfactory therapeutic utility. In addition, compliance considerations may favor the use of depot injectables as is the case for well-established antipsychotic maintainance therapy (see, for example, Larsen, 2003).

For the treatment of opiate addicts, naltrexone is used due to its antagonistic activity. Recognition of poor patient compliance has formed the basis for attempts to develop depot injectable narcotic antagonist delivery systems based on polymeric prodrug particles enabling constant release and effective levels of naltrexone *in vivo* for at least one month. The copolymer backbones employed consisted of different ratios of N^5-(3-hydroxypropyl)-L-glutamine (HPG) and L-leucine (Leu) to which naltrexone was linked by carbonate ester bonds (Negishi *et al.*, 1987) or through a succinic acid spacer arm (Bennett *et al.*, 1991) (Fig. 12). The biodegradable polymeric prodrugs were tested *in vivo* by determining their plasma naltrexone profiles after subcutaneous injection of the particulate formulations in rats. The authors emphasized that their analytical method did not provide for quantitation of the potential presence of prodrug intermediates. The naltrexone plasma profiles from three copolymers (HPG:Leu = 90:10, 70:30, and 50:50) containing a succinic acid spacer (Bennett, 1990c) are depicted in Fig. 16 which clearly demonstrates that change in copolymer backbone composition has a significant impact on drug release rates. After a slight initial burst, the rather hydrophobic 50:50 copolymer conjugate delivered naltrexone at a nearly constant level of approximately 1 ng/mL/mg conjugate, which was maintained for 3 weeks. Similar plasma profiles were found after subcutaneous administration of copolymer conjugates with naltrexone covalently attached by carbonate ester

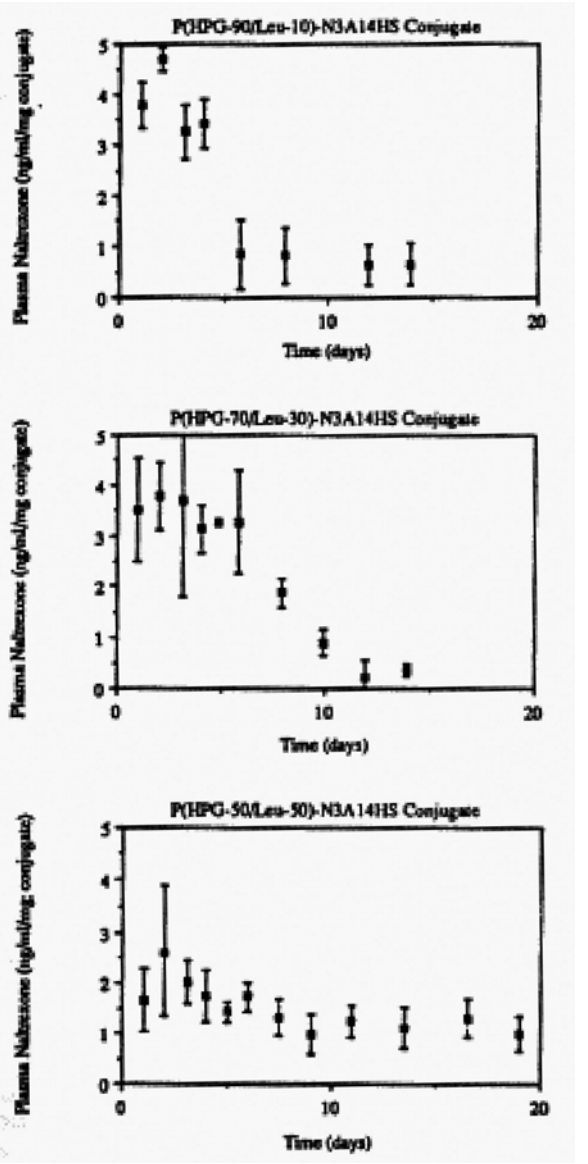


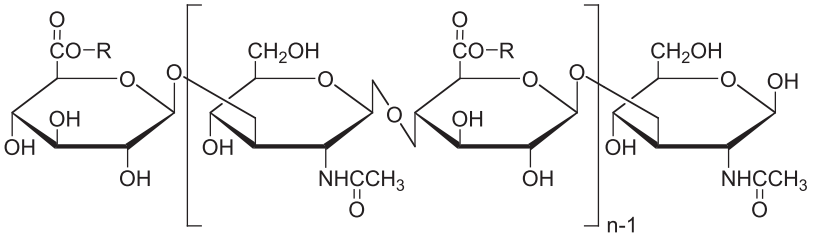
Figure 16. Naltrexone plasma levels following subcutaneous injection of N3A14HS-Copoly(HPG/Leu) conjugate particles in mice. Conjugates with different hydrophilic/lipophilic character were obtained by variation of the HPG/Leu ratio (e.g., 9:1, 7:3, and 5:5). Reproduced from Bennett (1990c) with permission of the author.

bonds (Bennett *et al.*, 1991). These data indicate the potential utility of solid macromolecular prodrug devices to provide systemic controlled delivery of highly potent drugs. Progress in this area may result from optimal control of manufacturing procedures and a better understanding of various processes taking place locally at the administration site (pH changes in the micro-environment, enzyme action, and fibrous capsule formation).

Intra-articular Injection

The synovial sack of major joints is surrounded by a cell lining that is not continuous. Consequently, the synovial fluid is directly contiguous with the interstitium of the blood vessel containing synovial tissue. Efflux of drug from joints after intra-articular instillation of a macromolecular prodrug results mainly from diffusion following the concentration gradient between the intra-articular site and the blood (Simkin *et al.*, 1993). Based on the general observation that the ability of macromolecules to pass biological barriers including the synovial membrane decreases with increasing molecular size, their use in intra-articular sites seems reasonable. Therefore, we have investigated the kinetics of degradation of various dextran-NSAID ester prodrugs in aqueous buffer and in different biological media (Larsen and Johansen, 1989). Essentially identical stabilities (drug release) were observed in buffer pH 7.4, 80% plasma, and human synovial fluid from an inflamed joint. Although the prodrugs provided a relatively slow drug release rate at physiological pH combined with a desirable high stability in aqueous solution in the pH range 4–5 *in vitro*, assessment of the feasibility of this potential intra-articular depot principle awaits pharmacokinetic assessment.

The development of water-soluble macromolecules as plasma expanders in man most often has been the starting point for designing suitable drug carrier conjugates. Alternatively, polymeric compounds naturally occurring in mammalian species have been of significant interest as transport vectors for drugs. This includes hyaluronic acid (HA), a mucopolysaccharide composed of altering residues of D-glucuronic acid, and N-acetyl-D-glucosamine (Fig. 17). In humans, HA is present in the joint synovial fluid, eye tissue fluids, and the skin. HA-derived scaffolds did not elicit any inflammatory response and are degraded completely within 4 months after implantation into rabbits (Radice *et al.*, 2000). Thus, HA derivatives have potential applications in drug delivery (Vercruysse and Prestwich, 1998; Abatangelo and Weigel, 2000). The steroidal alcohols hydrocortisone and methylprenisolone have been attached to HA in the form of ester linkages. Degradation kinetics of water-soluble conjugates in alkaline solution revealed specific base catalysis of hydrolysis (Benedetti *et al.*, 1990). Interestingly, the hydrocortisone derivative was only a very poor substrate for a porcine liver



R = H Hyaluronic acid

R = H, Steroid Hyaluronic acid ester (% esterification variable)

Figure 17. Structure of hyaluronic acid and a hyaluronic acid ester type prodrug.

esterase, even after prior presumed digestion of the polymeric backbone by hyaluronidase (Rajewski *et al.*, 1992). When poorly water-soluble partial steroid esters of HA were formed into films and microspheres, significant *in vitro* retardation of drug liberation was observed with drug release following neither zero-order nor square-root of time kinetics (Benedetti *et al.*, 1990; Kyyrönen *et al.*, 1992). The *in vivo* efficacy of microspheres formed from a methylprednisolone ester derivate was investigated using a rat model for adjuvant-induced arthritis. Preliminary experiments appeared encouraging (batch-to-batch variation of biological response was not reported), suggesting that compared with Depo-Medrol (an aqueous suspension of methylprednisolone acetate) injection of a comparable methylprednisolone dose in hyaluronate microspheres provided a less dramatic initial decrease in inflammation but a longer duration of action (Goei et al, 1992). It should be mentioned that Stella, Topp, and coworkers have investigated the hyaluronic acid-based (and a gellan-based derivative (Sanzgiri *et al.*, 1993)) macromolecular prodrugs as a formulation approach to achieve ophthalmic sustained delivery of steroids.

Collectively, rheumatic disorders including rheumatoid arthritis and osteoarthritis affect one-third of adults in the US and Europe; osteoarthritis by far is the most widespread rheumatic disorder, occurring in 7% of the population. In the case of arthritis, the annual cost to society was estimated to be approximately $US95 billion in the year 2000 (Matheson and Figgitt, 2001). Early use of DMARDs (disease-modifying anti-rheumatic drugs) is becoming more widely accepted to control the disease as quickly as possible once formal diagnosis has been made. Also, identification of pluripotent molecules may form the template for curative solutions in the area of joint degenerative diseases (Holland and Mikos, 2003). The implementation of these potent compounds, pharmacologically active at several targets at low concentrations, in therapy requires localized delivery of safe levels of the bioactive agents in a controlled manner. Therefore, intra-articular drug delivery strategies involving macromolecular prodrugs may add to the improvement of patient quality of life and may have significant impact on society in terms of medical care and lost wages.

Summary

The examples cited in this brief overview were selected to illustrate various aspects of the macromolecular prodrug approach to controlled drug release. Despite the substantial amount of knowledge gained about the behaviour of macromolecular prodrugs *in vitro*, relatively little information is available on the pharmacokinetic fate of such conjugates after different routes of administration. Although few in number, the published pharmacokinetic studies imply that application of macromolecular prodrugs could be of considerable interest for certain treatment modalities. Optimization of drug release profiles from formulated conjugates appears to be especially attractive. Progress in this exciting field necessitates a collaborative interdisciplinary effort involving minimally those involved in pharmaceutical technology, pharmaceutical chemistry, and pharmacology.

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2.4.3

Controlled Release - Proenzymes

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Table of Contents

List of Abbreviations

Arg.....arginine
Asp.....aspartic acid
caspase.....cysteine-dependent, aspartate-specific proteinase
El .....electrophile
Gly .....glycine
His .....histidine
Ile.....isoleucine
Met .....methionine
MMP .....matrix metalloproteinase
Nu .....nucleophile
Ser .....serine
TIMP.....tissue inhibitor of matrix metalloproteinases

Introduction

Prodrugs are familiar entities to readers of this volume, while proenzymes are a group of naturally occurring proteins that are of increasingly recognized biological significance (Saklatvala *et al.*, 2003). There is a structural resemblance between prodrugs and proenzymes, as Figure 1 illustrates. Both consist of two linked structural units. One of these units will eventually become the active drug in the case of prodrugs, or the active (mature) enzyme in the case of proenzymes. The other unit changes the properties of its partner unit while the two units remain linked.

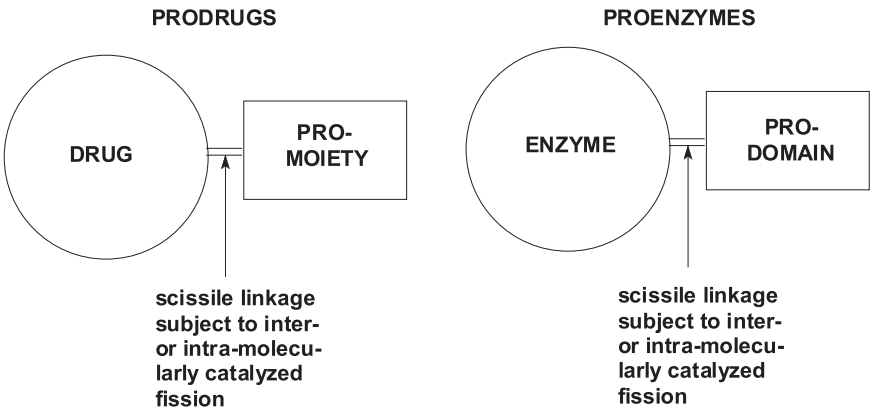

Figure 1. Comparison of prodrugs and proenzymes. Prodrugs (at left) are modified forms of drugs in which a promoiety has been attached to the drug to improve the characteristics of the drug as a pharmaceutical agent. The linkage of attachment is designed to allow fission under chosen circumstances so that the drug is liberated. Proenzymes (at right) are modified forms of enzymes that occur naturally. A polypeptide prodomain is attached during protein synthesis through a peptide linkage that may be cleaved hydrolytically under appropriate circumstances. Typically the cleavage is catalyzed by another enzyme (intermolecular catalysis) but could in principle be catalyzed by the enzyme part of the proenzyme (intramolecular catalysis).

In the case of prodrugs, the altered structural properties are intended to improve the pharmaceutical utility of the drug, for example by making transport properties more favorable for delivery to its target, by delaying release of the drug until its target is reached, or by protecting the drug against degradation. In the case of proenzymes, the effect of the prodomain is commonly to reduce or eliminate the catalytic activity that the mature enzyme possesses. The utility of such inhibition is easily apparent with proteolytic enzymes which, if they were present in active form at all times, might produce proteolysis damaging to their host organism or tissue. For example, the pancreatic digestive proteases are synthesized and stored in the pancreas in inactive, proenzyme forms (Fersht, 1998; Hedstrom, 2002). Proteolytic damage to the pancreas is thus averted, and indeed

pancreatitis can result from premature activation of the stored proteases. Defensive proteases synthesized by bacteria are also stored within the bacterial cell in inactive proenzyme forms and are activated only upon secretion. The presence of a prodomain may also facilitate correct protein folding (Bryan, 2002).

The purpose of this brief article is to call to the attention of those scientists who design and use prodrugs the potential utility of proenzyme-like entities. Synthetic analogs of proenzymes, it will be argued, could conceivably be employed in drug delivery.

Every reader will wish to consult the excellent previous review of Oliyai (1996), which covers highly germane points. Two papers cited by Oliyai appear to be particularly valuable and to have received too little attention (Smith *et al.*, 1981; Markwardt, 1989). Some of the contents of these reports will be mentioned at the end of the present chapter.

Proenzymes and their Activation

Proenzymes are also known as zymogens, emphasizing their capacity to generate enzymes under suitable conditions. When the prodomain is removed and the enzyme part of the proenzyme released to form, in some cases by conformational reorganization, the mature enzyme, the proenzyme is said to have been *activated*. The ways in which activation is accomplished vary from very simple processes to extraordinarily complex processes. The point can be illustrated by a few examples.

Pancreatic Serine Proteases (Fersht, 1998; Hedstrom, 2002)

These enzymes, as already noted, are generated and stored in proenzyme form in the pancreas. They are secreted into the duodenum, where the altered pH stimulates the activation process. The mature enzymes participate in the digestion of nutritional proteins, catalyzing the hydrolysis of the nutritional proteins to smaller peptide fragments and amino acids for absorption.

A typical pancreatic serine protease is α-chymotrypsin. The proenzyme form, chymotrypsinogen, is shown in Figure 2. The prodomain is an N-terminal fragment of 15 residues, with the scissile linkage between the prodomain and enzyme part of the proenzyme consisting of the peptide bond between the carboxyl group of Arg 15 and the amino group of Ile 16. As the figure shows, the prodomain lies closely upon the structure of its partner, and the interaction between the two produces substantial distortion in the enzyme part of the proenzyme. Some of the distortion occurs in the active site and renders the proenzyme nearly inactive as a proteolytic catalyst.

Activation of chymotrypsinogen is effected by trypsin, the mature form of trypsinogen, also secreted by the pancreas and activated by the enzyme enteropeptidase (enterokinase). Note that this scheme of activation of one protease by another which has been previously and independently activated has the character of a primitive *activation cascade*, a concept discussed below.

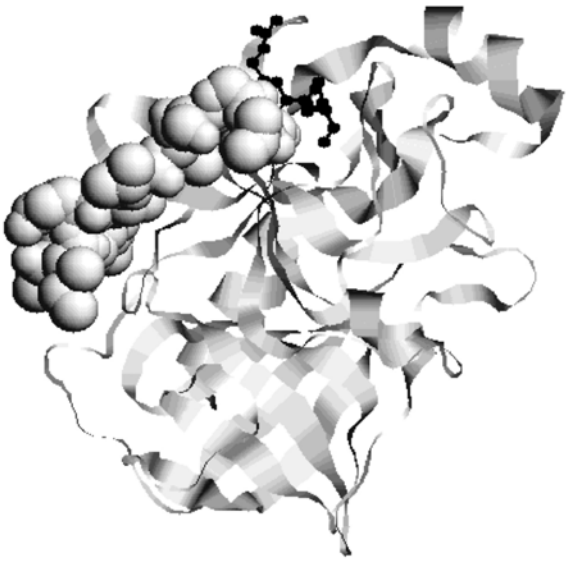

Figure 2. Chymotrypsinogen, the proenzyme form of the pancreatic serine protease chymotrypsin. The enzyme part of the structure is shown in ribbon representation and the prodomain in space-filling representation; the final residue of the prodomain (Arg 15) and the first residue of the mature enzyme (Ile 16) are shown in ball-and-stick representation. Trypsin, a protease that catalyzes the hydrolysis of peptide linkages between positively-charged residues such as Arg and any following residue, is the activating enzyme that catalyzes the hydrolysis of the Arg 15 – Ile 16 linkage. The prodomain is liberated and the mature enzyme is generated by a considerable reorganization of the enzyme part of the structure. The catalytically inactive chymotrypsinogen is thus converted to the catalytically active chymotrypsin. This illustration was produced from Protein Data Base file 2CGA with use of the program RasMol.

Trypsin specifically cleaves amide linkages that follow a residue the sidechain of which is positively charged. The Arg 15-Ile 16 bond of chymotrypsinogen is therefore a suitable site for tryptic hydrolysis. Following cleavage, and dissociation of the prodomain fragment, conformational reorganization of the enzyme fragment generates the mature enzyme. Part of the reorganization is portrayed in Figure 3, which contrasts the structure of the active-site region in the proenzyme (top) and in the mature enzyme (bottom). The most dramatic difference results from protonation of the α-amino group of Ile 16, following removal of the prodomain, which makes Ile 16 the N-terminus of mature chymotrypsin. The positive charge conferred by protonation allows the formation of a salt-bridge between the side-chain carboxylate of Asp 194 and the α-ammonium group of Ile 16. The new salt-bridge induces a sharp bend in the sequence from residue 192 to residue 195 and brings into proximity the backbone NH bonds of Ser 195 and Gly 193. These NH bonds now point toward the site that will be occupied by the negatively charged oxygen in the tetrahedral intermediate formed in the course of amide cleavage, catalyzed by the mature chymotrypsin. This structural feature is known as the “oxyanion hole.” Its absence in chymotrypsinogen and its

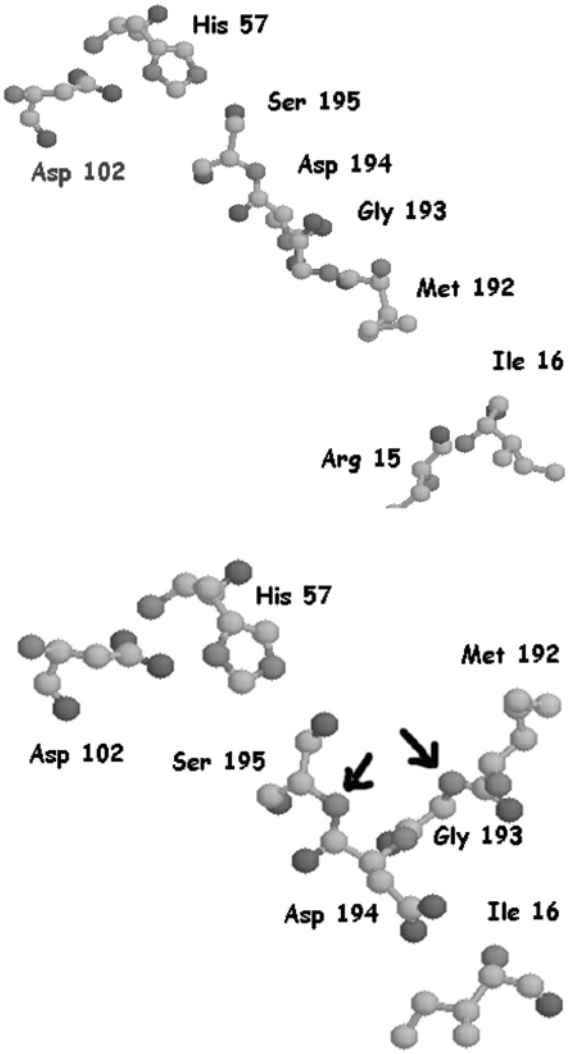

Figure 3. *Top panel:* An array of residues found in and near the active site of the mature enzyme α-chymotrypsin, here seen as they exist in the nearly inactive proenzyme, chymotrypsinogen. In the mature enzyme, the residues 102, 57, and 195 will form the "catalytic triad," and the backbone-NH bonds of residues 193 and 195 will form the "oxyanion hole." The prodomain of chymotrypsin consists of residues 1–15, of the which the final residue is seen at lower right, linked to residue 16. Fission of the 15-to-16 peptide bond liberates the prodomain and the enzyme part of the proenzyme; the latter then reorganizes to the mature α-chymotrypsin. *Lower panel:* The same residues as seen in mature α-chymotrypsin. A major reorganization of the structure has occurred, much of it stimulated by the liberation of the amino group of what is now the N-terminal residue of the mature enzyme, Ile 16. The amino group becomes protonated and forms a salt-bridge with the side-chain carboxylate of Asp 194. This induces, among other changes, the convergence of the backbone-NH groups of Ser 195 and Gly 193 (arrows) to form the "oxyanion hole." This illustration was produced from Protein Data Base files 2CGA and 4CHA with use of the program RasMol.

presence in mature chymotrypsin may account for a considerable part of the difference in catalytic activity between proenzyme and enzyme.

Activation Mechanisms

For proenzyme activation by proteolytic fission of the prodomain from the enzyme part of the proenzyme, the catalytic mechanisms at the atomic level correspond to one of those employed by the four classes of proteolytic enzymes. These classes are schematically illustrated in Figure 4. Chymotrypsin is a serine protease, Ser 195 (Figure 3) serving as the site of acylation, the combination of His 57 and Asp 102 providing both general-base and general-acid catalysis, and the oxyanion hole generating electrophilic stabilization of the tetrahedral intermediates and adjacent transition states. Cysteine proteases are generally similar to serine proteases but with a Cys residue becoming the site of acylation. Metalloproteases employ a metal center that increases the nucleophilicity of water by inducing dissociation of a proton to form a metal-stabilized hydroxide ion, and also electrophilically catalyzes attack at carbonyl and leaving-group departure. Aspartyl proteases possess a pair of aspartic-acid residues that act in a zwitterionic state to catalyze direct attack of water on amide carbonyl, followed by leaving-group expulsion. At the bottom of Figure 4, the molecular events in a typical catalytic sequence are shown. This sequence describes both the acylation and deacylation sequences for serine and cysteine proteases, which form an acylenzyme. The scheme is executed only once in each catalytic cycle for metalloproteases and aspartyl proteases, which stimulate the direct attack of water on the amide linkage.

More Complex Cases

There is no in-principle limit to the degree of complexity that can arise in proenzymes and their activation but an impressive example of complexity is offered by the activation of the proforms of matrix-metalloproteinases to their corresponding mature enzymes (Bode 2003). In this case, crystallographic studies have produced a very pleasing model. The activation event addressed by the model is the proteolytic liberation of the prodomain of pro-(matrix metalloproteinase 2) [proMMP2] by its activation protease, matrix metallo-proteinase 14 (MMP14).

MMP14, in addition to its Zn^{2+}-containing catalytic domain and other structures, possesses a transmembrane helical domain that anchors it to cell membranes, most of the molecule residing in the extracellular matrix, with only a small cytoplasmic domain inside. In the MMP2-activation complex, one such membrane-bound molecule of MMP14 is bound through its extracellular catalytic domain to a large polypeptide known as TIMP2. The TIMP (tissue inhibitors of metallo-proteinases) family are a group of specific endogenous inhibitors of the various MMPs, TIMP2 having a high affinity for MMP14. The MMP14:TIMP2 complex is thus proteolytically inactive, but is capable of assembling two other

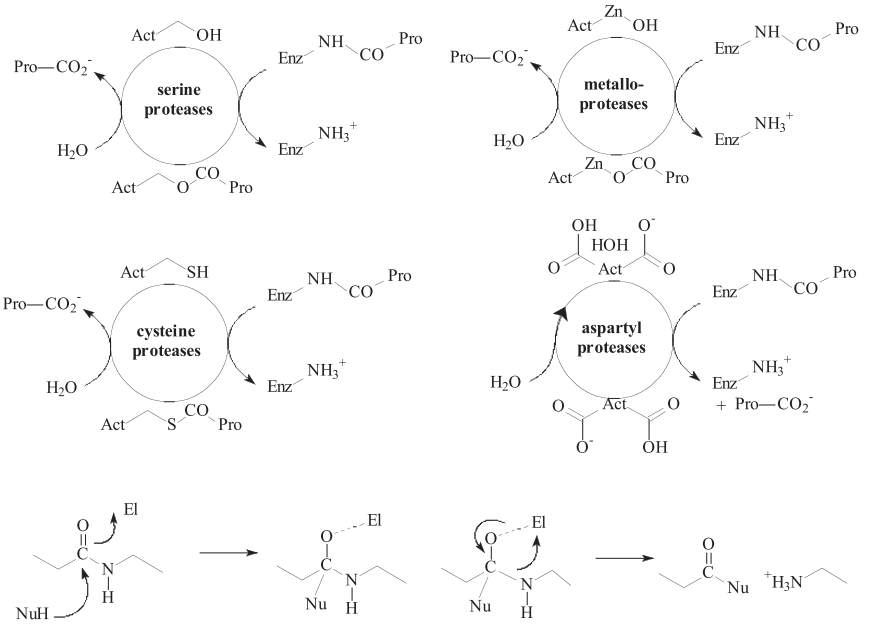

Figure 4. Schematic accounts of the catalytic cycles of the four classes of proteolytic enzymes, those most commonly involved in proenzyme activation by partial proteolysis. The basic chemistry is in all cases amide hydrolysis in which formation of a tetrahedral intermediate occurs by nucleophilic attack on the amide linkage, followed by expulsion of the amino leaving group (shown beneath the catalytic cycles). For serine proteases (Fersht, 1998; Hedstrom, 2002) and cysteine proteases (Brocklehurst *et al.*, 1998), the initial nucleophilic center is the hydroxyl group of serine or the sulfhydryl group of cysteine, respectively. Expulsion of the amino leaving group results in an acylenzyme, an ester or a thiolester, respectively. A second nucleophilic attack by water then leads to release of the second product and regeneration of the active enzyme. In metalloproteases (with Zn^{2+} as a typical metal; Bode, 2003), a metal-activated water molecule serves as nucleophile, while the metal center electrophilically activates the substrate carbonyl and assists the leaving group. The metal-carboxylate complex formally resembles an acylenzyme but nucleophilic attack by water is at the metal, not the carbonyl group. Aspartyl proteases (Dunn, 2002) contain an active-site pair of aspartyl carboxyl groups, the active form being the zwitterion, so that the anionic carboxylate can perform general-base catalysis, the neutral carboxyl general-acid catalysis. Shown beneath the catalytic cycles is a highly generalized indication of the molecular events in amide hydrolysis.

proteins in a specific relative orientation. One of these is a second, active molecule of MMP14, which is bound by an association of aligned transmembrane helices to the first molecule of MMP14, the binding reinforced by an association of the two cytoplasmic domains. The ternary complex thus generated, MMP14:TIMP2: MMP14, has a binding pocket between the TIMP2 component and the catalytic site of the second, active MMP14 component that precisely accommodates a molecule of proMMP2. The scissile linkage of the proMMP2 component is then presented directly to the MMP14 catalytic site and cleavage ensues.

Cascades of Proenzyme Activation

Cascade activation is a process in which an initial activation event produces a proteolytic enzyme that is a specific catalyst for activation of a second enzyme, which is a specific catalyst for activation of a third enzyme, etc. The final activation event in the cascade leads to the precipitation of a physiological event. Two illustrative examples are (a) the blood-coagulation cascade of serine proteases; and (b) the apoptosis cascade of the cysteine-protease caspases. These are treated separately below but are both summarized schematically in Figure 5.

Blood Coagulation (Jackson and Nemerson, 1980)

The cascade that links the initial events involving prekallikrein (a proenzyme) to the final formation of a blood clot is enormously more complex than the simple scheme for the so-called intrinsic pathway shown in Figure 5. There are numerous

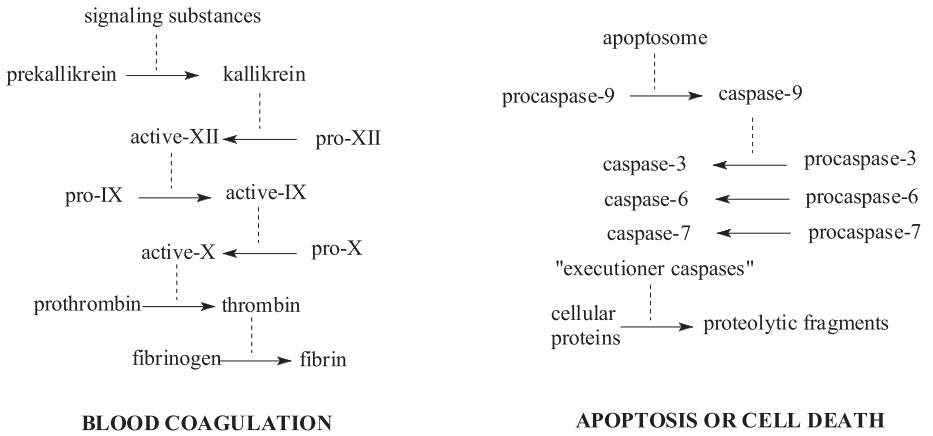

Figure 5. Abbreviated and schematic portrayals of the intrinsic cascades for blood coagulation (left) and apoptosis or cell death (right). The solid arrows represent a chemical conversion (e.g., of procaspase 3 to the mature caspase 3); the dashed lines represent a site of catalytic intervention (e.g., caspase 9 catalyzes the activation of procaspase 3 to caspase 3). The blood coagulation cascade can be initiated by cellular signalling species released in a variety of ways, these species stimulating the conversion of the proenzyme prekallikrein to the mature serine protease kallikrein. Kallikrein activates blood coagulation factor XII, leading into the serine-protease cascade of factors XII, IX, and X to prothrombin activation. Thrombin catalyzes the conversion of fibrinogen to fibrin, which aggregates to form a blood clot. Apoptosis can be produced by a cascade of cystein-dependent aspartate-specific proteinases, or caspases. These are cysteine proteases that cleave the peptide likage formed by the carboxyl group of an aspartate residue. The cascade leads to the "executioner caspases" that catalyze the proteolytic destruction of cellular proteins, producing cell death. Both pathways are far more complex than these schemes suggest. The blood-coagulation scheme was adapted from Jackson and Nemerson (1980). The apoptosis scheme was adapted from Boatright and Salvesen (2003).

ways in which the cascade of proenzyme-to-serine protease conversions can be initiated, there are numerous feedback loops omitted in Figure 5, nearly all the enzyme participants are strongly regulated through the concentrations of such cofactors as Ca^{2+} and of circulating, specific polypeptide inhibitors, and the pathway is coupled to an extrinsic pathway not shown at all. Nevertheless, even the oversimplified scheme of Figure 5 suggests the way in which the effect of a single initiating event can be amplified greatly by the unmasking of catalytic activities at successive points in the cascade. Together with the more complicated control features just mentioned, the cascade becomes a very sensitive device for controlling blood coagulation, a process with great defensive potential but also considerable potential danger to the host organism.

Apoptosis (Boatright and Salvesen, 2003)

Similarly, the scheme in Figure 5 for the intrinsic-pathway cascade leading to cell death is enormously oversimplified. There are at least two more pathways and again the regulation of the components and their interconversions is very complex. The point of the scheme is, however, that the proenzyme-to-enzyme cascade, here involving a series of cysteine proteinases, even in simplified form has the capacity of this kind of activation to produce high amplification and a sensitive off-on switch for an important but biologically sensitive result, namely cell death.

Proenzyme-like Prodrugs

It is only a short step at this point to envision the design of prodrugs that take advantage of existing proenzyme-activation capacities to achieve drug release. The concept is shown in Figure 6. One links either an enzyme or an analog of an enzyme that is the product of a known proenzyme-activation system to a promoiety. The linkage should be subject to scission by the natural proenzyme-activation system. Commonly, a simple peptide linkage with whatever adjacent structures are required to make the material a substrate of the proenzyme-activation system should suffice. Appropriate introduction of the construct into the physiological environment should then result in recruitment of the proenzyme-activation system and catalytic release of the enzyme/enzyme analog and the promoiety.

The pharmacologically active entity may in principle be either the enzyme/enzyme analog or the promoiety. Among the in-principle possibilities are:

- The enzyme/enzyme analog could be inert and serve only to deliver the promoiety as a drug itself to the site of action of the targeted proenzyme-activation system.
- The enzyme/enzyme analog could be any pharmacologically active protein as long as the activation system will tolerate the structure, and the promoiety could serve to stabilize the protein against denaturation or proteolysis.

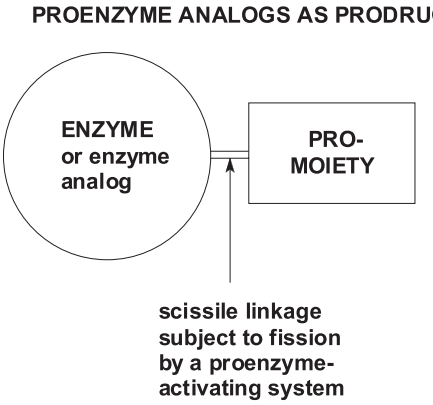

Figure 6. A construct in which the recruitment of a natural proenzyme-activation system could effect cleavage of a scissile linkage to release an enzyme/enzyme analog and a promoiety. See the text for further discussion.

- Constructs targeted on components of an activation cascade could in principle achieve unusual tissue distributions and pharmacokinetic characteristics.

It would be an understatement to note that the realization of such a method of drug delivery faces great obstacles so that effective design and construction of useful materials will require considerable ingenuity. The project appears, however, not *prima facie* impossible, and, indeed, approaches of this kind already have an encouraging history, as reviewed by Oliyai (1996).

Smith *et al.* (1981) demonstrated that serine-protease enzymes such as plasmin can be delivered for use as fibrinolytic agents in thrombotic disease if they are protected from endogenous inhibitors (and from producing unwanted proteolysis) by acylation of the active-site serine residue to form an acylenzyme. If the acyl moiety (the p-anisoyl group was used) is chosen so that an appropriate deacylation rate of the acylenzyme results, then binding to the clot, slow deacylation, and thrombolysis can be achieved. This and other similar work was later reviewed and discussed by Markwardt (1989).

Acknowledgments

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2.5.1

Targeting - Theoretical and Computational Models

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Table of Contents

List of Abbreviations

ADEPT .....Antibody-directed enzyme prodrug therapys
AUC .....Area under the concentration-time curve
Cl^{D}_{elim} .....Systemic clearance for parent drug
Cl^{PD}_{elim} .....Systemic clearance for prodrug
Cl^{PD}_{in}.....Prodrug transport rate in to target organ
Cl^{PD}_{out} .....Prodrug transport rate out of target organ
C_{max} .....Maximum concentration
DTI .....Drug targeting index
GDEPT .....Gene-directed enzyme prodrug therapy
IV .....Intravenous
K_m.....Michaelis-Menton constant
V_D .....Central parent drug related volume
VDEPT.....Virus-directed enzyme prodrug therapy
V_{max} .....Maximum prodrug conversion rate central volume
V'_{max} .....Maximum prodrug conversion rate target volume
V_{PD}.....Central prodrug related volume
V_{TD}.....Target site parent drug related volume
V_{TPD}.....Target site prodrug related volume

Introduction

Prodrugs are pharmacologically inactive compounds that undergo an *in vivo* chemical or enzymatic conversion to an active drug molecule to produce a therapeutic effect. An emerging application for prodrug technologies is their use for targeting drugs to specific tissues or cell types. The focus of this chapter is to review the potential use of prodrug technologies in achieving site-specific targeted delivery of drugs and to use computational models to illustrate conditions under which the targeted delivery can be achieved.

Conceptually, the use of prodrugs to overcome delivery barriers such as solubility or permeability as depicted in Scheme 1 in the first chapter is easily understood, and the criteria for determining the effectiveness of this approach are often easily measured for example, the increase in systemic bioavailability from administration of the prodrug relative to the parent drug when administered via the same route. However, following the time course of prodrug and drug can become complex when applying the prodrug concept to targeted drug delivery where multiple barriers may exist between the prodrug administration site and the drug target site. The existence of multiple barriers may provide for differing intercompartmental clearance rates for drug and prodrug, the potential for differing rates of prodrug to drug conversion in each compartment, and the potential for non-productive loss of drug and prodrug from non-target site volumes. Targeted prodrug delivery models that include these multiple factors can be cumbersome for simulations and can only represent a physiologically meaningful picture of drug distribution when developed for specific drugs/prodrugs with known clearance values, conversion rates, and distribution volumes. In contrast, simple models of targeted drug delivery are easily interrogated during simulations but should be used with an understanding that they are an oversimplification of the physiological system. Box summarized the situation when he wrote, "All models are wrong but some are useful" (Box, 1979); this should be kept in mind when contemplating this work.

Background

Site-specific delivery is the ultimate goal in all drug delivery research programs, where the optimal therapeutic benefit of a drug is obtained and unwanted effects are minimized. Site-specific delivery is particularly desirable for highly toxic compounds, such as those employed in the treatment of cancer; this topic is covered in greater depth elsewhere in this book. Two parameters commonly discussed in the assessment of the efficiency of drug targeting are *therapeutic advantage* and *drug targeting index*. The therapeutic advantage is an assessment of the benefits of targeting in relation to the therapeutic indexes of the targeted and non-targeted drug, where the therapeutic index of a drug is the ratio of the maximum tolerated dose and the minimum effective dose. (Kearney 1996)

$$Therapeutic\ Index = \frac{Maximum\ Tolerated\ Dose}{Minimum\ Effective\ Dose}$$

Equation 1.

$$Therapeutic\ Advantage = \frac{Therapeutic\ Index_{targeted\ drug}}{Therapeutic\ Index_{non-targeted\ drug}}$$

Equation 2.

In instances where the therapeutic advantage is significantly greater than unity, the targeted delivery system has successfully enhanced the therapeutic benefit of a drug without increasing adverse effects.

The drug targeting index was a parameter introduced by Hunt and colleagues (1988b) as an indicator of pharmacological efficiency and toxicity of targeted delivery systems; it serves as a measure of quantitative gain associated with targeting (Suzuki et al, 1996; Rowland and McLachlan, 1996). Calculations of the drug targeting index assume that the area under the concentration-time curve (AUC) at the target site and the toxicity site reflects the pharmacological activity and toxicity of the drug. In a review of pharmacokinetic considerations for targeted delivery, Suzuki and colleagues (1996) discuss examples of anti-cancer agents where drug activity is better correlated to drug exposure time to the cancerous cells rather than AUC; however, for many drugs activity and toxicity can readily be related to AUC at the site of effect. The drug targeting index (DTI) is defined as the ratio of drug delivered to the targeted and toxic sites when a targeted delivery system is employed divided by the same ratio when a non-targeted delivery method for the same drug is used. Drug targeting index values greater than unity reflect a benefit from the use of a targeted delivery system.

$$Drug\ Targeting\ Index = \frac{\left(AUC_{target\ site} \Big/ AUC_{toxic\ site}\right)_{targeted\ drug}}{\left(AUC_{target\ site} \Big/ AUC_{toxic\ site}\right)_{non-targeted\ drug}}$$

Equation 3.

Prodrug strategies can be employed to take advantage of specific enzymes, drug carriers, or physiological pH changes to achieve high local concentrations of parent drug, although this alone is insufficient to guarantee an improved therapeutic profile for a drug. The role of prodrugs in targeted delivery has been reviewed in the literature by a number of authors (Stella and Himmelstein, 1980, 1985a,b; Kearney, 1996; Han and Amidon, 2000; Ettmayer et al., 2004). The papers of Stella and Himmelstein discuss some of the pharmaceutical considerations of prodrugs in achieving site-specific drug delivery and they address in a quantitative manner whether prodrugs are capable of providing site-specific delivery of a parent (active) drug (Stella and Himmelstein, 1980, 1985a,b). Kearney (1996) reviews prodrug technologies developed to target specific tissues such as the brain, colon, kidney, and liver and reviews the use of antibody-directed enzyme prodrug therapy (ADEPT). Ettmayer and colleagues (2004) review marketed and investigational prodrugs and classify them according to the delivery or formulation barrier that necessitated their development. The reader is directed to this review for an in-depth discussion on the development of viable

clinical approaches to prodrug targeting of tissue and cell-specific enzymes and cell surface antigens. Additionally, the Ettmayer et al. review and that of Han and Amidon describe the clinical use of enzyme-prodrug therapies including ADEPT, gene-directed enzyme prodrug therapy (GDEPT) and virus-directed enzyme prodrug therapy (VDEPT). Yuan and colleagues have reported simulations of enzyme-prodrug therapy. (Yuan et al., 1991) These simulations were based on a simple two-compartment model including plasma and tumor and evaluated criteria such as mode of injection, time delay between antibody-enzyme conjugate and prodrug injection, binding affinities, prodrug conversion rates, and the relative size of the antibody-enzyme conjugate and prodrug doses. The reader is directed to the manuscript for a discussion of the therapeutic implications and model limitations.

As discussed by Stella and Himmelstein, it is important to choose relevant criteria for assessing the effectiveness of drug targeting via a prodrug. These criteria should necessarily be selected based on the clinical rationale for initially pursuing a prodrug alternative. For example, if a prodrug is being pursued to increase the amount of drug at a target site, then criteria such as the duration of time the drug remains above minimum effective levels should be evaluated. More general measures such as area under a concentration-time curve (AUC) or maximum concentrations (C_{max}) may also prove useful in such evaluations. If a prodrug approach is being undertaken to alleviate systemic toxicities by increasing drug levels at the target site relative to the general circulation or a specific toxic site, then enhancements in area under the target site level-time curve relative to sites of known toxicity or the general circulation are appropriate criteria to evaluate (such as DTI). The choice of approach should be based on the pharmacology/toxicology of the parent drug.

In their early work, Stella and Himmelstein developed a simple pharmacokinetic model to evaluate the key properties governing prodrug targeted delivery. Through a series of manuscripts, the Stella and Himmelstein model evolved from a simple, coupled two-compartment model for both drug and prodrug to a model including both extracellular and intracellular volumes at the target site for both the drug and prodrug. The initial coupled two-compartment model included central and target site compartments for drug and prodrug with enzymatic conversion of prodrug to drug in each compartment. The simulations and new findings presented in this manuscript will be based on this coupled, two-compartment model (see Figure 1).

In this hybrid classical/physiological model, the conversion of prodrug to parent compound is described by Michaelis-Menton kinetics and the prodrug is cleared via urinary excretion or non-productive hepatic metabolism. Transfer of compounds between the target site and the rest of the body was defined using clearance terms, which enabled the introduction of physiologically relevant variables such as organ blood flow and extraction coefficients. This model was then employed as a mathematical model for testing a variety of hypotheses related to the achievement of targeting via prodrugs.

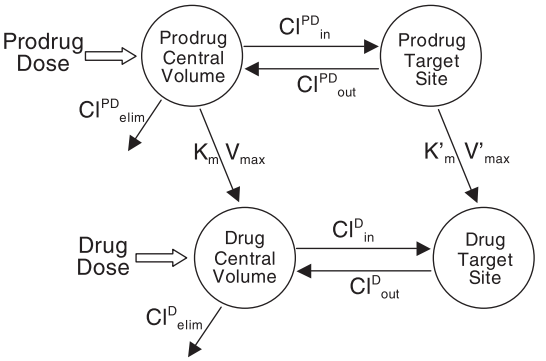

Figure 1. Model used in simulation for targeted delivery of drugs via prodrugs.

More specifically, the Stella and Himmelstein model assumes that the prodrug is introduced into the body as an intravenous bolus dose and distributes throughout a central prodrug-related volume (V_{PD}) and into a target site with a defined volume (VT_{PD}). The prodrug transport rates into and out of the target organ are linear and defined as clearance values Cl^{PD}_{in} and Cl^{PD}_{out}, respectively. The prodrug is converted to drug in the central volume and the target site via saturable Michaelis-Menton kinetics with physiologically relevant K_m and V_{max} values. For modeling purposes, the V_{max} and V'_{max} terms have been converted from the normal mass per unit time units to units reflecting activity per unit volume by dividing the V_{max} and V'_{max} values by the respective V_{PD} and VT_{PD} values. Analogous terms were defined for the parent drug central volume (V_D) and the target site (V_{TD}). Systemic clearance terms for the drug and prodrug (Cl^{D}_{elim} or Cl^{PD}_{elim}) were defined to account for excretion or metabolism of the respective species.

Through the use of this model, Stella and Himmelstein evaluated the parameters critical to attaining site-specific delivery of drug via a prodrug. Their work included varying key model parameters over a range of physiologically relevant values while holding other model variables constant to evaluate the effect on dependent variables, such as the concentration of drug at the target site. The reader is directed to the original papers for an in-depth discussion of the model.

As part of the present work, we will review the results Stella and Himmelstein found using the coupled, two-compartment model. The Stella and Himmelstein simulations made the assumption that the systemic (non-productive) clearance of the prodrug was zero, presumably due to limitation of processor power at the time. Using their model constants, we will evaluate the effect of prodrug clearance on drug levels at the target site. As in the previous work, we will not consider local input of drug or prodrug into the target tissue. We will also evaluate the critical parameters for targeted delivery when infusing either drug or prodrug to steady-state and following repeated bolus dosing. All simulations in the present work were conducted using SAAM II software using a Rosenbrock integrator algorithm (SAAM Institute, Inc., Seattle, WA). The current simulations also employed the

same values for the relative pharmacokinetic parameters as the Stella and Himmelstein simulations (see Table I).

Total Dose	350 mg
Drug Central Volume	50 L
Drug Target Site Volume	100 mL
Prodrug Central Volume	50 L
Prodrug Target Site Volume	100 mL
K_m and K'_m	10 μg/mL
V_{max}	10,000 μg/min
Cl^{D}_{elim}	250 mL/min
Cl^{PD}_{in} and Cl^{PD}_{out}	10 mL/min

Table 1. Simulation Model Parameters[1]
[1]All other parameters as described in the text

Model Based Simulations

Stella and Himmelstein demonstrated that for successful drug targeting via prodrugs there are at least three properties required of the prodrug and parent drug (Stella and Himmelstein, 1985a,b):

1. The prodrug must be readily transported to the target site and uptake must be reasonably rapid.
2. Once at the site, the prodrug must be selectively cleaved to the active drug relative to its conversions at other sites in the body.
3. The active drug, once selectively generated at the site, must be somewhat retained by the tissue.

While items 1 and 2 are apparent from inspection of the model, the impact of retention of drug at the active site as mentioned in item 3 may not be fully appreciated. As an illustration of the importance of retention of drug at the target site, we have re-run several of the simulations from the original manuscripts. Figure 2 shows the concentration of drug in the target site as a function of time following either bolus drug or prodrug input into the respective central volumes for a drug with either high ($Cl^{D}_{in} = Cl^{D}_{out}$ = 10 mL/min; Figs. 2A and 2B) or low ($Cl^{D}_{in} = Cl^{D}_{out}$ = 0.1 mL/min; Figs. 2C and 2D) permeability between the central volume and the target site. For this simulation, Cl^{PD}_{elim} was set to zero and V_{max} = 7140 μg/min as was done in the original Stella and Himmelstein simulations. All other parameters were as described in Table I. In effect, Fig. 2 represents a compilation of several figures from the Stella and Himmelstein papers. As seen in Figs. 2A and 2B, the use of a prodrug does not offer a large advantage over the parent drug for IV bolus dosing when the parent drug has high permeability between the central volume and the target site. For this simulation, the area under the concentration-time curve (AUC) for drug at the target site is only 32% higher

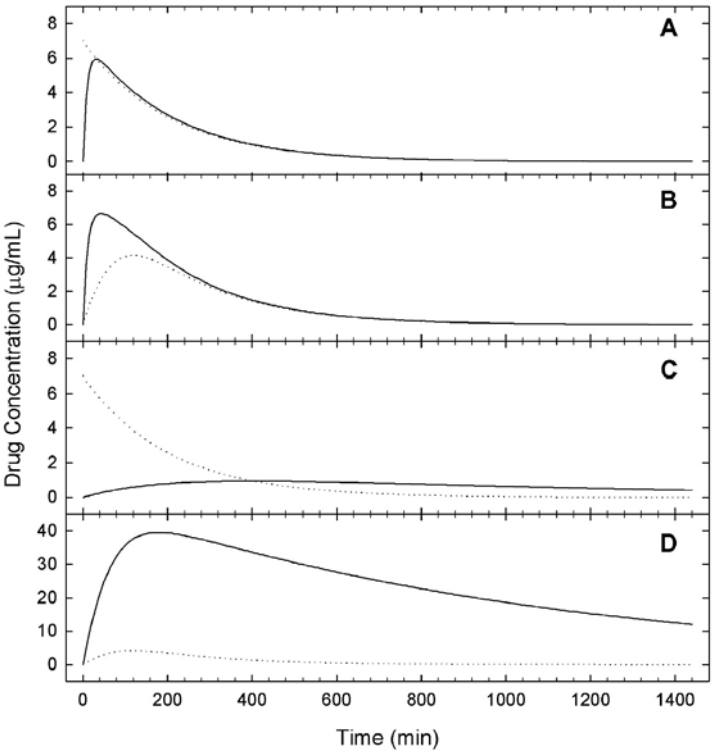

Figure 2. Plots of drug concentration in the central volume (dotted line) and target site (solid line) as a function of time for bolus drug or prodrug input. Panels A and B are for a drug with high permeability (Cl^{p}_{in} = Cl^{p}_{out} = 10 mL/min) between the central volume and target site with either drug (Panel A) or prodrug input (Panel B). Panels C and D are for a drug with low permeability (Cl^{p}_{in} = Cl^{p}_{out} = 0.1 mL/min) between the central volume and target site with either drug (Panel C) or prodrug input (Panel D). For these simulations Cl^{pd}_{elim} = 0 mL/min and V_{max} = 7140 μg/min. All other values as in Table I.

for prodrug input relative to drug input. However, an added benefit of the prodrug input may be seen for drugs with high target site permeability when the drug exhibits toxic side effects as the central volume exposure of drug is lower for the prodrug input in terms of maximum concentration (C_{max}; 7.00 μg/mL for drug input versus 4.13 μg/mL for prodrug input).

Relative to bolus IV drug input, a large increase in drug exposure at the target site is observed for prodrug input for drugs with low permeability between the central volume and the target site. This effect may be observed in Figs. 2C and 2D, where the AUC for drug at the target site following prodrug input is more than 350 times greater than from an equimolar dose of drug. This type of target site drug loading via a prodrug approach may be especially useful for cytotoxic drugs that do not have receptor-mediated pharmacological action. Stella and Himmelstein (1982) demonstrated this strong dependence of target site drug concentration on drug permeability between the central volume and the target site. The reader is directed to Fig. 2 of that manuscript and associated discussion

for additional detail on this subject. Later in this chapter we will take a closer look at the effect of drug retention at the target site on control of target site drug levels during drug and prodrug infusions or repeated bolus dosing.

Effect of Systemic Prodrug Clearance on Target Site Drug Concentration

One of the aspects that was not investigated in the earlier manuscripts is the effect of the systemic clearance rate of prodrug on the target site levels of drug. We know intuitively that systemic prodrug clearance will have an impact on target site drug exposure. Through model simulations we can investigate at what levels systemic prodrug clearance makes a significant impact on target site drug levels.

Figure 3 illustrates the effect of systemic prodrug clearance (Cl^{PD}_{elim}) on the AUC ratio for drug at the target site following bolus prodrug input at various values of systemic prodrug clearance relative to bolus prodrug input when systemic prodrug clearance is zero. The simulations were run for a drug that has low permeability between the central volume and the target site ($Cl^{D}_{in} = Cl^{D}_{out} =$ 0.1 mL/min) with selective conversion of the prodrug at the target site ($V'_{max} = 5$ V_{max}). As seen in Fig. 3, as the systemic prodrug clearance increases the utility of targeted prodrug delivery of drug to the target site decreases. At values of systemic prodrug clearance equal to the glomerular filtration rate (130 mL/min, assuming an extraction efficiency of 1), drug exposure at the target site is approximately 85% of the non-clearance prodrug value. For actively cleared prodrugs or prodrugs that are not selectively converted at the target site, the effect of systemic prodrug clearance on target site drug exposure may be significant.

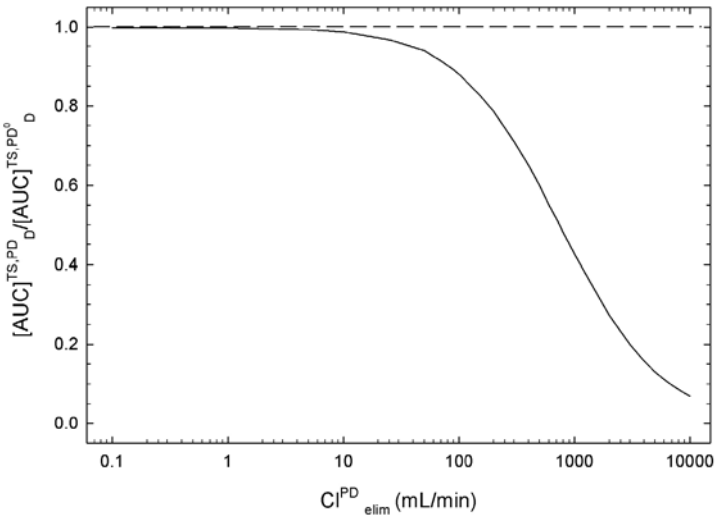

Figure 3. Plot of the ratio of AUC of drug at the target site following bolus prodrug input at varying prodrug clearance values ($[AUC]^{TS,PD}_{D}$) to bolus prodrug input with systemic prodrug clearance of zero ($[AUC]^{TS,PD°}_{D}$) as a function of systemic prodrug clearance. For these simulations $Cl^{D}_{in} = Cl^{D}_{out} = 0.1$ mL/min and $V'_{max} = 5$ V_{max}. All other values as in Table I.

The reduction in AUC of drug at the target site when the prodrug conversion is not target-site selective may be seen in Fig. 4, which shows prodrug-derived drug concentration at the target site as a function of time for a drug with high (Cl^{D}_{in} = Cl^{D}_{out} = 10 mL/min) or low (Cl^{D}_{in} = Cl^{D}_{out} = 0.1 mL/min) permeability between the central volume and the target site. For this simulation V_{max} equals 10,000 μg/min and V'_{max} equals 7140 μg/min. The systemic prodrug clearance for each permeability value was either 0 or 130 mL/min. At low drug target site permeability values, significant differences are observed in the time drug values are above set concentrations. This effect of systemic prodrug clearance may be especially important at times around the time of maximal concentration. As drug permeability increases these effects decrease.

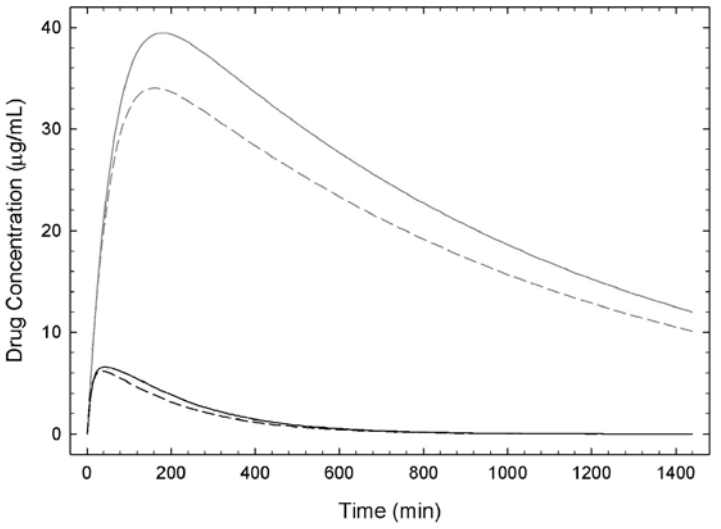

Figure 4. Plots of drug concentration in the target site as a function of time with systemic prodrug elimination (Cl^{PD}_{elim}) of 0 mL/min (solid line) or 130 mL/min (dashed line) for a drug with high permeability (Cl^{D}_{in} = Cl^{D}_{out} = 10 mL/min; black lines) or low permeability (Cl^{D}_{in} = Cl^{D}_{out} = 0.1 mL/min; grey lines) between the central volume and target site. For these simulations V'_{max} = 7140 μg/min. All other values as in Table I.

There are also instances where the systemic clearance of prodrug can totally negate the benefit of using a prodrug to increase the target site drug exposure relative to parent drug input. This effect may be seen in Fig. 5, which is a plot of the ratio of the target site drug exposure (AUC to 24 hours) from prodrug input relative to drug input as a function of drug permeability between the central volume and the target site. For this simulation, Cl^{PD}_{elim} = 130 mL/min and V'_{max} = 7140 μg/min. As can be seen in the figure, at Cl^{D}_{in} and Cl^{D}_{out} values greater than 10 mL/min the AUC ratio falls below unity, indicating that the prodrug approach offers no benefits over direct bolus drug input when using drug exposure at the target site as the evaluation criterion. This effect may be compared to that in Fig.

2 of the 1982 Stella and Himmelstein manuscript where Cl^{PD}_{elim} = 0 mL/min and the AUC ratio approaches unity, the lowest asymptote possible with no prodrug elimination. Again, the effect of systemic prodrug clearance on target site drug exposure would be more pronounced for highly cleared prodrugs.

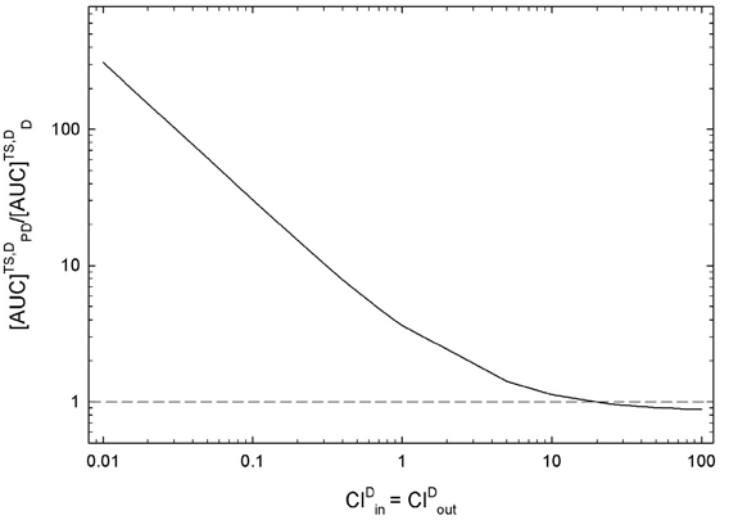

Figure 5. Plot of the ratio of AUC of drug at the target site following bolus prodrug input ($[AUC]^{TS,PD}_{D}$) to bolus drug input ($[AUC]^{TS,D}_{D}$) as a function of drug permeability between the central volume and the target site with systemic prodrug elimination (Cl^{PD}_{elim}) of 130 mL/min. For these simulations V'_{max} = 7140 μg/min. All other values as in Table I.

Drug Permeability Effects and Drug or Prodrug Infusion

Based on the results of multiple simulations of targeted drug delivery performed by Stella and Himmelstein (1985), drug targeting was defined as "the selective delivery and retention of a therapeutic agent to its site of action resulting in high target site, cell line, organ, etc. concentration of the therapeutic agent and a lowered drug burden to the rest of the body." While the inclusion of both delivery and retention in the definition of drug targeting is supported by their simulations employing IV bolus dosing, the benefits of drug retention at the target site for IV infusions or multiple bolus dosing (IV, pulmonary, oral) to steady-state are less certain. The source of this uncertainty is based on the fact that the lower the permeability of drug between the central volume and the target site (Cl^{D}_{in} and Cl^{D}_{out}; Fig. 1), the longer the time required to reach steady-state on infusion or multiple dosing. Therefore, the "benefit" of drug accumulation at the target site for drugs with low permeability between the central volume and the target site following prodrug dosing may lead to high and continually increasing drug levels during clinically relevant temporal dosing regimens.

For example, if we run simulations using the model employed for Fig. 2 using Cl^{p}_{in} = Cl^{p}_{out} = 10 mL/min for high permeability and 0.1 mL/min for low permeability, and infuse either drug or prodrug at 350 μg/min for 1000 min (total dose equivalent to bolus dose simulations), the effects are readily observable. Figure 6A is a plot of drug concentration in the central volume and target site as a function of time resulting from infusion of a high permeability drug into the central volume. The drug levels approach steady-state at the end of the infusion (1.39 μg/mL) and, as expected for a drug with high intercompartmental clearance, the exposures of both compartments to drug are almost identical. Figure 6B is a plot of drug concentration in the central volume and target site from an infusion of the prodrug of a highly permeable drug into its central volume. Again, steady-state drug levels are approached at the end of the infusion with target site steady-state levels being higher for the prodrug infusion relative to the drug infusion (1.74 versus 1.39 μg/mL, respectively) with nearly equivalent central volume drug exposures (AUC values of 1358 μg.min/mL versus 1369 μg.min/mL, respectively). Equivalent target site steady-state drug levels could be attained with a lower dose infusion of prodrug with an associated decrease in central volume drug exposure.

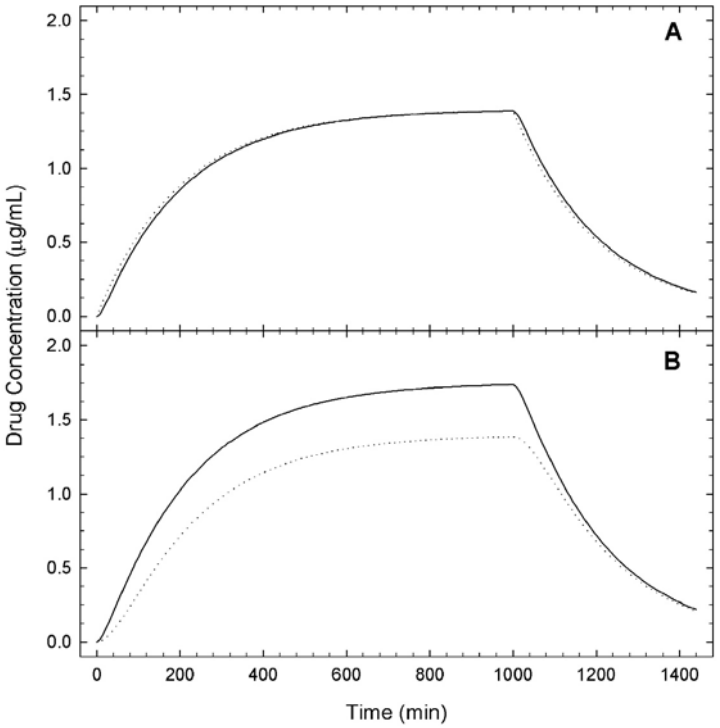

Figure 6. Plots of drug concentration in the central volume (dotted line) and target site (solid line) as a function of time for infusion (350 μg/min for 1000 min) of drug (Panel A) or prodrug (Panel B) for a drug with high permeability between the central volume and the target site (Cl^{p}_{in} = Cl^{p}_{out} = 10 mL/min). For these simulations Cl^{PD}_{elim} = 0 mL/min and V_{max} = 7140 μg/min. All other values as in Table I.

In contrast, Fig. 7A is a plot of drug concentration in the central volume and target site as a function of time for a low permeability drug infused into its central volume. The levels of drug in the central volume approach steady-state (1.39 μg/mL) at the end of the infusion; however, the drug levels at the target site are low and still increasing at the end of the infusion (C_{max} = 0.76 μg/mL). Additionally, the total 24-hour exposure of the target site to the drug is only 50% of that for the highly permeable drug. The inability to titrate to steady-state drug levels in reasonable times is even more evident for prodrug input of low permeability drugs. Figure 7B is a plot of drug concentration in the central volume and target site as a function of time that results from infusion of a prodrug of a low permeability drug. The drug concentrations at the end of the infusion are high (22.46 μg/mL) and continuing to rise. While the central volume drug exposure is almost equivalent to that for drug input (AUC to 24 hours of 1351 μg.min/mL versus 1369 μg.min/mL, respectively), the target site drug exposure over a 24-hour period from the prodrug infusion is almost 30 times greater than the drug exposure to the target site following an infusion of drug. As expected, analogous behavior is observed when the 350 mg dose is administered as multiple bolus doses, as depicted in Fig. 8A-D.

Based on these simulations, the inclusion of target site drug retention as a desirable property in the definition of drug targeting for infusion or multiple

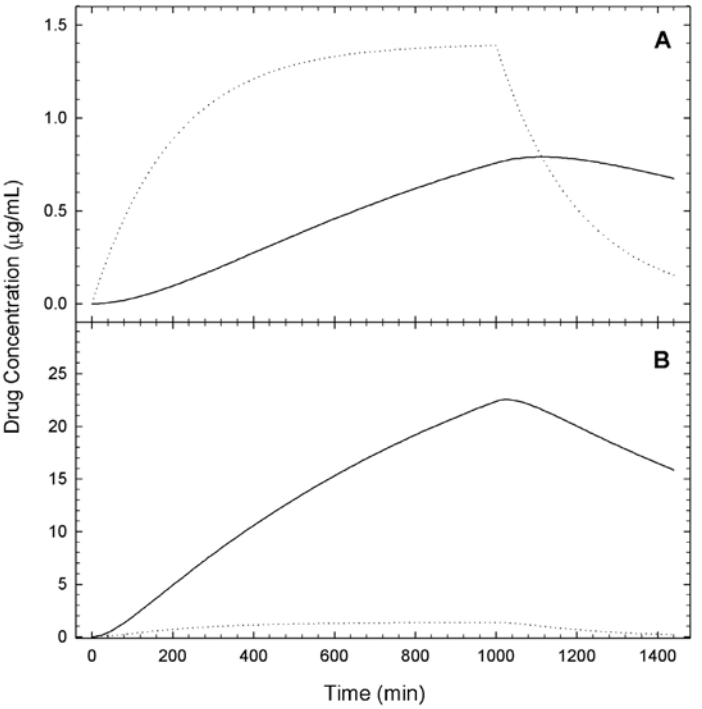

Figure 7. Plots of drug concentration in the central volume (dotted line) and target site (solid line) as a function of time for infusion (350 μg/min for 1000 min) of drug (Panel A) or prodrug (Panel B) for a drug with low permeability between the central volume and the target site (Cl^{p}_{in} = Cl^{p}_{out} = 0.1 mL/min). For these simulations Cl^{pd}_{elim} = 0 mL/min and V_{max} = 7140 μg/min. All other values as in Table I.

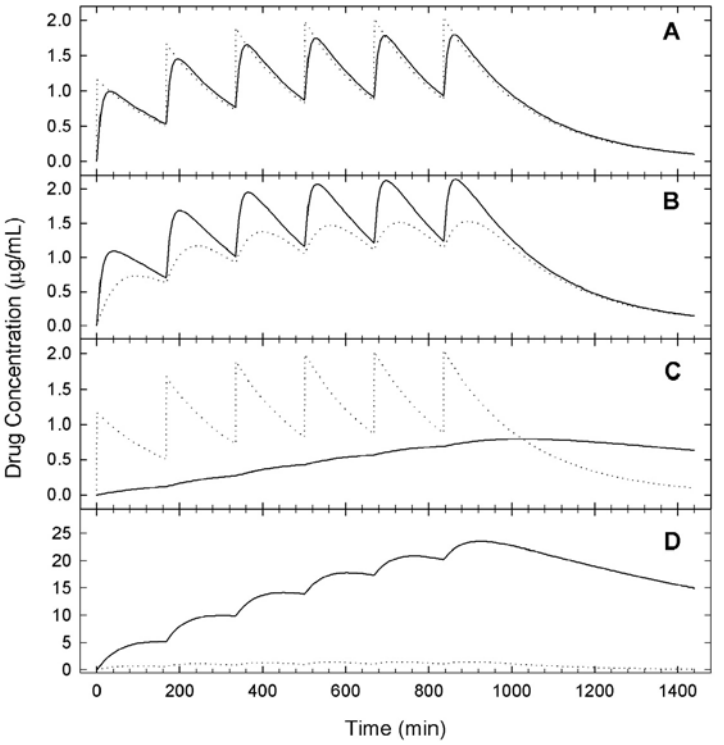

Figure 8. Plots of drug concentration in the central volume (dotted line) and target site (solid line) as a function of time for repeated bolus drug or prodrug input (58.33 mg every 167 min to a total dose of 350 mg). Panels A and B are for a drug with high permeability (Cl^{D}_{in} = Cl^{D}_{out} = 10 mL/min) between the central volume and target site with either drug (Panel A) or prodrug input (Panel B). Panels C and D are for a drug with low permeability (Cl^{D}_{in} = Cl^{D}_{out} = 0.1 mL/min) between the central volume and target site with either drug (Panel C) or prodrug input (Panel D). For these simulations Cl^{PD}_{elim} = 0 mL/min and V_{max} = 7140 μg/min. All other values as in Table I.

bolus dosing regimens is dependent on the desired time course exposure of the target site to drug. For example, if steady-state drug levels are to be held between a minimum effective level and a maximum tolerated level, then a drug with higher relative permeability between the central volume and the target site is desirable. In this instance, a prodrug may offer an advantage in that better target site to central volume drug exposure is attainable while still having the ability to titrate to steady-state drug levels. Alternatively, if maximum exposure of the target site to drug without concern for attaining steady-state drug levels is desirable, cytotoxic drugs, for example, then a targeting prodrug of a drug with low permeability between the central volume and the target site may be advantageous.

The utility and advantages of a prodrug approach for infused or multiple-dose drugs may be evaluated by observing the dependence of the target site/central volume drug AUC ratio from either drug or prodrug input as a function of drug permeability between the central volume and the target site.

Figure 9 was generated by determining the respective 24-hour AUCs for each compartment in the model depicted in Fig. 1 resulting from infusion of either drug or prodrug at 350 μg/min for 1000 min.

Several trends can be recognized when examining Fig. 9. As the permeability values for drug infusion decrease from 1 mL/min, the exposure of the target site to drug relative to the central volume also decreases as a result of a larger relative contribution from systemic drug clearance. Such a situation may not be optimal for a drug with toxic side effects that mirror systemic drug levels. A prodrug approach may be an alternative in these situations as the AUC ratio for prodrug infusion increases as permeability values decrease from 1 mL/min. However, the use of prodrugs to attain steady-state levels of drug at the target site becomes increasingly difficult as permeability decreases, and the prodrug approach may only be useful for high target-site drug loading.

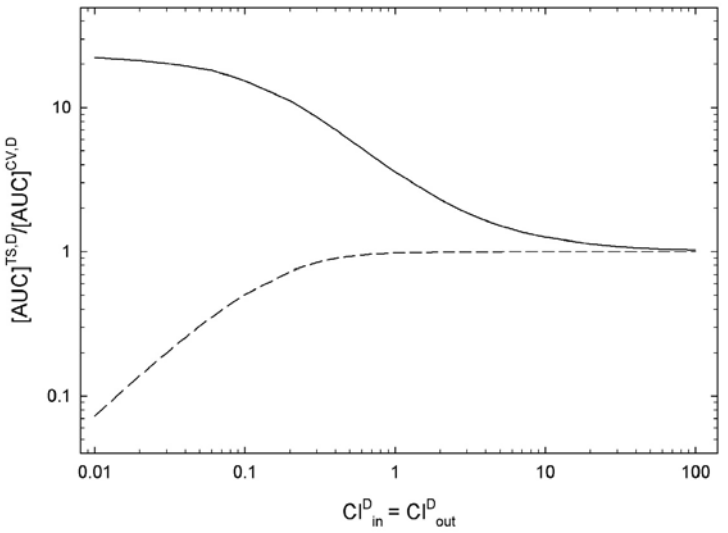

Figure 9. Plot of the ratio of AUC of drug at the target site ($[AUC]^{TS,D}$) relative to the central volume ($[AUC]^{CV,D}$) following infusion (350 μg/min for 1000 min) of drug (dashed line) or prodrug (solid line) as a function of drug permeability between the central volume and the target site ($Cl^{D}_{in} = Cl^{D}_{out}$). For these simulations V'_{max} = 7140 μg/min. All other values as in Table I.

At intermediate values of drug permeability (1–10 mL/min), the prodrug approach offers both the ability to titrate steady-state target site drug levels and a favorable target site-to-central volume drug exposure ratio. In this region, the prodrug approach provides both targeting to the target site and the ability to reduce potential toxic side effects associated with the central volume drug concentration relative to infusion of the parent drug. As drug permeability values increase from 10 mL/min, the advantages of prodrug targeting decrease. Physiologically, as these values increase above this level, the target site would have

to be either a highly perfused organ with high extraction efficiency or a site with active uptake of drug. Under these circumstances the benefits of prodrug targeting collapse and offer minimal advantage over parent drug infusion. This effect is observed as the superimposition of the AUC ratio values for prodrug and drug infusion in Fig 9.

Begin with the End in Mind

As demonstrated by these simulations, the effect of drug retention at the target site during infusion or multiple dosing to steady state can be beneficial or detrimental to successful drug therapy. Like the importance of choosing relevant criteria for assessing the effectiveness of drug targeting via a prodrug, knowledge of the required target site drug concentration-time profile as it relates to pharmacological action and systemic toxicity is important. Based on these simulations and the work of Stella and Himmelstein and others, the definition of drug targeting may be refined to include "the selective delivery of a therapeutic agent to the site of action in a manner that maximizes the pharmacological effect and minimizes the toxilogical burden to the rest of the organism." Armed with this understanding of the interplay between drug properties, relative clearance behavior, and conversion rates, the development scientist can embark on a rational road to targeted prodrug development.

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2.5.2

Targeting - Cancer – Small Molecules

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Table of Contents

List of Abbreviations

5-FU 5-fluorouracil
9-ACPT 9-aminocamptothecin
9-NCPT 9-nitrocamptothecin
ADAPT .....antibody-directed abzyme prodrug therapy
ADEPT .....antibody-directed enzyme prodrug therapy
AMC 7-amino-4-methylcoumarin
BN/GRP .....bombesin/gastrin-releasing peptide
APN .....aminopeptidase N (= CD13)
BDEPT .....bacterial-directed enzyme prodrug therapy
Boc .....*tert*-butoxycarbonyl
Chg .....cyclohexylglycine
CPT .....camptothecin
Dox .....doxorubicin
ECM .....extracellular matrix
EPR .....enhanced permeability and retention effect
FMPA .....fusion protein-mediated prodrug activation
GDEPT .....gene-directed enzyme prodrug therapy
HA .....hyaluronic acid
poly-HPMA .....poly[N-(2-hydroxypropyl)methacrylamide]
LDL .....low-density lipoprotein
LH-RH .....luteinizing hormone-releasing hormone
mAb .....monoclonal antibody
MDEPT .....macromolecular-directed enzyme prodrug therapy
MDR .....multi-drug resistance
Mel .....melphalan
Mmc .....mitomycin C
MMP .....matrix metalloproteinase
MTD .....maximum tolerated dose
MTX .....methotrexate
PABA .....para-aminobenzyl alcohol
PABC .....para-aminobenzyloxycarbonyl
PAI-1 .....plasminogen activator inhibitor 1
PDEPT .....Polymer-directed enzyme prodrug therapy
PEG .....poly(ethylene) glycol
Plg .....plasminogen
PLGA .....poly(lactic-co-glycolic acid),
PSA .....prostate specific antigen
SERPIN .....serine protease inhibitor
SST .....somatostatin
t-PA .....tissue-type plasminogen activator
TPZ .....tirapazamine
u-PA .....urokinase-type plasminogen activator
u-PAR .....urokinase-type plasminogen activator receptor
VDEPT .....virus-directed enzyme prodrug therapy

Cancer Chemotherapy

Except for heart and coronary disease, cancer is now the principal cause of death in the Western world. Despite extensive cancer research to find improved drugs and treatments, the average chance of being cured of cancer is augmented every year by only 0.5 percent. Cancer comprises a broad group of diseases characterized by uncontrolled and independent proliferative growth of tumor cells (Alberts *et al.*, 1994). In cancer, malignant tumors invade surrounding tissue and give rise to formation of secondary tumors (metastases). The ability to metastasize is largely responsible for the lethality of malignant tumors. Surgery and radiotherapy are mostly used for treatment when a tumor is localized to a certain tissue. When metastasis has occurred, chemotherapy becomes an important weapon against cancer.

Cancer chemotherapy, first experimented with in 1943 by employing mustard gas alkylating agents (DeVita *et al.*, 1997), is typically associated with severe side effects because highly toxic compounds are used. This can be explained by the fact that most chemotherapeutic agents interfere with the cell division process. Not only cancer cells but also dividing healthy cells are tackled by the agent, with undesired side effects such as nausea, vomiting, diarrhea, hair loss, and serious infection as a consequence (Boyle and Costello, 1998).

Most chemotherapeutic agents used for treatment of cancer have a small therapeutic window (or therapeutic index), meaning that they cause severe, sometimes life-threatening side effects, while often little therapeutic effect is seen. Drug concentrations that would completely eradicate the tumor cannot be reached because gastrointestinal tract and bone marrow toxicity are dose limiting. As a consequence, a lower dose of the agent is given, which diminishes the chance of achieving a complete cure.

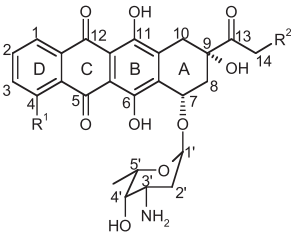
Another problem associated with chemotherapy is that tumor cells can develop resistance against anticancer agents after prolonged treatment (Volm, 1998). Tumors often develop resistance mechanisms against more than one chemotherapeutic agent; this is called multi-drug resistance (MDR). MDR can already be effectuated by treatment with a single drug, which can activate the P-glycoprotein pump, a cellular excretion system (Gerlach *et al.*, 1986). Resistant tumors are not often curable because the resistant cells can survive and proliferate after treatment with low-dose chemotherapeutic agents. The remission of tumor burden is of limited duration and of variable degree, and re-growth and spread can be even more malignant.

Lack of selectivity of chemotherapeutic agents is a major problem in cancer treatment. For this exact reason it is necessary to develop targeted delivery approaches for chemotherapeutic agents. A targeted conjugate derivative of an anticancer agent, which delivers the toxic agent at the tumor site, in the vicinity of or inside tumor cells, may possess a significantly improved therapeutic window with respect to the parent agent.

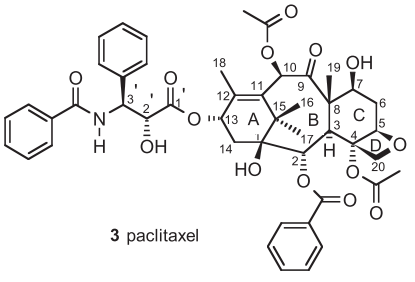
Anticancer Agents

Several classes of antitumor compounds are used for treatment of cancer and can thus be employed as parent agents for targeted delivery. Currently, about 40 cytotoxic agents are registered as anticancer agents. often they comprise complicated natural compounds, and they can be classified on the basis of their biochemical mechanism of action or their origin (Tannock and Hill., 1998). Classes of chemotherapeutic agents are: i) alkylating agents (*e.g.*, nitrogen mustard, melphalan, mitomycin C, duocarmycins), ii) antimetabolites (*e.g.*, methotrexate, 5-fluorouracil), iii) natural products and their derivatives (*e.g.*, anthracyclines, vinca alkaloids, taxanes, camptothecins), and iv) miscellaneous agents (*e.g.*, cisplatin). Although all of these agents have been employed by researchers as parent agents in tumor-activated prodrug therapy, most efforts in the field of targeted conjugates have been pursued using anthracyclines, taxanes, and camptothecins as parent agents.

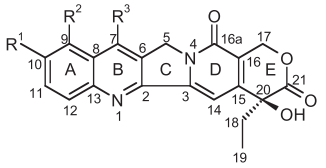
The anthracyclines (Figure 1) are members of an important class of cytotoxic agents that have been used for many years in the treatment of many different types of cancer (Priebe, 1995). In fact, doxorubicin (Dox, **1**, also known as Adriamycin®) is considered one of the most active single anticancer agents because of its broad antitumor spectrum. In the late 1950s daunorubicin (Dau, **2**, also known as daunomycin) was first isolated (Di Marco *et al.*, 1964), and several years later Arcamone was the first to isolate doxorubicin (Arcamone *et al.*, 1969). Many analogs have been synthesized and tested since that time, but doxorubicin is still

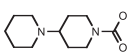


1 doxorubicin: R^1 = OMe; R^2 = OH
2 daunorubicin: R^1 = OMe; R^2 = H



3 paclitaxel



4: R^1 = R^2 = R^3 = H: camptothecin (CPT)
5: R^1 = R^3 = H, R^2 = NH_2: 9-aminocamptothecin (9-ACPT)
6: R^1 = OH, R^2 = $(CH_3)_2NHCH_2$, R^3 = H: topotecan
7: R^1 =  , R^2 = H, R^3 = CH_3CH_2: irinotecan (CPT-11)

8: R^1 = OH, R^2 = H, R^3 = CH_3CH_2: SN-38

Figure 1. Daunorubicin, doxorubicin, paclitaxel, and camptothecins as representative anticancer agents.

the anthracycline most frequently used in the clinic (Weiss, 1992). Other anthracyclines include idarubicin, epirubicin, mitoxantrone, and actinomycin D.

The anthracyclines act upon the cell division process via multiple mechanisms of action (Priebe, 1995). These mechanisms comprise interaction with DNA topoisomerase II, causing single and double strand breaks (the most important mechanism) (DeVita *et al.*, 1997), intercalation between DNA base pairs perpendicular to the helical axis, and binding of the cell membrane (Tannock and Hill, 1998). Another portion of the biological activity of anthracyclines comprises their *in vivo* bioreductive activation to a semiquinone radical. This radical generates superoxide (O_2^-) and hydroxyl radicals that can damage cell membranes and DNA. The most recently discovered mode of action of anthracyclines is their ability to induce apoptosis (programmed cell death) (Dunkern and Mueller-Klieser, 1999).

The anthracyclines suffer from severe dose-limiting side effects, with cumulative cardiotoxicity being the most serious. The fact that only limited doses of anthracycline can be given to the patient hampers complete eradication of the tumor and contributes to the development of multidrug resistance.

The two most important members of the taxane class of anticancer drugs are the clinically used paclitaxel (**3**, Figure 1) and docetaxel (proprietary names Taxol® and Taxotere®, respectively). Several other taxane derivatives have been evaluated in clinical trials. Paclitaxel is used against ovarian, breast, non-small cell lung and lung cancer, and AIDS-related Kaposi's sarcoma. It was first isolated in the late 1960s from the bark of Taxus brevifolia and was considered one of the most promising anticancer agents of the last decade (Nicolaou *et al.*, 1994; Farina, 1995). Interest in this complex molecule increased particularly when it was discovered in the late 1970s that paclitaxel stabilizes microtubules by inhibiting the microtubule depolymerization process (Schiff *et al.*, 1979; Amos and Lowe, 1999), a unique mechanism of action (Kingston, 2001). Much later, it was discovered that paclitaxel can induce apoptosis (Wang *et al.*, 2000) through binding the anti-apoptotic protein Bcl-2 (Rodi *et al.*, 1999). Finally, paclitaxel can interact with DNA (Krishna *et al.*, 1998), although it has not yet been revealed to what extent this mechanism contributes to its activity. Paclitaxel is certainly not an ideal anticancer drug. Apart from causing serious side effects that are typically associated with chemotherapy (Panchagnula, 1998), a low water-solubility is characteristic of paclitaxel. The medium in which it is administered, Cremophor EL, causes hypersensitivity reactions (Szebeni *et al.*, 1998).

The isolation of camptothecin (**4**; CPT) from Camptotheca acuminata and its structure determination was reported in 1966 (Figure 1) (Wall *et al.*, 1966). The camptothecins (CPTs) belong to a relatively new class of promising anticancer agents, of which two derivatives have reached the clinic for treatment of ovarian and colon cancer: topotecan (**6**; Hycamtin®) and irinotecan (**7**; CPT-11; Camptosar®), respectively (Kehrer *et al.*, 2001). Irinotecan **7** is an inactive prodrug derivative of the cytotoxic CPT derivative SN-38 (**8**). CPT derivatives such as 9-nitrocamptothecin (9-NCPT) and 9-aminocamptothecin (**5**; 9-ACPT) have also reached the clinical trial stage. Both CPT and 9-ACPT have shown outstanding

preclinical effectiveness (Giovanella *et al.*, 1991; Potmesil, 1994). As in the case of paclitaxel, interest in the camptothecin class of agents increased in the early 1980s when their mechanism of action was discovered. CPTs are the only agents introduced into the clinic that are directed to topoisomerase I. Their exact molecular mechanism of action has not yet been completely unraveled (Kerrigan and Pilch, 2001). Topoisomerase I is an enzyme that is involved in the relaxation of superhelical DNA. It breaks and religates single strand DNA during the DNA replication process in the nucleus (Hertzberg *et al.*, 1989; Pommier, 1993). CPTs are able to trap the covalent cleavage intermediate, the cleavage being the critical step in this catalytic cycle (Wang, 1985). Specific inhibition of the religation step results in stabilization of the topoisomerase I-DNA cleavable complex (Kerrigan, 1994), the crystal structure of which has been reported (Redinbo *et al.*, 1998). CPTs possess several undesired properties (Kehrer *et al.*, 2001): i) they cause side effects that usually go together with cancer chemotherapy, such as severe diarrhea, myelosuppression, and hemorrhagic cystitis; ii) they are poorly water-soluble; and iii) they contain a 6-membered lactone ring that can be opened under physiological conditions to yield a relatively inactive carboxylate derivative.

Anticancer Prodrug Therapy and Targeting Strategies

Prodrug Therapy

In 1908, Paul Ehrlich, one of the main founders of chemotherapy, received the Nobel Prize for his idea of the 'magic bullet.' He meant by this an active chemical substance that is delivered only at the desired site in the body. A promising approach to overcoming side effects of anticancer agents and achieving a more tumor-selective treatment is prodrug therapy. In this concept, a cytotoxic drug is incorporated in a non-toxic derivative, the prodrug. Upon administration of the prodrug, a selective activation at the tumor site, for example an enzymatic cleavage, must trigger regeneration of the toxic parent drug. The released agent must subsequently be able to kill the cancer cells. Thus, a tumor-activated prodrug is a therapeutically inactive derivative of a therapeutically active drug, which can be converted chemically and/or enzymatically to the parent drug in the tumor environment. The tumor-selective activation should be effectuated by exploitation of properties that distinguish neoplastic cells from normal cells (Dubowchik and Walker, 1999; de Groot *et al.*, 2001a; Damen *et al.*, 2001) .

For site-specific activation, differences in physiological conditions such as hypoxia, pH, the presence of tumor-specific receptors and antigens, and the presence of tumor-associated enzymes can be exploited. Extensive research has been performed to develop new targeting concepts and to design new prodrugs for a range of tumor-associated targets (Dubowchik and Walker, 1999; Damen *et al.*, 2001; de Groot *et al.*, 2001a; Huang and Oliff, 2001) .

As discussed in other sections of this book, prodrugs can be applied to: i) enhance bioavailability and passage through biological barriers, ii) increase

duration of pharmacological effects, iii) decrease toxicity and adverse reactions, iv) and improve stability and solubility (Bundgaard, 1991). Targeting of drugs to a specific organ or tissue (for example, in cancer) can be added to this list. A prodrug approach becomes of even greater interest when multiple properties of the parent agent can be improved at the same time. For example, when a peptide is conjugated as a promoiety (part of the prodrug that is attached to the parent drug) to a parent drug, both specificity and water-solubility may be improved. Although decreased side effects is a major advantage of targeted prodrug therapy in itself, it also potentially enables administration of the low-toxic and selective conjugate in higher doses than the parent drug. As a result, it would allow for a more efficacious treatment of cancer.

In general, prodrugs designed for tumor-activated prodrug therapy must fulfill several requirements. The most important requirements are (Carl, 1983):

i) The tumor-associated biomolecule must be present in significantly elevated levels in tumor tissue compared to normal tissue. Its level must be high enough to generate cytotoxic levels of free drug in the tumor.
ii) Prodrug activation at sites distant from tumor tissue must be minimal.
iii) The prodrug must be a good substrate or possess high binding affinity for the tumor-associated biomolecule.
iv) Ideally, the prodrug is significantly less toxic than the parent drug.
v) The prodrug must not be rapidly excreted from the body and must not enter cells randomly. Therefore, it must possess suitable polarity.

The difficulty in targeted chemotherapy is in delivering high enough concentrations of cytotoxic drug to the target site to completely eradicate the tumor. In modern drug development, targeting of cytotoxic drugs to the tumor site can be considered one of the primary goals.

Prodrug Monotherapy

Anticancer prodrug monotherapy is defined as chemotherapy in which prodrugs are used that are designed for direct activation or recognition by a tumor-associated factor, such as hypoxia, an enzyme or a receptor. In prodrug monotherapy, the administered prodrug is directly and site-specifically cleaved to yield the parent compound (Figure 2). The difference between this and other prodrug therapies, discussed in the next paragraph, is that monotherapy is a one-step therapy: only the prodrug is administered. In anticancer prodrug monotherapy, a tumor-associated factor (a phenotypic difference between tumor and normal tissue) is responsible for prodrug recognition/activation. Tumor-associated factors can be a low pH, the natural abundance of certain enzymes in tumor tissue, or the natural existence of regions in solid tumors where low oxygen tension is present, which results in enhanced activity of reductive enzymes.

Furthermore, receptors or antigens that are expressed in elevated levels in tumor tissue can be targeted.

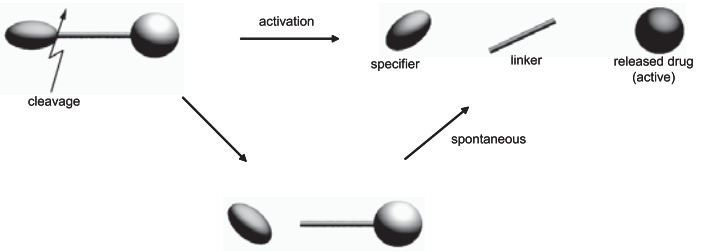


Figure 2. Prodrug cleavage triggers drug release. A releasable linker may facilitate activation.

For this approach to work, a target biomolecule is needed that is produced by tumor cells or generated in tumor tissue and, at least as important, is not present or hardly present in normal tissue. Although the identification of all the molecular targets that characterize cancers is at present incomplete, more details regarding phenotypic differences between cancer and healthy cells are expected to be revealed by future research (Huang and Oliff, 2001).

Two-step prodrug therapies

In two-step prodrug therapy, the enzyme that is supposed to activate the prodrug administered in the second step of the therapy must be targeted to tumor tissue in the first step. Enzymes can be directed to the tumor site or be expressed selectively at the tumor site in several manners. In the concept of antibody-directed enzyme prodrug therapy (ADEPT) (Bagshawe, 1987, 1995; Senter *et al.*, 1988), a specific enzyme can be localized in the first step using an antibody-enzyme conjugate. The monoclonal antibody (mAb) must specifically bind a tumor-associated antigen on the cell surface. Overexpressed tumor-specific antigens can often be found in a wide range of human malignant tumor cells (Panchal, 1998). After localization of the enzyme and clearance of non-localized conjugate, the second step of the therapy is administration of the prodrug, which is a substrate for the localized enzyme.

In another two-step prodrug therapy, a gene encoding for the enzyme is targeted to tumor cells in the first step; this is called gene-directed enzyme prodrug therapy (GDEPT) (Niculescu-Duvaz *et al.*, 2004). When a retrovirus is used to deliver the gene to the tumor cell, the therapy is called virus-directed enzyme prodrug therapy (VDEPT) (Huber *et al.*, 1991). In bacterial-directed enzyme prodrug therapy (BDEPT), spores of genetically modified anaerobic bacteria, which can germinate only in hypoxic regions of the tumor can be employed (Lemmon *et al.*, 1997). The bacterium then produces the programmed enzyme selectively at the tumor site. Polymer- or macromolecular-directed enzyme prodrug therapy (PDEPT/MDEPT) makes use of the enhanced permeability and retention effect (EPR) (Maeda and Matsumura, 1989) in tumor tissue, which can

lead to accumulation of an enzyme that is conjugated to a polymer (Melton *et al.*, 1999). If a humanized catalytic antibody (abzyme) is used, the therapy is referred to as antibody-directed abzyme prodrug therapy (ADAPT) (Miyashita *et al.*, 1993). Another technique called fusion protein-mediated prodrug activation (FMPA) (Bosslet *et al.*, 1994) uses DNA recombinant technology to prepare fusion proteins that contain both the antibody and the enzyme. Bispecific antibodies that bind both to the tumor cell antigen and the enzyme can also be employed for prodrug activation. In all concepts mentioned above, the prodrug is administered in a second step after prior localization of a specific enzyme in the first step of the therapy. A number of polymeric drug delivery conjugates as well as ADEPT and GDEPT therapies are currently being evaluated in clinical trials.

Releasable Linkers in Targeted Conjugates

In this section as well as in other sections throughout this book, (tumor-activated) prodrugs in which a releasable (self-elimination) linker (or spacer) is incorporated between a specifier (targeting unit part of prodrug or conjugate that is (enzymatically) removed) and the parent drug are discussed. Two main reasons for application of releasable linker systems in prodrugs and bioconjugates are facilitation of (enzymatic) activation, and incorporation of appropriate linkage chemistry. In a prodrug designed for enzymatic activation, steric burden caused by a bulky specifier or a bulky parent drug (or both) can (partially) block efficient enzymatic cleavage. In Figure 2, the principle of drug release from a self-elimination spacer-containing conjugate is schematically depicted.

Two types of self-elimination spacers can be distinguished. The first concerns self-elimination spacers that eliminate as a consequence of shifting conjugated electron pairs, ultimately leading to expulsion of the leaving group (the drug). We call this spacer type 'electronic cascade linker.' The second releasable spacer type, the 'cyclization linker.' involves self-elimination spacers that release the drug following an intramolecular cyclization reaction.

The most prominent example of an electronic cascade spacer is the 1,6-elimination linker that was developed in the early 1980s by Katzenellenbogen and colleagues (Figure 3) (Carl *et al.*, 1981). Following enzymatic hydrolysis of the

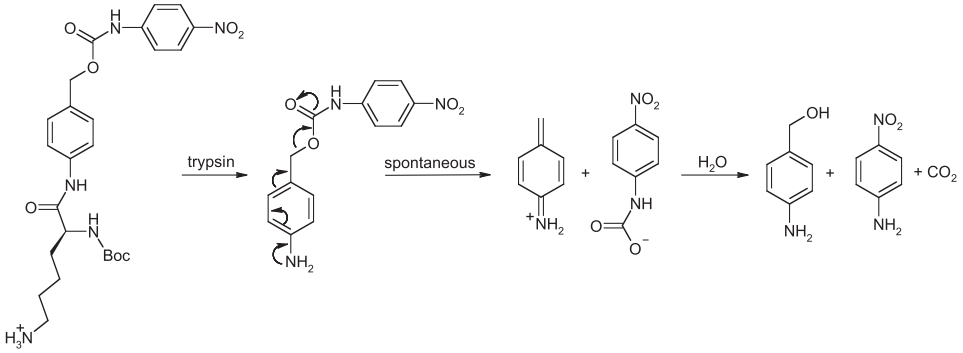


Figure 3. First application of the 1,6-elimination electronic cascade linker in prodrug design.

lysine residue by trypsin, the resulting strongly electron-donating 4-aminobenzyl group triggers a 1,6-elimination to release the model compound 4-nitroaniline. This 1,6-elimination spacer can be considered as one of the most versatile self-immolative connectors that can be incorporated in drug conjugates.

When the electron-donating group and the leaving group on the benzene ring are positioned ortho with respect to one another, 1,4-elimination can take place in a similar fashion. Other 1,4-elimination linkers have also been reported (Rivault *et al.*, 2004). The 1,4- and 1,6-elimination processes can occur when an electron-donating amino or hydroxyl group is generated from a masked amino or hydroxyl functionality.

The second self-elimination spacer type concerns the cyclization spacer, of which several variations exist. One prominent example is the ethylene diamine spacer that, after removal of the specifier R^1, intramolecularly cyclizes to yield a cyclic urea derivative and the liberated parent drug HOR^2 (Figure 4, A) (Saari *et al.*, 1990). Because some cyclization spacers possess long half-lives of cyclization, bulky substituents have been introduced on the spacer with the goal of conformationally pre-orientating the functional groups involved in the cyclization reaction and achieving a favorable enthalpy and entropy effect for ring closure (Thorpe-Ingold effect) (Eliel, 1962). According to this principle, the trimethyl lock spacer is a frequently used cyclization spacer (Figure 4, B) (Nicolaou *et al.*, 1996; Wang *et al.*, 1997). Following activation, an intramolecular cyclization leads to the formation of a lactone and release of free drug R^2H.

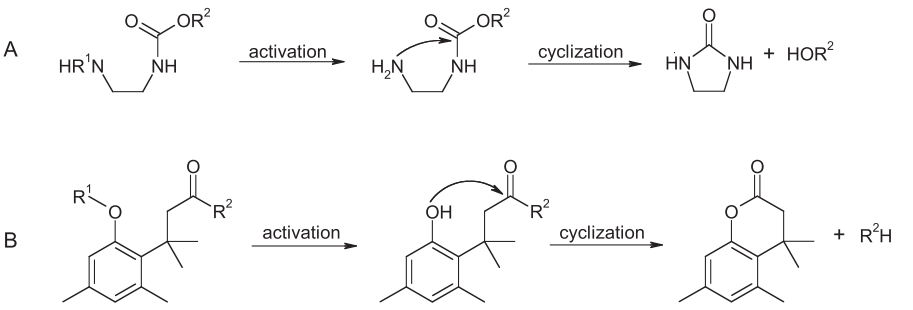


Figure 4. Prodrug activation, intramolecular spacer cyclization and drug release.

The need for incorporation of a linker in between the parent drug and the specifier seems to be a common theme for conjugates designed for enzymatic activation. Several structurally different enzymes cleaved spacer-containing prodrugs much more readily than the corresponding prodrugs lacking a spacer. Evidence for this recurring theme was obtained, for example, with substrates for the enzyme β-glucuronidase. A glucuronide directly coupled to epirubicin was inert toward β-glucuronidase cleavage, whereas a 1,6-elimination spacer-containing doxorubicin analog did show substrate behavior for the enzyme (Desbène *et al.*, 1998; Papot *et al.*, 1998; Madec-Lougerstay *et al.*, 1999). Another β-glucuronide carbamate prodrug of daunorubicin was only a moderate substrate

for this enzyme (Leenders *et al.*, 1995b), whereas incorporation of a spacer drastically enhanced the affinity of the enzyme for the prodrug (Leenders *et al.*, 1995a; Houba *et al.*, 1996). Incorporation of a spacer between 5-fluorouracil (5-FU) and the glucuronide specifier dramatically enhanced the rate of hydrolysis by glucuronidase (Madec-Lougerstay *et al.*, 1999). Doxorubicin and Mmc prodrugs designed for cleavage by cathepsins and lacking a linker were resistant to enzymatic activation, whereas incorporation of a linker led to drug release for both parent drugs (Dubowchik and Firestone, 1998; Dubowchik *et al.*, 1998).

We and others have reported novel releasable linker chemistries and concepts. For example, we have developed releasable linkers for which both the length and the chemistry are tunable (de Groot *et al.*, 2001b). Advantages are that efficiency of drug release can be improved and that different linker chemistries are available for different drug molecules. Depending on the drug's functional group used for linkage, the linkers enable conjugation via appropriate chemistry, preferably through stable carbamate linkages. Senter *et al.* have reported another interesting electronic cascade releasable linker system, which is applicable for conjugation via the drug's aromatic hydroxy group (Toki *et al.*, 2002).

A novel releasable linker concept has been recently reported simultaneously by two groups, among which is our own group (de Groot *et al.*, 2003; Amir *et al.*, 2003). In the same year, a third group reported their work on the same concept (Li *et al.*, 2003). Branched self-elimination linker systems release multiple leaving groups upon a single activation event. Double release and triple release spacers were developed for which proof of principle has been delivered. Multiple generations of multiple release spacers can be coupled to one another to yield dendrimeric multiple release conjugates, which we termed 'cascade-release dendrimers' (Figure 5).

The multiple release spacers in these exploding dendrimers fall apart into the corresponding separate monomers and release all drug molecules upon a single

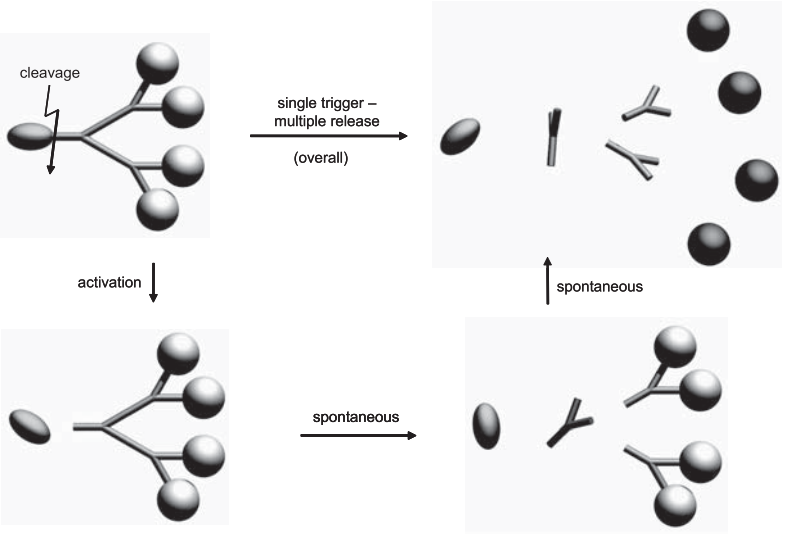


Figure 5. A 'cascade-release dendrimer.' containing two generations of double-release linker monomers, releases all end groups (drug molecules) following a single activation event.

activation (enzymatic cleavage). Proof of this has been reported for complete release of four paclitaxel molecules as end groups from a cascade-release dendrimer prodrug containing two generations of double release self-elimination linkers. This type of branched releasable linker system may prove useful in construction of single-activation/multiple release prodrugs (Meijer and van Genderen, 2003).

Tumor-associated (Enzyme) Targets and Prodrugs

pH, Hypoxia, and β-Glucuronidase Targeted Prodrugs

pH-sensitive Prodrugs

Under normal physiological conditions, the pH of plasma and tissues is maintained slightly above neutral pH. Many tumors, however, possess hypermetabolic activity and/or may be in a hypoxic state (decreased oxygen level). These phenomena induce a localized decrease of pH, which can be 0.7 to 1.0 pH units lower than the normal physiological pH. This difference in pH can be exploited for drug targeting. Recent research in the area of pH-sensitive prodrugs has been performed mostly with antibody-drug conjugates (Lam *et al.*, 2003) and drug-polymer conjugates (Kratz *et al.*, 1999; Tomlinson *et al.*, 2003).

Hypoxia-activated Prodrugs

Many solid tumors contain a malformed vasculature (Vaupel *et al.*, 1989). As a result of this, some cells are too distant from a blood vessel and die (necrotic area). There also exists a transition area in which a low oxygen tension is present, but the cells in this region are still viable. This area is called the chronic hypoxic area (Brown, 1999). Due to the highly irregular blood flow, some regions inside the tumor suffer from temporary cessation of blood flow and lack of oxygen (acute hypoxia) (Brown, 2000b). The presence of regions with acute or chronic hypoxia in tumors is a major problem in cancer treatment (Rauth *et al.*, 1998). Firstly, hypoxic cells are more resistant to damage by radiation therapy. Oxygen is an important radiation sensitizer and the low oxygen concentration in hypoxic cells greatly reduces the efficacy of the treatment. Secondly, tumor hypoxia is also a problem for chemotherapeutic treatment. Not only do hypoxic cells receive a diminished amount of oxygen, also concentrations of nutrients such as glucose are lower in hypoxic tissue. This causes cells to stop or slow down their rate of progression through the cell cycle. Since most anticancer agents are more effective against rapidly dividing cells, the antitumor agent will affect these quiescent cells far less. It is important to note that these agents have to diffuse from the blood vessel to hypoxic tissue, and many anticancer drugs, because of their reactivity, are limited in their diffusion range. Tumor hypoxia is also responsible for the amplifi-

cation of genes conferring drug resistance. In addition, hypoxia may play a crucial role in malignant progression by direct mutations (Reynolds *et al.*, 1996), metastasis (Sundfor *et al.*, 1998), and angiogenesis (the formation of new blood vessels) (Schweiki *et al.*, 1992).

The low oxygen levels can, on the other hand, be turned into a therapeutic advantage. Apart from being selective to tumor cells, a drug that is toxic only to hypoxic cells would overcome the resistance of hypoxic tumor cells toward standard therapy. Hypoxia-selective cytotoxic agents can be used in combination therapy with conventional anticancer chemotherapeutic agents. The hypoxia-selective drug preferentially kills the hypoxic tumor cells, leaving the aerobic tumor cells unaffected, while the other agent would eradicate the aerobic tumor cell population (Denny and Wilson, 1993).

Activation of hypoxia selective prodrugs arises from bioreduction in the absence of oxygen in hypoxic areas. Therefore, hypoxia selective cytotoxic drugs are referred to as bioreductive drugs. Most hypoxia selective drugs are designed for reduction by endogenous reducing enzymes also present in aerobic cells. Usually, these enzymes give rise to a one-electron adduct, which is back oxidized in the presence of molecular oxygen. Further reduction of the prodrug to the active drug is therefore restricted to hypoxic tissue. Activation can also occur by tumor-associated reductive enzymes, such as DT diaphorase (Ross *et al.*, 1993).

The three most commonly used reducible moieties are quinones (Dirix *et al.*, 1996; Jaffar *et al.*, 1998; Gharat *et al.*, 1998), N-oxides (Brown, 1993) and (hetero)aromatic nitro groups (Siim *et al.*, 1997). One of the most important members of bioreductive quinone drugs is mitomycin C (Mmc), which is used to treat patients. The most promising member of the N-oxide bioreductive drugs (White *et al.*, 1989; Mann and Shervington, 1991; Henderson *et al.*, 1996; Wilson *et al.*, 1996; Raleigh *et al.*, 1998, 1999; Highfield *et al.*, 1998, 1999) is tirapazamine (TPZ) (Wang *et al.*, 1992; Brown, 2000a), which showed encouraging results in a phase III clinical trial in advanced non-small cell lung cancer in combination with cisplatin (Brown, 2000b).

In another approach, the nitroaromatic group is utilized as an electronic switch; reduction is followed by electronic cascade fragmentation to release the drug (Figure 6) (Jenkins *et al.*, 1990; Skarsgard *et al.*, 1995; Siim *et al.*, 1997; Shyam *et al.*, 1999; Lee and Wilson, 2000; Reynolds *et al.*, 2000). Reduction of a nitroaromatic function can also be used to induce an intramolecular cyclization reaction and subsequent release of the parent compound (Sykes *et al.*, 1999).

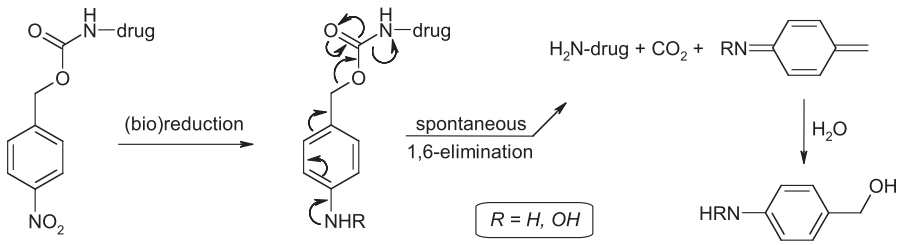


Figure 6. Release of the parent drug after reduction and 1,6-elimination in analogous fashion with respect to Figure 3.

Most of these prodrugs contain a nitrobenzyl carbamate moiety (as in Figure 6), which after reduction triggers a 1,6-elimination. The application of reduction and subsequent fragmentation of nitrobenzyl carbamates and nitrobenzyl carbonates in this type of prodrug originates from protective group chemistry (Johnston *et al.*, 1978). The bioreductive nitrobenzyl carbamate triggers have been combined with a variety of parent drugs, such as derivatives of aniline mustard, actinomycin D, Dox, Mmc, and enediynes. Substituents on the aromatic ring may tune the redox properties of the nitroaromatic system, although strongly electron-withdrawing substituents may reduce the fragmentation rate. Also, nitro heteroaromatic prodrugs have been described or proposed, in which the electron-withdrawing heteroatom significantly increases the redox potential of the nitro substituent, which is therefore reduced more easily (Everett *et al.*, 1999; Hay *et al.*, 1999, 2000; Parveen *et al.*, 1999) .

β-*Glucuronidase-activated Prodrugs*

The potential of β-glucuronidase as a target enzyme for activation of prodrugs in selective chemotherapy has been established for a long time (Sperker *et al.*, 1997). Already in 1966 a correlation was found between therapeutic response to administration of aniline mustard in tumor-bearing mice and tumor-associated β-glucuronidase activity (Connors and Whisson, 1966). This was explained by the fact that the aniline mustard was metabolized to highly toxic 4-hydroxyaniline mustard, which was subsequently converted to a considerably less toxic glucuronide in the liver. Tumor-selective cytotoxicity was generated when the glucuronide derivative was converted into the toxic 4-hydroxyaniline mustard parent compound by β-glucuronidase in the tumor (Connors and Whisson, 1966). Inflammatory cells that are present in necrotic areas of tumor tissue are the source of β-glucuronidase, a lysosomal enzyme (Mürdter *et al.*, 1997). Lysosomal β-glucuronidase is liberated extracellularly in high local concentrations in necrotic areas of human cancers (Bosslet *et al.*, 1998). It has been demonstrated that the presence of extracellular β-glucuronidase is required for effective prodrug monotherapy with β-glucuronide prodrugs (Cheng *et al.*, 1999).

Many β-glucuronide antitumor prodrugs have been developed for application in ADEPT. However, these prodrugs can also be applied in prodrug monotherapy targeting high levels of β-glucuronidase in necrotic tumor tissue. β-Glucuronide prodrugs without a spacer have been reported (Roffler *et al.*, 1991; Leenders *et al.*, 1995a; Chen *et al.*, 1997; Bakina *et al.*, 1997) with a(n) (un)substituted fragmentation spacer (Desbène *et al.*, 1998; Papot *et al.*, 1998; Madec-Lougerstay *et al.*, 1999), or with a self-elimination cyclization spacer (Leenders *et al.*, 1995a; Houba *et al.*, 1996; Schmidt *et al.*, 1997; Lougerstay-Madec *et al.*, 1998). When a bulky anthracycline molecule was used as parent drug, it was discovered that incorporation of a self-elimination spacer between drug and specifier dramatically increased the rate of β-glucuronidase activation (Haisma *et al.*, 1994). Following β-glucuronide hydrolysis, the spacer spontaneously dissociates to release the drug. Without incorporation of a spacer, the glucuronide moiety is a poor substrate for

β-glucuronidase (Leenders *et al.*, 1995b). The structures of two spacer-containing β-glucuronide prodrugs are depicted in Figure 7. The spacers of both prodrugs eliminate via 1,6-elimination.

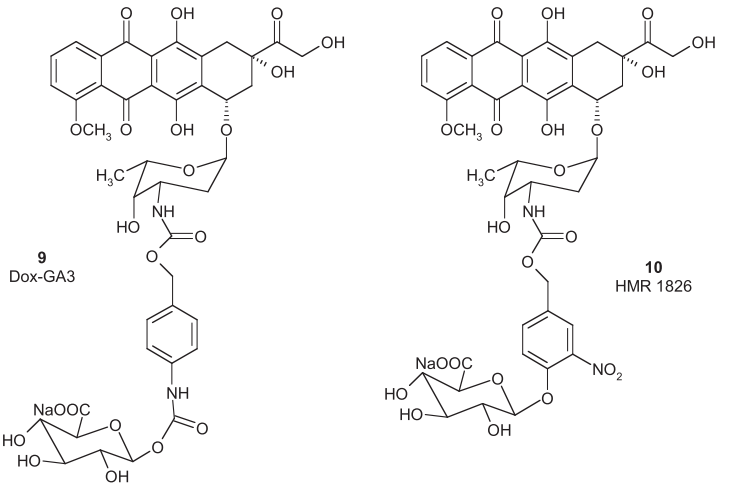


Figure 7. Releasable linker-containing β-glucuronide prodrugs.

Prodrug **9** (Dox-GA3) was developed in our group (Leenders *et al.*, 1999), whereas prodrug **10** (HMR 1826) was developed by French colleagues (Florent *et al.*, 1998). In both of these doxorubicin prodrugs, the parent drug is linked to the promoiety via its amino function. This functional group of the anthracyclines is often used for derivatization to obtain analogs with decreased toxicity. HMR 1826 (10) has been extensively investigated *in vivo* as a prodrug for monotherapy. It showed a higher therapeutic *in vivo* efficacy in comparison with parent drug, and in addition it has been shown to possess a 7-fold increased selectivity index with respect to doxorubicin (Múrdter *et al.*, 1997). It was 100-fold less cardiotoxic than doxorubicin (Platel *et al.*, 1999). Interesting *in vivo* results have also been obtained with β-glucuronyl carbamate-based prodrugs. The β-glucuronyl carbamate-based daunorubicin prodrug (structurally corresponding to doxorubicin prodrug **9**) induced higher *in vivo* tumor growth inhibition and delay than did the parent daunorubicin (Houba *et al.*, 1998) and higher tumor-selective accumulation (Houba *et al.*, 1999). Doxorubicin prodrug **9** is characterized by a substantially higher maximum tolerated dose (MTD) in comparison with parent doxorubicin (showing decreased toxicity), and it induced improved tumor growth inhibition compared to doxorubicin (Houba *et al.*, 2001) . Paclitaxel is another anticancer agent possessing a relatively low therapeutic index, and there is a high need for an improved delivery system for this molecule (Panchagnula, 1998). One can use the 7-hydroxyl group as a handle for attachment of the promoiety, although this functional group is less important for biological activity than the 2'-hydroxyl function (Figure 1). The main advantage of using the 7-hydroxyl group as a handle is the lower susceptibility of paclitaxel-7-esters and 7-carbonates to

ubiquitous enzymes in comparison with paclitaxel-2'-esters and 2'-carbonates. β-Glucuronidase-activated paclitaxel-2'-ester prodrugs with cyclization spacers have been reported by our group (de Bont *et al.*, 1997). β-Glucuronide prodrugs of 9-aminocamptothecin (9-ACPT) have also been synthesized, in which the 9-amino function was connected through a carbamate linkage to the spacer-glucuronic acid conjugate (Leu *et al.*, 1999). These prodrugs were characterized by increased water-solubility with respect to parent drug, susceptibility toward cleavage by β-glucuronidase *in vitro* and reduced cytotoxicity in comparison with parent drug. Recently, etoposide served as parent drug for a β-glucuronidase-activated prodrug (Schmidt and Monneret, 2003).

A limitation of β-glucuronide prodrugs in monotherapy may be that they are not activated throughout the tumor tissue, but only at sites of inflammatory cell infiltration (Huang and Oliff, 2001). Another drawback is their low circulation half-life, thus fast excretion. In fact, glucuronidation is one of the body's natural mechanisms to excrete (toxic) compounds from the body. Despite these limitations, and although a number of β-glucuronide prodrugs were originally designed for ADEPT, the prodrug monotherapy results obtained with the two prodrugs depicted in Figure 7 show that the concept holds promise.

Tumor-associated Protease Targets and Protease-cleavable Prodrugs

Tumor-specific Targets for Prodrug Therapy

Especially during the past decade considerable insight has been gained into how proteolytic systems play a role under pathological conditions and how they are involved in tumor invasion and metastasis. There is an increasing body of literature that links production of certain proteases to tumor malignancy (Yamashita and Ogawa, 1997). Mostly, proteolytic activity is required for tumor cells when they invade other tissue and form metastases (Reuning *et al.*, 1998; Ghosh *et al.*, 2000). A primary, un-metastasized tumor is encapsulated in a so-called extracellular matrix (ECM), which consists of proteins. The ECM facilitates organization of cells into more complex functional units, such as tissues and organs (Vu, 2001). In order to form metastases, the primary tumor must get through this matrix and must degrade it to make cell migration possible (Lijnen, 2000). Enhanced expression of proteolytic enzymes enables neoplastic cells to disseminate to distant sites (Yamashita and Ogawa, 1997). A number of protease families, such as cathepsins (Szpaderska and Frankfater, 2001), the urokinase-type plasminogen activator (u-PA) system (Schmitt *et al.*, 2000), and matrix metalloproteinases (MMPs) (Kleiner and Stetler-Stevenson, 1999; Overall and López-Otín, 2002; Huang *et al.*, 2002), are responsible for proteolytic ECM degradation. Recent studies indicate that proteases are not only involved in metastasis, but also in earlier stages of tumor progression at primary sites (Koblinski *et al.*, 2000). Most

recent efforts to design and synthesize enzymatically cleavable prodrugs have focused on peptide prodrugs as substrates for specific proteolytic enzymes.

Cathepsin B

Cathepsins are cysteine proteases that are present in relatively high levels in mammalian lysosomes. Cathepsins, and especially cathepsin B, are overexpressed in tumors, and they may play a crucial role in cancer invasion by directly degrading ECM proteins (Elliott and Sloane, 1996; McKerrow *et al.*, 2000). An intracellular form of cathepsin B has been reported to contribute to ECM degradation (Szpaderska and Frankfater, 2001). Proteases inside tumors cells are also believed to participate in local proteolysis by digestion of phagocytosed ECM (Koblinski *et al.*, 2000), and cathepsin B may be one of them.

Plasmin and the u-PA System

Plasmin is a principal enzyme involved in fibrinolysis, and it digests the fibrin network of blood clots. However, it is also widely accepted that this serine protease is intimately associated with metastatic spread of tumor cells (Yamashita and Ogawa, 1997). Many studies have been reported in which the role of u-PA and the serine protease plasmin in tumor growth was investigated. There exists substantial evidence that the serine protease plasmin plays a key role in tumor invasion and metastasis (Yamashita and Ogawa, 1997; Reuning *et al.*, 1998; Irigoyen *et al.*, 1999; Ghosh *et al.*, 2000). Active plasmin is formed on the tumor cell surface from the inactive pro-enzyme plasminogen (Plg, Figure 8). In 1976 it was discovered that u-PA is produced and released from cancer cells (Astedt and Holmberg, 1976). Tumor-associated cell bound u-PA that is produced by the cancer and/or stromal cells can cleave plasminogen, resulting in formation of active plasmin locally at or near the surface of tumor cells (Figure 8) (Hewitt and Danø, 1996) .

Many tumor cell lines and tumors have a significantly higher u-PA level than their normal counterparts (Quax *et al.*, 1990; Foekens *et al.*, 2000) and u-PA has been shown to be correlated with invasive behavior. u-PA has also been shown to be a strong prognostic factor for reduced survival and increased relapse in many types of tumors (Ganesh *et al.*, 1994; Ganesh *et al.*, 1996; Emeis *et al.*, 1997; Schmitt *et al.*, 1997). The urokinase-type plasminogen activator receptor (u-PAR) plays an important role by binding u-PA to the cell (Preissner *et al.*, 2000). Plasmin activity remains localized because cell-bound urokinase can convert cell-bound plasminogen into active plasmin, which remains bound to the cell. Cell-bound active plasmin is not inhibited. Active urokinase and active plasmin do not occur in the blood circulation since they are rapidly inhibited by serine protease inhibitors (SERPINs) such as plasminogen activator inhibitor 1 (PAI-1) and α_2-antiplasmin (Winman *et al.*, 1979; Holmes *et al.*, 1987; Plow and Miles, 1990), respectively, which block the active site. Furthermore, tissue-type plasminogen activator (t PA), which can, as u-PA, convert plasminogen into plasmin, is rapidly

cleared from the bloodstream by the liver (King, 1994). The endogenous plasminogen activators are maintained at low levels in the circulation.

Plasmin also plays an active role in angiogenesis (Reijerkerk *et al.*, 2000). The absence of plasmin in mice (plasminogen knock-out mice) delays tumor invasion and angiogenesis. The lack of either u-PA, t-PA, or u-PAR did not affect tumor invasion and vascularization *in vivo*, probably because generation of plasmin is still possible (Bajou *et al.*, 2001). These data demonstrate the key role of plasmin in tumor growth.

Because proteolytically active plasmin is localized at tumor level and it is generated at the end of the proteolytic cascade, it can be considered a useful target enzyme for exploitation in tumor-activated prodrug therapy.

Matrix Metalloproteinases (MMPs)

MMPs comprise the second key proteolytic system involved in tumor growth and metastasis (Polette *et al.*, 2004). They are a family of zinc-dependent endoproteinases and are present in elevated levels in malignant tumors (MacDoughall and Matrisian, 1995; Curran and Murray, 2000). The MMP family of proteases contains approximately 20 members. Especially MMP-2 and MMP-9 have been correlated to tumor growth (McKerrow *et al.*, 2000; Hanemaaijer *et al.*, 2000) and angiogenesis (Brooks *et al.*, 1996; Fang *et al.*, 2000; Boger *et al.*, 2001; Pepper, 2001; Silletti *et al.*, 2001). MMPs are probably involved in early alterations that lead to tumor formation (Koblinski *et al.*, 2000). They are believed not only to remodel and degrade ECM proteins but also to be associated with angiogenesis (Nelson *et al.*, 2000; Overall and López-Otín, 2002).

Interactions Between Specific Enzymes and Receptors in Tumor Growth

Several tumor-associated factors, such as the u-PA system, MMPs, cathepsins, integrin receptors, and hypoxic factors, are firmly linked with one another and do not act upon tumor growth independently. During tumor growth, tumor cells interact with one another and with the ECM (Liotta and Kohn, 2001). It is believed that complex mutual interactions between proteases exist *in vivo* to enable tumor cells to break through the ECM (Koblinski *et al.*, 2000). Also during angiogenesis the ECM is continuously remodeled by balanced degradation and resynthesis. Substantial research is conducted on the complex interplay between proteolytic enzymes and the way in which they regulate one another's activation (Lijnen, 2000; Koblinski *et al.*, 2000). Each proteolytic enzyme has specificity for cleaving a subset of ECM molecules, and collectively they catalyze proteolysis of all ECM components (Vu, 2001). Components of the u-PA system and matrix metalloproteinases are tightly associated (Figure 8) (Inuzuka *et al.*, 2000). For example, u-PA-mediated plasmin formation may contribute to activation of pro-MMPs into MMPs (for example, in the case of MMPs 3, 9, 12, and 13) (Lijnen, 2000). On the other hand, cathepsins may activate the u-PA system (Elliott and Sloane, 1996), by conversion of pro-u-PA to u-PA (Kobayashi *et al.*, 1991).

This molecular cross-talk at the invasion front between serine- and metalloprotease activation pathways may be essential to promote tumor invasion and metastasis through both basement membrane and ECM (Ghosh *et al.*, 2000). Probably, concerted action of the different proteases is required to degrade basement membrane and the ECM to allow escape of tumor cells from the primary site. Extensive research is necessary to further unravel the interplay between enzymes in different proteolytic systems.

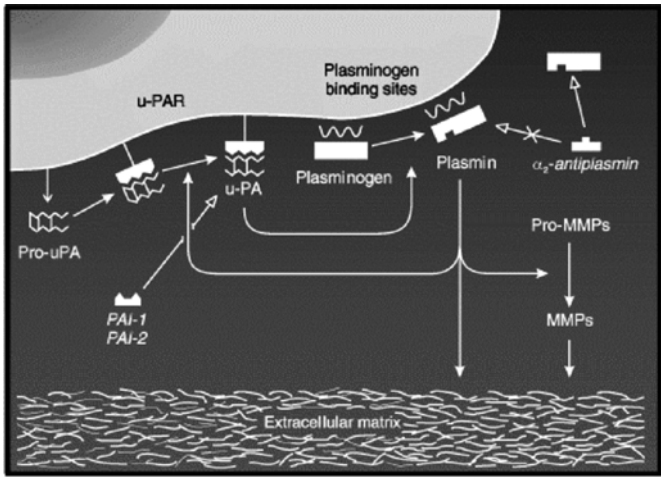


Figure 8. Generation of proteolytic activity on the tumor cell surface[1].

[1]Barrett, A.J.; Rawlings, N.D.; Woessner, J.F. Handbook of Proteolytic Enzymes 1998, Academic Press, London UK.

Degradation of the ECM is not the only activity that is necessary for tumor invasion. Not only in tumor growth but also in normal physiology, contacts between cells and the ECM regulate fundamental cellular processes, such as growth, survival, differentiation, motility, and signal transduction (Vu, 2001). Such interactions of cells with the ECM can take place through cell surface receptors. For example, adhesive interactions between tumor endothelial cells and the ECM are crucial in angiogenesis. This adhesion between the cell membrane and the ECM can be mediated by the diverse family of integrin receptors. Differences in integrin expression between normal and malignant tissue have been extensively reported, and adhesive interactions mediated by the integrins are a requirement for metastasis formation (Meyer and Hart, 1998).

Not only does cross talk between proteolytic systems occur, also mutual interactions between tumor-associated proteases and receptors exist (Figure 9). For example, u-PA and u-PAR are implicated not only in proteolytic events but also in cell adhesion by regulating integrin function (andreasen *et al.*, 2000; Preissner *et al.*, 2000). In fact, u-PA plays a multifunctional role: it is involved in proteolysis and invasion, motility, angiogenesis, proliferation, migration, and adhesion (Schmitt *et al.*, 2000). Urokinase receptor/integrin complexes are functionally involved in adhesion (May *et al.*, 1998; Simon *et al.*, 2000; Tarui *et al.*, 2001; Van

der Pluijm *et al.*, 2001). u-PAR can, as integrins, act as a receptor for vitronectin, an ECM component. The balance between concentrations of u-PA, u-PAR, PAI-1, and integrins may provide a regulating switch for invasion (Hapke *et al.*, 2001).

Integrins are also known to guide matrix-degrading processes by regulation of expression and activation of the MMPs (Brooks *et al.*, 1996; Hofmann *et al.*, 2000a,b; Ivaska and Heini, 2000; Silletti *et al.*, 2001). The $\alpha_v\beta_3$ integrin cell surface receptor in this manner regulates both matrix degradation and motility (Brooks *et al.*, 1996). Thus, integrins interact with both MMPs and the u-PA system. Adhesive and proteolytic events are tightly associated (May *et al.*, 1998; Simon *et al.*, 2000; Van der Tarui *et al.*, 2001; Pluijm *et al.*, 2001).

Angiogenesis is also linked to hypoxia, as hypoxic conditions can stimulate angiogenesis. Hypoxia induces an increase in expression of both u-PAR (Graham *et al.*, 1998, 1999; Kroon *et al.*, 2000; Maity *et al.*, 2000) and PAI-1 (Fitzpatrick and Graham, 1998), and it can induce expression of α_v integrins. Endothelial cells themselves can thus increase their angiogenic potential in response to hypoxic conditions (Graham *et al.*, 1998, 1999; Kroon *et al.*, 2000; Maity and Solomon, 2000).

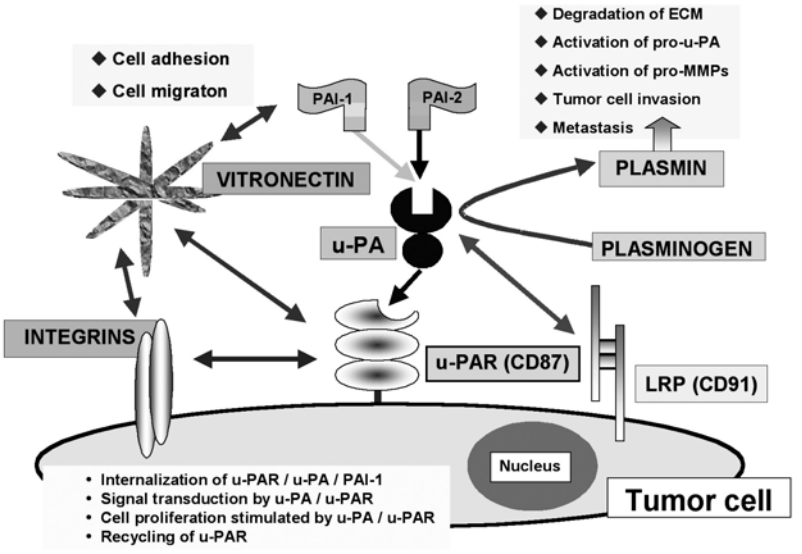


Figure 9. Mutual interaction between the u-PA system, MMPs, and integrin receptors[2].
[2]Schmitt, M.; Wilhelm, O.G.; Reuning, U.; Krüger, A.; Harbeck, N.; Lengyel, E.; Graeff, H.; Gänsbacher, B.; Kessler, H.; Bürgle, M.; Stürzebecher, J.; Sperl, S.; Magdolen, V. Fibrin. Proteol. 2000, 14, 114-132. Prof. Schmitt kindly provided the electronic version of Figure 9.

In conclusion, complicated mutual interactions exist between the tumor-associated enzymes and between tumor-associated enzymes and tumor-associated receptors. Considerable future research will be necessary to further unravel the specific mechanisms by which these enzymes and receptors regulate tumor growth.

The tumor-associated occurrence of the enzymes discussed in this section may serve as a target for development of selective inhibitors to arrest metastasis and invasion (Dunbar *et al.*, 2000; Koblinski *et al.*, 2000; Rosenberg, 2000; Van Noorden *et al.*, 2000; Hidalgo and Eckhardt, 2001; Muehlenweg *et al.*, 2001). Inhibitors against plasmin (Sanders and Seto, 1999; Okada *et al.*, 2000a,b; Quax *et al.*, 2000), u PA (Sperl *et al.*, 2000; ; Verner *et al.*, 2001; Wilson *et al.*, 2001) , u-PAR (Bürgle *et al.*, 1997), PAI-1 (Folkes *et al.*, 2001), and MMPs (Barta *et al.*, 2000; Haq *et al.*, 2000; Maekawa *et al.*, 2000; Hicklin *et al.*, 2001; Overall and López-Otín, 2002) have been reported. However, it seems that some considerations need to be taken into account when using protease inhibitors. Although it may seem paradoxical, high levels of the physiological u-PA inhibitor PAI-1 have been correlated to a poor prognosis for some cancers. PAI-1 is essential for cancer cell invasion and has been reported to promote and regulate tumor angiogenesis (Bajou *et al.*, 1998, 2001; McMahon *et al.*, 2001). Proteases and their inhibitors play a delicate role in the precise control of the ECM microenvironment (Vu, 2001). An intricate balance between ECM breakdown and deposition is needed for normal physiology, and too much but also too little of a protease may lead to disease (Brooks *et al.*, 2001). Blocking a certain protease with an inhibitor may also induce other proteolytic enzymes to compensate for the lack of activity of one particular protease. Single or combined deficiency of u-PA and t-PA did not lead to impaired tumor angiogenesis *in vivo*, suggesting that compensatory mechanisms are active (Bajou *et al.*, 2001). Treatment with the MMP inhibitor batimastat induced formation of liver metastases, overexpression of MMPs, and upregulation of angiogenesis factors (Krüger *et al.*, 2001). MMP-9 expression has been reported to be stimulated by a synthetic broad spectrum MMP inhibitor (Maquoi *et al.*, 2002). In another study, the MMP inhibitor galardin was found to massively up-regulate expression of several MMPs, suggesting that compensatory regulatory systems existed also in this case (Lund *et al.*, 1999).

Uncontrolled use of inhibitors against proteinases such as u-PA and perhaps also against MMPs might not have the desired tumor suppressive effects (Bajou *et al.*, 2001). Instead of using protease inhibitors for therapeutic intervention, the use of protease-activated prodrugs may offer an interesting alternative. When protease activity is exploited only for prodrug activation, the enzymatic balance may be left undisturbed. Enzymes such as cathepsin B, plasmin, MMPs, and prostate-specific antigen can be useful target enzymes for the design and synthesis of antitumor prodrugs.

Prodrugs Activated by Tumor-associated Proteases

Aminopeptidase-activated Prodrugs

A series of prodrugs of methotrexate (MTX) has been reported, in which the 2-amino group has been derivatized with amino acids (Smal *et al.*, 1995). Aminopeptidases localized at tumor level (Menrad *et al.*, 1993; Saiki *et al.*, 1993)

should convert these prodrugs to generate free MTX. 2-L-Pyroglutamyl-MTX showed buffer stability and aminopeptidase activation. Although tumor-associated aminopeptidases exist, unspecific release of parent drug through proteolysis by ubiquitous aminopeptidases in plasma is likely to be a major disadvantage of this series of prodrugs.

Prostate-specific Antigen-activated Prodrugs

As the average life expectancy in the Western world increases, so does the incidence of prostate cancer. The extracellular serine protease prostate-specific antigen (PSA) is a prostate cancer-associated enzyme. PSA is produced by prostate glandular cells, whereas in the circulation this enzyme is inactivated. As a result, enzymatically active PSA is present only in prostate cancer tissue.

Substrate specificity of PSA was investigated using peptide derivatives of 7-amino-4-methylcoumarin (AMC) containing up to seven amino acids coupled via an amide bond to the peptide C-terminus. The sequence His-Ser-Ser-Lys-Leu-Gln (HSSKLQ) was selected because of specificity and serum stability (Denmeade *et al.*, 1997). This sequence was incorporated in a hexa- or heptapeptide doxorubicin prodrug (Denmeade *et al.*, 1998), in which the C-terminal carboxylic acid was coupled to the primary amine of doxorubicin. PSA was unable to hydrolyze the peptide bond between hexapeptide and drug. When an additional leucine (Leu) residue was incorporated between the hexapeptide and the drug, PSA was able to induce release of Leu-Dox. The heptapeptide prodrug showed *in vitro* selective cytotoxicity upon incubation with PSA-producing human prostate cancer cells and non-PSA-producing control cells, indicating that PSA mediated Leu-Dox release. *In vivo* activity was reported in nude mice bearing PSA-producing PC-82 human prostate cancer xenografts (Khan and Denmeade, 2000). The leucine-containing heptapeptide-doxorubicin derivative was less toxic than free doxorubicin and induced a more significant decrease in tumor size compared to control treatment. Leu-Dox itself has already been shown to possess activity against cancer cell lines. Also, a PSA-cleavable heptapeptide-thapsigargin conjugate proved to be a substrate for PSA. The released drug, an apoptosis-inducing agent, inhibited growth of prostate cancer xenograft tumors (Denmeade *et al.*, 2003).

In vivo efficacy has also been obtained with the PSA-activated peptide-doxorubicin conjugate N glutaryl-(4-hydroxyprolyl)-Ala-Ser-Chg-Gln-Ser-Leu-Dox (Chg: cyclohexylglycine) (Garsky *et al.*, 2001). PSA cleaves between the Gln and Ser residues. How the remaining residues, in particular serine, are to be removed after PSA cleavage remains unclear. The conjugate was shown to be 15 times more effective than parent doxorubicin in inhibition of growth of human prostate cancers in nude mice when both compounds were evaluated at their MTD concentration (Defeo-Jones *et al.*, 2000). Furthermore, tumor-selective localization of doxorubicin after prodrug administration was demonstrated (Wong *et al.*, 2001). The conjugate liberated Leu-Dox and Dox. In nude mice with tumors that did not produce PSA, the conjugate showed no significant reduction of tumor weight. A control conjugate containing a D glutamine residue was not readily cleaved by PSA.

Cathepsin-activated Prodrugs

As early as the 1980s, amino acid and dipeptide derivatives of daunorubicin, such as Leu-Dau and Ala-Leu-Dau, were reported (Baurain *et al.*, 1980; Masquelier *et al.*, 1980). Aminopeptidases have been suggested as activating enzymes, although cathepsins may be responsible for activation as well (Huang and Oliff, 2001). In the early 1990s it was shown that the efficacy of doxorubicin against human ovarian, breast and lung carcinomas could be increased by attachment of an L-leucine residue to the amino group of the drug (Boven *et al.*, 1992; De Jong *et al.*, 1992). Several reports have appeared discussing recent *in vivo* studies with N-L-leucyl-doxorubicin (Leu-Dox) against melanoma xenografts (Breistol *et al.*, 1998). Cathepsin B is presumed to be a candidate enzyme for the activation of the prodrug to free doxorubicin (Huang and Oliff, 2001). Leu-Dox showed an increased therapeutic window when compared to doxorubicin (Breistol *et al.*, 1999).

Dubowchik *et al.* (1997) reported the synthesis of dipeptide derivatives of doxorubicin, Mmc, and paclitaxel designed for activation by lysosomal cathepsin B. The dipeptide derivatives were connected to the 7-OH group of paclitaxel or to the amino group of doxorubicin via a para-aminobenzyl alcohol (PABA) self-elimination spacer. The 7-hydroxy group of paclitaxel was chosen as an attachment site because esters and carbonates at this position of paclitaxel are sterically more protected against unspecific hydrolysis, in comparison with the 2'-position. However, the low importance of the 7-hydroxyl function of paclitaxel for cytotoxic activity may result in residual cytotoxic activity of paclitaxel conjugates linked through the 7-position.

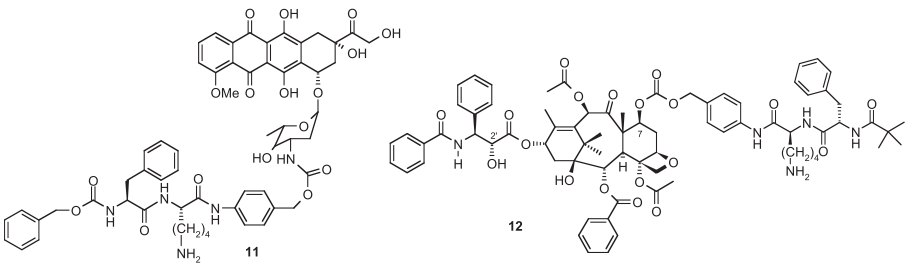


Figure 10. Cathepsin B sensitive prodrugs of paclitaxel and doxorubicin.

A model study of several dipeptide prodrugs of doxorubicin (such as **11**, Figure 10) as substrates for lysosomal cathepsin B was subsequently reported (Dubowchik and Firestone, 1998). Prodrugs were synthesized and incubated with cathepsin B to determine rates of drug release. It was found that prodrugs with a R-Phe-Lys sequence coupled to doxorubicin via a para-aminobenzyloxycarbonyl (PABC) linkage gave the fastest cathepsin B activation rates, R being, for example, a protecting group. Interestingly, without incorporation of a 1,6-elimination linker between peptide and drug, no drug release was observed. The possibility of targeting these peptide prodrugs to extracellular cathepsin B was postulated. In a

second model study by the Dubowchik group, paclitaxel and Mmc were explored for their suitability as parent drugs in cathepsin B-sensitive peptide conjugates (Dubowchik *et al.*, 1998). A Z-Phe-Lys-PABC-2'-paclitaxel prodrug was prepared, but for reasons of ubiquitous cleavage by esterases, derivatization of paclitaxel at the 7-position was preferred. Unexpectedly, the 7-substituted Boc-Phe-Lys-PABC-7-paclitaxel (Boc = *tert*-butoxycarbonyl) prodrug (**12**, Figure 10) appeared to be more effectively cleaved by cathepsin B than the 2'-linked prodrug. In the case of Mmc, the aziridine nitrogen was chosen for connection with the promoiety, yielding the prodrug Boc-Phe-Lys-PABC-Mmc. As demonstrated for the cathepsin-activated doxorubicin prodrug, the Mmc prodrug lacking a 1,6-elimination spacer was also resistant to cleavage by cathepsin. Half-lives of cathepsin B cleavage of the doxorubicin and Mmc prodrugs were considerably shorter than the half-lives for cleavage of the paclitaxel prodrugs, indicating that paclitaxel as a parent drug imposes more steric crowding on enzymatic prodrug activation than Dox or Mmc.

Plasmin-activated Prodrugs

The idea of targeting peptide prodrugs to the tumor-associated protease plasmin was first proposed in 1980 by Carl and Katzenellenbogen (Carl *et al.*, 1980). A D-Val-Leu-Lys tripeptide connected to the amino functions of AT-125 or phenylene diamine mustard (prodrugs **13** and **14**, respectively, Figure 11) could be cleaved to generate free drug upon treatment with plasmin.

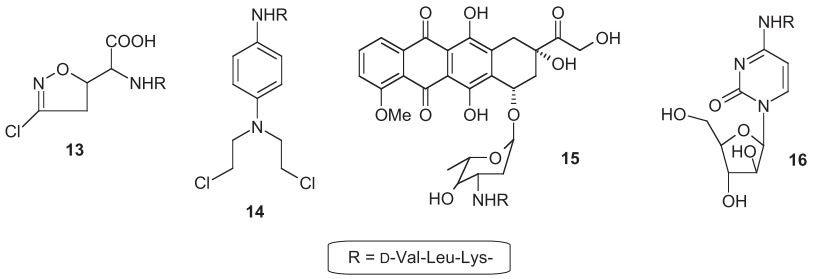


Figure 11. First anticancer prodrugs designed for activation by the serine protease plasmin.

The D-configuration of the N-terminal amino acid should prevent undesired proteolysis by ubiquitous enzymes. A 5- to 7-fold increase of selective toxicity of prodrugs against u-PA-producing cells in comparison with cells containing a low level of u-PA was observed. Addition of a plasmin inhibitor was shown to decrease the cytotoxicity of the prodrug in the u-PA-producing cells. Subsequent *in vivo* experiments with these prodrugs led to disappointing results (Chakravarty *et al.*, 1983a).

The first doxorubicin prodrug designed for activation by plasmin has been reported in the early 1980s (Chakravarty *et al.*, 1983b). The D-Val-Leu-Lys-Dox prodrug **15** (Figure 11) showed a 7-fold increased selective cytotoxicity against u-

PA-producing cells in comparison with normal cells containing a low level of u-PA. Although the prodrug showed enhanced selectivity, its activation by plasmin was highly inefficient (Chakravarty *et al.*, 1983b). Most likely, the steric burden imposed by doxorubicin prevented plasmin from cleaving substrate **15**.

In the early 1990s, a plasmin-activated prodrug of the anti-leukemia agent 1-β-D-arabinofuranosylcytosine was reported (**16**, Figure 11) (Balajthy *et al.*, 1992). This prodrug proved to be effectively cleaved by plasmin, and it showed a higher antiproliferative activity than the parent drug against L1210 leukemia cells. The presence of a plasmin inhibitor dramatically decreased the activity. The major drawback of this prodrug was its low plasma stability.

More recently, plasmin served as the target enzyme for design and synthesis of tripartate prodrugs of alkylating agents that consisted of a tripeptide specifier coupled to the drug via a cyclization spacer (Lauck-Birkel *et al.*, 1995; Eisenbrand *et al.*, 1996). In the presence of plasminogen, *in vitro* prodrug cytotoxicity significantly increased. Both D-Ala-Phe-Lys and D-Val-Leu-Lys were coupled to N-nitroso-urea as parent drugs, via a cyclization spacer (Eisenbrand *et al.*, 1996) (Figure 12).

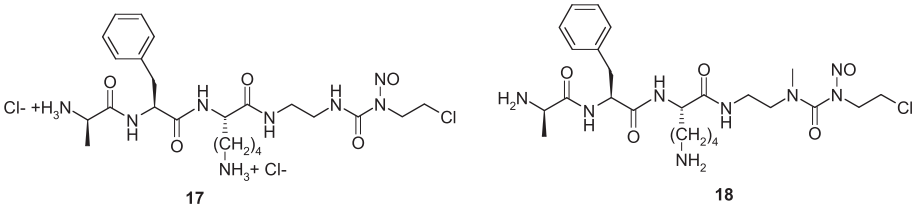


Figure 12. Cyclization spacer-containing peptide prodrugs of N-nitroso-urea.

The synthesized prodrugs contained an ethylene diamine (**17**) or a monomethylated ethylene diamine spacer (**18**). After cleavage by plasmin, a cyclization reaction led to the formation of a pentacyclic urea derivative, imidazolidin-2-one, with the concomitant expulsion of free parent drug. Prodrug **18**, containing the methyl-substituted spacer, was more stable than **17**. The methyl-substituted prodrug was more cytotoxic upon incubation with two cell lines in the presence of plasminogen than in its absence, indicating plasmin-mediated prodrug activation. In addition to the depicted D-Ala-Phe-Lys prodrugs, the corresponding D-Val-Leu-Lys prodrugs have also been synthesized, showing similar half-lives of prodrug activation. Plasmin-activated prodrugs of the anthracyclines daunorubicin and doxorubicin have been developed by our group. The prodrugs contain a self-immolative 1,6-elimination linker to enable proteolytic activation by plasmin (de Groot *et al.*, 1999). This is in contrast to plasmin-activated anthracycline prodrugs lacking a linker, which are not cleaved by plasmin (Chakravarty *et al.*, 1983b). The linker-containing prodrugs have displayed considerable *in vitro* selective cytotoxicity for plasmin-generating u-PA-transfected MCF-7 breast cancer cells, whereas they were considerably less toxic for MCF-7 control cells. Plasmin-activated paclitaxel-2'-carbamate prodrugs have also been developed in

our group (de Groot *et al.*, 2000). Paclitaxel-2'-carbamates are particularly interesting because a free 2'-hydroxyl group is important for biological activity and because, in general, carbamate linkages are more stable *in vivo* than esters and carbonates. We have also designed and applied novel elongated releasable linker systems to conjugate different toxic agents via different linker chemistries to a targeting unit, in this case a plasmin substrate (de Groot *et al.*, 2001b). We reported plasmin-activated doxorubicin and paclitaxel prodrugs containing two or three electronic cascade 1,6-elimination spacers (Figure 13). Prodrugs in which the novel spacer systems were incorporated in between a plasmin substrate and the parent drug proved to be activated significantly faster by plasmin in comparison with the corresponding prodrugs containing spacer systems of conventional length. In addition, several of these prodrugs showed a markedly decreased *in vitro* cytotoxicity. In preliminary *in vivo* studies, the plasmin-activated doxorubicin prodrugs were much less toxic than the parent doxorubicin, while they showed substantial antitumor efficacy (Devy *et al.*, 2004). A significant improvement of the therapeutic window of the plasmin-activated prodrug over the parent compound has also been observed in other animal models. These linker systems may be generally employed for contributing to appropriate drug release characteristics of prodrugs and bioconjugates.

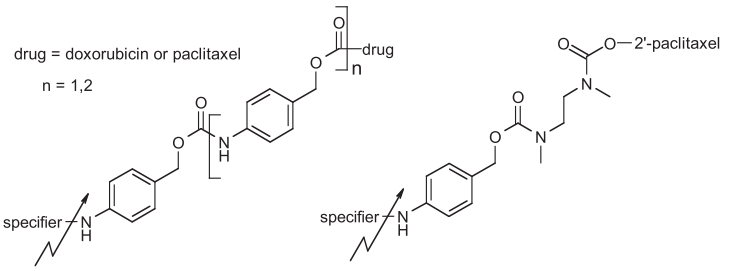


Figure 13. Prodrugs with elongated spacer systems consisting of multiple electronic cascade and/or cyclization spacers.

Although u-PA is less frequently chosen than plasmin as target enzyme for prodrug activation, conjugates that are activated by u-PA have been reported (Kurtzhals *et al.*, 1989). Tumor-selective targeting of anthrax toxin to u-PA has been described (Liu *et al.*, 2001a, 2003). An advantage of using plasmin for prodrug activation compared to u-PA is that one molecule of u-PA can generate multiple equivalents of enzymatically active plasmin.

Matrix metalloproteinase-activated prodrugs

Prodrugs can also be designed for specific activation by MMPs (also designated collagenases). In the late 1970s, a Z-Pro-Leu-Gly-Pro-Gly specifier coupled to nitrogen mustard was reported to be cleaved between Leu and Gly by collagenase (Marquisee and Kauer, 1978). No uniform *in vivo* antitumor activity

was demonstrated, presumably due to lack of drug release from the liberated tripeptide-drug conjugate. Although the specific mechanism of drug release was not addressed, polymeric prodrugs of Mmc containing the peptide sequences Gly-Phe-Ala-Leu and Gly-Phe-Leu-Gly-Phe-Leu between polymer and drug have been reported to release drug in the presence of collagenase IV (MMP 2) (Soyez *et al.*, 1996a). MMP activation of a low-toxic hexapeptide (Pro-Gln-Gly-Ile-Mel-Gly) that contained the parent drug melphalan (Mel) has also been described (Timár *et al.*, 1998). MMP cleavage generated Ile-Mel-Gly, which was more toxic than the hexapeptide, but liberation of free melphalan was not reported. An octapeptide conjugate released Ile-Ala-Gly-Gln-Dox upon MMP activation (Kratz *et al.*, 2001). Again, liberation of unconjugated parent drug was not observed. Not only a range of MMP-activated prodrugs that has been published but also some unpublished MMP-cleavable prodrugs prepared in our own lab release free parent drug with difficulty.

Other Protease-activated Prodrugs

Although the specific enzymes that are responsible for drug release are in some cases unknown, prodrugs with certain peptide sequences have been reported to release free parent compound upon tumor-selective activation. Ala-Leu-Ala-Leu-Dau was published in the early 1980s (Trouet *et al.*, 1982). Ala-Leu-Ala-Leu conjugates are presumably cleavable by lysosomal enzymes (Dubowchik *et al.*, 2002). More recently, tetrapeptide derivatives of doxorubicin (β-Ala-Leu-Ala-Leu-Dox) with or without an N-succinyl group were shown to be less toxic *in vivo* and showed enhanced antitumor efficacy in comparison with doxorubicin (Fernandez *et al.*, 2001; Trouet *et al.*, 2001). Doxorubicin release has been proposed to occur after several consecutive proteolytic cleavage steps. CD10, a cell surface metalloprotease, was shown to extracellularly cleave the succinyl prodrug to generate Leu-Dox, which is converted intracellularly into free Dox (Pan *et al.*, 2003).

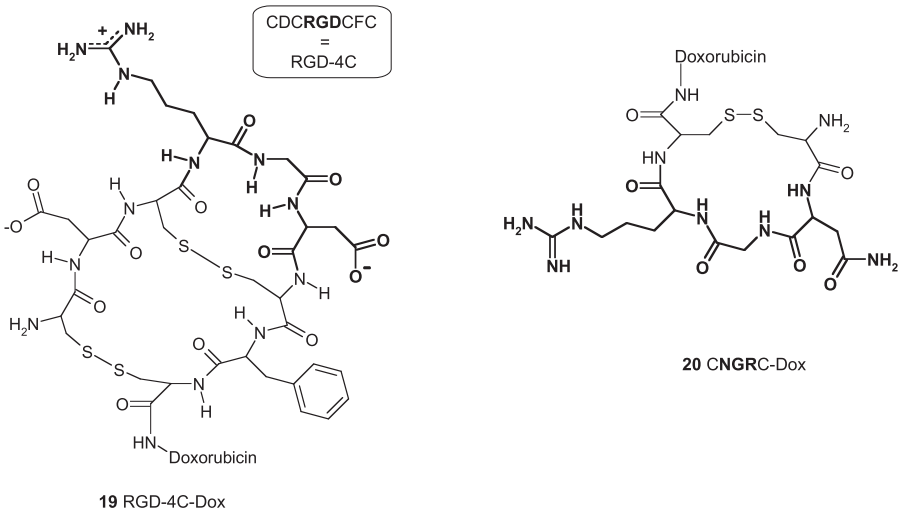
Receptor Binding Prodrugs

Adhesion Receptors

A relatively new approach for the treatment of solid tumors is anti-angiogenic therapy, in which tumor vasculature is the target for therapy (Carmelite and Jain, 2000; Eatock *et al.*, 2000; Thorpe, 2004). In 1971, Folkman proposed that tumor growth and metastasis are angiogenesis-dependent. Angiogenesis is an essential process for the growing tumor, as it must assure its blood supply (Folkman, 1971, 2000). A tumor that grows beyond 1–2 mm in diameter needs an independent blood supply for nutrition and oxygen, whereas the neovascularization itself is important for a tumor to release cells into the circulation. Hence, blocking angiogenesis can be a strategy to arrest tumor growth. Instead of attacking the tumor cells themselves, one can attack these newly formed blood vessels by way of

attacking the vascular endothelial cells (Begent, 1984; Burrows and Thorpe, 1994). Vascular targeting has a clear advantage because of its amplification mechanism: relatively many tumor cells are dependent on capillary endothelial cells. This is one of the reasons why development of integrin antagonists has received broad attention (Curley *et al.*, 1999). For example, Ruoslahti *et al.* identified several peptides that home to tumor vasculature by means of phage display techniques. After injection of phage display peptide libraries in nude mice bearing human breast cancer xenografts, identification of recovered phage from tumors was possible, revealing several tumor homing peptide motifs. Two of these motifs, CDCRGDCFC (RGD-4C) (which contains two disulfide bridges), and CNGRC (with one disulfide bridge), were conjugated to doxorubicin (prodrugs **19** and **20**, respectively, Figure 14), and the conjugates were used for treatment of mice bearing human breast carcinoma cells (Arap *et al.*, 1998). The peptide conjugates of doxorubicin proved less toxic and substantially more efficacious than the free parent drug. The mechanism of drug release from the peptide conjugates was not discussed, but it is possible that the conjugate is degraded after internalization by the cell. The RGD-4C peptide binds selectively to αvβ3 integrin adhesion receptors expressed in tumor vasculature (Ruoslahti, 1996; Giancotti and Ruoslahti, 1999). This receptor is involved in and required for angiogenesis (Brooks *et al.*, 1994). Integrin receptor proteins play a crucial role in cancer; they mediate contact between cells and between cell and extracellular matrix (Mizetewski, 1999). The process of angiogenesis depends on these adhesive interactions of vascular cells (Brooks *et al.*, 1994). Because αv integrins are expressed by many human tumors, peptides and peptide mimetics that selectively bind to this class of receptors could be useful for future anticancer (pro)drug development. Therapies that target the integrins for inhibition of tumor growth, metastasis and angiogenesis are being explored (Mizetewski, 1999).

Our group has developed a bifunctional prodrug that contains a specifier with dual tumor-specificity. Both the integrin binding RGD-4C sequence and a



19 RGD-4C-Dox

20 CNGRC-Dox

Figure 14. Receptor binding doxorubicin conjugates RGD-4C-Dox and CNGRC-Dox.

plasmin-cleavable substrate sequence are incorporated in the doxorubicin prodrug (de Groot *et al.*, 2002). The dual specificity motive may potentially enhance the prodrug's tumor-recognition potential.

The other peptide in Figure 14, monocyclic CNGRC, appeared to be a potent inhibitor of the tumor-associated protein CD13, also referred to as aminopeptidase N (APN) (Pasqualini *et al.*, 2000).

Additional evidence for the usefulness of the conformationally restricted RGD and NGR peptides was found when peptides containing 21 or 26 residues, which consisted of the CNGRC or RGD-4C sequence connected to pro-apoptotic peptides, were synthesized (Ellerby *et al.*, 1999). Following internalization, these compounds induced selective *in vivo* apoptosis, reduction of tumor volume and longer survival of mice. Screening of phage display peptide libraries continues to prove a powerful technique for identification of tumor homing peptides.

Folate Receptors

The folate receptor is a membrane glycoprotein that binds folic acid with high affinity. It is a marker for ovarian carcinomas (Campbell *et al.*, 1991) and it is overexpressed in several other tumors. Although monoclonal antibodies against these receptors have been used for tumor targeting purposes, folic acid can be used as well as a targeting ligand (Reddy and Low, 1998; Leamon *et al.*, 1999; Sudimack and Lee, 2000; Leamon and Low, 2001; Liu *et al.*, 2001b; Steinberg and Borch, 2001; Lu *et al.*, 2004). Selective toxicity of several folic acid conjugates of toxins or antisense oligodeoxynucleotides (Li *et al.*, 1998) for cells expressing folate receptors has been reported (Leamon and Low, 1992; Leamon *et al.*, 1993; Ladino *et al.*, 1997).

Hormone Receptors

To increase the selectivity index of cytotoxic compounds, they can be conjugated with hormone receptor binding molecules (Schally and Nagy, 1999). Alkylating agents have been coupled to estrogenic steroid molecules (Konyves *et al.*, 1984), whereas daunorubicin has been coupled to β-melanocyte-stimulating hormone (Varga, 1985). Schally and Nagy have worked on hormone receptor targeted chemotherapy. They reported luteinizing hormone-releasing hormone (LH-RH) conjugates containing different chemotherapeutic agents that showed *in vitro* selectivity (Bajusz *et al.*, 1989; Janáky *et al.*, 1992). Growth inhibition of prostate cancers in rats was achieved with LH-RH analogs of anthraquinone and MTX, whereas peptide or drug alone was less effective (Pinski *et al.*, 1993). Also, conjugates in which a decapeptide LH-RH agonist was connected to doxorubicin or to the potent doxorubicin derivative 2-pyrrolinodoxorubicin have been reported. The peptide was connected via a 14-O-hemiglutarate spacer to the anthracycline (Nagy *et al.*, 1996, 2000). The prodrugs were less toxic than the parent drugs, and induced higher tumor growth inhibition in prostate and breast cancers in mice (Jungwirth *et al.*, 1997; Miyazaki *et al.*, 1999; Kahán *et al.*, 2000) .

The LH-RH Dox conjugate also proved efficacious against androgen-sensitive prostate cancers (Letsch *et al.*, 2003).

In a similar manner, octapeptide antagonists for the bombesin/gastrin-releasing peptide (BN/GRP) receptor were coupled via a hemiglutarate spacer to the 14-hydroxyl function of doxorubicin or 2-pyrrolinodoxorubicin (Nagy *et al.*, 1997). The bombesin conjugates showed a dramatic cell killing effect on receptor expressing cells and induced selective tumor growth inhibition of lung carcinoma in nude mice (Kiaris *et al.*, 1999). Others have reported the synthesis of poly(ethylene glycol) conjugates of paclitaxel, in which a heptapeptide was incorporated, designed for binding to the BN/GRP receptor (Safavy *et al.*, 1999). The peptide-containing conjugate was more toxic than unconjugated paclitaxel against cells containing BN receptors, although a possible disadvantage of these conjugates is the linkage of the taxane via a 2'-succinate ester, which may be susceptible to cleavage by unspecific esterases.

Significant *in vivo* results have also been reported for a cytotoxic somatostatin (SST) analog containing MTX as a parent dug, designed for targeting SST receptors (Radulovic *et al.*, 1992). Also, octapeptide SST analogs of doxorubicin and 2-pyrrolinodoxorubicin, connected again via a 14-O-hemiglutarate spacer, were less toxic and more effective in tumor growth inhibition than the corresponding anthracycline parent drugs (Nagy *et al.*, 1998). A major disadvantage of the hormone receptor targeted prodrugs that are coupled via a hemiglutarate to the parent anthracycline is their susceptibility to carboxylesterases and possibly other esterases in serum. Partial enzymatic cleavage of the 14-ester function of the hemiglutarate conjugates can lead to premature unspecific release of parent drug in the circulation of animals (Nagy *et al.*, 2000).

Other Receptors

Anticancer drugs may be delivered to the tumor site by using low-density lipoprotein (LDL) as a vehicle. These micelle-like structures are recognized by specific cell surface receptors. The use of LDL as a macromolecular drug delivery vehicle has been extensively reviewed (Firestone, 1994; Dubowchik and Walker, 1999).

Several human cancer cell lines are known to overexpress hyaluronic acid (HA) receptors. HA is a linear polysaccharide. HA-paclitaxel bioconjugates that showed selective toxicity toward HA-receptor expressing cell cultures have been reported (Luo and Prestwich, 1999). A possible disadvantage of these conjugates is that paclitaxel was linked to the polysaccharide via a 2'-succinate ester linkage.

After a preclinical study with (polymeric) conjugates of doxorubicin that contained galactosamine for targeting the asialoglycoprotein receptor, effective hepatic targeting was achieved in a patient (Julyan *et al.*, 1999) .

In another study, a (polymeric) doxorubicin conjugate, which contained the EDPGFFNVE nonapeptide, was investigated (Omelyanenko *et al.*, 1999). This peptide served as binding epitope to the CD21 receptor, which is involved in virus

attachment to human B-lymphocytes. The nonapeptide-containing conjugates possessed specific cytotoxicity to malignant T- and B-cells.

Targeted Drug Delivery via Macromolecular Prodrugs

Although macromolecular anticancer prodrugs will be discussed in detail in the next section, for the sake of completing the overview of scope of tumor-activated conjugates, they are also briefly discussed in this section.

Polymeric Drug Delivery

Polymeric drug delivery is an area that has gained considerable attention in recent years, and extensive effort has been directed to develop prodrugs containing polymer based transport forms (Duncan, 2003; Marcucci and Lefoulon, 2004). These compounds generally show extended circulating life *in vivo* and may make use of the EPR effect observed in tumor tissue (Maeda and Matsumura, 1989; Soyez *et al.*, 1996b). This effect causes polymeric substances of sufficient size to localize in tumors. EPR is a consequence of discontinuous (leaky) and poorly formed tumor endothelium and poor lymphatic drainage, which leads to trapping of polymeric material inside tumor tissue (passive targeting). As previously indicated, also two-step PDEPT, in which a polymer carries a prodrug-activating enzyme to tumors, does benefit from this passive targeting effect (Satchi *et al.*, 2001; Satchi-Fainaro *et al.*, 2003). As early as 1975, Ringsdorf proposed pharmacologically active polymers of which the properties could be varied by incorporation of 'comonomer' units (Ringsdorf, 1975). In his model, several functional units, such as a targeting moiety, a solubilizing moiety, and a drug (optionally connected to the polymer via a linker) could be incorporated in a polymer. The polymer in macromolecular prodrugs can be a biodegradable carrier such as polyglutamic acid, or a polyester such as poly(lactic-co-glycolic acid, PLGA). Polymers such as poly[N-(2-hydroxypropyl)methacrylamide] (poly-HPMA) (Vasey *et al.*, 1999; Duncan *et al.*, 2001) and poly(ethylene) glycol (PEG) (Greenwald, 2001; Greenwald *et al.*, 2000) are also frequently used. In polymeric drug delivery conjugates, Gly-Phe-Leu-Gly often serves as a lysosomally cleavable peptide sequence between drug and polymer (Dubowchik *et al.*, 2002). Currently, a number of polymeric prodrugs are being tested in clinical trials (Duncan *et al.*, 2001). A problem in some polymeric prodrugs is inefficient drug release, which may play a decisive role in the final toxicity/efficacy of the polymeric construct (Rihová *et al.*, 2001). Incorporation of appropriate linker chemistry may improve drug release properties in such cases (Soyez *et al.*, 1996b).

Antibody-drug Conjugates

For the purpose of targeted delivery of anticancer agents, antibodies have been equipped with drugs, radioisotopes, and cytotoxins. Particularly, monoclonal antibodies (mAbs) that specifically bind tumor-associated antigens have been used

for this purpose. The antibody conjugates that release drug or toxin can be regarded as macromolecular prodrugs. A number of these compounds have shown disappointing results in the past in clinical studies (Nagy *et al.*, 1998). Antibody-drug conjugates that were initially developed were probably not sufficiently efficacious. This may be due to the fact that antibody-drug conjugates often contain only a few drug molecules per antibody and that tumor cells may express only a limited number of antigen molecules.

In order to amplify the effect of localization of an antibody to the tumor cell antigen, approaches such as ADEPT were proposed, in which one localized enzyme molecule can activate multiple prodrug molecules. This approach could be considered an improved approach with respect to antibody-drug conjugates. The ADEPT strategy knows limitations (Melton *et al.*, 1999). The antibody-enzyme conjugate may be immunogenic or may not localize to the desired extent. Also, the clearance of unbound mAb-enzyme conjugate (before administration of prodrug in the second step) can be inadequate.

Currently, there is a renewed interest in the antibody-drug conjugate type of targeted cytotoxics. For example, the anticancer antibody-drug conjugate Mylotarg® has received marketing approval in 2000, and a number of antibody-drug conjugates are currently in preclinical and clinical development (Lam *et al.*, 2003; Doronina *et al.*, 2003; Mao *et al.*, 2004). One strategy to enhance the efficiency of mAb-drug conjugates is employing branched linkers that enable conjugation of multiple drug molecules to a single antibody functional group. Alternatively, highly potent cytotoxic agents are conjugated to the antibody. Especially in the latter case, it is important to incorporate a (releasable) linker moiety in between mAb and drug that is stable in the circulation but labile when the conjugate has reached the tumor site. Cleavage of the drug can happen intracellularly or extracellularly, depending on whether or not a conjugate is internalized.

Prodrug Strategy Considerations

'The magic bullet': scientists have been searching for it for decades. The magic anticancer bullet should ideally kill tumor cells and at the same time spare normal cells. Targeting of prodrugs and conjugates containing a tumor-specific targeting unit appears to be a promising strategy to selectively attack cancer cells. The enormous potential of non-toxic prodrugs in the concept of anticancer prodrug therapy is widely recognized. Enzymes, such as β-glucuronidase, PSA, cathepsin B, plasmin, and MMPs, may be interesting targets for tumor-activated prodrug therapy. Polymeric conjugates, which have the ability to passively target to tumor tissue, may be developed, with or without an additional targeting ligand. Antibody-drug conjugates and receptor-binding drug constructs comprise another type of prodrug that exploits the occurrence of tumor-specific antigens and receptors.

Properties of a prodrug conjugate can be chosen to a large extent on a molecular level. Many different strategic combinations are possible in which

aspects can be varied, such as: monotherapy or two step therapy; recognition by enzyme, receptor, or both; low-molecular-weight or large (biomolecular) construct; intracellular or extracellular drug release; incorporation of a linker or no linker; releasable or non-releasable linker; type of cell targeted—for example, tumor endothelial cells or tumor cells, etc. Scientists have tried to find and develop appropriate molecular prodrug designs to maximize chances for successful anticancer therapy based on mentioned aspects.

Advantages of low-molecular weight prodrugs for monotherapy may be that they are not likely to induce an immunogenic response, and that they may easily penetrate tumor tissue. High molecular-weight conjugates, for example whole antibodies, may penetrate tumors poorly, and less than one percent may eventually reach the tumor (Boyle and Costello, 1998). However, if a conjugate contains a large (bio)molecule as targeting agent, for example a tumor-homing antibody or a polymer, circulation half-life may be significantly higher. If a prodrug is cleaved extracellularly, the parent agent should have sufficient affinity to enter cells and should not leak away to a large extent from the tumor site and kill healthy cells. An advantage of extracellular prodrug activation is the bystander killing effect (Sahin *et al.*, 1990). This feature in prodrug therapy refers to the drug being able to kill a tumor cell that does not necessarily bear the tumor-homing motif. Advantages of prodrug monotherapy over two-step therapies are that it is simpler, less costly and more convenient for the patient. In two-step therapies, both steps must be optimized in order to get efficient treatment. On the other hand, two-step therapies open up strategies that are not possible in monotherapy. For example, non-human enzymes can be targeted to tumors, for example, in the form of an antibody-enzyme conjugate. The exogenous enzyme can convert the prodrug substrate with high specificity in the tumor.

Tumor-targeted prodrugs and bioconjugates must meet a number of criteria in order to be successful in attacking tumor cells while leaving healthy cells undisturbed. A first important criterion is conjugate stability in the circulation. This means that the prodrug should be stable against ubiquitous hydrolysis by enzymes present in healthy tissue, organs and plasma. The components of which a prodrug is composed have to be assembled in such a way that the product is processed exclusively in the tumor environment to release free parent drug upon arrival. Proper choice of the chemistries that link the components together is considered crucial. A second major criterion is that the drug can be efficiently released once conjugate localization in the tumor environment has taken place. Incorporation of a linker between the targeting moiety and drug molecule can contribute to increased efficiency of prodrug activation. In numerous examples, parent drug is not released from the prodrug if the targeting moiety is connected directly to a functional group of the drug molecule. Introduction of intelligent releasable linkers in small-molecule prodrugs as well as in polymeric drug delivery constructs and antibody-drug conjugates may allow fast and tumor-specific conjugate hydrolysis and drug release.

It can be expected that future research will further unravel the process of tumor growth from the biological point of view. This should yield extended insight

into which biomolecules are the most appropriate targets for anticancer (prodrug) therapy. Discovery of location and function of specific enzymes, receptors, membrane proteins, and antigens is expected to result in an increasing number of available handles for therapeutic intervention, for example the use of prodrug conjugates.

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2.5.3

Monoclonal Antibody Drug Conjugates for Cancer Therapy

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List of Abbreviations

AML.....acute myeloid leukemia
mAb .....monoclonal antibody
MMAE .....monomethylauristatin E
MTD.....maximum tolerated dose
Phe-Lys .....phenylalanine-lysine
Val-Cit.....valine-citrulline

Introduction

A great deal of interest has surrounded the use of monoclonal antibodies (mAbs) and mAb-based regimens for cancer therapy (Dubowchik and Walker, 1999; Carter, 2001; Meyer and Senter, 2003; Trail *et al.*, 2003; Guillemard and Saragovi, 2004). The rationale for such work is conceptually appealing since mAbs are known to exhibit higher specificity profiles than conventional anticancer drugs and should, therefore, be safely administered and potentially quite effective. Indeed, several mAbs and mAb-based reagents are now clinically approved, including Rituxan (rituximab) for non-Hodgkin's lymphoma (King and Younes, 2001), Panorex (edrecolomab) for colorectal carcinoma (Schwartzberg, 2001), Herceptin (trastuzamab) for metastatic breast cancer (Yarden and Sliwkowski, 2001), and Avastin for colorectal carcinoma (Ferrara *et al.*, 2004). The only clinically approved mAb-drug conjugate is Mylotarg (gemtuzumab ozogamicin). Bexxar (tositumomab), and Zevalin (ibritumomab) are clinically approved mAb-radioisotope conjugates used for the treatment of lymphomas (Hernandez and Knox, 2004). Many other mAbs and mAb-conjugates are in advanced clinical trials (Carter, 2001; Schultes and Nicodemus, 2004).

It has been demonstrated that mAb-drug conjugates for cancer therapy are advantageous compared to unconjugated mAbs, since many mAbs that are selective for tumor cell-surface antigens have little cytotoxic activity. However, several limitations to using mAbs as drug carriers have been identified, including physiological barriers to mAb extravasation and intratumoral penetration, mAb immunogenicity, normal tissue expression of the targeted antigen, low drug potency, inefficient drug release from the mAb, and difficulties in releasing drugs in their active states (Jain, 1990; Dubowchik and Walker, 1999; Payne, 2003). As a result, much research has been focused on chimeric and humanized mAbs that are relatively non-immunogenic and have high affinities for tumor associated antigens, mAbs that are efficiently internalized into cells once they bind to the target antigen, engineered mAbs that are designed for efficient drug delivery, new drugs with high potencies, and linker technology to accommodate these novel agents. This chapter will describe mAb-drug conjugates that have shown particular promise in preclinical tumor models and are either moving toward or are currently in clinical trials.

Acid-labile Linkers for Drug Conjugation

Upon binding to cell-surface antigens, many mAbs are internalized through a process known as receptor-mediated endocytosis, which carries the mAb into lysosomes that are both acidic and rich in proteolytic enzymes (Dubowchik and Walker, 1999). Considerable attention has been directed at developing linkers that are relatively stable at neutral pH but undergo hydrolysis under the mildly acidic (circa pH 5) conditions within the lysosomes. Acid labile functionalities used for drug attachment include hydrazones (Dubowchik and Walker, 1999), hydrazines and thiosemicarbazones (Kaneko *et al.*, 1991), trityl groups (Patel *et al.*, 1995), cis-

aconityl spacers (Shen and Ryser, 1981), and orthoesters, acetals, and ketals (Srinivasachar and Neville, 1989). The cleavable linker system that has been most extensively exploited contains the hydrazone functionality.

Several methods are available for producing mAb-drug conjugates through hydrazone bond formation. The simplest method is to introduce the hydrazone functionality as a appendage of the drug and attach the entire complex to reactive functionalities on mAbs. Alternatively, aldehydes and ketones can be introduced onto the mAb through treatment with sodium periodate, which oxidizes gem-diols on mAb sugars. Addition of hydrazido drug derivatives leads to the formation of hydrazones that are relatively stable at neutral pH but labile under acidic conditions (Laguzza *et al.*, 1989; Apelgren *et al.*, 1990; Hinman *et al.*, 1993). One advantage of this methodology is that the mAbs are modified regiospecifically, since the carbohydrates on mAbs are largely restricted to the Fc region. However, the oxidation method leads to a variety of reactive species, and the resulting hydrazones are poorly defined. In addition, the oxidative conditions used for hydrazone formation can also lead to methionine oxidation, which can be detrimental to mAb binding activity (Hamann *et al.*, 2002b). To circumvent this problem, bifunctional crosslinking reagents that contain aldehyde or ketone functionalities can be attached to mAb lysine residues and can then be used for subsequent modification. This approach permits much greater control over the relative hydrolysis rates of the hydrazone bond. Aromatic ketones are most useful in this approach because the resulting hydrazones are stable for several days under neutral pH conditions but are much less stable at pH 5 (Apelgren *et al.*, 1990, 1993; Hamann *et al.*, 2002a,b).

The only clinically approved mAb-drug conjugate contains an acid-labile hydrazone linker. Mylotarg (gemtuzumab ozogamicin) is a radical departure from previously described conjugates in that the cytotoxic element, N-acetyl-gamma

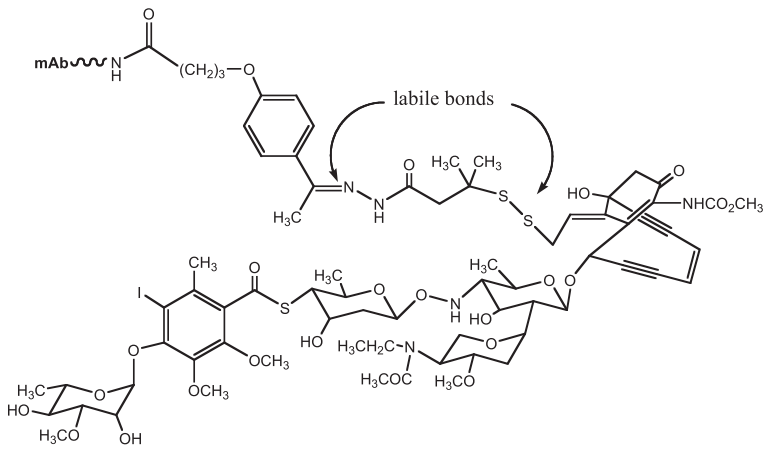

Figure 1. Mylotarg is currently the only clinically approved mAb-drug conjugate used in cancer chemotherapy. The mAb component consists of hP67.6, recognizing the CD33 antigen on acute myelogenous leukemia. The calicheamicin drug component is released by hydrazone hydrolysis, followed by disulfide bond reduction (Bross *et al.*, 2001; Damle and Frost, 2003).

calicheamicin, is structurally and mechanistically unrelated to any anticancer drug used in clinical medicine (Damle and Frost, 2003). The drug is a minor groove binder that produces DNA double strand breaks with an IC_{50} in the low ng/mL range (Hamann *et al.*, 2002a,b). The linkage between the mAb and the drug incorporates two labile bonds, a hydrazone and a sterically hindered disulfide, as shown in Figure 1. It is believed that the hydrazone is cleaved before the disulfide is reduced.

The mAb moiety in Mylotarg is a humanized form of P67.6, a murine mAb that binds to the CD33 antigen present on myeloid cells, notably transformed cells of acute myeloid leukemia (AML) patients. CD33 has been shown to internalize and is not present on pluripotent stem cells (van der Jagt *et al.*, 1992). Any non-transformed myeloid cells eliminated by the conjugate can therefore be replaced. Greater than 80% of AML patients express the CD33 antigen (Appelbaum *et al.*, 1992).

Mylotarg consists of a 1:1 mixture of hP67.6, a humanized IgG4, with hP67.6 conjugated to 4–6 moles N-acetyl-gamma calicheamicin, providing an average drug loading ratio of 2–3 drugs/mAb (Bross *et al.*, 2001). In conjugates prepared with murine P67.6, the hydrazone linkage was generated through periodate oxidation of the mAb carbohydrate, followed by condensation of the resulting putative aldehydes with an acyl hydrazide derivative of N-acetyl-gamma calicheamicin. However, the humanized form of the mAb, hP67.6, lost binding activity upon periodate treatment, possibly because of a sensitive methionine residue in the antigen-binding region (Hamann *et al.*, 2002a). Consequently, the conjugate was formed by reacting the drug-hydrazone-linker complex with mAb lysine residues. The hydrazone in Mylotarg is a derivative of p-hydroxyacetophenone, which underwent 6% hydrolysis in 24 h at pH 7.4 and was cleaved quantitatively under the same conditions at pH 4.5 (Hamann *et al.*, 2002a). The role that conjugation technology can play in efficacy was further underscored by the finding that humanized lysine-linked conjugate was substantially more potent (45-fold on HL-60 cells) and more selective (70-fold on HL-60 cells) *in vitro* and somewhat more active *in vivo* than the murine carbohydrate-linked version.

A Phase I clinical trial of Mylotarg in refractory or relapsed AML patients demonstrated tolerable levels of toxicity at doses up to 9 mg/m^2 (Sievers *et al.*, 1999). Responses were documented in 8 of 40 patients. Toxic effects included fever and chills, reversible elevation of liver enzymes, myelosupression with severe neutropenia, and veno-occlusive disease (Sievers *et al.*, 1999; Giles *et al.*, 2001). The CD33 antigen was saturated in treated patients within 30 min at the 9 mg/m^2 dose. Response correlated with a combination of antigen saturation and low tumor cell efflux activity. The serum half-life of the conjugate was found to be 38 ± 21 h. Immune response to the calicheamicin was detected in 2 of 40 patients, but no anti-hP67.7 response was observed.

In the Phase II trial, patients were given up to 3 doses at 9 mg/m^2 at intervals of 14 to 28 days; the overall response rate was 30%, with a relapse-free survival time of 6.8 months (Sievers *et al.*, 2001). In May 2000, Mylotarg was clinically

approved for the treatment of AML. As this is the first such mAb-drug conjugate, it represents a significant breakthrough in the field.

The technology used in developing Mylotarg has recently been extended to include a new calicheamicin conjugate that binds to the CD22 antigen on normal B-cells and on B-cell lymphomas (Damle and Frost, 2002; DiJoseph *et al.*, 2004, 2005). More recently, the Lewis^Y antigen on carcinomas of the breast, colon, lung, ovaries, and prostate has been targeted with the Hu3S193-N-acetyl calicheamicin immunoconjugate, an agent that is active in several xenograft carcinoma models at well-tolerated doses (Boghaert *et al.*, 2004). The effects were immunologically specific in most of the models, indicating that selective delivery of the potent drug to tumors mediates antitumor activity. In addition, the activities obtained suggest that the hydrazone and disulfide bonds within the drug-linker complex are sufficiently stable to allow for effective drug delivery in tumor models that are much less accessible than leukemia models such as AML. However, it should be noted that maximum localization of antibody conjugates in solid human tumors takes between 24 and 48 h (Meredith *et al.*, 2003), which may require significantly greater linker stability than that of the hydrazone present in Mylotarg.

Several other hydrazone-linked conjugates have been reported. Among the most highly investigated are hydrazone derivatives of doxorubicin, a modestly potent approved anticancer drug with multiple mechanisms of activity. Conjugates of doxorubicin were prepared by linking a maleimido hydrazone derivative of doxorubicin to the anticarcinoma chimeric mAb cBR96 through a hydrazone that was selected for stability at pH 7 and drug release at pH 5 (Trail *et al.*, 1993). Conjugates with structures represented in Figure 2 were formed by reduction of the BR96 interchain disulfides and by adding the maleimido drug derivative. As many as 8 drugs could be attached to each mAb with complete retention of binding activity. This would suggest that the antibody chains remain associated in the absence of covalent bonds. It was possible to append more than 8 drugs/mAb using branched linkers (King *et al.*, 2002).

Preclinical studies with BR96-doxorubicin demonstrated immunologically specific cures at well-tolerated doses in both mice and rats (Trail *et al.*, 1993).

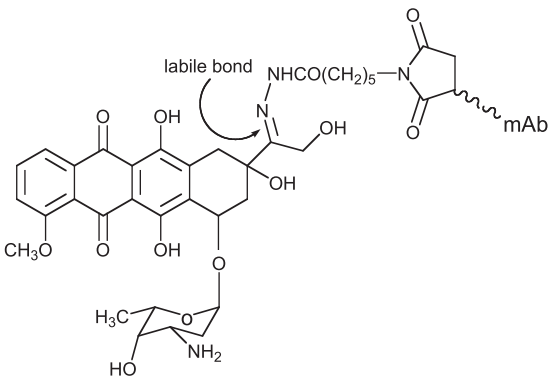

Figure 2. mAb-Doxorubicin hydrazone conjugates are designed to release doxorubicin within acidic intracellular vesicles. The hydrazone bond is relatively stable at neutral pH but labile under acidic conditions (Trail *et al.*, 1993).

However, the amount of conjugate needed to achieve these effects was very high (>100 mg conjugate/kg), reflecting the relatively low potency of the targeted drug. Pharmacokinetic studies indicated that the intratumoral drug concentration was much higher in animals treated with conjugate than in animals treated with the maximum tolerated dose (MTD) of unconjugated doxorubicin (Mosure *et al.*, 1997)

In a Phase I clinical trial, the MTD of BR96-doxorubicin was found to be 600–700 mg/m^2 with gastrointestinal dose-limiting toxicities (Saleh *et al.*, 2000) The half-life of drug release from circulating conjugate was approximately 43 h, which is suboptimal, given that the half life of the mAb in circulation was approximately 12 days. The conjugate was marginally active in this trial.

A subsequent Phase II trial (Tolcher *et al.*, 1999) confirmed that the response rate was low, with gastrointestinal dose-limiting toxicities. This study showed that unconjugated BR96 mAb elicited the same toxicities as the conjugate, suggesting that normal tissue cross-reactivity and mAb-mediated activities might have contributed to the toxicity. One of the noteworthy findings in this study was that active drug was detected within tumor masses, providing support for the concept of using mAbs for anticancer drug delivery.

The results from these clinical studies prompted further investigation into the use of conjugates in combination with other drugs. One such study demonstrated that paclitaxel strongly synergized with BR96-doxorubicin (Trail *et al.*, 1999). It was found that this combination was highly effective in lung, colon, and breast tumor xenograft models. Synergistic activities between BR96-doxorubicin and docetaxel were also reported, and a mechanism for synergy was proposed based on conjugate-mediated G2 cell-cycle arrest, leading to enhanced sensitization of the cells to taxanes (Wahl *et al.*, 2001). The promising *in vitro* and *in vivo* synergy studies provide the basis for ongoing clinical trials in which BR96-doxorubicin is combined with chemotherapeutic agents.

Disulfide-Linked Conjugates

Seminal work with mAb-protein toxin conjugates, such as mAb-ricin A chain conjugates, strongly suggested that disulfide linkers permitted reversible drug attachment and significant selectivity because thiol concentrations are much higher inside of cells than in serum (Vitetta *et al.*, 1993). However, based on the observation that the disulfides were cleaved in serum, investigations led to the development of hindered disulfides that were more stable in circulation but still susceptible to intracellular reduction (Thorpe *et al.*, 1988). Since this work, hindered disulfides have played a significant role as linkers for mAb-drug conjugates.

Cantuzumab mertansine (Figure 3) is the most advanced disulfide-bound drug conjugate. It is composed of the humanized mAb huC242 conjugated to the highly potent anti-tubulin agent DM1, a derivative of the natural product maytansine. HuC242, which was humanized using a technique known as “variable domain resurfacing” (Roguska *et al.*, 1996), recognizes a glycoform of MUC1

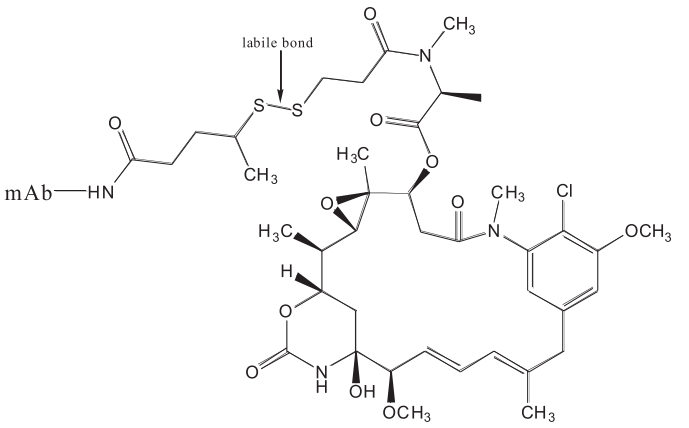

Figure 3. The structure of a mAb-DM1 conjugate illustrates how a derivative of maytansine can be attached to mAbs through a hindered disulfide-containing linker. The drug is released through disulfide bond reduction (Liu *et al.*, 1996).

known as CanAg, which is strongly expressed on most pancreatic, biliary, and colorectal cancers and on 40–55% of non-small cell lung, gastric, uterine, and bladder cancers. CanAg is internalized upon binding to the conjugate.

DM1 is highly potent, with an IC_{50} in the picomolar range. To prepare DM1 conjugates, lysine amino groups on the mAb were first acylated with NHS esters of thiopyridyl disulfides. DM1, activated by treatment of a disulfide precursor with DTT and HPLC purified, was attached through disulfide exchange, releasing the thiopyridyl chromophore. A secondary methyl group adjacent to the disulfide provides steric stabilization of the disulfide *in vivo*. In nude mice bearing COLO 205 xenografts, cures were obtained at 16 mg mAb component/kg/day for 5 days (Liu *et al.*, 1996).

Results from a Phase I dose escalation clinical trial have been reported (Tolcher *et al.*, 2003). Doses were escalated in the trial from 22 to 295 mg/m^2, which was the approximate MTD. Analyses of serum samples from these patients indicated that the disulfide bond linking the drug and mAb cleaved in circulation and that the conjugate was cleared more rapidly than unconjugated mAb. The net result was an increase in the ratio of mAb to conjugate in the blood over a period of days after each injection. While evidence for minor responses was obtained, it is likely that the therapeutic potential of huC242-DM1 will be improved by increasing the systemic stability of the linker used to join the drug to the mAb.

CC1065, a potent minor groove binder alkylating agent, was also conjugated to mAbs through the hindered disulfide linkages as described for DM1 (Chari *et al.*, 1995). The resulting conjugates contained an average of 4–5 drugs per mAb and were highly potent *in vitro* with IC_{50} values in the range of 10 ng/mL. In SCID mouse models, the acute LD_{50} was 14 mg conjugate/kg, and the MTD was 5 mg conjugate/kg when administered over 5 consecutive days. In a disseminated lymphoma model, the conjugate provided a 2.7-fold increase in median survival time at its MTD, while drug alone and non-binding control conjugates provided

substantially smaller increases in median survival time. Although the therapeutic window was very modest, the results provide further evidence that potent cytotoxic agents linked to mAbs through disulfide bonds have efficacy in preclinical models.

Another example of the use of disulfide linkers for conjugate formation includes taxoid anticancer drugs attached to mAbs against the epidermal growth factor receptor (Ojima *et al.*, 2002). A form of paclitaxel was used that was 100–1000 times more potent than the parent drug on drug-resistant cell lines. The drug was conjugated through the same hindered disulfide used to link DM1 to mAbs, and the resulting conjugate was active at sub-saturating concentrations. Tumor growth in nude mouse xenograft models was completely inhibited with a dose of 10 mg conjugate/kg given on five consecutive days, a dose that elicited no toxic side-effects. The taxoid conjugate described warrants further study, since the class of drugs is of clinical interest and the particular drug chosen is highly potent. The stability of the linker disulfide bond in circulation may not be optimal, given what was found with the mAb-DM1 conjugate containing the same linker technology. The potential that these and related (Miller *et al.*, 2004) taxoids have to circumvent common drug-resistant pathways requires further study and should be of considerable interest, given that this was identified as an issue with Mylotarg (Sievers *et al.*, 1999).

A final example of a disulfide-linked conjugate involves a variation of Mylotarg, in which calicheamicin was linked to the murine anti-MUC1 mAb, CTM01 (Hinman *et al.*, 1993) through mAb lysines *via* the same sterically hindered disulfide bond that is present in Mylotarg. There was no hydrazone moiety in this particular conjugate because it was found that carcinoma cells do not require the acid-labile linkage for full drug activity. CTM01-calicheamicin showed potent and specific anti-tumor efficacy in preclinical models of ovarian carcinoma. A version of this conjugate using a humanized version of the CTM01 was tested in a Phase I clinical study (Gillespie *et al.*, 2000). The MTD was 16 mg/m^2, and there was some evidence for minor clinical responses.

Peptide-linked Conjugates

The use of peptides as conditionally stable linkers for drugs to mAbs is of interest since enzymatic hydrolysis produces linkers of considerably greater systemic stability than those that are chemically cleaved by water or small molecular weight reducing agents. The peptides are designed for high serum stability and rapid enzymatic hydrolysis once the mAb-conjugate is internalized into lysosomes of target cells. This approach is particularly attractive since the proteases that lead to drug release are mainly intracellular and are not nearly as active outside the cells, due to their pH optima and inhibition by serum protease inhibitors.

Initial work in this area conscripted cathepsin B for drug release as this is one of the most abundant of the lysososomal enzymes. A number of mAb-peptide-doxorubicin conjugates were prepared, and it was shown that efficient drug release required the incorporation of a self-immolative spacer between the peptide and

doxorubicin (Dubowchik and Firestone, 1998). Several dipeptides were found to be suitable, based on serum stability in serum and rapid cleavage by lysosomal extracts or purified cathepsin B. The structures of the peptide-linked doxorubicin conjugates are shown in Figure 4.

The first peptide-linked antibody drug conjugates were BR96-phenylalanine-lysine-p-aminobenzyl-doxorubicin (BR96-Phe-Lys-Dox) and the corresponding valine-citrulline derivative, BR96-Val-Cit-Dox; they were found to be serum stable and underwent rapid cleavage by cathepsin B and lysosomal extracts, leading to the release of doxorubicin (Dubowchik *et al.*, 2002a). The stability characteristics of these conjugates were evident from *in vitro* assays, in which high levels of specificity were observed even upon prolonged treatment. Higher potency was obtained with bivalent linkers that allowed increased levels of doxorubicin substitution (Dubowchik et al, 2002b). However, the resulting conjugates, having approximately 16 drugs/mAb, were non-covalently aggregated due to the hydrophobic nature of the drug. To circumvent this, hydrophilic ethyleneglycol hydrazides were appended in a reversible manner to the free carbonyl group of doxorubicin, and the resulting highly substituted conjugates were mostly monomeric (King *et al.*, 2002). This approach is quite interesting as two independent events, proteolysis and acid-catalyzed hydrazone hydrolysis, must take place for intracellular drug activation. It is not known if having two such mechanisms for drug release allows enhanced specificity and lower toxicity.

This work was extended to include other drugs (Figure 4) such as paclitaxel (Dubowchik *et al.*, 1998), mitomycin C (Dubowchik *et al.*,1998)), camptothecin (Walker *et al.*, 2002), tallisomycin (Walker *et al.*, 2004), and auristatin family members (Doronina *et al.*, 2003; Francisco *et al.*, 2003). The latter class is of particular interest, because of their pronounced activities on a wide range of tumor types. The auristatins are structurally related to dolastatin 10, a

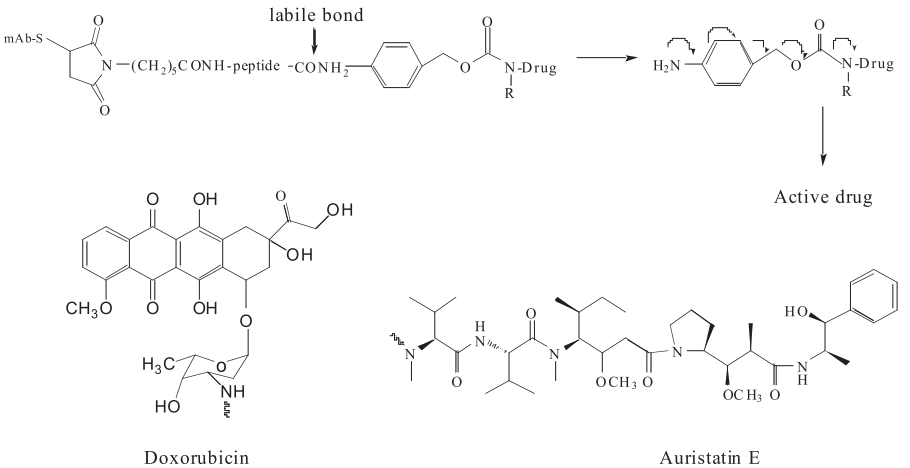

Figure 4. Structures of mAb-drug conjugates incorporating enzymatically cleavable peptide linkers. The drug is released upon hydrolysis of the peptide, followed by [1,6]-fragmentation of the aminobenzylcarbamate. Several drugs have been attached to mAbs using this linker strategy (Dubowchik *et al.*, 2002a; Doronina *et al.*, 2003).

pentapeptide natural product that has been the subject of several human clinical trials for cancer therapy (Saad *et al.*, 2002). Molecules in this family inhibit tubulin polymerization and may also lead to intratumoral vascular damage (Otani *et al.*, 2000). The activities are generally 100–1,000 times more potent than doxorubicin. Additional distinguishing characteristics of the auristatins are that they are prepared in large quantities through total synthesis and, unlike calicheamicin and the DNA alkylating agents described earlier, the drugs tend to be exceedingly stable.

Peptide derivatives of the synthetic auristatin derivative MMAE were prepared using methodologies similar to those described for the peptide-linked doxorubicin conjugates (Doronina *et al.*, 2003). mAb-Val-Cit-MMAE and mAb-Phe-Lys-MMAE conjugates were prepared through interchain mAb disulfide reduction, producing conjugates with approximately 8 drugs/mAb (Figure 4). Unlike the doxorubicin conjugates, mAb-peptide-MMAE conjugates effected immunologically specific cell kill at concentrations well below that required for antigen saturation. The conjugates were highly stable in human serum, with projected half-lives of greater than 80 days. By comparison, the hydrazone-linked mAb-AEVB conjugate released free drug with a half-life of only 2 days in plasma. Thus, the peptide linker is much more stable than an optimized hydrazone linker.

In vivo studies in both carcinoma and hematologic tumor xenograft models demonstrated that the mAb-Val-Cit-MMAE conjugate was highly effective, immunologically specific, and well-tolerated. Studies were reported with cAC10-Val-Cit-MMAE, a conjugate that bound to the CD30 antigen on hematologic malignancies, in which cures of established tumors were obtained at doses as little as 1/60th the MTD (Figure 5). Therapeutic windows this high have not been

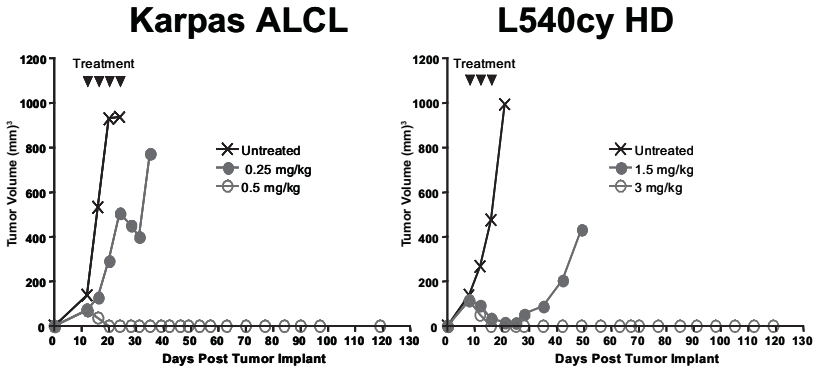

Figure 5. Therapeutic effects of cAC10-Val-Cit-MMAE in nude mice with subcutaneous Karpas anaplastic large cell lymphoma and L540cy Hodgkin's disease human tumor xenografts. The conjugates bind to the CD30 antigen present on both of these tumor models. Mice were treated with conjugates according to the schedule indicated by the arrows. The quantities indicated are based on the total protein content, and were well below the maximum tolerated dose of 30 mg total conjugate/kg of body weight. The higher doses shown led to cures of established tumors in all of the animals treated. The data are derived from Doronina (2003) and Francisco (2003) and coworkers.

reported in the drug conjugate field, and the results underscore the importance of drug potency, linker design, and mAb trafficking in developing conjugates with optimal chances for therapeutic efficacy.

Conclusions

The potential of mAb-drug conjugates for treating human cancers is apparent from the approval and acceptance of Mylotarg for the treatment of AML. Mylotarg is active in the clinic even though the linker and drug are unstable and the conjugate is susceptible to the multidrug resistance phenotype. This review has focused on several areas of conjugation technology that are designed to address these shortcomings. These include recent work leading to the generation of new classes of cytotoxic agents that are quite stable and readily available, linker systems that are stable in the circulation and allow the efficient release of drug at the target site, and conjugation methodologies that provide highly uniform products. Further studies are needed to produce re-engineered mAbs that are optimized for selective drug attachment and intratumoral access. It is also possible that engineered mAbs can be selected to lower the systemic toxicities of mAb-drug conjugates by minimizing metabolism within sensitive tissues. By combining these advances, new generations of mAb-drug conjugates with activities in cancers that are resistant to known therapeutic modalities may evolve.

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2.5.4

Antibody-Directed Enzyme Prodrug Therapy

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Table of Contents

List of Abbreviations

ADEPT .....Antibody-Directed Enzyme Prodrug Therapy
hCG.....human Chorionic Gonadotrophin
CPG2 .....Carboxypeptidase G2
CEA .....Carcinoembryonic Antigen
A5CP .....A5B7-F(ab')2-CPG2 Conjugate
CMDA.....4-[(2-Chloroethyl)[2(mesyloxy)ethyl]amino] benzoyl-L-glutamic Acid Prodrug
ZD2767P .....4-[N,N-bis(2-Iodoethyl) amino] phenoxy-carbonyl-L-glutamic Acid Prodrug

Introduction

Antibody-directed enzyme prodrug therapy (ADEPT) was conceived as a means of reducing the toxicity of chemotherapy and improving its efficacy by generating the cytotoxic agent at tumor sites (Figure 1). It was proposed that this could be done by using an antibody directed at a tumor-associated antigen to convey an enzyme to tumor sites and, when the enzyme had cleared from the blood, to give a low toxicity prodrug from which a high toxicity drug would be released by enzymic catalysis within tumors. The active drug would be a small molecule able to diffuse within a tumor and thus overcome the heterogeneity in tumor marker expression that was seen as a potential limitation to conjugates of cytotoxic agents and antibodies. At the outset it was envisaged that the antibody-enzyme complex could be genetically engineered and that the drug generated would have a very short half-life (Bagshawe, 1987).

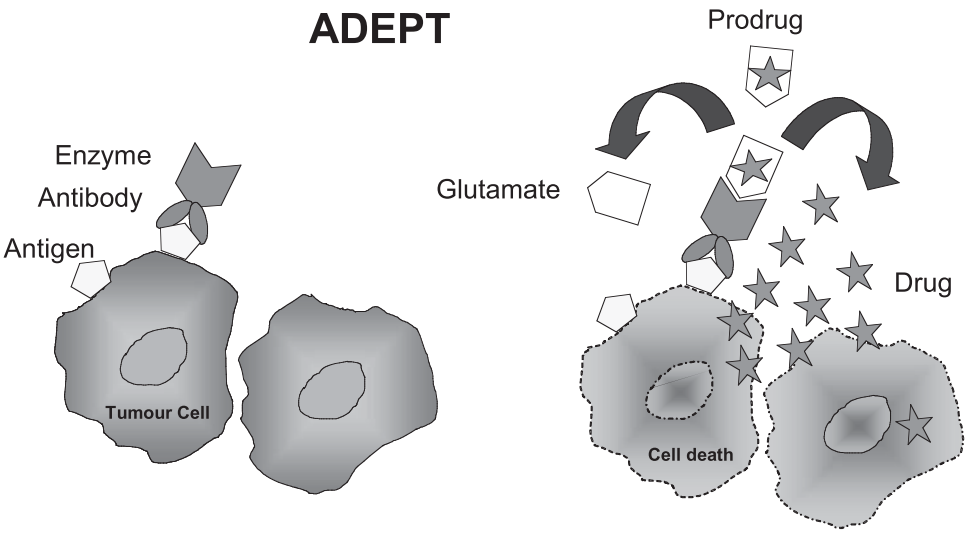

Figure 1. Illustrates the basic principle of ADEPT

At the present time (April 2005), reports of clinical experience with ADEPT have come only from the London group that first described this approach, and the first part of this review is an account of the experience of this group. This is followed by a review of the preclinical studies reported from other groups.

The London Group's Studies

Following the studies of Mach *et al.* (1974) and Goldenberg *et al.* (1978), the London group based at Charing Cross Hospital Medical School had several years experience with the tumor localisation of intravenously injected radiolabelled antibodies. Later, it was found that radiolabelled antibody-enzyme conjugates

behaved in a fashion virtually identical to that of radiolabelled antibodies. That is, the high uptake of the radiolabelled preparations by tumors stood out on gamma camera scans against the lower background of radioactivity in the blood. Intact antibodies and conjugates made with intact antibodies were observed to be retained in the blood for several days; this led to the use of antibody fragments that cleared more rapidly from the blood whilst still remaining active for several days in tumors (Begent *et al.*, 1980).

The issue of clearing enzyme from blood was highlighted by early preclinical studies using a choriocarcinoma xenograft that was associated with a high circulating level of the antigenic target, human chorionic gonadotrophin (hCG), in the blood and a colonic carcinoma xenograft (LS174T) that had no detectable antigenic target, carcinoembryonic antigen in the blood, although it was expressed on the cells. In the choriocarcinoma model, enzyme activity located in the xenografts and cleared quickly from the blood, allowing prodrug to be given between 52 and 76 h later and effecting eradication of most of the xenografts. By contrast, giving the prodrug at the same time in the LS174T model as in the choriocarcinoma model resulted in fatal toxicity, presumably because of persistence of enzyme in blood. The prodrug could only be given in the LS174T model 6–7 days after the antibody-enzyme conjugate, by which time enzyme levels in the tumor had fallen too low for effective therapy (Bagshawe *et al.*, 1988, Bagshawe, 1989).

The solution lay in the use of a murine monoclonal antibody directed at the enzyme that inactivated the enzyme and accelerated its clearance from blood. To prevent the anti-enzyme antibody from lingering in the blood and possibly acting on tumor-located enzyme, it was galactosylated, thus ensuring its rapid removal from blood via hepatic receptors. When this was given 19 h after the antibody enzyme conjugate, the prodrug could be given a few hours later; significant growth delay was then observed in the colon carcinoma model (Bagshawe, 1989, Sharma *et al.*, 1990, 1991, Sharma 1994).

The choice of enzyme was a major issue. It was argued that the enzyme should have no human homologue so as to achieve the required specificity of action and that human enzymes would not have the high turnover rate that seemed desirable to achieve a high rate of turnover of prodrugs. Some bacterial enzymes have the capacity for high turnover rates of substrates but carry the penalty of immunogenicity. Whereas there was no prospect of avoiding the need for specificity, it seemed possible that there would, in time, be solutions to the problem of immunogenicity. The enzyme carboxypeptidase G2 (CPG2) from Pseudomonas sp (Sherwood *et al.*, 1985) that had been cloned and produced in Escherichia coli (Minton *et al.*, 1983) and that catalyses the hydrolytic cleavage of glutamate from folates was adopted for the purpose.

The requirements for suitable prodrugs were also under consideration at the outset. The use of CPG2 meant that the conversion of a cytotoxic drug to a prodrug would be achieved by glutamation. It was judged to be important that the drug should not be subject to the common mechanisms of drug resistance such as P-glycoprotein exclusion or gene amplification. These considerations pointed to

alkylating agents that appeared to be less susceptible to these effects than the other classes of cytotoxic agents (Frei *et al.*, 1988). The first prodrugs to be made for an ADEPT system were, therefore, N-mustard prodrugs for activation by CPG2 (Springer *et al.*, 1990). Although it was thought that the active drug should have a short half-life, the earliest drugs fell well short of this characteristic (Antoniw *et al.*, 1990), and it was only after the first clinical trials that its importance was fully appreciated. Again, the early clinical trials pointed to the desirability of prodrugs that are soluble in aqueous solution, and the early drug that was used was soluble only in dimethyl sulphoxide.

First Pilot Scale Clinical Trials

These first clinical trials were carried out in 1990–1994 on patients with advanced colorectal cancer whose disease had progressed after failing to respond to 5-fluorouracil and folinic acid. The studies were carried out with fully informed consent of the patients. There were ten males and nine females aged 28–68 years with histologically confirmed adenocarcinomas of colon, rectum, or appendix. Plasma CEA levels ranged from 26–16,420 μg/L. Hepatic metastases were evident upon CT scan in ten patients and nine of these had a pelvic mass or ascites. Most had metastases at multiple sites.

The agent used was a murine monoclonal antibody A5B7-F(ab')2 directed at CEA. This had been conjugated using bifunctional agents to CPG2; this conjugate will be referred to here as A5CP. Although radiolabelled A5B7 had been used clinically as a diagnostic targeting agent, it had not been used previously in a therapeutic role. CPG2 had been given to two patients with severe methotrexate toxicity, but no details had been provided by the clinicians involved. Neither the clearing antibody (SB43) nor the prodrug 4-[(2-chloroethyl)[2(mesyloxy)ethyl] amino]benzoyl-L-glutamic acid (CMDA) had been given to patients previously. Additional variables were the duration of the intravenous infusions of A5CP and the clearing antibody SB43. There was a question as to whether to give the prodrug by slow infusion or as a series of bolus injections. It was, therefore, not possible to start with a fixed pre-determined protocol. A dose escalation study with the prodrug alone was carried out on seven patients in the dose range 0.2–2.4 g/m2. Toxicity was minimal, with WHO grade 2 toxicity nausea and vomiting at the highest dose level. Enzyme levels in blood were measured and prodrug was given only after the enzyme became undetectable.

With the full protocol and CMDA being given as a series of bolus injections, there was no toxicity below a CMDA dose totalling less than 2.0 g/m^2. Three patients received 2.7–3.9 g/m^2; two of these had partial responses and one had a mixed response. Disease progression recurred after 4–6 months, and one patient survived for 22 months. All of these patients had grade 4 myelotoxicity with recovery after 16 days.

A second group of five patients received CMDA by continuous infusion, and one who received 4.0 g/m^2 of CMDA had a partial response with stable disease for 22 months. This group was compromised by the later finding that the batch of

A5CP used failed to localise in xenografts. All the patients treated up to this point developed anti-murine and anti-bacterial antibodies within 12 days.

A final group of six patients additionally received cyclosporin starting 48 h before the A5CP infusion with the objective of receiving more than one cycle of therapy. CMDA was given by continuous infusion. Two patients received two cycles of therapy and two others received three cycles. There were two partial responses with stable disease for 8 and 18 months, respectively. Two patients who received oral cyclosporin died from myelotoxicity and renal failure soon after therapy. The other four patients received cyclosporin intravenously, but all four developed anti-murine and anti-bacterial antibodies by 21 days (Bagshawe *et al.*, 1995; Bagshawe and Begent, 1996).

In a second pilot scale trial carried out at the Royal Free Hospital Medical School, ten patients were entered and received the same agents and cyclosporin. The A5CP was labelled with ^{131}I to confirm tumor localisation of the conjugate, and the dose of conjugate was halved from 20,000 units/m^2 used in the first trial to 10,000 units/m^2. A single bolus of CMDA at 200mg/m^2 was given daily for three days.

Four patients responded, and there was one partial response with near disappearance of a biopsy-proven deposit adjacent to a colostomy and disappearance confirmed by CT scan of hepatic metastases. Biopsies of liver metastases in four cases gave tumor-to-blood ratios for enzyme activity >10,000:1 (Napier *et al.*, 2000).

The Lessons from Pilot Clinical Trials

These studies gave valuable information about the way forward. Despite strenuous efforts to produce uniform conjugates of the antibody and enzyme it seemed clear that there was some variation between batches; however, this was a problem that would have to await the development of fusion protein technology.

What Caused the Observed Myelosuppression?

Following the first trial there was uncertainty about the cause of the myelosuppression that was observed. It seemed possible that the HPLC methodology then available failed to detect low levels of enzyme activity in blood. However, it was found that the active drug had a half-life that was in the range 20–30 min (Antoniw *et al.*, 1990; Martin *et al.*, 1997) so that even when generated only at tumor sites it could leak back into the blood. The finding of very high tumor-to-blood ratios after giving the clearing antibody in the second trial indicated that leaking back of drug from tumor sites into blood was the cause of myelosuppression.

Prodrug and Drug Characteristics

Apart from the long half-life of the drug generated from CMDA that had to be tackled, there was also the problem of CMDA's solubility. It could only be given in relatively large amounts of dimethyl sulphoxide (DMSO), and it was felt, though not proven, that this contributed to nausea and vomiting. So, a desirable characteristic of the prodrug is also its solubility in aqueous solutions. It was evident from these studies that CMDA was itself relatively nontoxic; this allowed relatively large doses to be given, but the drug generated from it was not as toxic as needed although it was clearly capable of causing myelosuppression. The differential toxicity between prodrug and drug is clearly of vital importance since the toxicity of the prodrug determines the amount that can be given and, therefore, the concentration of drug generated from it.

It was recognised that the agents used in these early studies were relatively crude but that with due diligence more refined agents would be produced.

The Clearing Antibody

The clearing antibody had proved highly effective in the clinic as it had in the mouse model. Given by slow intravenous infusion there was no evidence of immune complex problems. There was every expectation that the murine monoclonal antibody could be replaced by a human anti-enzyme antibody. It was therefore disappointing, even calamitous, that the three-stage ADEPT system was subsequently judged to be too complex and the clearing system was eliminated, even though it was clear from the earlier evidence that ADEPT would not work without it. The main emphasis was placed on a new prodrug that generated a much more active cytotoxic agent than CMDA and that had a half-life too short to measure (Springer *et al.*, 1995; Blakey *et al.*, 1995; Monks *et al.*, 2001). A trial was therefore conducted in which some 27 patients received A5CP and, after enzyme in blood had fallen to very low levels, ZD2767P prodrug was given. As was predictable, enzyme levels in tumors would also have fallen by the time prodrug was given, and no clinical or radiological responses were observed (Francis *et al.*, 2002).

It may be that ZD2767P is the ideal prodrug for ADEPT, but it is unfortunate that the optimum half-life of the active drug in the ADEPT concept has not been studied. Diffusion of a small molecule within a large tumor mass can be assumed to take a finite time, and it is possible that the half-life of the active drug can be too short as well as too long.

Fusion Proteins

There was a ray of hope that came in the form of fusion proteins. The principle is to encode the gene for the antibody with that encoding the enzyme and to overexpress the resulting product in a suitable expression system. The first antibody-enzyme fusion protein was described by Bosslet *et al.* (1992). The fusion

protein used by the London group incorporates a ScFv anti-CEA antibody (MFE) with CPG2 (Chester *et al.*, 1994) to give a fusion protein MFE23-CPG2 which, when expressed in *Pichia Pastoris*, is glycosylated (Medzihradszky *et al.*, 2004). The first advantage of this development is the avoidance of the inherent variability of chemical conjugates. Fusion proteins also lend themselves to large-scale production. The relatively small size of such a fusion protein favours tumor penetration and rapid clearance from blood, but it has a much shorter residence time in tumors than the earlier chemical conjugates so that a new strategy was required for ADEPT.

The glycosylated MFE-CPG2 gave tumor to blood ratios up to 1600:1 at 6 hours after administration, so it became possible to administer prodrug in the time window available between the clearance of enzyme from blood and clearance from tumor and to repeat the cycle at short intervals (Sharma *et al.*, 2005).

Studies had been carried out on the epitopes on CPG2 to determine whether these could be modified to reduce its immunogenicity (Spencer *et al.*, 2002). The incorporation of a His-tag into the fusion protein was found to have masked one of the dominant epitopes so that some patients failed to develop antibodies (Sharma *et al.*, 2001 Mayer *et al.*, 2004).

The Snag

All this was encouraging, but there is a snag. The snag is individual variability. Although the fusion protein clears from the blood in a matter of hours, there is variability between individual patients in the time it takes after the fusion protein is given for blood enzyme to become low enough to make it safe to give the prodrug. With the short time window for prodrug to be given, it is necessary to know as soon as it is safe to do so. This necessitates frequent blood samples and HPLC measurements to be carried out immediately on each of them.

The Way Forward

It seems that omission of the clearance step with an anti-enzyme antibody is much more complicated than its inclusion. A three-step ADEPT system is less complicated than many conventional chemotherapy protocols in everyday use. Therefore, re-incorporation of the clearance step would allow prodrug to be given at a standard time when enzyme concentration in the tumor would be at or close to its peak. The new clearing antibody would ideally be human, enzyme-inactivating, and designed for rapid clearance. It would also be interesting to see the effect of administering such a human antibody only a few hours after the fusion protein on the immune response of the patient to the CPG2 component.

Preclinical Studies by Other Groups

Most groups in the field have made prodrugs from known cytotoxic agents already approved for clinical use. Other enzymes of microbiological origin with no human homologues that have been used in ADEPT systems include β-lactamase, cytosine deaminase and penicillin G amidase (reviewed in Senter and Springer 2001, Bagshawe *et al.*, 2004).

β-lactamase enzymes cleave the B-lactam rings of prodrugs bonded to cephalosporin; a range of prodrugs has been constructed around this enzyme. These include a nitrogen mustard (Alexander *et al.*, 1991), doxorubicin (Vrudhula *et al.* (1995), vinblastine (Meyer *et al.*, 1993), taxol (Rodrigues *et al.*, 1995) and carboplatin (Hanessian and Wang 1993).

Cytosine deaminase conjugates have been used to convert 5-fluorocytosine to 5-fluorouracil, resulting in a 17-fold increase in the concentration of 5-fluorouracil in tumors compared with systemic administration of 5-fluourouracil (Wallace *et al.*, 1994).

Some groups have used enzymes of microbiological origin that have human homologues, including β-glucuronidase and nitroreductase. These have no advantage in terms of immunogenicity over those described above, but β-glucuronidase is potentially usable because it has a different pH optimum and better turnover rate than the human form. Nitroreductase of bacterial origin differs in its substrate activity from the human form.

Enzymes of human origin have the advantage of being nonimmunogenic but, of those proposed, β-glucuronidase is not very efficient at physiological pH and a mutated carboxypeptidase was not stable. A human propyl endopeptidase that activated an N-protected glycine melphalan prodrug is more promising (Heinis *et al.*, 2004).

When ADEPT was first proposed, there was speculation that molecular engineering would allow the development of so-called "Abzymes," that is antibodies with catalytic activity (Bagshawe, 1989). Progress has been slow, but a recent report of an aldolase antibody that releases etoposide from an etoposide prodrug and has proved effective in a murine neuroblastoma model may be the beginning of new developments in this area. (Rader *et al.*, 2003).

Fusion proteins have been described by other groups. These include an anti-melonoma antibody ScFv combined with B-lactamase for the conversion of 5-fluorocytosine to 5-fluorouracil. Another combines an anti-CEA antibody with human placental B-glucuronidase expressed in baby hamster ovary cells to convert a doxorubicin prodrug (Bosslet *et al.*, 1992) and another fuses an antibody directed at tumor necrosis with human B-glucuronidase (Biela *et al.*, 2003).

The accelerated clearance of enzyme from blood by means of antibody directed at the enzyme was confirmed by Kerr *et al.* (1993) with a cytosine deaminase enzyme. Polyethylene glycol modified proteins have undergone accelerated clearance by an anti-polyethylene glycol antibody (Cheng *et al.*, 1999).

The immunogenicity issue arising from murine antibodies and bacterial enzymes has been solved so far as the antibody components are concerned by the use of human or humanised antibodies. The potential of immunosuppressive

agents has not been tested beyond the first clinical trials described above. Later, immunosuppressive agents may be more effective than cyclosporin but their use in the ADEPT concept would be an added complication with the potential for additional toxicity.

The possibility of using human enzymes and human catalytic antibodies remains, and the work of the London group with carboxypeptidase G2 suggests that a progressive reduction in immunogenicity may be achievable.

Prodrugs

Many prodrugs were synthesised in the pre-ADEPT era for activation by endogenous enzymes, and many of these were alkylating agents (Wilman, 1986). Some of the potential advantages of alkylating agents have already been discussed, but evidence that they achieve log linear tumor cell kill over a wide dose range may be advantageous for ADEPT.

A cephalosporin nitrogen mustard carbamate prodrug has been described (Alexander *et al.*, 1991) and a melphalan prodrug that was activated by a penicillin-G amidase (Vrudhula *et al.*, 1993). A p-hydroxyaniline mustard glucuronide prodrug was activated by an antibody glucuronidase conjugate and effected cures in mice bearing AS-30D tumors (Cheng *et al.*, 1997). An E-coli nitroreductase has been used to activate prodrugs of dinitrobenzamide (Anzelark *et al.*, 1998). The generation of 5-fluorouracil from 5-fluorocytosine has been reported from several groups using cytosine deaminase conjugates (Wallace and Senter 1994, Aboagye *et al.*, 1998, Deckert *et al.*, 2003)

Doxorubicin has been the focus of other studies (Vrudhula *et al.*, 1993, Florent *et al.*, 1998). Other prodrugs with interesting properties include a derivative of camptothecin that was linked by an aromatic spacer to glucuronic acid. (Leu *et al.*, 1999). A series of compounds with half lives ranging from 0.9 to180 min that released paclitaxel and a derivative was found to be less toxic than the parent compound (Wrasidlo *et al.*, 2002). A prodrug of palytoxin that is activated by penicillin G amidase was a thousand times less toxic than the parent compound and released the drug extracellularly with high potency (Bignami *et al.*, 1992).

Conclusions

It is clear that a wide range of antibodies, enzymes and prodrugs have been considered in the ADEPT context. Many of them have shown interesting properties *in vitro* and in mouse models. Whilst mouse models have proved to be invaluable in studies of conjugate location at tumor sites and in exemplifying the need to clear enzyme from blood before giving the prodrug they cannot reveal all the requirements for success in the clinic. As in other areas of research it would have been advantageous if all the effort that has gone into these agents had been co-ordinated. The problem is of course the wide range of disciplines that is required to mount a successful ADEPT program from concept to the clinic. One might have hoped that the pharmaceutical industry would collaborate to improve

upon the encouraging early clinical results rather than impose artificial and uninformed constraints that have delayed its successful development by many years. As already indicated, a three stage ADEPT system is less complex to administer than many current chemotherapy protocols and the pharmaceutical world's infatuation with the idea of a single highly effective anti-cancer drug remains as illusory as ever.

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2.5.5

Prodrugs for Liver-targeted Drug Delivery

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Table of Contents

List of Abbreviations

HBV.....hepatitis B virus
HCV.....hepatitis C virus
NTP.....nucleoside triphosphate
NMP.....nucleoside monophosphate
AGP.....asialoglycoprotein
LDL.....low density lipoprotein
HDL.....high density lipoprotein
NCTP.....sodium taurocholate cotransporting polypeptide
OATP.....organic anion transporting polypeptides
OCT.....organic cation transporter
OAT.....organic anion transporter
MDR.....multidrug resistance protein
MRP.....multidrug resistance-associated protein
CYP.....cytochrome P_{450}
PMEA.....9-(2-phosphonylmethoxyethyl)adenine
POM.....pivaloyloxymethyl
araC.....cytarabine
dCK.....deoxycytidine kinase
araA.....arabinose adenine

AZT.....azidothymidine
ADA.....adenosine deaminase
RBC..... red blood cell
WHV .....woodchuck hepatitis virus
PDE.....phosphodiesterase.

Introduction

The liver is the organ in the body primarily responsible for maintaining a continuous supply of glucose, lipids, and other important metabolic substrates to peripheral tissues (Kahl, 1999). The liver also helps to control the circulating levels of certain amino acids, ketone bodies, free fatty acids, lipoproteins, and some hormones (e.g., insulin, steroids, T_3) and plays an important role in the detoxification and excretion of various xenobiotics. Chronic liver diseases such as viral hepatitis and liver cancer, as well as some metabolic diseases associated with the liver such as non-alcoholic steatohepatitis, injure the liver. Chronic liver injury can lead sequentially to impaired liver function, liver failure, and death. Drugs for treating liver diseases are often limited by tolerability and extra-hepatic safety concerns, which prevent their use at maximally effective doses. Targeting drugs to the liver may circumvent some of these limitations and enable more effective therapies. This chapter reviews the liver-targeting strategies explored to date and, more specifically, the strengths and weaknesses of various prodrug strategies used for liver-specific drug delivery.

Liver Structure and Function

The liver is a large lobular organ in the abdominal cavity supplied by blood from the portal vein (75% of blood volume) and the hepatic artery (25% of blood volume) (Jones, 1996). Sinusoids, which are the capillaries of the liver, form the intralobular vascular network that drains the blood into the hepatic venules and then into the hepatic vein. Sinusoids differ from normal capillaries in that they are larger and are lined with both endothelial cells and cells known as Kupffer cells, which are liver-associated macrophages that phagocytize particulates,

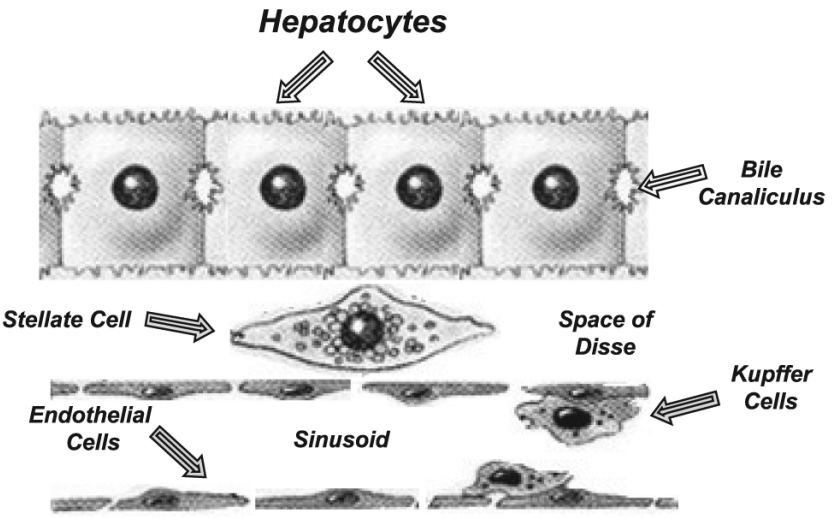


Figure 1. Schematic of a liver cross-section. Adapted and reprinted from Bissell DM, Cell-matrix Interaction and Hepatic Fibrosis. *Prog Liver Dis* 1990; 9:143–155 with the permission of the author.

bacteria and erythrocytes (Figure 1). The endothelial cells in the sinusoid form a porous wall with average gaps of 1000 Å (fenestrae), which helps to promote rapid exchange of substrates between the plasma and the space of Disse (perisinusoidal space).

Across the space of Disse is a continuous layer of hepatocytes. Hepatocytes represent the major cell type in the liver, accounting for approximately 60% of the total liver cell population and 78% of liver volume (Jones, 1996). In addition to containing most of the metabolic pathways and enzymes used to detoxify xenobiotics, hepatocytes are also the cells infected by liver-targeted viruses and the cells that are transformed to tumor cells in the largest proportion of patients with primary liver cancer. Nutrients, xenobiotics, and proteins are readily taken up by hepatocytes following diffusion across the space of Disse, which in the normal liver contains only a low density matrix (basement membrane), stellate cells, and hepatocyte microvilli. Following liver injury, however, stellate cells (1.4% of liver volume) are activated and produce a fibrillar matrix consisting predominantly of collagen that ultimately leads to closure of the endothelial fenestrae and decreased hepatocyte exposure (Friedman, 1993).

Products generated within hepatocytes are effluxed into the sinusoid and/or the biliary canalicular space. Bile canaliculi are formed between two adjacent hepatocytes using a specialized cell membrane domain (13% of total membrane surface). Bile flows from the bile canaliculi into the bile ductules and then through larger interlobular bile ducts before emerging from the liver via the hepatic duct proper.

Drug Metabolism and Excretion Pathways

Uptake

The liver typically represents the organ in the body associated with the highest drug exposure due in large part to its high blood flow (1.5 L/min, 27% of cardiac output in humans) and high vessel permeability as well as its anatomical location, which necessitates that orally administered drugs pass through the liver prior to entering the systemic circulation. The liver and especially the hepatocyte are designed to rapidly take up nutrients, xenobiotics and drugs from the blood. Lipophilic molecules readily enter hepatocytes through passive diffusion, whereas negatively and positively charged compounds use cell surface transporters to enter hepatocytes (van Montfoort *et al.*, 2003). Even large macromolecules such as proteins are able to interact with hepatocytes since the fenestrated endothelium enables passage out of the blood vessel. Binding of macromolecules to various cell surface receptors often triggers a process known as receptor-mediated endocytosis, which leads to internalization of vesicles containing the receptor complex. Intracellular trafficking of these vesicles to endosomes and lysosomes results in dissociation of the macromolecule and, in some cases, its degradation by acid-catalyzed chemical reactions or by enzymes in these compartments capable of degrading proteins, lipids, and polysaccharides.

Metabolism

Following uptake by hepatocytes, drugs are often metabolized to water-soluble metabolites that are excreted through the bile or kidneys. Metabolism typically entails an oxidation, reduction, or hydrolytic reaction (Phase I) to generate a metabolite that subsequently undergoes drug conjugation (Phase II). Cytochrome P_{450}s (CYPs) catalyze most of the oxidations that lead to hydroxylation or epoxidation of organic compounds (Lewis, 2003). Reductases and hydrolases, including esterases, peptidases, amidases, acetyl transferases, and epoxide hydrolases, catalyze the remaining Phase I biotransformations. Drug conjugation reactions (Phase II biotransformations) frequently follow Phase I metabolism and result in the formation of glucuronides, glutathione conjugates or other water soluble conjugates (e.g., sulfates).

While many of the enzymes catalyzing Phase I- and Phase II-type transformations are also expressed in other tissues, especially the kidneys and the gastrointestinal tract, the liver is typically the dominant drug metabolism organ. This dominance is due to the high levels of these metabolizing enzymes in the liver coupled with the high exposure of most drugs to the liver as a consequence of its size and blood flow. In the liver, sinusoidal blood crosses different microenvironments as it flows from terminal portal venule to the terminal hepatic venule. These microenvironments lead to large metabolic heterogeneity (Katz and Jungermann, 1993). Hepatocytes in the periportal region (Zone 1) are exposed to higher drug levels due in part to their greater surface-to-volume ratio. In contrast, the perivenous hepatocytes are associated with higher levels of both Phase I and Phase II enzymes and are consequently more susceptible to oxidative damage and toxicity due to glutathione depletion.

Excretion

Drugs taken up by the liver are excreted, frequently following intrahepatic transformation, into either the circulation or the bile. Most drugs returned to the sinusoid are eliminated renally, especially if they are low molecular weight, highly hydrophilic compounds. Elimination via the bile entails transport of drugs out of the hepatocyte and into the bile canaliculi (Kullak-Ublick *et al.*, 2000). Carrier proteins and transport proteins expressed on the sinusoidal (basolateral) membrane of the hepatocyte differ from the proteins expressed on the bile canalicular surface (apical), leading to directional transport of some drugs from the circulation to the bile.

Liver Diseases

The most common chronic liver disease is viral hepatitis, which worldwide largely comprises patients infected with hepatitis B virus (HBV) (350 million) and patients infected with hepatitis C virus (HCV) (125 million). These viruses infect hepatocytes and result in inflammatory responses that lead to chronic liver injury.

Liver injury from viral infections, as well as from chronic ingestion of alcohol, acute exposure to high doses of acetaminophen, or exposure to high levels of iron or environmental toxins leads to fibrosis, i.e., the overproduction of extracellular matrix. Chronic fibrosis can affect blood flow and liver function and ultimately result in liver cirrhosis, which is associated with a high risk of death from either liver failure or primary liver cancer. Drugs for treating HBV and HCV are available but fail to prevent the onset of liver disease in the majority of patients. No drugs are currently approved to treat patients with primary liver cancer or liver cirrhosis.

Other diseases associated with the liver include a variety of metabolic diseases such as diabetes, hyperlipidemia, metabolic syndrome and, possibly, obesity. Chronic hyperglycemia is the hallmark feature of type 2 diabetes, which is partially due to the overproduction of glucose by the liver. Hypercholesterolemia arises from increased synthesis and/or decreased metabolism of cholesterol, which principally involves pathways residing in the liver. Obesity and metabolic syndrome are commonly associated with fatty liver disease and its more severe form known as non-alcoholic steatohepatitis (NASH), which over time can lead to liver cirrhosis and an increased risk of acute liver failure and hepatocellular carcinoma (Suriawinata and Fiel, 2004). While drugs are available for treating most metabolic diseases, drug efficacy is often compromised by dose-limiting side effects, which prevent the majority of patients from achieving optimal disease control.

Nucleoside-Based Drugs

Efforts to target drugs to the liver have focused primarily on nucleosides (Meijer *et al.*, 1992; Meijer and Molema, 1995), largely because of their well-known antiviral activity and the knowledge that nucleosides as a class of drugs often exhibit a wide variety of extra-hepatic toxicities, including neuropathies, nephropathies, and myelosuppression. Another potential benefit projected for liver targeting is improved antiviral activity, which to date appears less than the efficacy associated with nucleoside-based drugs used to treat non-hepatic viral infections (e.g., HIV, herpes).

One reason for the inability of many nucleosides to exhibit good activity against HBV and HCV stems from their poor conversion in the liver to the nucleoside triphosphate (NTP) (Yamanaka *et al.*, 1999). The NTP is the biologically active metabolite of most nucleosides responsible for inhibiting viral replication either by directly inhibiting the viral DNA or RNA polymerase or by acting as a DNA or RNA chain terminator. Nucleosides are converted to the NTP through a series of phosphorylations (Figure 2). The first phosphorylation converts the nucleoside to the nucleoside monophosphate (NMP) and is catalyzed by a nucleoside kinase. Conversion of the NMP to the NTP is catalyzed by nucleotide kinases. In the majority of cases, the poor conversion of the nucleoside to the NTP is attributed to the initial phosphorylation by the nucleoside kinase, which in contrast to nucleotide kinases, exhibits substantial intolerance to

structural variation and a more limited tissue distribution (Arner and Eriksson, 1995).

To circumvent this problem, several strategies were devised to bypass the nucleoside kinase and directly deliver the NMP (Figure 2). One strategy entailed use of certain phosphonic acids that act as NMP mimetics (De Clercq *et al.*, 1986) and are capable of entering the hepatocyte through anion transporters. A second, more widely used strategy employs prodrugs of NMPs that are cleaved intracellularly (Krise and Stella, 1996; Freeman and Ross, 1997; Schultz, 2003). In many cases, the NMP prodrugs produced higher NTP levels and improved antiviral activity in cell studies relative to the corresponding nucleoside. As will be discussed in the following sections, a few of these prodrugs also led to increased liver NTP levels and improved antiviral activity in animal studies.

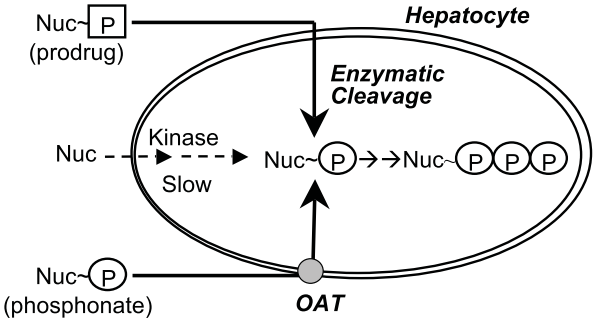


Figure 2. Strategies for bypassing the nucleoside kinase. Certain nucleosides are poorly converted to the NMP. NMP prodrugs cross the cell membrane and are cleaved intracellularly to the corresponding NMP, whereas phosphonic acid analogues of the NMP enter the cell via anion transporters.

Liver Targeting Strategies

Organ-specific drug targeting is a well recognized potential strategy for either increasing drug efficacy, improving drug safety, or both (Tomlinson, 1987). However, despite several decades of research, few drugs that depend on tissue targeting to gain a therapeutic benefit have advanced into the clinic. The most commonly used strategies rely on antibodies and other macromolecules to target the vascular bed of specific tissues through binding to tissue-specific antigens expressed on the surface of the endothelium (Arap *et al.*, 1998; Ruoslahti, 2002). Drugs conjugated to these proteins are exposed to extravascular sites if the drug-conjugate is able to cross the endothelial barrier, undergo efficient uptake by the target cell and, subsequently, cleave intracellularly to the active drug. Alternatively, the drug-conjugate could be cleaved within the vascular bed and the drug taken up by the tissue, provided that uptake is faster than washout by the bloodstream (Stella and Himmelstein, 1980; Tomlinson, 1990).

The liver may represent the organ with the greatest potential for organ-specific drug delivery in part because of its fenestrated endothelium which enables

macromolecules to pass through the endothelial barrier and directly interact with hepatocytes and other liver-specific cells (e.g., Kupffer cells, stellate cells). In addition, the liver is the organ in the body primarily responsible for drug uptake and metabolism; it therefore possesses a variety of cell surface carrier and transport proteins as well as metabolizing enzymes.

Successful targeting of drugs to the liver requires not only efficient uptake and conversion of the drug-conjugate or prodrug to the biologically active drug but also mechanisms for retaining the drug in the liver for a period of time sufficient for the drug to accumulate to pharmacologically active levels. Moreover, for liver targeting to improve drug safety, drug exposure to extrahepatic tissues must be reduced. Reduced hepatic exposure can occur through directional transport of drug conjugates or prodrugs from the circulation into the hepatocyte and then, following cleavage, excretion of the active drug via the bile. Alternatively, drug exposure can be reduced as a result of intrahepatic metabolism of the drug to an inactive metabolite following cleavage of the prodrug or drug conjugate.

In this section, the liver proteins responsible for drug uptake, metabolism, and excretion are described as well as their potential as targets for liver-specific drug delivery.

Receptors

Drugs conjugated to macromolecules that bind to liver-specific receptors deliver drugs to intracellular sites following receptor-mediated endocytosis and cleavage of the drug conjugate (Meijer and Molema, 1995). Receptors on hepatocytes include the asialoglycoprotein (AGP) receptor, as well as receptors for various proteins, including lipoproteins (LDL and HDL), insulin, epidermal growth factor, transferrin, transforming growth factor-beta, and immunoglobulins such as IgA. Kupffer cells have receptors for agalactoglycoprotein, α2-macroglobulin, fibronectin, and complement factors. Endothelial cells have receptors for agalactoglycoprotein, sulfated polysaccharides, and immune complexes as well as scavenger receptors that recognize negatively charged proteins. Activated stellate cells express the mannose 6-phosphate/insulin-like growth factor II receptor (Beljaars *et al.*, 2002).

The AGP receptor (Stockert, 1995) is the receptor most commonly exploited for liver-specific drug delivery based on its high expression levels in the liver relative to other tissues (Meijer and Molema, 1995; Wu *et al.*, 2002). Successful delivery of therapeutic drug levels using a receptor-mediated approach is dependent on a multitude of factors, including receptor expression levels in the diseased tissue, receptor internalization rate, the loading capacity of the carrier molecule, and the efficiency of drug conjugate cleavage inside the hepatocyte.

Transporters

An alternative strategy for liver-specific drug delivery targets a class of proteins called transporters that are expressed on the cell surface of liver cells.

Transporters on hepatocytes in the liver and tubular cells in the kidney facilitate uptake and excretion of charged compounds into the bile and urine, respectively (Kullak-Ublick, 1999; van Montfoort *et al.*, 2003). Several families of transporters have been identified and characterized. Most well-studied is the sodium-dependent bile acid transporter family now known as the Na^+-taurocholate cotransporting polypeptide (NCTP in humans) family, which is expressed on the basolateral surface of hepatocytes where it functions as the transporter for conjugated bile acids and sulfated steroids. Other transporter families expressed on hepatocytes and important for uptake include the sodium-independent bile acid transporters known as the organic anion-transporting polypeptides (OATPs in humans), the organic cation transporters (OCTs) (Jonker and Schinkel, 2004), and the organic anion transporters (OATs, humans) (Figure 3). OATP1 has broad substrate specificity and is associated with the uptake of conjugated and unconjugated bile acids, steroid hormones, thyroid hormone, and drugs such as enalapril and pravastatin. OCTs are important in the uptake of various quaternary amines, monoamine neurotransmitters, thiamine, and cimetidine. OATs are predominantly expressed in the kidney, except for OAT2, which is expressed in liver where it is responsible for uptake of prostaglandins, salicylate, and other negatively charged compounds.

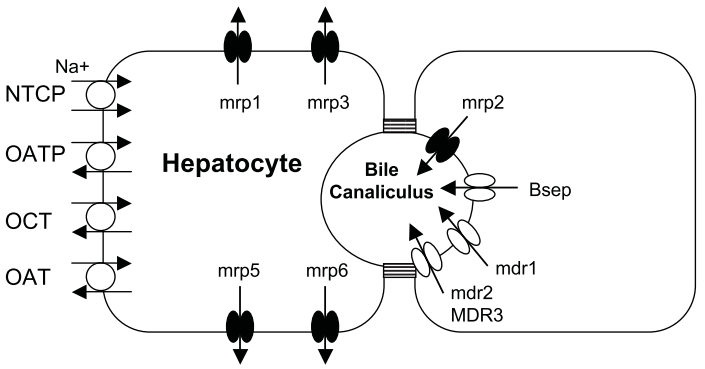


Figure 3. Transport proteins on the sinusoidal and canalicular membranes of the hepatocyte. Adapted and reprinted from Kullak-Ublick GA, Regulation of Organic Anion and Drug Transporters of the Sinusoidal Membrane. *J Hepatol* 1999; 31:563–573 with permission from The European Association for the Study of the Liver.

Negatively and positively charged compounds are effluxed from hepatocytes into either the sinusoid or the bile (Figure 3). Efflux into the sinusoid uses transporters that are bi-directional (e.g., OATs, OATPs, OCTs) whereas efflux into the bile uses transporters that belong to the *ABC* transporter superfamily and are expressed on the bile canalicular membrane. Included in this superfamily of transporters are the multidrug resistance proteins (MDR, e.g., MDR1 or P-glycoprotein) and the multidrug resistance-associated proteins (MRP, e.g., MRP2) (Kullak-Ublick *et al.* 2000).

Hepatocyte-specific drug delivery using liver transporters is achieved with drugs or prodrugs that are recognized and efficiently transported by transporters on the sinusoidal membrane (e.g., NTCP transporter). Successful delivery of therapeutic drug levels using a prodrug strategy requires the prodrug to be sufficiently stable in the gastrointestinal tract and circulation to enable uptake by the liver via the transporters. Moreover, the prodrug must undergo cleavage inside the hepatocyte at a rate faster than the rate of prodrug efflux into the circulation or the bile.

Enzymes

A third strategy for liver-specific drug delivery uses prodrugs that are cleaved by liver enzymes. Prodrugs of this type have proven to be difficult to find, presumably because they require enzymes that: 1) are expressed predominantly in the liver; 2) catalyze a reaction useful for prodrug cleavage; and 3) efficiently cleave the prodrug moiety independent of the drug. Relative to other organs, the liver expresses a large number of enzymes catalyzing a wide array of reactions as part of the liver's role in phase I and phase II biotransformations. In some cases, the enzyme is expressed predominantly in the liver (e.g., CYPs) while in other cases, a specific isoenzyme of a more widely distributed enzyme that can be exploited for liver targeting is expressed in the liver (e.g., carboxyesterase that cleaves capecitabine; Shimma *et al.*, 2000). Numerous other enzymes exist that are less liver specific (e.g., deaminases, phosphoramidases, etc.) but could be used for liver targeting if other features on the prodrug promote preferential uptake by the liver (molecular weight, charge, recognition by various liver-specific transporters, etc.).

HepDirect Prodrugs

Cytochrome P_{450}s (CYPs) are largely expressed in the liver and represent a family of oxidases with broad substrate specificities that catalyze reactions useful for prodrug cleavage. Over a dozen CYP families and 50 genes (Ingelman-Sundberg, 2002) are present in the human liver where they function as a primary oxidative metabolic pathway for a variety of endogenous substrates (e.g., testosterone, bile acids) as well as over 50% of the marketed drugs (Gibbs and Hosea, 2003). The major isozyme in humans is CYP3A4, which accounts for approximately 28% of the total CYP activity (Shimada *et al.*, 1994). CYP3A4 is expressed in the liver and to a lesser extent the small intestine (de Waziers *et al.*, 1990). Low levels (<2% of the liver) are also consistently found in the large intestine and stomach.

A prodrug strategy dependent on a CYP-catalyzed cleavage reaction was recently described and shown to result in high liver-specific drug delivery (Erion *et al.*, 2004a,b). The prodrugs, which are named HepDirect™ prodrugs, are substituted cyclic 1,3-propanyl esters of phosphates or phosphonates designed to undergo a single enzyme-catalyzed oxidative cleavage reaction. Mechanistic

studies indicated that cleavage of HepDirect prodrugs with a C4 aryl group begins with hydroxylation of the C4 methine and that the hydroxylation reaction is catalyzed predominantly by CYP3A4 in human liver microsomes (Erion *et al.*, 2004a) (Figure 4). Formation of the C4 hydroxylated product results in rapid ring opening and an intermediate that is converted to the NMP. Conversion to the NMP occurs via a base-catalyzed β-elimination reaction; however, when the NMP is a phosphate, the diester intermediate could also be hydrolyzed to the NMP intracellularly via a phosphodiesterase-catalyzed reaction.

HepDirect prodrug SAR studies showed that the C4 aryl group is essential for prodrug cleavage, most likely as a result of the increased susceptibility of benzylic hydrogens to CYP-catalyzed oxidation (Erion *et al.*, 2004a). Efficient prodrug cleavage is also highly dependent on the relative configuration between C4 and phosphorus but not on the absolute configuration at C4 or the NMP structure. Results from the kinetic studies also suggested that HepDirect prodrugs would likely undergo extensive metabolism in humans since catalytic efficiencies for prodrug cleavage were similar to or better than the efficiencies reported for most other CYP-metabolized drugs (e.g., ifosfamide).

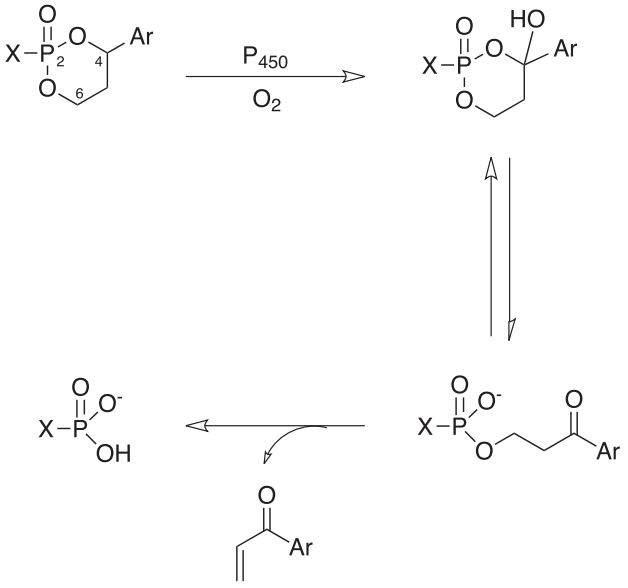


Figure 4. HepDirect prodrug cleavage mechanism and products. HepDirect prodrugs diffuse into hepatocytes and undergo a CYP3A-catalyzed oxidation. Rapid and irreversible ring-opening of the hydroxylated product leads to the intermediate monoacid, which breaksdown to the corresponding NMP and an aryl vinyl ketone. The NMP is further converted to the NTP whereas the vinyl ketone is trapped by glutathione. Adapted and reprinted from Erion MD, Liver-Targeted Drug Delivery Using HepDirect™ Prodrugs. *J Pharmacol Exp Ther* 2005; 312:554–560 with permission from The American Society for Pharmacology and Experimental Therapeutics.

Liver targeting using the HepDirect prodrug strategy was demonstrated for a structurally diverse set of NMPs. The high liver specificity was attributed in large part to the high liver specificity of CYP3A4 expression coupled with the high stability of HepDirect prodrugs in aqueous solutions, blood, and non-hepatic tissues other than the gastrointestinal tract. High prodrug stability enabled the prodrug to remain intact after administration and make multiple passes through the liver. Another important factor contributing to the high liver specificity was related to the anionic nature of the prodrug cleavage intermediates and products, which are unlikely to exit the hepatocyte via passive diffusion.

HepDirect prodrugs of NMPs achieve high liver NTP levels for the reasons cited above as well as for others attributed to the nucleoside. For example, HepDirect prodrugs of NMPs, where the corresponding nucleoside is poorly phosphorylated by intracellular nucleoside kinases (e.g., lamivudine, Erion *et al.*, 2004a; cytarabine, Erion *et al.* 2004b) generate high liver NTP targeting because the HepDirect prodrug is converted in the liver to the NMP, which effectively bypasses the nucleoside kinase and enables conversion to the NTP. Extrahepatic exposure is limited by the lack of HepDirect prodrug conversion outside of the liver and the inability of poorly phosphorylated nucleosides generated by intrahepatic dephosphorylation of the NMP to escape the liver and undergo phosphorylation in non-liver tissues. High liver specificity is also possible for HepDirect prodrugs of NMPs that undergo either increased biliary excretion or rapid intrahepatic metabolism to an inactive metabolite.

While a CYP3A4-dependent strategy for liver-specific drug delivery has many attractive features, it also is associated with several potential limitations. One concern is related to CYP3A expression in the gut and the potential for prodrug cleavage in the intestine, resulting in limited oral bioavailability and/or toxicity. While CYP3A activity in the intestine is significantly less than in the liver, its overall impact on HepDirect prodrug metabolism is still unclear. Results from animals administered the HepDirect prodrug pradefovir showed very low levels of the active drug in the intestine relative to the liver, high oral bioavailability across several species (rats, dogs, and monkeys), and no GI toxicity (Erion *et al.*, 2004b; Lin *et al.*, 2004b). Moreover, studies evaluating portal prodrug and drug levels after oral administration as well as the portal/systemic extraction ratio suggested that intestinal CYP3A plays at most a minor role in prodrug conversion (Lin *et al.*, 2004b).

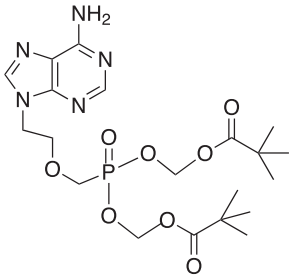
Another potential CYP3A4-related concern centers on CYP3A4 levels in diseased liver, which are often reduced in patients with liver cirrhosis (Villeneuve and Pichette, 2004) and are about half normal in primary liver tumors (Zhang *et al.*, 2000). A third concern is the potential for large intra- and interpatient differences in drug levels due to differences in CYP3A expression (Lin and Lu, 2001). Differences for CYP3A expression typically range across patients from three- to tenfold, which is substantially less than that of other CYPs (e.g., CYP2 class) (Shimada *et al.*, 1994). Moreover, minimal differences in expression are observed across gender, race, and age. The largest CYP3A-related concern that may hinder use of HepDirect prodrugs in certain diseases is the potential for

significant drug-drug interactions. Since HepDirect prodrugs are rapidly cleaved by CYP3A4 and the prodrug cleavage products are unlikely to interact with CYP3A4, the largest drug-drug interaction potential exists in combinations of HepDirect prodrugs and drugs that inhibit CYP3A4, e.g., the antifungals and the HIV drug ritonavir.

A possible non-CYP related limitation of HepDirect prodrugs containing a C4-aryl group is the generation of an aryl vinyl ketone byproduct. Vinyl ketones as a compound class are associated with significant toxicity, including both cytotoxicity and genetic toxicity (Neudecker *et al.*, 1989). Toxicity is attributed to alkylation of essential proteins and DNA. Intracellular glutathione detoxifies vinyl ketones through a 1,4-addition reaction that is rapid and quantitative in tissues associated with millimolar glutathione levels such as the liver and gut (Dinkova-Kostova *et al.*, 2001). Consequently, drugs that undergo metabolism to a highly reactive vinyl ketone in the liver (e.g., acetaminophen) exhibit good safety as long as glutathione levels remain above 0.5-1 mM (*ca*. 20% of normal liver levels) (Mitchell *et al.*, 1973). Activation of HepDirect prodrugs by CYP3A leads to the generation and retention of the ring-opened intermediate (Figure 4) inside CYP3A-containing cells, thereby confining production of the prodrug byproduct to cells that contain glutathione. Studies designed to test the acute safety of HepDirect prodrugs showed that even at high doses only a transient 25% reduction in hepatic glutathione levels was observed despite substantial prodrug turnover (Erion et al, 2004b). Moreover, unlike acetaminophen, prodrug turnover produced no evidence of liver toxicity as judged by both serum liver enzyme levels and liver histology. While the lack of liver toxicity may reflect rapid detoxification by intracellular glutathione, it may also reflect an overall lower toxicity potential of aryl vinyl ketones as suggested by results in glutathione-depleted hepatocytes treated with a HepDirect prodrug (Erion *et al.*, 2004b) and results from an embryotoxicity study with phenyl vinyl ketone (Hales *et al.*, 1989). In addition to these results, no byproduct-related toxicity has been observed to date in animal toxicology studies as well as in both in vitro and in vivo genetic toxicology studies (Lin *et al.*, 2004b; unpublished results).

Pradefovir

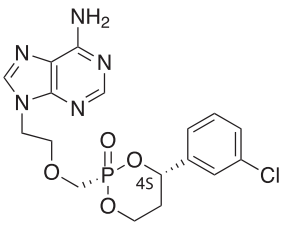
HepDirect prodrugs of the antiviral agent 9-(2-phosphonylmethoxyethyl) adenine (PMEA) were studied in an effort to find a drug candidate with improved therapeutic potential relative to the recently approved HBV drug adefovir dipivoxil (Hepsera™, Gilead Sciences) (Dando and Plosker, 2003). Adefovir dipivoxil is the bispivaloyloxymethyl (POM) prodrug of PMEA (**1**), which, unlike HepDirect prodrugs, is cleaved by esterases. Esterases are expressed throughout the body and, as a consequence, reduce adefovir dipivoxil exposure to the liver and increase PMEA exposure to extrahepatic organs. Exposure of PMEA to the kidneys is associated with kidney toxicity, which proved to be dose-limiting in Phase 3 clinical studies (Marcellin *et al.*, 2003). In one study, patients treated with a 30 mg/day dose of adefovir dipivoxil exhibited a significantly greater reduction



Structure 1. Hepsera

in HBV DNA levels than patients treated with a 10 mg/day dose. The higher dose, however, showed evidence of kidney toxicity whereas the 10 mg/day dose was devoid of toxicity and, as a result, was the dose approved by the FDA. These findings suggested that targeting PMEA to the liver using HepDirect prodrugs might, at a minimum, recapture the efficacy lost by dosing adefovir dipivoxil at the 10 mg/day dose while retaining a similar or better safety profile.

The HepDirect prodrug of PMEA, MB06866 (**2**), now known as pradefovir, was shown to target PMEA and PMEA-related metabolites to the liver relative to adefovir dipivoxil (Erion *et al.*, 2004b). Liver targeting was attributed in part to the stability of pradefovir in blood and most non-hepatic tissues. Non-hepatic tissue stability increases prodrug exposure to the liver and, consequently, its conversion to PMEA and decreases prodrug cleavage in non-hepatic tissues. The net result in rats was a large increase in the liver/kidney (12-fold) and liver/intestine (84-fold) targeting ratio for pradefovir relative to adefovir dipivoxil (Figure 5). The enhanced liver/intestine ratio is noteworthy given that the intestine is the only other organ that expresses CYP3A at appreciable levels (de Waziers et al, 1990). This enhancement was attributed in part to the lower intestinal specific activity of CYP3A relative to esterase activity, which results in less HepDirect prodrug conversion in the intestine and more prodrug entering the portal vein in transit to the liver. Liver targeting was confirmed in subsequent studies in the rat using whole body autoradiography as well as studies in male cynomolgus monkeys (Lin *et al.*, 2004b).



Structure 2. Pradefovir

The increased liver/kidney ratio was postulated to arise from differences in the route of PMEA clearance for PMEA in the circulation compared to PMEA generated in hepatocytes (Erion *et al.*, 2004b). Anionic compounds undergo both

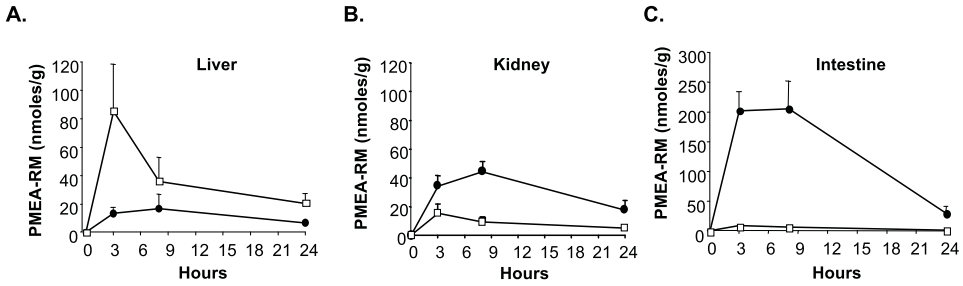


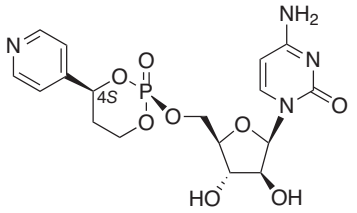
Figure 5. The concentration of radioactivity (DPM/ g tissue) in terms of PMEA-related metabolites (PMEA-RM) (PMEA + PMEAp + PMEApp, nmol/g tissue) in liver (A), kidney (B) and small intestine (C) determined after oral administration of MB06866 (□) or adefovir dipivoxil (●) to normal fasted rats at a 30 mg/kg PMEA equivalent dose. Adapted and reprinted from Erion MD, Liver-Targeted Drug Delivery Using HepDirect™ Prodrugs. *J Pharmacol Exp Ther* 2005; 312:554–560 with permission from The American Society for Pharmacology and Experimental Therapeutics.

renal and biliary clearance depending on transport efficiencies of the transporters on the basolateral and apical surfaces of hepatocytes and renal tubular cells (van Montfoort *et al.*, 2003). Because PMEA administered i.v. is cleared largely by the kidney, the increase in the liver/kidney ratio with pradefovir suggests that PMEA in the circulation has limited ability to enter the liver (de Vrueh *et al.*, 2000) and PMEA generated in the hepatocyte is effluxed into both the circulation via bi-directional anion transporters on the sinusoidal membrane and the bile via MRPs on the biliary canalicular membrane. Efflux of PMEA by MRP-4 and MRP-5 is reported to occur in cultured rat microglia (Dallas *et al.*, 2004). Biliary clearance reduces systemic exposure to PMEA since the high anionic charge of PMEA likely impedes reabsorption of PMEA transferred to the intestine from the bile and therefore enterohepatic recirculation.

Several Phase I and two 28-day proof-of-concept studies of pradefovir have been completed in HBV patients. The studies show that pradefovir is both orally bioavailable in humans and converted to PMEA. The pharmacokinetic profile of pradefovir also appears favorable based on the plasma levels of pradefovir and PMEA, the low variability in these levels, and their dose-proportionality (Chao *et al.*, 2004).

MB07133

A second HepDirect prodrug undergoing clinical evaluation is MB07133 (**3**), a HepDirect prodrug of cytarabine monophosphate (araCMP). Cytarabine (araC) is a well-known oncolytic nucleoside used for several decades to treat acute myelocytic leukemia. In leukemic cells, araC is converted to araCTP, which inhibits cell proliferation through inhibition of DNA polymerases as well as through chain termination following incorporation into the growing DNA strand. AraCTP is also produced in bone marrow cells, resulting in concomitant bone marrow toxicity. In contrast, araCTP levels are low in most other tissues, including



Structure 3. MB07133

liver and solid tumors, due in part to the low levels of the kinase that phosphorylates araC to araCMP, namely, deoxycytidine kinase (dCK) (Arner and Eriksson, 1995).

Unlike araC, MB07133 targets araCTP production to the liver and greatly reduces araCTP levels in bone marrow, where there is no CYP3A activity. High levels of araCTP are produced in the liver because the prodrug enters cells independent of nucleoside transporters and cleavage of MB07133 produces araCMP. Delivery of araCMP effectively bypasses the step limiting araCTP production from araC while simultaneously avoiding metabolism by cytidine deaminase. Cytidine deaminase is expressed at high levels in the liver and is responsible for the rapid deamination of araC to the inactive metabolite araU (Camiener and Smith, 1965). Studies in rodents comparing araC and MB07133 showed that MB07133 resulted in high araCTP levels in the liver and undetectable levels in the bone marrow while araC produced high levels of araCTP in the bone marrow and undetectable levels in the liver (Erion *et al.*, 2004b) (Figure 6). The

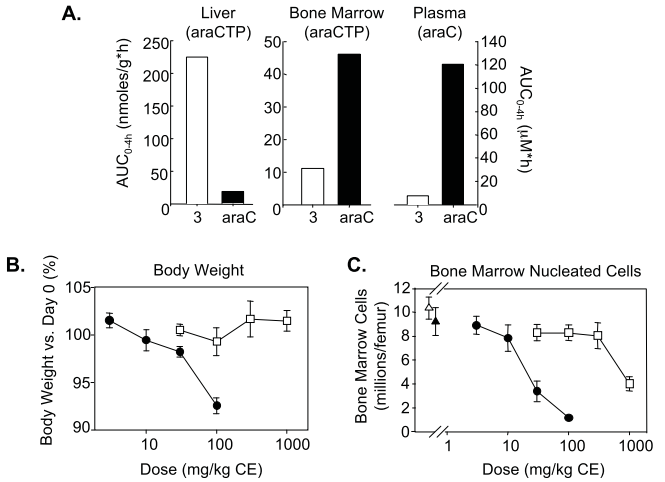


Figure 6. (*A*) Mean liver and bone marrow araCTP $AUC_{0\text{-}4h}$ and plasma araC $AUC_{0\text{-}4h}$ determined from samples collected after i.p. injection of a 100 mg/kg araC equivalent dose of MB07133 (**3**) or araC to normal mice. (*B-C*) Dose-response of HepDirect prodrug of araCMP (□) and araC (●) in male mice for body weight (*B*) and bone marrow nucleated cells (*C*) measured on day 5 following once daily dosing. Untreated (▲) and vehicle treated (△) mice. Adapted and reprinted from Erion MD, Liver-Targeted Drug Delivery Using HepDirect™ Prodrugs. *J Pharmacol Exp Ther* 2005; 312:554–560 with permission from The American Society for Pharmacology and Experimental Therapeutics.

net effect of the HepDirect prodrug was an increase of at least 45-fold in the liver/bone marrow ratio for araCTP exposure.

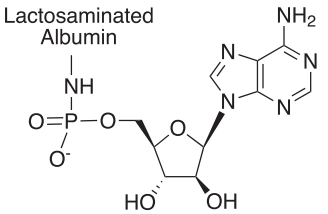
Liver targeting led to an improvement in safety as demonstrated by the decreased bone marrow suppression and death in mice treated with MB07133 relative to araC (30-fold shift in the dose-response). The finding of bone marrow suppression at high MB07133 doses correlated with the presence of araC in the plasma. AraC is presumably derived from araCMP produced in the liver that undergoes subsequent intrahepatic dephosphorylation. A portion of the araC produced inside the hepatocytes is deaminated and effluxed into the circulation as araU. The rest is presumably effluxed as araC, which at high doses of MB07133 reaches levels that are toxic to the bone marrow (Erion *et al.*, 2004b).

Prodrugs Targeting Asialoglycoprotein Receptors

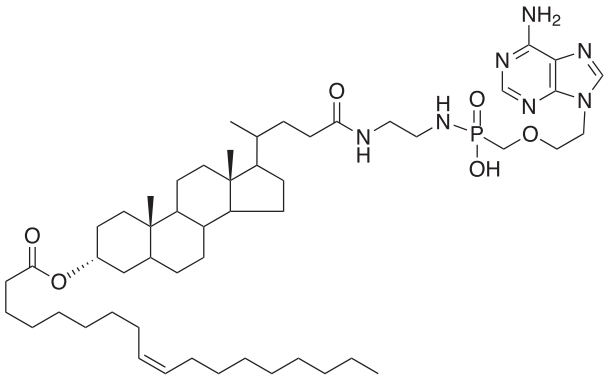
Over the past 25 years, the most common strategies for liver-specific drug delivery are based on drug carriers targeting the asialoglycoprotein (AGP) receptor (Meijer and Molema, 1995; Wu *et al.*, 2002). This receptor recognizes galactose- and N-acetyl-galactosamine-terminated glycoproteins. Most often the carriers are naturally occurring glycoproteins such as asialofetuin and asialoorsomucoid administered in their desialylated form. Alternatively, carriers can be proteins such as albumin and lipoproteins that are randomly derivatized with sugar groups to enhance recognition and uptake by the AGP receptor and avoid uptake by non-parenchymal cells via the scavenger receptor.

Glycoprotein-drug conjugates are typically made by forming an acid labile bond between the drug and a surface amino group on the protein. For example, araAMP was conjugated to lactosaminated albumin to form a drug conjugate (**4**) containing 12 molecules of araAMP connected via phosphoramide bonds. Rapid uptake by hepatocytes and transport to the lysosome putatively leads to phosphoramide bond cleavage and production of araAMP (Fiume *et al.*, 1997). Studies in mice and woodchucks demonstrated selective liver uptake and improved efficacy and/or safety relative to araA. Moreover, the conjugate was shown in human clinical trials to result in reduced viremia without causing the araA-related neurotoxic side effects (Fiume *et al.*, 1988).

A more recent example used a reconstituted lactosylated high-density lipoprotein (HDL) carrier containing PMEA conjugated with lithocholic acid-3α-oleate (**5**) (Bijsterbosch *et al.*, 2001). Studies in anesthetized rats injected with



Structure 4. araAMP Conjugate

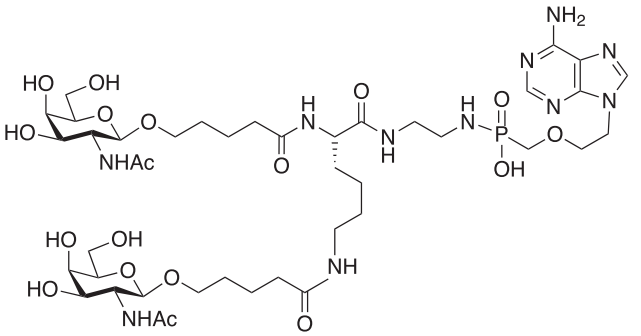


Structure 5.

[^{3}H]-labeled conjugate showed that the reconstituted carrier was rapidly taken up by the liver and, more specifically, hepatocytes (89% of the hepatic uptake) (de Vrueh *et al.*, 2000). Thirty minutes after administration of the conjugate 69% of the label was associated with the liver whereas the kidney showed less than 2%. However, analysis of cytosolic metabolites obtained from the liver 5 h after dosing showed that free PMEA and phosphorylated forms of PMEA represented only a minor fraction of the label (<5%). These results may suggest either that cleavage of the conjugate is slow or that the PMEA generated by acid-catalyzed cleavage in the lysosome is not readily transported to the cytosol. Moreover, no data were provided demonstrating that liver-specific uptake of the conjugate translated to either improved efficacy or safety relative to intravenously administered adefovir dipivoxil.

Numerous other drugs have been delivered to the liver using carriers specific for the AGP receptor, including antivirals such as AZT, trifluorothymidine, and ribavirin; antineoplastic agents such as araC, daunorubicin, doxorubicin, methotrexate, and ricin; antiparasitic agents such as allopurinol and primaquine; and various other agents, including diagnostic agents (Meijer and Molema, 1995). While high liver uptake is typically shown in these studies (usually by monitoring tissue distribution of the radiolabeled drug/drug conjugate), few of these studies demonstrated liver specificity as determined either by measuring drug levels in liver relative to non-liver tissues or by demonstrating improved efficacy and/or safety.

Since protein-based drug carriers are administered parenterally and often are associated with high manufacturing costs and poor loading capacities, various simple polymers have been explored as alternative carriers (Meijer and Molema, 1995). The polymers are usually rich in sugars recognized by the AGP receptor and ideally are biodegradable and non-immunogenic. The most commonly used polymer is poly-L-lysine, which is readily modified with galactose, lactose, or *N*-acetylgalactosamine for targeting the hepatocyte and mannose for targeting Kupffer and endothelial cells. More recently, low molecular weight bi- and trivalent cluster glycosides were reported to bind to the AGP receptor with high affinity (Biessen *et al.*, 2000). A prodrug of PMEA (**6**) produced a tenfold



Structure 6.

enhancement in liver levels relative to PMEA. Moreover, the accumulation of the PMEA prodrug in extrahepatic tissues, including kidney, was substantially reduced. Conversion of the prodrug was purportedly in the lysosome where the half-life was estimated to be approximately 100 min.

While the results generated over the past thirty years using drug conjugates of proteins, various polymers and low molecular weight glycosides targeting the AGP receptor have shown some promise, it is important to note that no AGP-targeted drug conjugate has proceeded into advanced clinical trials. Poor oral bioavailability, manufacturing difficulties, immunogenicity, and chemical instability greatly limit their potential as drug candidates (Meijer and Molema, 1995). Moreover, therapeutic drug levels depend on the expression of the AGP receptor, which is often reduced in liver diseases (e.g., primary liver tumors). Other factors that limit drug levels include the internalization rate of the AGP receptor, which is estimated to be maximally five million glycosylated particles per hour, and the intrahepatic conversion of the conjugate to the free drug. Accordingly, therapeutically successful drug delivery using the AGP receptor strategy will most likely require drugs with high potency and carriers with high loading capacities.

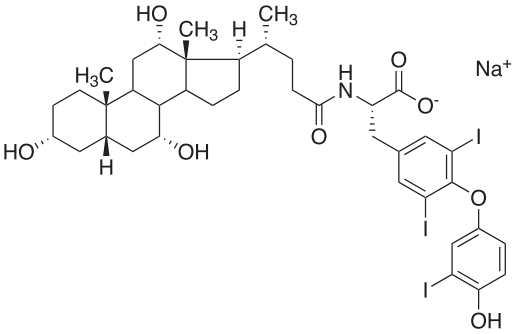
Prodrugs Targeting Bile Acid Transporters

Bile acids are acidic sterols produced by the liver to aid absorption of fats and fat-soluble vitamins. Once absorbed and present in portal blood, bile acids are rapidly taken up by hepatocytes via membrane transporters. Transport of bile acids across the sinusoidal membrane leads to accumulation in the cytosol followed by excretion into the bile through transporters on the bile canalicular membrane (Kullak-Ublick *et al.*, 2000). The bile acid transporters on the sinusoidal membrane include the NTCP (sodium-dependent) and OATP (sodium-independent) transporters. NTCP transporters are expressed exclusively in the liver although a structurally related transporter also exists in the intestine. NTCP-mediated bile acid transport is unidirectional and occurs by an active transport mechanism.

Given the liver specificity of NTCP and its high capacity for transporting bile acid conjugates, bile acid-containing prodrugs were evaluated as a potential

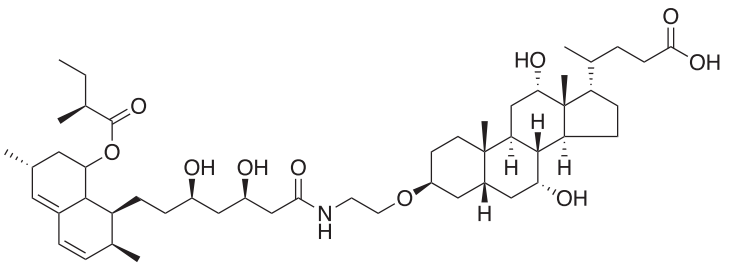
strategy for liver-specific drug delivery. The first examples were reported by Kramer *et al.* (1992). In this study, the cytostatic drug chloramucil was covalently linked via an amide bond to 7α,12α-dihydroxy-3β-(ω-aminoalkoxy)-5β-cholan-24-oic acid. Studies in hepatocytes showed that, unlike chloramucil, the prodrug inhibited taurocholate accumulation. However, despite hepatocyte uptake, no products from prodrug cleavage could be demonstrated. Intact prodrug was excreted into the bile, suggesting that the amide bond was stable in the liver and that the prodrug was a likely substrate for transporters on the bile canalicular membrane. Nevertheless, the results demonstrated that the bile acid component could affect drug distribution and elimination since chloramucil is normally eliminated via the kidneys.

Bile acids were also coupled via an amide bond to a prolyl 4-hydroxylase inhibitor (Kramer *et al.*, 1992) and the thyroid hormone L-T_3 (**7**) (Stephan *et al.*, 1992). As with chloramucil, no prodrug conversion was evident for either compound following their transport into hepatocytes. The prodrug of L-T_3, however, did show increased cholesterol lowering and reduced cardio-stimulation relative to L-T_3; this suggests that a small proportion of the prodrug may cleave in hepatocytes, resulting in liver-targeted delivery of L-T_3.



Structure 7. CGH 509A

HMG CoA reductase inhibitors were also conjugated to bile acids using an aminoethyl spacer between the carboxylate of the statin and the 7α hydroxy of the bile acid (**8**) (Petzinger *et al.*, 1995). The prodrug was shown to interact with the hepatocyte and ileocyte bile acid uptake systems. Cholesterol biosynthesis was inhibited by the prodrug in Hep G2 cells as well as in vivo one hour following intravenous administration (Kramer *et al.* 1994). The bile acid-based prodrug produced small increases in drug levels in the liver and up to tenfold lower levels in non-hepatic tissues. No data were reported demonstrating cleavage of the amide bond. Instead, the authors showed that the prodrug inhibited cholesterol synthesis in the liver but not in the small intestine whereas mevinolin inhibited both liver and intestinal cholesterol synthesis. These results provide some evidence for liver targeting, assuming that inhibition of cholesterol synthesis by the prodrug depends on prodrug cleavage.



Structure 8. S 2887

As illustrated by some of the above examples, the main difficulty encountered to date with bile acid-containing prodrugs is discovering an optimal linker between the bile acid and the drug. A linker that is resistant to cleavage (e.g., amide bond) results in excretion of the intact prodrug into the bile while a linker that is readily cleaved (e.g., ester) may fail to target the liver due to extrahepatic cleavage. Other concerns with the bile acid transporter strategy include the potential for decreased liver uptake in patients with liver diseases due to decreased expression of bile acid transporters (e.g., primary liver cancer) and the potential for significant drug-drug interactions between bile acid-containing prodrugs and drugs that use the NCTP transporter (e.g., bumetanide, furosemide, and verapamil).

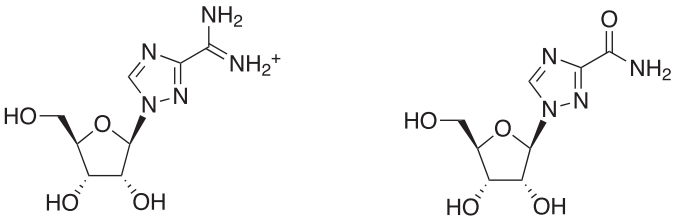
Other Prodrug Strategies

The liver contains a large number of enzymes that catalyze a wide variety of reactions. While most of these enzymes are not liver specific, liver-targeting remains possible if the prodrug distributes preferentially to the liver. Extraction of drugs by the liver is naturally high relative to other tissues as a result of the liver's high blood flow and high vessel permeability as well as its anatomical location, which requires orally administered drugs to pass through it prior to entering the systemic circulation. Additional factors can contribute to preferential liver distribution, including prodrug properties that favor liver uptake (e.g., high molecular weight, structural features recognized by liver transporters, etc.).

In this section, several prodrugs that are reported to generate the active drug primarily in the liver despite being cleaved by an enzyme expressed in both liver and non-hepatic tissues are reviewed.

Viramidine

Viramidine (**9**) is reported to be a liver-targeted prodrug of ribavirin with potential to reduce the incidence of hemolytic anemia while maintaining or enhancing antiviral activity (Lin *et al.*, 2003). Ribavirin (**10**) is a marketed nucleoside used in combination with interferon (INF)-α or pegylated INFα to treat patients with HCV. The pharmacokinetic profile of ribavirin exhibits rapid absorption and distribution throughout the body (Ferrara *et al.*, 1981). Ribavirin



Structures 9 and 10. (9) Viramidine and (10) Ribavirin

forms phosphorylated metabolites in most tissues, resulting in a large intracellular pool and a prolonged elimination phase ($t_{1/2}$ = 79 h) (Glue, 1999). In the red blood cell (RBC), the absence of phosphatases to dephosphorylate the phosphorylated metabolites leads to a long intracellular half-life (*ca*. 40 days) and to accumulated levels that result in dose-limiting hemolytic anemia in a significant proportion of patients.

Viramidine is converted to ribavirin by adenosine deaminase (ADA) (Wu *et al.*, 2003), an enzyme ubiquitously distributed throughout mammalian tissues, including liver and RBCs (Van der Weyden and Kelly, 1976). Rats treated with a single dose of [^{14}C]-viramidine showed slightly lower levels of ribavirin and phosphorylated metabolites of ribavirin in liver compared to rats treated with [^{14}C]-ribavirin. A slightly greater decrease in these metabolites was observed in RBCs, suggesting at most twofold liver targeting for viramidine in the rat (Lin *et al.*, 2004a). Greater liver targeting was observed in the monkey following 10 days of drug administration (Lin *et al.*, 2004a). In this study, radioactivity was threefold higher in the liver and twofold lower in RBCs in monkeys treated with viramidine relative to monkeys treated with ribavirin. Analysis of the metabolic profiles showed that a large proportion of the increase in the radioactivity in the liver was due to higher concentrations of the deribosylated ribavirin and the presence of viramidine and phosphorylated forms of viramidine. Nevertheless, even with these differences, ribavirin triphosphate appeared to be increased by approximately 50% in the liver and decreased twofold in the RBC. The lower RTP levels in the RBC correspond to the decreased haematopoetic toxicity found in a 28-day monkey study (Lin *et al.*, 2003), which, if replicated in humans, may result in a significant reduction in the incidence of anemia in patients treated with INF and viramidine.

The mechanism for the improved liver targeting of viramidine in the monkey is unknown, but may be related to differences in drug distribution and subsequent metabolism. Ribavirin enters the RBC via the nitrobenzylthioinosine-sensitive nucleoside transporter (Glue, 1999). No phosphorylated viramidine metabolites are observed in the RBCs in rats or monkeys, so either RBCs fail to take up and/or phosphorylate viramidine or viramidine is rapidly deaminated in the RBC. Accordingly, the liver targeting that is observed in the monkeys is postulated by the authors to possibly arise from the reduced distribution of viramidine into the RBC and, consequently, the increased availability of viramidine to the liver (Lin *et*

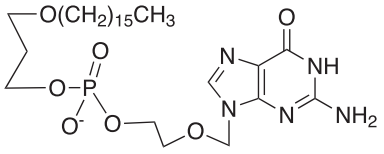
al., 2004a). The reduced RBC distribution may be related to viramidine's positively charged amidine, which could hinder uptake by the RBC via the nucleoside transporter while enhancing uptake by the hepatocyte via OCTs. Interestingly, OCTs are primarily expressed in the liver in humans while more widely distributed in rodents (Jonker and Schinkel, 2004).

Phospholipid Prodrugs

A series of phospholipid prodrugs discovered by Hostetler was used for targeting certain nucleosides to the liver. Prodrug cleavage is reported to involve a multistep process involving enzymes expressed in the liver as well as other tissues. Based on studies in CEM cells exposed to phosphatidyl azidothymidine (AZT), prodrug cleavage begins with two deacylations catalyzed by phospholipase A and lysophospholipase (Hostetler et al, 1991). The intermediate, glycerol-3-phospho AZT, is subsequently cleaved by cellular phosphodiesterases to produce the NMP, which in turn is converted to the corresponding NTP.

Liver targeting is reported for phospholipid prodrugs of several nucleoside antivirals. In the first study, tritiated dioleoylphosphatidyl dideoxycytidine was administered in liposomes intraperitoneally to mice and found to produce 40-fold higher drug levels than dideoxycytidine in the liver based on liver tritium levels over a 24-h period (Hostetler *et al.*, 1994). No increase in tritium levels was found in the sciatic nerve, which is the target tissue of ddC-related toxicity. In the second study, 1,2-dipalmitoylphosphatidyl dideoxyguanosine was administered in liposomes (i.p.) to woodchucks experimentally infected with the woodchuck hepatitis virus (WHV) and shown to result in 23- to 46-fold reduced WHV DNA levels after 4 weeks of treatment (Korba *et al.*, 1996). Liver targeting was inferred from the antiviral activity of dideoxyguanosine, which reduced WHV DNA levels only 2.2- to 10.4-fold. More recently, liver targeting was reported for a prodrug of acyclovir, namely 1-*O*-hexadecylpropanediol-3-*P*-acyclovir (**11**), which showed a 95% reduction in WHV DNA whereas acyclovir had no effect at a 5.3-fold higher molar dose (Hostetler *et al.*, 2000).

The magnitude of liver targeting achieved by phospholipid prodrugs of nucleosides is not entirely clear from the above work, especially since tissue NTP levels and plasma nucleoside levels were not reported. Moreover, the prodrug class is reported to be effective against viruses (e.g., CMV, HIV and HSV) that infect non-hepatic cells suggesting either that the prodrug distributes to and is activated by other tissues or that the nucleoside generated following prodrug



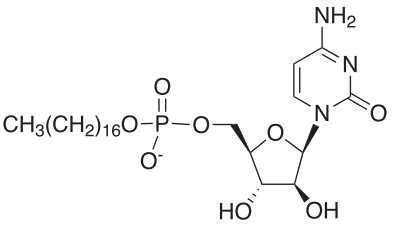
Structure 11. HDP-P-acyclovir

activation leaks out of the liver and into non-hepatic tissues. The mechanism of liver targeting is also unclear. The enzymes involved in prodrug activation are distributed throughout the body. Some liver targeting likely arises from administration of the prodrugs in liposomes, since liposomes are known to distribute to the liver. Hepatocytes take up liposomes if their size is less than 100 nm whereas larger liposomes are preferentially taken up by endothelial cells and Kupffer cells through the scavenger receptor (Meijer and Molema, 1995). Alternatively, the negatively charged phospholipid may enable preferential uptake by hepatocytes via organic anion transporters. Once inside the hepatocytes, kinase bypass is achieved upon generation of the glycerol-3-phosphonucleoside since hepatocytes contain phosphodiesterases (PDEs), which readily cleave most phosphate diesters. Cleavage could occur at either ester to produce the NMP and/or the corresponding nucleoside with production of the latter limiting NTP production and possibly compromising liver targeting.

YNK-01

The prodrug of araCMP, YNK-01 (**12**), concentrates in the liver following oral administration, possibly as a result of its amphophilic character (Suto *et al.*, 1997). Prodrug activation is reported to entail an initial CYP-mediated oxidation of the *w*-methyl of the stearate chain (Yoshida *et al.*, 1990). The resulting carboxylate is then degraded in 2-carbon units through peroxisome-mediated β-oxidation. The shorter alkyl chain esters subsequently undergo PDE-catalyzed cleavage, which likely produces both araCMP and araC, depending on which P-O-C bond is hydrolyzed.

Initial interest in YNK-01 was as an araC prodrug capable of producing high levels of plasma araC following oral administration (Kuhr *et al.*, 2000). Unlike araC, YNK-01 is not a substrate for cytidine deaminase, an enzyme that rapidly deaminates and inactivates araC. Prodrug cleavage led to sustained production of araC and to efficacy in patients with acute and chronic myelogenous leukemia along with simultaneous araC-related toxicities (myelosuppression, gastrointestinal toxicity) (Kuhr *et al.*, 2000). YNK-01 was also studied in patients with hepatocellular carcinoma based on knowledge that YNK-01 cleaves in the liver to araCMP, which might produce high levels of araCTP in the tumor by bypassing dCK. An initial clinical study using YNK-01 gave promising results (Suto *et al.*, 1997), which were not confirmed in a subsequent study, possibly because of dose-limiting araC-related side effects.



Structure 12. YNK-01

Summary

Chronic liver diseases remain poorly treated with current therapies and would greatly benefit from prodrugs capable of targeting drugs to the liver. Liver targeting of certain drugs, especially nucleosides active against viral hepatitis, has been demonstrated largely using strategies that capitalize on proteins expressed predominantly in the liver. In many cases, liver targeting was achieved through liver-selective uptake by receptors (e.g., AGP receptor) and transporters (e.g., bile acid transporter) expressed predominantly on the sinusoidal membrane of the hepatocyte. More recently, a series of CYP3A-activated prodrugs, termed HepDirect, were described and shown to target the liver by capitalizing on the high expression of CYP3A in the liver and the ability of the hepatocyte to retain the prodrug cleavage intermediates. Using the HepDirect prodrug strategy, the antiviral PMEA and an oncolytic nucleoside araC were targeted to the liver as a means to improve both their efficacy and safety. Both drug candidates are currently undergoing human clinical trials with results expected over the next few years that could demonstrate the therapeutic benefits of liver targeting.

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2.5.6

Prodrug Approaches for Drug Delivery to the Brain

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Table of Contents

List of Abbreviations

ADA .....Adenosine deaminase
ADP .....Adenosine diphosphate
AIDS .....Acquired immunodeficiency syndrome
AMPA...(S)-2-Amido-3-(3-hydroxy-5-methylisoxazole-4-yl) propionic Acid
APD .....9-(β-D-1,3-Dioxolan-4-yl)-2 aminopurine
AUC.....Area under the curve
AZT .....Zidovudine
BBB.....Blood-brain barrier
CDS.....Chemical delivery system
CLin.....Influx clearance
CL out.....Efflux clearance
CNS.....Central nervous system
CSF .....Cerebrospinal fluid
DCHQC .....6,7-dichloro-3,4-dihydro-3-oxo-2-quinoxalinecarboxylic acid
DCKA.....5,7-Dichlorokynurenic acid
ddA.....2',3'-Dideoxyadenosine
ddC .....2',3'-Dideoxycytidine
ddG .....2',3'-Dideoxyguanosine
ddI.....2',3'-Dideoxyinosine
ddP .....Dideoxypurine
DPD .....Dihydropyrimidine dehydrogenase
DPDPE.....[D-Pen2, D-Pen5]-enkephalin
DXG .....9-(β-D-1,3-dioxolan-4-yl) guanine
(+)-EHNA .....erythro-9-(2-hydroxy-3-nonyl) adenine
GABA.....γ-Aminobutyric acid
GDEPT .....Gene-directed enzyme prodrug therapy
GLUT1 .....Glucose transporter
GSH.....Glutathione
F-ddA.....2'-Fluoro-2',3'-dideoxyadenosine
F-ddI.....2'-Fluoro-2',3'-dideoxyinosine
5-FC 5-Fluorocytosine
5-FU 5-Fluorouracil
HIV .....Human immunodeficiency virus
HPLC .....High-performance liquid chromatography
K7DA.....[Lys7] dermorphin
LAT1 .....Large amino acid transporter
L-dopa .....β-(3,4-Dihydroxyphenyl)-L-alanine
MAO .....Monoamine oxidase
MP.....Monophosphate
mRNA.....Messenger ribonucleic acid
MRP .....Multidrug resistance-associated protein
MTIC .....5-(3-Methyl-triazen-1-yl) imidazole-4-carboxamide
NADH .....Nicotinamide adenine dinucleotide
NADPH .....Nicotinamide adenine dinucleotide phosphate

NMDA .....N-Methyl-D-aspartate
NSAID.....Non-steroidal anti-inflammatory drugs
OAT.....Organic anion transporter
OATP.....Organic anion transporter polypeptide
OX26.....Monoclonal antibody to the rat transferrin receptor
P-gp .....P-glycoprotein
RT.....Reverse transcriptase
SATE.....S-acyl-2-thioethyl
TP.....Thymidine phosphorylase
TRH .....Thyrotropin-releasing hormone

Introduction
The Multifunctional Challenge of the Blood-Brain Barrier

The microvasculature of the human brain consists of approximately 400 miles of capillaries with a surface area of about 100 square feet (Pardridge, 1991). Because the capillary network in the brain is so extensive, Pardridge has suggested that "nearly every neuron is virtually perfused by its own microvessel" (Pardridge, 2002). Yet, despite this extensive surface area and the fact that the blood-brain barrier (BBB) is comprised of a cell monolayer having a thickness of only 200–300 nm, the impermeability of this barrier is widely acknowledged, and methods to circumvent it have been the subject of many reviews (Rapoport, 1976; Rapoport *et al.*, 1979; Fenstermacher and Rapoport, 1984; Neuwelt, 1989; Pardridge, 1991, 1993, 2001; Bradbury, 1992; Drewes and Betz, 1993; Anderson, 1996).

The prodrug approach continues to be one of the most rewarding avenues for overcoming the blood-brain barrier, but the challenges imposed by the BBB seem to have increased as appreciation for the multifunctional nature of the barrier has evolved. The BBB is no longer viewed as simply a passive permeability barrier but as a well-organized dynamic interface that actively and selectively regulates both the uptake of molecules from the blood into the brain and efflux from the brain parenchyma back into the systemic circulation (Tamai and Tsuji, 1996, 2000). For a prodrug to be successful in improving brain delivery it may need to penetrate a complex gauntlet comprised of the physical barrier imposed by the endothelial cell lipid bilayer membrane, the enzymatic blood-brain barrier that may result in premature prodrug and/or drug metabolism during passage through the endothelial cell, and the active resistance provided by various efflux transport processes designed to shuttle molecules from the brain back into the blood. This complex gauntlet is the first topic of this chapter.

It may be helpful to keep in mind that while the primary role of the BBB is a protective one, knowledge of these protective mechanisms may also present the prodrug design chemist with potential ideas for enhancing drug delivery by using the same processes. For example, the exploitation of specific metabolic enzymes within the blood-brain barrier may provide a means for site-selective BBB prodrug bioconversion. Transporters residing within the capillary lumen may present opportunities for enhancing drug uptake using transporter substrates as promoieties.

The Passive Permeability Barrier

The brain microvasculature differs from that in peripheral organs in that BBB endothelial cells are joined by highly resistant tight junctions that block paracellular diffusion (Reese and Karnovsky, 1967; Brightman and Reese, 1969; Brightman, 1977). Tight junctions not only preclude paracellular transport but also maintain the polarity of active transport processes by restricting lateral diffusion of proteins in plasma membrane lipid bilayers, thereby preserving the asymmetric distributions of membrane-bound enzymes and transporters within

the endothelial cell (Betz *et al.*, 1980; Brightman and Tao-Cheng, 1993). Unlike peripheral capillaries, BBB capillaries also exhibit minimal pinocytosis (Rapoport, 1976), leaving transcellular diffusion across the lipid bilayers of the endothelial cell as the only passive route available.

The Enzymatic Blood-Brain Barrier

A wide variety of metabolic enzymes are localized within capillary endothelial cells, creating an enzymatic blood-brain barrier. Alkaline phosphatase (Gomori, 1941; Djuricic and Mrsulja, 1977) and γ-glutamyl transpeptidase (Djuricic and Mrsulja, 1977; Dallaire *et al.*, 1991) are among the most enriched in capillary endothelial cells and are, therefore, often used as marker enzymes for microvessels. An example of the role of capillary enzymes in contributing to an enzymatic barrier for drug molecules is the combined action of aromatic L-amino acid decarboxylase, catechol-O-methyltransferase, and monoamine oxidase on the fate of neurotransmitter amines or their precursors such as L-dopa (Bertler *et al.*, 1966; Hardebo and Owman, 1979, 1980; Hardebo *et al.*, 1979, 1980). Important enzymes that are localized in cerebral capillaries include various esterases and peptidases (Djuricic and Mrsulja, 1977; Brecher *et al.*, 1978; Brownlees and Williams, 1993), oxidases and reductases (Ghersi-Egea *et al.*, 1988, 1994; Minn *et al.*, 1991), drug-conjugating enzymes (Ghersi-Egea *et al.*, 1988, 1994; Minn *et al.*, 1991), and enzymes involved in purine metabolism (Betz, 1985; Mistry and Drummond, 1986; Schrader *et al.*, 1987; Moriwaki *et al.*, 1999).

The Active Efflux Barrier

There is growing appreciation for the importance of active efflux transport as a significant component of the BBB. P-glycoprotein (P-gp) (Schinkel and Jonker, 2003) has become the prototype representative of the BBB efflux transporters following early evidence for the gene encoding for P-glycoprotein in endothelial cells of the blood-brain barrier (Cordon-Cardo *et al.*, 1989) and the finding that P-glycoprotein is predominantly expressed in brain capillaries (Jette and Beliveau, 1993; Beaulieu *et al.*, 1996, 1997). The development of a mouse strain lacking the mdr1a gene encoding for P-glycoprotein provided the most convincing evidence for the important role of P-gp-mediated efflux at the blood-brain barrier (Schinkel *et al.*, 1994, 1996; Kim *et al.*, 1998).

The identification and determination of the functional role of efflux transporters at the BBB is currently a highly active area of investigation (Lee *et al.*, 2001; Sun *et al.*, 2003). In addition to P-gp, other efflux transporters that may contribute significantly to the blood-brain barrier include the multidrug resistance-associated proteins (MRPs) (Huai-Yun *et al.*, 1998; Regina *et al.*, 1998; Seetharaman *et al.*, 1998; Zhang *et al.*, 2000; Lee *et al.*, 2001; Kusuhara and Sugiyama, 2002) and various organic anion transporters (OATs) (Kusuhara *et al.*, 1998; Sugiyama *et al.*, 1999; Gao *et al.*, 2000; Lee *et al.*, 2001; Ohtsuki *et al.*, 2002; Eraly *et al.*, 2003; Hasegawa *et al.*, 2003; Kikuchi *et al.*, 2003; Mori *et al.*, 2003; Ohtsuki *et al.*, 2003; Sun *et al.*, 2003).

Prodrug Design for CNS Delivery— A Multi-Dimensional Design Problem

A typical problem drug candidate having poor CNS uptake is a polar molecule. For such a molecule, instinct would guide one to assume that access to the brain is the principal problem to be solved via the prodrug approach. This can usually be accomplished quite readily by reversibly modifying the polar molecule to mask one or more polar functional groups using promoieties that render the overall compound more lipophilic. Yet, despite the fact that improved prodrug access generally results from such modifications, seldom will such a one-dimensional approach lead to a successful therapeutic agent, nor will such an approach be likely to provide enhanced concentrations of parent drug in the brain.

The difficulty is that multiple criteria must be satisfied to achieve site-specific drug delivery to the central nervous system or, for that matter, to any organ. The unique barrier properties of the blood-brain barrier makes the challenge more daunting in some respects, although in certain cases the impermeability of this barrier offers the potential of exquisite site-selectivity if this barrier can be surmounted to deliver a drug which is then trapped on the brain side of the barrier. The general criteria for targeted drug delivery have been outlined in many reviews (Stella and Himmelstein, 1980, 1982, 1985; Tomlinson, 1987; Boddy *et al.*, 1989; Anderson, 1996; Pardridge, 2001, 2002) and elsewhere in this book. These general criteria are (a) prodrug access to the intended site of action; (b) selective bioconversion at the site of action; and (c) prolonged retention within the target. Not all of the criteria must be satisfied in a single prodrug to achieve an improvement in CNS delivery, however, as examples below will demonstrate.

The Classical Prodrug Approach: Lipophilic Esters

Esterase-Activated Prodrugs

Historically the ester bond has been the predominant linkage utilized in prodrug design for CNS delivery and it is likely to be the initial choice considered by the prodrug chemist for several reasons: (1) ester formation results in the masking of a polar hydroxyl or anionic carboxyl residue, thus increasing the lipophilicity and passive membrane permeability of the parent drug; (2) esters are relatively stable in solid formulations in the absence of water and can be stabilized in aqueous solution via pH adjustment and other techniques; and (3) esterases are assumed to be ubiquitous in tissue so that it is reasonable to assume that bioconversion will occur readily after prodrug administration.

The carboxylesterases (EC 3.1.1.1) are representative of a broader class of hydrolases designated as class 3 enzymes by the International Union of Pure and Applied Chemistry. They are widely distributed in various tissues, with high levels in the liver, kidney, testis, lung, and plasma (Rooseboom *et al.*, 2004). Because of the widespread distribution of carboxylesterases in various tissues and, as later

evidence will show, relatively low activity in brain tissue, it would appear to make no sense to use esters to target drugs to the CNS. Yet, some esterase-activated prodrugs have proven to successfully enhance drug concentrations in the brain. Success is more likely when the parent compound exhibits negligible CNS uptake—then the challenge is not so much a matter of targeting the drug to the brain but simply reducing selectivity in tissue uptake so that brain uptake is no longer negligible.

An important, yet frequently ignored consideration in the design of esterase-activated prodrugs and in the selection of animal models for their evaluation is the fact that species-to-species differences in esterase activity are significant. Generally, nonspecific esterase activity decreases progressively from mouse<rat<rabbit<dog<human (Krisch, 1971; Morikawa *et al.*, 1976; Dixon and Webb, 1979; Quon *et al.*, 1988; Minagawa *et al.*, 1995). Not surprisingly, the monkey appears to be the best animal model for predicting esterase activity in humans (Cook *et al.*, 1995).

Parent Drug Hydrophilicity/Lock-In Potential

Is there an ideal parent candidate for the classical lipophilic prodrug approach? Despite the numerous attempts to improve CNS delivery via ester formation, the success of this strategy has been decidedly mixed. While many factors are involved in the design of a successful esterase-activated prodrug, the odds are improved if the parent drug is an appropriate candidate for this approach. The ideal parent drug candidate is one that exhibits poor permeability across the blood-brain barrier in both directions and therefore has the potential for being "locked in" brain tissue once it is formed. If this criterion is met, then the prodrug need only access the brain tissue and undergo conversion to the parent drug. Selectivity in terms of the bioconversion in brain tissue may not be necessary.

Nipecotic acid prodrugs serve as useful examples of the potential advantages of simple ester formation in improving CNS uptake for what would appear to be the right type of drug candidate. Nipecotic acid (Figure 1) is a potent inhibitor *in vitro* of neuronal and glial uptake of γ-amino butyric acid (GABA), a major inhibitory neurotransmitter in many brain regions. With pKa values of 3.86 and 10.28 for its carboxyl and amino substituents (Krogsgaard-Larsen and Johnston, 1975), nipecotic acid is zwitterionic at physiological pH and therefore devoid of activity *in vivo* due to its negligible penetration across the BBB (Lodge *et al.*, 1977; Frey *et al.*, 1979; Wang, 2003).

Figure 1 describes the situation for a polar, hydrophilic drug such as nipecotic acid with respect to CNS delivery and illustrates the potential advantage afforded by even simple esters if they are able to cross the BBB and undergo conversion in brain tissue. If the efflux of nipecotic acid from the brain tissue mirrors its uptake, any prodrug that enters the brain and undergoes bioconversion to the parent drug within brain tissue will produce nipecotic acid that is "locked-in" due to its inability

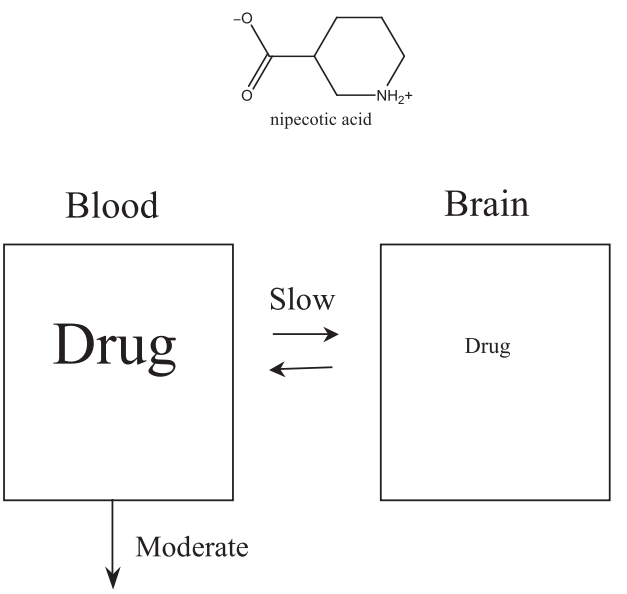


Figure 1. Illustration of the limited CNS access expected for a polar hydrophilic molecule such as nipecotic acid after an intravenous bolus injection.

to passively diffuse across the BBB. Parent compounds of this type are excellent candidates for simple lipophilic ester prodrugs.

Nipecotic acid lacks a strong UV-absorbing chromophore, which makes more difficult the generation of pharmacokinetic information that can be utilized to interpret the pharmacodynamic activity. Consequently, the earliest studies of prodrugs of nipecotic acid relied on anticonvulsant activities in mice or *in vitro* measurements of inhibition of GABA uptake into mouse whole-brain minislices (Crider *et al.*, 1982, 1984; Hinko *et al.*, 1984, 1988). Without bioconversion data, confusion arose in these early studies as to whether or not the anticonvulsant activities observed *in vivo* were due to the intact esters or to brain tissue esterase activation (Shek, 1994). Clearly, it would be important to the drug design chemist to know if bioconversion to nipecotic acid was a requirement or not.

Onset times for bicuculline-induced convulsions (in mice) versus the logarithms of nipecotic acid prodrug bioconversion $t_{1/2}$ in serum peaked at a $t_{1/2}$ (calculated for 100% serum) of ~10 min or less (Altomare *et al.*, 1988), implying that bioconversion is indeed a prerequisite for a pharmacological effect and that there is an optimum lability for activity. Such an optimum lability is anticipated if the prodrugs are themselves inactive. Extremely rapid bioconversion would produce the impermeable nipecotic acid in the bloodstream prior to CNS entry while too slow bioconversion would not provide therapeutic concentrations of the active drug in the brain as the prodrug conversion rate in brain tissue must be sufficient to compete with the overall clearance of the prodrug from the systemic circulation. Without pharmacokinetic data and quantitative modeling of the transport and conversion kinetics, one cannot design an optimal prodrug.

Ester Lability Design

Altomare *et al.* (1988) tried to address the conversion issue for prodrugs of nipecotic acid by comparing the serum hydrolysis rates of a series of substituted X-phenyl esters with their anticonvulsant activity *in vivo*. They also developed quantitative structure-activity relationships to predict prodrug hydrolysis rates in buffer and in 10% human serum. Values for log k_{OH} at 37°C and pH 7.4 were correlated (r = 0.953) with the Hammett σ^- substituent constant according to Eq. (1):

$$\log k_{OH} = 0.87(\pm 0.15)\sigma^- + 3.51(\pm 0.08)$$

Equation 1.

Ester hydrolysis rates in 10% human serum at pH 7.4 and 37°C were approximately an order of magnitude higher and could also be correlated with σ^- yielding a similar slope (0.82 ± 0.28, r = 0.853). This suggests that the sensitivity of the esterase-catalyzed reaction to electronic effects of various substituents in the nipecotic esters closely resembles the sensitivity found for nucleophilic attack by hydroxide ion. However, an improved correlation for the serum-catalyzed hydrolysis (r = 0.953) was obtained when the Hansch hydrophobic substituent constant π was included (Eq. (2)), indicating that interaction of the ester with a hydrophobic region of the enzyme also has an impact.

$$\log t_{1/2} = -0.99(\pm 0.18)\sigma^- - 0.21(\pm 0.09)\pi + 2.25(\pm 0.10)$$

Equation 2.

Although steric effects were probably similar in the above series, they also play a major role in enzymatic hydrolysis reactions. Buchwald and Bodor (1999) recently found that the inaccessible solid angle around the ester carbonyl oxygen ($\Omega_h^{O=}$), an indicator of steric effects, proved to be the most relevant parameter for estimating human blood hydrolysis rates of prodrugs and other ester-containing drugs. The overall equation they employed, shown below in Eq. (3), also included the electronic charge on the carbonyl carbon ($q_{C=}$) and the overall lipophilicity as obtained from the calculated log of the octanol/water partition coefficient of the compound (QlogP) as additional important parameters.

$$\log t_{1/2} = -3.805 + 0.172\Omega_k^{O=} - 10.146q_{C=} + 0.112Q\log P$$

Equation 3.

Wang (2003) (see also Wang *et al*., 2005) compared three simple ester prodrugs (ethyl, butyl, and neopentyl esters) for their ability to enhance brain uptake of nipecotic acid and monitored the pharmacokinetics and brain distribution of the intact prodrug and nipecotic acid formed after intravenous and intranasal administration of the butyl ester. The half-lives for hydrolysis of the esters in whole rat blood were extremely short ($t_{1/2}$ = 3.1 min (ethyl ester), 4.7 min (butyl ester), and 8.5 min (neopentyl ester) and, consistent with this finding, the half-life for the butyl ester *in vivo* (rats) was < 5 min. For the butyl ester, Wang estimated

V_{max} and K_m in brain homogenate to be 47 ± 31 ng/min/g with a K_m of 3.7 μg/g, yielding a V_{max}/K_m value of 0.013 min^{-1} and $t_{1/2}$ of ~55 min, > tenfold slower than the bioconversion of the butyl ester in blood. Thus, the butyl ester fell far short of meeting criterion (b), selective bioconversion in the brain tissue. Yet, despite these very short half-lives in blood and the unfavorable brain/blood bioconversion rates, the brain/blood ratios of nipecotic acid produced at 10 min were 0.84, 1.17, and 0.87, respectively, for the ethyl, butyl, and neopentyl esters indicating substantial improvement in brain delivery. Wang suggested that the hydrolysis rate in brain tissue was the limiting factor governing nipecotic acid delivery to the brain via the prodrug approach.

Intravenous self-administration of a prodrug would be an inconvenient option for a patient suffering from impending seizures, chronic pain and/or anxiety. Given the systemic lability of the prodrugs described above and the high levels of carboxylesterase activity in the liver and intestinal tissue, however, oral administration would probably deliver only the parent compound to the circulation. Therefore, Wang compared the brain uptake of nipecotic acid following intravenous delivery with that following nasal delivery. Figure 2 shows the brain concentrations of nipecotic acid following nasal and intravenous administration of nipecotic acid (10 mg/kg) or butyl nipecotate (4.7 mg/kg nipecotic acid equivalents). The AUCs of nipecotic acid in brain were comparable after intravenous and intranasal administration and dramatically improved when compared to nipecotic acid itself. Although the butyl ester could no longer be detected in brain tissue after 10 min, the author concluded that this was not necessarily the result of efficient ester hydrolysis but, rather, due to the rapid disappearance of the ester from brain tissue, primarily by back-diffusion into blood.

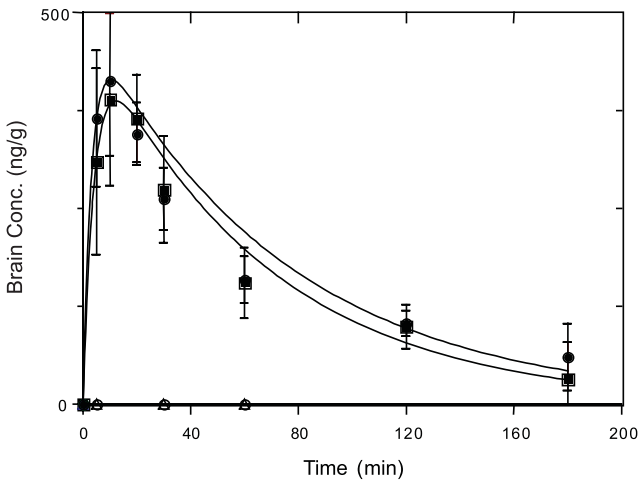


Figure 2. Brain concentrations of nipecotic acid following nasal and intravenous administration of nipecotic acid (10 mg/kg) or butyl nipecotate (4.7 mg/kg nipecotic acid equivalents) (Wang, 2003) (see also Wang *et al*., 2005). Key: ○, intravenous nipecotic acid; △, intranasal nipecotic acid; ●, intravenous prodrug; ■, intranasal prodrug.

The half-life for elimination of nipecotic acid from the brain was determined to be ~80 min, consistent with this drug being "locked-in" to some extent once formed in brain tissue. However, given the zwitterionic nature of this drug and its poor passive membrane permeability a longer retention in brain tissue would have been expected (see "Overcoming Efflux Transport").

Bioconversion Selectivity

The above example illustrates that simple esterase-activated prodrugs may be effective in delivering certain drugs to the brain, provided that the parent drugs are poorly able to permeate and therefore "locked-in" the brain tissue compartment to some degree once formed. However, the difficulty of selecting a pro-moiety having optimal bioconversion rates in both brain tissue and the systemic circulation remains a significant obstacle even for hydrophilic candidates. Very rapid hydrolysis in the systemic circulation will provide insufficient time for prodrug to enter the CNS while very slow biotransformation in brain tissue may lead to insufficient steady-state levels of drug in the brain and, therefore, limited CNS activity. The bioconversion rates of prodrugs are determined to some extent by the properties of the parent compound.

Low brain tissue esterase activity. A recurring theme in the design of esterase-activated prodrugs to enhance brain/plasma concentration ratios has been the relatively low activity of non-specific esterases in brain tissue in comparison to liver and blood. Anderson *et al.* (Anderson *et al.*, 1992; Morgan *et al.*, 1992) evaluated the bioconversion rates of a series of 5'-esters of 2',3'-dideoxyinosine in both rat plasma and brain tissue homogenate (Table 1) . All of the compounds exhibited rapid hydrolysis in plasma but the brain tissue/plasma hydrolysis rates were generally well below 1.0. *In vivo* studies in rats (Anderson *et al.*, 1990a, 1992)

Prodrug	O/W PC	t1/2 (brain) (min)	t1/2 (plasma) (min)	Brain/Plasma Bioconversion Rate Ratio	Steady-state Enhancement Factor*
5'-Acetate	0.125	1.2	0.4	0.33	-
5'-Isobutyrate	1.04	1.2	0.1	0.083	-
5'-Butyrate	1.29	1.6	0.04	0.025	1.0
5'-Pivalate	4	8.8	0.65	0.071	3.1
5'-Valerate	4.5	1.8	0.03	0.017	-
5'-Benzoate	4.6	20	0.4	0.02	-

Table 1. Properties of various 5'-esters of 2',3'-dideoxyinosine. (The low brain tissue/plasma bioconversion rate ratios render these prodrugs less effective in delivering ddI to the brain).

*Steady-state ratio of ddI_{brain}/ddI_{plasma} from prodrug vs. from intravenous ddI.

revealed no difference in steady-state brain concentrations of 2',3'-dideoxyinosine (ddI) after infusions of the 5'-butyrate in comparison to ddI alone and only a modest (~twofold) increase in brain concentration ratios of ddI after infusion of the 5'-pivalate.

Durrer *et al.* (1991) examined 16 esters of nicotinic acid for their potential to improve brain activity of this neurotransmitter by generating quantitative structure-metabolism relationships in both subcellular fractions of rat liver and brain tissue. While K_m values were similar in both tissues for a given substrate, V_{max} values in liver exceeded those in brain tissue by ~2 orders of magnitude. The lack of optimal bioconversion kinetics may also account for the limited success reported by Cooper *et al.* (1987a,b) to develop esters of L-dopa (which is itself a prodrug of dopamine) with improved brain delivery. They evaluated 14 compounds, but none exhibited superior potency or a longer duration of action than L-dopa itself although they did offer an advantage in water solubility. Kinetic studies would probably have confirmed that none of the prodrugs evaluated possessed optimal bioconversion characteristics. Badir *et al.* (1991) also concluded that a series of monoesters of valproic acid were inferior to valproic acid itself due to their rapid systemic bioconversion.

The challenge of peptides. Peptide delivery to the brain is a particularly challenging problem. Peptide transport across the blood-brain barrier is restricted due to molecular size constraints, the hydrophilic nature of peptides, which can be attributed largely to their ionizable and hydrogen-bonding residues, and the susceptibility of peptides to rapid degradation by peptidases localized within the capillary endothelium (Djuricic and Mrsulja, 1977; Brecher *et al.*, 1978; Hersh *et al.*, 1987; Solhonne *et al.*, 1987; Turner *et al.*, 1987; Bodor *et al.*, 1992; Brownlees and Williams, 1993). Although there are exceptions, simple esterase-activated prodrugs are often ineffective in improving peptide delivery to the brain despite their increased lipophilicity. For example, although lipophilic monoester prodrugs of several dicarboxylic acid angiotensin-converting enzyme inhibitors (e.g., enalapril, ramipril, zofenopril (a thioester), and fosinopril) were shown to access the central nervous system and inhibit brain-converting enzyme in a lipophilicity-dependent manner (Gohlke *et al.*, 1989; Ranadive *et al.*, 1992), Nordstrom *et al.* (1993) demonstrated that ramipril (the ethyl ester of ramiprilate) and ramiprilate produced the same CSF concentrations of ramiprilate after intravenous administration, suggesting that there was no prodrug advantage in this case.

In an effort to improve the brain delivery of a model opioid peptide (H-Tyr-D-Ala-Gly-Phe-D-Leu-OH), Borchardt's group (Bak *et al.*, 1999; Wang *et al.*, 1999; Ouyang *et al.*, 2002) synthesized three types of sequentially labile cyclic peptides (Figure 3) that would require esterase activation as the initial step in their bioconversion. However, an evaluation of the prodrugs in an *in situ* perfused rat brain model (Chen *et al.*, 2002) indicated that certain undesirable characteristics significantly limited their ability to deliver the parent peptide to the brain. Most importantly, these prodrugs were bioconverted much more rapidly in rat plasma than in brain homogenate and were rapidly cleared from the liver *in vivo* (Yang *et*

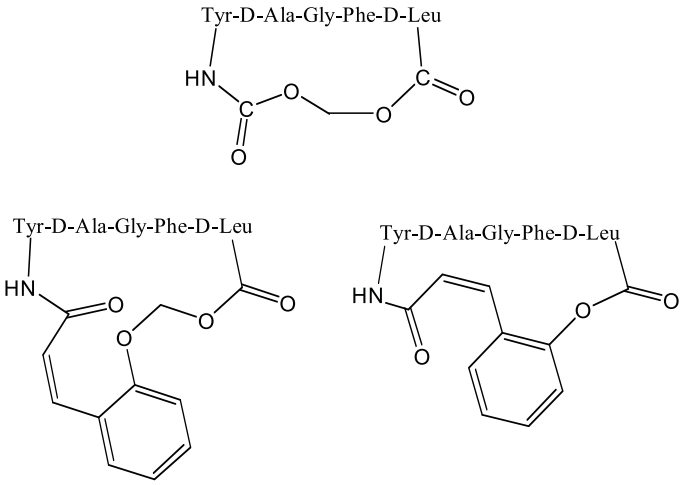


Figure 3. Three types of sequentially labile, esterase activated prodrugs of an opioid peptide intended for delivery of the peptide to the CNS (Bak *et al.*, 1999; Wang *et al.*, 1999; Ouyang *et al.*, 2002).

al., 2002). Chen *et al.* (2002) concluded that the chemical linkers would need to be modified to increase bioconversion rates in brain and reduce bioconversion in blood. All three prodrugs were also shown to be limited in their brain uptake by efflux transporters, particularly P-gp (see "Overcoming Efflux Transport").

Despite the disadvantageous combination of peptide hydrophilicity and instability in the presence of brain peptidases, simple esters to enhance CNS activity of peptides have been successful in isolated cases. The peptide analog thiorphan is a potent inhibitor of neutral endopeptidase, one of two zinc metallopeptidases involved in the *in vivo* inactivation of endogenous enkephalins (Malfroy *et al.*, 1978; Guyon *et al.*, 1979). Endopeptidase inhibition has therefore been investigated as a means for producing analgesia with reduced side effects

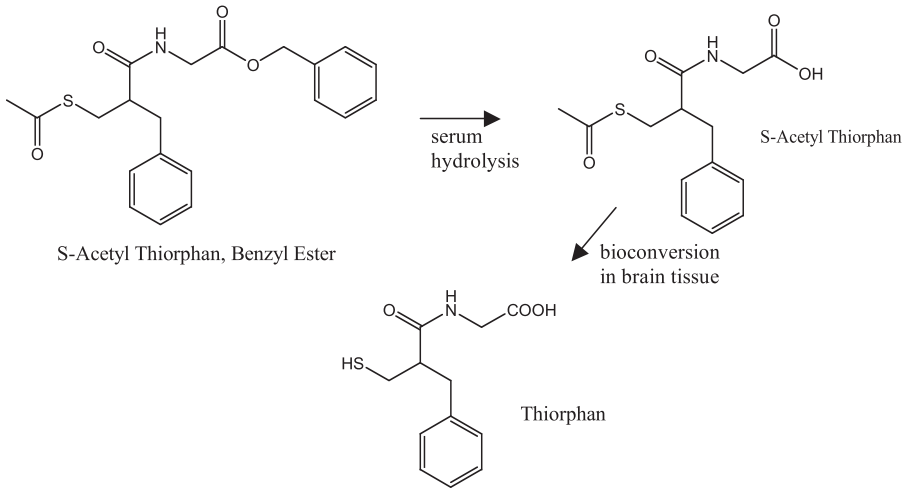


Figure 4. Postulated reaction sequence for a double prodrug of thiorphan (Lambert *et al.*, 1993).

(Chipkin, 1986; Roques and Fournie-Zaluski, 1986; Barnard *et al.*, 1991). Thiorphan itself, however, is active only at very high doses because it does not cross the blood-brain barrier readily (Roques *et al.*, 1980).

S-acetyl thiorphan and its benzyl ester (acetorphan), both exhibit higher analgesic activities in mice after parenteral administration than thiorphan itself despite their reduced *in vitro* inhibitory activities toward neutral endopeptidase (Lecomte *et al.*, 1986; Lambert *et al.*, 1993). Lambert *et al.* (1993) observed that the activities of S-acetylthiorphan and acetorphan were comparable despite the differences in their lipophilicity. They suggested (Figure 4) that the benzyl ester hydrolyzes rapidly in serum and that S-acetylation alone provided sufficient lipophilicity to enhance blood-brain barrier penetration.

More recently, Prokai-Tatrai *et al.* (2003) demonstrated that long-chain primary alcohol esters of the TRH analog pGlu-Glu-Pro-NH_2 improved its analeptic activity in mice, perhaps due to the prolonged retention of the parent peptide once in brain tissue.

The γ-aminobutyric acid analog baclofen, a zwitterion with very poor uptake into brain tissue and CSF (Deguchi *et al.*, 1995), would appear to be an excellent candidate for esterase-activated prodrugs to improve its delivery to the brain due to its lock-in potential. Leisen *et al.* (2003) therefore recently synthesized 5 alkyl esters of baclofen with octanol/water partition coefficients ranging from 27- to 240-fold higher at pH 7.3 than baclofen itself. Despite high brain tissue/blood ratios of the intact ester, the methyl ester of baclofen did not lead to increased brain concentrations of baclofen. This was attributed to lower than expected hydrolysis in the brain. However, another factor that may have contributed was the active efflux of baclofen. Deguchi *et al.* (1995) reported that the clearance of baclofen from brain tissue, CL_{out}, exceeds CL_{in} by 40-fold. Thus, the despite the appearance that baclofen would be a good candidate for enhanced CNS delivery via esterase-activated prodrugs due to its zwitterionic nature and presumed lock-in potential, this is not the case.

Overcoming Efflux Transport

Several of the parent compounds discussed in the previous section were found to present a more difficult challenge because they are not only polar molecules but evidently substrates for efflux transporters. As a class, the antiviral nucleosides serve as useful examples to illustrate this added challenge. These compounds have been popular candidates for ester prodrug design because they are quite polar and, in most cases, exhibit very low CNS uptake. Often there is a strong therapeutic rationale for achieving high brain concentrations of these agents. For example, despite the significant improvements attained in the treatment of AIDS using combination therapy, many of the drugs used to treat AIDS exhibit poor CNS penetration. The demonstrated presence of HIV in brain tissue, the correlation between the development of symptoms of HIV dementia and brain penetration of the dideoxynucleoside reverse-transcriptase inhibitors, and the concern that the brain may serve as a sanctuary site for HIV and a possible source

of continuous reinfection and development of resistant virus has led researchers to explore a variety of prodrug approaches for improving brain concentrations of these drugs.

Shown in Figure 5 are the structures of several nucleoside RT inhibitors along with their percent uptake into CSF relative to their plasma concentrations reported in human studies. Some of these estimates vary significantly because they are based on single time points. Those representing determinations at steady-state (marked with an asterisk) are believed to be more reliable. These values indicate that zidovudine (AZT), the most lipophilic with a log $P_{octanol/water}$ of ~0, also exhibits the highest CSF concentration at steady-state. Nevertheless, the CSF/plasma percentage for zidovudine (24%) is significantly below 100% (Pizzo *et al.*, 1988). Groothuis and Levy (1997) estimated that the brain concentration following a 200 mg oral dose of AZT would be five times below the lowest concentration needed to effectively inhibit HIV replication. Since increasing the dose would also increase bone marrow toxicity (Pizzo, 1990), prodrugs or other approaches for increasing the CNS concentration of AZT have been aggressively pursued. The need is still more acute for the other nucleoside RT inhibitors, some of which (e.g., ddI and ddC) exhibit significantly lower brain uptake than AZT (Sawchuk and Yang, 1999).

The dideoxynucleoside RT inhibitors exhibit low brain and CSF concentrations in part because of their poor permeation across the blood-brain barrier resulting from their polar nature. A more important factor is that these compounds appear to be maintained at low concentrations in the brain by efflux transport systems or in some cases possibly by metabolism in brain tissue. Carrier-

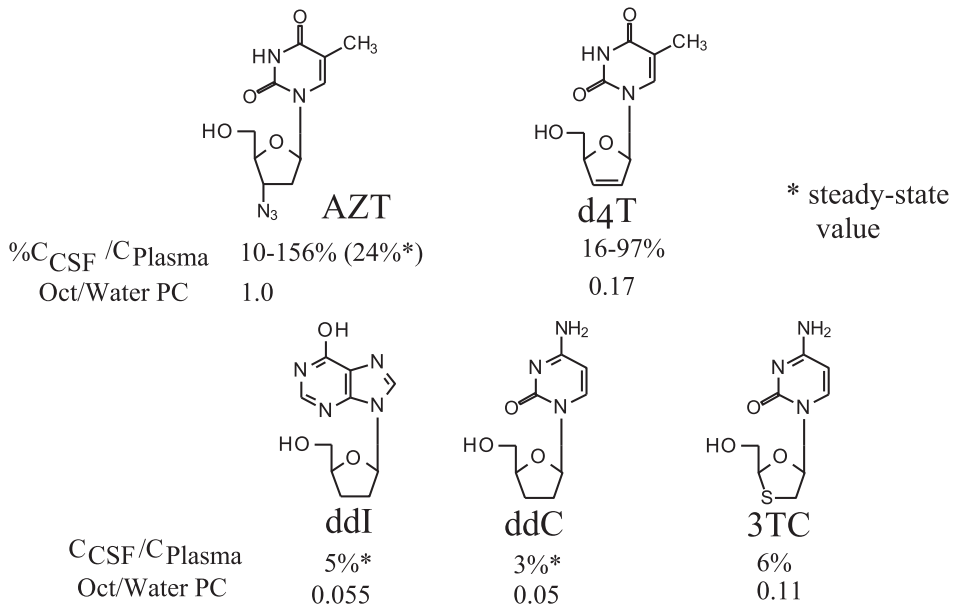


Figure 5. Range of reported values for % C_{CSF} /C_{Plasma} and octanol/water partition coefficients for several dideoxynucleoside reverse-transcriptase inhibitors. (Values marked with an asterisk were determined at steady-state.)

mediated efflux of ddI (Anderson *et al.*, 1990b; Galinsky *et al.*, 1991; Hoesterey *et al.*, 1991; Takasawa *et al.*, 1997) and AZT (Hedaya and Sawchuk, 1989; Galinsky *et al.*, 1990; Wong *et al.*, 1992; Dykstra *et al.*, 1993; Tuntland *et al.*, 1994; Wang and Sawchuk, 1995; Wang *et al.*, 1995; Takasawa *et al.*, 1997) results in relatively rapid clearance of these compounds from the brain. The brain tissue efflux of both AZT (Wang *et al.*, 1995; Takasawa *et al.*, 1997) and ddI (Galinsky *et al.*, 1991; Takasawa *et al.*, 1997) have been shown to be inhibited by probenecid, an organic anion transporter inhibitor. Wada *et al.* (2000) and others (Hasegawa *et al.*, 2003) demonstrated that rOAT1 and rOAT3 are both able to transport dideoxynucleosides and that these transporters, along with the organic anion transporter polypeptides (OATPs), are inhibited by probenecid. OAT1, OAT3, OATP2, and OATP3 have been localized in brain tissue (Sun *et al.*, 2003). Mori *et al.* (2003) demonstrated that rOAT3 resides on the basolateral side of brain capillary cells where it presumably facilitates efflux from brain tissue by promoting dideoxynucleoside uptake into the capillary cells. Other transporters (e.g., the equilibrative nucleoside transporters) may also be involved.

Figure 6 illustrates the delivery problem when a parent compound exhibits slow uptake and is also the substrate for an efflux-transporter. Lipophilic prodrugs that display high permeability must still undergo rapid bioconversion in brain tissue to significantly increase the steady-state concentrations of parent drug due to this rapid efflux term.

A variety of ester prodrugs of the anti-HIV dideoxynucleosides (e.g., zidovudine (AZT), 2',3'-dideoxyinosine, etc.) and other antivirals (e.g., 5-iodo-2'-deoxyuridine, ganciclovir, etc.) have been evaluated, allowing the exploration of the effects of the parent drug and pro-moiety on various prodrug properties such as lipophilicity, stability in plasma, and bioconversion in brain tissue in the hope that these properties could be related to the *in vivo* enhancement in delivery of the parent drug to the brain. AZT has been a very popular template for the

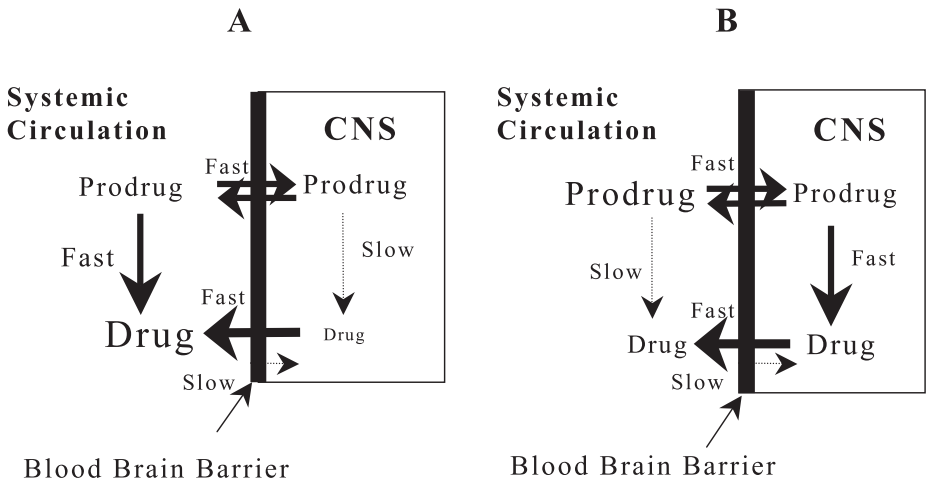


Figure 6. Efflux transport makes prodrug design for CNS delivery more difficult. (A) Slow prodrug bioconversion combined with efflux transport of parent drug; (B) Rapid prodrug bioconversion competes with the efflux transport rate.

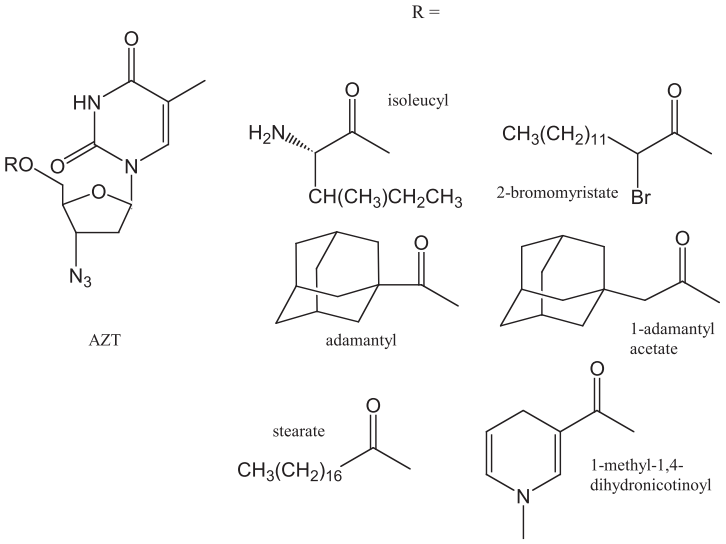


Figure 7. Lipophilic 5'-O-ester prodrugs of AZT.

exploration of various ester prodrugs (Parang *et al.*, 2000) but with only modest benefits in terms of expected improvement in therapy. If a fivefold enhancement in CNS concentration of AZT is set as the benchmark (based on the estimate of Groothuis and Levy (1997)), nearly all prodrug approaches attempted for this drug thus far have fallen short of the mark.

Aggarwal *et al.* (1990) prepared a variety of 5'-O-esters of AZT with the aim of identifying esters with increased lipophilicity and stability in plasma esterases while hopefully retaining adequate hydrolysis rates in target cells. The isoleucyl ester (Figure 7), which emerged as one of the most promising of those investigated, represents a prototypical esterase-activated prodrug of AZT (Lupia *et al.*, 1993). Even though it possesses an ionizable α-amino substituent, this prodrug has a tenfold higher lipophilicity than AZT itself at pH 7.4 (Aggarwal *et al.*, 1990). At pH 7.4 this amino group would be largely protonated and therefore strongly electron withdrawing, which would be expected to activate the ester linkage toward nucleophilic attack. The finding that the prodrug resulted in more rapid uptake of AZT into CSF in rabbits than when AZT itself was administered combined with the absence of intact prodrug in the CSF even at 5 min following intravenous administration led the authors to suggest that the prodrug is converted rapidly to AZT after crossing the blood-CSF barrier. Yet, the CSF/plasma ratios of AZT were twofold higher at 30 min from AZT alone and comparable at 75 min. Brain/plasma ratios were comparable at 30 min, but at 75 min the prodrug showed a modest (~twofold) advantage.

Optimum lipophilicity for CNS delivery?

Since prodrugs with enhanced lipophilicity have been shown to provide advantages in CNS delivery, perhaps the trend could be further exploited by using

fatty acid esters or other highly lipophilic promoieties. Indeed, some of the more intriguing (and puzzling) successes have come from extremely lipophilic prodrug esters. Is it then the case that the higher the lipophilicity the better from the standpoint of CNS delivery?

The antiviral properties of the adamantane moiety and its potential as a brain-directed drug carrier led Tsuzuki *et al.* (1994) to prepare several adamantane conjugates of AZT and evaluate their BBB penetration. The adamantoyl ester and the 1-adamantaneacetate (Figure 7) were estimated to have ca. 250-fold greater lipophilicity than AZT itself. Other compounds evaluated, including 3 adamantoylaminoalkyl carboxylates varying in length of the alkyl spacer and the stearic acid (C-18) ester, also exhibited very high lipophilicities. All of the compounds except the 1-adamantaneacetate were converted to AZT within 30 min in rat plasma. The increased stability of the 1-adamantaneacetate in rat plasma ($t_{1/2}$ = 3.3 h) led the authors to conclude that this compound would be the most promising prodrug for delivering AZT to the brain. (This is not intuitively obvious in the absence of brain tissue conversion data, however. Perhaps the authors would not have made the same prediction in advance of their results.) They also noted that, in contrast to its relatively rapid hydrolysis in rat plasma, the 1-adamantane acetate remained 100% intact over 4 h in human plasma.

Despite their slow bioconversion in plasma, the highly lipophilic adamantane prodrugs (Figure 7) produced their highest concentrations of AZT in brain tissue (in rats) at 15 min. At this time point the brain concentration/plasma ratios of AZT were 0.14 from AZT, 0.33 from the adamantoyl ester, 0.24 from the 1-adamantaneacetate, and 0.06 from the stearate. The conclusion of this study was that the adamantane moiety may be a useful brain-directed carrier for poorly absorbed drugs, although the enhancement in brain/plasma ratios was modest.

Parang *et al.* (1998a,b) evaluated the CNS delivery potential of the 2-bromomyristate of AZT (Figure 7) in mice in comparison to AZT alone and again concluded that highly lipophilic prodrugs seem to offer advantages in enhancing the brain distribution of AZT. They reported that the $AUC_{brain/blood}$ ratios for AZT after administration of either AZT or the 2-bromomyristate were 0.05 and 0.23, respectively, implying a >fourfold advantage for the prodrug.

Some observations cited in the above studies are difficult to reconcile with the properties of AZT and the features necessary to improve its delivery via the prodrug approach as illustrated in Figure 6. For example, Parang found that the peak brain concentrations of AZT were higher than those produced by AZT and were exhibited within the first 15 min, suggesting rapid bioconversion of the prodrug in brain tissue. However, Parang *et al.* (1998c; 2000) demonstrated that the 2-bromomyristate of AZT undergoes hydrolysis much more rapidly in rat plasma than in rat brain homogenate. Slow prodrug conversion in brain tissue combined with rapid prodrug permeation into and out of brain tissue should not have produced significantly elevated brain concentrations of AZT in comparison to AZT alone due to the problems illustrated in Figure 6.

One potential complicating factor that is important to address in studies of prodrugs for improving CNS delivery is the difficulty of quenching prodrug

bioconversion at the time of sacrifice of the animal and removal of the brain, particularly if hydrolysis in brain tissue is facile. Unfortunately, the extent to which a lipophilic prodrug may continue to undergo hydrolysis during processing of brain tissue is usually not addressed in publications, so it is difficult to ascertain whether or not the process has been adequately validated. Lipophilic prodrugs present at high concentrations in brain tissue may exhibit the appearance of rapid bioconversion and superior delivery of the parent compound due to further hydrolysis during processing of the brain tissue.

Deguchi and coworkers (Deguchi *et al.*, 2000; Deguchi and Morimoto, 2001) synthesized the 1,3-diacetyl-2-ketoprofen glyceride (Figure 8) with the expectation that blocking the carboxyl group of the NSAID ketoprofen would increase BBB permeation and lead to higher brain tissue concentrations for the treatment of neurodegeneration in Alzheimer's disease. They demonstrated in mice that, while no prodrug could be detected in brain interstitial fluid, the area under the brain/plasma vs. time curves of ketoprofen increased ~fivefold following administration of the triglyceride ester in comparison to ketoprofen administration alone (Figure 8). This improvement was attributed to the 1,400-fold increase in lipophilicity provided by the prodrug and rapid conversion in the brain.

They used microdialysis to monitor interstitial concentrations of the prodrug and ketoprofen in brain tissue, and thus did not have to contend with the possible problem of quenching esterase reactivity during the isolation and processing of brain tissue. However, the insertion of microdialysis probes does cause damage to tissue in the vicinity of the probe that may alter the brain uptake of polar molecules (Morgan *et al.*, 1996) and could potentially alter the local tissue esterase activity.

To confirm the rapid bioconversion of the prodrug in brain tissue Deguchi *et al.* (2000) also monitored the time courses of prodrug, ketoprofen, and ketoprofen

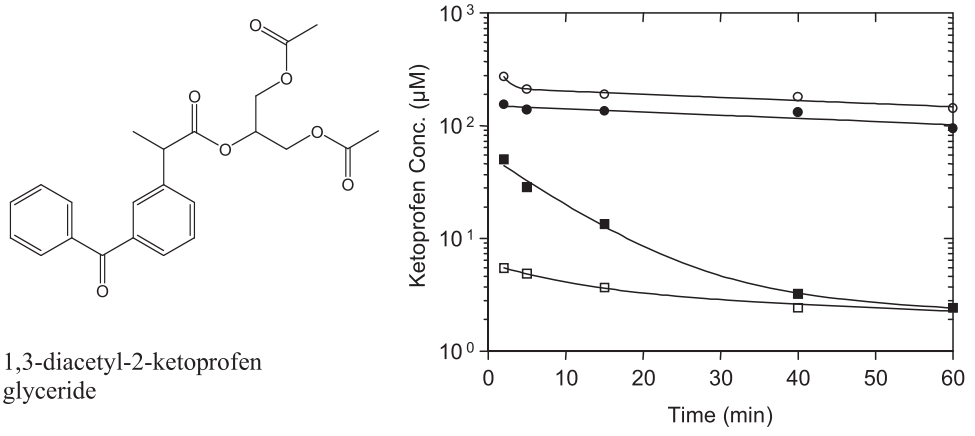


Figure 8. Plasma (○,●) and brain (□,■) concentrations of ketoprofen after i.v. administration of ketoprofen or 1,3-diacetyl-2-ketoprofen glyceride in mice (40 μmol/kg) (Deguchi *et al.*, 2000; Deguchi and Morimoto, 2001). Closed symbols are ketoprofen concentrations from prodrug.

metabolites in brain tissue after a 6 s *in situ* perfusion of prodrug through the external carotid artery at concentrations of 5–16 mM. The brain tissue hydrolysis of prodrug was highly concentration-dependent but the $t_{1/2}$ for prodrug conversion was < 2 min at all concentrations. Ketoprofen and other NSAIDS are substrates for OAT1 (Apiwattanakul *et al.*, 1999) and CL_{out} for ketoprofen from brain interstitial fluid exceeds CL_{in} by ~sevenfold (Deguchi *et al.*, 2000). Consequently, the increased brain concentrations realized from the triglyceride prodrug were short-lived, decreasing to those obtained from ketoprofen itself within 40 min.

Numerous additional examples of highly lipophilic esters including steroid esters, glycerides, drug-phospholipid conjugates to improve the CNS delivery of γ-aminobutyric acid (GABA), L-dopa, and other centrally acting drugs have been described (Shashoua *et al.*, 1984; Jacob *et al.*, 1985, 1990; Hesse *et al.*, 1988; Lambert, 2000). Nevertheless, others have suggested that the successful design of lipophilic esters with the ability to enhance parent drug concentrations in the brain is not a matter of maximizing lipophilicity but rather optimizing this property. Kawaguchi *et al.* (1990) synthesized a series of 10 aliphatic acid esters of zidovudine varying in chain length from the acetate (C2) to the stearate (C18) and determined their hydrolysis rates in aqueous buffers at pH 2–9 and in the presence of mouse plasma, and liver, intestine, and kidney homogenates. The hydrolytic rate constants of these esters at pH 2 and pH 9 were nearly constant with increasing chain length, declining only gradually by factors of 2.3 at pH 2 and 2.9 at pH 9. On the other hand, the rate constants for enzyme-catalyzed hydrolysis varied dramatically, increasing by >3 orders of magnitude from the acetate (C2) to the decanoate (C10) then declining by the same amount from the decanoate to the stearate (C18). Thus, there appears to be an optimum chain length for bioconversion rate, which may be due largely to a steric factor as predicted in Eq. (3).

Tsuzuki *et al.* (1994) found that the 5'-stearate ester of AZT produced AZT concentrations in brain tissue well below those from administration of AZT itself, indicating that the bioconversion of this ester in brain tissue may have been inadequate. However, they also found that the intact ester uptake into brain tissue was quite low; they attributed this to an excessive lipophilicity for optimal permeability. Brain uptake of highly lipophilic compounds can be limited by binding to plasma proteins and by unfavorable partitioning from the endothelium into the underlying tissue, both of which increase with lipophilicity. Various factors that may contribute to an optimal lipophilicity for CNS delivery have been reviewed (Robinson and Rapoport, 1986, 1992; Greig, 1989). In several instances, poor brain uptake of lipophilic prodrugs has been attributed to plasma protein binding (Rosowsky *et al.*, 1982; Greig *et al.*, 1990a,b). Raub *et al.* (1993) developed a biophysical-kinetic model to account for the contributions of protein binding and membrane partitioning to passive permeability of highly lipophilic molecules across the BBB.

Recently, efflux by the P-glycoprotein (P-gp) transporter has been identified as an additional factor limiting the uptake of lipophilic prodrugs. Leisen *et al.* (2003)

found that the affinity of baclofen ester prodrugs for P-gp correlated with their lipophilicity parameters. Killian and Chikhale (2000), noting that the permeability-area products for brain entry of their reductively activated prodrugs of melphalan and acivicin did not correlate with their relative lipophilicity, demonstrated an effect of prodrug on the brain uptake of verapamil, a P-glycoprotein substrate. Chen *et al.* (2002) found that a series of cyclic prodrugs of a model opioid peptide (H-Tyr-D-Ala-Gly-Phe-D-Leu-OH) exhibited membrane permeability coefficients in an *in situ* rat brain perfusion model that were no higher than that of the parent peptide despite having cLogP values ranging from 3.3–5 in comparison to the parent peptide's cLogP of –0.32. In the presence of GF120918, a potent P-gp inhibitor, the permeabilities of the prodrugs increased by 120–300-fold and an improved correlation with lipophilicity was established. Witt *et al.* (2001) considered the possibility that P-gp inhibition by poly(ethylene glycol) may have been involved in the improved analgesia of a pegylated prodrug of the opioid analog [D-Pen^2, D-Pen^5]-enkephalin (DPDPE), but the studies necessary to test this possibility were not conducted.

Finally, it is important to recognize that there will be a diminishing return associated with increasing lipophilicity when accompanied by a molecular size increase. Pardridge has suggested that there is a molecular weight cut-off of 500 daltons for delivery to the brain (Pardridge, 2002). While this is not absolute, biophysical studies of permeability across lipid bilayer membranes have demonstrated that there is a heightened selectivity to permeant size associated with lipid bilayers due to the ordered nature of the lipid chains (Lieb and Stein, 1986; Xiang and Anderson, 1994, 1998).

Locked-In Intermediates of Active Drugs

Redox trapping—"Chemical Delivery Systems"

Previously, the ideal candidate for improved brain delivery via simple ester prodrugs was described as a polar molecule with such poor BBB permeability that it would be "locked-in" brain tissue once bioconversion occurred. However, many drugs that might appear to be in this category on the basis of their physico-chemical properties turn out to have higher than expected clearance from brain tissue due to metabolism or efflux transport processes. Many other compounds that may exhibit less than optimal brain uptake are not sufficiently hydrophilic to be locked into the brain tissue once regenerated. The design of prodrugs that readily enter the brain but undergo rapid conversion within brain tissue to trapped or "locked-in" intermediates is a popular strategy to improve CNS delivery in such instances. Bodor and coworkers (Brewster and Bodor, 1992; Bodor, 1994, 1995; Bodor and Buchwald, 1999; Prokai *et al.*, 2000) coined the term "chemical delivery system" to distinguish this approach, which requires multiple steps for conversion to the active drug, from prodrugs requiring a single activating step. (The term prodrug can include derivatives that are activated in

multiple steps, however. Such sequentially labile derivatives are often referred to as "double" (Bundgaard, 1989) or "triple" (Tsukamoto *et al.*, 2001) prodrugs.)

Dihydropyridine-Pyridinium Redox Pair.

The most frequently employed moiety in the chemical delivery system approach is the dihydropyridine-pyridinium (1, 4-dihydrotrigonellinate-trigonellinate) redox pair. The "lock-in" feature is achieved through enzymatic oxidation to form a cationic intermediate that is trapped in the brain but rapidly lost from the periphery, reducing systemic toxicity (Figure 9). Depending on the rate constant for regeneration of the active parent drug in the brain, it is possible to achieve a sustained pharmacological activity using this approach, as demonstrated for the estradiol system (Anderson *et al.*, 1986; Tapfer *et al.*, 2004), which is now in clinical studies for hormone replacement therapy. Two applications of the chemical delivery system approach will be summarized in this chapter—brain targeting of antiviral dideoxynucleosides and delivery of peptides to the CNS.

Brain targeting of antiviral drugs. Not surprisingly given the wide interest in improving the CNS uptake of anti-HIV RT inhibitors, a brain-targeted chemical delivery system for zidovudine (AZT-CDS) has been developed and evaluated in depth (Aggarwal *et al.*, 1990; Chu *et al.*, 1990a; Little *et al.*, 1990; Brewster *et al.*, 1991; Gallo *et al.*, 1991; Lupia *et al.*, 1991; Brewster *et al.*, 1993, 1995, 1997). Shown in Figure 9 are the general structure of the AZT-CDS (1-methyl-1,4-

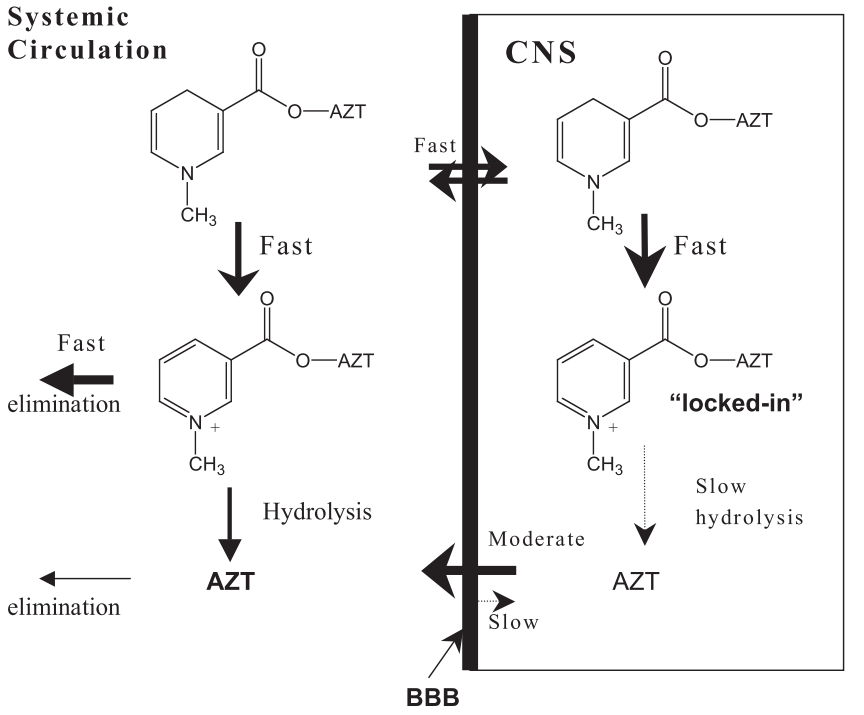


Figure 9. Illustration of redox-trapping. Generation of a "locked-in" cationic intermediate in the CNS produced from the 1-methyl-1,4-dihydronicotinate ester of AZT.

dihydronicotinate ester) and the sequence of events leading to the improved brain delivery of AZT. The targeting advantage is achieved by oxidation of the promoiety to the quaternary ammonium methylnicotinate salt, which is then trapped in the brain until hydrolysis of the ester linkage releases AZT.

Studies in several species have demonstrated improved CNS delivery. In mice, Chu *et al.* (1990a) reported a tenfold increase in the area under the brain concentration versus time curve for AZT after treatment with AZT-CDS in comparison to AZT alone. Three- to fourfold increases were observed in rats and rabbits (Little *et al.*, 1990; Brewster *et al.*, 1991) while in dogs the levels of AZT in brain were nearly twofold higher at 30 min and 3.3-fold higher at 90 min after intravenous administration of AZT-CDS (Brewster *et al.*, 1997). Lupia *et al.* (1993) compared the AZT-CDS with the isoleucinyl ester and found that the AZT-CDS provided only modestly higher brain/plasma ratios than the simple ester prodrug. At 30 min, the brain/plasma AZT ratios in rabbits were 0.58 from AZT alone, 1.09 from AZT-CDS, and 0.52 from the isoleucinyl ester. At 75 min both the AZT-CDS and isoleucinyl ester yielded ratios approximately twofold higher than AZT alone.

Even when a trapped intermediate is generated, the steady-state concentration of parent drug in the brain is still determined by the relative rates of regeneration of parent drug and its elimination from brain tissue. As the most lipophilic of the nucleoside RT inhibitors and a substrate for efflux transporters, AZT has been one of the more challenging candidates for any prodrug approach. It is not clear whether any of the approaches explored would produce steady-state concentrations in brain tissue sufficient to inhibit viral replication. The high variability in results from one lab to another was at least partially accounted for in a study by Brewster *et al.* (1995), who found that higher doses of the AZT-CDS produced disproportionately higher brain levels of AZT. They suggested that this was due to saturation of active brain efflux at the higher doses.

Brewster *et al.* (1994) evaluated the same carrier moiety for improving the CNS delivery of ganciclovir for the treatment of cytomegalovirus infection in the brain. Ganciclovir (log PC = –1.95) is considerably more polar than AZT (log PC ~ 0) and exhibited a brain/blood concentration ratio of only 0.063 when ganciclovir was administered to rats. The ganciclovir-CDS was found to be 55 times more lipophilic and delivered an area under the brain concentration versus time curve fivefold higher than that achieved from ganciclovir. The higher brain tissue concentration combined with reduced blood levels yielded a brain/blood concentration ratio of 2.54, dramatically higher than that from ganciclovir itself. The sustained levels of ganciclovir in the brain (2.7 μM) were thought to be sufficient to inhibit viral replication (IC50 = 0.5–3 μM).

Molecular packaging for brain delivery of an enkephalin analog. The same redox strategy has been employed to deliver various neuropeptides or peptide precursors to the brain, including enkephalin analogs (Bodor *et al.*, 1992), kyotorphin analogues (Chen *et al.*, 1998), and thyrotropin-releasing hormone analogs (Prokai *et al.*, 1999). However, the attachment of the redox moiety to the NH_2 terminus of a peptide does not provide sufficient lipophilicity to promote brain uptake (Bodor and Prokai, 1995), and the trapped intermediate with an

unmodified carboxy terminus would not be protected from various exo- and endopeptidases in the blood-brain barrier (Brownlees and Williams, 1993). Cholesteryl ester formation at the C-terminus substantially increases the overall lipophilicity and, prior to its removal, also protects the peptide from recognition by peptidase enzymes. The resulting conjugate consists of a peptide that is buried within a much larger and more lipophilic molecular package, as illustrated for the enkephalin analog, Tyr-D-Ala-Gly-Phe-Leu (YAGFL), in Figure 10 (Bodor *et al.*, 1992). Brain uptake and oxidation to the trapped pyridinium ion is followed by the removal of cholesterol by esterases or lipases within brain tissue. From that point on the peptide is susceptible to a variety of peptidases, many of which lead to unwanted decomposition products as depicted in Figure 10. To facilitate conversion to the desired product, the authors inserted an alanyl spacer at the N-terminus of the peptide such that the action of dipeptidyl peptidase would produce the parent peptide as its primary product. Further metabolism of the trapped intermediate also had to be reduced by replacing the terminal L-leucine with D-leucine. This system produced evidence for prolonged (>5 h) analgesia in rats after systemic administration.

Retrometabolic design of a TRH brain delivery system. Thyrotropin-releasing hormone (TRH, pGlu-L-His-L-Pro-NH_2) elicits a number of interesting neuropharmacological effects that may be useful in the management of various neurologic disorders (Yarbrough, 1983; Faden *et al.*, 1989; Sunderland *et al.*, 1989; Lampe *et al.*, 1991; Itoh *et al.*, 1994). However, its action as an exogenous neuropeptide is limited by its short $t_{1/2}$ of ~ 5 min (Bassiri and Utiger, 1973) and its inability as well as that of its analogs to penetrate the blood-brain barrier

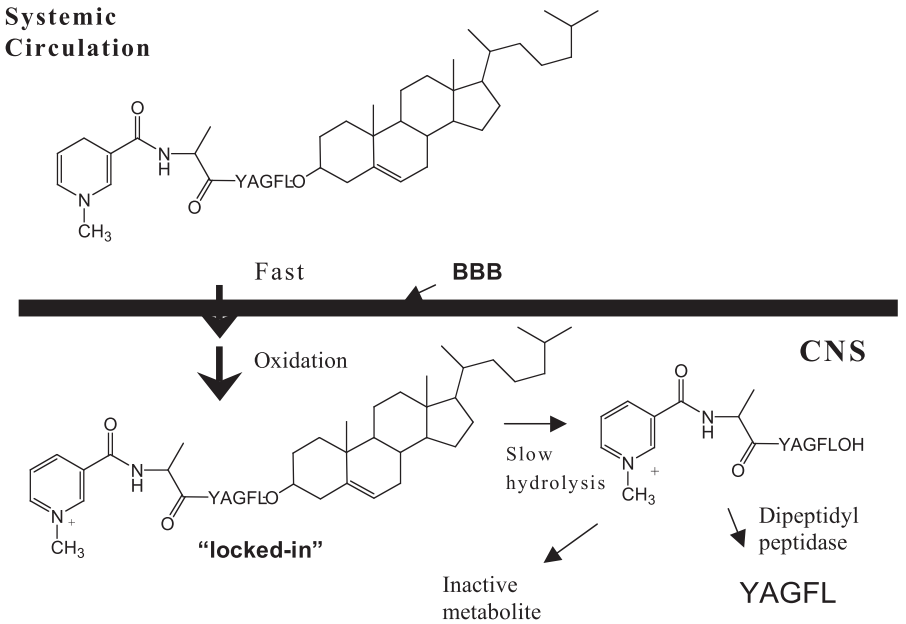


Figure 10. Brain delivery of a Leu-enkephalin analog using redox-trapping and retrometabolic design (Prokai-Tatrai *et al.*, 1996).

(Zlokovic *et al.*, 1988; Zlokovic, 1995) attributable to its low lipophilicity (Bodor and Prokai, 1995).

Prokai *et al.* (1994, 1999) applied the concept of retrometabolic design to improve the CNS delivery of TRH. This analog contains no free N-terminal amino and therefore cannot be directly linked to the 1,4-dihydrotrigonellyl moiety. The biosynthesis of TRH involves a large precursor polyprotein that contains multiple sequences of Gln-His-Pro-Gly (Jackson, 1989; Richter *et al.*, 1994). The glycyl residue is transformed to an amide by peptidyl-glycine α-amidating monooxygenase (Bradbury *et al.*, 1982; Husain and Tate, 1983) while glutaminyl cyclase converts the glutaminyl residue to pyroglutaminyl (Busby *et al.*, 1982; Fischer and Spiess, 1987). These observations led Prokai *et al.* (1999) to synthesize the compounds shown in Figure 11. The slow step in the metabolic sequence for the generation of the TRH analog from these compounds was demonstrated *in vitro* to be the peptidase-catalyzed conversion of the trapped T^+-Spacer-Gln-Leu-Pro-NH_2 to Gln-Leu-Pro-NH_2. All of the compounds shown in Figure 11 were found to be more potent than the parent peptide in reducing the barbiturate-induced sleeping times in mice, with the Pro-Ala spacer producing the greatest effect—a 56% decrease in sleeping time in comparison to vehicle control.

Several groups have observed that the dihydropyridine-modified prodrugs are difficult to purify and formulate due to their facile oxidation in air oxygen (Shanmuganathan *et al.*, 1994), acid-catalyzed hydration of the C_5-C_6 double bond (Pop *et al.*, 1984), and isomerization to the 1,2-dihydro-derivatives (Sheha *et al.*, 2003). Carelli *et al.* (1996) and Sheha *et al.* (2003) have described new redox carriers that have potential advantages.

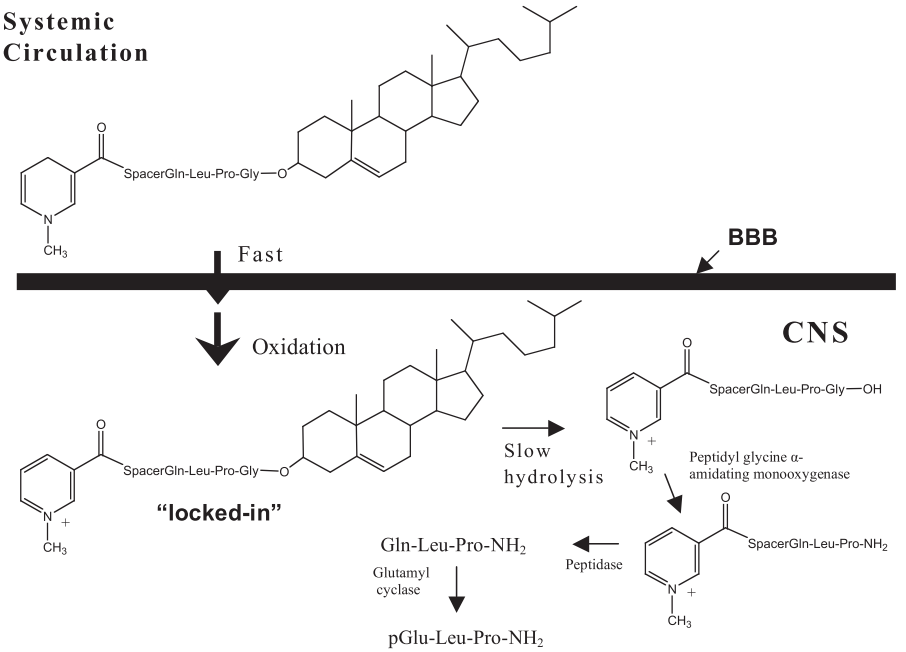


Figure 11. Metabolism based brain targeting system for a thyrotropin releasing hormone analogue (pGlu-Leu-Pro-NH_2). The spacer consisted of either a single amino acid (Ala, Pro) or a dipeptide (Ala, Pro, or Ala-Pro combinations) (Prokai *et al.*, 1999).

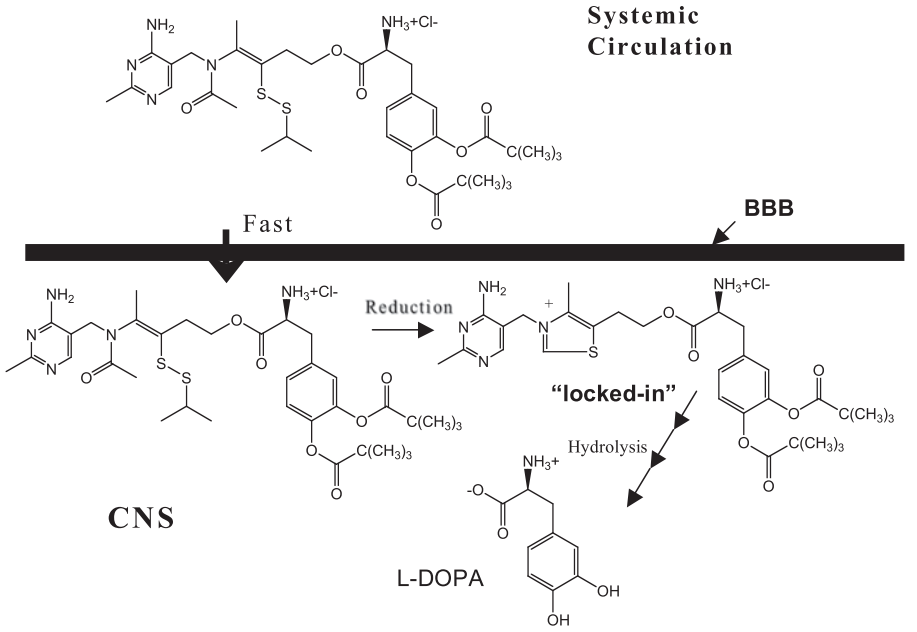


Figure 12. Generation of a "locked-in" thiazolium intermediate via a thiol-mediated ring-closure reaction to improve the CNS delivery of L-DOPA (Ishikura *et al.*, 1995).

Quaternary Thiazolium Intermediate Formation

Ishikura *et al.* (1995) developed an alternative "lock-in" strategy based on a redox ring-closure to form a quaternary thiazolium intermediate, such as that which exists in the thiamine molecule. They evaluated this approach as a means of improving the CNS uptake of L-dopa, as illustrated in Figure 12. The initial reaction leading to ring closure involves generation of a free thiol by reaction with glutathione or another endogenous thiol. Half-lives for generation of the quaternary thiazolium from a series of thioesters, thiocarbonates, and two disulfides were compared in 25% rat or human blood and 25% rat brain tissue homogenate (Yoshikawa *et al.*, 1999). All but one of the thioesters and thiocarbonates were converted preferentially in blood, with blood:brain bioconversion rate ratios varying from 2.3- to 25-fold. Interestingly, the only exception was the extremely lipophilic hexadecanoyl ester which exhibited a more rapid bioconversion in brain tissue, with a blood-brain rate ratio of 0.55. The two disulfides exhibited very rapid and preferential bioconversion in 25% brain tissue with half-lives <0.5 min. Intravenous administration of the 2-propyldithio derivative in rats (shown in Figure 12) produced elevated brain concentrations of dopa, with an AUC in brain tissue 30-fold higher than that achieved following intravenous administration of L-dopa.

A similar approach was applied to the CNS delivery of 6,7-dichloro-3,4-dihydro-3-oxo-2-quinoxalinecarboxylic acid (DCHQC), a receptor antagonist for the (S)-2-amido-3-(3-hydroxy-5-methylisoxazole-4-yl)propionic acid (AMPA) receptor for glutamate, and 5,7-dichlorokynurenic acid (DCKA), an antagonist of

the N-methyl-D-aspartate (NMDA) receptor. Although DCKA and DCHQC are structurally similar, ester-linked conjugates of DCHQC were unstable in plasma and neither the conjugates nor DCHQC could be detected in brain after intravenous administration of conjugates. DCKA conjugates, on the other hand, appeared to be too stable.

Anionic "locked-in" intermediates

Phosphinates

The above examples achieved the goal of generating a trapped intermediate in brain tissue via formation of a quaternary cation. However, alternative methods of generating trapped intermediates have also been evaluated. Chen *et al.* (2001) developed the dual phosphinic inhibitors of the enkephalin-degrading enzymes neutral endopeptidase and aminopeptidase N, one of which is shown in Figure 13, but found that they were only weakly active when administered intravenously, evidently due to the presence of three highly polar functional groups. Double prodrugs using a benzyl ester to mask the terminal carboxylate and various phosphinic protecting groups were designed to promote the CNS delivery and improve the duration of antinociceptive action of the dual inhibitors. The S-acetyl derivative (Figure 13) provided an extended analgesia over a 2-h period. In rat serum hydrolysis of the benzyl ester occurred rapidly (more rapidly than in brain homogenate) while the phosphinic ester was converted more slowly in both tissues. Possibly the hydrolysis of the benzyl ester in brain tissue to produce a

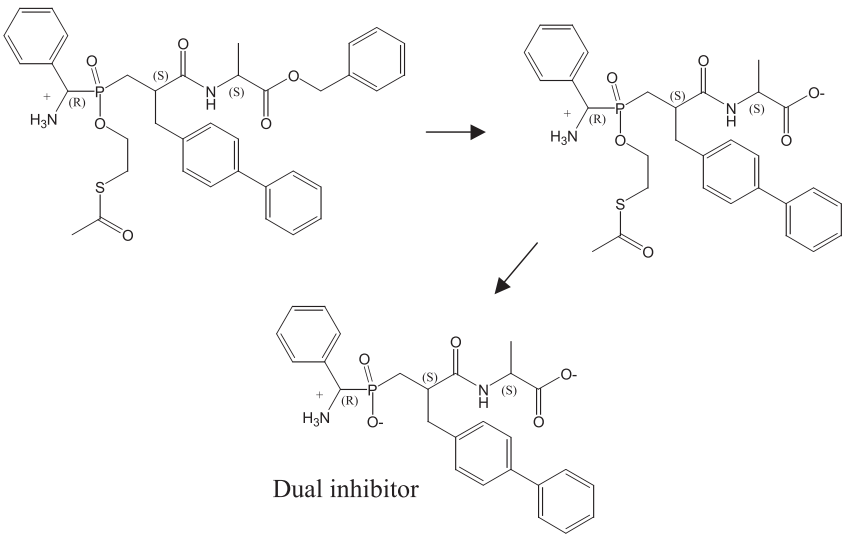


Figure 13. Bioconversion sequence in rat brain tissue for a double prodrug of a dual inhibitor of the enkephalin degrading enzymes, neutral endopeptidase and aminopeptidase N (Chen *et al.*, 2001).

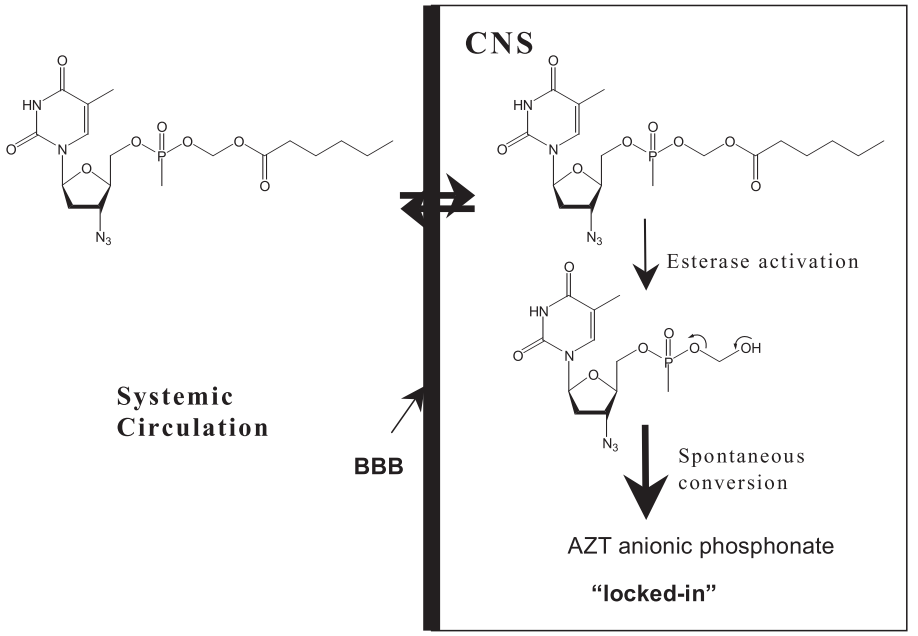


Figure 14. Generation of a trapped AZT-phosphonate in the CNS using esterase activation followed by a spontaneous loss of formaldehyde (Somogyi *et al.*, 1998).

trapped zwitterion, followed by slow biotransformation to the active inhibitor could account for the prolonged duration of action of this prodrug.

Phosphonates

Bodor and colleagues have designed phosphonate carriers with the aim of generating anionic "locked-in" intermediates for the CNS delivery of testosterone (Somogyi *et al.*, 1997) and zidovudine (AZT) (Somogyi *et al.*, 1998), as illustrated in Figure 14 for the AZT prodrug. Esterase-catalyzed removal of the hexanoate ester occurred rapidly in 20% tissue homogenates (rabbit) with half-lives of 3.8 min in blood, 2.9 min in brain tissue, and only 0.17 min in liver homogenate; this was facilitated by the use of an acyloxyalkyl spacer to minimize steric hindrance at the ester linkage. *In vivo*, the anionic AZT-P^- levels in brain tissue exceeded blood concentrations and decreased only gradually over a 48-h period. This proved that an anionic "lock-in" mechanism could work despite the presence of organic anion efflux transporters (vide infra) at the blood-brain barrier. Alkaline phosphatase was found to cleave the P-O bond to generate AZT but at a rate that was too slow to produce sufficiently high levels in the brain.

Phosphates.

The antiviral 2',3'-dideoxynucleosides used in the treatment of HIV infection are themselves prodrugs that must be activated intracellularly to their 5'-triphosphates (Furman *et al.*, 1986; Balzarini *et al.*, 1987; Hao *et al.*, 1988, 1990). The

initial phosphorylation step by intracellular nucleoside kinases to produce the 5'-monophosphate is often dependent on the host species, cell type, and the phase of the cell cycle (Balzarini *et al.*, 1989; Perno *et al.*, 1989; Gao *et al.*, 1993, 1994; Shirasaka *et al.*, 1995) and may also be limited by poor affinity of the dideoxynucleosides for the cellular kinases (Balzarini *et al.*, 1987; Starnes and Cheng, 1987; Johnson *et al.*, 1988; Johnson and Fridland, 1989). Thus, the ideal prodrug for delivery of anti-HIV nucleosides to the brain would deliver the 5'-monophosphates intracellularly (and only to infected cells). The derivatization of phosphates, phosphonates, bisphosphonates, and phosphinates has been addressed in another chapter of this book, and nucleotide prodrugs, in particular, have been reviewed elsewhere (Jones and Bischofberger, 1995; Parang *et al.*, 2000).

While numerous publications have described nucleotide prodrugs designed to more efficiently deliver antiviral monophosphates intracellularly, only a few studies have specifically addressed the CNS delivery potential of such prodrugs. Henin *et al.* (1991) described the synthesis, transmembrane transport, and antiviral activity of a series of lipophilic glycosyl phosphotriesters in which AZT monophosphate was coupled to a carbohydrate (D-glucose, D-mannose, and ethyl D-mannopyranoside) and a hexadecyl chain. Namane *et al.* (1992) demonstrated that the (mannopyranosidyl) ethyl phosphotriester produced high brain concentrations of primarily AZT 5'-phosphate after its oral administration. The total AUC of AZT derivatives in brain tissue exceeded that obtained from AZT administration by >1000-fold, and even at 48 h the total AZT derivative concentration in the brain was 50-fold higher than the minimum AZT concentration needed for anti-HIV activity. These advantages reflect the lipophilic character of the prodrug and the likelihood that both the polar (mannopyranosidyl) ethyl posphodiester and AZT 5'-phosphate may be trapped in brain tissue once formed.

More recently, the S-acyl-2-thioethyl (SATE) group has emerged as a potential phosphate protecting group leading to the intracellular release of 5'-monophosphates of dideoxynucleosides. Lefebvre *et al.* (1995) synthesized the bis(t-butyl-SATE prodrug of AZT monophosphate (AZTMP) shown in Figure 15 that was subsequently evaluated in mice by Tan *et al.* (2000) for its potential to deliver AZT monophosphate to the brain. A probable pathway for bioconversion of the bis(SATE) phosphotriester to the 5'-phosphate is shown in Figure 15. Although not depicted in Figure 15, after carboxylesterase-mediated thioester hydrolysis the 2-thioethyl moiety may cyclize to form episulfide which has been shown to be toxic (Jones and Bischofberger, 1995). Following intravenous administration of the prodrug, high concentrations of both prodrug and AZTMP were detected in brain at 3 min, but these concentrations declined rapidly and could no longer be detected at 30 min. AZT concentrations peaked in brain at 30 min but the terminal half-life of AZT in brain tissue was prolonged relative to brain concentrations after intravenous administration of AZT itself by 2–4-fold. While the overall AUC for AZT in brain tissue was not significantly enhanced after prodrug administration, the distribution ratio (AUC_{brain}/AUC_{blood}) was nearly doubled in comparison to AZT. The prolonged half-life for AZT in brain tissue following prodrug administration is consistent with intracellular delivery of

AZTMP, which may not have been easily detected by the procedures employed due to their sensitivity and to possible phosphate ester hydrolysis during tissue homogenization by highly efficient plasma membrane phosphatases (Van Hoof *et al.*, 1997). Samtani *et al.* (2004) recently demonstrated that artifacts in pharmacokinetic profiles of corticosteroids due to *in vitro* hydrolysis of their phosphate ester prodrugs could be prevented by treatment with enzyme inhibitors such as sodium arsenate.

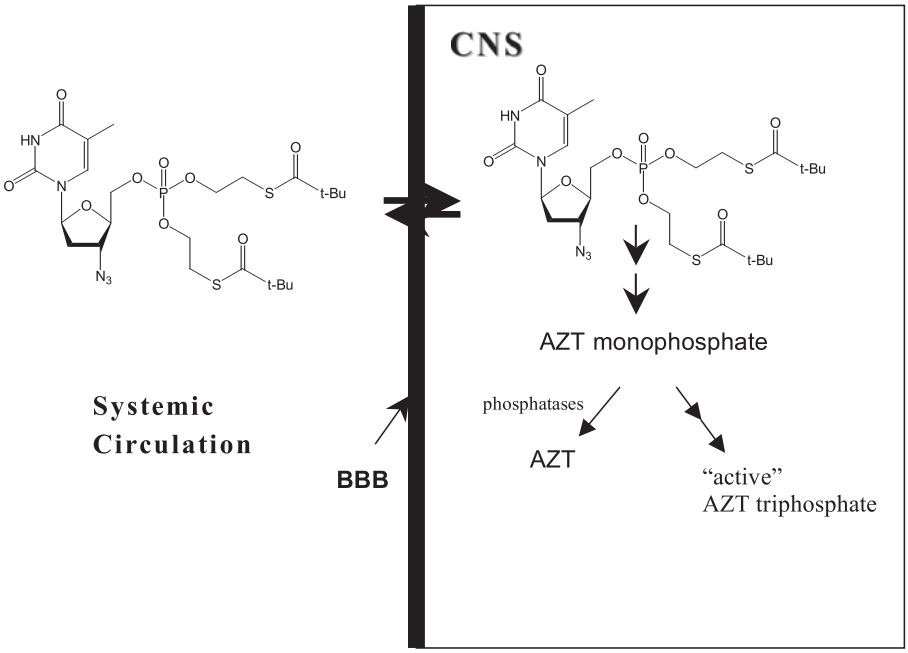


Figure 15. Brain delivery of AZT monophosphate using the bis(t-butyl-SATE) phosphotriester prodrug (Tan *et al.*, 2000).

Schlienger *et al.* (2000) suggested that the slow removal of the 2nd SATE group from the mono(SATE) phosphodiester may limit the formation of 5'-monophosphates from the phosphotriester prodrug. They replaced one of the SATE groups with various aryl groups to produce the phenyl(t-butyl-SATE) phosphotriester and several tyrosinyl(t-butyl-SATE) phosphotriesters, suggesting that the tyrosinyl derivatives may be substrates for the large neutral amino acid transporter system. This hypothesis awaits further study.

Alternatives to Esterase-Activation

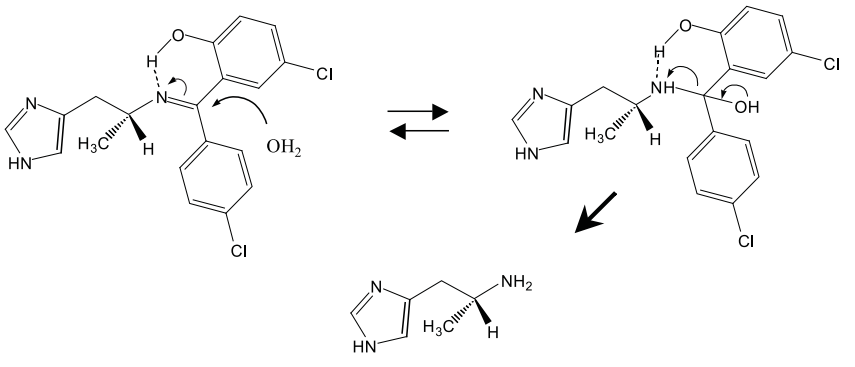
The number of both non-enzymatic and enzymatic alternatives to esterase-mediated prodrug activation is at this point limited only by the imagination. The examples provided below are not intended to be exhaustive but rather illustrative of approaches that have been explored to improve CNS uptake and, depending on the parent drug and the application, might be effective.

Chemical (Non-Enzymatic) Activation

Reliance on brain tissue esterases or other brain tissue enzymes for prodrug bioconversion may be necessary to achieve prodrugs with both adequate formulation stability and rapid conversion *in vivo*. On the other hand, the variability in enzyme activities from species to species and the difficulty in identifying enzyme systems that activate prodrugs with site specificity in the CNS give reason to search for non-enzymatic bioconversion strategies. While such approaches are unlikely to provide brain-selective bioconversion, they may offer delivery advantages over esterase-activated or other prodrugs that are converted preferentially in the periphery over the brain.

Azomethine Prodrugs—(R)-α-Methylhistamine

A recent illustration of the use of chemically activated prodrugs to improve brain penetration is the design of azomethine prodrugs of (R)-α-methylhistamine (Krause *et al.*, 2001), a potent and selective histamine H3-receptor agonist (Figure 16). Because of its strong basicity, (R)-α-methylhistamine exhibits poor penetration across the blood-brain barrier. (R)-α-methylhistamine also exhibits high affinity for histamine-N-methyltransferase and, consequently, methylation is suspected as the cause of this drug's short half-life of only 3 min (Rouleau *et al.*, 1997). The strategy employed to promote both oral bioavailability of the prodrug and CNS delivery of the active drug was to reversibly mask the primary amine by forming a lipophilic azomethine. The imine bond, the result of a classical condensation reaction between a ketone and a primary amine, was demonstrated to undergo hydrolysis to the active form in aqueous buffers (pH 1 and 7.4) and in plasma. The hydrolysis rates in plasma and in liver homogenate were generally similar to the rates in pH 7.4 buffer, indicating that these prodrugs are converted chemically, not enzymatically (Rouleau *et al.*, 1997; Krause *et al.*, 2001).



(R)-α-methylhistamine

Figure 16. Non-enzymatic conversion of an azomethine prodrug of (R)-α-methylhistamine (Krause *et al.*, 2001).

A series of 28 azomethine prodrugs varying in electron-withdrawing substituents on either aromatic ring, steric hindrance, and lipophilicity exhibited widely varying percentages of conversion to parent drug at 30 min, ranging from 1.3 to 100%. Following oral administration, those prodrugs that exhibited >85% hydrolysis in 30 min at pH 1 did not provide detectable plasma levels of intact prodrug. Compounds undergoing less than 10% conversion at pH 7.4 in 30 min produced unacceptably low plasma concentrations of the parent drug, even when they were well absorbed. For those candidates that met the oral bioavailability criteria, CNS ratios calculated from the AUCs of (R)-α-methylhistamine in brain tissue relative to plasma varied from <1% to 39.3%. The authors found within a subset of benzophenone azomethines that CNS penetration of intact prodrug correlated with lipophilicity ($r^2 = 0.714$). In turn, the brain/plasma (R)-α-methylhistamine AUC ratios correlated with CNS penetration of prodrug ($r^2 = 0.907$), suggesting that within this structurally related series of prodrugs with moderate bioconversion rates in plasma, those with higher log P values produced higher cerebral cortex levels of released (R)-α-methylhistamine.

Base-catalyzed Ring Opening of Temozolomide

Temozolomide (Figure 17) is an antitumor prodrug that has exhibited promising activity in patients with high-grade glioma. Glioblastoma tumor cells are particularly aggressive in terms of infiltrating the surrounding healthy brain tissue so that tumor resection must be followed by radiation and often chemotherapy. Temozolomide has been shown to reduce tumor cell invasion in co-culture with primary brain cell aggregates by inducing apoptosis and senescence (Gunther *et al.*, 2003). Clinical trials of temozolomide for the treatment of high-grade glioblastomas have shown increases in progression-free survival (Bower *et al.*, 1997; Janinis *et al.*, 2000).

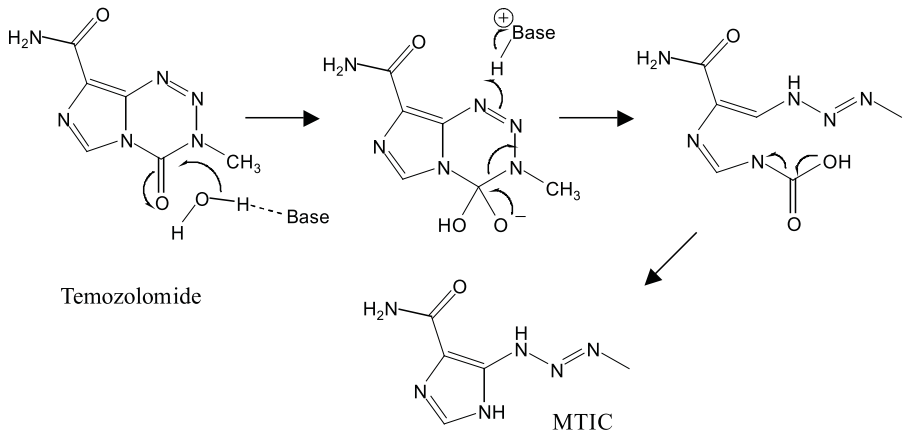


Figure 17. Non-enzymatic, base-catalyzed activation sequence for the conversion of temozolomide to the antitumor agent MTIC (Denny *et al.*, 1994).

The mechanism of chemical activation of temozolomide is via base-catalyzed ring opening to form the bioactive agent 5-(3-methyl-triazen-1-yl) imidazole-4-carboxamide (MTIC) as shown in Figure 17. In pH 7.4 phosphate buffer at 37°C, temozolomide has a half-life of 1.83 h (Denny *et al.*, 1994) while its mean plasma half-life in patients was reported to be 1.81 h (Newlands *et al.*, 1992). The good oral bioavailability and clinical activity of this prodrug against glioma appears to be the result of a combination of its acid stability, blood-brain barrier penetration, and tissue-independent bioconversion at an appropriate rate, although Denny *et al.* (1994) also speculated that a small pH gradient between healthy brain tissue and glioma may provide some advantage for prodrug conversion in tumor tissue.

Oxidoreductase-Activated Prodrugs for CNS Delivery

A variety of class 1 oxidoreductase enzymes found within brain tissue (Minn *et al.*, 1991; Rooseboom *et al.*, 2004) have been targeted for prodrug bioconversion, including aldehyde oxidase, monoamine oxidases, the cytochromes P450, NADPH-cytochrome P450 reductase, and xanthine oxidase. These enzymes are located in many tissues but their activities in brain tissue are in many cases relatively low and may vary from species to species. Aldehyde oxidase (EC 1.2.3.1), for example, was detected at moderate levels in rat brain by immunohistochemical staining but was absent in human brain tissue (Moriwaki *et al.*, 1996, 2001). The cytochrome P450 enzyme superfamily (EC 1.14.14.1) is present in brain tissue but at levels far below those in liver (Minn *et al.*, 1991; Rooseboom *et al.*, 2004).

Dihydropyridine Oxidation

The brain oxidation of a dihydropyridine moiety to pyridine has been discussed previously as a frequently employed method for generating a trapped intermediate from which a sustained drug release within the brain could be achieved. A similar approach has proven effective when the drug itself contains a pyridinium or related group. For example, Sasaki *et al.* (2001) employed a redox activation strategy to generate a quinolizinium cation active drug in brain tissue from a tetrahydro derivative. Erb *et al.* (1999) tested the dihydropyridine form of N-methylnicotinamide, 1,4-dihydro-N-methyl-nicotinamide, as a possible prodrug because N-methylnicotinamide, a choline transporter inhibitor with potential utility for increasing choline levels in the brain, is a quaternary cation and therefore does not permeate across the blood-brain barrier. The prodrug produced fourfold increases in plasma concentrations and 20-fold increases in brain concentrations of N-methylnicotinamide 2 h after administration. The authors found that the oxidation of their dihydropyridine in liver was reduced by inhibitors of cytochrome P450 enzymes while such inhibitors did not affect oxidation in brain tissue. This led the authors to suggest that the oxidation of dihydropyridines in brain tissue is by NADH transhydrogenases rather than a cytochrome P450 enzyme, as also suggested by Bodor and Brewster (1983).

Monoamine Oxidase—N-Dealkylation of Milacemide

Monoamine oxidase (MAO, EC 1.4.3.4) is a mitochondrial enzyme localized within the cerebral capillary endothelial cells (Lai *et al.*, 1978; Hardebo and Owman, 1979, 1980; Hardebo *et al.*, 1980; Betz and Goldstein, 1981; Kalaria and Harik, 1987; Minn *et al.*, 1991), residing on both luminal and abluminal membranes (Inomata *et al.*, 1984). MAO catalyzes the oxidative deamination of primary, secondary, and tertiary amines, including monoamine neurotransmitters such as dopamine, epinephrine, norepinephrine, serotonin, and tyramine (Finberg and Youdim, 1983; Minn *et al.*, 1991). Prodrugs of both monoamine oxidase A and B have been evaluated for their potential to enhance CNS delivery (Yu and Davis, 1990, 1991; Yu *et al.*, 1994; Flaherty *et al.*, 1996).

Milacemide (2-n-pentylaminoacetamide), is a secondary monoamine with anticonvulsant, mood elevating, and memory enhancing effects (Van Dorsser *et al.*, 1983; Roba *et al.*, 1986; Saletu *et al.*, 1986; Handelmann *et al.*, 1989; Schwartz *et al.*, 1991). These actions of milacemide have been attributed to a prodrug effect. MAO B catalyzes the N-dealkylation of milacemide to form glycinamide, ultimately leading to the formation of glycine, a naturally occurring amino acid neurotransmitter (de Varebeke *et al.*, 1988; Semba *et al.*, 1993). De Varebeke *et al.* (1988) found a higher V_{max} for milacemide conversion by MAO-B in rat brain than in liver and CSF/serum AUC ratios of glycinamide in rats were 5.6, 6.0 and 5.5 at 100, 200, and 400 mg/kg i.p. doses, respectively, in rats, illustrating the ability of milacemide to provide impressively high CSF/serum ratios of glycinamide (Semba *et al.*, 1993). Using microdialysis, Doheny *et al.* (1996) found that whereas glycinamide concentrations rose linearly and in a dose-dependent manner in both hippocampus and frontal cortex after milacemide administration to rats, glycine concentrations rose only in the hippocampus. O'Brien *et al.* (1991) found that α-methylmilacemide is also an active anticonvulsant although it is not a substrate for monoamine oxidase, thus casting doubt on whether or not bioconversion is required for activity.

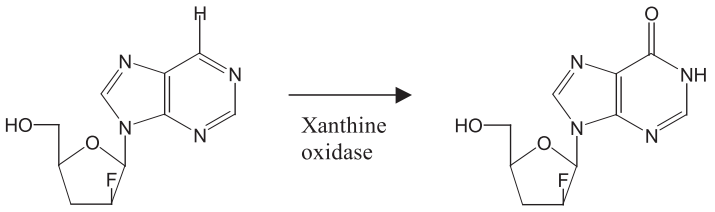
Prodrug approaches that rely on elevated activities of capillary endothelial cell enzymes for favorable bioconversion may risk premature bioconversion in the intestine due to the high activities of at least some of the same enzymes in intestinal mucosa. Consistent with this expectation, the oral bioavailability of milacemide in humans was found to be low due to a 90% first pass metabolism (Roba *et al.*, 1986). An additional concern from the standpoint of prodrug design is that MAO levels in cerebral microvasculature appear to fluctuate significantly from species to species, with rats having activities >tenfold higher than human, mouse, pig, and guinea pig (Kalaria and Harik, 1987).

Xanthine Oxidase—Activation of Antiviral Prodrugs

Xanthine oxidase, a constitutive enzyme present in all cells involved in purine metabolism, catalyzes the oxidation of a large number of substrates, including hypoxanthine and xanthine (Bray, 1975). Although its highest levels are in liver and intestinal mucosa (Al-Khalidi and Chaglassian, 1965), xanthine oxidase is also

present at high concentrations in BBB capillary endothelial cells (Betz, 1985; Lindsay *et al.*, 1991; Terada *et al.*, 1991; Moriwaki *et al.*, 1999).

Krenitsky *et al.* (1984) utilized xanthine oxidase activation to enhance the oral bioavailability of acyclovir by synthesizing 6-deoxyacyclovir, which was 18 times more soluble than acyclovir (Jones *et al.*, 1987). They found that 6-deoxyacyclovir was oxidized by both xanthine oxidase and aldehyde oxidase, but only the xanthine oxidase reaction produced acyclovir. Shanmuganathan *et al.* (1994) attempted to use the same approach to enhance the brain delivery of the 2',3'-dideoxynucleosides ddI and 2'-F-ara-ddI. The ddI prodrug was too unstable in acidic media, so the authors emphasized the 2'-F-ara-ddP prodrug of 2'-F-ara-ddI in their studies, depicted in Figure 18. Oral administration of 2'-F-ara-ddI in mice did not produce detectable levels of drug in brain tissue even though the serum AUC after oral 2'-F-ara-ddI was similar to those produced from either oral or i.v. 2'-F-ara-ddP. Both oral and intravenous administration of 2'-F-ara-ddP produced nearly the same 2'-F-ara-ddI AUC in brain tissue, suggesting that xanthine oxidase bioconversion in brain tissue could enhance CNS delivery. The oral bioavailability of 2'-F-ara-ddP was only 60.7%, yet the AUC for 2'-F-ara-ddI in brain tissue was the same after i.v. or oral prodrug administration. This is difficult to rationalize if the CNS uptake is due to a prodrug effect.



2'-F-ara-ddP 2'-F-ara-ddI

Figure 18. Xanthine oxidase mediated conversion of 2'-F-ara-ddP to 2'-F-ara-ddI (Shanmuganathan (above Figure 18) *et al.*, 1994).

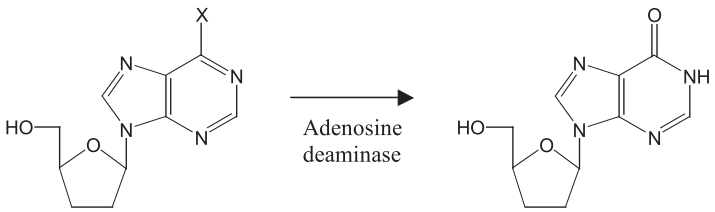
Manouilov *et al.* (1997) prepared 9-(β-D-1,3-dioxolan-4-yl)-2-aminopurine (APD) as a prodrug of the antiviral agent 9-(β-D-1,3-dioxolan-4-yl)guanine (DXG). They found that their prodrug was very stable in mouse brain homogenate (t1/2= 46.2 h) compared to liver homogenate where the half-life was only 0.34 h. (The 2'-F-ara-ddP prodrug evaluated by Shanmuganathan (above) exhibited a shorter t1/2 in mouse liver homogenate (3.54 min) but also very slow and incomplete conversion in brain homogenate.) The oral bioavailability of APD was only 41%, yet the AUC for DXG in serum and brain were similar after either oral or i.v. administration of the prodrug, indicating no demonstrable advantage of the prodrug for CNS delivery. The mixed outcomes produced when two groups employed the same strategy for similar compounds and the relatively low brain activity of xanthine oxidase in comparison to the liver suggest that this approach is unlikely to be of general utility.

Transferase-activated Prodrugs for CNS Delivery

Adenosine Deaminase

Adenosine deaminase (ADA), a ubiquitous catabolic enzyme involved in purine salvage in nearly all tissues of animals (Brady and O'Donovan, 1965; Ho *et al.*, 1980) and humans (Van der Weyden and Kelley, 1976; Ho *et al.*, 1980), catalyzes the irreversible hydrolytic deamination of adenosine or deoxyadenosine to produce inosine and ammonia (Zielke and Suelter, 1971; Van der Weyden and Kelley, 1976). Brain tissue levels of adenosine deaminase exceed those in whole blood (Brady and O'Donovan, 1965; Van der Weyden and Kelley, 1976) and appear to be concentrated in brain microvessel endothelial cells (Mistry and Drummond, 1986; Schrader *et al.*, 1987; Johnson and Anderson, 1996) although the highest levels are in the spleen and intestine (Brady and O'Donovan, 1965; Van der Weyden and Kelley, 1976; Mohamedali *et al.*, 1993).

The relatively high brain tissue activity of ADA has led several groups to consider lipophilic ADA-activated prodrugs (Figure 19) of the dideoxynucleoside reverse transcriptase inhibitors as a means of improving the CNS activity of this class of agents. 2',3'-Dideoxyadenosine (ddA), one of the earliest anti-HIV dideoxynucleosides to be evaluated, undergoes extremely rapid ADA-catalyzed deamination to 2'3'-dideoxyinosine (ddI),. Thus, Russell and Klunk (1989) were unable to detect intact ddA in mouse plasma after an oral dose. In rhesus monkeys the estimated plasma elimination half-life for ddA is 24 s (Hawkins *et al.*, 1995).



X = NH_2, F, Cl, Br, I

2',3'-dideoxyinosine

Figure 19. Adenosine deaminase activation of lipophilic prodrugs of ddI (Anderson *et al.*, 1992; Morgan *et al.*, 1992).

Although ddI was ultimately developed as a therapeutic anti-HIV agent in place of ddA, ddI itself is a relatively polar molecule with an octanol/water partition coefficient of 0.055 (Barchi *et al.*, 1991), approximately 15-fold less lipophilic than AZT, and poor CNS entry. In rats, the steady-state concentration of ddI in cerebrospinal fluid was reported to be only 2% of that in plasma (Anderson *et al.*, 1990b; Galinsky *et al.*, 1991) while in rhesus monkeys the percentage in CSF obtained from AUC ratios was 4.8% (Hawkins *et al.*, 1995). Studies of the influence of ddI on dementia in HIV-infected children suggested

similarly low CSF values of <5% and highly variable effects on IQ scores (Brouwers, 1991; Butler *et al.*, 1991; Balis *et al.*, 1992). Moreover, ddI is highly unstable under acidic conditions (Anderson *et al.*, 1988) complicating its oral delivery, and ddI is also a substrate for purine nucleoside phosphorylase, which may further contribute to its low CNS penetration (Johnson and Anderson, 1996, 2000).

Recognizing these deficiencies, Marquez *et al.* (1987; 1990) prepared the acid stable 2'-fluorinated analogs of ddA and ddI. F-ddA is tenfold more lipophilic than ddA, and the 2'-fluoro substituent reduces the ADA deamination rate by approximately 20-fold (Driscoll, 2000). Steady-state brain parenchyma/plasma concentration ratios for F-ddI were approximately fivefold higher from intravenous infusions of F-ddA in rats compared to infusions of F-ddI alone (Singhal *et al.*, 1996). Johnson *et al.* (1996) demonstrated in bovine brain microvascular endothelial cell cultures that the localization of ADA in the blood-brain barrier could account for the enhanced brain delivery observed. In rhesus monkeys, the AUC CSF/plasma ratio for total dideoxynucleoside (prodrug plus metabolite) was increased from 0.017 after an i.v. bolus dose of F-ddI to 0.06 after F-ddA administration (Roth *et al.*, 1999), in reasonable agreement with the results obtained in rats.

Similarly, Wen *et al.* (1995) evaluated (-)-6-aminocarbovir as an ADA-activated prodrug of (-)-carbovir. The brain/blood carbovir concentration ratio in rats increased from 0.032 after an i.v. infusion of carbovir to 0.08 after i.v. infusion of the prodrug.

While all of the *in vivo* studies of the CNS delivery potential of 6-amino-substituted prodrugs of dideoxynucleosides have indicated some degree of improvement over the 6-HO-substituted parent compounds, the degree of improvement was modest. Although NH_2 is the preferred leaving group for ADA-catalyzed hydrolysis, the increase in lipophilicity provided by this substituent is only ~tenfold. Recognizing that other substituents can also be removed by ADA (Chassy and Suhadolnik, 1967; Driscoll, 2000), several groups have explored a variety of alternatives including 6-halo, 6-mercapto, 6-alkylamino, and 6-alkoxy substituents as promoieties (Marquez *et al.*, 1987, 1990; Chu *et al.*, 1990b; Shirasaka *et al.*, 1990; Barchi *et al.*, 1991; Murakami *et al.*, 1991; Burns *et al.*, 1993; Ford *et al.*, 1995; Driscoll *et al.*, 1996). An examination of the relative hydrolysis rates of various derivatives in these publications reveals that adenosine deaminase is quite selective in terms of the permitted size of the substituent at the 6-position for rapid bioconversion. In the F-ddI prodrug series, for example, the relative ADA-catalyzed hydrolysis rates for various substituents at the 6-position were (NH_2 - = 100): F-, 202; NO_2-, 58; Cl-, 2.0, Br-,1.7; CH_3NH- , 0.9; CH_3O-, 0.6; I-, 0.3.

Other than the 6-amino substituted prodrugs, the 6-halo prodrugs have generated the greatest interest due to their increased lipophilicity combined with moderate bioconversion rates. Anderson and coworkers evaluated the enhancement in central nervous system uptake of ddI after administration of the 6-Cl-, 6-Br-, and 6-I-dideoxypurine prodrugs in rats (Anderson *et al.*, 1992; Morgan *et al.*, 1992), demonstrating that the brain tissue concentrations of ddI

were significantly elevated after i.v. infusion of 6-Cl-ddP (fivefold) and 6-Br-ddP (twofold) in comparison to an infusion of ddI, while 6-I-ddP led to a fourfold lower brain tissue concentration due to a corresponding reduction in plasma concentrations. These outcomes correspond to observed half-lives for conversion in rat brain tissue homogenate at 37°C of 19, 20, and 178 min, respectively. It appears that the slow conversion of 6-I-ddP in both brain tissue and blood could account for the lower tissue levels of ddI, although the ratios of ddI concentration in brain parenchyma-to-plasma after 30 min prodrug infusions were 0.67, 0.40, and 0.48 for 6-Cl-ddP, 6-Br-ddP, and 6-I-ddP, respectively, while the corresponding ratio for ddI administration was only 0.02. The 6-Cl and 6-I-ddG derivatives of 2',3'-dideoxyguanosine (ddG) also gave higher CSF-to-plasma percentages for ddG following an intravenous dose in rhesus monkeys (6-Cl, 24%; 6-I, 17%, ddG, 8.5%) (Hawkins *et al.*, 1995).

The 6-Cl derivative of F-ddI was also evaluated in rats although there was no comparison to an F-ddI-only control (Driscoll, 2000). However, Singhal *et al.* (1997) had previously shown that the steady-state ratio of F-ddI concentration in CSF normalized to the plasma concentration is only 1.1%. Infusion of the 6-Cl prodrug at a twofold higher infusion rate produced comparable CSF concentrations of F-ddI but lower plasma concentrations due to the slow hydrolysis rate of the 6-Cl derivative. This led to an enhanced steady-state F-ddI CSF/plasma concentration ratio approaching nearly 10%.

Anderson *et al.* (1992; 1996) combined model simulations with experimental determinations of the CNS delivery of two series of prodrugs of ddI, a set of 5'-acyl esters and a set of 6-halo-dideoxypurines (ddPs), to illustrate the importance of selective bioconversion to enhance CNS delivery. The prodrug parameters compared were their octanol/water partition coefficients, rate constants for bioconversion in rat blood, and rate constants for bioconversion in rat brain tissue homogenate Significant overlap existed in the octanol/water partition coefficients within the two series, indicating that similar access of prodrug to the brain could be realized within the two sets and that the brain tissue bioconversion rates did not differ greatly between the two sets. However, the two series did differ dramatically in their conversion in blood or plasma, with the 5'-esters undergoing very rapid hydrolysis in plasma ($t_{1/2}$ generally < 1 min) in contrast to the 6-halo-substituted prodrugs, which are quite stable in blood ($t_{1/2}$ > 4 h). The higher rates of 6-halo-ddP conversion in brain tissue homogenate relative to blood are consistent with the favorable distribution of ADA in the cerebral microvasculature (Johnson and Anderson, 1996). The simulations predicted that the 6-halo-substituted prodrugs would exhibit higher CNS/plasma concentrations than the 5'-esters, as observed experimentally, confirming the importance of optimal bioconversion rates and higher conversion rate selectivity in brain tissue relative to the systemic circulation.

Although ADA is preferentially localized in cerebral capillary endothelial cells, its highest levels are found in the spleen, intestine, lung, and colon (Ho *et al.*, 1980). The high intestinal activity may seriously limit commercialization of prodrugs that must also exhibit oral bioavailability. Anderson *et al.* (1995)

reported that the oral bioavailability of the intact 6-Cl-ddP prodrug was 7% ± 3% compared to a portal bioavailability of 97% ± 11%, suggesting either poor absorption or extensive gut wall metabolism. Poor absorption was ruled out by the appearance of >50% of the dose as ddI in the systemic circulation after an oral dose of the prodrug, confirming that premature activation by intestinal ADA occurs. The same authors were able to demonstrate in perfusion studies in the mesenteric vein-cannulated rat ileum model that it is possible to inhibit local ADA-mediated bioconversion of the 6-Cl-ddP riboside (Singhal and Anderson, 1998; Singhal *et al.*, 1998) and 2'-F-ddA (DeGraw and Anderson, 2001) while at the same time avoiding significant inhibition of systemic ADA by selecting an appropriate enzyme inhibitor and inhibitor concentration. The inhibitor that exhibited the optimal characteristics for local ADA inhibition was *erythro*-9-(2-hydroxy-3-nonyl) adenine ((+)-EHNA).

Thymidine Phosphorylase/Cytidine Deaminase

Thymidine phosphorylase (TP) catalyzes the reversible conversion of thymidine to thymine and deoxyribose-1-phosphate and is, therefore, involved in nucleotide salvage (Rooseboom *et al.*, 2004). Although thymidine phosphorylase is present in many tissues, its activity is elevated in solid tumors but with considerable inter-patient variability (Miwa *et al.*, 1998). Blanquicett *et al.* (2002) recently demonstrated a 6.2-fold elevation of TP in glioblastoma multiforme relative to normal human brain tissue and further showed that TP mRNA could be increased by ~70-fold 4 days after radiation treatment.

Induction of thymidine phosphorylase may be advantageous in the treatment of brain tumors when used in conjunction with administration of a TP-activated prodrug, such as capecitabine. Capecitabine (Figure 20) is a triple prodrug of 5-fluorouracil (5-FU) that requires initial activation by carboxylesterase followed by conversion to 5'-deoxy-5-fluorouridine-catalyzed by cytidine deaminase and, finally, conversion to the cytoxoic agent 5-FU by thymidine phosphorylase.

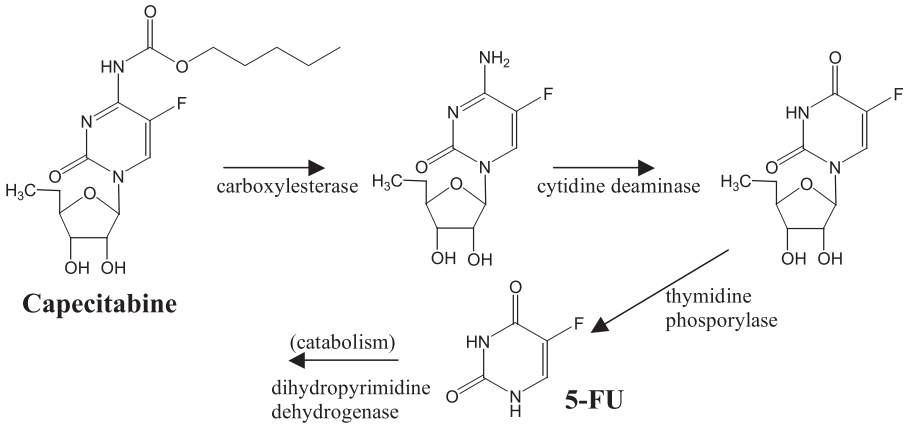


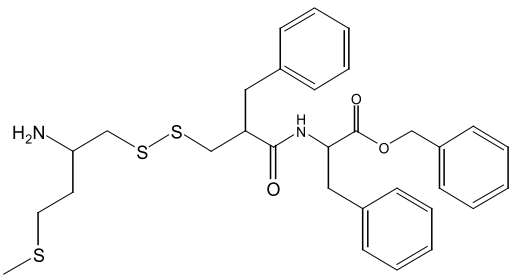
Figure 20. Bioconversion of the triple prodrug capecitabine to the antitumor agent 5-FU (Tsukamoto *et al.*, 2001).

Tsukamoto *et al.* (2001) showed that the antitumor efficacy of capecitabine is determined largely by three factors: 1) tumor-specific activation by TP; 2) 5-FU elimination in the tumor by the enzyme dihydropyrimidine dehydrogenase (DPD); and 3) the tumor blood flow rate. Whereas Blanquicett *et al.* (2002) found that radiation produced ~ 70-fold elevation in TP mRNA expression, DPD levels were unaffected so that the TP/DPD ratio in tumors could be dramatically increased. A recent case study reported the successful treatment of brain metastasis with capecitabine (Wang *et al.*, 2001).

Glutathione/Glutathione S-Transferase

Glutathione S-transferase (EC 2.5.1.18) plays an important role as a detoxifying enzyme, catalyzing the formation of drug conjugates with the tripeptide glutathione (GSH). It is present in virtually all tissues with especially high activities (in rats) in the testis, liver, intestine, and kidney. Brain cytosol activity is also high in the rat (DePierre and Morgenstern, 1983). Microsomal mRNA expression in human tissues is highest in the liver, kidney, and colon but brain levels are also high (Estonius *et al.*, 1999). As shown by the examples below, glutathione and/or glutathione S-transferase in brain tissue may also be exploited for prodrug activation.

In seeking more potent aminopeptidase N inhibitors capable of crossing the blood-brain barrier, Fournié-Zaluski *et al.* (1992a) observed that various β-amino thiols were potent inhibitors *in vitro* but inactive as antinociceptive agents upon i.v. administration in mice unless they were administered in their disulfide prodrug forms. Incubation of the disulfides in rat plasma serum at 37°C for 1 h had no effect, while under the same conditions the disulfides were cleaved in rat brain homogenate. Since inhibition of only one of the two aminopeptidases leads to only modest analgesic responses, the same group also developed mixed inhibitor-prodrugs by covalently linking thiol inhibitors of both aminopeptidase N and neutral endopeptidase (Fournie-Zaluski *et al.*, 1992b). One series of mixed inhibitor-prodrugs utilized a disulfide bridge between the free mercapto groups of both inhibitors. The most active of these mixed inhibitor-prodrugs, RB 101 (Figure 21) exhibited threefold greater analgesic potency after i.v. administration

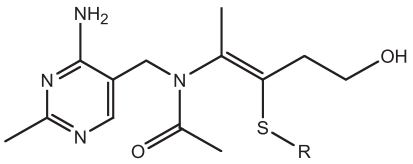


RB101

Figure 21. Structure of RB101, a brain tissue glutathione/glutathione S-transferase-activated prodrug of a dual inhibitor of enkephalin-metabolizing enzymes (Fournie-Zaluski *et al.*, 1992).

than that of a similar combined dose of the two constitutive inhibitors (Fournie-Zaluski *et al.*, 1992b; Noble *et al.*, 1992). HPLC studies indicated that the disulfide was resistant to serum enzymes but cleaved by brain homogenates in a saturable process that was dependent on protein concentration. These results suggest that the conversion was enzyme-mediated, although they did not show that glutathione S-transferase was responsible for the conversion.

Yoshikawa *et al.* (1999) compared the stabilities of a series of thioester, thiocarbonate, and disulfide prodrugs of thiamine in human and rat blood and rat brain (Table 2). In accordance with results discussed previously, the esterase-activated prodrugs exhibited slower bioconversion in brain tissue homogenate than in rat blood. The highly lipophilic hexadecanoic acid ester, which hydrolyzed modestly faster in brain tissue albeit relatively slowly in both blood and brain tissue, appears to be an exception. Most striking is the observation that the disulfides were converted quite rapidly to the parent thiols in both blood and brain homogenates, with preferential conversion in brain tissue. This conversion reaction was believed to involve intracellular glutathione or hemoglobin (Ishikura *et al.*, 1995). The same reaction was employed as the initial activation step in the design of drug delivery systems based on intramolecular thiolate-mediated ring closure to form a "locked-in" quaternary thiazolium intermediate described earlier (Figure 12).



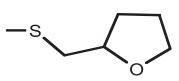
R	$t_{1/2}$ (brain) (min)	$t_{1/2}$ (blood) (min)	Brain/Blood Bioconversion Rate Ratio
-COOMe	110	27	0.25
-COOEt	62	21	0.34
-COOBenzyl	173	7	0.04
-COEt	7	3	0.43
$-CO(CH_2)_4CH_3$	50	4	0.08
$-CO(CH_2)_7CH_3$	12	3	0.25
$-CO(CH_2)_{14}CH_3$	23	42	1.83
$-SCH(CH_3)_2$	0.5	1	2
	<0.5	0.5	>1

Table 2. Conversion half-lives in rat blood and brain tissue homogenate and brain/blood (rat) bioconversion rate ratios for various thiamine prodrugs at 37°C. (Yoshikawa *et al.*, 1999)

The disulfide bond has emerged as a favored linker for brain delivery of chimeric peptide macromolecules consisting of a drug molecule (e.g., neuropeptide) linked to a targeting vector (e.g., antibody) that have been proposed to facilitate brain uptake via receptor-mediated transcytosis (Pardridge, 1991, 1993; Bickel and Kang, 1999; Bickel *et al.*, 2001). An example of this approach is described in the section "Transporter/Receptor-Mediated Prodrug Delivery."

Gene-Directed Enzyme Prodrug Therapy (GDEPT)

The ubiquitous distribution of most endogenous enzymes limits the targeting potential of prodrug strategies that rely on activation by endogenous brain tissue enzymes. Gene therapy offers the potential to achieve specific tissue targeting by introducing a gene encoding an exogenous prodrug-bioconverting enzyme specifically into the tissue being targeted. A particularly active area of research in this area has been directed toward site-specific prodrug activation in brain tumors.

5-Fluorocytosine (5-FC) has been evaluated for potential gene-directed enzyme prodrug therapy for brain tumors in several reports (Mullen, 1992; Dong *et al.*, 1996; Ge *et al.*, 1997; Aghi *et al.*, 1998; Wang *et al.*, 1998). 5-FC is a nontoxic prodrug that is activated to 5-fluorouracil by bacterial cytosine deaminase, an enzyme that is not found in mammals. As illustrated in Figure 22, the GDEPT concept involves intratumoral administration of a suitable vector containing the gene encoding cytosine deaminase to induce gene expression in tumor cells. Systemic administration of 5-FC is followed by passage of the prodrug across the blood-brain barrier, conversion within the genetically modified tumor cells to 5-FU, and further metabolism by endogenous kinases to the cytotoxic phosphorylated nucleotide monophosphate and triphosphate, which damage both DNA and RNA.

Rehemtulla *et al.* (2002) described several potential advantages that cytosine deaminase gene therapy might have over other approaches for CNS malignancies such as gliomas. First, 5-FU is not a cell-cycle specific cytotoxic agent so cell-killing will not be limited to dividing tumor cells. Secondly, 5-FC has high bioavailability and penetrates the blood brain barrier (Bourke *et al.*, 1973; Block and Bennett, 1974). Thirdly, 5-FU also can diffuse across cell membranes thus increasing the likelihood for improved cell killing due to a bystander effect even in tumor cells that do not express cytosine deaminase. Finally, 5-FU is also a potent radiosensitizer (Pu *et al.*, 1995).

In another frequently adopted approach, thymidine kinase gene therapy is followed by coadministration of the prodrug ganciclovir, which is phosphorylated by thymidine kinase resulting in blockage of DNA replication and induction of apoptosis (Ishii-Morita *et al.*, 1997). Several gene therapy studies using this strategy have been conducted in animals (Ezzeddine *et al.*, 1991; Culver *et al.*, 1992; Ram *et al.*, 1993; Izquierdo *et al.*, 1995) and in humans (Klatzmann *et al.*, 1998; Shand *et al.*, 1999). The first large randomized controlled Phase III clinical trial of gene therapy for the treatment of gioblastoma multiforme utilizing

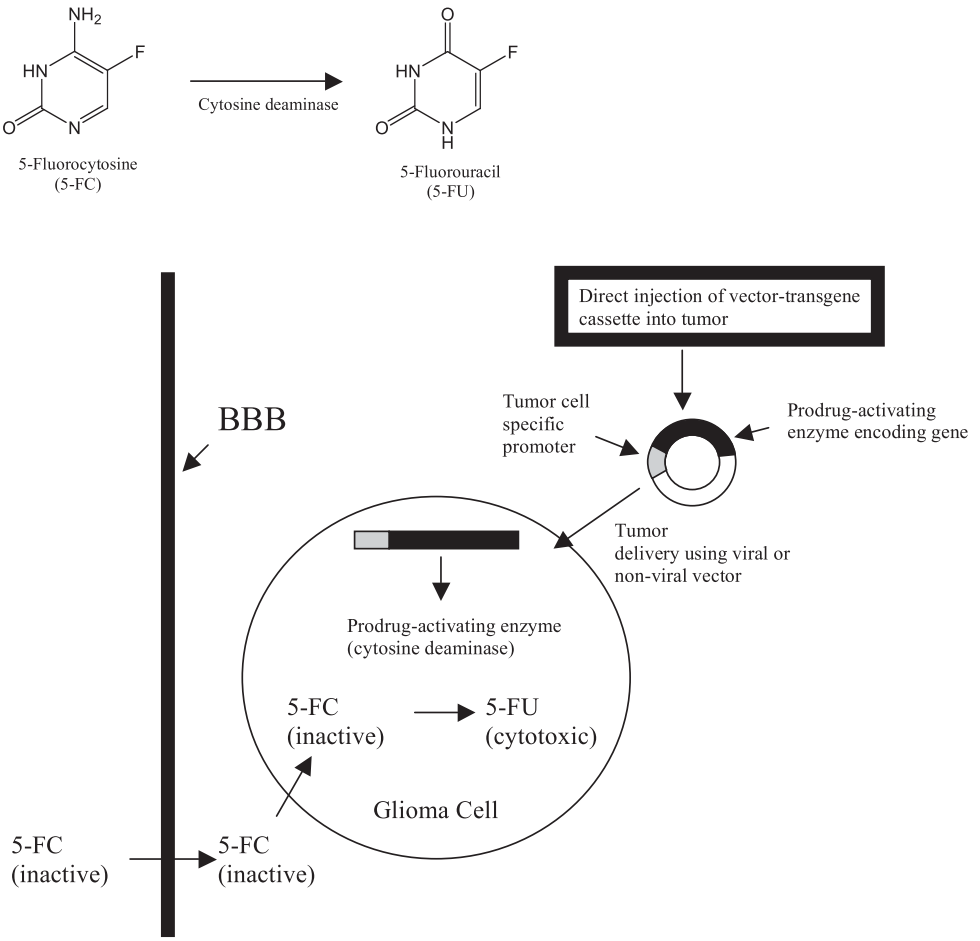


Figure 22. Illustration of the GDEPT (gene-directed enzyme prodrug therapy) approach for site-specific activation of a cytotoxic drug (5-FU) to brain tumor cells by transfecting tumor cells with a foreign prodrug-activating enzyme encoding gene.

retrovirus-mediated transduction of glioblastoma cells with the herpes simplex virus thymidine kinase gene, administered immediately after surgical resection of the tumor, produced no significant benefit in survival (Rainov, 2000). This failure was attributed to the poor transduction rate of the locally administered vector-producing cells employed. Vector-producing cells survive for only limited periods of time after tumor implantation due to host humoral and cellular immune responses (Shand *et al.*, 1999; Rainov, 2000). Methods to extend survival of vector-producing cells (Nafe *et al.*, 2003) and to improve the gene transfer vectors continue to be active areas of research (Jacobs *et al.*, 2002; Rehemtulla *et al.*, 2002; Wang *et al.*, 2003). Inherently migratory neural stem cells also appear to be promising delivery vehicles for brain tumor targeting of prodrug activating enzyme-producing genes (Aboody *et al.*, 2000; Brown *et al.*, 2003).

Transporter/Receptor-Mediated Prodrug Delivery

Receptor-Mediated Transcytotic Pathways

Macromolecule drug carrier-conjugates may be useful for the delivery of oligopeptides or other large molecule drugs such as antisense agents, recombinant proteins, or gene medicines (Pardridge, 1993, 2001, 2002). Macromolecular delivery systems take advantage of naturally occurring BBB membrane proteins by binding on the cell surface followed by receptor-mediated or adsorptive endocytosis and exocytosis from the blood-brain barrier cells into brain tissue. For example, the transferrin receptor is present at high concentrations on the brain capillary vascular endothelium (Jefferies *et al.*, 1984). A mouse monoclonal antibody to this receptor, OX26, as well as drug-antibody conjugates have been shown to cross the blood-brain barrier (Friden *et al.*, 1991; Saito *et al.*, 1995).

The OX26 monoclonal antibody has proven to be one of the more successful model vectors for drug delivery to the brain (Pardridge, 1991). Bioreversible coupling of different peptide ligands to the vector was achieved by biotinylating the peptides using a disulfide-based coupling strategy. Binding of the biotinylated peptide to the targeting vector was made possible by using a covalent OX26-avidin conjugate as illustrated in Figure 23.

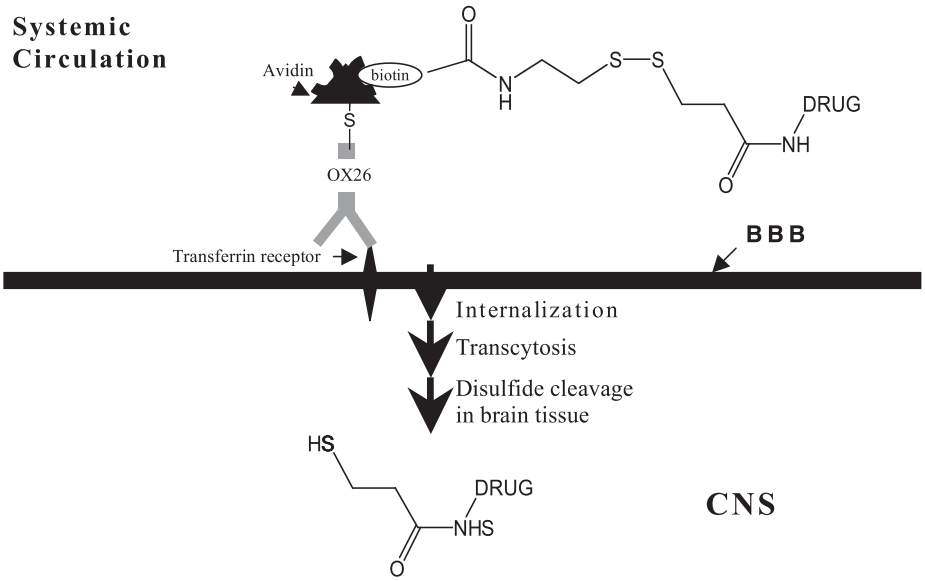


Figure 23. Schematic of an avidin-biotin linked chimeric peptide (Pardridge, 1993; Bickel *et al.*, 2001). The peptide is covalently attached to biotin through a disulfide linkage. Avidin is linked to OX26, a monoclonal antibody to the rat transferrin receptor, through a thioether bond. Delivery of the peptide drug to the brain requires the following steps: (1) binding of the antibody-drug conjugate to the BBB transferrin receptor; (2) receptor-mediated internalization and transcytosis of the drug carrier-conjugate; (3) disulfide cleavage to release the peptide drug.

For this approach to achieve the goal of enhancing the delivery of a therapeutic peptide to the brain, several issues regarding the linker strategy had to be resolved. Bickel *et al.* (1995) considered these issues in the delivery of the μ-opioid neuropeptide [Lys^7] dermorphin (K7DA). First it was shown that the intact chimeric peptide conjugate was inactive, establishing that a reversible linker would be required, while the K7DA peptide with a mercaptopropionate group attached to the Lys^7 moiety retained its activity. Second, the linker had to be sufficiently stable in the circulation *in vivo* to allow sufficient time for uptake into the brain and stable during passage across the blood-brain barrier while undergoing conversion to the active peptide after transcytosis. An intracerebroventricular injection of the fully intact antibody-drug conjugate produced a delayed but dose-related analgesic response in rats consistent with disulfide bond cleavage in brain tissue. *In vitro* studies demonstrated that cleavage also occurred with dithiothreitol, and earlier experiments had demonstrated that the disulfide linker cleavage occurred rapidly in brain tissue homogenate (Pardridge *et al.*, 1990b). HPLC analysis of plasma and brain tissue 60 min after an i.v. injection of radiolabeled chimeric peptide-antibody conjugate indicated that the conjugate was largely intact in plasma while the predominant radiolabel-containing fragment in brain tissue was the product of disulfide bond cleavage. Perfusions of the antibody-drug conjugate in the carotid artery also produced peptide uptake and 40% disulfide cleavage in brain tissue within the 10-min brain perfusion. Isolated brain microvessels did not cleave the conjugate. This was consistent with previous studies demonstrating that disulfide cleavage does not occur in endosomes (Feener *et al.*, 1990) because it is the cytosol where high levels of glutathione are localized (Lodish and Kong, 1993). The authors considered the possibility that the brain uptake after i.v. administration of the conjugate could be attributed to the uptake of the low molecular weight disulfide cleavage product produced in the circulation, but they did not separately explore the brain uptake of this metabolite.

An earlier study of a disulfide-linked avidin-OX26 conjugate with a vasoactive intestinal peptide analog separately evaluated the effect of systemic administration of the avidin-OX26 targeting vector alone, the biotinylated-SS-peptide analog alone, and the complete conjugate (Bickel *et al.*, 1993). Only the disulfide-linked, peptide-targeting vector conjugate produced a significant increase in cerebral blood flow, demonstrating for the first time *in vivo*, according to the authors, that this strategy could be used to deliver neuropeptides to the brain to achieve a pharmacological effect.

Small Molecule Transporters

Apart from receptor- or adsorptive-mediated transcytosis for large molecules, the delivery of low molecular weight drugs may be facilitated by "piggy-backing" through attachment to a small molecule nutrient molecule. Small, hydrophilic nutrients including hexoses, low molecular weight carboxylic acids, organic cations, a variety of L-amino acids, peptides and nucleosides enter the brain via

blood-brain barrier uptake transporters (Smith, 1993; Tamai and Tsuji, 1996, 2000; Lee *et al.*, 2001). A limited number of these have been successfully exploited as carriers for prodrugs, although success has been mixed for several possible reasons. First, any prodrug that is a substrate for one of the blood-brain barrier uptake transporters must compete with high affinity endogenous substrates, the transport of which is presumably the etiological basis for the presence of the carrier. Secondly, some of the transporters may have very low capacity and therefore are efficient carriers only at low plasma concentrations of substrate. Finally, the carrier may be quite selective for its intended substrates and may not recognize prodrugs in which the drug portion is a large molecule.

Two of the more frequently exploited transporters will be discussed below.

Large Amino Acid Transporter (LAT1)

The supply of large neutral amino acids in the brain is regulated by the large amino acid transporter (LAT1), which is selectively expressed in the blood-brain barrier (Boado *et al.*, 1999). L-dopa, a derivative of dopamine, represents a prototypical example of a prodrug substrate for LAT1 (Wade and Katzman, 1975). Dopamine is poorly transported across the blood-brain barrier and also rapidly degraded during its passage by monoamine oxidase (Hardebo and Owman, 1979; Hardebo *et al.*, 1979). L-Dopa undergoes LAT1-mediated BBB uptake and is decarboxylated to the neurotransmitter dopamine by L-amino acid decarboxylase, which is localized in the capillary endothelium (Hardebo *et al.*, 1979). However, because L-dopa is also extensively (95%) metabolized in the peripheral circulation, prodrugs of dopa with improved CNS delivery are still needed.

A recent example of the successful application of LAT1-mediated prodrug uptake is the demonstration of the facilitated uptake of 4-chlorokynurenine and subsequent conversion in brain tissue to potent NMDA receptor antagonist 7-chlorokynurenic acid (Figure 24) (Hokari *et al.*, 1996). N-Methyl-D-aspartate (NMDA) receptor antagonists are of interest because of the important roles these receptors play in neuronal damage induced by the release of excitatory amino acids following head injuries, seizures, and stroke. In the early stages of neurodegeneration, brain levels of the NMDA glycineB receptor antagonist kynurenic acid are elevated due to a surge in its formation and release in activated astrocytes (Gramsbergen *et al.*, 1997; Ceresoli-Borroni *et al.*, 1999). Kynurenic acid is formed from an endogenous precursor, kynurenine, by the kynurenine aminotransferases (Varasi *et al.*, 1996; Guidetti *et al.*, 2000), which are identical to glutamine transaminase K and α-aminoadipate transaminase (Cooper, 2004). Analogs of kynurenic acid such as 7-chlorokynurenic acid and 5,7-dichlorokynurenic acid exhibit superior potency and selectivity for the NMDA receptor *in vitro* but minimal brain penetration (Leeson and Iversen, 1994). Their precursors, L-4-chlorokynurenine and L-4,6-dichlorokynurenine, are prodrugs that are also converted in astrocytes to the corresponding active drugs (Salituro *et al.*, 1994). Lee and Schwarcz (2001) demonstrated that 4-Cl-kynurenine administration

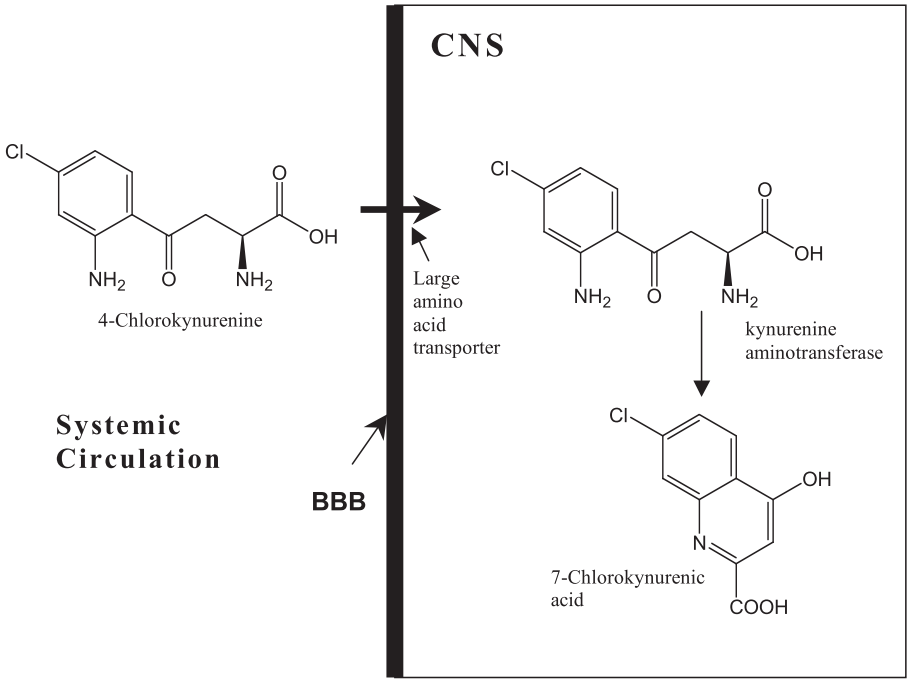


Figure 24. Facilitated brain uptake of 4-chlorokynurenine is followed by kynurenine transaminase catalyzed bioconversion to 7-chloorokynurenic acid, a potent NMDA receptor antagonist (Hokari *et al.*, 1996).

provides enhanced 7-Cl-kynurenic acid formation disproportionately at the site of an emerging excitoxic lesion by targeting the activated astrocytes. Similarly, Wu *et al.* (2002) showed using microdialysis that systemic administration of the convulsant kainic acid resulted in enhanced intracellular production of 7-Cl-kynurenic acid from its prodrug, leading to elevated extracellular concentrations.

Glucose Transporter (GLUT 1)

GLUT 1 is the predominant glucose transporter present in the blood-brain barrier (Pardridge *et al.*, 1990a; Farrell and Pardridge, 1991; Tamai and Tsuji, 2000) and has recently been an attractive transporter for prodrug delivery. An approach described previously for improving the CNS delivery of AZT monophosphate involved the synthesis of the polar (mannopyranosidyl)ethyl phosphodiester of AZT (Henin *et al.*, 1991; Namane *et al.*, 1992). The unexpectedly enhanced delivery of this polar phosphodiester was attributed to a carrier-mediated process but this was not conclusively demonstrated.

Bonina *et al.* (2003) prepared a set of derivatives of L-dopa and dopamine linked through a succinic acid spacer to either the C-3 position of glucose or the C-6 position of mannose. *In vitro* hydrolysis half-lives in rat plasma were quite slow for all prodrugs, ranging from 3–5 h. The succinates were inactive while all four prodrugs were active in reducing morphine-induced locomotion in mice.

The C-3 glucose ester of L-dopa and C-6 mannose ester of dopamine (Figure 25) were the most potent. Both dopamine derivatives were equally active in reversing the reserpine-induced hypolocomotion in rats, and more active than either L-dopa or the L-dopa prodrugs. Oldendorf (1971) had explored glycosylated dopamine derivatives much earlier but found no significant modification of spontaneous activity in reserpinized mice. Bonina (2003) suggested that the time frame of the analyses or a difference in the rat and mouse in either transporter or hydrolytic activity may be involved. These investigators (Battaglia *et al.*, 2000; Bonina *et al.*, 2000) have also applied the glycosylation strategy to improve the CNS delivery of 7-chlorokynurenic acid.

Fernandez *et al.* (2003) explored a wider range of glycosylated dopamine derivatives, including succinyl linked esters at the C-6, C-3, and C-1 positions of glucose or carbamate-linked prodrugs at the same positions (see Figure 25 for an example). They compared the stabilities of these prodrugs as well as their ability to inhibit glucose transport using human erythrocytes. The C-6 derivatives displayed the highest affinities for GLUT-1, but the anomeric carbamates at C-1 (Figure 25) exhibited a moderate affinity for GLUT-1, adequate stability in rat plasma ($t_{1/2}$ = 1.3 h), and more rapid dopamine release in brain extract than the other carbamates ($t_{1/2}$ = 12 h).

The molecular size of the drug that can be attached to glucose without destroying its affinity for GLUT-1 is not yet known. Rouquayrol *et al.* (2001) extended the strategy to a series of glucose-containing prodrugs of the anti-HIV protease inhibitors saquinavir, indinavir, and nelfinavir but did not conduct studies of GLUT-1 affinity. They concluded that these prodrugs had promising therapeutic potential, "provided that their bioavailability, penetration into the HIV sanctuaries, and/or the liberation of the active free drug from the carbamate prodrugs are improved."

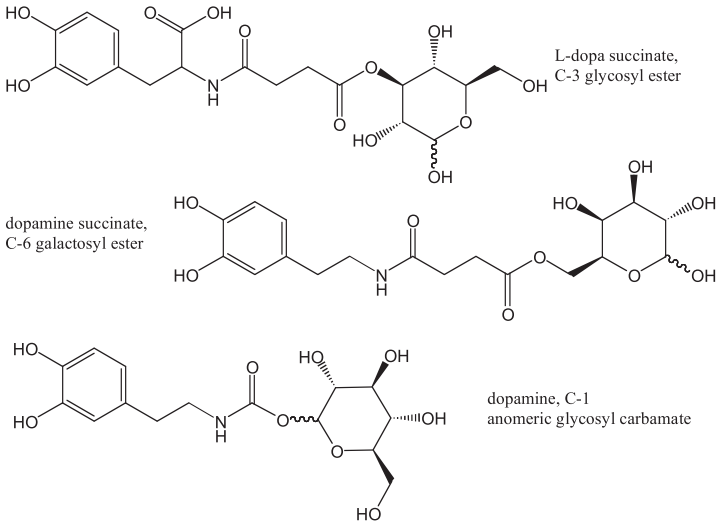


Figure 25. Glycosyl and galactosyl derivatives of L-dopa and dopamine designed as substrates for the blood-brain barrier GLUT-1 transporter (Bonina *et al.*, 2003; Fernandez *et al.*, 2003).

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2.5.7

Lymphatic Absorption of Orally Administered Prodrugs

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List of Abbreviations

CM.....chylomicrons;
DHT .....dihydrotestosterone
DHTU.....dihydrotestosterone undecanoate
G-3-P.....glycero-3-phosphate
PLA_2 .....phospholipase A_2
T.....testosterone
TG.....triglyceride
TU .....testosterone undecanoate
VLDL.....very low density lipoproteins
VPA.....valproic acid

Introduction

Orally administered drugs may gain access to the systemic circulation via absorption into the portal blood or by transport through the intestinal lymphatic system. In the majority of cases, absorption via the portal blood is the predominant pathway as portal blood flow, relative to intestinal lymph flow, is orders of magnitude higher (approximately 500-fold). However, for some highly lipophilic compounds, their association and interaction with enterocyte-derived lymph lipoproteins may be sufficient to overcome the differences in relative blood/lymph flow rates resulting in the lymphatics becoming a quantitatively important drug transport pathway.

Hepatic first pass metabolism may act to limit the amount of portally absorbed drug that ultimately reaches the systemic circulation. In contrast, drugs that are absorbed via the lymphatic system avoid first pass hepatic metabolism as the lymphatic system drains directly into the systemic circulation (via the thoracic duct) at the junction of the left internal jugular and subclavian veins. Therefore, a clear advantage of intestinal lymphatic drug transport is the possibility of bypassing hepatic first-pass metabolism. Intestinal lymphatic drug transport may also provide delivery advantages including the capacity to delivery specifically to the lymph and lymphoid tissue, and potentially the ability to modulate the rate of delivery of material via the lymphatics to the systemic circulation. The pivotal role of the lymphatics as a central part of the immune system, and specifically as a conduit for the dissemination of metastases from a number of solid tumours, makes targeted delivery to the lymphatics particularly attractive.

Historically, there have been relatively few studies of drugs transported through the intestinal lymphatics because (i) there have been various animal model limitations associated with the study of lymph transport, and (ii) prior to the utilisation of higher throughout screening technologies in drug discovery, many of the emerging drug candidates were not sufficiently lipophilic to be lymphatic transport candidates. However, the widespread adoption of higher throughput screening techniques have led to identification of increasingly lipophilic drug candidates, which when coupled with better understanding of transport via the intestinal lymphatics, has now enabled the rational exploration of the potential contribution of lymphatic drug transport to a drug candidate's overall bioavailability.

In parallel with the increased interest in the delivery and absorption of highly lipophilic drugs, there has been continued interest in the use of lipophilic prodrugs as a means of enhancing their target cell permeability as well as improving their bioavailability by specifically enhancing their absorption and transport via the intestinal lymphatics.

The intestinal lymphatics are a specialised absorption pathway for lipids and lipidic derivatives as well as a number of highly lipophilic xenobiotics and drugs including DDT (Sieber, 1976; Charman and Stella, 1986a; Charman *et al.*, 1986), benzo[a]pyrene (Laher *et al.*, 1984), PCBs (Busbee *et al.*, 1985), cyclosporin (Ueda *et al.*, 1983), naftifine (Grimus and Schuster, 1984), probucol (Palin and Wilson, 1984), mepitiostane (Ichihashi *et al.*, 1992a,b), lipophilic vitamins and derivatives

(Kuksis, 1987), testosterone undecanoate (Coert *et al.*, 1975; Noguchi *et al.*, 1985; Shackleford *et al.*, 2003) and halofantrine (Porter *et al.*, 1996a,b; Khoo *et al.*, 2002, 2003). Whilst these compounds have widely varying structures, it is possible to identify the relevant features that support lymph transport thereby defining the primary design strategies relevant for prodrugs intended to be absorbed via the intestinal lymphatics. The key features include the following:

(i) High lipophilicity: prodrugs can be designed to impart very high lipophilicity (*e.g.*, log P values higher than 5 and significant solubility in a triglyceride lipid), resulting in increased partitioning and association with enterocyte-derived lipoproteins.

(ii) Molecular specificity: prodrugs can be designed to advantageously incorporate key structural features of natural lipids thereby providing a potential mechanism to exploit endogenous absorption and biosynthetic pathway for lipids such as triglycerides and phospholipids.

Early approaches to prodrug design were often based on the simplistic premise that because the primary driving force for lymphatic transport was lipophilicity, this could be readily achieved through reversible derivatisation with (for example) a long-chain alkyl fatty acid. Whilst this strategy benefited from utilisation of relatively simple chemistries (esters/amides), it was limited by the lack of hydrolytic and enzymatic selectivity of the pro–moieties and pre-absorptive hydrolysis often limited the utility of these approaches. To address these limitations, more recent approaches have utilised more complex, yet specific chemistries, designed to mimic the structure of endogenous or dietary lipids with the goal of the compounds becoming associated with, and possibly incorporated into, the endogenous lipid processing pathways.

This review seeks firstly to briefly describe some of the historical approaches employed in attempts to target prodrugs to the intestinal lymphatics. Secondly, it aims to describe some more recent examples of chemistry and lymphatic transport models that enhance our understanding of lymphatic transport and how it may contribute to the overall systemic delivery of orally administered prodrugs.

Prodrug Approaches to Lymphatic Delivery

Post-hoc analysis of the structural and physicochemical properties of compounds known to be transported by the intestinal lymphatics indicate that the "threshold" physicochemical requirements include high lipophilicity evidenced by a log P > 5 and lipid solubility in a long chain triglyceride (or equivalent) of >50 mg/mL (Charman and Stella, 1986b; Charman, 1992). Additionally, if the compound is a salt of a highly lipophilic free acid or free base, then the profile of the neutral form should also be considered (Khoo *et al.*, 2002; Taillardat-Bertschinger *et al.*, 2003). For prodrugs, it is possible that lymphatic transport of candidates not meeting these physicochemical criteria may occur if they are structurally similar to lipids, and are substrates for the natural lipid processing pathways. To consider effective prodrug strategies, the following brief consid-

eration of the processes involved in the digestion, absorption and lymphatic transport of orally administered lipids provides the basis as to why these design criteria are typically applied.

Lipid Digestion, Absorption and Lymphatic Transport: Implications for Lymphatic Prodrug Design

Intestinal absorption of the products of lipid digestion is the result of three sequential processes: (i) dispersion of ingested lipids into a coarse emulsion of high surface area, (ii) enzymatic hydrolysis of the fatty acid esters (primarily triglyceride lipid) at the oil/water interface, and (iii) dispersion of the lipid digestion products into an absorbable form. Formation of the coarse dispersion occurs in the stomach where pre-duodenal (gastric/lingual) lipase hydrolyses a fraction of the ingested lipid to more amphiphilic species such as di-glycerides and fatty acids, which then act as surfactants to stabilise the crude emulsion formed by the shear associated with gastric emptying. The remaining triglyceride and diglycerides are subsequently hydrolysed within the proximal small intestine to mono-glyceride and fatty acid by pancreatic lipase which acts at the triglyceride/water interface after activation by co-lipase and bile salts. The products of lipid digestion are then assembled into various intestinal colloidal species which are stabilised by the bile salts and phospholipid released by the gall-bladder in response to the presence of lipid within the duodenum.

The movement of lipid digestion products into the enterocyte requires transport through the unstirred water layer to the brush-border surface of the enterocytes. The efficiency of transport to the enterocyte is reliant on diffusion of these components within colloidal structures, as their aqueous solubility is too low to support sufficient mass transport. The mechanism of uptake of the lipid digestion products across the apical membrane of the enterocyte is not fully understood, although it appears to involve both active and passive processes (Stremmel *et al.*, 1985; Stremmel, 1988; Thomson *et al.*, 1993; Schoeller *et al.*, 1995a,b; Poirier *et al.*, 1996).

Once within the enterocyte, 2-monoglycerides and fatty acids are rapidly and efficiently incorporated into a number of lipid processing pathways (Fig. 1). As a 'rule of thumb', medium chain fatty acids (*e.g.*, C12 and below) are transported directly into portal blood, whereas longer chain length fatty acids and 2-mono-glygerides (*e.g.*, C18 and greater) are re-synthesised within the enterocyte to triglyceride primarily via the mono-acyl glycerol pathway involving direct and sequential acylation of 2-monoglyceride by CoA-activated fatty acids. However, it should be noted that the mono-acyl glycerol pathway is not the sole source of TG, as the glycero-3-phosphate (G-3-P) pathway produces *de novo* TG under conditions of low lipid load, and indeed this pathway is inhibited under conditions where MG is readily available, such as in the postprandial state (Nordskog *et al.*, 2001).

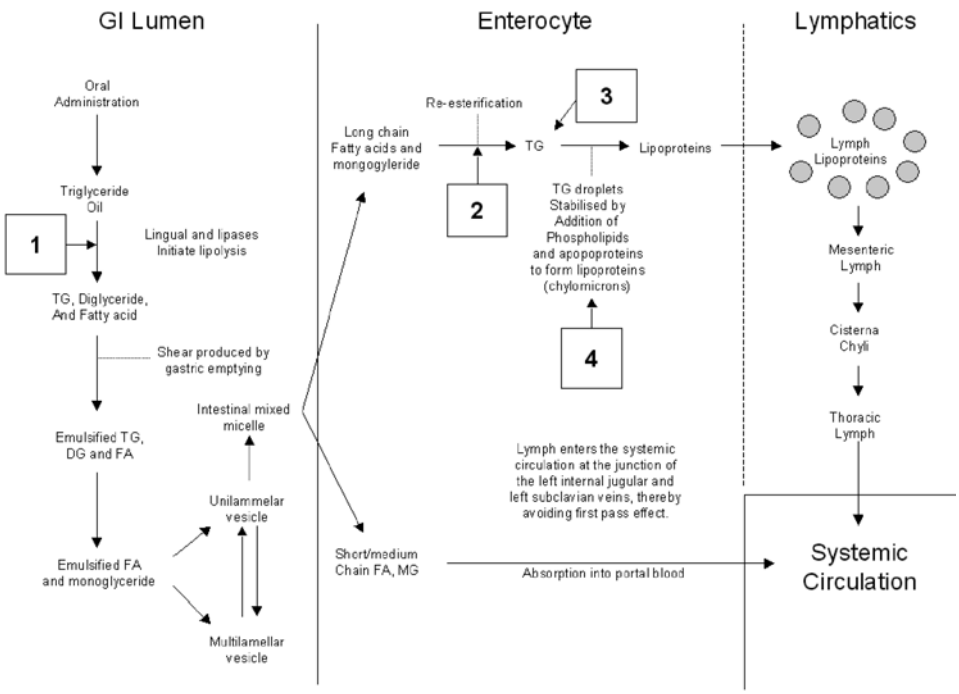

Figure 1. Schematic diagram describing the sequential steps in the digestion of lipids and subsequent absorption via the portal blood and intestinal lymphatics. The points at which prodrugs based on various design strategies become incorporated into those pathways prior to the eventual prodrug transport into lymph are shown in numbers and are defined as follows: (1) Fatty acids are cleaved from glyceride and phospholipid prodrugs by hydrolytic enzymes (lipases and phospholipases) within the GI tract, after which the resultant di-glycerides and lysophospholipids are incorporated into the processing pathways that exist for this class of dietary lipids. (2) Monoglyceride prodrugs once absorbed into the enterocyte are subject to re-esterification to form triglyceride-like prodrugs. (3) "Simple" lipophilic prodrugs (typically esters and ethers) once absorbed associate with the lipophilic lipid processing domains where the prodrug preferentially partitions into the triglyceride core prior to (or during) the point at which the TG is assembled into chylomicrons. (4) Phospholipid prodrugs associate with lymph lipoproteins at the point where natural phospholipids and apoproteins are added as stabilising agents to the surface of the developing lipoprotein.

Irrespective of the biosynthetic pathway from which they are derived, triglycerides are processed progressively through various intracellular organelles where the surface of the developing colloid (lipoprotein) is stabilised by the ordered addition of phospholipids (which are absorbed or synthesised *de novo* in a series of specific enzymatic processes) and various apoproteins. Under conditions of low lipid load (*i.e.*, fasting conditions), the primary lipoproteins produced by the small intestine and incorporating TG manufactured by the G-3-P pathway are very low density lipoproteins (VLDL), whereas under conditions of high lipid load (*e.g.*, after a meal) the predominant lipoproteins produced which incorporate TG

synthesised by the mono-acyl glycerol pathway are chylomicrons (CM) (Nordskog *et al.*, 2001). Following their assembly, VLDL and CM fuse with the basolateral membrane of the intestinal cell and are released into the lamina propria where they are preferentially absorbed via the open capillaries of the mesenteric lymphatics rather than into intestinal blood vessels. The exclusive movement of VLDL and CM into mesenteric lymphatic capillaries rather than into blood vessels is due to the fact that lymphatic capillaries lack a basement membrane and are therefore "permeable" to the large colloids, whereas blood vessels possess tight inter-endothelial junctions and a continuous basal lamina that preclude facile access of colloidal lipoproteins. The reader is referred elsewhere for more extensive discussion of the differences in the endothelial structure of blood vessels and the lymphatic circulation (Swartz, 2001).

Although the various processes contributing to lymphatic drug transport are not fully characterised, it is clear that specificity for access is defined by association with the lipoproteins assembled within the enterocyte, and that it is lipoprotein association (and their physical exclusion from access to blood capillaries) that leads to specific lymphatic access. From consideration of the processes involved in the digestion and absorption of natural lipids, it is apparent that there are points at which a prodrug could become incorporated into, or associated with, endogenous lipid processing pathways such that the prodrug is a substrate for transport into lymph.

Figure 1 describes some possible interactions of the prodrug/drug with the hydrolytic and endogenous lipid processing pathways. For a prodrug where the targeting strategy is dependent on the lipophilicity of the administered compound, incorporation into lipoproteins presumably occurs within the enterocyte during triglyceride synthesis and assembly into the enterocyte-derived lipoproteins. However, this process is not well described and there is a paucity of data describing the relative importance of simple partitioning behaviours and the role of intracellular binding and transfer proteins in driving drug-lymph lipoprotein association. Alternatively, a prodrug may be designed for incorporation with the biochemical pathways of lipid digestion such that the prodrug is initially hydrolysed by intestinal lipases prior to being re-acylated with natural fatty acids and subsequently incorporated into lipoproteins. Additionally, a prodrug whose structure mimics phospholipids might be similarly expected to become involved with the phospholipid hydrolysis/re-acylation pathway such that the phospholipid-based prodrug is eventually incorporated into the lipoprotein surface during intracellular lipoprotein assembly.

Prodrugs have been prepared for a variety of drug classes using the above described synthetic strategies and these are briefly reviewed in the following sections. It should be noted that in many cases, there has not been definitive demonstration of the prodrug being transported into lymph following oral administration. In such cases, lymphatic transport has either been assumed or inferred on the basis of indirect evidence such as increased plasma drug/prodrug concentrations. In only few examples have studies been conducted in thoracic or mesenteric duct-cannulated animals to definitely demonstrate lymphatic

Drug	Prodrug	Rationale for Design of Prodrug	Evidence of Lymphatic Transport of Prodrug?	References
Testosterone	Testosterone undecanoate	Improved Oral Bioavailability	Direct	(Coert *et al.*, 1975; Horst *et al.*, 1976; Noguchi *et al.*, 1985; Shackleford *et al.*, 2003)
Epitiostanol	Mepitiostane (epitiostanol 17-methyloxycyclopentyl ether)		Direct	(Ichihashi *et al.*, 1991a, 1992a,b; Ichihashi *et al.*, 1991b)
Propranolol	Propranolol palmitate		Indirect	(Vyas *et al.*, 1999)
Closantel	Closantel palmitate	Improved oral bioavailability and targeting of lymphatics to treat lymph-resident disease	Not investigated	(Loiseau *et al.*, 1997)
Zidovudine (AZT)	AZT-5'-esters (Butyrate, Laurate, Oleate)	Targeting of lymphatics to treat lymph-resident disease	Direct	(Bibby *et al.*, 1996)
Vitamin A (Retinol)	Retinyl palmitate	Enhanced drug stability	Direct	(Fernandez and Borgstrom, 1990)
Vitamin E (a-Tocopherol)	Various α-Tocopherol esters (acetate, palmitate, acid succinate, nicotinate, α-hydroxybenzoate, pivalate)		Direct	(Nakamura *et al.*, 1975)
Indomethacin	Indomethacin Farnesyl ester	Reduced G.I. irritation	Direct	(Mishima *et al.*, 1990)

Table 1. Examples of some drugs and their ester/ether-linked lipophilic prodrugs.
Definitions: "Direct" indicates lymphatic prodrug absorption was evidenced by measurement of prodrug concentrations in lymph following oral prodrug administration to thoracic or mesenteric lymph-duct cannulated animals; "Indirect" means there was no direct evidence of lymphatic prodrug absorption, however it was assumed to have occurred on the basis of increased plasma concentrations or increased therapeutic affect following oral prodrug administration; "Not Investigated" means neither direct nor indirect evidence pertaining to possible role of the lymphatic absorption of this prodrug was provided.

transport of the administered prodrug, however such studies are essential to assess the utility of synthetic prodrug strategies and thereby enable the optimisation of such approaches.

Lipophilic Esters/Ethers

Perhaps the simplest strategy for targeting lipophilic prodrugs to the lymphatic transport pathway after oral administration is derivatisation of simple ester or amide linkages with (typically) straight chain or cyclic alkyl groups. A number of examples of this approach are summarised in Table 1, and the reader is referred to previous reviews (Stella and Pochopin, 1992; Charman and Porter, 1996) for more extensive discussion of various lipophilic prodrugs.

This relatively simple approach has been used to increase the oral bioavailability of a number of drugs by avoiding extensive hepatic first-pass metabolism (*e.g.*, testosterone, epitiostanol, propranolol and closantel) or by increasing chemical stability within the GI tract (*e.g.*, Vitamin A and Vitamin E). This strategy has also been used in attempts to reduce GI irritation (arising from GI exposure to free drug such as indomethacin) and to increase drug targeting to lymphoid tissue (*e.g.*, the macrofiliaricidal, closantel and the anti-HIV agent, zidovudine).

In general, lipophilic derivatisation via simple chemical approaches has typically failed to produce extensive lymphatic transport (*i.e.*, the majority of the administered dose does not appear in thoracic lymph) which has been ascribed to extensive pre-absorptive hydrolysis of the prodrug. However, in some cases the available clinical data suggests that in spite of the often limited extent of lymphatic transport, this approach may be sufficient to afford (positive) therapeutic outcomes that were otherwise not possible. Therefore, prodrug approaches based on lipophilic derivatisation using simple chemistries should not necessarily be dismissed, particularly when the goal of a prodrug is to achieve relatively low systemic concentrations of highly potent drugs (such as hormones). This concept is well illustrated by the clear utility of testosterone undecanoate which produces clinically relevant therapeutic concentrations of testosterone which arise from lymphatic transport of the undecanoate ester. This example is described in detail in the section on Testosterone Undecanoate.

Glyceride Prodrugs

Examples of prodrugs based on the strategy of utilising the naturally existing biosynthetic pathways of dietary lipids (Fig 2) are presented in Table 2, and the reader is referred to previous reviews (Stella and Pochopin, 1992; Charman and Porter, 1996; Lambert, 2000) for more extensive discussions of this approach. As discussed earlier, triglycerides within the intestinal lumen are subject to lipase-mediated pre-absorptive hydrolysis to yield a combination of fatty acids and sn-2 monoglyceride which are then absorbed into the enterocyte, re-acylated and incorporated into lymph lipoproteins. As it is the sn-2 monoglyceride derived from dietary lipids that remains intact within the intestinal lumen, is absorbed into the enterocyte, processed and then transported into lymph, it is not surprising

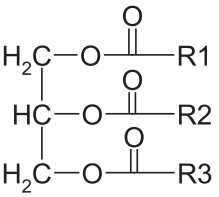

Figure 2. Generic chemical structure of a glyceride.

Drug	Position of Drug Attachment To Glyceride Promoiety	Rationale for Design of Prodrug	Evidence of Lymphatic Transport of Prodrug?	References
LK-A	sn-1	Improved oral bioavailability	Direct	(Sugihara and Furuuchi, 1988)
L-Dopa	sn-2		Direct	(Garzon-Aburbeh *et al.*, 1986)
Bupranolol	sn-2		Not investigated	(Mantelli *et al.*, 1985)
Phenytoin	sn-1 and sn-2		Not investigated or Indirect	(Scriba, 1993a, b; Scriba *et al.*, 1995a,b,c)
Closantel	sn-2	Improved oral bioavailability and targeting of lymphatics to treat lymph-resident disease	Not investigated	(Loiseau *et al.*, 1997)
Niclosamide	sn-2	Targeting of lymphatics to treat lymph-resident disease	Not investigated	(Elkihel *et al.*, 1994)
Melphalan	sn-2		Indirect	(Deverre *et al.*, 1992b; Loiseau *et al.*, 1994)
γ-aminobutyric acid (GABA)	sn-2		Not investigated	(Deverre et al., 1989; Deverre *et al.*, 1992a)
Chlorambucil	sn-2		Direct	(Garzon-Aburbeh *et al.*, 1983; Loiseau *et al.*, 1997)

Table 2. Examples of some drugs and their ester/ether-linked lipophilic prodrugs.
Definitions: "Direct" indicates lymphatic prodrug absorption was evidenced by measurement of prodrug concentrations in lymph following oral prodrug administration to thoracic or mesenteric lymph-duct cannulated animals; "Indirect" means there was no direct evidence of lymphatic prodrug absorption, however it was assumed to have occurred on the basis of increased plasma concentrations or increased therapeutic affect following oral prodrug administration; "Not Investigated" means neither direct nor indirect evidence pertaining to possible role of the lymphatic absorption of this prodrug was provided.

Drug	Position of Drug Attachment To Glyceride Promoiety	Rationale for Design of Prodrug	Evidence of Lymphatic Transport of Prodrug?	References
Valproic acid	sn-2	Enhanced CNS penetration	Indirect	(Mergen *et al.*, 1991)
γ-aminobutyric acid (GABA)	sn-2		Not investigated	(Hesse *et al.*, 1988; Jacob *et al.*, 1987, 1990; Jacob *et al.*, 1985; Shashoua *et al.*, 1984)
γ-vinyl-GABA	sn-2+sn-3		Not investigated	(Jacob *et al.*, 1990)
Thiorphan	sn-2		Indirect	(Lambert *et al.*, 1995)
Aspirin	sn-2	Reduced G.I. Irritation	Direct	(Carter *et al.*, 1980; Kumar and Billimoria, 1978; Paris *et al.*, 1979, 1980a)
Indomethacin	sn-1		Not investigated	(Paris *et al.*, 1980b)
Naproxen	sn-1 and sn-2		Direct	(Sugihara *et al.*, 1988)
NSAIDs (Ketoprofen, aclofenac, ibuprofen, desmethyl-naproxen)	sn-2		Not investigated	(Paris and D.G., 1982)

Table 2 (continued).

that the majority of examples presented in Table 2 have the drug attached at the 2-position of the glycerol backbone. More specifically, pre-absorptive glyceride processing within the GI tract involves selective hydrolysis of lipids attached at the 1- and 3-positions of the glycerol backbone, therefore, drug attachment at the sn-2 position should conceptually avoid pre-absorptive release of the free drug within the GI lumen. In cases where the free drug is known to cause GI irritation (as with many NSAIDs), sn-2 rather than sn-1 attachment of the drug to the glycerol backbone should reduce the potential for such irritation following oral prodrug administration, although it should not be assumed that drug attachment at the sn-2 position is an exclusive pre-requisite for successful targeting of a prodrug to the lymphatics. Being an enzymatic process, lipase-mediated cleavage at the sn-1 position will only occur when the specific steric and chemical requisites for binding to the catalytic site of lipases are met, therefore sn-1 attachment may prove to be a successful lymph targeting strategy if gastric and duodenal lipases

are unable to cleave the drug from the glyceride promoiety due to a steric or chemical incompatibility during pre-absorptive processing. There are a number of examples of lymphatically transported glyceride prodrugs with the drug attached at the sn-1 position of the glyceryl backbone, one example being LK-903, a glyceride prodrug for the hypolipidemic compound LK-A, and factors affecting the lymphatic transport and GI hydrolysis of that prodrug have been reviewed elsewhere (Stella and Pochopin, 1992).

Phospholipid Prodrugs

Phospholipids (Fig. 3) present within the intestinal lumen are derived from dietary and biliary sources. Prior to absorption, the fatty acid at the 2-position of the phospholipid is hydrolysed under the stereospecific action of pancreatic phospholipase A_2 (but not by gastric or duodenal lipase) to produce a fatty acid and the corresponding sn-1 lysophospholipid. Once absorbed, a fraction of the sn-1 lysophospholipid is subsequently re-esterified within the enterocyte to the respective phospholipid by lysophoshphatidylcholine acyl transferase. The phospholipid so formed is then incorporated into the lipoprotein assembly pathway and becomes an integral part of the lipoprotein surface components. The most likely site of prodrug transport will be the lipoprotein surface region due to the amphiphilic nature of the reformed phospholipid, and the capacity for transport will be based on the high surface area of the small sized lipoproteins (*e.g.*, 85–500 nm).

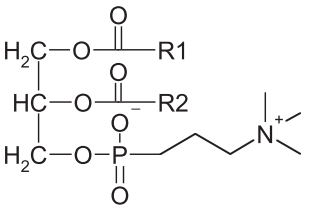

Figure 3. Generic chemical structure of a phospholipid.

There has been an increasing number of prodrugs designed to utilise the natural processing pathways for phospholipids (Table 3). While the features of those design strategies (both biopharmaceutic and pharmacological) have been reviewed elsewhere (Stella and Pochopin, 1992; Charman and Porter, 1996; Lambert, 2000; Morris-Natschke *et al.*, 2003), it is important to note that in the majority of examples presented in Table 3, phospholipid prodrugs have been designed with the drug attached to the phosphate group of the phospholipid backbone. This has presumably been done to minimise pre-absorptive drug release (because of the specificity of PLA_2 to hydrolyse at the sn-2 position), thereby leaving the drug-lysophosholipid analogue available for absorption and intercalation into the phospholipid processing pathway.

Drug	Position of Drug Attachment To Phospholipid Promoiety	Rationale for design of prodrug	Evidence of Lymphatic Transport of prodrug?	References
Cidofovir	sn-2 'phospholipid mimic'	Improved oral bioavailability and reduced toxicity	Indirect	(Ciesla *et al.*, 2003)
Forscarnet	sn-3 phosphate		Not investigated	(Beadle *et al.*, 1998; Kini *et al.*, 1997)
Fluorouridine	sn-3 phosphate	Targeting of lymphatics to treat lymph-resident disease	Direct	(Sakai *et al.*, 1993)
Zidovudine (AZT)	sn-3 phosphate		Not investigated	(Kucera *et al.*, 2001)
Acyclovir	sn-3 phosphate		Indirect	(Hostetler *et al.*, 1997)
Ibuprofen	sn-1, sn-2, sn-1+sn-2	Proof-of-concept for prodrug class	Not investigated	Kurz and Scriba, 2000)
Valproate	sn-1, sn-2, sn-1+sn-2		Not investigated	(Kurz and Scriba, 2000)
Valproate	sn-2 (SPD421)	Specificity of drug release at site of action (i.e., the CNS)	Direct	see DP-VPA

Table 3. Examples of some drugs for which phospholipid or phospholipid-like prodrugs have been prepared.

Definitions: "Direct" indicates lymphatic prodrug absorption was evidenced by measurement of prodrug concentrations in lymph following oral prodrug administration to thoracic or mesenteric lymph-duct cannulated animals; "Indirect" means there was no direct evidence of lymphatic prodrug absorption, however it was assumed to have occurred on the basis of increased plasma concentrations or increased therapeutic affect following oral prodrug administration; "Not Investigated" means neither direct nor indirect evidence pertaining to possible role of the lymphatic absorption of this prodrug was provided.

One example of the successful targeting of a prodrug to the biosynthetic and absorptive pathways of phospholipids involves dipalmitoylphosphatidylfluorouridine (DPPF, a phospholipid prodrug of 5-fluorouridine), where oral administration of DPPF to mice (300 mg/kg in aqueous solution) resulted in the appearance of DPPF, 5-fluorouridine and two related compounds in thoracic lymph (Sakai *et al.*, 1993). The related compounds were identified as 1-palmitoyl-2-arachidonylphosphatidylfluorouridine (PAPF) and 1-palmitoyl-2-linoleoylphosphatidylfluorouridine (PLPF), with their appearance being consistent with the successful incorporation of DPPF into the pre-absorptive 2'-hydrolysis and re-acylation pathways for phospholipids.

While attachment of the drug to the phosphate group of a phospholipid backbone may be an effective way to reduce pre-absorptive drug release (by avoiding PLA_2-mediated hydrolysis), attachment at that position should not be considered an exclusive prerequisite for effective lymphatic targeting as the intact prodrug (or its product of reacylation) may be transported into lymph if the linkage between the phospholipid pro-moiety and the drug does not fulfil the steric or chemical requirements for it to be a substrate for phospholipase A_2. This concept is highlighted by the results of a recent study with a series of phosphatidylcholine prodrugs of ibuprofen where it was found that only those prodrug analogues with a fatty acid in the sn-2 position were degraded by pancreatic PLA_2 (Kurz and Scriba, 2000). An sn-2 attached phospholipid prodrug that is currently under clinical (Phase II) development is DP-VPA (Bialer *et al.*, 1999, 2001, 2002; Fisher and Ho, 2002; Labiner, 2002; Isoherranen *et al.*, 2003), a prodrug of valproate, and experiments have recently been conducted in our laboratories with DP-VPA to establish the extent of its lymphatic absorption following oral administration. The results of those experiments are presented in the section on DP-VPA.

Recent Examples of the Utility of Lipid Prodrugs for Lymphatic Delivery

To fully characterise the lymphatic absorption of an orally administered prodrug, it is necessary to study the absorption process in an animal model where (a) the physiology of the gastrointestinal tract is comparable to that of the human, (b) where there is a postprandial response comparable to that observed in human, and (c) where human-relevant sized dosage forms can be utilised (Edwards *et al.*, 2001). Furthermore, as discussed briefly in a previous section, lymphatic transport can only be definitively demonstrated by direct measurement of prodrug/drug concentrations in lymph collected from mesenteric or thoracic duct-cannulated animals.

Studies of lymphatic drug/prodrug absorption have traditionally been performed in mesenteric lymph duct-cannulated rats, and while the rat model has been used successfully to study factors such as the impact of different formulations and the role of prodrug lipophilicity as determinants of prodrug lymph transport (Noguchi *et al.*, 1985), there are some complexities associated with extrapolation of the data to humans. For example, the quantity of formulation (and lipid) typically dosed to rats is considerably larger (on a mL/kg basis) than would be administered to humans, it is not possible to administer actual human-relevant sized formulations and the rat does not have a postprandial response reflective of humans.

To address these limiting issues, and to examine the lymphatic transport of a series of compounds after pre- and postprandial administration, we recently developed a triple-cannulated dog model (Khoo *et al.*, 2001). In this model, all thoracic lymph is collected and therefore the rate of drug transport (calculated as the product of drug concentration and lymph flow rate) of any compound present

in lymph corresponds directly to the rate at which that analyte would be delivered into the systemic circulation in an "intact" (*i.e.*, non-cannulated) animal. Analysis of lymph concentrations of prodrug is used to directly calculate the absolute fraction of the administered dose absorbed via the intestinal lymphatics. Furthermore, cannulation of the cephalic vein enables determination of the systemic exposure arising from portally absorbed prodrug/drug while blood sampled from a portal vein cannula provides insight into the extent of pre-portal (lumenal and enterocyte-based) metabolism and hepatic first-pass extraction.

This model has been used successfully to study factors such as the impact of food and formulation on the lymphatic absorption of various lipophilic drugs and drug candidates. With regards to prodrugs, the model has been used to study the lymphatic absorption of a lipophilic ester prodrug (testosterone undecanoate) and a phospholipid prodrug (DP-VPA), and the results of those studies are presented in the following two sections.

Testosterone Undecanoate

Orally administered testosterone (T) is ineffective in the treatment of male androgen deficiency syndromes due to extensive pre-systemic first-pass metabolism (Daggett *et al.*, 1978). Following oral administration, the high lipophilicity of the ester prodrug, testosterone undecanoate (TU) (Fig 4) causes the prodrug to target the lymphatic absorption pathway (Coert *et al.*, 1975; Noguchi *et al.*, 1985) affording delivery of TU into the systemic circulation via the lymph. Liberation of free testosterone in the systemic circulation (through the action of plasma esterases) has been shown to result in a positive therapeutic outcome (Hirschhauser *et al.*, 1975; Maisey *et al.*, 1981; Shackleford *et al.*, 2003). TU is therefore an excellent example of a prodrug were lymphatic absorption imparts a significant increase in systemic prodrug/drug exposure by avoiding hepatic first-pass metabolism.

Whilst the appearance of orally administered TU in thoracic lymph had been observed both in rats (Coert *et al.*, 1975; Noguchi *et al.*, 1985) and man (Horst *et al.*, 1976), until recently, the absolute fraction of orally administered TU entering the systemic circulation via the intestinal lymphatics had not been evaluated.

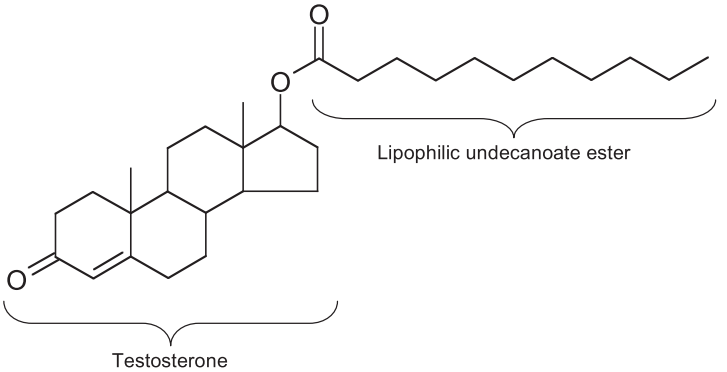

Figure 4. Chemical structure of testosterone undecanoate.

Recently, we investigated the quantitative contributions of lymphatically absorbed TU to systemic T exposure (Shackleford *et al.*, 2003). In this study, TU was administered orally to postprandial thoracic lymph duct-cannulated dogs and the lymph and systemic serum concentrations of TU and T, as well as the reduced metabolites 5-α-dihydrotestosterone undecanoate (DHTU) and 5-α-dihydrotestosterone (DHT) were determined over the 10-12 h post-dosing period. The study design employed a stable isotope methodology where isotopically labelled prodrug (^{2}H-TU) was administered intravenously concomitant with each oral TU dose enabling determination of the absolute bioavailability of portally absorbed TU. Furthermore, insertion of a portal vein cannula allowed for analyte determination in portal blood to provide insight into the extent of pre-systemic prodrug metabolism.

Following oral administration of TU, the prodrug was subject to extensive presystemic hydrolysis and metabolism as evidenced by the time profiles of analyte concentrations in portal serum (Fig 5). However, significant concentration profiles of both TU and DHTU were observed in thoracic lymph. The transport profiles depicting the rate and cumulative lymphatic transport of lymph triglyceride (TG), TU and DHTU are presented in Figure 6. The mean (± SE) fraction of administered TU transported into thoracic lymph was 3.20 ± 0.46 %. The absolute bioavailability of portally absorbed TU (calculated using systemic concentrations of TU and ^{2}H-TU) was 0.054 ± 0.029%. In this study, it was not possible to estimate the absolute fractional conversion of ^{2}H-TU to ^{2}H-T and this precluded calculation of the absolute mass of T reaching the systemic circulation as a consequence of lymphatic TU transport. However, model-independent pharmacokinetic analysis indicated that after oral administration of TU in the intact animal, 83.6 ± 1.6 % of systemically available T results from systemic hydrolysis of lymphatically transported TU.

Additional insight into the disposition of TU (and DHTU) within the enterocyte was possible by examining the time course profiles of the rate of lymph transport of TG, TU and DHTU (Fig. 6). While the maximum rate of lymphatic TU transport occurred during the 1–2 h post-dosing period, the maximal rate of transport of TG and DHTU occurred during the 2–3 h post-dosing period.

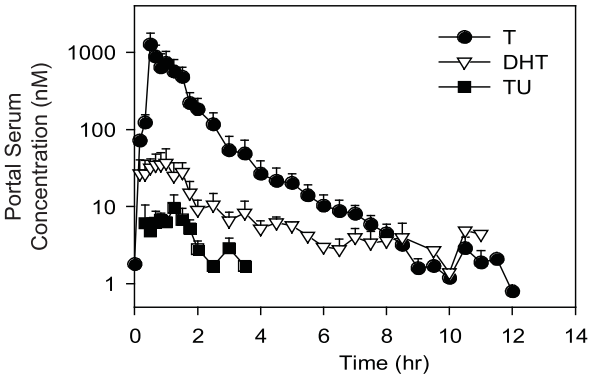

Figure 5. Mean (± SE) portal serum concentrations (nM) of T, DHT and TU after oral administration of TU to postprandial lymph duct-cannulated dogs (n = 4)

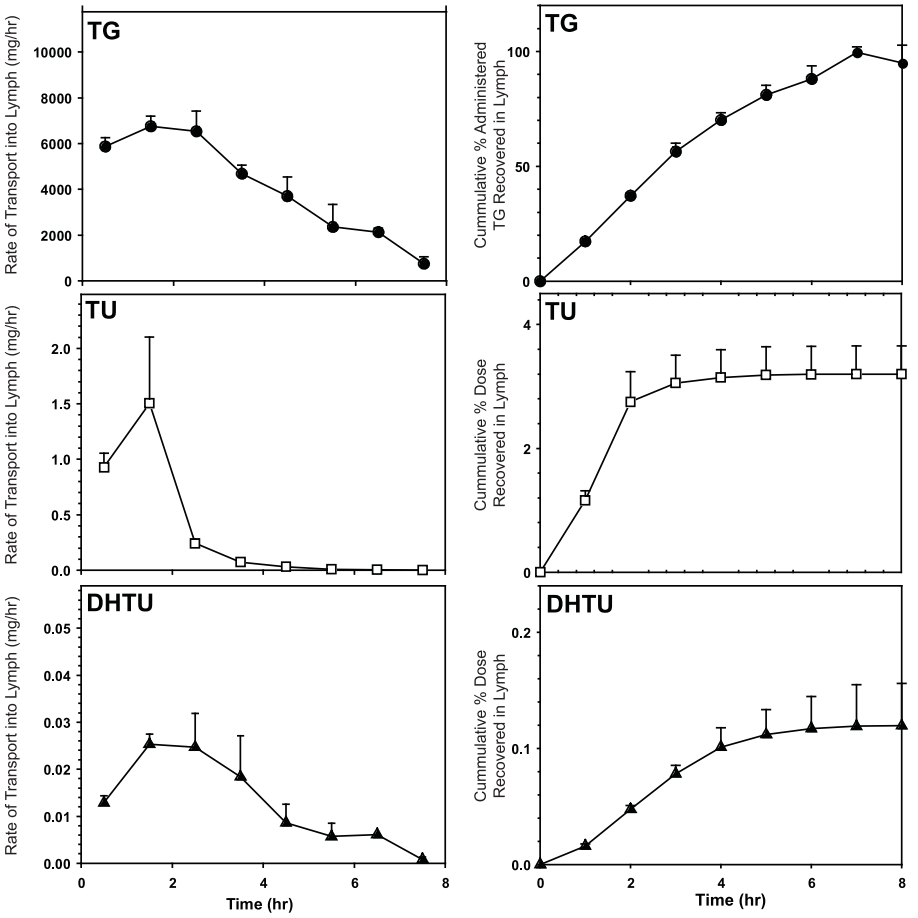

Figure 6. Mean (± SE) rate (mg/hr) and cumulative (% of dose) thoracic lymphatic transport of TG , TU and DHTU after oral administration of 80 mg of TU to postprandial lymph duct-cannulated dogs (n = 4).

Furthermore, the rate of lymphatic TU transport declined rapidly after reaching its maximal transport rate, whereas the rate of TG and DHTU transport declined more gradually. The change in the relative rates of TG and TU transport after the 1–2 h post-dosing period is consistent with the relative decrease in TU transport most likely reflecting a reduction in the available mass of TU within the enterocyte for incorporation into lymph chylomicrons. In contrast, the prolonged transport of DHTU in parallel with TG transport suggests a longer residence time for DHTU within the enterocyte lipid-processing microdomains, due to either differences in metabolic stability or different enterocyte-based processing mechanisms. The issue of intracellular drug processing as it relates to the competing pathways of metabolism, lymphatic transport and portal blood absorption has received little attention. However, in our laboratory, recent data from assessment of the lymphatic transport of DP-VPA, a phospholipid prodrug of valproic acid (VPA), has provided insight into the likely differences in processing

between phospholipid-related prodrugs and glyceride-related systems, and this example is described below.

DP-VPA

DP-VPA (Fig. 7), also called SPD-421, is a specifically designed phospholipid prodrug of valproic acid (VPA) under development for the treatment of partial and generalized epileptic seizures (Bialer *et al.*, 1999, 2001, 2002; Fisher and Ho, 2002; Labiner, 2002; Isoherranen *et al.*, 2003). DP-VPA is designed to penetrate the CNS as the intact prodrug and specifically release VPA at the epileptic focus through the hydrolytic activity of phospholipase A_2 (PLA_2). The release of free valproate is designed to occur in response to paroxysmal neuronal activity by increased PLA_2 activity that accompanies epileptic seizure. If successful, the targeted delivery of VPA at the site of epileptic seizure would result in lower doses

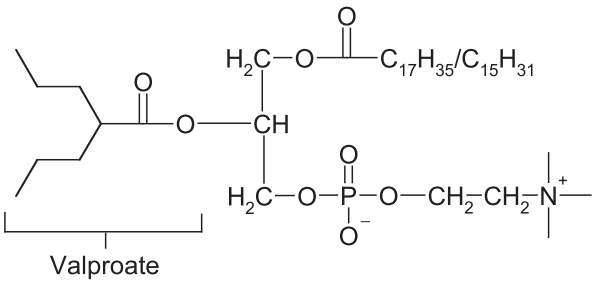

Figure 7. Chemical structure of DP-VPA.

being necessary to achieve protection from seizures thereby reducing the potential for toxic side effects otherwise associated with higher systemic exposure of valproate.

The structural similarity of DP-VPA to natural phospholipids suggested that a significant proportion of an oral dose may gain access to the systemic circulation via the intestinal lymphatics after incorporation into endogenous phospholipid processing pathways. This contention was consistent with preclinical pharmacokinetic studies in rats and Phase I human studies where it was found that the oral bioavailability of DP-VPA was subject to a marked food effect where the systemic exposure in both species was increased following postprandial administration of the prodrug (Bialer *et al.*, 2002).

In order to establish that lymphatic absorption was an important determinant of DP-VPA pharmacokinetics, we conducted some initial proof-of-concept studies in thoracic duct-cannulated greyhounds. In these preliminary experiments, DP-VPA (~485–552 mg/dog) was administered to thoracic duct-cannulated greyhounds 30 min after they each consumed 690 grams of commercial dog food

(~34 gm triglyceride lipid). Total thoracic lymph was collected in discrete samples for the 10 h post-dosing period and the concentrations of VPA-related material and TG in each sample were determined by LC-MS and a spectrophotometic assay, respectively. From these initial studies, 10–18% of the administered DP-VPA was found to have been transported into thoracic lymph following postprandial oral administration (Fig. 8), and while the majority of the material recovered in the lymph corresponded to intact DP-VPA, the presence of closely related structural congeners in lymph suggested that DP-VPA had also been incorporated into the biosynthetic lipid processing pathways (i.e. that the parent prodrug had been subject to hydrolysis and re-esterification).

Interestingly, when the relative rates of lymphatic transport of DP-VPA and TG were compared (Fig. 9), there was a very high degree of correlation which continued throughout the 10 h post-dosing period. Such a correlation is in contrast with that observed for TU, and what has been reported for Hf (Khoo *et al.*, 2001), as in those cases, the rate of lymphatic drug transport declined markedly prior to such changes in the rate of TG transport. It seems likely that the disparity in behaviour of DP-VPA, compared with TU and Hf, results from differences in the mechanism by which each of these compounds access lymph lipoproteins. In the case of TU and Hf, association between drug and the

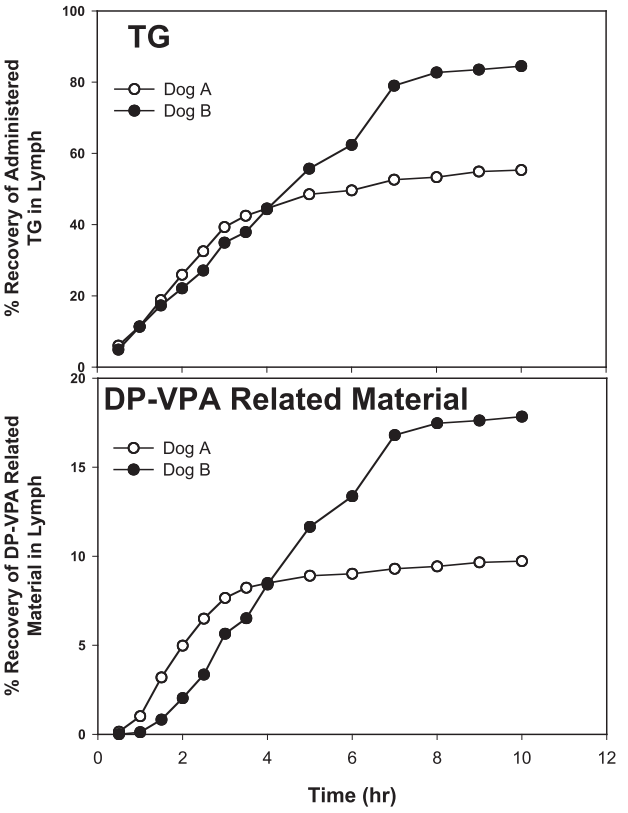

Figure 8. Cumulative lymphatic transport of TG- and DP-VPA-derived material following oral administration of DP-VPA to two postprandial lymph duct-cannulated dogs.

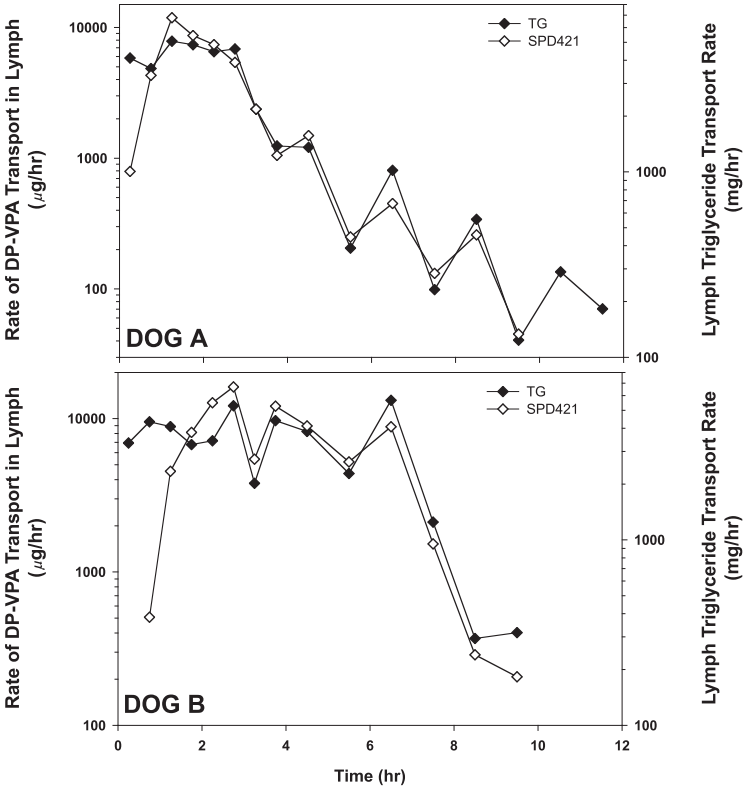

Figure 9. Rate of lymphatic transport vs. time of TG and DP-VPA derived material following oral administration of DP-VPA to two postprandial lymph duct-cannulated dogs.

developing lipoproteins in the enterocyte is primarily driven by partitioning between the lipid core of the lipoproteins (primarily chylomicrons) and the aqueous environment of the enterocyte. It is reasonable to assume that the proportion of the drug dose partitioned into lipoproteins will be lymphatically transported, and conversely, the proportion partitioned into the cytoplasm is available for absorption into the portal blood. Therefore, the time profile for the rate of lymph transport of TU or Hf will reflect both the mass of drug in the enterocyte (which drives partition into the lipoproteins) and the throughput of lipid through the enterocyte. Conversely, since DP-VPA is specifically incorporated into the phospholipid biosynthetic and processing pathways, the mass of drug driving the partition process becomes less important since lipoprotein association is a more specific association. Hence, the time profile of drug and lipid transport into the lymph is expected to be more closely related to, and will more closely reflect, the time-course for the processing and transport of total lipid into lymph.

Food and Formulation Considerations

Regardless of the mechanism of association of drugs or prodrugs with lymph lipoproteins, it is apparent that appreciable intestinal lymphatic transport is

dependent on an appropriate lipid source to drive lipoprotein assembly. Whilst maximal drug transport into lymph is likely to occur postprandially, it is less apparent what the minimum lipidic requirement for appreciable lymphatic transport is. Recently, Bagchus *et al.* (2004) determined the plasma AUC values for T in 24 post-menopausal volunteers following oral administration of TU (80 mg) in a crossover study with administration of 4 different meals (lipid content of either 0.6 g, 5 g, 19 g or 44 g) and it was found that an upper limit of T exposure was achieved with as little as 19 g of lipid. The "standard western diet" contains approximately 120 g lipid/day, and realising that a standard packet of potato crisps contains ~15 g of lipid, it appears likely that a standard diet (or a small, lipid-rich 'snack') can adequately support lymphatic transport of lipophilic drugs and prodrugs. However, further studies are required to confirm the general applicability of TU-food intake data to other lymphatically transported compounds.

While the postprandial administration of a lipophilic drug/prodrug is a useful mechanism to support lymph lipoprotein assembly and lymphatic drug transport, there can be issues associated with 'take with food' patient instructions. These include the often variable perception of what constitutes 'food', especially in patients where the desire to ingest a fatty meal may be diminished, or where regular high fat food intake to support daily or multiple daily doses in unavailable. We have recently studied the lymphatic drug transport of Hf after fasted administration of a single unit dose form containing approximately 0.6 g of triglyceride lipid. In this study, the lymphatic transport of Hf was approximately 50% of the maximal extent observed after administration of a high fat meal containing 35 g lipid (Khoo *et al.*, 2003). These data indicate that whilst attention must be given to administering compounds designed for lymphatic transport with an appropriate lipid source, it is possible even under fasting conditions to stimulate lymphatic transport, provided an appropriate lipid-based formulation is employed. Further discussion of the design aspects of lipid-based formulations is beyond the scope of the current review, however, the interested reader is directed to the following reviews for more information (Humberstone and Charman, 1997; Porter and Charman, 2001a,b; O'Driscoll, 2002).

Summary and Perspectives

There have historically been relatively few examples of drugs for which the intestinal lymphatics have been a primary route of absorption. However, recent trends in compound design suggests an increased likelihood for lymph transport and that the use of lymph directing prodrugs may more effectively exploit the inherent delivery advantages. Increased knowledge of lipid processing and the lipoprotein assembly/transport process should make it possible for prodrugs to be more effectively incorporated into those pathways. However, there are some issues that the prodrug designer should bear in mind when targeting the lymphatics. Firstly, the issue of possible cardiac effects (*e.g.*, QT_c prolongation) should be considered if high lymph concentrations of prodrug (with an inherent QT_c

liability) result in localised high plasma concentrations in the vicinity of the heart, as the thoracic lymph duct empties into the systemic circulation at the junction of the left internal jugular and subclavian veins (McIntosh *et al.*, 2003). Secondly, although a number of prodrugs have been designed and synthesised in order to target lymph-resident diseases (*e.g.*, filiaricidal infection and lymph-resident viruses such as HIV), an often overlooked issue is that lymphatically absorbed lipophilic and glyceride prodrugs are associated almost exclusively with the internal lipid core of lipoproteins. As such, the free concentration of prodrug within lymph plasma will likely be low until lipase enzymes and lipid transfer proteins act to transfer lipids (and presumably drugs) out of the circulating lipoproteins. In other words, targeting of prodrugs for intestinal lymphatic transport and local lymphatic targeting is somewhat paradoxical. as in spite of the designed prodrug potentially having enhanced lymphatic transport due to association with the lipoprotein, this may result in decreased release locally within the lymphatics for subsequent conversion to the active drug. However, in the case of phospholipid prodrugs, if they are primarily associated with the surface of enterocyte-derived lipoproteins, this may ultimately provide a means of delivery to the surface of target cells if taken up via specific receptor-mediated mechanisms.

In summary, there are numerous examples where prodrugs have been designed to be absorbed by the intestinal lymphatics and thereby exploit the advantages associated with this route of absorption. However, in spite of the number of such prodrugs, there are relatively few examples where the extent of lymphatic transport and the contribution of that transport to systemic prodrug/drug exposure has been definitively demonstrated using thoracic or mesenteric lymph duct-cannulated animal models. Direct demonstration of prodrug appearance in lymph following oral administration is the best means to guide, and then ascertain, whether the lymph-targeting prodrug strategy has been successful. Utilisation of these recently available animal models will be highly beneficial to provide a more complete understanding of the importance of lymphatic prodrug absorption as an increasingly useful prodrug strategy.

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2.5.8

Colonic Delivery

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Table of Contents

List of Abbreviations

5-ASA.....5-Aminosalicylic acid
BPAA.....Biphenylylacetic acid
C/B .....Ratio of distal colon wet weight to body weight
CDS .....Colonic damage score
cfu.....Colony-forming unit
CyD .....Cyclodextrin
GI .....Gastrointestinal
HPMA.....*N*-(2-Hydroxypropyl)methacrylamide
IBD.....Inflammatory bowel diseases
MPO.....Myeloperoxidase
T/B .....Ratio of thymus wet weight to body weight
TNBS .....2,4,6-Trinitrobenzenesulfonic acid

Introduction

There are a number of colonic diseases that could be treated more effectively using a colon-specific delivery system. These include ulcerative colitis, colorectal cancer, and Crohn's disease (Hanauer and Kirsner, 1988; Riley, 1993). The local delivery of drugs such as anti-inflammatory agents, anticancer agents, and antibiotics to the colon should permit lower dosing resulting in fewer side effects and increasing therapeutic efficacy. The principal goals of colon-specific delivery after oral administration are, first, to avoid absorption and biodegradation of drugs in the upper intestine such as in the stomach and small intestine where acid- or enzyme-labile drugs are degraded and most small drug molecules are absorbed and, second, to release drugs site-specifically in the lower intestine such as in the cecum and colon. With these goals in mind, many different colon-specific drug delivery systems have been investigated during the last decade (Friend, 1992; Rubinstein, 1995; Hovgaard and Brønsted, 1996; Kinget *et al.*, 1998; Sinha and Kumria, 2001). Among these, the most important are film-coating of drug formulations with pH- or pressure-sensitive polymers, coating with bacterial degradable polymers, delivering drugs from time-dependent formulations or biodegradable matrices, and delivering drugs from small molecule prodrugs or polymeric conjugates. The prodrug approach to colonic delivery is currently used to treat inflammatory bowel diseases (IBD), in which active drugs are liberated by the action of bacterial enzymes such as azo-reductase, glucosidases, and glucronidases in the colon (Friend, 1992; Sinha and Kumria, 2003). In this section, we focus our discussion on the prodrug approaches to colon-specific drug delivery of anti-inflammatory drugs such as 5-aminosalicylic acid (5-ASA) and steroids used for the management of IBD.

Physiological Factors

The gastrointestinal (GI) tract is divided into the stomach, the small intestine and the large intestine. The entire length of the human GI tract is about 500–700 cm with a surface area of 200,000 cm^2; the length of the duodenum is 20–30 cm, that of the jejunum is 150–250 cm, that of the ileum is 200–300 cm, and that of the colon is 90–150 cm (Gruber *et al.*, 1987; Faigle, 1993). The small intestine consists of the duodenum, jejunum, and ileum; the surface of these tracts is covered with villi and microvilli that increase enormously the mucosal surface area and contain many digestive enzymes and transporter systems. Therefore, almost all nutrients and small foreign molecules, such as drugs, are absorbed from the small intestine or are degraded to small absorbable molecules that then enter into systemic circulation. Because of the high surface area of the small intestines, this constitutes a significant barrier to the delivery of prodrugs to the colon in an intact form.

The pH of the intestinal fluids affects the efficacy of colonic drug delivery after oral administration (Rubinstein, 1995). The pH of the resting human stomach is 1.3–1.7, but increases to 5–6 after ingestion of a meal. The duodenal

pH in fasted humans is about 6.4. The pH of the colon varies depending on the types of food ingested. For example, the pH drops to the range of 5.0–6.5 because of the acidification of colonic contents by the products of bacterial fermentation, particularly in the right colon compared with the left colon. The strong acidic fluid of the stomach is one of the biggest obstacles to the delivery of intact acid-labile prodrugs to the colon. These pH changes affect the degree of ionization of weak acidic and basic produgs and their chemical stability.

The transit time of prodrugs in the GI tract significantly affects the efficacy of the colonic delivery system (McLeod and Tozer, 1992; Tozer *et al.*, 1995). The transit time through the GI tract varies depending on various factors such as the GI motility, the quality and quantity of food ingested, and physiological factors (Davis *et al.*, 1986; Coupe *et al.*, 1991). The average overall transit time from the mouth to the anus in humans is 24–72 h. In general, the transit time from the mouth to the small intestine in healthy human adults is 0.5–2 h, whereas it can be delayed to 3–6 h and 5–8 h after the intake of light meals and heavy meals, respectively. It is longer for solid meals than for liquids. The transit time from the stomach to the large intestine is 2–4 h, and that from the small intestine to the anus is 6–48 h. Therefore, colon-specific prodrugs should be chemically stable until they arrive in the colon, but the long transit time in the colon gives sufficient time for release of an active drug from the prodrug. The intestinal transit times vary markedly between different animal species. Rats and mice have an overall transit time (20–30 h) and a small intestinal transit time (4–5 h) similar to those of humans (Pettersson *et al.*, 1976; Gruber *et al.*, 1987). Further, age, diseases, and other physiological conditions alter these transit times, resulting in changes in composition and number of bacterial microflora in the GI tract, as described in the following section. Therefore, variability in the transit time and the bacterial microflora should always be kept in mind not only in the design and development of colon-specific delivery systems but also in their administration or dosing regimens.

Biological Factors

The bacterial flora of animal GI tracts is a very complex ecosystem that contains various aerobic and anaerobic microorganisms (Scheline, 1973). The enzymes produced by these microorganisms can significantly metabolize endogenous and exogenous compounds, thus affecting the release of drugs from their produgs in the GI tract.

Human saliva contains about 10^7 colony-forming units (cfu)/mL of aerobic and anaerobic bacteria. These bacteria can then enter the stomach when the saliva is swallowed (Haeberlin and Friend, 1992). The number of bacteria in the human stomach decreases, however, to 10^2–10^3 cfu/mL in the empty state owing to its acidic conditions, and the gastric microflora is predominantly aerobic. This cfu number varies depending on the presence of food in the stomach and changes in pH. The nature and number of bacteria in the proximal small intestine are similar to those in the stomach. On the other hand, the bacterial concentration

steeply increases in the lower part of intestine, ileum, cecum, and rectum (10^{10}–10^{11} cfu/mL), where anaerobic bacteria predominantly exist. Approximately one third of fecal dry weight consists of bacteria, in which as many as 400 different bacteria species are found. The predominant species in the colon are bacteroids, bifidobacterium and eubacterium. Various enzymes produced by these bacteria play an important role in the metabolism of endogenous and exogenous compounds in this region of the intestines. For example, dietary fiber is not digested in the stomach and small intestine but it is degraded to small saccharides by sugar-degrading enzymes produced by colonic microflora. These initial products are then fermented to volatile short chain fatty acids such as acetate, propionate, and butyrate. These short chain fatty acids produced from dietary fiber roughage are utilized as a carbon source for microorganisms in the colon, and it has been postulated that their deficiency causes ulcerative colitis (Scheppach *et al.*, 1992; Butzner *et al.*, 1996). Several prodrugs of butyric acid have been prepared with aim of treating distal ulcerative colitis and colorectal cancer (Nudelman *et al.*, 1992; Pouillart *et al.*, 1992; Hirayama *et al.*, 2000).

There is a large interspecies difference in GI flora (Hawksworth *et al.*, 1971). Coprophagic animals such as rats and mice have large numbers of bacteria along the whole GI tract in relatively high concentrations (10^{6}–10^{8} cfu/mL even in the stomach and small intestine). On the other hand, the stomach and small intestine of rabbits and guinea pigs have much lower bacterial concentrations than those of rats and mice and have microflora similar to that of humans. The large intestine has higher concentrations and larger numbers of bacteria than the stomach and small intestine in all animals. From the viewpoint of bacteria distribution in the GI tract, therefore, rabbits and guinea pigs may be more suitable than rodents as models for humans in the evaluation of the prodrug approach for colon-specific delivery.

Colon-Specific Delivery Prodrugs

As described above, an ideal prodrug approach for colonic delivery is to develop a prodrug that is transported intact through the stomach and small intestine but is hydrolyzed to release an active drug by action of enzymes produced by bacterial microflora of the colon. Some examples of the prodrug approach for colon-specific delivery are shown in this section.

Azo-linked Prodrugs

The azo linkages are cleaved to form a pair of amines by the action of azoreductases produced by anaerobic bacteria in the colon (Scheline, 1973). This reduction occurs favorably in the anaerobic environment created by bacteria in the cecum and colon. Most of the bacteria in the human intestine are capable of reducing azo linkages, releasing active drugs from azo prodrugs. Prontosil (Figure 1) is the first azo drug used for treatment of streptococcal infections in animals; its azo bond is split to release a sulfonamide by cecal bacteria. A prodrug of

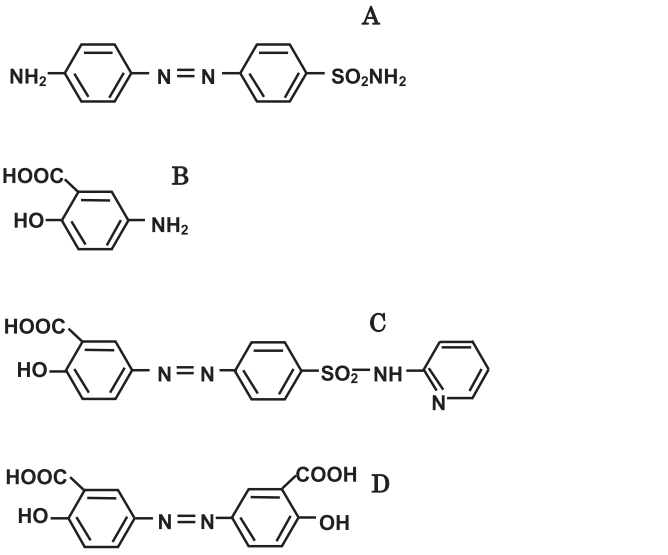

Figure 1. Prontosil (A), 5-ASA (B), Sulfasalazine (C) and Olsalazine (D).

sulfapyridine bound to 5-ASA through the azo linkage, sulfasalazine (Figure 1), was developed to deliver the sulfonamide site-specifically to the colon for the treatment of intestinal infection; this is because 5-ASA is known to have an affinity for inflamed connective tissue. Later, this drug was found to be effective in the treatment of ulcerative colitis; its pharmacological activity is attributable to the 5-ASA moiety that is released after cleavage of the azo linkage. Several azo derivatives of 5-ASA are shown in Figure 1, where olsalazine is a dimer of 5-ASA linked by the azo bond, releasing 2 moles of 5-ASA after the cleavage (Ryde, 1992; Rubinstein, 1995). This prodrug was developed to avoid the release of non-therapeutic moieties such as the sulfapyridine moiety of sulfasalazine, and is now marketed in the US, the UK, and other countries (Nilsson *et al.*, 1995). These azo

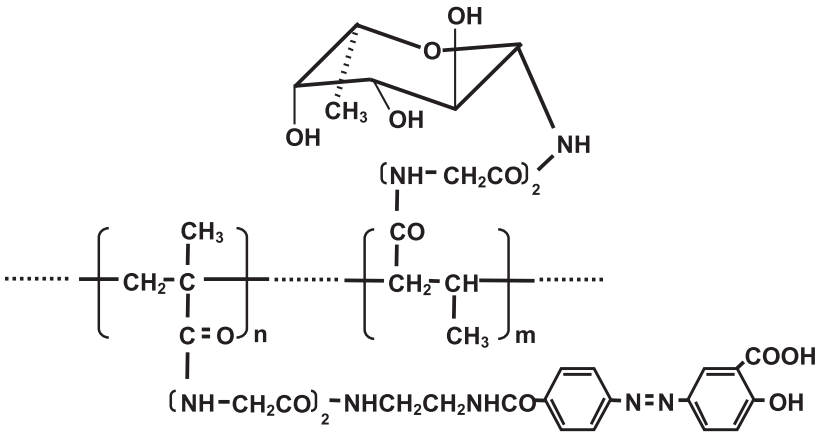

Figure 2. HPMA Polymer Azo-linked to 5-ASA and Fucosylamine.

prodrugs are poorly absorbed across the small intestine and are reduced to release 5-ASA in the colon.

As a logical consequence, various azo derivatives have been prepared to develop colon-specific delivery systems. For example, one of the most interesting polymeric prodrugs is the water-soluble *N*-(2-hydoxypropyl)methacrylamide copolymer (HPMA polymer) azo-linked to 5-ASA, developed by Kopeãek *et al.* (Rathi *et al.*, 1991; Kopeãek and Kopeãekova, 1992). This polymer was further made mucoadhesive by introducing a pendant amino-saccharide moiety such as fucosylamine (Figure 2); thus, this polymer is able to anchor to mucosal lectins of the colon and release 5-ASA after reduction of the azo bond in the colon. Further, azo-crosslinked polymers have been evaluated as a coating for the colonic delivery of insulin and some drugs (Saffran *et al.*, 1986). This coating is degraded by the action of azoreductases and releases the drugs site-specifically in the colon.

Amino Acid Prodrugs

Nakamura *et al.* (1992a,b,c) prepared some conjugates of salicylic acid linked covalently with amino acids such as alanine, methionine, tyrosine, and glutamic acid (Figure 3) with the aim of developing a potent prodrug of salicylic acid to prolong its blood concentration after the metabolism by intestinal microorganisms. These amino acid moieties were incorporated to avoid absorption from the stomach and small intestine. However, the methionine prodrug was absorbed from the upper duodenal tract, without being metabolized to salicylic acid, when administered to rabbits. The tyrosine prodrug was hydrolyzed in the small intestinal mucosa of rabbits, and thus it is probably difficult to deliver the prodrug to the lower part of the GI tract without hydrolysis. On the other hand, the L-alanine and glutamic acid prodrugs were hydrolyzed only in the contents of the lower GI tracts such as cecum and colon but not in the upper ileum. When these prodrugs were orally administered to rabbits, the active moiety of the prodrugs, salicylic acid, was detected 2 h after administration and reached the maximum blood level at 10–18 h after the dose. This delayed appearance of salicylic acid in the blood after oral administration is typically seen from colonic delivery systems

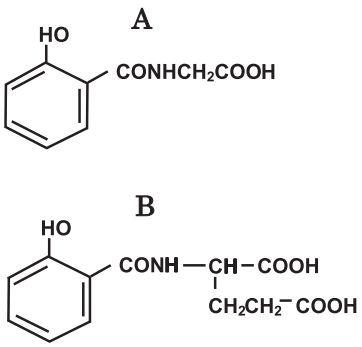

Figure 3. Salicylic Acid Prodrugs Linked to Alanine (A) and Glutamic Acid (B) through an Amide Bond.

because it takes several hours for the proposed prodrug/system to reach the colon. The 5-ASA/amino acid prodrug is also reported to work as a colon-specific delivery prodrug for 5-ASA (Jung *et al.*, 2000). These results indicate that small polar or ionic amino acids may be useful as promoieties for colon-specific delivery prodrugs.

Glucoside and Glucuronide Prodrugs

There are a large number of plant glycosides such as flavonoids, amygdalins, and sennosides. Further, many exogenous and endogenous substances are metabolized to water-soluble glucuronides in mammals and are excreted as the conjugates. These glycosides and glucuronides are polar compounds and are generally poorly absorbed from the GI tract. However, the intestinal microflora is capable of metabolizing these conjugates (Scheline, 1973). As a result, when these conjugates reach the lower portions of the GI tract, they are hydrolyzed to liberate the aglycones by the action of colonic bacteria. This conversion can sometimes have a toxicological effect in humans when plants are taken orally (Brown, 1977). Many drug glucuronides are excreted by the liver via the bile to the upper GI tract. There they can be hydrolyzed to the parent drug and reabsorbed from the tract and enter the systemic circulation; this has been referred to as enterohepatic circulation. The estimated bacterial β-D-glucosidase activities in human are 0.0001, 0.004, and 78 μmole of *p*-nitrophenol-β-D-glucoside degraded per h per g intestinal contents for the proximal small intestine, the distal small intestine, and cecum/colon, respectively. Similarly, bacterial β-D-glucuronidase activities are 0.00002, 0.0009, and 9.0 μmole of phenolphthalein-β-D-glucoside degraded per h per g intestinal contents, respectively (Hawksworth *et al.*, 1971). These results suggest that glucosides and glucuronides can work as colon-specific delivery prodrugs, *i.e.*, they can survive passage through the stomach and small intestine but liberate the active drug in the colon by enzyme-catalyzed hydrolysis of bacteria.

Prednisolone- and dexamethasone-21-β-glucosides and budenoside-, dexamethasone-, and menthol-β-D-glucuronides have been prepared (Friend and Chang, 1985; Friend 1992; Nolen and Friend 1994; Nolen *et al.*, 1995). The colon-specific release of these prodrugs was confirmed by *in vitro* and *in vivo* experiments. The budenoside conjugate (Figure 4) showed superior pharmaco-

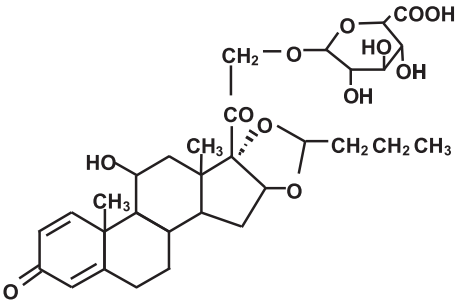

Figure 4. Budenoside-β-D-glucuronide.

logical activity compared to that of the parent drug with decreased adrenal suppression side effects.

Cyclodextrin Prodrugs

Cyclodextrins (CyDs) are cyclic oligosaccharides usually consisting of six to eight glucose units, which are called α-, β-, and γ-CyDs, respectively. The most important characteristic of CyDs is their ability to form inclusion complexes with various drug molecules, where the drug is included in the CyD cavity. The inclusion complexation of CyDs is utilized for improvement of the physicochemical and biological properties of drugs (Duchêne, 1991; Stella and Rajewski, 1997; Uekama *et al.*, 1998; Uekama, 2004).

CyDs are known to be poorly hydrolyzed by some glycosidases (see discussion below) and only slightly absorbed in passage through the stomach and small intestine because of their high polarity. However, they are fermented by some colonic microflora into small saccharides and thus absorbed as maltose or glucose in the large intestine (Antenucci and Palmer, 1984; Flourié *et al.*, 1993). Most bacteroid strains isolated from the human colon are capable of degrading CyDs as evidenced by their ability to grow on CyDs, using them as the sole carbon source, and by the stimulation of cyclodextranase activity by exposure to CyDs. This biodegradation property of CyDs is useful as a colon-targeting carrier, and thus CyD prodrugs can serve as a part of the promoiety for site-specific delivery of drugs to the colon. We have designed the CyD conjugates of non-steroidal anti-inflammatory drugs, biphenylylacetic acid (BPAA) and ketoprofen, a steroidal drug, prednisolone, and a short-chain fatty acid, *n*-butyric acid. These and other prodrugs for colonic delivery of the anticancer agent 5-fluorouracil are shown in Figure 5 (Hirayama *et al.*, 1996, 2000; Uekama *et al.*, 1997; Minami *et al.*, 1998; Yano *et al.*, 2001a,b, 2002; Kamada *et al.*, 2002).

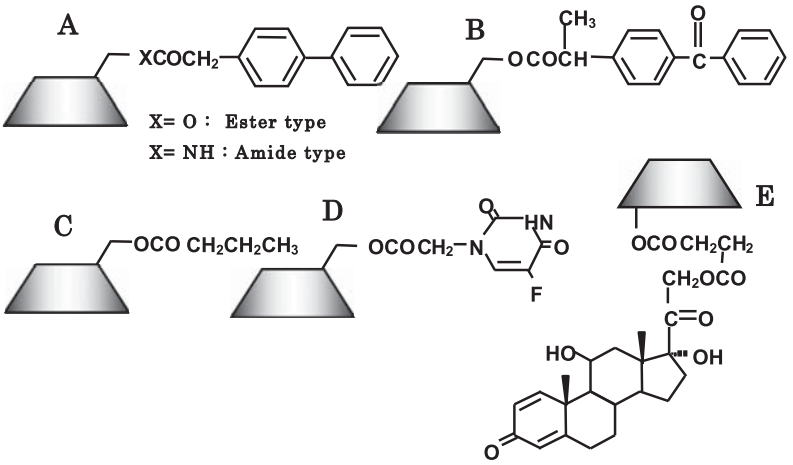

Figure 5. CyD Produgs Linked to Drugs through Ester or Amide Bonds.
(A) BPAA conjugates, (B) Ketoprofen conjugate, (C) *n*-Butyric acid conjugate, (D) 5-Fluorouracil conjugate, (E) Prednisolone conjugate.

We prepared the amide- and ester-type conjugates of biphenylylacetic acid (BPAA) with CyDs (Uekama *et al.*, 1997). The release profiles of BPAA after incubation of the ester conjugates in rat GI tract contents, intestine and liver homogenates, and blood in isotonic buffer solutions were compared with those of the ethyl ester of biphenylylacetate, a simple ethyl ester of BPAA. Ethyl biphenylylacetate was easily hydrolyzed in liver and gastrointestinal homogenates and also in blood, whereas it was quite stable in cecal and colonic contents. In sharp contrast, the α- and γ-CyD ester conjugates released BPAA quantitatively in cecal and colonic contents, while they liberated no appreciable drug on incubation with the other fluids. The CyD amide conjugates hardly released BPAA at all, but liberated only the maltose/BPAA or small oligosaccharide/BPAA conjugates, probably because the amide linkage is chemically enzymatically stable. These results suggest that the CyD rings of the prodrugs are hydrolyzed to the linear saccharides in the cecal and colonic contents but not in the contents of the stomach and the small intestine. In the case of the ester conjugates, the resulting maltose/BPAA or small oligosaccharide/BPAA conjugates are further hydrolyzed to BPAA, whereas for the amide conjugates the hydrolysis stops at the ring-opening step, as shown in Figure 6. Therefore, two enzymes are involved in the drug release of the CyD conjugates, *i.e.*, first, sugar-degrading enzymes and, second, ester-hydrolyzing enzymes. This consecutive hydrolysis mechanism was demonstrated in hydrolyses of the *n*-butyric acid/β-CyD ester conjugate and the 5-fluorouracil/β-CyD conjugate catalyzed by α-amylase (Aspergillus oryzae) and carboxylic esterase (porcine liver) (Hirayama *et al.*, 2000).

Figure 7 shows the serum levels of BPAA after oral administration of the BPAA/α- and γ-CyD ester conjugates in rats, compared with levels of the drug alone. The serum levels BPAA from the α- and γ-CyD conjugates increased after a lag time of about 3 h and reached maximum levels at about 8–9 h; this also resulted in a significant increase in the extent of bioavailability. The extent of bioavailability from the α- and γ-CyD conjugates was about 4 and 5 times larger

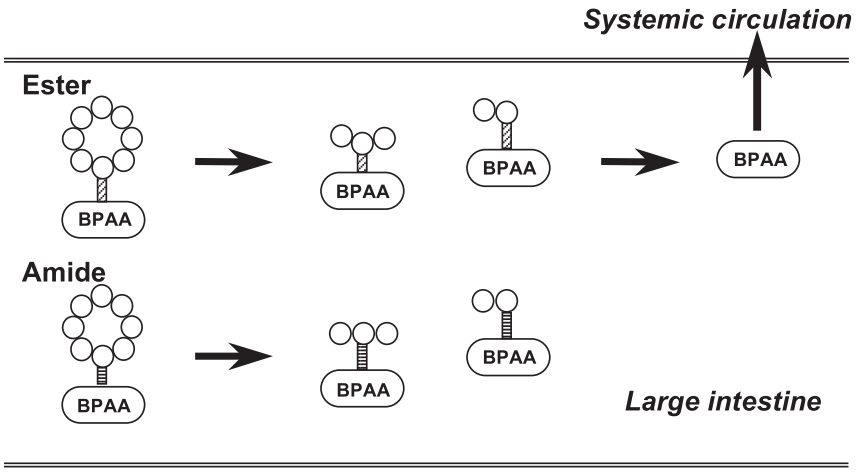

Figure 6. BPAA Release in Large Intestine after Oral Administration of BPAA/γ-CyD Ester and Amide Conjugates. Open circle: Glucose unit in CyD molecule.

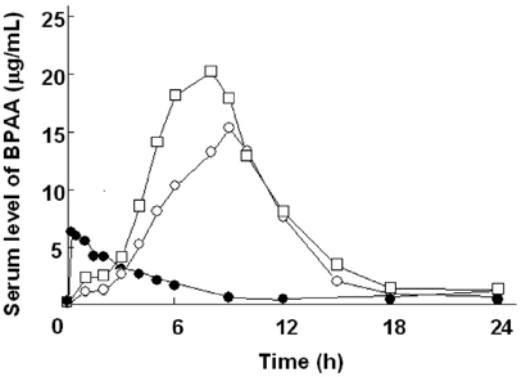

Figure 7. Serum Levels of BPAA after Oral administration of BPAA and it's α-CyD and γ-CyD Ester Conjugates (equivalent to 10 mg/kg BPAA) in rats.
Closed circle: BPAA alone, open circle: α-CyD conjugate, open square : γ-CyD conjugate.

than that of BPAA alone, respectively. The anti-inflammatory effect of the BPAA/γ-CyD ester conjugate after oral administration was evaluated using the model of carageenan-induced acute edema in rat paw. Because BPAA is produced after the conjugate has reached the cecum and colon, the anti-inflammatory effect of the conjugate was much higher than that of the drug alone and was highest 12h after the administration.

Glucocorticoids are known to be effective in the treatment of various inflammatory and allergic disorders and have been used to treat ulcerative colitis patients. However, oral and intravenous administrations of glucocorticoids to patients with severe ulcerative colitis are restricted because of the undesirable systemic side effects such as adrenosuppression, immunosuppression, hypertension, and osteoporosis. To reduce such side effects, rectal application has been used to treat ulcerative colitis. However, chronic use of glucocorticoids in high doses often causes systemic side effects even after topical application. One of the methods to overcome this problem, is to develop a prodrug that is poorly absorbed from intestinal tracts and slowly releases the active drug at the site of action, the colon. The colon-specific drug delivery system may be particularly useful for the treatment of ulcerative colitis. Therefore, we prepared the prednisolone-appended α-CyD conjugate where the drug is covalently bound to the secondary hydroxyl groups of α-CyD by an ester linkage through a spacer of succinic acid, as shown in Figure 5 (Yano *et al.*, 2001a,b, 2002). Interestingly, the ester conjugates are very soluble (> 50%w/v) in water, the solubility being > 1000 times those of prednisolone and its hemi-succinate ester. This is in contrast to the decreased solubility of CyD derivatives conjugated at the primary hydroxyl groups. For example, the aqueous solubility of the BPAA and *n*-butyric acid conjugated at the primary hydroxyl group of β-CyD was about 1/10 that of the parent drug.

The prednisolone-appended α-CyD ester conjugate was orally administered to 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis rats, and its anti-

inflammatory and systemic adverse effects were compared with those of prednisolone alone and α-CyD alone (Yano *et al.*, 2002). The results are shown in Figure 8. The colonic damage score (CDS), the ratio of distal colon wet weight to body weight (C/B), and the myeloperoxidase (MPO) activity were evaluated as measures of the therapeutic effect of prednisolone, whereas the ratio of thymus wet weight to body weight (T/B) was evaluated as a measure of the side effects of the drug. Healthy rats gave a CDS of 0, a C/B ratio of 0.0018, a MPO activity of 0.00011 units/mg tissues, and a T/B ratio of 0.0014. In the case of the control experiment, in which the saline solution without drugs was administered after the TNBS treatment, the CDS value, the C/B ratio and the MPO activity were 8.2, 0.0069, and 0.0115 units/mg tissue, respectively. The solution of α-CyD alone (2.2% w/v) without drugs gave therapeutic indices similar to those of the control experiments, indicating no anti-inflammatory effects of the CyD on the TNBS-induced colitis under the experimental conditions. On the other hand, oral administration of prednisolone alone and the prednisolone/α-CyD conjugate (equivalent doses of 10 and 20 mg/kg of prednisolone) significantly decreased the CDS, the C/B ratio, and the MPO activity, indicating higher anti-inflammatory

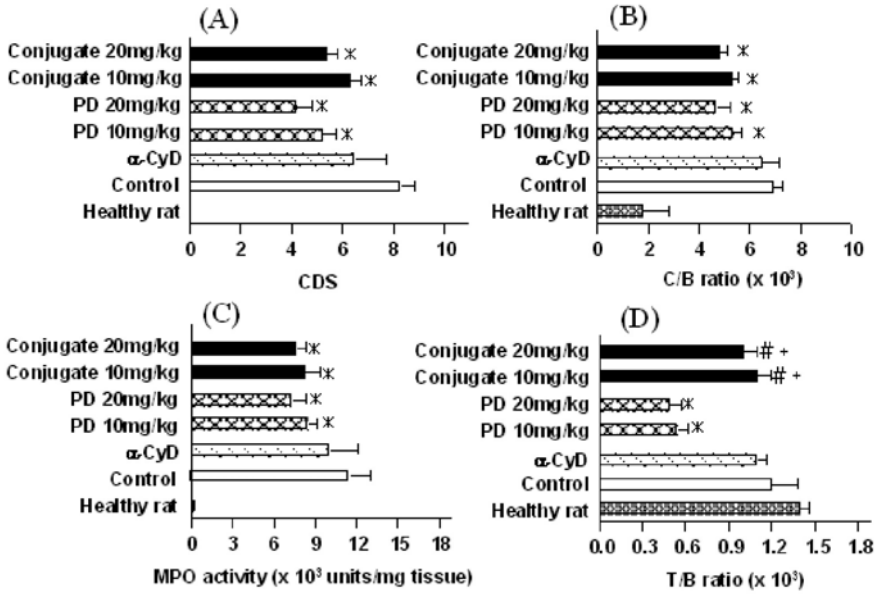

Figure 8. CDS (A), C/B Ratio (B), MPO Activity (C) and T/B Ratio (D) after Oral Administration of Prednisolone and Prednisolone/α-CyD Conjugate to TNBS-induced Colitis Rats.

Control: Administration of 0.5% methylcellulose solution without drugs to TNBS-induced colitis rats.

α-CyD: Administration of α-CyD (2.2%w/v) solution without drugs to TNBS-induced colitis rats.

Each value represents the mean ± S.E. of 4–9 rats.

*: $p < 0.05$ vs. control, #: $p < 0.05$ vs. prednisolone (10 mg/kg), +: $p < 0.05$ vs. prednisolone (20 mg/kg).

activity. The CDS value and the MPO activity of the conjugate and prednisolone alone were similar. The thymus atrophy is known to be a typical systemic adverse effect from steroid therapy. The administration of prednisolone alone gave the smallest T/B ratio ($4–5 \times 10^{-4}$), indicating a significantly higher adverse effect. On the other hand, the prednisolone/α-CyD conjugate gave a larger T/B ratio, which was the same as that (1.2×10^{-3}) of the control experiment, indicating a small systemic adverse effect. These results suggest that the conjugate can alleviate the adverse effects of prednisolone without reducing its therapeutic effect.

In vitro and *in vivo* hydrolysis studies indicated that the conjugate maintains local concentration in the colon at a low but constant level for a long period due to the slow ester hydrolysis. The high anti-inflammatory effect with the low adverse effect was observed also in intracolonic administration of the ester conjugate to TNBS-induced colitis rats (Yano *et al.*, 2001a,b). Therefore, CyDs can serve as promoieties for colon-specific targeting prodrugs, and the CyD prodrug approach can provide a versatile means for construction of colon-specific delivery systems for certain drugs.

Conclusion

Because it has the potential to give the highest efficacy of drugs with minimal adverse effects, site-specific drug delivery, *i.e.*, targeting, is one of the biggest goals currently challenging pharmaceutical scientists. In the design of colon-specific delivery prodrugs for oral administration, it is most important to deliver the prodrug in an intact form to the colon while minimizing absorption and degradation in the upper intestine. Further, the liberation of an active drug from the prodrug in the colon should be triggered by external stimuli that are intrinsic to the colon. Among these stimuli are pH, motility, pressure, electric potential, bacteria, and enzymes of the colon. The prodrug approach for colon delivery may be useful not only for anti-inflammatory agents but also for anti-cancer agents and protein/peptide drugs (Bai *et al.*, 1995). A comprehensive understanding of the physiology and biology of the colon, as well as its biochemistry, will provide insight into and, perhaps, clinical successes in the design of colon-specific prodrugs.

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Part III

Functional Group Approach to Prodrugs

3.1

Prodrugs of Carboxylic Acids

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Table of Contents

List of Abbreviations

ACE .....Angiotensin-converting enzyme
bid .....twice a day
HMG-CoA.....3-hydroxy-3-methylglutaryl-Coenzyme A
L-Dopa L-3,4-dihydroxyphenylalanine
logD .....partition coefficient on a logarithmic scale between octanol and water at a given pH value, typically at a pH of 7.4
mGluR2 .....metabotropic glutamate receptor 2
NSAIDs .....non-steroidal anti-inflammatory drugs
PEPT1.....oligopeptide transporter 1
PGF_{2a}.....Prostaglandin F_{2a}
qid.....four times a day

Introduction

Carboxylic acids are often present as a functional group of effective medicines. They are involved in specific charge-charge interactions and are thus often critical for the binding of agents to their targets. The carboxylic acid functional group adds to the hydrophilicity of the drug as well as to its polarity and this may impede the bioavailability. Most carboxylic acids have a pKa value of about 3.5 to 4.5 and thus these compounds are ionized (deprotonated) under physiological conditions. Quite often, the logD values of these agents drop below the range of logD values of well absorbed drugs (logD > 0). These properties of carboxylic acids in active principles has been well recognized for many years and a number of practical solutions have been identified with the most common one being that of the ester modification. This brief review will concentrate on successful prodrug strategies for carboxylic acids, with particular emphasis on compounds which have reached the market, and will not cover ester prodrugs of alcohols and phenols.

The great majority of prodrugs of carboxylic acids are of the ester type. The attractiveness of using esters comes from the ready availability of a diverse set of alcohols and phenols and the ubiquitous distribution of esterases and peptidases to liberate the active principle in the *in vivo* setting. Thus the physical as well as the kinetic properties of the ester prodrug can be tailored to the specific acid at hand to optimize the absorption, chemical and/or enzymatic conversion and distribution of the drug. A special case of an ester prodrug is that of an internal lactone formed with a hydroxyl group in the appropriate proximity of the carboxylic acid. There are very few other prodrug strategies for carboxylic acids, which have seen clinical development or have reached the market. One of these is Losartan, a primary alcohol that undergoes oxidative metabolism to the corresponding carboxylic acid. There are to-date no commercially successful prodrugs of carboxylic acids of the amide variety. This is a direct consequence of the rate of metabolic conversion of a typical amide to the carboxylic acid, which is generally significantly slower than that of an ester.

The area of ester prodrugs has been extensively reviewed in recent publications (Beaumont *et al.*, 2003; Testa and Mayer, 2003). This review focuses on commercially successful examples of prodrugs of carboxylic acids and attempts to delineate some of the guiding principals in the selection process for particular modifications.

Ester Prodrugs

Methyl Esters

The methyl ester is the simplest ester modification of a carboxylic acid. The conversion to the acid releases methanol, which is not the most benign alcohol. Methanol is converted metabolically by stepwise oxidation to formaldehyde and to formic acid. The toxicity of methanol has been extensively reviewed (Kostic and

Dart, 2003; Tephly, 1991) and shown to be due mainly to the accumulation of its secondary metabolite, formate. The major toxicity manifestations are acidosis and blindness, however high systemic concentrations of methanol are required to show these effects. Therefore it is not surprising that methyl esters are found in two types of drugs: a) as a prodrug moiety of low dose compounds, such as prostaglandins and b) in drugs in which the methyl ester is required for activity and either a slow conversion to the carboxylic acid is the goal or is a compound with a short duration of action. Within the prodrug class, the methyl esters are found most often in sterically unencumbered positions to facilitate conversion to the carboxylic acid. Illustrative Examples are shown in Table 1.

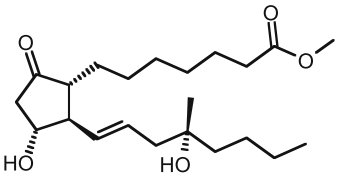
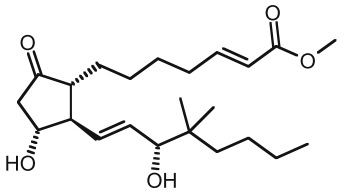
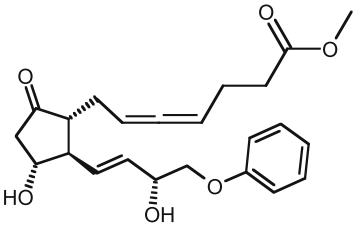
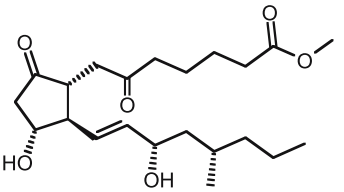
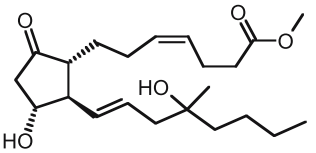
Name	Structure	References
Misoprostil		Monk and Clissold, 1987
Gemeprost		Green, 1984
Enprostil		Goa and Monk, 1987
Ornoprostil (Ronoprost)		Inoue and Kajiyama, 1986
Enosiprost		Allan et al., 1987

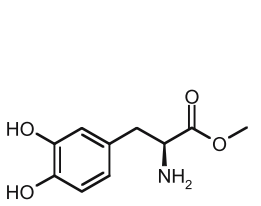
Table 1. Prostaglandin Methyl Esters

Prostaglandins undergo a number of metabolic transformations, in part due to the highly lipophilic nature of these compounds. The metabolic transformations start in the intestine and continue through the absorption process, before reaching the liver and the first pass metabolism in that organ. As a consequence, only a small fraction of an oral dose of a typical prostaglandin reaches the systemic circulation. This has made the development of prodrugs difficult. In a series of papers, a group at Upjohn reported on alkyl and aromatic esters of prostaglandins and detailed their physical (melting point, solubility) and pharmacokinetic properties (Morozowich *et al.*, 1979; Anderson and Conradi, 1980). These investigations showed that the pharmacokinetic properties are dominated by metabolism and the type of the ester prodrug had only a minor influence on exposure. It is for this reason that the prostaglanding prodrug field is dominated by the most simple of esters, the methyl esters. Their facile conversion to the acid is for instance demonstrated by the pharmacokinetic properties of misoprostil (Cytotec), the most widely used prostaglandin methyl ester (Monk and Clissold, 1987). The methyl ester is not detectable in human plasma after oral administration at typical doses of 0.2 to 1.2 mg per day (bid or qid), with the acid the dominant species, which undergoes further, slower metabolism. The primary half life of the acid in humans is 1.5 hrs.

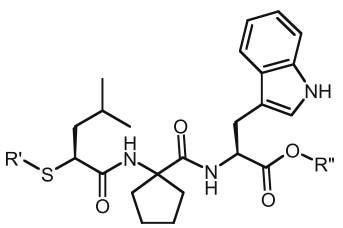
A great variety of esters of L-Dopa (L-3,4-dihydroxyphenylalanine) have been investigated (Testa and Mayer, 2003. p. 426-427). Only the methyl ester, Levomet, has reached the market. As discussed by Testa and Mayer (2003), the rate of enzymatic hydrolysis by carboxyesterase does not generally vary much with the size of primary and secondary esters and only the tert-butyl ester is protected from both enzymatic and chemical hydrolysis *in vivo*; the latter due to extensive protein binding.

An interesting, orally active, double prodrug (CGS 37808) of the endothelin-converting enzyme-1, neutral endopeptidase 24.11 and angiotensin-converting enzyme (ACE) inhibitor CGS 35601 has been characterized (Trapani *et al.*, 2004). While the parent showed only activity after iv administration, the prodrug provided cardiovascular effects after oral administration in rats. Detailed pharmacokinetic data has not yet been published.

The foregoing discussion may suggest that all esters, in particular methyl esters, act as prodrugs for carboxylic acids. This is certainly not the case and there are a number of agents in which the methyl ester is critical for activity, with the corresponding acid being inactive. Often such active esters are found in short



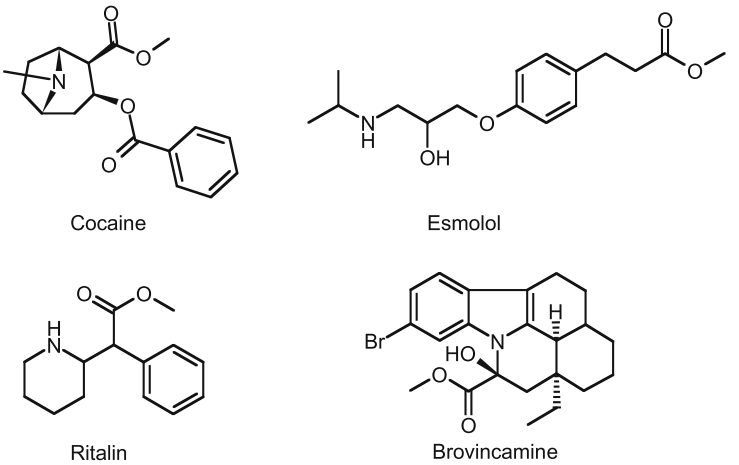
Levomet



CGS 35601: R'= R" = H
CGS 37808: R'= Ac; R" = Me

Structures of Levomet, CGS 35601 and CGS 37808

acting therapeutic agents that undergo rapid hydrolysis to the acid by chemical as well as enzymatic means. Examples of such agents are cocaine, the ultra-short acting β-blocker Esmolol, the attention-deficit / hyper-activity disorder drug Ritalin and the cerebral vasodialator Brovincamine. The methyl ester in Brovincamine is quite hindered, achieves good systemic exposures in man and declines with bi-phasic half lives of 1 h and 5h (Mayo *et al.*, 1985). The major metabolite is the corresponding acid. For Ritalin, an extended release formulation has been developed to prolong the action of the drug (Lyseng-Williamson and Keating, 2002).



Structures of Cocaine, Esmolol, Ritalin and Brovincamine

Ethyl Esters

Ethyl esters are the most widely used alkyl ester prodrug. First of all, there are no toxicity concerns stemming from the prodrug moiety, ethanol, as the concentrations of ethanol resulting from metabolic cleavage of ethyl ester containing pharmaceuticals are quite low, even for drugs taken in gram quantities. While for the prostaglandins, methyl esters are the preferred ester, it is the ethyl ester for a large variety of Angiotensin-converting Enzyme (ACE) inhibitors. Many compounds in this class have two carboxylic acid groups. Interestingly, in this subclass it is the mono ester, which is used as the preferred prodrug and not the di-ester. The reason is that the hydrophobic properties need to be balanced with the solubility of the compounds. Second, and more importantly, the mono-acids are substrates of the intestinal transporters PEPT1 leading to higher absorption rates than what would be expected based on the lipophilicity and anticipated passive transport through cellular membranes of these compounds (Moore *et al.*, 2000). It has been shown that in addition to a proline like carboxylic acid group at one end of the molecule, the carbonyl group forming the peptide like bond to the alanine like residue is critical for recognition by the transporter (Schoenmakers *et al.*, 1999). Representative samples, all maintaining the critical recognition elements for the transporter, are shown in Table 2. Vabeno and colleagues (2005) recently described a more general conformational model for

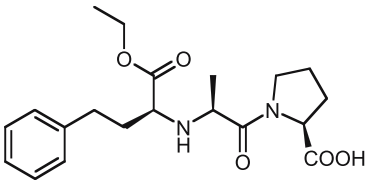
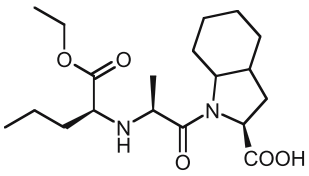
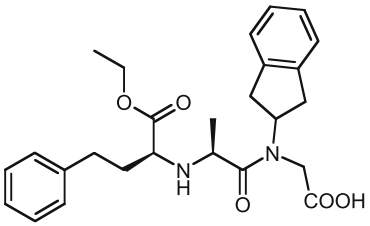
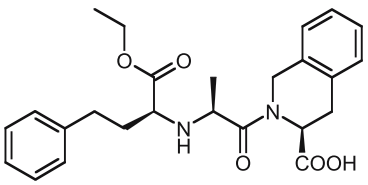
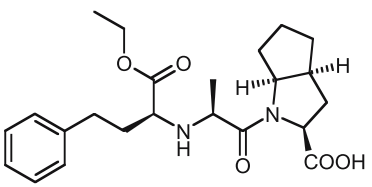
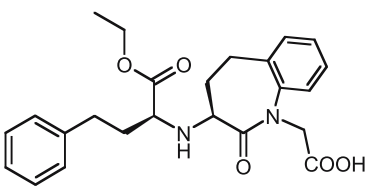
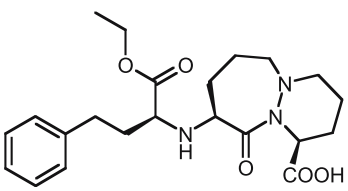
Name	Structure	References
Enalapril		Tood and Heel, 1986
Perindopril		Macfadyen, Lees and Reid, 1990
Delapril		Hutt *et al.*, 1994
Quinapril		Wadworth and Brogden, 1991
Ramipril		Todd and Benfield, 1990
Benazepril		Waldmeier *et al.*, 1991
Cilazapril		Natoff *et al.*, 1990

Table 2. Ethyl Esters of ACE inhibitors

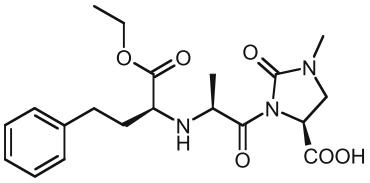
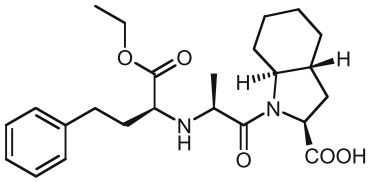
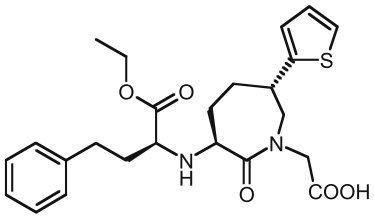
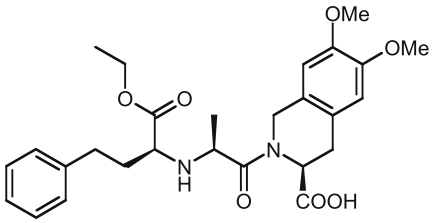
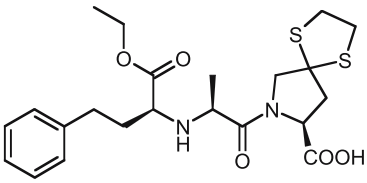
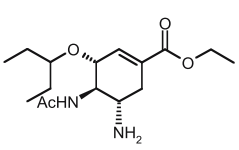
Name	Structure	References
Imidapril		Tood and Heel, 1986
Trandolapril		Macfadyen, Lees and Reid, 1990
Temocapril		Hutt *et al.*, 1994
Moexipril		Wadworth and Brogden, 1991
Spirapril		Todd and Benfield, 1990

Table 2. Ethyl Esters of ACE inhibitors

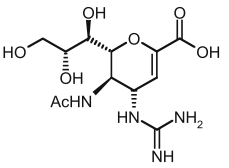
hPEPT1 substrates, which should aid in the design of prodrugs targeting this transporter.

Ethyl esters have also been employed to increase the lypophilicity of drugs without the concomitant benefit of an active transport system. Some of the most successful examples are shown below. The ethyl ester of Oseltamivir increases the oral bioavailability of the parent in man from less than 5% to 80% (He *et al.*, 1999) and allows this anti-influenza antiviral agent to be administered orally while the more polar competitor compound, Zanamivir, is administered clinically by intranasal dosing (Waghorn and Goa, 1998). Tazarotene is a topically administered retinoic acid derivative and is rapidly cleaved in the skin to the

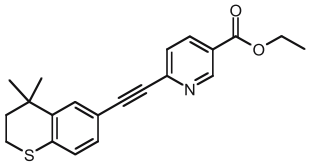
biologically active acid, tazarotenic acid (Tang-Liu *et al.*, 1999). Tazarotenic acid undergoes rapid further metabolism, including sulfoxide formation, and the systemic bioavailability is only about 1% of the total topical dose.



Oseltamivir (Tamiflu)



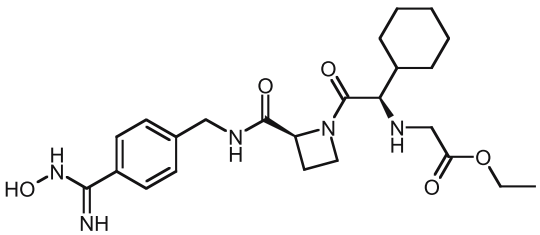
Zanamivir (Relenza)



Tazarotene

Structures of Oseltamivir, Zanamivir and Tazarotene

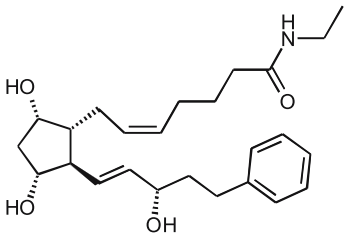
An example of a 'double' prodrug containing an ethyl ester is the thrombin inhibitor, Ximelagatran (Sorbera *et al.*, 2001). Following absorption, the ethyl ester needs to be hydrolyzed and the hydroxy-benzamidine reduced to the benzamidine to liberate the active species melagatran (see also benzamidine chapter in this book). Ximelagatran is 80-times more permeable in Caco-2 cells than melagatran and has a 20% bioavailability in man, a 2.7- to 5.5-fold increase over megalatran.



Structure of Ximelagatran

Isopropyl Esters

A number of prostaglandins have been developed for ocular application to reduce intra-ocular pressure. All examples developed thus far are isopropyl esters, which offer for this application the best pharmacokinetic properties, at least in animal models (Rabbit eye). However, it is difficult to assess the benefit of these



Structure of Bimatoprost

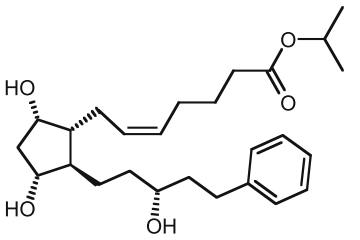
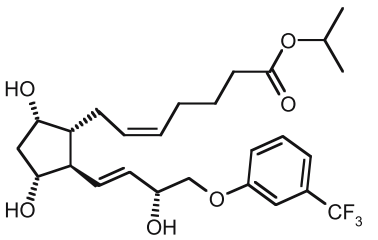
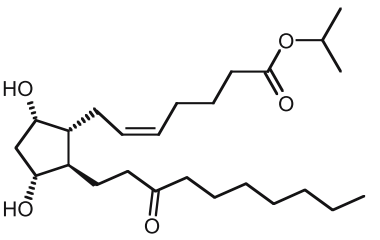
Name	Structure	References
Latanoprost		Watson, 1999
Travoprost		Sorbera and Castaner, 2000; Waugh and Jarvis, 2002
Unoprostone isopropyl ester		Linden, 2001

Table 3. Isopropylesters

prodrugs in humans as these agents are given topically in very low concentrations (0.001% in ocular solutions). Representative examples are shown in Table 3.

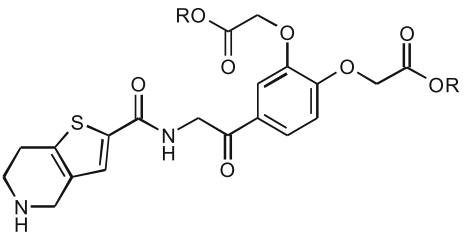
The closely related ethyl amide Bimatoprost, also a ocular agent given topically, is not a prodrug of the corresponding $PgF_{2\alpha}$ acid. This compound has profound ocular hypotensive effects on its own, and this may mask the slow metabolic conversion to the acid (Woodward *et al.*, 2004).

n-Butyl Esters

The *n*-butyl ester examples serve to illustrate different aspects of prodrug research, one detailing the special application of nasal delivery and the other the limitations caused by efflux pumps. In the first example, the *n*-butyl ester of nipecotic acid is shown after intranasal application to rats to provide plasma and brain levels of nipecotic acid equivalent to an iv application of the same prodrug (Wang *et al.*, 2005). The administration of the parent, nipecotic acid, under the same conditions gave lower brain exposures. This is explained by the longer terminal half life and the larger volume of distribution of nipecotic acid after administration of the prodrug.

R = H: ME3277

R = *n*-butyl: ME3229



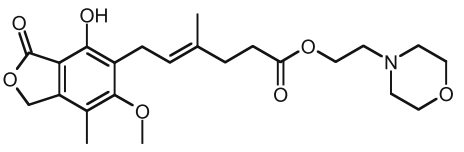
Structures of ME3229 and ME3277

Limitations to prodrugs has been demonstrated in the case of ME3277, a glycoprotein IIb/IIIa antagonist. The bis *n*-butyl ester ME3229 failed to lead to improved exposures in rodents and this was attributed to a ATP dependent efflux pump, distinct from P-glycoprotein (Pgp) and the organic anion transporter, MRP2, acting on the parent di-acid ME3277 (Okudaira *et al.* 2000a,b). Apparently, cleavage of the bis *n*-butyl esters is rapid, occurs already in enterocytes, and the liberated di-acid is effluxed efficiently. This demonstrates a significant limitation to prodrug design as the undesirable pharmacokinetic properties of the parent can not be overcome due to strong energy dependent efflux transport of the parent.

Other Alkyl Esters

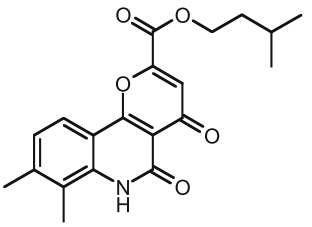
Different alkyl ester prodrugs of a number of carboxylic acid drugs have been investigated in some detail (Testa and Mayer, 2003), however very few have been studied clinically or have reached the market. Some of the most studied agents is the group of 'phenyl propionic acids' of the non-steroidal anti-inflammatory drugs (NSAIDs). The goal of these investigations was to create effective anti-inflammatory agents devoid of gastrointestinal side effects (Moerk and Bundgaard, 1992) as the parent acids already enjoy good to excellent oral bioavailabilities. This has generally not been successful as the gastrointestional side-effects could not be eliminated by either prodrugs, nor by enteric-coated or sustained-release formulations (Davies, 1999; Rothstein, 1998). It is therefore not surprising that none of these alkyl prodrugs of NSAIDs have reached the market. However, two guaiacol esters have found commercial utility due to the special property of the prodrug moiety, guaiacol; see the aryl esters section below.

The morpholinoethyl ester of mycophenolic acid is employed in the successful immunosuppressive agent CellCept®. The selection of this particular aminoalkylester modification resulted from a detailed investigation into the



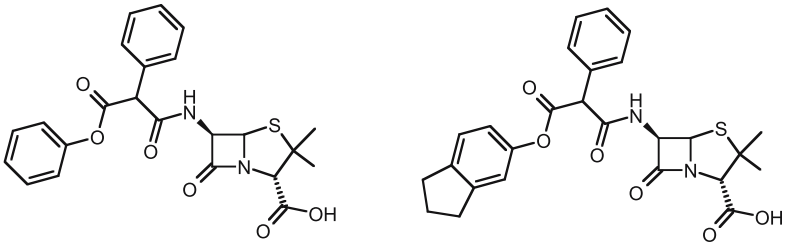
Structure of Mycophenolate mofetil

solubility and conversion rates of a number of potential prodrugs of either the phenolic or the carboxylic acid functional group (Lee *et al.*, 1990). The prodrug has greatly improved solubility at lower pH values and is rapidly converted to mycophenolic acid in plasma. The prodrug demonstrated a remarkable two-fold increase in bioavailability compared to the parent in the monkey.



Structure of Repirinast

Repirinast is the isoamyl ester of the pharmacologically active acid, the antiallergy compound MY-1250, also know as BAY-w 8199 (Beermann *et al.*, 1992). The parent acid was not orally active in experimental animals and ester prodrugs were evaluated, resulting in the selection of the isoamyl ester for development (Morinaka *et al.*, 1981). The ester is not detectable in serum after oral administration suggesting rapid cleavage during absorption as the ester is quite stable under acidic conditions (Beermann *et al.*, 1992). The bioavailability of this ester in man, as measured by the parent acid, is quite variable and is influenced by food, with a (2 fold) increase observed in the fed versus the fasted state (Schaefer *et al.*, 1993).

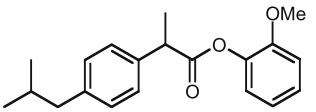


Carfecillin Carindacillin

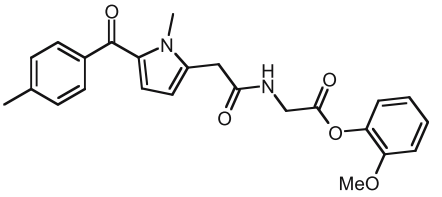
Structures of Carfecillin and Carindacillin

Aryl Esters

Esterification with phenols provides aryl esters and it would be expected that on average these would be less chemically stable than alkyl esters. It is therefore not surprising that only a limited number of aryl ester prodrugs have been investigated in detail. Among the early examples are the prodrugs of the antibiotic Carbenicillin, carfecillin (phenyl ester) and carindacillin (indanyl ester).



Ibuprofen guaiacol ester



Amtolmetin guacil

Structures of Ibuprofen guaiacol ester and Amtolmetin guacil

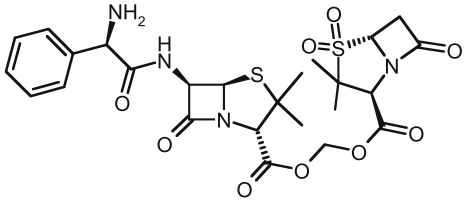
Both prodrugs showed similar improvements of the oral bioavailability (Modr *et al.*, 1977) and this was later attributed to activity of the monocarboxylic acid transporter at least for carindacillin (Li *et al.*, 1999).

Guaiacol esters have been employed for two NSAIDs, ibuprofen and tolmetin. The two prodrugs, ibuprofen guaiacol ester (Barillari *et al.*, 1982) and amtolmethin guacil (Tubaro *et al.*, 1995) show improved pharmacokinetic properties and a decrease in gastrointestinal irritation. This is attributed to the activity of the prodrug moiety guaiacol, which also inhibits prostaglandin synthesis on its own (Fossati *et al.*, 1991).

(Acyloxy)alkyl or [(Alkoxycarbonyl)oxy]methyl Esters

Alkyl esters of β-lactam antibiotics are generally cleaved too slowly *in vivo* to provide suitable exposures of the parent carboxylic acid of the antibiotic. This was attributed to steric hindrance around the carbonyl carbon in these compounds. In a landmark paper by Jansen and Russell (1965), acyloxyalkyl esters of benzylpenicillin were introduced and shown to undergo rapid, enzymatic conversion *in vivo* to the parent antibiotic. This approach has now been used numerous times, particularly for β-lactam antibiotics. Table 4 shows a set of representative examples. In practical terms, there is no significant difference between the (acyloxy)alkyl and the [(alkoxycarbonyl)oxy]alkyl esters in that both are cleaved efficiently *in vivo*.

The cleavage of the prodrug moiety is initiated at the distal carbonyl group which, in a stepwise fashion, leads to the elimination of the distal carboxylic acid followed by the 'central' aldehyde, either formaldehyde or acetaldehyde, and the free acid of the antibiotic. The overall cleavage process is quite efficient and



Structure of Sultamicillin

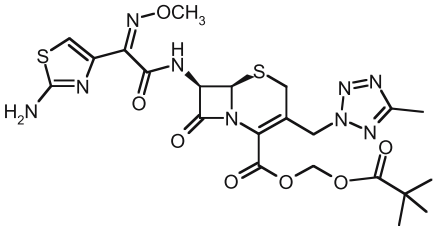
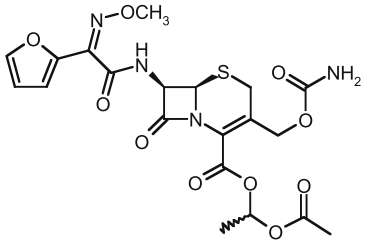
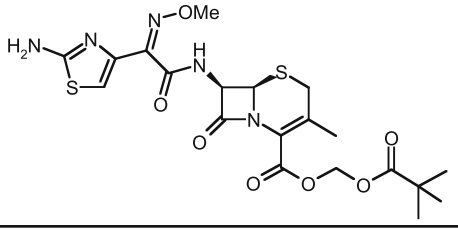
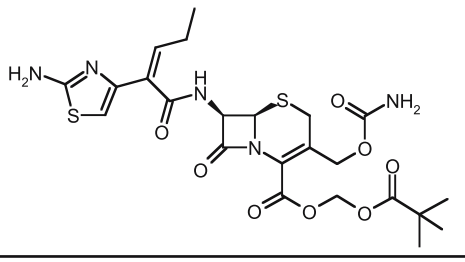
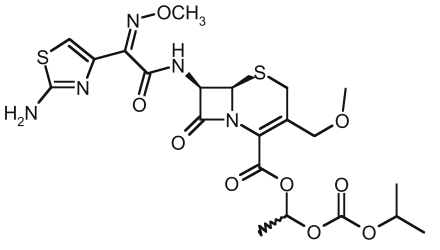
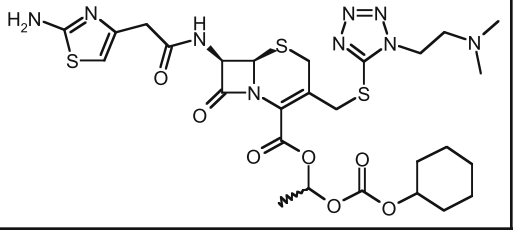
Name	Structure	References
Cefteram Pivoxil		Shimaka, 1987
Cefuroxime Axetil		Perry and Brogden, 1996
Cefatamet Pivoxil		Blouin and Stoeckel, 1993
Cefcapene Pivoxil		Fujimoto, 2002
Cefpodoxime Proxetil		Frampton et al., 1992
Cefotiam Hexetil		Imada and Hirai, 1995

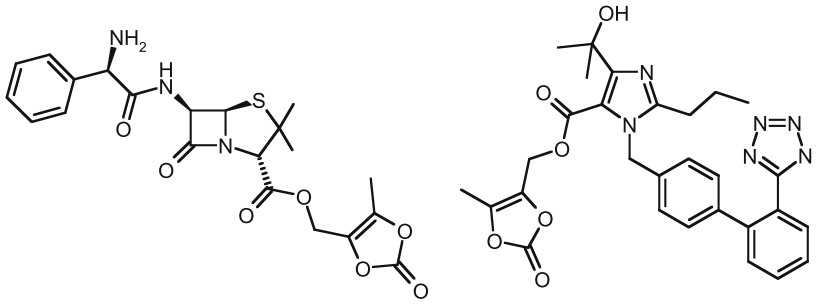
Table 4. Acyloxymethyl Esters and related Prodrugs

occurs either in intestinal epithelial cells or rapidly in plasma to furnish the antibiotic. Significant concerns have been raised about the release of 'toxic' aldehydes from these prodrugs, however these concerns typically overstate the actual toxicity of these short-lived and reactive species (Cascieri and Clary, 1992). One concern in using acetaldehyde as the aldehyde in these prodrugs is the introduction of a new chiral center, which in the case of chiral active acids, often leads to the formation of a difficult to control mixture of diastereomers.

A special prodrug of this kind is the mutually beneficial modification of the β-lactam antibiotic Ampicillin and the β-lactamase inhibitor Sulbactam to form Sultamicillin (Friedel *et al.*, 1989). Upon oral administration, the combination drug is efficiently absorbed and rapidly hydrolyzed to provide approximately three times higher plasma concentration of Ampicillin compared to an equivalent dose of the antibiotic alone. In addition, the β-lactamase inhibitor is released in equivalent amounts to Ampicillin leading to an enhanced antibacterial effect of this combination (Lode *et al.*, 1989).

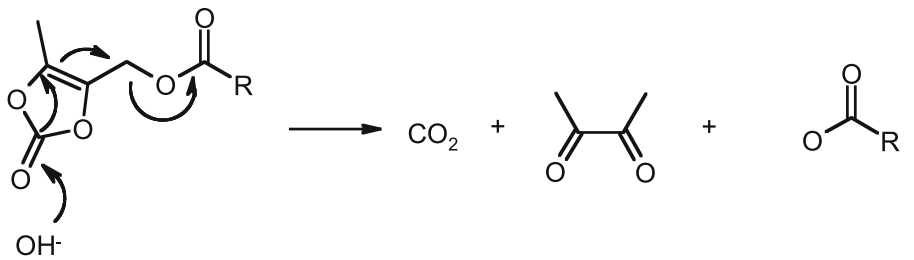
(Oxodioxolyl)methyl Esters

The achiral (5-methyl-2-oxo-1,3-dioxolen-4-yl)methyl esters have been shown to be chemically as well as plasma labile. Two examples of this ester prodrug modification have reached the market: the penicillin Lenampicillin (Saito *et al.*, 1984) and the ACE inhibitor Olmesartan medoxomil (Benicar) (Warner and Jarvis, 2002). Basic hydrolysis according to Scheme 1 leads to the parent drug, CO2 and dimethylglyoxal through the facile addition of hydroxide (or water) to

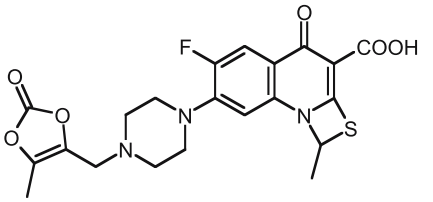


Lenampicillin Olmesartan Medoxomil (Benicar)

Structures of Lenampicillin and Olmesartan Medoxomil (Benicar)



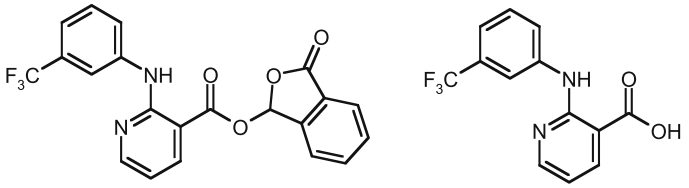
Scheme 1



NM441; Paraoxonase susceptibal prodrug of a fluoro-quinolone antibiotic

Structure of NM441

the activated carbonate like carbonyl group. In addition, it has been demonstrated for Lenampicillin that conversion in plasma is mediated by the enzyme, paraoxonase. Paraoxonase is a serum protein which hydrolyzes multiple classes of substrates, including esters, lactones and organophosphorous compounds, including paraoxon (Gan *et al.*, 1991). This approach can also be employed to modify functional groups other than acids as demonstrated by the antibacterial agent prulifloxacin (NM441). Also in this case, paraoxonase plays a major role in the hydrolysis (Tougou *et al.*, 1998)



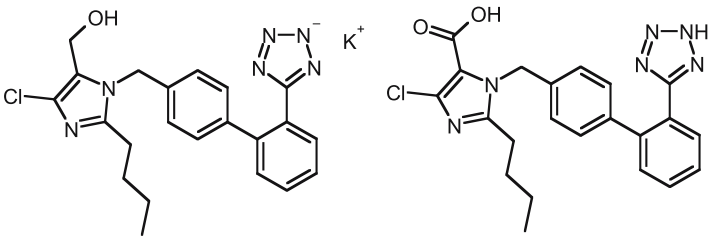
Structures of Talniflumate and Niflumic Acid

Other Esters

A special structural prodrug type is found in Talniflumate, the prodrug of niflumic acid, an anti-inflammatory agent sold in Argentina and other countries for over 20 years and is now being evaluated as a agent for the treatment of cystic fibrosis (Knight, 2005). Niflumic acid is a cyclooxygenase-2 inhibitor and in addition inhibits excess mucus production. The prodrug provides a modest benefit in this case by leading to more rapid absorption as indicated by higher plasma levels 30 min after oral dosing in healthy male volunteers (1.67 ± 0.54 μg/mL vs. 0.38 ± 0.15 μg/mL). However the total area under curve (AUC) as well as the half life was found to be essentially identical.

Oxidative Activation of Carboxylic Acid Prodrugs

There are a few examples of prodrugs, which are converted by oxidation to the active carboxylic acid. The prominent example is the active metabolite of Losartan, E3174. Both Losartan and E3174 have affinity at the Angiotensin II

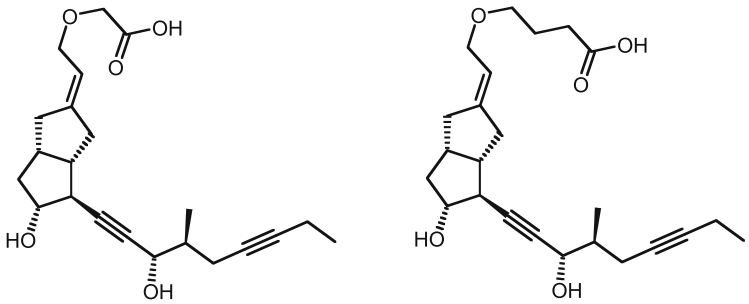


Losartan Potassium E3174

Structures of Losartan Potassium and E3174

receptor with the metabolite possessing 10 fold greater affinity and 15 to 20 times higher functional potency as an antagonist than the parent (Goa and Wagstaff, 1996).

The oral bioavailability of Losartan in man is about 33%, a result of significant first pass metabolism and not due to limitations in absorption. The conversion to the metabolite occurs already during uptake from the intestinal lumen and the Cytochrome P450 enzyme CYP2C9 is mainly responsible for this conversion (Stearns *et al.*, 1995). Higher exposure levels of the metabolite are observed in most individuals and the carboxylic acid metabolite has a longer half life in humans. Despite this successful example oxidative conversion of primary alcohols to the corresponding carboxylic acids is not a generally useful approach because of the low capacity and variability (polymorphic nature) of the oxidative pathways.



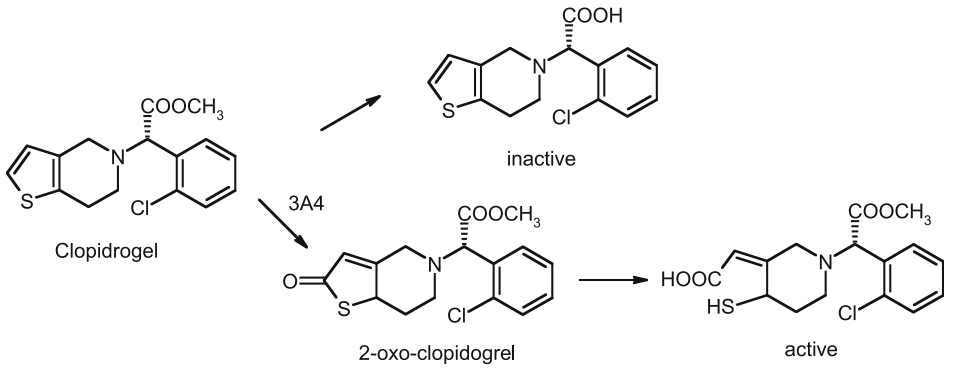
Cicaprost Eptaloprost

Structures of Cicaprost and Eptaloprost

A ultimately unsuccessful approach has been described by Hildebrand (1993) on the Cicaprost prodrug Eptaloprost. The goal of this effort was to create a sustained delivery of the prostacyclin mimetic Cicaprost through β-oxidation of a prodrug. Eptaloprost was studied in rat, monkey and man, however neither in animals nor in man could an improved systemic profile of Cicaprost be demonstrated. It is noteworthy that Cicaprost is well protected from further β-oxidation by the side chain oxygen in its β-position.

The intriguing example of Clopidogrel has been extensively reviewed. In humans, most of the dose (~ 85%) of Clopidogrel is converted to the inactive acid.

Cytochrome P450 3A4 converts most of the remaining Clopidogrel to 2-oxo-clopidogrel, which undergoes spontaneous hydrolysis to the highly active, unstable thiol (Clarke and Waskell, 2003). The thiol binds covalently through a disulfide linkage to platelet ADP receptors, which explains the anti platelet aggregation properties of Clopidogrel. The methyl ester of the active metabolite is essential for activity. It is unlikely that such a prodrug strategy could be designed a priori.

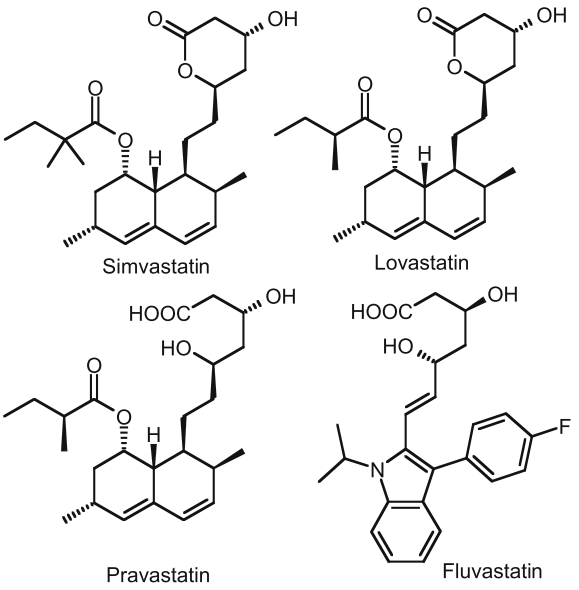


Scheme 2. Metabolic Activation of Clopidrogel

Lactones

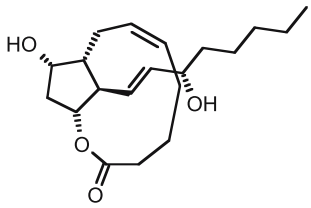
The great majority of lactone prodrugs can be found in the 'statins', inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase). The four commercially important examples, Simvastatin, Lovastatin, Pravastatin and Fluvastatin are evenly divided between lactone (prodrug) and open forms of these β,δ-dihydroxy acids. Simvastatin and Lovastatin are administered as lactone prodrugs, while Pravastatin and Fluvastatin are dosed in the active, open form (Desager and Horsman, 1996). The lactones are converted *in vivo* by carboxyesterase; a reaction which is fully reversible. The lactones have lower aqueous solubilities compared with the corresponding open forms and are not necessarily better absorbed. Analysis of any available data is complicated by the high first pass metabolism of these agents, particularly the lactones, belying the fact that they are rapidly and readily absorbed. Unfortunately, none of the examples have been tested in man in both the lactone and the open, active form and thus it is not possible to arrive at firm conclusions of the optimum form of these agents. However, comparative data is available from animal studies (Reinoso *et al.* 2002). In the case of the lovastatin, the open form (di-hydroxy acid) has higher bioavailability in dogs, rats and monkeys compared with the lactone form (Duggan *et al.*, 1989).

The lack of a clear distinction between lactones and open forms demonstrates not only the importance of balancing solubility and lipophilicity, but also the influence of first pass metabolism to bioavailability.



Structures of Simvastatin, Lovastatin, Pravastatin and Fluvastatin

A large ring lactone prodrug of a prostaglandin, the 1,11-lactone of prostaglandin $F_{2\alpha}$, has been evaluated for the treatment of ocular hypotension and found to be better tolerated than the free acid but comparable to the corresponding 11,15-bis-pivaloylester of $PGF_{2\alpha}$ (Chien *et al.*, 1997). No further details have emerged from this effort in the literature.



Structure of 1,11-lactone of prostaglandin F2α

Discussion

The examples described above show that in order to improve the pharmacokinetic properties it is not always necessary, and sometimes detrimental, to modify the polar and typically charged carboxylic acid functional groups. Care most be taken to balance solubility and polarity of the pharmaceutical agent to achieve optimal absorption. Not surprisingly, the most dramatic improvements in oral bioavailability is achieved through esterification of rather polar active principles, such as Olsetamivir (Tamiflu) or β-lactam antibiotics. In these examples, the prodrug modification removes a zwitterionic species, which in part may explain

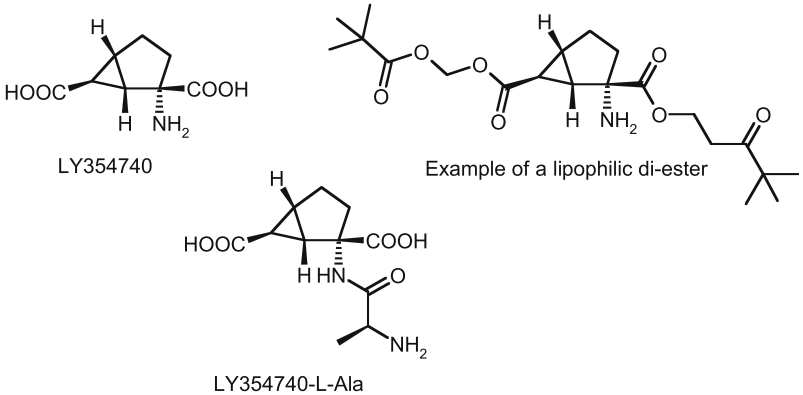
the significant improvements observed through esterification. The least successful area is the prodrug efforts for classical NSAIDs. The parent acids have good pharmacokinetic properties on their own and the goal of the prodrug effort was to reduce the gastro-intestinal side effects. Except for the special guaiacol esters containing prodrug moieties with acitivities on their own, all other prodrugs have failed to reach the market as they are unable to improve the side effect profile of the parent.

A number of prodrugs benefit from an active uptake transport. The ACE inhibitors, in the form of their mono ethyl esters are substrates of the PEPT1 transporter, while the aryl ester of β-lactam antibiotics, Carfecillin and Carindacillin, are substrates for the monocarboxylic acid transporter. On the other hand, lactone formation in the class of the statins has at best only a modest effect and may in some cases be detrimental to oral bioavailability. In these cases, esterifaction in the form of lactonization, increases the lipophilicity and also reduces the solubility of these rather lipophilic compounds.

There are a few examples of prodrugs being activated by oxidative mechanism. The hurdles are clear: most oxidative enzymes are polymorphic in man and thus the conversion may show individual differences, as has been demonstrated for Losartan making dose estimation difficult.

Prodrug modification of carboxylic acids have to be approached with caution. It is imperative to first assess the lipophilicity and solubility of the parent acid in order to develop a prodrug strategy. Simply increasing the lipophilicity while reducing solubility will in most cases be counter productive.

Equally important is the consideration of potential active transport mechanism as shown with the ACE inhibitor class. A more recent example from the Lilly labs demonstrates this very nicely. The mGluR2 agonist LY354740 is a amino dicarboxylic acid and thus a very polar compound. This compound was subjected to a detailed prodrug analysis. First a number of lipophilic di-esters were prepared with modest success (< 20% bioavailability in rats). The breakthrough came with the preparation of the L-alanine amide prodrug (LY354740-L-Ala). This is still an amino dicarboxylic acid, however it is a substrate



Structures of LY354740, LY354470-L-Ala and prototypic LY354740 di-ester prodrug

of the PEPT1 transporter, undergoes ready enzymatic hydrolysis and therefore provides the highest bioavailability of the parent (Moher *et al.*, 2003). Again this example demonstrates the importance of assessing active transport systems as well as solubility and lipophilicity in devising prodrugs of carboxylic acids.

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3.2

Prodrugs of Alcohols and Phenols

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List of Abbreviations

ACE .....Angiotensin-converting enzyme
ADEPT .....Antibody-directed enzyme prodrug therapy
AUC .....Area under curve
$C_{max,ss}$ .....Maximum concentration of drug at steady-state
GDEPT .....Gene-directed enzyme prodrug therapy
IC_{50} .....Inhibitory concentration at 50% inhibition
IV .....Intravenous
L-dopa L-3,4-dihydrophenylalanine
PEPT-1 .....Oligopeptide transporter 1
NSAIDs .....Non-steroidal anti-inflammatory drugs
$t_{1/2}$ .....Half-life

Introduction

The rationale for the development of prodrugs is the delivery of a higher concentration of the pharmacologically active drugs into the systemic circulation and to its site of action. In general, poor systemic blood levels after drug administration are due to pharmaceutical or physiological causes, leading to either poor absorption or first-pass effects such as metabolism.

Drugs containing hydroxyl groups, alcohols, and phenols can have a variety of physical/ chemical properties that have advantages and disadvantages. Too many hydroxyl groups often imparts polar properties to such molecules, and phenolic drugs in particular are often subject to phase II metabolism (see below). On the other hand, the presence of hydroxyl groups provides a handle for prodrug intervention whereby the properties of the parent drug can be manipulated. For example, acylation, alkylation, or reduction could lead to a less polar prodrug while phosphorylation can lead to a more soluble prodrug.

Although many drugs are efficiently absorbed from the gastrointestinal tract, they often exhibit limited systemic bioavailability due to first-pass metabolism or are inactivated before reaching the systemic circulation. This first-pass metabolism is well documented in drugs bearing the phenolic hydroxyl group, resulting in low bioavailability after oral administration and, thus, limiting their usefulness. The inactivation of these drugs in the gut and/or liver is due to sulfation, glucuronidation, or methylation of the hydroxyl group (George, 1981; Longcope *et al.*, 1985). One approach to circumvent the high first-pass metabolism of alcohols or phenols is to administer the drug orally as a prodrug that can minimize the metabolism in the gastrointestinal tract and liver. A prerequisite for such a strategy would be to ensure that conversion of the prodrug occurs in an organ other than the intestine or the liver (Svensson and Tunek, 1988; Lokind *et al.*, 1991). If this bioconversion occurs within the intestine or the liver, the active parent drug may be subsequently metabolized, thus offering no protection to the hydroxyl functional group.

An additional aim of the prodrug strategy is to transiently mask polar groups within a drug molecule, resulting in an increased lipophilicity of the molecule and thereby promoting membrane permeability. Esterification of the hydroxyl group(s) has been one of the preferred prodrug strategies to achieve such an objective. By appropriate esterification of the hydroxyl group(s), it is possible to obtain derivatives with desirable lipophilicity as well as *in vivo* lability; the latter is influenced by electronic and steric factors, and whether the ester is a good substrate for enzymatic lability.

Although both carboxylic acids and alcohols can be derivatized to their ester derivatives, the esters of carboxylic acids have had more clinical success. This may be due to a favorable pharmacokinetic effect obtained as a result of masking the significantly more acidic and, therefore, more polar carboxylate anion (p*Ka* 3.5–4.5) in comparison to a phenol (p*Ka* 8–11) or alcohol (p*Ka* 15–17), which are uncharged at physiological pH. Nevertheless, in selected cases, systematic selection of the promoiety has resulted in prodrugs of the hydroxyl group that

have successfully overcome physiological barriers that hindered delivery of the parent drug.

In addition to facilitating intestinal absorption or cellular permeation, a number of prodrugs of hydroxyl group-containing anti-cancer drugs have been targeted toward molecular receptors on tumor cells for site-specific activation. The activation of such prodrugs makes use of physiological or metabolic differences between normal and tumor cells. These prodrugs aim to minimize systemic toxicity of the parent cytotoxic drugs. A few have successfully achieved their objective, one example being irinotecan hydrochloride (see structure **58**), which is used clinically in the treatment of cancers of the colon or rectum.

This chapter is not intended to be an exhaustive review, but aims to provide an overview of the various prodrug design strategies designed to modify the hydroxyl group with special emphasis on the physicochemical properties and pharmacokinetics of the representative compounds.

Aliphatic and Aromatic Ester Prodrugs of Alcohols and Phenols

The use of ester prodrugs has been reasonably successful in optimizing the delivery characteristics of alcohol or phenol parent drugs. Some of these objectives include an enhancement of aqueous solubility and stability (Johansen and Bundgaard, 1981; Graul *et al.*, 1997) to provide a sustained-release derivative of the parent drug (Jann *et al.*, 1985), and improvement in permeation across biological membranes by altering the lipophilic character of a drug (Tsuzuki *et al.*, 1994).

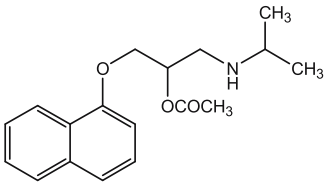
The esterification of a hydroxyl group on the parent drug is favored if the resultant prodrug possesses improved characteristics, including moderate chemical stability, which permits formulation with adequate shelf-life and *in vivo* conversion to the parent drug in the presence of esterases (Williams, 1985; Leinweber, 1987). An ideal ester prodrug should possess adequate water solubility at the pH of maximum stability and adequate stability to allow long-term storage (>2 years). In addition, these prodrugs should undergo rapid and quantitative conversion *in vivo* to release the parent drug. However, their use is not necessarily without problems, as exemplified by some succinate esters with limited aqueous stability (Rattie *et al.*, 1970) and incomplete and/or slow conversion *in vivo* to the parent drug as reported for esters of chloramphenicol (Ambrose, 1984) and metronidazole (Johansen and Larsen, 1984; Larsen *et al.*, 1988).

While there are numerous reviews on ester prodrugs of the hydroxyl functional group-containing drugs, this section will be restricted to selected aliphatic and aromatic promoieties and commenting on their suitability to enhance the bioavailability of the parent drug. One such series that has been extensively evaluated as promoieties for alcohols and phenols is the acyl ester series. Optimizing the physical and chemical properties of the incorporated carboxylic acid promoiety is critical in customizing the absorption, and chemical

and enzymatic lability of the prodrug, which significantly influences the bioavailability of the parent drug *in vivo*.

Aliphatic Esters

Acyl groups that have been incorporated as promoieties for the hydroxyl group range from lower alkyl groups to long-chain fatty acids, with interesting qualitative structure-metabolism relationship observed within this homologous series. One of the simplest acyl groups incorporated is the acetate promoiety. As observed in the case of propranolol, the prodrug *O*-acetylpropranolol (**1**) yielded higher blood levels of propranolol after oral administration to beagle dogs or rats than after an equivalent dose of propranolol hydrochloride; this is because it can successfully overcome the extensive first-pass metabolism of the parent drug in the liver (Anderson *et al.*, 1988). Propranolol has two optical isomers, dextro (*R*)- and levo (*S*)-propranolol; the latter is about 100-fold more potent as a β-blocker than the (*R*)-isomer (Barrett and Cullum, 1968). Therefore, the prodrug *O*-acetylpropranolol is a racemic mixture and, as expected, an enantioselective enzymatic hydrolysis is observed. In 90% human serum, the hydrolysis rate of the (*R*)-isomer ester was about threefold faster than that of the (*S*)-isomer (Takahashi *et al.*, 1990) whereas the opposite enantioselectivity was observed in rat intestine and liver tissue homogenate (Takahashi *et al.*, 1992). This selective hydrolysis of the ester prodrug of propranolol suggests that the more stable (*S*)-isomer may be present longer in human blood. An implication of this finding is that substrate enantioselectivity must be considered when evaluating prodrugs attached to a chiral parent drug.



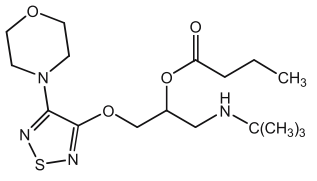
(1)

Structure 1.

In a study to examine the effect of carboxylesterase-mediated hydrolysis of homologous acyl esters of propranolol, Irwin and Belaid (1988) observed that the hydrolysis rate was directly proportional to the length of the carbon chain. Thus, the hexanoyl ester undergoes faster enzymatic hydrolysis than does the acetyl ester, an observation that was reported earlier by Johansen and Larsen (1985) while evaluating various aromatic and aliphatic acyl promoieties of metronidazole. In a similar study to evaluate the relationship between enzymatic lability and the extent of corneal and conjunctival penetration of a series of alkyl, cycloalkyl, and

aryl ester prodrugs of the nonselective β-adrenergic antagonist timolol, Chien *et al.* (1991) observed that, while the straight chain-alkyl and unsubstituted cycloalkyl esters were enzymatically hydrolyzed more rapidly than their corresponding branched and substituted analogs, they penetrated the cornea and conjunctiva more readily than did branched chain esters of comparable lipophilicity. For example, *O*-butyryl timolol (**2**) hydrolyzed more rapidly than *O*-isobutyryl ester (**3**), *O*-valeryl timolol (**4**) hydrolyzed more rapidly than *O*-pivaloyl (**5**) and *O*-neopentanoyl (**6**), and *O*-cyclobutanoyl timolol (**7**) hydrolyzed more rapidly than *O*-1'-methyl (**8**) and *O*-2'-methylcyclopropranoyl ester (**9**). The corneal and conjunctiva penetration of all of these ester prodrugs, regardless of their enzymatic lability, varied parabolically with lipophilicity. Therefore, it seems that enzymatic lability is a factor in addition to lipophilicity that may be utilized to improve target tissue drug absorption by a homologous series of ester prodrugs.

In addition to using monoalkyl esters as the promoiety, the synthesis and biological evaluation of diesters has also been performed, as in the case of



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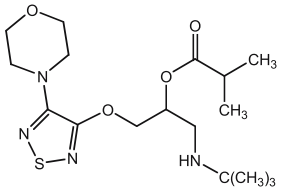

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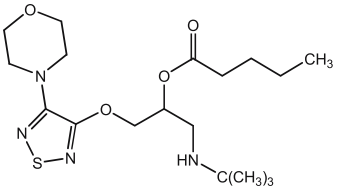

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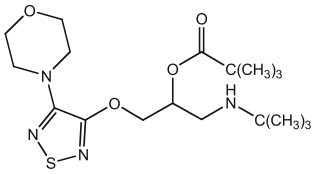

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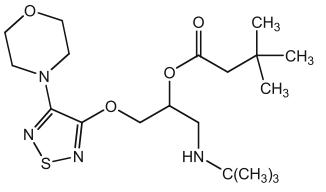

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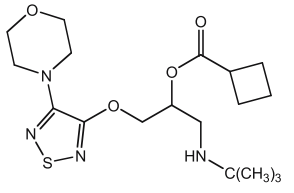

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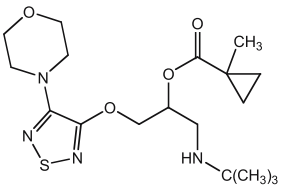

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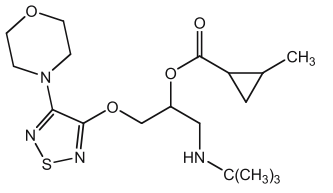
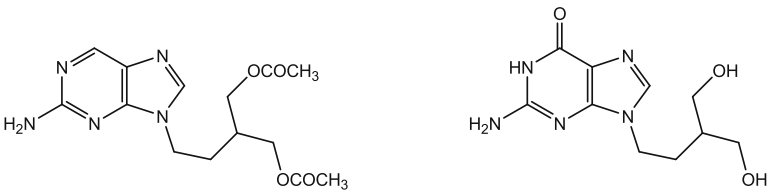


(9)

Structures 2-9.

famciclovir (**10**), the orally administered diacetate ester prodrug of the antiviral agent penciclovir (**11**) (Filer *et al.*, 1994). Following oral administration to human subjects, 10 is well absorbed, yielding an absolute bioavailability of penciclovir of 77% (Luber and Flaherty, 1996). Famciclovir is rapidly and extensively metabolized by hydrolysis of the esters and oxidation at C-6 to penciclovir, which reaches peak plasma concentration in plasma within 0.75 h. This prodrug, with its comparable efficacy, similar adverse effect profile, and less frequent dosing regimen compared to acyclovir, represents an alternative for the treatment of herpes zoster and genital herpes in immunocompetent adults.

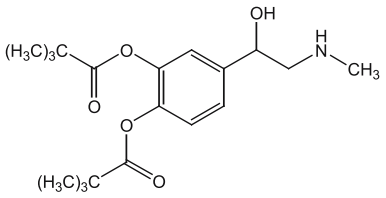


(10) (11)

Structures 10-11.

Branched acyl groups as promoieties have also been incorporated into prodrugs specifically for their higher lipophilicity and slower chemical and enzymatic hydrolysis in comparison to the corresponding linear analogs. Among such branched acyl groups, the trimethyl acetic acid, or pivalic acid, has been the most commonly used to derivatize the hydroxyl group. Indeed, pivalic acid combines reduced reactivity with high lipophilicity, which may be advantageous in specific prodrug strategies. In one such application directed toward ophthalmic drug delivery, the dipivalyl acid diester of epinephrine, dipivefrine (**12**), penetrates the cornea 17-fold faster than does epinephrine (**13**) due to its 600-fold higher lipophilicity in comparison to that of epinephrine at pH 7.2 (Hussain and Truelove, 1976; Wei *et al.*, 1978). As a consequence, a smaller topical dose of 12 achieves a similar therapeutic level with a much reduced systemic level of 13. However, the incorporation of the dipivalyl ester does not always prove to be favorable, as observed in the case of the *O*-pivaloyl ester (**14**) of the β-blocker oxprenolol, which was found to be too stable for ocular use against glaucoma ($t_{1/2}$ in aqueous humor $\approx$ 700 min.) (Jordan, 1998).

Higher alkyl chain homologous, especially fatty acids, have been utilized to generate lipophilic prodrugs such as the haloperidol decanoate (**15**), a sustained-release prodrug of the neuroleptic haloperidol (Beresford and Ward, 1987). Administration of **15** in sesame oil results in slow release of haloperidol, which is therapeutically beneficial when patients require medication for extended periods of time. The peak plasma concentration of an intramuscularly administered haloperidol decanoate occurs within 6 days, and plasma concentration decreases with an apparent half-life of three weeks. The high lipophilicity of this ester prodrug results in its binding to blood and tissue proteins, which minimizes the



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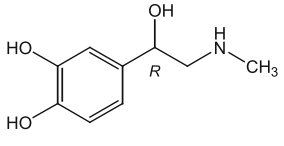

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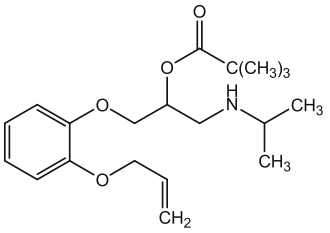
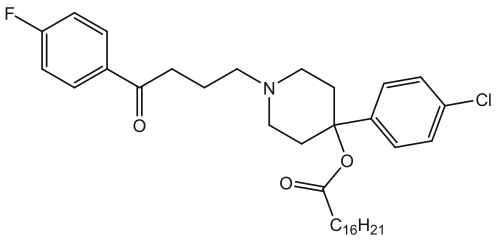


(14)

Structures 12-14.

enzymatic hydrolysis and thereby extends its *in vivo* effects. Fluphenazine is another therapeutically administered antipsychotic; it is available as both an enanthate (**16**) and a decanoate ester (dissolved in sesame oil). The enanthate



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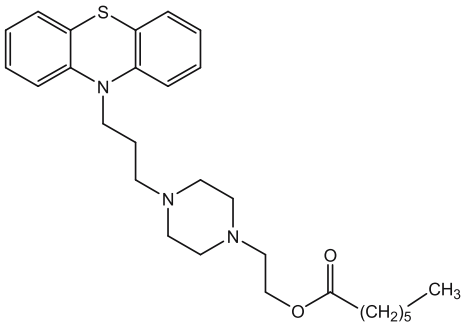


(16)

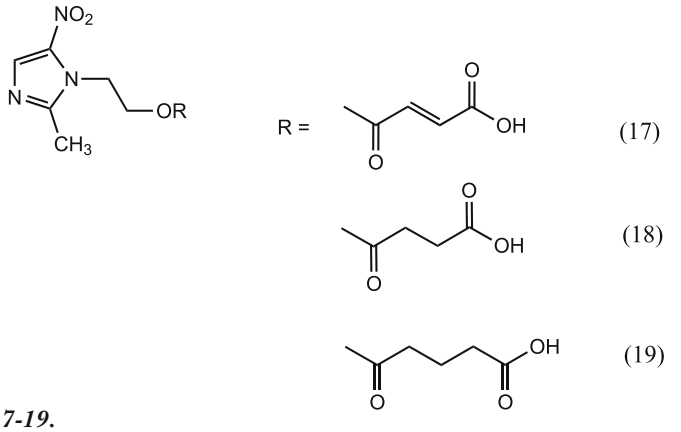
Structures 15-16.

ester produces peak plasma concentration within 2–3 days and thereafter declines with an apparent elimination half-life of 3–4 days, while the decanoate ester produces a peak plasma concentration within 24 h after a single intramuscular injection and then declines with an apparent elimination half-life ranging from 6 to 10 days (Jann *et al.*, 1985).

Hemiesters of Dicarboxylic Acids

While an increase in the degree of branching or the length of the acyl side-chain results in lipophilic prodrugs, there have been prodrug attempts to incorporate an ionizable acyl promoiety to increase aqueous solubility. The focus of this section is the hemiesters of dicarboxylic acids that have been evaluated for enhancing the aqueous solubility of chloramphenicol (Brent *et al.*, 1980), hydrocortisone (Garrett, 1962), methylprednisolone (Anderson and Taphouse, 1981), metronidazole (Johansen and Larsen, 1984), and propranolol (Garceau *et al.*, 1978) and a few other drugs.

The bioavailability of such prodrugs varies significantly after parenteral administration with different extents of bioconversion observed, as has been reported for steroids (Melby and St. Cyr, 1961; Derendorf *et al.*, 1985) and chloramphenicol (Nahata and Powell, 1981; Burke *et al.*, 1982). The bioconversion of metronidazole from its monoesters of maleic acid, succinic acid, and glutaric acid in buffer solutions and biological media have provided some interesting observations (Larsen *et al.*, 1988; Vermeersch *et al.*, 1990). Metronidazole hemimaleinate (**17**) and the hemisuccinate ester (**18**) were not ready substrates for plasma esterases ($t_{1/2}$ ≈300–600 h), while a 50-fold enhancement in hydrolysis of the hemiglutarate ester (**19**) was observed in plasma ($t_{1/2}$ ≈16.4 h) in comparison with buffer ($t_{1/2}$ ≈813 h) under physiological conditions (pH 7.4 and 37°C). This lack of reactivity of esters such as **17** and **18** that contain a terminal carboxylate or sulfonate moiety toward esterases has been previously documented (Anderson *et al.*, 1985a) and, hence, it was not unusual. However, the marked increase in hydrolysis of **19** was interesting. This enhancement in rate may be due to an increase in spatial separation of the terminal carboxylate from the ester bond undergoing enzymatic hydrolysis or an altered conformation of the promoiety,



Structures 17-19.

which may facilitate the ester bond hydrolysis. These data point out an interesting structure-reactivity relationship among such hemiesters and may be valuable in the evaluation process for promoiety selection of other alcohols and phenols.

Aromatic Esters

The concluding part of this section will focus on the incorporation of aromatic acyl groups, especially those derived from benzoic acid, in prodrugs of the hydroxyl functional group. The incorporation of aromatic acids as promoieties is favored, as the hydrolysis rate of the ester linkage may be altered depending on the substituents on the aromatic ring. In one such attempt to find timolol ester prodrugs combining high chemical stability in aqueous solutions with facile enzymatic conversion by esterase in the eye to liberate timolol, unsubstituted and substituted benzoate esters were among many promoieties evaluated (Bundgaard *et al.*, 1988a). The chemical and enzymatic stability of these prodrugs varied widely and is a function of both steric and electronic effects within the ester acyl group.

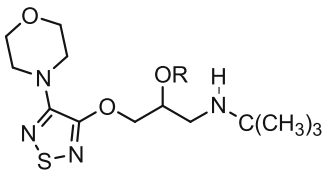
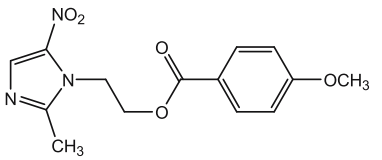
 R = (R = H: Timolol)	**Buffer $t_{1/2}$ (h)**	**80% Human Plasma $t_{1/2}$ (h)**
PhCO	2.0	4.4
2–CH_3–C_6H_4CO	10	52
4–CH_3–C_6H_4CO	4.9	20
2–CH_3O–C_6H_4CO	4.1	7.3
4–CH_3O–C_6H_4CO	4.9	20
2–CH_3COO–C_6H_4CO	0.51	*Not Reported*
2–$PhCOOCH_2$–C_6H_4CO	4.5	*Not Reported*
2–NH_2–C_6H_4CO	36.6	70
2–CH_3NH–C_6H_4CO	41.8	*Not Reported*

Table 1. Chemical and enzymatic hydrolysis of ester prodrugs of Timolol at pH 7.4 and 37°C (Bundgaard *et al.*, 1988a).

However, no clear relationship emerged that may account for the chemical and enzymatic hydrolysis rates. Within the series, a lower ester hydrolysis rate is observed in 80% human plasma compared to phosphate buffer without plasma, indicating that this rate-retarding step may be due to binding of the prodrugs to plasma proteins (see Table 1). Among the promoieties evaluated, the substituted benzoate esters were the most chemically stable. To conclude, the 2-aminobenzoate and 2-methylaminobenzoate derivatives are the most stable ($t_{1/2}$, pH 7.4 buffer, 37°C ≈ 35–40 h), with an estimated shelf–life of about 1 year at 25°C in a solution at pH 4.0.

As mentioned earlier, the substituents on the aromatic ring can strongly influence the rate of ester bond hydrolysis, and this effect is most pronounced for *p*-substituted electron-donating or -withdrawing groups. This effect is best exemplified by comparison of chemical hydrolysis rates of the *p*-methoxybenzoate (**20**) and *p*-nitrobenzoate (**21**) ester prodrugs of metronidazole (Johansen and Larsen, 1985). The strong electron-withdrawing p-nitro substituent on the aromatic ring makes the carbonyl of the ester bond undergoing hydrolysis more electrophilic, which favors an attack of the hydroxide nucleophile and results in the substantially shorter half-life of **21** ($t_{1/2}$ ≈ 52 h) versus that of **20** ($t_{1/2}$ ≈ 2740 h) in buffer solution at 37°C and pH 7.4. Therefore, the choice of substituents on the aromatic promoiety is critical for both the rate and extent of enzymatic hydrolysis of the prodrugs, and this structure-activity relationship will be of great benefit in designing prodrugs customized for specific applications.



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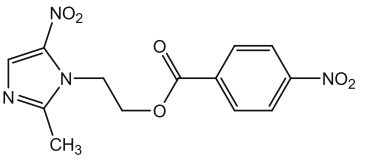


(21)

Structures 20-21.

The utility of any potential ester prodrug is dependent on its pharmacokinetic properties, tissue distribution, and bioconversion rates to liberate the parent drug. Ester prodrugs are generally substrates for ubiquitous esterases and, therefore, any prodrug designed for tissue- or site-specific delivery must be resistant to plasma esterases but undergo conversion by the tissue- or organ-selective intracellular enzymes, which continues to be truly challenging.

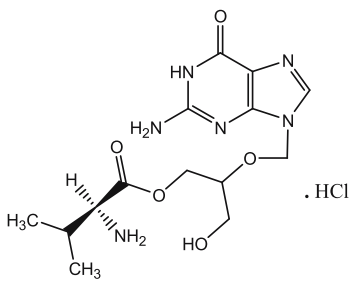
α-Amino Acid and Cyclic Amino Acid Esters of Alcohols and Phenols

The incorporation of an ionizable α-amino acid or a short-chain aliphatic amino acid ester promoiety is a useful prodrug strategy to increase the aqueous

solubility of water-insoluble drugs containing a hydroxyl group. Some of the examples include the α-amino acid ester prodrugs of taxol (Mathew *et al.*, 1992), acyclovir (Beauchamp *et al.*, 1992), metronidazole (Mahfouz and Hassan, 2001), and HIV protease inhibitors (Gaucher *et al.*, 2004).

Ideally these α-amino acid ester prodrugs should attain high aqueous solubility at the pH of optimum stability and maintain this stability to allow long-term storage (>2 years), yet should convert quantitatively *in vivo* to release the parent drug. Whereas successful amino acid ester prodrugs are currently used clinically (ex. valacyclovir hydrochloride), this strategy has not been without its share of problems. Although these prodrugs undergo facile hydrolysis by plasma enzymes (Bundgaard *et al.*, 1984a; Cho and Haynes, 1985), they display poor aqueous stability as represented by esters of metronidazole (Bundgaard *et al.*, 1984b), acyclovir (Colla *et al.*, 1983), paracetamol (Kovach *et al.*, 1981; Jensen and Bundgaard, 1991) and corticosteroids (Johnson *et al.*, 1985).

The reason for this instability in mildly acidic aqueous solutions (pH 3–5) is due partly to the strongly electron-withdrawing effect of the protonated amino group that activates the carbonyl atom of the ester linkage toward hydroxide ion attack and partly to the intramolecular catalytic effect of the amino group (protonated or unprotonated) on the ester cleavage (Bundgaard *et al.*, 1984b). This chemical instability issue can be minimized either by extending the amino group from the ester linkage as described by Anderson *et al.* (1985b) or by incorporating an amino acid such as L-valine that possesses a side chain with an optimal hydrophilic/hydrophobic balance combined with high chemical stability at physiological pH. As a result, incorporating a L-valine amino acid in the promoiety has led to the development of two clinically successful prodrugs, namely, valganciclovir hydrochloride (Valcyte™, Roche) (**22**), the L-valyl ester prodrug of ganciclovir (Reusser, 2001; Martin *et al.*, 2002), and valacyclovir hydrochloride (Zovirax®, GlaxoSmithKline) (**23**), the L-valyl ester prodrug of acyclovir (Beutner, 1995). The chemical stability of **23** at physiological conditions ($t_{1/2} \approx 13$ h, pH 7.4, 37°C) contrasts with its rapid and extensive *in vivo* hydrolysis to acyclovir, indicating a significant metabolism in different animal species (de Miranda *et al.*, 1981). Additionally, an efficient hydrolytic activation of **22** and **23** has been demonstrated in a Caco-2 cell culture model by biphenyl hydrolase-like protein (BPHL), a novel human serine hydrolase which may also be present in the epithelial intestinal cells in humans (Kim *et al.*, 2003).



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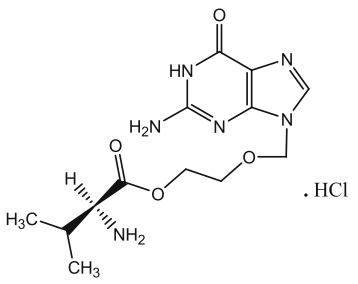


(23)

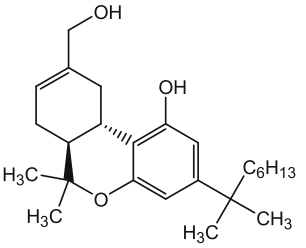
Structures 22-23.

The absolute bioavailability in humans of acyclovir and ganciclovir from orally administered valacyclovir and valganciclovir is 54.2% (Soul-Lawton *et al.*, 1995) and 60% (Jung and Dorr, 1999), respectively, a three- to tenfold increase over the poor bioavailability of their parent drugs, *per se*. In fact, orally administered **22** or **23** produce plasma levels similar to those attained with IV administration of ganciclovir or acyclovir, respectively (Soul-Lawton *et al.*, 1995). This increase in oral bioavailability of the parent drugs is a result of favorable transport of the prodrugs into the gut-lumen by an endogenous oligopeptide transporter (PEPT-1) (Ganapathy *et al.*, 1998; Han *et al.*, 1998, Sugawara *et al.*, 2000), thereby leading to enhanced blood levels of the parent drugs after systemic bioconversion. This enhanced uptake of the prodrugs has been further investigated using human cell-lines, confirming the observation that these prodrugs are recognized and transported into mucosal cells across the intestinal lumen via PEPT-1 for subsequent hydrolysis by intracellular esterases (de Vrueh *et al.*, 1998; Guo *et al.*, 1999).

These oligopeptide transporters are highly expressed in the intestine and are involved in the transport of di/tri-peptides and drugs with dipeptide like structures, such as the β-lactam antibiotics and ACE-inhibitors (Tsuji and Tamai, 1996; Rubio-Aliaga and Daniel, 2002). Additionally, an over-expression of these oligopeptide transporters in some cancer epithelial cells has been reported (Gonzalez *et al.*, 1998; Nakanishi *et al.*, 2000), thereby generating a possibility of providing enhanced oral absorption and/or improved targeting of potential anticancer drugs to these tumors (Landowski *et al.*, 2005). However, it certainly would be a challenge to administer these anticancer drugs orally for selective activity toward tumor cells, as these cytotoxic drugs may potentially be taken up by PEPT-1 transporters in non-cancer cells and by PEPT-2 in the kidneys, resulting in undesirable systemic toxicity due to rapid cleavage.

In addition to the efforts with α-amino acid promoieties, there have been prodrug attempts to incorporate amino acid esters containing tertiary or quaternary nitrogen heterocycles (Pop *et al.*, 1996) and esters of cyclic amino acids (Altomare *et al.*, 2003) to enhance the aqueous solubility of lipophilic drugs. Dexabinol (**24**), a synthetic nonpsychotropic cannabinoid, is a noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist (Feigenbaum *et al.*, 1989) that was evaluated for the treatment of brain damage associated with ischemia or trauma. The high lipophilicity of **24** precludes its use in therapeutically desirable aqueous formulations administered intravenously. The N-morpholino propionate (**25**) and N-methylpiperazino butyrate (**26**) prodrugs linked selectively to the allylic hydroxylic group are sufficiently stable in aqueous solutions but are unlikely prodrug candidates due to their poor conversion to parent drug in human plasma (less than 8% conversion to **24** after 24 h incubation) (Pop *et al.*, 1996).

In a follow-up study, Pop *et al.* (1999) evaluated trialkylammonium acetoxymethyl ester prodrugs of **24**. Most of these prodrugs (**27–32**) were soluble in water (≈ 50 mg/mL). This increase in aqueous solubility of these prodrugs, compared to the water-insoluble parent drug, is due to the presence of a permanently ionized ammonium group.



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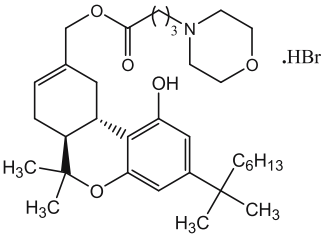

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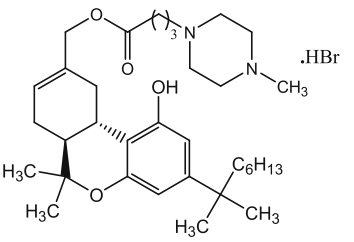
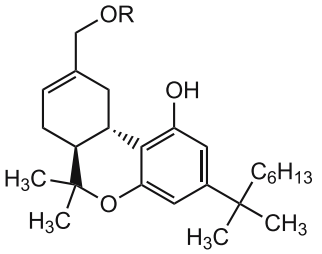


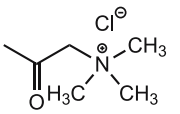
(26)

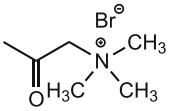
Structures 24-26.

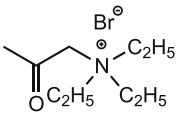
The chemical stability of the prodrugs is inversely dependent on the pH of solution, as indicated by the increase in half-life ($t_{1/2}$) of **27** from 14.6 days at pH 7.4 to 26.1 days at pH 5.5. Unfortunately, these prodrugs undergo a very rapid esterase-catalyzed hydrolysis *in vitro* and *in vivo* to generate **24**. As these highly water-soluble prodrugs lack long-term stability in aqueous solutions, they may be stored as lyophilized powders that can be reconstituted prior to administration.

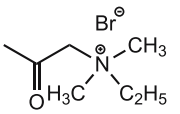
Propofol (**33**) is an intravenously administered anesthetic agent that rapidly and smoothly induces anesthesia (Bryson *et al.*, 1995) but is associated with pain when being administered in human subjects (Tan and Onsiong, 1998). The exact cause of pain is unclear, but seems to be related to the effect of **33** on the vascular endothelium. Many strategies have been evaluated to reduce this discomfort, including various combinations of lidocaine with propofol, the use of meperidine, metoclopromide, and short acting opioids, as well as altered rates and sites of injection. Moreover, due to its formulation as a lipid-based emulsion, **33** suffers from a number of limitations, such as physical instability, potential for causing embolism, and the requirement for strict aseptic handling (Bennett *et al.*, 1995). These drawbacks have generated the need for a clinically desirable aqueous formulation of propofol. As a part of this endeavor, the prodrug technology has been specifically evaluated with an emphasis on minimizing discomfort to patients by prolonging the duration of therapeutic activity and enhancing the aqueous solubility of propofol (Morimoto and Barker, 1999; Sagara *et al.*, 1999; Stella *et al.*, 2000; Hendler, 2002). In one such attempt to develop water-soluble propofol

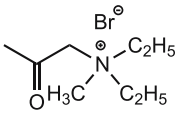


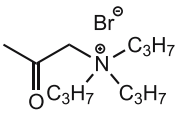
R =  (27)

 (28)

 (29)

 (30)

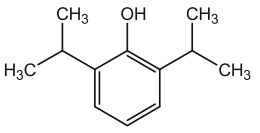
 (31)

 (32)

Structures 27-32.

prodrugs, Altomare (2003) evaluated four cyclic esters of propofol incorporating α-L-proline (**34**), and the three positional isomers of piperidine carboxylic acids, pipecolinic acid (**35**), β-nipecotic acid (**36**) and γ-isonipecotic acid (**37**) in their promoieties.

Within this prodrug series, an inverse relationship of aqueous solubility and melting point exists, as demonstrated by the substantially higher aqueous solubilities of **34** (0.3 g/mL) (189–192°C) and **36** (0.46 g/mL) (154–156°C) in comparison to those of **35** (0.011 g/mL) (225–228°C) and **37** (0.026 g/mL) (234–236°C), which reflect differences in crystal lattice energy. These prodrugs are chemically stable at pH 7.4 with a $t_{1/2} \approx 6$ h, and are substrates for enzymatic hydrolysis to release propofol. The L-proline ester **34** is the most promising derivative, possessing high aqueous solubility and high chemical stability at physiological pH, which might permit development of a freeze-dried formulation for parenteral administration.



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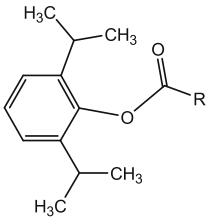

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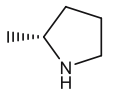

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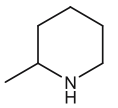

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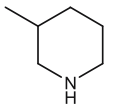

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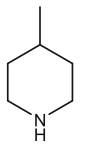


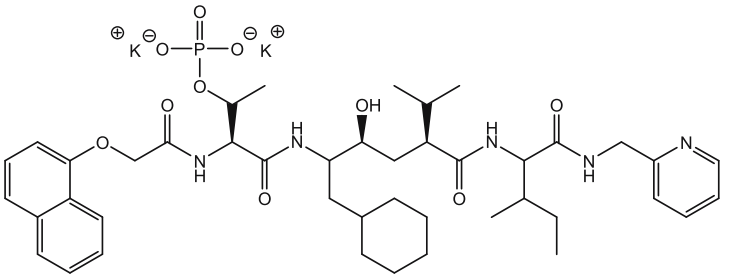
(37)

Structures 33-37.

Phosphate Ester Prodrugs of Alcohols and Phenols

Incorporation of a phosphate group has successfully overcome numerous drug delivery problems that might have compromised the therapeutic utility of potential drugs (Sinkula and Yalkowsky, 1975; Stella, 1996; Ellis *et al.*, 2004). These prodrugs are structurally formed by either direct incorporation of a phosphate moiety into the hydroxyl group of a parent drug in the form of a phosphomonoester or attaching it to the parent drug via a chemical linker.

The first and second acid dissociation constants result in p*Ka* values of the phosphate moiety in the ranges of 1.8–2.0 and 6.5–6.8, respectively. Consequently, the promoiety is ionized at physiological pH which results in significantly increased aqueous solubility of poorly soluble phenol and alcohol group-bearing parent drugs (Yoshimura *et al.*, 1978; Loo *et al.*, 1981; Cho *et al.*, 1982; Mantyla *et al.*, 2004). In one such application, the extremely low aqueous solubility (<3 μg/mL) of peptidomimetic HIV protease inhibitors increased considerably (>10 mg/mL) at pH 7 after incorporating a phosphate promoiety as shown by **38** (Chong *et al.*, 1993). This marked increase in solubility was also reported by Girotra *et al.* (1992) (from less than 0.1 mg/mL to greater than 30 mg/mL) for the phosphate ester prodrug **39** of a platelet-activating factor (PAF) antagonist.



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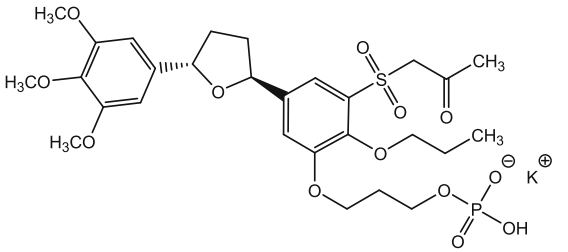


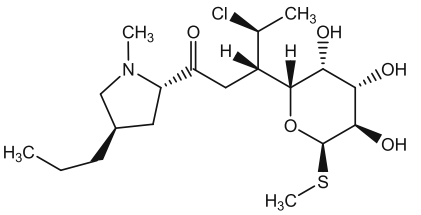
(39)

Structures 38-39.

These phosphomonoester prodrugs are chemically very stable with extremely long shelf-lives and undergo an alkaline phosphatase (EC 3.1.3.1)-catalyzed bioconversion *in vivo* to release the parent alcohol or phenol drug and inorganic phosphate. This enzyme is ubiquitous in the human body and cleaves the phosphomonoesters at the phosphorus-oxygen bond with high catalytic efficiency (Garattini *et al.*, 1985; Gani and Wilkie, 1995). Numerous phosphomonoester prodrugs have shown good *in vitro* (Kearney and Stella, 1992; Safadi *et al.*, 1993; TenHoor and Stewart, 1995) and *in vivo* (Hare *et al.*, 1975; Gunnarsson *et al.*, 1984; Mollmann *et al.*, 1985) conversion to parent drug in presence of alkaline phosphatases.

Clindamycin (**40**) is an antibiotic used in the treatment of gram-positive bacterial infections, including bacteria associated with acne. When clindamycin hydrochloride proved too irritating to use parenterally (Novak *et al.*, 1970), the 2-phosphate ester was developed (Riebe and Oesterling, 1972). Clindamycin phosphate (**41**), clinically approved for parenteral administration and topical application (Cambazard, 1998), is inactive *in vitro*, but is rapidly hydrolyzed *in vivo* to **40**. After a short-term intravenous infusion of **41,** peak serum levels of active clindamycin are reached. The prodrug **41** disappears rapidly from the serum with an average elimination half-life of 6 min. However, the serum elimination half-life of **40** is about 3 h in adults and $2^1/_2$ h in pediatric patients (DeHaan *et al.*, 1973). After intramuscular injection of **41**, peak levels of active clindamycin are reached within 3 h in adults and 1 h in pediatric patients (Roberts *et al.*, 1978).

Fluconazole (**42**) (Diflucan®), Pfizer's broad-spectrum antifungal agent, is efficacious in the treatment of serious fungal infections (Richardson *et al.*, 1990;



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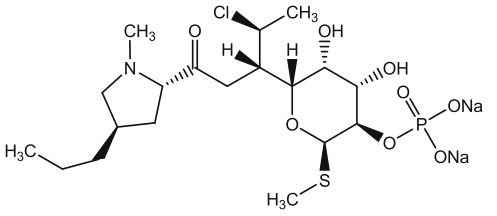
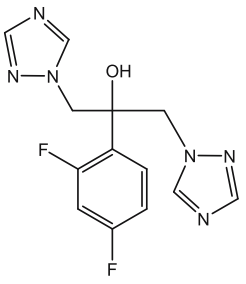


(41)

Structures 40-41.

Troke, 1997); it is available as tablets for oral administration, as a powder for oral suspension, and as a sterile solution for intravenous use. The tertiary alcohol in **42** is phosphorylated to yield fosfluconazole (**43**), a highly water-soluble (>300 mg/mL), chemically stable prodrug of fluconazole (Bentley *et al.*, 2002). This increased aqueous solubility of **43** permits smaller infusion volumes of high concentration, which results in rapid achievement of a therapeutically desirable steady-state plasma concentration of **42**. In healthy humans, the estimated mean bioavailability of **42** from intravenous administration of **43** is 97%, with a $C_{max,ss}$ ratio of 98%. The prodrug undergoes extensive bioconversion; less than 1% of the administered dose is excreted unchanged in urine, and the majority (86%) is eliminated in the urine as **42** (Sobue *et al.*, 2004). Although the t_{max} of **42** is reduced in individuals with hepatic impairment (3.1 h vs. 4.8 h), indicating a more rapid conversion in the impaired subjects, there is no statistically significant change in the pharmacokinetic parameters of **42** (Sobue *et al.*, 2005). These results suggest that no dose adjustment is required when **43** is administered to subjects with mild-to-moderate hepatic impairment.



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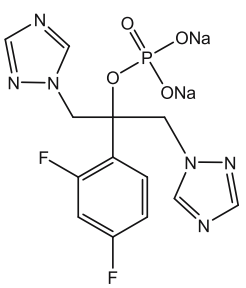
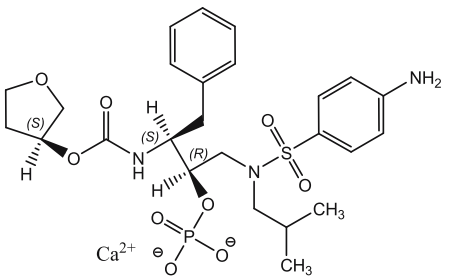


(43)

Structures 42-43.

Fosamprenavir (**44**) (Lexiva®, GlaxoSmithKline/Vertex) is an orally administered prodrug of amprenavir (**45**) approved by the FDA as an inhibitor of the human immunodeficiency virus (HIV) protease. Although the oral absorption of **45** is rapid, with C_{max} being achieved in 1–2 h (Sadler *et al.*, 1999; Veronese *et al.*, 2000), a dosing regimen of eight capsules twice daily is required to maintain effective plasma concentrations. This substantial dosing schedule may be a concern for patients, especially those following a highly active antiretroviral therapy (HAART) regimen.

The water-soluble (0.31 mg/mL) calcium salt of **44** was preferred from a formulation point of view and was synthesized to enhance oral bioavailability of **45** with a lower excipient-to-drug ratio, thereby reducing the burden of the daily dosing regimen and greatly improving patient compliance.



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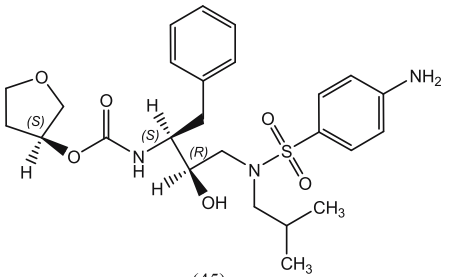


(45)

Structures 44-45.

Upon oral administration, **44** is rapidly hydrolyzed to **45** in the epithelial cells of the intestine with no systemic absorption of the prodrug (Furfine *et al.*, 2004). In a 6-week randomized controlled trial in HIV-infected adults, 1200 mg of **45** twice daily was compared with twice-daily 1395 mg or 1860 mg doses of **44**. In comparison with amprenavir, the two fosamprenavir doses delivered equivalent steady-state values for area under the plasma amprenavir concentration-time curve (AUC), a ≈ 30% lower amprenavir $C_{max,ss,}$ and a dose-dependent increase in plasma amprenavir concentration at the end of the dosing interval (Wood *et al.*, 2004). The lower peak plasma concentration of **45** after dosing with **44** reduces the gastrointestinal side effects associated with administration of **45** alone. Thus, after an orally administered dose with fewer numbers of tablets, **44** achieves the therapeutically relevant concentration of **45** (see Table 2).

The phosphomonoester prodrug strategy has also been successfully applied toward phenols (Rodriguez *et al.*, 1999; Yagi *et al.*, 1999; Pettit and Lippert, 2000). One such example, among others, is etoposide phosphate (**46**) (Etopophos®, Bristol-Myers Squibb), the water-soluble phosphate prodrug of etoposide (**47**), a semi-synthetic podophyllotoxin-derived antineoplastic agent (Saulnier *et al.*, 1994). The low aqueous solubility of **47** (<0.2 mg/mL, pH 1.3–8.0) (Shah *et al.*, 1989) coupled with its chemical instability necessitates the use of excipients to avoid precipitation in an injectable solution (Beijnen *et al.*, 1991). The enhanced

Participants (No.)	Treatment regimen	AUC∞ (μg.h/mL)	C_{max} (μg/mL)	t_{max} (h)[a]	$t_{1/2}$ (h)
Healthy adult	FPV 1400 mg (2 x 700 mg tablets) fasted	19.05	4.26	1.5	–
Volunteer (31)	FPV 1400 mg (2 x 700 mg tablets) fed[b]	19.37	4.51	2.0	–
Adults with HIV infection[c] (Wood *et al.*, 2004) **(53)**	FPV 1395 mg (3 x 465 mg tablets)	22.8	4.64	2.5	7.7
	FPV 1860 mg (4 x 465 mg tablets)	42.3	7.94	2.0	7.9
	APV 1200 mg (8 x 150 mg capsule)	24.6	7.19	1.3	9.6

Table 2. Mean pharmacokinetic parameters of amprenavir after single–dose administration of fosamprenavir (FPV) or amprenavir (APV) to healthy adult volunteers or adults with HIV infection. Reproduced from (Chapman *et al.*, 2004).

[a]Median values.

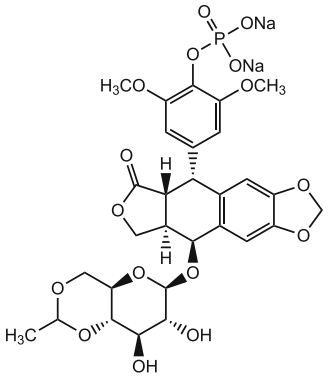
[b]Patients received FPV with a standard high–fat meal.

[c]All patients also received abacavir 300 mg and lamivudine 150 mg twice daily.

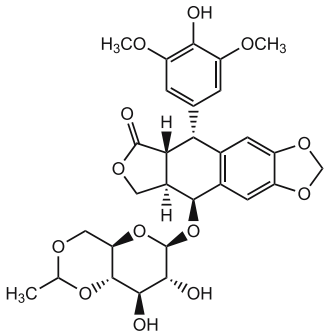
AUC∞ = area under the plasma amprenavir concentration–time curve from zero to infinity; C_{max} = maximum plasma amprenavir concentration; $t_{1/2}$ = elimination half–life; t_{max} = time to C_{max}

aqueous solubility of **46** reduces the potential for precipitation following dilution and during intravenous administration of **47**. Etoposide phosphate is rapidly and quantitatively converted to etoposide after intravenous administration, resulting in pharmacokinetic equivalence with etoposide (Budman *et al.*, 1994; Fields *et al.*, 1995; Sessa *et al.*, 1995; Mummaneni *et al.*, 1996). Hande (1998) has reviewed the development and clinical status of **46** and **47**.

An alternate approach to achieving local activation of a prodrug in tumor cells is the use of enzyme immunoconjugates. In one such methodology, called antibody-directed enzyme prodrug therapy (ADEPT), antigens expressed on tumor cells are used to target enzymes to the tumor site (Bagshawe, 1987). In initial applications of the ADEPT approach using alkaline phosphatase, **46** was efficiently activated to **47** by a monoclonal antibody/alkaline phosphatase (L6-AP) immunoconjugate which was bound to the surface of antigen-positive tumor cells (Senter *et al.*, 1988). As a control to estimate the toxicity of the prodrug **46**, *in vitro* studies with human colon carcinoma cells H3347 demonstrated that **46** is 100-fold less toxic than **47**. However, when **46** was preincubated with the H3347 cell



(46)



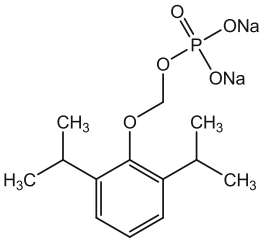
(47)

Structures 46-47.

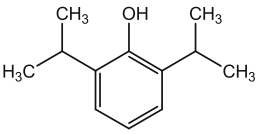
surface-bound L6-AP immunoconjugate, the prodrug was converted to the cytotoxic parent drug etoposide **47**. Similar results were observed *in vivo* after implantation of H3347 cells in nude mice and subsequent administration of L6-AP immunoconjugate followed 18–24 h later by administration of the prodrug **46**. The antitumor response observed was superior to that of **46** or **47** administered individually, suggesting that this may be due to a greater accumulation of cytotoxic **47** at the tumor site than could normally be achieved by systemic administration of **47**.

The major prerequisite for a successful phosphate prodrug is adequate chemical stability and quantitative *in vivo* conversion to the pharmacologically active drug. However, not all phosphate prodrugs undergo the desired rapid conversion to the parent drug; for example, phosphomonoesters of secondary and tertiary alcohols undergo slower rates of enzymatic conversion *in vitro* (Kearney and Stella, 1992; Safadi *et al.*, 1993; Vyas *et al.*, 1993). The reason for this slower enzymatic catalysis might be a sterically hindered substrate. To enhance this rate of conversion, the phosphonooxymethyl (POM) prodrugs series was developed, which incorporates a methylene spacer to extend the phosphate moiety from the sterically hindered hydroxyl group of the parent drug. Such prodrugs release the parent drug via a two-step conversion process, a rate-limiting enzyme-catalyzed dephosphorylation followed by a fast chemical breakdown of the hemiacetal intermediate. In addition to its use with alcohols and phenols, this approach has been successful in drug molecules that do not possess a hydroxyl group, such as the prodrug of phenytoin, fosphenytoin (Varia *et al.*, 1984).

GPI 15715 (**48**) (Aquavan® injection, Guilford Pharmaceuticals Inc.), the water-soluble, chemically stable phosphonooxymethyl prodrug of propofol **49**, is currently undergoing advanced stage clinical trials as an intravenously administered sedative-hypnotic agent for minor surgeries. The formulation of **49** had disadvantages, including pain at the site of injection (Nakane and Iwama, 1999) and possible cardiovascular effects (Doursout *et al.*, 2002). Unlike **49**, which is formulated in a lipid-based emulsion, **48** is formulated in a clear aqueous



(48)



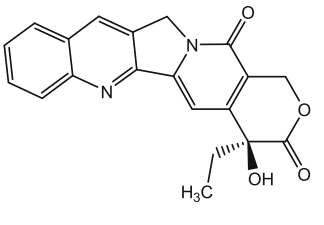
(49)

Structures 48-49.

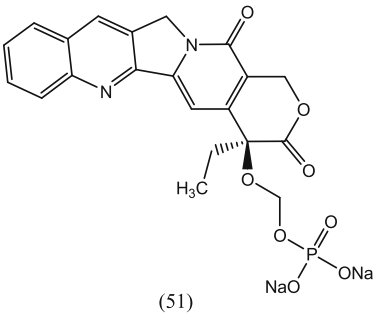
solution and rapidly undergoes bioconversion to **49** by alkaline phosphatase. In early clinical studies, **48** was characterized by a rapid onset and recovery and effective anesthesia at doses that were not associated with cardiovascular or other side effects (Fechner *et al.*, 2003).

The POM technology has been successfully applied to hydroxynaphthoquinones (Mantyla *et al.*, 2004) and alcohols (Golik *et al.*, 1996) as well as phenols. The POM strategy (Stella *et al.*, 2000) was used to increase the low aqueous solubility of camptothecin (2–3 μg/mL) (Wall *et al.*, 1966) and to minimize the E-ring hydrolysis. Camptothecin (**50**), with its intact E–ring lactone, is tenfold more potent than the open carboxylate form (Wani *et al.*, 1986; Hertzberg *et al.*, 1989); however, due to an equilibrium between the two forms, the inactive carboxylate form predominates at physiological pH (Wall *et al.*, 1966). Incorporating the POM promoiety at the 20-OH resulted in stabilization of the lactone E-ring against hydrolysis and generated a highly water-soluble (13.1 mg/mL, pH 4.0) prodrug **51**, which is stable (t_{90} ≈40 days, 25°C) and undergoes bioconversion *in vivo* to release **50**, a potent topoisomerase I inhibitor.

In summary, the incorporation of a phosphate moiety to create water-soluble phosphomonoester or phosphonooxymethyl prodrugs of alcohols or phenols has, in many cases, improved the pharmacokinetic properties of the parent drug. The evaluation criteria for such prodrugs should include high chemical stability and rate of bioconversion *in vivo*.



(50)



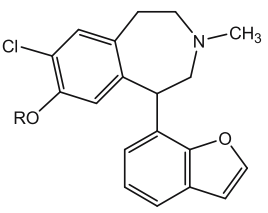
(51)

Structures 50-51.

Carbamate Ester Prodrugs

Carbamates are esters of carbamic acid with substitution at both the amino and carboxylate terminals (R'-NH-CO-OR); they have been preferentially utilized in the design of prodrugs as a means of achieving first-pass and systemic hydrolytic stability. Upon hydrolysis, these carbamate esters release the parent phenol or alcohol drug (ROH) and carbamic acid (R'-NH-COOH), which, being chemically unstable, breaks down to the amine (R'-NH_2) and CO_2.

The mechanism of OH-catalyzed hydrolysis of these carbamate esters (R'-NH-CO-OR) is strongly dependent on both the p*Ka* of the proton on the leaving group (ROH) and the degree of substitution on the nitrogen of the carbamate ester (Christenson, 1964; Bundgaard *et al.*, 1988b). Since phenols have a lower p*Ka* than alcohols, carbamate esters of phenols are generally more chemically labile than those of alcohols. In the case of alcohols, both the *N*-monosubstituted and *N,N*-disubstituted carbamates are chemically stable toward hydrolysis. In phenols, *N,N*-disubstituted carbamates are chemically stable, whereas *N*-monosubstituted carbamates are the most labile toward chemical hydrolysis. This comparison in the chemical stability of phenol carbamate esters is best illustrated by the prodrugs of two dopaminergic 7-hydroxy[3]benzazepines **52** and **53** (R = H) (Hansen *et al.*, 1991). The carbamate prodrugs were synthesized with the aim of protecting the parent phenols against first–pass metabolism following oral administration. As expected, the *N*-monosubstituted carbamates (R = allyl-NH-CO, $PhCH_2$-NH-CO, etc.) were found to be highly unstable at pH 7.4 and 37ºC, with half-lives of hydrolysis between 4 and 40 min in buffer and from 0.7 to 4.6 min in human plasma, thus proving too chemically and enzymatically unstable for further evaluations. The *N,N*–disubstituted carbamates (R = R'R"NCO, R' = Me or Et; R" = Me, Et, i-Pr, etc.), on the other hand, were extremely stable at pH 7.4 both in buffer and plasma solutions, with less than 5% degradation in 4 days. In fact, these prodrugs showed a potent inhibition of plasma butyrylcholinesterase (EC 3.1.1.8) but were less potent inhibitors of the specific erythrocyte acetylcholinesterase (EC 3.1.1.17). Although these prodrugs were stable in plasma, these carbamate esters undergo rapid bioconversion to the phenolic parent drug in the liver, as exhibited in liver microsomes from mouse and rats. This bioconversion is suggested to occur via direct carboxylesterase-mediated hydrolysis and cytochrome P450-catalyzed hydroxylation to give an N-hydroxymethyl derivative that spontaneously decomposes to the N-monomethylcarbamate (Scheme 1). The



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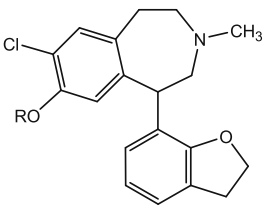
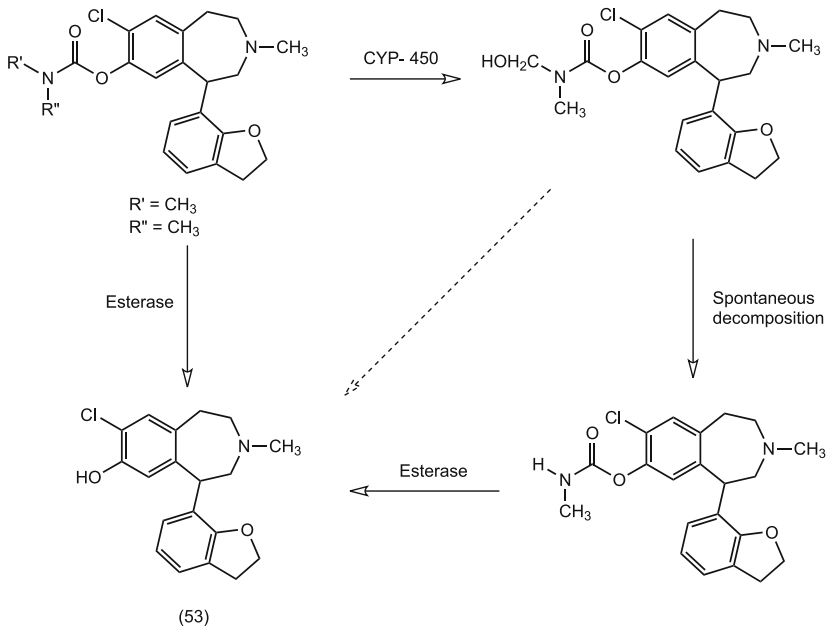


(53)

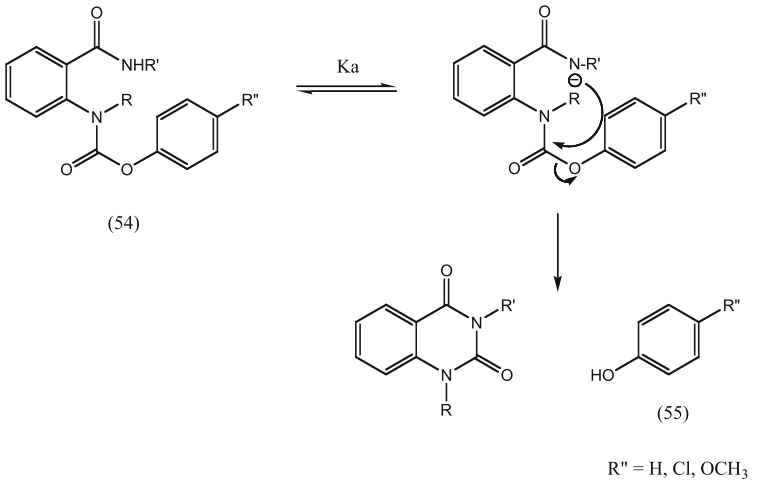
Structures 52-53.



Scheme 1.

authors concluded that the *N,N*-disubstituted carbamates may be potentially useful prodrugs for 7-hydroxy[3]benzazepines due to their favorable chemical and enzymatic stability.

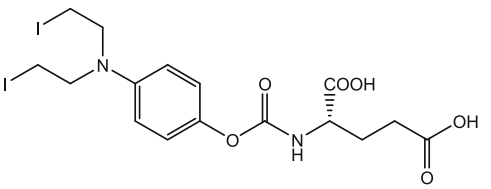
As illustrated above, carbamate prodrugs undergo conversion to their parent drug either by enzyme-catalyzed hydrolysis or a non-enzymatic/chemical process. However, the enzyme-catalyzed conversion of prodrugs is subject to biological variations, which has led to non-enzymatic/chemical approaches to generate the parent drug. In a few of these strategies, the prodrug-to-drug conversion occurs either by an intramolecular cyclization-elimination reaction (Saari *et al.*, 1990a;



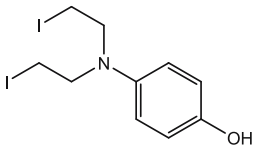
Scheme 2.

Thomsen and Bundgaard, 1993; Thomsen *et al.*, 1994) or an elimination-addition mechanism (Hansen *et al.*, 1992). Scheme 2 illustrates a simplified cyclization-elimination reaction of anthranilamides **54** to release substituted model phenols **55** (Thomsen and Bundgaard, 1993).

These carbamate prodrugs are stable at pH 1–6 but undergo base-catalyzed cyclization in neutral and alkaline pH to 2, 4-quinazolinediones with concomitant release of parent phenol. The rate of cyclization is affected by the steric and polar properties of the carboxamido substituent (R') and the *pKa* of the phenol. Therefore, by selecting appropriate substituents it is possible to obtain prodrugs that cyclize at a practical rate at physiological pH. However, most of these strategies for non-enzymatically activated prodrugs have had limited therapeutic application due to a combination of competing enzymatic reactions interfering with the intramolecular cyclization process (Fredholt *et al.*, 1995), and a significant increase in molecular weight of the derivatized drug, which adversely affects absorption of the prodrug through biological membranes (Lipinski *et al.*, 1997). Most of the therapeutically relevant carbamate prodrugs have been designed as substrates of specific enzymes. One of the promising prodrugs undergoing clinical trials is ZD2767P (**56**) (Francis *et al.*, 2002) as a part of the ADEPT approach (Springer *et al.*, 1995). ADEPT is a site-specific therapy in which a prodrug is selectively activated at the tumor site by an enzyme, which has been targeted to the tumor by an antibody-enzyme conjugate (Bagshawe, 1987). The di-iodophenol mustard glutamate prodrug ZD2767P is hydrolyzed at the carbamate bond by the enzyme carboxypeptidase G2 (CPG2), which releases the potent anti-cancer drug di-iodophenol mustard (**57**), resulting in tumor regression and prolonged growth delay in a colorectal tumor model (Blakey *et al.*, 1996). When tested in the human cell line LoVo, the prodrug was 100-fold less cytotoxic than the parent drug. Moreover, the half-life ($t_{1/2}$) of the drug is approximately 2 min, which is sufficient



(56)

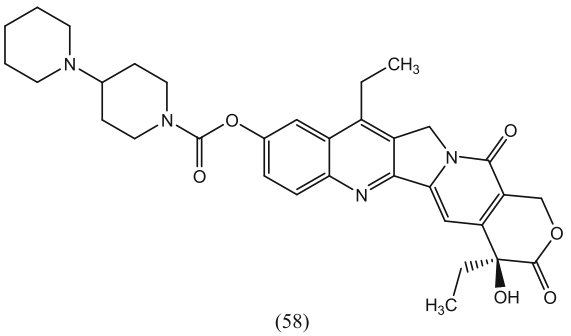


(57)

Structures 56-57.

for diffusion into the tumor cell from the local release site and to minimize peripheral toxicity.

Irinotecan hydrochloride trihydrate (**58**) (Camptosar®, Pfizer) is a water-soluble carbamate prodrug of the lipophilic antineoplastic agent SN-38 (**59**), which is 1000-fold more potent than irinotecan *in vitro* as an inhibitor of type-I DNA topoisomerase (Kunimoto *et al.*, 1987). Human liver microsomal carboxylesterases, CES 1A1 and CES2, cleave the ester bond in irinotecan, releasing the ionizable piperidinopiperidine promoiety and SN-38, the active form of the drug (Slatter *et al.*, 1997).



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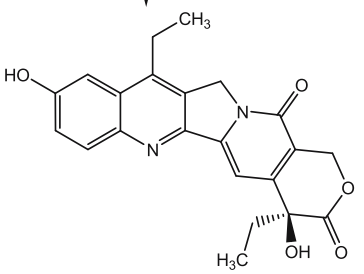


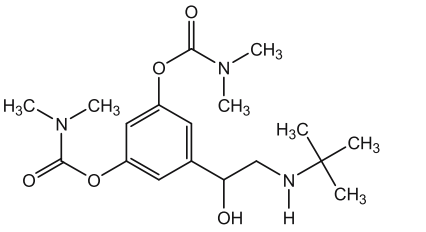
(59)

Structures 58-59.

Currently, irinotecan hydrochloride in combination with 5-fluorouracil and leucovorin is approved by the FDA as a first-line therapy in the treatment of metastatic carcinoma of the colon or rectum. Irinotecan hydrochloride is clinically administered as a short intravenous infusion (0.5–1.5 h). The peak plasma concentration of the prodrug is attained at the end of the infusion period (Slatter *et al.*, 2000), with a rapid decrease thereafter due to multiple distribution and elimination pathways (Sparreboom *et al.*, 1998).

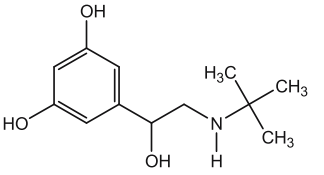
The therapeutic activity of a drug can be prolonged either by minimizing the rate of enzymatic hydrolysis of its prodrug or by decreasing the rate of metabolism of the drug. One of the best examples of clinically administered prodrugs is bambuterol (**60**) (Bambec®, Astra-Zeneca), a bis-dimethyl carbamate prodrug of the beta2-adrenoreceptor agonist terbutaline (**61**), which is used as a bronchodilator in the management of asthma. Bambuterol, which is adrener-

gically inactive, is rapidly hydrolyzed by butyrylcholinesterase in human plasma to generate a monocarbamate metabolite and, subsequently, terbutaline (Svensson and Tunek, 1988; Tunek *et al.*, 1988). Additionally, a CYP-450-catalyzed hydroxylation of a *N*-Me group followed by a *N*-demethylation, results in metabolites that eventually form terbutaline (Sitar, 1996).



(60)

Butyrylcholinesterase

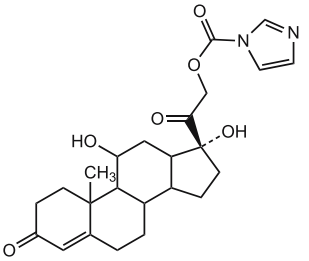


(61)

Structures 60-61.

Once they are bound to butyrylcholinesterase, the carbamate groups on bambuterol decrease the turnover rate of its metabolizing enzyme, thereby decreasing the conversion rate of bambuterol to the active drug terbutaline (Olsson and Svensson, 1984). During the process of enzymatic hydrolysis of **60**, a catalytic serine residue becomes carbamoylated. Release of carbamic acid to regenerate the active enzyme occurs slowly; as a result, inhibition of the butyrylcholinesterase by bambuterol typically lasts for approximately two days, after which full recovery of the enzyme occurs. This slow hydrolysis of bambuterol results in long therapeutic activity of terbutaline and produces an internal depot from which terbutaline is slowly released. Consequently, the clinical efficacy of orally administered once-daily bambuterol is equivalent to that of twice-daily sustained-release terbutaline (Fugleholm *et al.*, 1993).

While carbamate prodrugs of phenols are therapeutically relevant, carbamate prodrugs of alcohols have not been practical due to their high stability. However, an exception to this observation is the activated steroidal imidazole-1-carboxylic acid ester prodrugs of hydrocortisone **62** and testosterone **63** that undergo facile hydrolysis in aqueous buffer solutions (Klixbull and Bundgaard, 1983).



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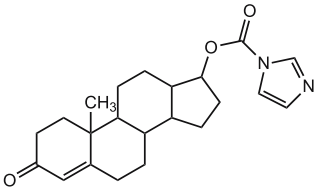


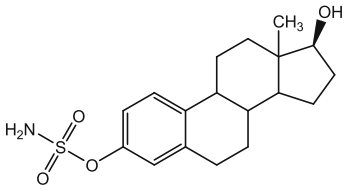
(63)

Structures 62-63.

Under physiological conditions, the half-lives of hydrolysis of the hydrocortisone and testosterone prodrug are 8 min and 65 h, respectively. This substantially longer half-life of the testosterone carbamate prodrug in 80% human plasma is due to derivatization at a sterically hindered secondary alcohol, which reduces the susceptibility of the compound to enzymatic hydrolysis. These prodrugs are not substrates for enzymatic catalysis in human plasma, but undergo chemical hydrolysis to release the parent drug. The protonation of the acyl-imidazole group (p*Ka* ≈ 3.5) enhances the solubility of these prodrugs relative to the parent steroid in acidic aqueous solutions. Thus, the imidazole-1-carboxylic ester prodrug strategy may be useful for oral delivery of poorly soluble drugs that contain an alcohol functional group.

Sulfamate Ester Prodrugs of Alcohols and Phenols

The incorporation of a sulfamate group into the steroid nucleus is a result of the search for novel, orally active estrogens for female fertility control. Orally administered natural and synthetic estrogens used as contraceptives and in Hormone Replacement Therapy (HRT) undergo extensive first-pass metabolism in the liver resulting in suboptimal bioavailability and excess hepatic toxicity (Lobo and Cassident, 1992). Incorporation of a sulfamate promoiety (-OSO_2NH_2) at the metabolically labile 3-hydroxyl of the estrogen steroid nucleus generates sulfamic acid prodrugs **64** and **65** of estradiol and ethinyl estradiol, respectively. These prodrugs possess higher systemic oral estrogenic activity and significantly reduced hepatic estrogenicity in comparison to the parent steroids (Elger *et al.*, 1995).



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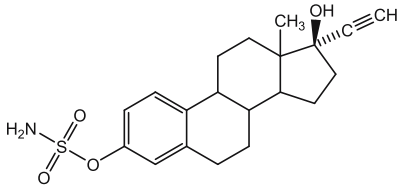


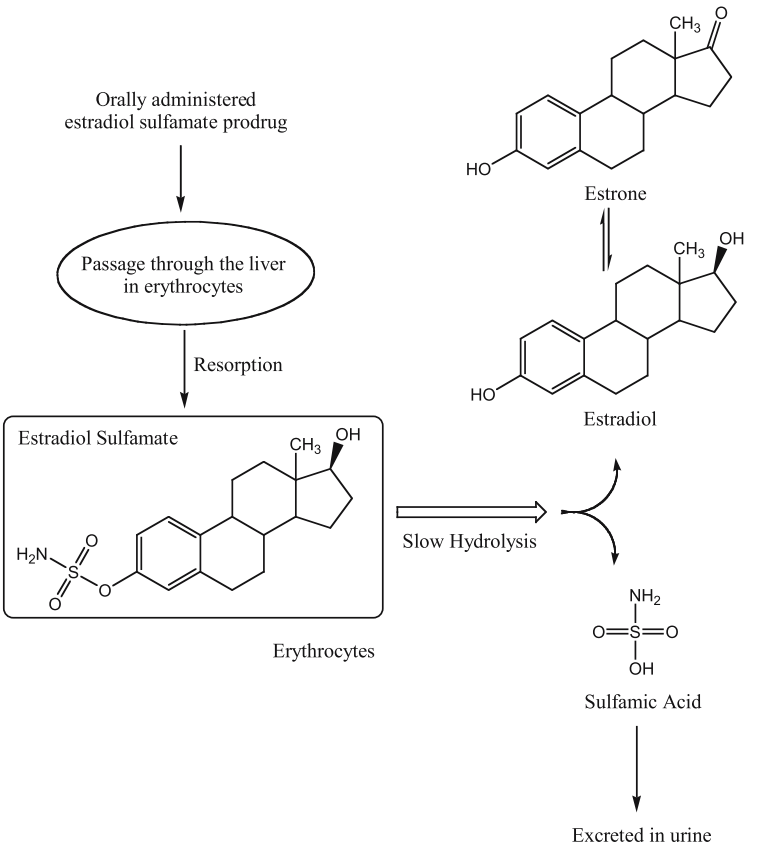
(65)

Structures 64-65.

This enhanced oral estrogenic activity of the sulfamate derivatives is due to their sequestration into red blood cells (RBC), resulting in their transit through the liver without undergoing first-pass metabolism. The preferential uptake into the RBC of these derivatives is due to their affinity for the enzyme carbonic anhydrase, which is present in high concentration in RBC. Sequestration of the derivatives, especially estradiol sulfamate into RBC, alters neither the erythrocytic oxygen transport nor the blood gases and acid-base balance parameters, indicating that such binding would not impair oxygen delivery to the body (Bauer *et al.*, 2003).

This binding to RBC also prevents the sulfamate prodrugs from exerting undesirable estrogenic activity in the liver (Elger, 2001). As shown in Scheme 3, after passage through the liver, the prodrug undergoes systemic hydrolysis in the membranes of erythrocytes, releasing estrogen and sulfamic acid. The well-documented mechanisms of steroid metabolism proceed after hydrolysis of the sulfamate promoiety.

The development of steroidal estrogen prodrugs offers a superior option to overcome limitations of natural and synthetic steroidal estrogens used in current contraceptive regimens. In this context, the orally administered sulfamate steroidal estrogen prodrugs might offer a drug release pattern similar to either transdermal or parenterally administered estrogens.



Scheme 3.

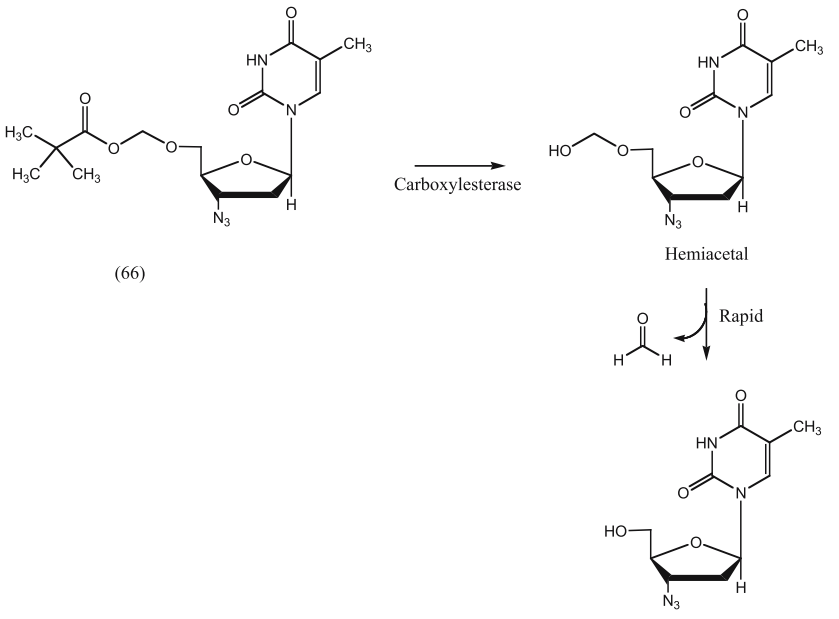
A comprehensive review by Winum *et al.* (2005) lists several potential drugs that have been designed with *O*-substituted, *N*-substituted, and di/tri-substituted sulfamates with a wide range of therapeutic activity. However, there is no indication that these sulfamate group-incorporated derivatives of biologically active molecules act as prodrugs.

Ether Prodrugs of Alcohols and Phenols

Incorporation of a non-ionizable aliphatic or an aromatic ether promoiety into the hydroxyl group of phenols or alcohols results in prodrugs with enhanced lipophilicity (Winkelmann *et al.*, 1988; Hammer *et al.*, 1996) and/or improved pharmacokinetic properties (Ashida *et al.*, 1993; Carty *et al.*, 1993).

The increased lipophilicity of ether prodrugs favors permeation into cellular membranes and the blood-brain barrier, an aspect particularly relevant for certain classes of therapeutic agents. Hammer *et al.* (1996) introduced a methylene linker between the ribose 5'-hydroxyl of certain anti-HIV active nucleosides and a lipophilic carboxylic acids, thus generating prodrugs **66** that could permeate the blood-brain barrier by passive diffusion more favorably than parent drug 3'-azido-3'-deoxythymidine (AZT) **67**. After permeating into the blood-brain barrier, the lipophilic ester moiety is hydrolyzed by carboxylesterases, yielding a chemically unstable hemiacetal intermediate that then rapidly dissociates to release the active nucleoside as depicted in Scheme 4.

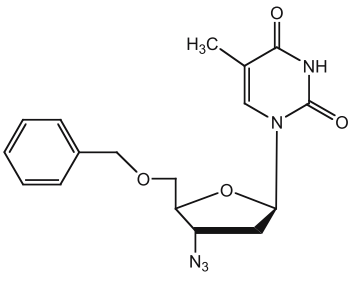
Although the concept seemed interesting, these prodrugs display a lower potency in the anti-HIV test system in comparison to the parent drug. The proper interpretation of the test data requires knowledge about the activity of



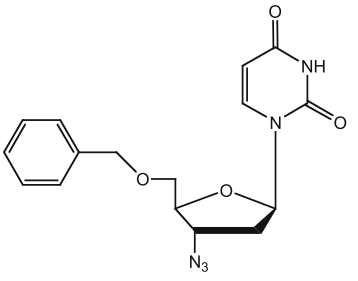
(66) (67)

Scheme 4.

carboxylesterase and the rate of release of parent drug in the test system. Thus, the result is not entirely surprising as the active polymerase inhibitor is not the nucleoside but, rather, its corresponding triphosphate. An extension of the strategy to deliver anti-HIV nucleosides to the brain, 5'-O-benzyl and glucose prodrugs of 3'-azido-3'deoxythymidine (AZT) and 3'-azido-2',3'-dideoxyuridine (AZdU) were evaluated for *in vitro* stability and *in vivo* pharmacokinetic studies in mice (Doshi *et al.*, 1993). The 5'-O-benzyl prodrugs of AZT (**68**) and AZdU (**69**) are stable in phosphate buffer (pH 7.4), but undergo relatively rapid hydrolysis in homogenates of the mouse brain ($t_{1/2} \approx$ 1–2 h) and liver ($t_{1/2} \approx$ 0.3–2 h). Upon intravenous administration of these prodrugs in mice, the brain:serum ratio of the area under the concentration time-curve (AUC), a parameter indicative of prodrug uptake into brain, was tenfold higher in comparison with the parent drug. Although these prodrugs penetrated the blood-brain barrier, no parent drug was detected in the brain, suggesting that the prodrugs were converted to metabolites other than the parent nucleoside drugs.



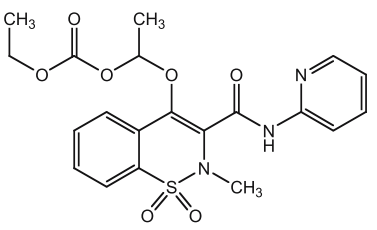
(68)



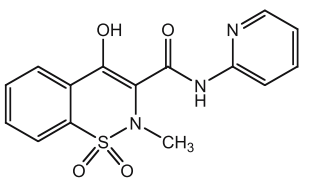
(69)

Structures 68-69.

Ampiroxicam (**70**), an ethoxycarbonyl ether prodrug of piroxicam (**71**), has been specifically developed to minimize the gastric irritation caused by piroxicam after oral administration in humans (Carty *et al.*, 1993). Piroxicam is an acidic enol-bearing non-steroidal anti-inflammatory drug (NSAID), which acts by inhibiting the enzyme cyclooxygenase (CO), the initial enzyme in the pathway for conversion of arachidonic acid to prostaglandins (Flower, 1974). The same mechanism is responsible for the characteristic side effect of gastrointestinal irritation, which may be caused by a direct topical effect and/or by the drug



(70)



(71)

Structures 70-71.

circulating systemically. Incorporating a promoiety at the enol functionality leads to elimination of the prostaglandin synthesis inhibitory activity, resulting in improved gastric tolerance (Lombardino and Wiseman, 1972; Falkner *et al.*, 1990).

In human subjects, **70** undergoes quantitative and rapid conversion to **71** because there is no statistically significant difference in their AUC values after equivalent doses (Table 3). Since **70** is stable to chemical hydrolysis at pH 1 ($t_{1/2} \geqslant$ 50 h) and undergoes slow hydrolysis ($t_{1/2} \geqslant$ 8 h) at pH 6.0–8.5, the conversion to **71** most likely occurs during absorption through the intestinal wall (Falkner *et al.*, 1990).

		Ampiroxicam		
Parameters	Piroxicam 20 mg	27 mg	40.5 mg	54 mg
Mean AUC (0–144) (μg.h/mL)	131 ± 29	122 ± 35	120 ± 31[b]	125 ± 29[b]
Mean C_{max}	2.2 ± 0.4	1.7 ± 0.4	1.7 ± 0.4[b]	1.7 ± 0.4[b]
Mean t_{max}	2.9 ± 2.3	4.4 ± 2.7	5.5 ± 4.7	5.3 ± 3.2
Mean half–life[c] (h)	59	59	55	59

Table 3. Comparison of mean pharmacokinetic parameters of piroxicam after oral administration of piroxicam or ampiroxicam to man[a] (Falkner *et al.*, 1990).

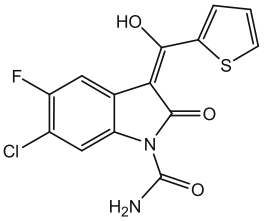
[a]Data are mean values ± SD for 22 subjects

[b]Normalized to a 27 mg dose (20 mg piroxicam equivalent)

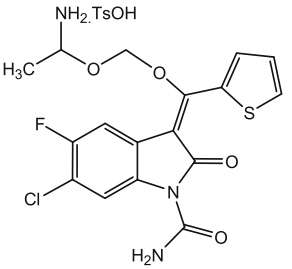
[c]Calculated from mean elimination rate constant

A result of incorporating the ethoxycarbonyl promoiety at the ionizable 4-hydroxy enol (p*Ka* 5.1) in **71** is the decrease in the aqueous solubility. The longer t_{max} value of **71** after administration of **70** than after an equivalent dose of **71** is the result of the slower desolution of **70** in the intestine prior to absorption (Table 3).

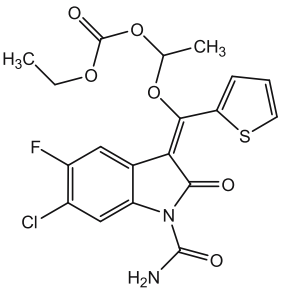
In cases where the promoiety prevents the ionization of a functional group on the parent drug, incorporating an ionizable group or heteroatom within the promoiety might overcome the parent drug's transient loss in aqueous solubility. This was the strategy utilized by Robinson *et al.* (1996) in evaluating numerous (acyloxy)methyl and [(aminoacyl)oxy]methyl ether derivatives as potential prodrugs of an antirheumatic oxindole **72**. Ether-linked prodrugs of **72** were selectively prepared to overcome the possible hydrolytic instability of carbonyl-linked compounds such as ester and carbonates due to the good leaving group ability of the enolate of **72** (p*Ka* 3.5).



(72)



(73)



(74)

Structures 72-74.

As expected, the ionizable amino group on the promoiety of the [(aminoacyl)oxy]methyl series resulted in an increase in aqueous solubility, as seen by a >300-fold increase in solubility of (α-L-alanyloxy)-methyl ether tosylate (**73**) (150 μg/mL, pH 6.5) over the non-ionizable lactate ether (**74**) (0.38 μg/mL, pH 6.5), and a fourfold enhancement in solubility over the parent drug **72** (40 μg/mL, pH 6.2). Consequently, the success in achieving acceptable bioavailability of **73** in rats, dogs, and monkeys is a result of the ionizable functionality in the promoiety, which compensates for masking the ionizable enol hydroxyl group.

Intramolecular-activated Prodrugs of Alcohols and Phenols Based on Cyclization-Elimination and Electronic Cascade Strategies

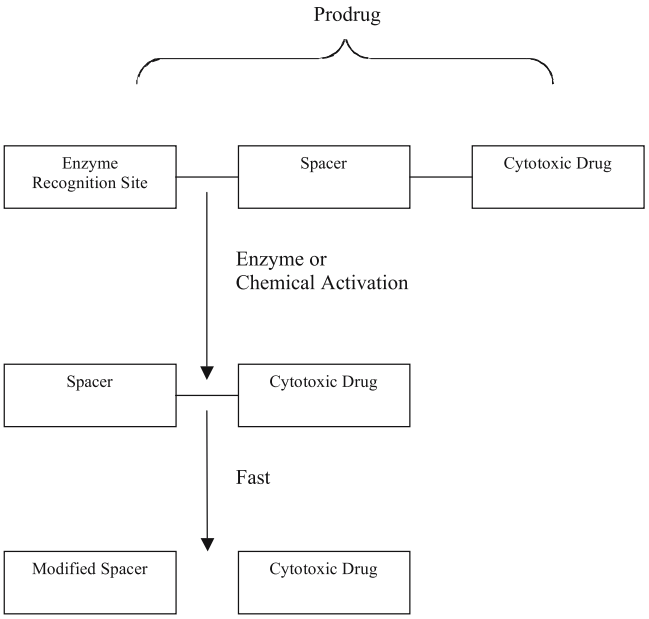
Although chemotherapeutic agents remain the major systemic treatment of malignancies, they have limited efficacy against metastasized tumors. This reduced therapeutic activity of cytotoxic agents is due in part to a combination of insufficient drug concentration inside the tumor cells and increased activity of efflux pumps on the tumor cells, which leads to the development of multidrug resistance.

The clinical efficacy of chemotherapeutic agents can be enhanced by achieving a more selective delivery to malignant cells, thereby reducing systemic toxicity. In this aspect, the prodrug approach has been quite promising in its ability to deliver anticancer drugs selectively to tumor tissues. Such prodrugs may

incorporate a specifically designed promoiety activated either by tumor-specific proteins as in the ADEPT (Tietze and Feuerstein, 2003) and GDEPT (Springer and Niculescu-Duvaz, 2000) approach or by a site-specific activation as in the case of prodrug monotherapy (de Groot *et al.*, 2001a). These prodrugs may also be activated in response to an altered chemical environment in the tumors, such as pH (Tannock and Rotin, 1989) or intracellular oxygen concentration (Naylor and Thomson, 2001), to efficiently release the cytotoxic drug.

In all of these strategies, the specifically 'constructed' prodrug must be recognized by an enzyme or undergo a chemical transformation to liberate the drug. While in the prodrug monotherapy (PMT), activation of the prodrug is catalyzed either by an altered physicochemical property of the tumor cell or by tumor-localized enzymes such as β-glucoronidase, the ADEPT strategy requires prior administration of an enzyme to initiate the conversion of prodrug to drug. Therefore, to facilitate such an enzymatic catalyzed conversion, the promoiety includes a spacer that serves to extend the enzyme binding site from the drug (or the effector) (Scheme 5). The design of both the enzyme recognition site and the drug is dependent on the mechanism of activation of the prodrug and the targeted physiological feature of the tumor cell. Once activated by a specific enzyme, the spacer undergoes a self-elimination reaction to release the parent drug.

Exemplifying the approach shown in Scheme 6, two examples of prodrugs utilizing self-eliminating spacers are discussed here. The first type involves electron-rich moieties that initiate an intramolecular cyclization reaction to eliminate the drug; these are referred to as *cyclization-elimination* spacers (Scheme 6a). The second example involves spacers that undergo elimination as a result of shift in conjugated electron pairs, ultimately causing the release of the parent

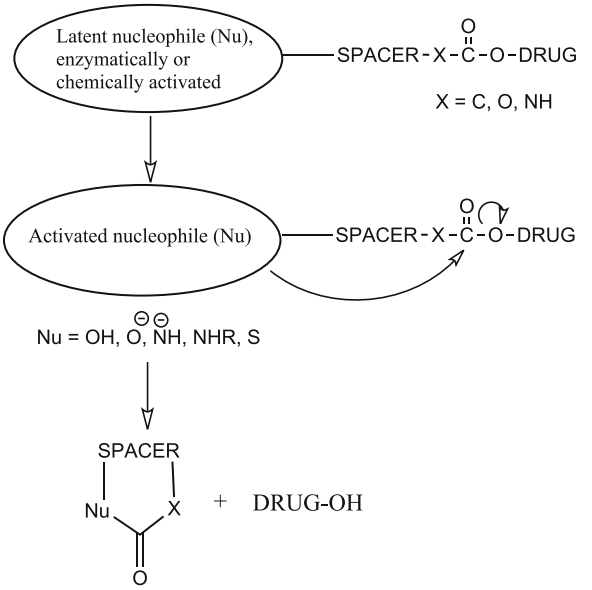


Scheme 5.

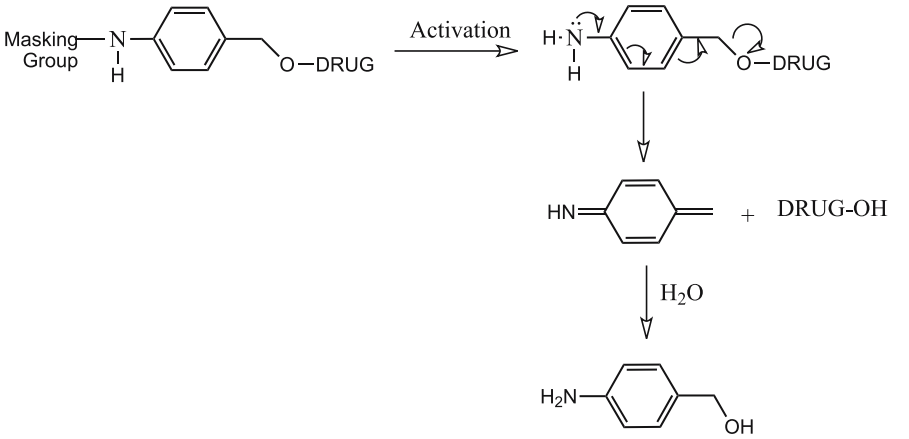
alcohol or phenol drug. These spacers are referred to as *electronic cascade spacers* (Scheme 6b).

The discussion in this section will be limited to prodrugs incorporating spacers that undergo either a cyclization-elimination reaction or an electronic cascade to release the parent alcohol or phenol. This discussion is not intended to serve as an exhaustive review, as this topic has been widely referenced and reviewed, but will emphasize the critical design features and bioconversion pathways of prodrugs that are linked to the hydroxyl functionality of the parent drug.

(**A**). Cyclization-elimination Spacers



(**B**). Elimination-cascade spacers

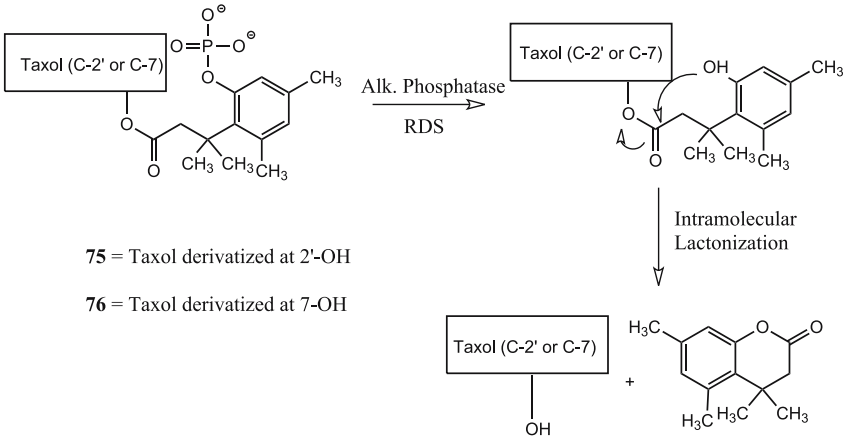


Scheme 6 (a & b).

Prodrugs Incorporating a Spacer Facilitating a Cyclization-Elimination Reaction to Release the Parent Drug.

As shown in Scheme 6a, the general principle of the activation strategy involves an enzymatic or chemical activation of a masked nucleophile, which then initiates an intramolecular attack on an electron-deficient carbonyl to eliminate the hydroxyl-bearing drug. To facilitate this conversion, the design of the prodrug must include features that favor both the cyclization of the spacer and facile elimination of the parent drug.

One such design feature is the incorporation within the promoiety of a 'trimethyl lock,' which is known to greatly facilitate a lactonization reaction (Milstien and Cohen, 1972). Upon activation of the hydroxyl group, substituted o-hydroxylphenylpropionic acid derivatives undergo a spontaneous intramolecular lactonization reaction to release the group attached to the carboxyl functionality; this is a favorable consequence of the trimethyl lock substitution pattern. Such a system has been utilized to develop amine (Amsberry and Borchardt, 1991) and esterase-sensitive prodrugs for peptides (Wang *et al.*, 1997). In one such approach to develop prodrugs for alcohols, Ueda *et al.* (1993) synthesized and evaluated two phosphonooxyphenyl propionate esters of taxol linked at either the 2'-OH (**75**) or the 7-OH (**76**) positions. These water-soluble (>10 mg/mL), alkaline phosphatase-sensitive prodrugs undergo conversion to release taxol within 25 min under physiological conditions. *In vivo* studies demonstrated that **75** possesses efficacy similar to that of taxol itself against the murine solid tumor (M109) model.



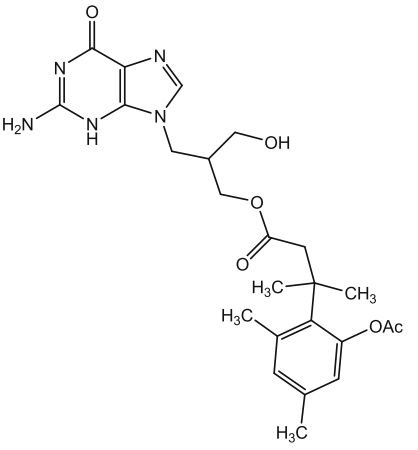
75 = Taxol derivatized at 2'-OH

76 = Taxol derivatized at 7-OH

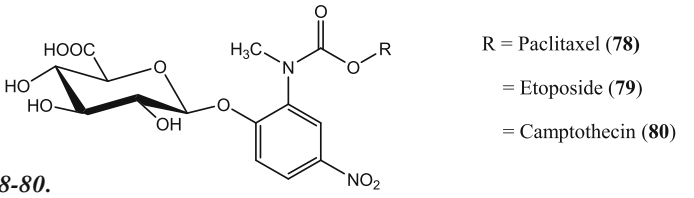
Structures 75-76.

In an attempt to improve oral bioavailability of an antiviral drug ganciclovir, an esterase–sensitive prodrug was synthesized by using the 'trimethyl lock' system (Dillon *et al.*, 1996). The prodrug **77** displayed a favorable fourfold increase in oral bioavailability over the parent drug in rats, thereby indicating potential *in vivo* applications of this 'trimethyl lock' system.

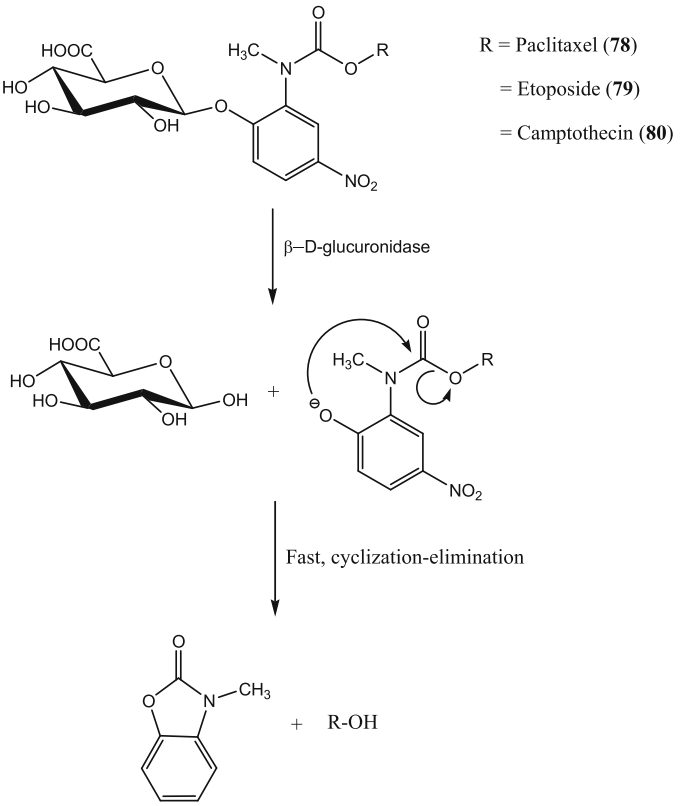
Another specifically designed spacer that facilitates a cyclization-elimination reaction to release parent drug is the structurally modified form of N-(substituted



Structure 77. (77)



Structures 78-80.



Scheme 7.

2-hydroxyphenyl) carbamate, incorporated in the glucuronide-based prodrugs of paclitaxel (Schmidt *et al.*, 2001), etoposide (Schmidt and Monneret, 2003), and camptothecin (Angenault *et al.*, 2003).

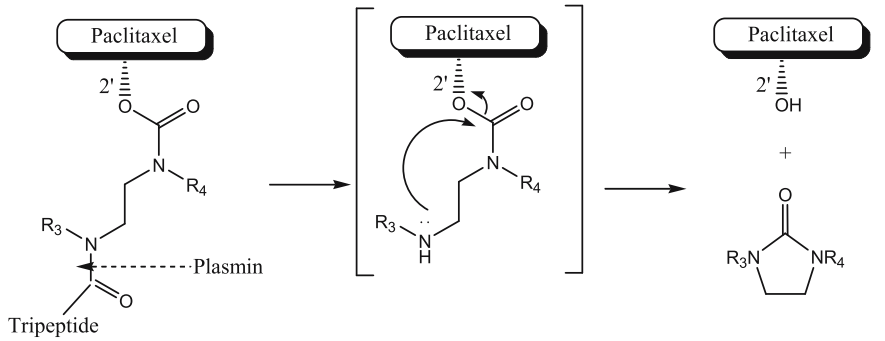
The prodrugs release the parent alcohol or phenol drug specifically at the tumor site by a mechanism depicted in Scheme 7. After enzymatic cleavage of the glycoside by β-glucuronidase, the phenolate anion initiates an intramolecular cyclization to eliminate the carbamate bond-linked drug with concomitant release of a cyclized spacer.

In comparison to paclitaxel, the paclitaxel prodrug **78** is about 2000-fold more water-soluble (10 mM vs. 5 μM), and is adequately stable in human serum at 37ºC with a half-life of 45 h. Whereas the IC_{50} value for paclitaxel measured against LoVo cells (human colon cancer cell line) is 90 nM, the prodrug **78** is about 700-fold less cytotoxic with an IC_{50} value of 65 μM. Although **78** undergoes a β-glucuronidase mediated conversion *in vitro*, fairly high concentrations are required for fast release of the parent drug, which are likely to be difficult to achieve under *in vivo* conditions.

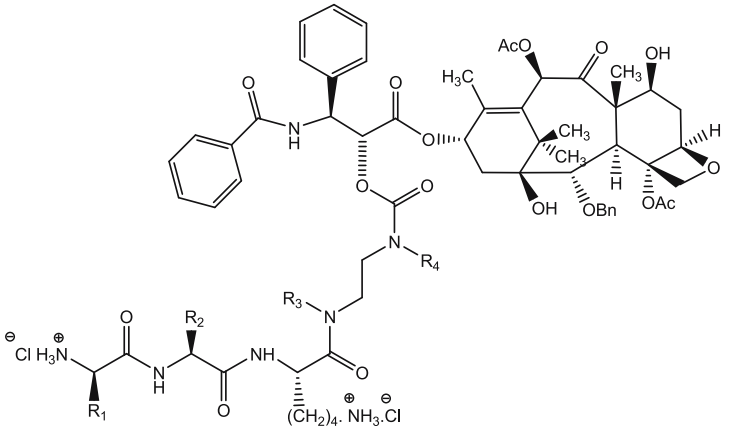
A more recent application of the *N,N'*-dimethyl ethylenediamine spacer, previously utilized by Saari *et al.* in evaluating cyclization-elimination-based prodrugs of phenols (1990a) and alcohols (1990b), has been directed toward the development of prodrugs as a part of the ADEPT activation strategy. When activated by a specifically targeted enzyme, the terminal amino group on the spacer is activated and initiates an intramolecular cyclization reaction to eliminate a phenol (Lougerstay-Madec *et al.*, 1998) or alcohol (Zhang *et al.*, 2005) parent drug with concomitant release of the cyclized spacer.

In one such application, de Groot *et al.* (2000) incorporated the *N,N'*-dimethyl ethylenediamine spacer to evaluate 2'-carbamate- and carbonate-linked prodrugs of paclitaxel for specific activation by tumor-associated protease plasmin. Since the proteolytic active form of plasmin is located in the tumor, linking a cytotoxic drug to a plasmin substrate may result in tumor-selective delivery. On the basis of this rationale, after plasmin hydrolysis the spacer is expected to undergo spontaneous cyclization to yield a cyclic urea derivative (*N,N'*-dimethyl imidazolidinone), thereby releasing paclitaxel, as illustrated in scheme 8.

Although these prodrugs were chemically stable, only **84** and **85** were converted to paclitaxel in the presence of plasmin. The half-lives of enzymatic



Scheme 8.

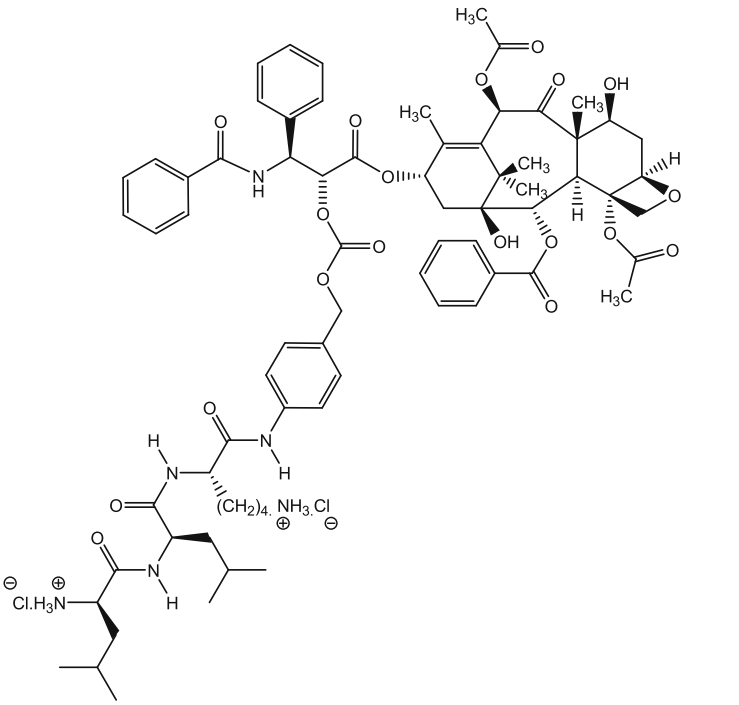


(**81**). $R_1 = CH_3$, $R_2 = CH_2Ph$, $R_3 = R_4 = H$

(**82**). $R_1 = CH_3$, $R_2 = CH_2Ph$, $R_3 = R_4 = CH_3$

(**83**). $R_1 = CH(CH_3)_2$, $R_2 = CH_2CH(CH_3)_2$, $R_3 = R_4 = CH_3$

(**84**). $R_1 = CH_3$, $R_2 = CH_2Ph$, $R_3 = H$, $R_4 = CH_3$



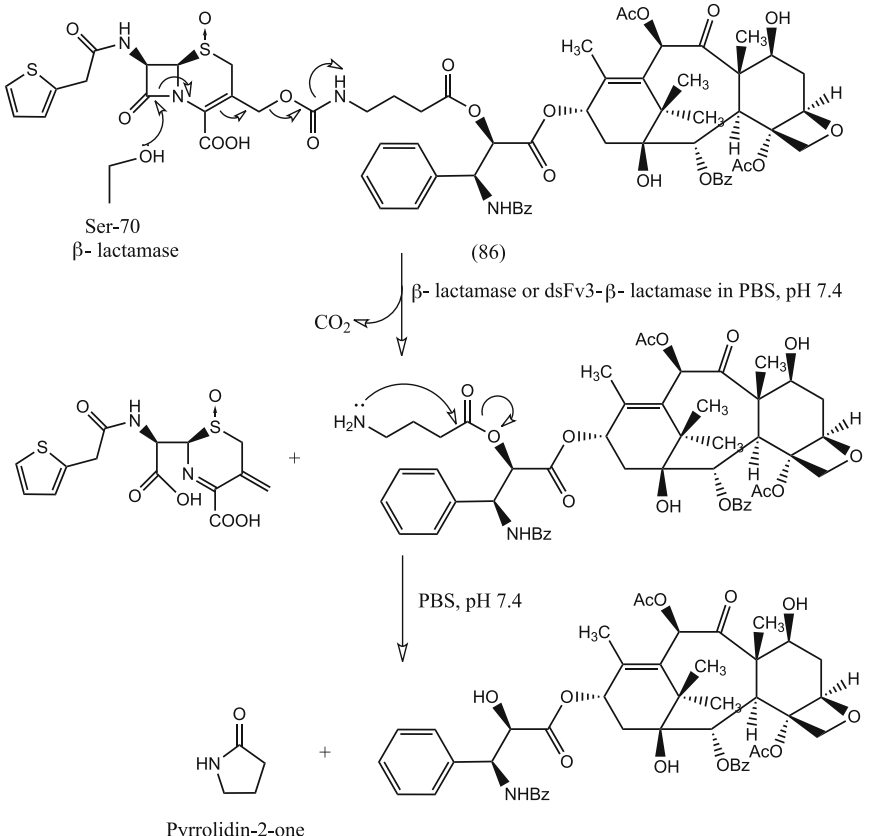
(85)

Structures 80-85.

hydrolysis for **84** and **85** were 3.5 and 42 min, respectively. While the 1,6-elimination of the spacer of **85** was instantaneous, the half-life of spacer cyclization of **84** after peptide cleavage was 23 h, which is unfavorable for any potential *in vivo* application, as the drug-linker complex might diffuse out from the site of cleavage before the parent drug is released.

Enzyme-activated prodrugs combined with an antibody-enzyme fusion protein may enhance the anti-tumor efficacy of antibodies and minimize undesirable systemic toxicity of conventional cytotoxicity drugs. In one such application, the enzyme β-lactamase binds to an antibody and generates a fusion protein (dsFv3-β-lactamase), which selectively activates a cephalosporin-linked paclitaxel prodrug **86** (Rodrigues *et al.*, 1995). This cepham prodrug of paclitaxel (PROTAX) is synthesized by substituting the C-3' position of cephalothin with 2'-(γ-aminobutyryl) taxol. As shown in Scheme 9, hydrolysis of **86** by β-lactamase rapidly releases 2'-(γ-aminobutyryl) taxol, followed by a rate-limiting self-immolation of the spacer to generate paclitaxel.

PROTAX **86** is approximately tenfold less toxic than paclitaxel against the breast cancer tumor cell line SK-BR-3 *in vitro*, but is equally active following prolonged activation with β-lactamase. However, two of the major concerns with PROTAX are the potential for direct ester hydrolysis by endogenous esterases and the slow internal intramolecular cyclization rate ($t_{1/2}$ = 16 h) of the intermediate

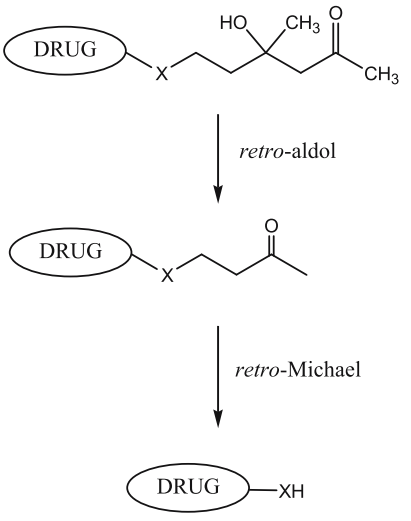


Scheme 9.

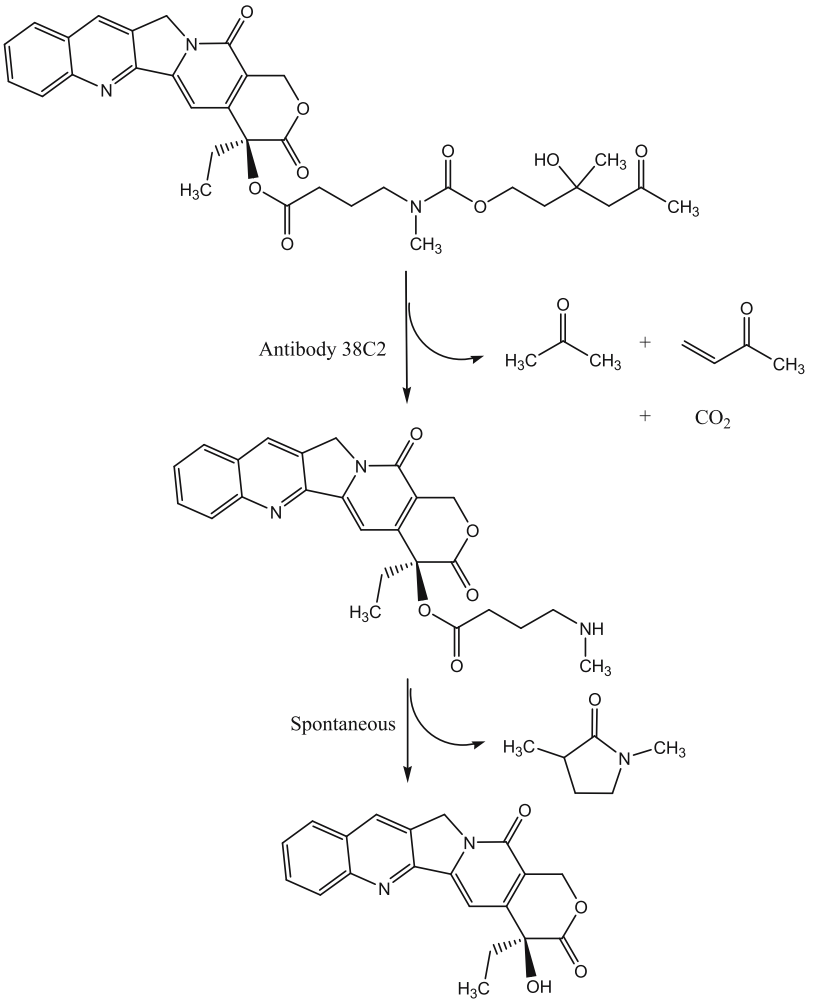
to release paclitaxel. Although in a follow-up study, Vrudhula *et al.* (2003) attempted to modify the chemistry of the linker to ensure a more rapid release of paclitaxel, the rate-limiting cyclization of the linker ($t_{1/2} \approx 50$–230 min) still proved to be an issue that might compromise any possible *in vivo* applications of such a strategy.

In a continuing effort to deliver chemotherapeutic agents, site-specific activation by catalytic antibodies may hold promise. As these antibodies can be easily manipulated for human application, novel prodrug-activating enzymes devoid of immunogenicity can potentially be prepared for repeated clinical application (Bagshawe, 1989). One such antibody is the aldolase antibody 38C2 (Barbas *et al.*, 1997), which activates a specifically designed prodrug by catalyzing a retro-aldol/retro-Michael reaction sequence (Shabat *et al.*, 1999). Unlike natural aldolase enzymes, which typically process phosphorylated sugars, the antibody aldolase 38C2 processes hydrophobic substrates and can catalyze a retro-aldol involving tertiary aldols (List *et al.*, 2000). Upon decarboxylation of the trigger portion on the promoiety by the aldolase antibody, a free amine group in the spacer undergoes spontaneous lactamization to release the parent drug (see Scheme 10b).

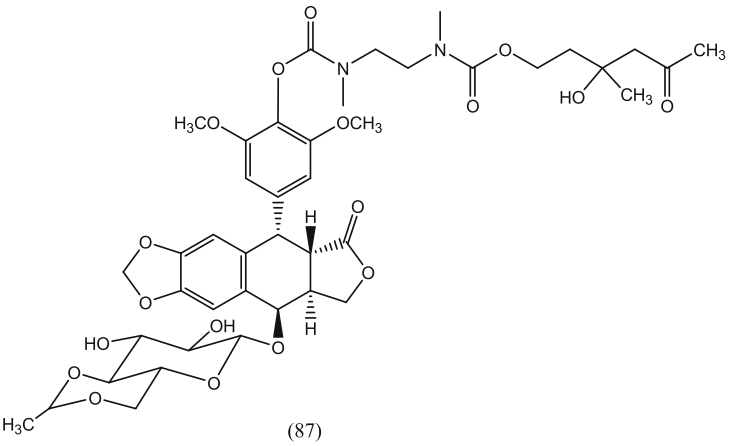
This interesting prodrug activating strategy has been utilized in evaluating prodrugs of etoposide (Haba *et al.*, 2005) and camptothecin (Pessah *et al.*, 2004; Haba *et al.*, 2005) for site-specific delivery. In one such approach by Shabat and co-workers (2001), the etoposide prodrug (**87**) was found to be 100-fold less toxic than etoposide in an *in vitro* assay when evaluated in the NXS2 neuroblastoma cell-line, while its therapeutic activity was regained when activated by the catalytic antibody 38C2. The proof of concept to demonstrate the specific antibody-catalyzed *in vivo* activation of **87** was performed in murine neuroblastoma based on the NXS2 cell-line. Mice receiving both an intratumorally injected catalytic antibody and intraperitoneally injected **87** revealed a dramatic reduction in tumor



Scheme 10a.



Scheme 10b.



(87)

Structure 87.

growth in contrast to the control group, which received each agent as monotherapy or in phosphate buffer-saline. Furthermore, no systemic toxicity was observed in mice treated with 1250 mg of **87**, a 30-fold increase over the dose of etoposide used. These studies demonstrate that localized activation of the prodrug is superior to systemic administration of a cytotoxic drug in achieving a high concentration of drug at the tumor site while, at the same time, minimizing any undesirable toxicity.

Prodrugs Incorporating a Spacer Facilitating an Electronic Cascade, Resulting in Elimination of the Parent Drug

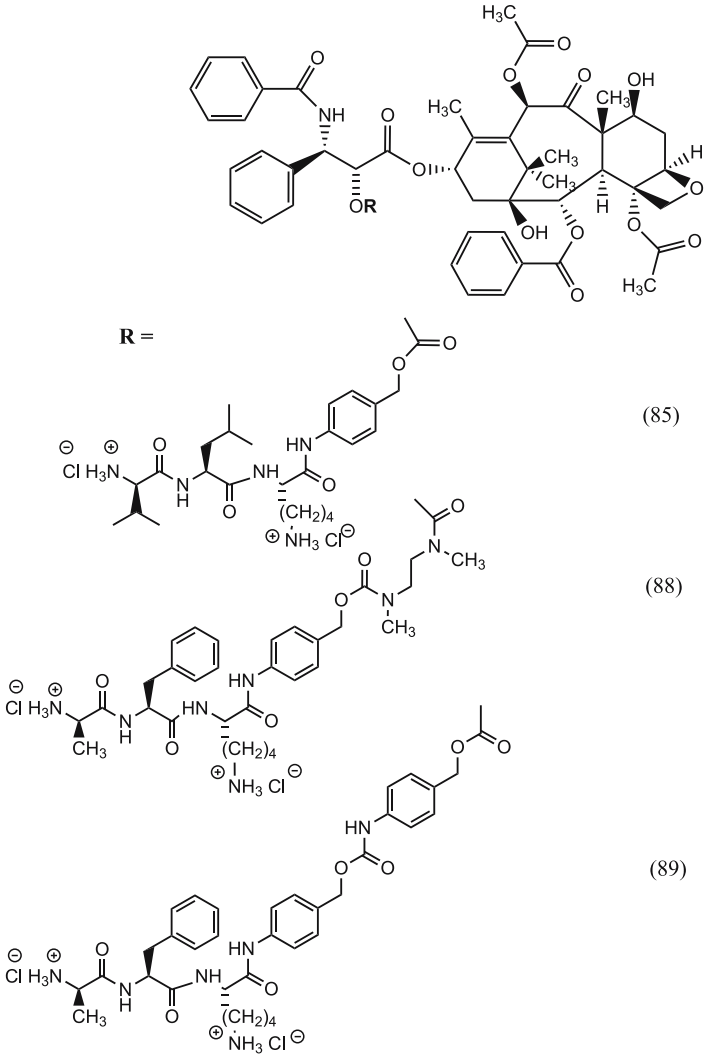
This section summarizes prodrug strategies of alcohols that incorporate a spacer favoring an electronic cascade mechanism to release a hydroxyl bound drug, as illustrated in Scheme 6(b). The activation of an electron-donating group in the promoiety causes a shift in the conjugated electron pairs that results in elimination of the parent alcohol or phenol hydroxyl-bearing cytotoxic drug. A 1,4- or a 1,6-elimination process can occur when the electron-donating group generated from a masked amino or hydroxyl functional group and a good leaving group like a halide, carbamate or carboxylate are positioned *ortho* or *para* to one another on a central aromatic ring. A concomitant formation of quinone methide or quinonimine methide occurs when an amino group or an hydroxyl group is activated and undergoes an 1,6-elimination process (Wakselman, 1983).

Typically, electronic cascade spacers eliminate faster upon activation (or unmasking) than do cyclization spacers, which are often characterized by a rate-limiting elimination step of the parent drug. One of the most versatile spacer utilized in such electronic cascade strategies has been the *p*-aminobenzyl alcohol (PABA) system due to its instantaneous elimination upon unmasking the amine (Carl *et al.*, 1981). In contrast, the *p*-hydroxybenzyl electronic cascade spacers require electron-withdrawing substituents on the phenyl ring to facilitate an elimination reaction (Florent *et al.*, 1998). In cases where the spacer undergoes rapid elimination after activation, the rate-limiting step is either the chemical or enzymatic activation of the prodrug that determines the overall efficiency of drug release.

Para-aminobenzyl alcohol has been utilized to spatially separate biologically active drugs from the site of enzymatic cleavage. This bifunctional spacer is attached to the hydroxyl functional group of parent drug either via an ether (Toki *et al.*, 2002), a carbamate, and/or a carbonate linkage (de Groot *et al.*, 2001b, 2002), while an amide bond links its amino functional group to a peptide sequence to facilitate an efficient and site-selective release of the pharmacologically active drug. In presence of a hydrolyzing enzyme, for example plasmin, the prodrug undergoes a spontaneous elimination to release the chemically unmodified drug and the modified spacer.

In one such application, de Groot *et al.* (2001b) evaluated the effect of spacer length and chemistry of linkage (carbonate or carbamate) of spacers at the 2'-OH of paclitaxel for target-selective delivery. In presence of the enzyme plasmin, the

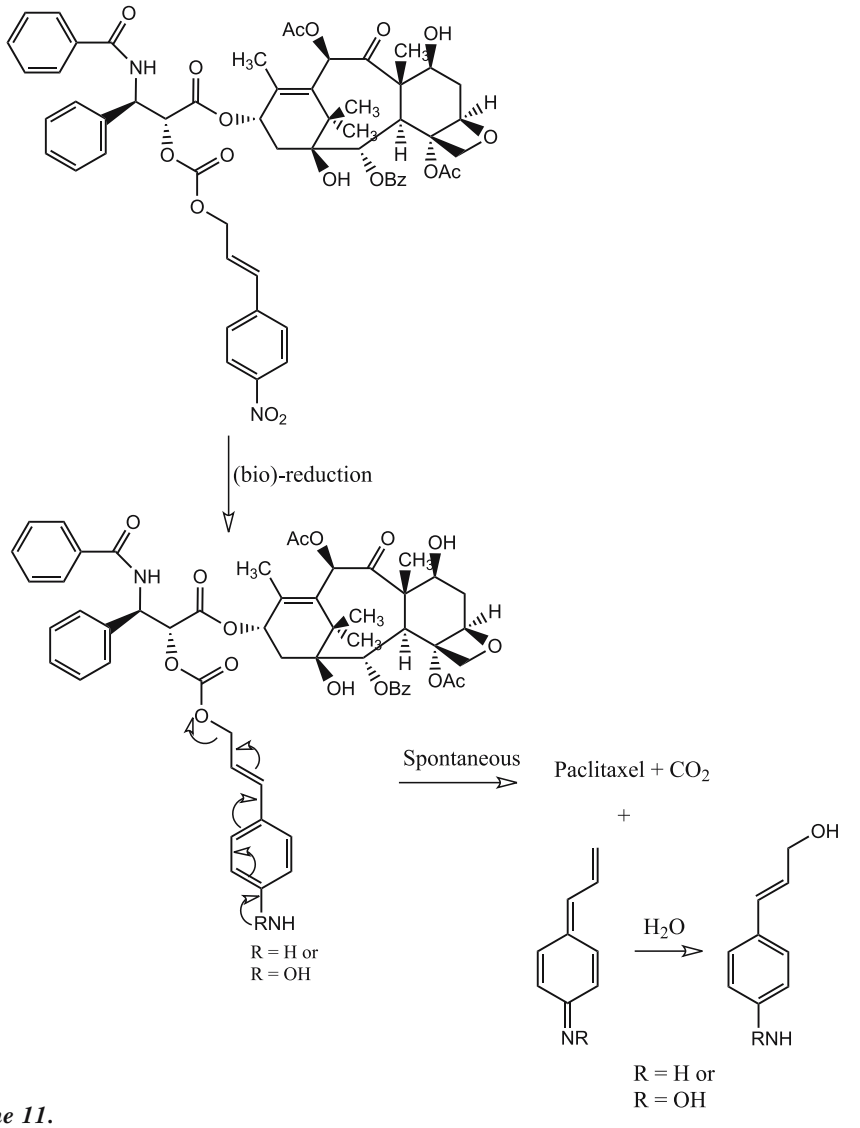
prodrugs **85**, **88**, and **89** undergo a 1,6-elimination to release paclitaxel with concomitant release of the modified spacer components and carbon dioxide. As expected, upon incubation with plasmin, the double electronic cascade spacer containing prodrug **89** released paclitaxel with a sixfold increased rate ($t_{1/2}$ act = 7.5 min) in comparison with the single spacer-containing prodrug 85 ($t_{1/2}$ act = 42 min); this indicates a favorable enzymatic activation rate, particularly for sterically hindered substrates such as paclitaxel. The prodrug **89** incorporating an extended spacer also displays a higher *in vitro* cytotoxicity profile, which might be due either to a facilitated release of paclitaxel or to the prodrug's susceptibility to ubiquitous esterases that cleave the promoiety-drug bond. This instability of an ester linkage may be overcome by linking the promoiety via a more enzymatically stable carbamate bond. The markedly lower cytotoxicity (20-fold) of the double spacer-containing 2'-carbamate prodrug **88** compared to that of **85** indicates that linking



Structures 85, 88-89.

the promoiety to paclitaxel via a carbamate bond may provide enhanced prodrug stability toward undesirable enzymatic hydrolysis.

The prodrug strategy has also been evaluated for delivery of cytotoxic drugs to solid tumors. The inefficient development of blood vessels in these solid tumors results in hypoxic regions within the tumor population (Lin *et al.*, 1972). This hypoxic condition favors a reducing environment, which presents a possibility for designing prodrugs that are selectively activated under such bioreductive environments (Ross *et al.*, 1996); one such successful example is the prodrug tirapazamine (Brown, 1993). Bioreductive prodrugs undergo activation to cytotoxic agents that are often radical intermediates. These intermediates are rapidly oxidized back to nontoxic prodrugs in normal tissues with high levels of oxygen, whereas a less efficient quenching occurs in solid tumors, resulting in solid-tumor selective toxicity (Lin *et al.*, 1972). This selectivity is clinically



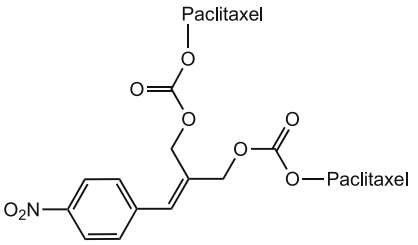
Scheme 11.

beneficial, since these hypoxic solid tumors are more resistant to radiotherapy, requiring a two- to threefold higher radiation dose (Denny and Wilson, 2000).

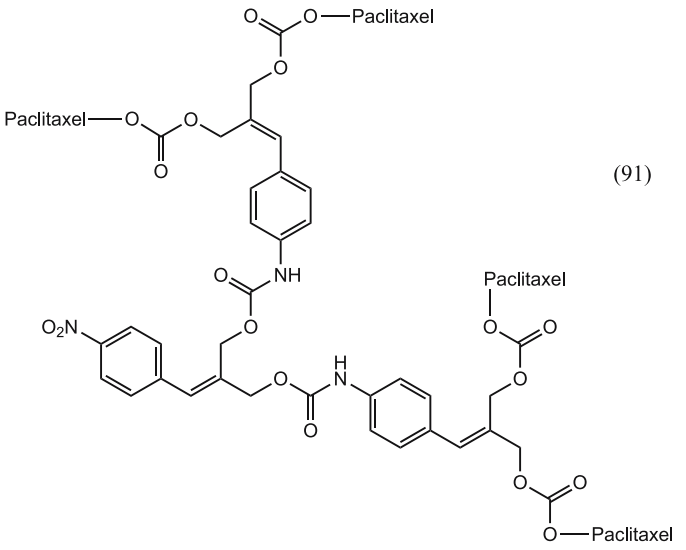
In one such application to deliver bioreductive prodrugs of paclitaxel to hypoxic solid tumors, Damen and co-workers (2002) linked a promoiety bearing nitroaromatic and/or nitro heteroaromatic substituents as bioreductive triggers to the 2'-OH of paclitaxel. As illustrated in Scheme 11, these prodrugs released paclitaxel after nitro group reduction and subsequent 1,6- or 1,8-elimination.

These prodrugs are stable in Tris buffer (pH 7.4, 37°C) for approximately 24 h. However, in human tumor cell lines, they undergo a nonspecific hydrolysis by esterases, resulting in marginally decreased cytotoxicity compared to paclitaxel, thereby eliminating any possible *in vivo* applications of this prodrug strategy.

One of the new emerging concepts in nanotechnology has been the application of dendrimer assemblies in drug delivery (Padilla De Jesús *et al.*, 2002; Shamis *et al.*, 2004). Dendrimers are tree- or star-shaped polymers about 1–2 nanometers in size, with a 'trigger,' a central core, supporting framework, and multiple terminal endgroups that are specifically constructed to support drugs or antibodies. These biologically active endgroups can be released by either a 1,4- (Shamis *et al.*, 2004) or a 1,8-elimination (de Groot *et al.*, 2003) mechanism after a chemical or an enzymatic activation of the 'trigger' molecule. Utilizing a 1,8-elimination-disassembling subunit, de Groot synthesized dendrimers that release



(90)

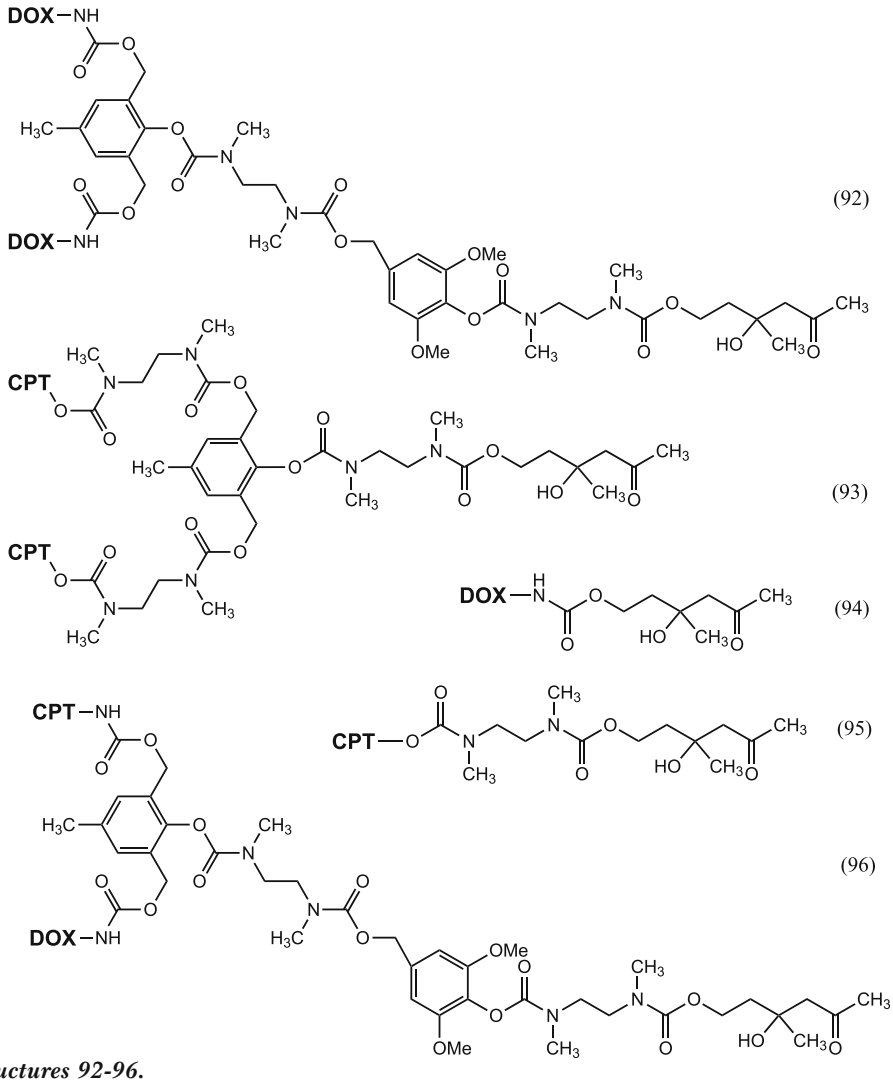


(91)

Structures 90-91.

two (**90**) and four (**91**) equivalents of paclitaxel upon reduction of the nitro group to the amine under mild conditions (Zn/AcOH). The release of these 2'-hydroxyl-bound paclitaxels from the dendrimer occurs in less than 30 min, with no indication of degradation or formation of side products occurring, thereby proving the validity of this concept.

Shamis and co-workers (2004) used a 1,4-eliminating subunit to synthesize and evaluate homo- and heterodimeric prodrugs that release two equivalents of doxorubicin, camptothecin, or both. The trigger molecule is the retro-aldol/retro-Michael substrate of antibody 38C2, which is attached to the central core of the dendrimeric prodrug via a self-eliminating linker. These prodrugs were evaluated using a cell-growth inhibition assay of the Molt-3 leukemia cell line. When activated by the catalytic antibody 38C2, an increase in activity (lower IC_{50} value) was observed for the two homodimeric dendritic prodrugs **92** and **93** in comparison with the analogous monomeric prodrugs **94** and **95**. This effect can



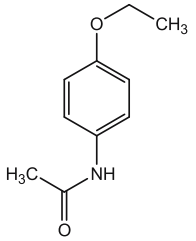
Structures 92-96.

be explained by the number of enzymatic cleavage steps required to activate the prodrugs; two cleavage steps are required for the monomeric prodrugs, while only one is needed for the homodimeric prodrug. More significant was the 50-fold higher growth inhibition displayed by the heterodimeric prodrug **96** than by the combination of two monomeric prodrugs **94** and **95**, indicating the possibility to achieve a synergistic release of these two cytotoxic drugs. Importantly, the toxicity of the heterodimeric prodrug 96 in the absence of the catalytic antibody 38C2 is similar to that measured for the combination of the monomeric prodrugs.

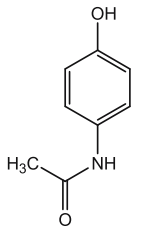
The dendrimer concept seems intriguing, but it will certainly require more development work before any *in vivo* applications will be tested. In addition to finding ways to retain the dendrimer within tissues, one of the most critical barriers to overcome would be the identification of the source to activate the trigger molecule within a living cell. If such a biological agent is identified, say, an enzyme, then it might be possible to selectively activate the dendrimer assembly within specific tissues, such as tumors.

Oxidation/Reduction Leading to Hydroxylation

Earlier in this chapter, ethers were mentioned as protective groups for drugs with alcohol-containing functional groups. O-dealkylation of ethers is the result of an oxidative metabolism process leading to the release of the drug and an aldehyde. An historical example is phenacetin (**97**) as a prodrug of acetaminophen (**98**). Here, the prodrug was actually found to be more toxic than active parent drug; as a result, phenacetin was removed from the market in favor of acetaminophen. Acetaminophen not only displays superior analgesic activity compared to phenacetin but, in addition, does not produce methemoglobinemia and hemolytic anemia. Phenacetin is converted to approximately a dozen metabolites by *O*-deethylation, *N*-deacetylation, and hydroxylation processes (Veronese *et al.*, 1985). The *N*-hydroxyphenatidine metabolite of **97** has been shown to be responsible for the formation of methemoglobin and hemolysis of red blood cells (Jensen and Jollow, 1991). The use of oxidative/reductive events leading to drug regeneration for a prodrug is well appreciated but is not used as extensively as might be expected. One major reason would seem to be concerns about genetic and environmentally determined levels of various P450 isozymes responsible for prodrug conversion (Ingelman-Sundberg, 2001).



(97)

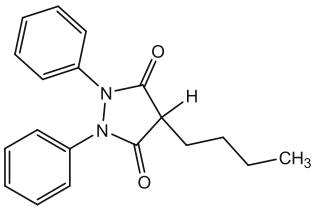


(98)

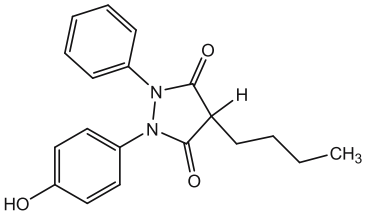
Structures 97-98.

How were phenacetin and acetaminophen initially identified as pain relievers? They both owe their origin to the observation that acetanilide was identified as a chemical with therapeutic value (Brodie and Axelrod, 1948). Although found to be too toxic, the *p*-hydroxy metabolite of acetanilide, acetaminophen, was active. In this example, the hydroxy group was introduced via *p*-hydroxylation, a common metabolism for aromatic compounds. The idea of using aromatic or aliphatic hydroxylation to "activate" a molecule to a drug has not been a common one. More often than not, examples available in the literature are the result of serendipity.

A case in point is the oxidation of phenylbutazone (**99**) to oxyphenbutazone (**100**) (Aarbakke *et al.*, 1977). Both molecules were marketed as non-steroidal anti-inflammatory agents. The active metabolite **100** shows superior analgesic activity in comparison with phenylbutazone and is said to cause less gastric irritation.



(99)

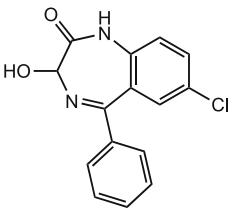


(100)

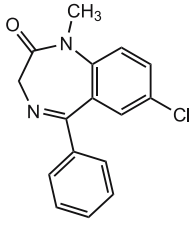
Structures 99-100.

Oxazepam (**101**) is the common active metabolite of chlordiazepoxide, halazepam, and diazepam (**102**) (Caccia and Garattini, 1990). However, unlike other benzodiazepines, oxazepam undergoes glucuronidation and, therefore, has a shorter half-life ($t_{1/2} \approx 5.8$ h) than any of its precursors (Sonne *et al.*, 1988). As a result of its pharmacokinetic profile, oxazepam is clinically prescribed as a short-acting benzodiazepine in the management of anxiety disorders or for short-term relief from symptoms of anxiety.

A detailed investigation into the metabolic processes and pharmacokinetics of a new drug is now common and required by regulatory agencies. Identifying the sites for active metabolite formation is important not only for explaining any



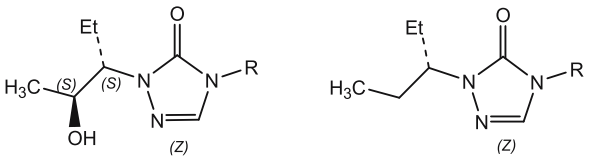
(101)



(102)

Structures 101-102.

potential toxicity but also for predicting therapeutic outcomes. An interesting recent case is the discovery of posaconazole (**103**), the hydroxylated analog of the orally active broad spectrum antifungal Sch 51048 (**104**). *In vitro* and *in vivo* susceptibility studies showed that posaconazole has excellent activity against a wide spectrum of pathogenic fungi including *Candida spp., Cryptococcus neoformans* (Galgiani and Lewis, 1997), *Blastomyces dermatitidis* (Sugar and Liu, 1996), and several other fungal pathogens (Espinel-Ingroff, 1998). In contrast to the established triazoles, posaconazole has fungicidal activity against yeasts and molds, including itraconazole- and fluconazole-resistant yeasts. It is our understanding that the hydroxylated analog **103** was detected during *in vitro/in vivo* metabolism screening (Bennett *et al.*, 2006). Nevertheless, the incorporation of a polar alcohol functionality improves the hydrophilicity of the molecule and additionally provides a 'synthetic handle' to prepare additional water-soluble prodrugs for intravenous administration when the oral route of administration is impractical (Bennett *et al.*, 2000). Posaconazole (Noxafil®) has recently been approved in the European Union as an oral suspension for the treatment of invasive fungal infections.



(103) (104)

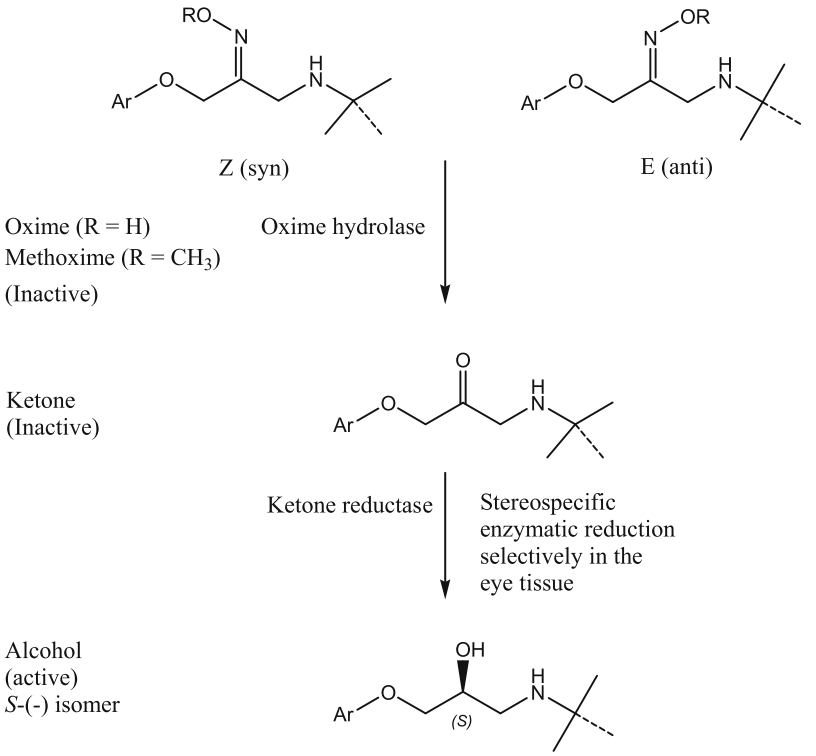
Structures 103-104.

The examples just discussed represent cases of serendipity, whereby active oxidatively derived hydroxy-metabolites were identified and found to possess better or alternative physicochemical properties or bioproperties than the original molecule.

The hydroxyl functionality can also be derived from reduction of a ketone or an aldehyde. An example is from the work of Bodor (1988; El-Koussi and Bodor, 1989) seen while evaluating the delivery of intraocular pressure (IOP)-reducing β-adrenergic blocking agents to the eye. In these compounds, a β-amino oxime or an alkyloxime functional group was used to replace the corresponding β-amino alcohol pharmacophore (Scheme 12)

These oximes exist in either the syn (*Z*) or the anti (*E*) configuration and are hydrolyzed within the iris-ciliary body of the eye. The ketone intermediate is subsequently stereospecifically reduced to produce the desirable S-(–) stereoisomer of the respective β-adrenergic blocker. Thus, activation of the drug results from the reduction of the ketone intermediate in the final step.

The rationale proposed for the site- and stereospecific delivery of these β-adrenergic anti-glaucoma drugs into the iris-ciliary body is a metabolic process



Scheme 12.

common to lipophilic ketone precursors of β-amino alcohols. However, administration of the ketone precursors is not desirable due to their chemical instability in aqueous solutions. Hence, to stabilize the ketone intermediate, they are converted to their respective oximes, which undergo enzymatic hydrolysis in the eye to eventually release the active drug.

It seems that the use of oxidative/reductive metabolic pathways may be under utilized as a means of delivering hydroxyl group-containing drugs to the body. However, the reluctance to use this methodology is grounded in the lack of quantitative predictability between animal species and concerns about extensive inter-individual variability for these metabolic pathways.

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3.3

Prodrugs of Amines

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Introduction

The amine group is one of the most frequently found functional groups in today's armory of commercially available drugs. Like many pharmaceuticals, compounds containing an amine can have physicochemical attributes that present obstacles to their safe and effective delivery to desired sites of action. Amines are generally considered to be amenable to derivatization reactions and thus provide a "synthetic handle" that can be exploited in chemical modifications. As a result, numerous prodrugs of amines have been evaluated in an effort to overcome formulation and delivery barriers, which include low aqueous solubility, toxicity of the vehicle, poor membrane permeability, chemical and metabolic instability, and lack of specificity

An amine, by definition, is considered to be any hydrocarbon derivative of ammonia. Amines are generally classified as primary, secondary, tertiary or quaternary, depending on the degree of hydrocarbon substitution, and they can be further classified as aliphatic, aromatic or heterocyclic. Aliphatic amines typically have alkyl substituents whereas aromatic amines are coupled to at least one aromatic group. Heterocyclic amines have a nitrogen contained within a ring system, which may be partially or fully unsaturated.

An important physicochemical characteristic of amines is their basicity, or propensity to accept a proton and form the respective conjugate acid, according to the Brönsted-Lowrey classification. Accordingly, the pKa value associated with an amine is a reflection of its ability to share the associated lone pare of electrons in the formation of a bond with hydrogen. This pKa value is influenced by electronic and steric properties as well as the hybridization state. Electron-withdrawing substituents decrease the strength of the base (*i.e.*, lower pKa). Alternatively, electron-donating substituents (*i.e.*, methoxy) increase the basicity and assist in stabilization of the positively charged conjugate acid. These influences on basicity can occur either through inductive or resonance effects. Steric factors can hinder hydrogen bond formation and result in reductions in basicity. Amines bonded to bulky alkyl groups will generally have lower pKa values than corresponding amines with more exposed lone pairs of electrons. The hybridization state of the amine is also an important determinant in basicity as it reflects how close electrons are to the nitrogen nucleus. Aliphatic sp^3 hybridized amines are generally more basic than sp^2 hybridized heterocyclic amines.

From a synthetic point of view, it is also important to consider the nucleophilicity of the amine. Basicity and nucleophilicity often correlate with one another; however, it is important to realize the distinction between these two properties. Basicity, expressed by pKa values, is an equilibrium constant for reversible protonation whereas nucleophilicity refers to the rate of a chemical reaction at an electrophilic center. Many of the same factors that influence pKa will influence nucleophilicity; however, as previously mentioned, steric factors often lead to greater reduction in chemical reactivity than they do in basicity.

Produgs highlighted in this chapter were created in order to overcome many different barriers. It is important to consider how the addition of the promoiety will change the properties of the parent drug and how this fits in with the intended

purpose. One important consideration is the influence of derivatization on the pKa of the parent drug. Typically, derivatizations of amines will result in reductions in both basicity and nucleophilicity. Reductions in basicity would be favorable for improving the rate of diffusion across biological membranes since at a given pH the percent of the molecule existing in the unionized state will increase. Reductions in nucleophilicity have been exploited in efforts to improve the chemical stability of parent amines that are prone to intermolecular aminolysis reactions.

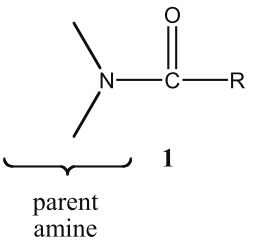
It is also important to focus on the physicochemical and structural properties of the promoiety itself. Some promoieties are considered hydrophilic and are designed to improve the water solubility of parent drugs. Conversely, others are lipophilic and are designed to improve membrane permeability. Another, less obvious, effect of amine derivatization is changes in intermolecular interactions between molecules in the crystalline state. Prodrugs that decrease the extent of these interactions will be expected to have increased dissolution rates and possibly higher water solubility. Therefore, it is conceivable that a prodrug could have both increased water solubility and membrane permeability relative to the parent amine.

Additionally, structural design of promoieties has been optimized in order to make prodrugs with improved specificity for target sites. This has been accomplished by designing promoieties that are substrates for enzymes specifically localized at the desired sites of action. Obviously, one must consider all of these contributions collectively when designing a successful prodrug.

With this introduction, the remainder of this chapter will focus on specific chemical strategies to derivatize amines. The prodrug strategies reviewed here will be categorized according to the type of promoiety employed rather than the intended use of the prodrug. There are several excellent and comprehensive reviews that pertain to prodrug strategies for amines that follow a similar format. The most extensive reviews, although somewhat dated, were compiled by Pitman (1981) and Bundgaard (1985). Since these early reviews, several book chapters and reviews have focused on this topic as well (Roche, 1987; Sloan, 1992; Hu 2005). Testa and Mayer (2003) have also recently published a book devoted to the hydrolysis mechanisms of prodrugs that also include various amine prodrugs.

This chapter includes examples of some earlier work on prodrugs covered in previous reviews to give a historical perspective and for the sake of completeness. However, emphasis is given to newer prodrug concepts as well as how some of the more classical approaches have been used and optimized in recent years.

N-Acyl derivatives



Structure 1.

Acylated amines are defined here as the category of prodrugs that take on the general structure 1. When R is a hydrocarbon, the prodrug is a true acyl prodrug; however, very few successful examples of such prodrugs exist in the literature. Simple acylation of amines leads to the creation of amides, which are well known for their resistance to both chemical and enzymatic hydrolysis.

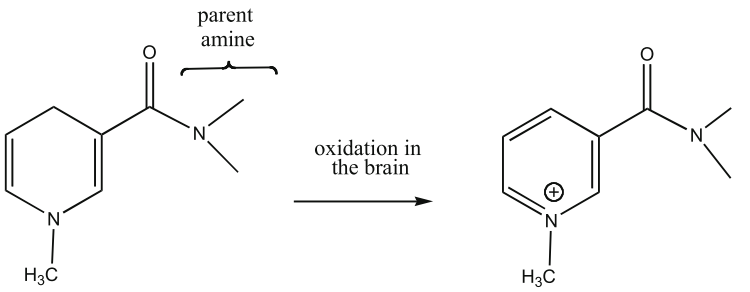
Prodrugs of dopamine (**1**, R = glycosyl) were synthesized and were shown to be too stable for use as prodrugs in *in vivo* mice studies (Fernandez *et al.*, 2000). Zhu *et al.* (2001) have tried to capitalize on this slow release profile; they coupled a model primary amine to oxidized cellulose and evaluated its usefulness as a prodrug for sustained release purposes. The authors found that the hydrolysis in buffer and in rat liver homogenate was minimal even after days of incubation, thus illustrating the stability of this hindered amide linkage.

Both amidases and esterases exist that can facilitate the hydrolysis of certain amide prodrugs (Testa and Mayer, 2003). Typically, these enzymes have a fairly narrow window of substrates that they can hydrolyze. Steric factors, both at the amine and acyl ends of the amide, play a significant role in affecting the enzymes' activity. Stark *et al.* (2001) synthesized a prodrug (**1**, R = palmityl) on the primary amine of (R)-α-methylhistamine and showed that it was subject to a relatively fast *in vivo* hydrolysis in studies with mice. The specific enzyme responsible for the accelerated hydrolysis was not identified.

The lability of amides in buffer can be relatively high depending on the characteristics of the parent amine. The chemical hydrolysis is pH dependent and has been shown to increase if the lone pair of electrons associated with the acylated amine are substantially delocalized. This was demonstrated with a variety of amide prodrugs of theophylline and allopurinol, which have half-lives of less than 30 min at pH 7.4 (Higuchi *et al.*, 1971; Lee *et al.*, 1979; Bundgaard and Falch, 1985).

Bodor and colleagues have pioneered work in central nervous system (CNS) targeted delivery of prodrugs (**2**) which contain an amide linkage (Simpkins and Bodor, 1994). This strategy has been applied to phenyethylamine and dopamine (Bodor and Farag, 1983a,b).

These prodrugs were designed specifically to enhance penetration of amines across the blood-brain barrier (BBB) into the CNS. Amines, normally ionized at physiological pH, have difficulty crossing the BBB unless an endogenous



2 **3**

Structures 2 and 3.

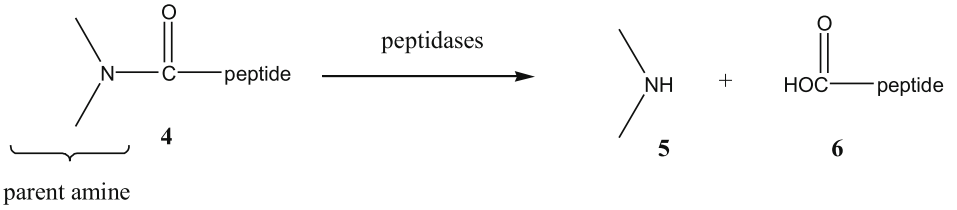
transport mechanism is involved. Linking such drugs to dihydropyridine gave the prodrugs the ability to penetrate all membranes, including the BBB. The prodrug is subsequently oxidized enzymatically to the quaternary salt (**3**) in the CNS. The quaternary intermediate is considered to be membrane impermeable and thus "trapped" in the brain. Slow hydrolysis of the amide then yields sustained delivery of the parent amine in the brain.

In the previous strategy, slow hydrolysis *in vivo* was acceptable because the clearance of the prodrug from the CNS was extremely slow due to the quaternary nature of the oxidized salt. In most other prodrug applications, faster *in vivo* conversion is required. A number of creative strategies have been described that have led to N-acylated prodrugs with dramatically accelerated bioreversion characteristics. The majority of these approaches utilize two different methods for increasing hydrolysis rates. The first strategy exploits peptidases and the second utilizes an intramolecular cyclization approach to catalyze the cleavage of the amide. These strategies are highlighted in the following two sections.

Peptidase-assisted Cleavage

Peptidase proteolytic activity can be found in all biological tissues and fluids. One strategy to facilitate the *in vivo* hydrolysis is to design prodrugs that are substrates for these enzymes. This strategy is depicted in Scheme 1.

The first prodrugs designed to be substrates for peptidase-assisted cleavage utilized γ-glutamic acid. Prodrugs of dopamine, L-dopa, and sulfamethoxazole (**4**, R = L-glutamine) were readily hydrolyzed after *in vivo* administration in a variety of animals (Orlowski and Wilk, 1977; Wilk *et al.*, 1978). Interestingly, the



4 **5** **6**

Scheme 1.

enzyme responsible for the cleavage, γ-glutamyl transpeptidase, is highly concentrated in kidneys. Such prodrugs have been shown to provide kidney-specific release of parent amine (**5**) and the conjugated peptide or amino acid (**6**). However, Drieman *et al.* (1993) demonstrated that the strategy was not universally applicable to all drugs coupled to L-glutamine. Some prodrugs studied were either susceptible to kidney active transport mechanisms or were not substrates for γ-glutamly transpeptidase.

Other amino acid and peptide derivatives of amines have been shown to be enzymatically labile to varying degrees. Some were designed to be substrates of plasmin, a protease that is shown to have higher activity in tumor cells (Evers *et al.*, 1982). The anticancer agents acivicin, phenylenediamine and doxorubicin were all derivatized with the tri-peptide D-Val-Leu-Lys to create prodrugs that were shown to have increased conversion to the respective parent compounds in cancer cell lines compared to non-transformed fibroblasts (Carl *et al.*, 1980; Chakravarty *et al.*, 1983a,b).

Tumor-selective delivery of anticancer drugs by a prostate-specific antigen (PSA)-targeted peptide conjugates of drugs has been described. Wong *et al.* (2001) investigated a prodrug of doxorubicin containing the substrate for PSA, a seven amino acid peptide. The peptide was conjugated to the primary amine of doxorubicin in an attempt to specifically localize the free drug to prostate cancer cells, which have high expression of PSA. Following intravenous administration of the prodrug in a nude mouse xenograft model of human prostate cancer, a 2.5-fold increase in tumor-associated doxorubicin was observed relative to equimolar injections of parent drug.

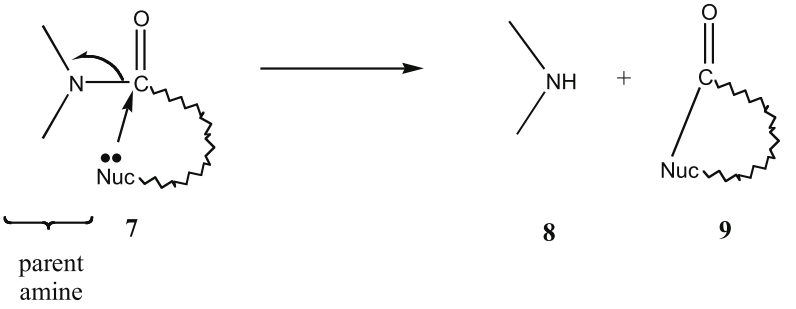
In an attempt to improve water solubility of amines, Pochopin and co-workers (1994) evaluated the utility of amino acid prodrugs of dapsone. The authors compared the *in vivo* conversion kinetics in rabbits of prodrugs that contained L and D amino acids. The D amino acid prodrugs had half-lives in the range of 30 min to 1 h whereas L forms had corresponding values of less than 2 min. This work suggests that the peptidase responsible for cleavage is stereospecific. Another possibility is that different enzymes are responsible for the different prodrug isoforms, as the authors noted, and that D-amino acid analogs were not labile in whole blood samples while L-forms were.

Several prodrugs utilizing peptidase assisted cleavage have been commercially quite successful. Examples include N-acetyl-L-cysteine and midodrine. N-Acetyl-L-cysteine is probably the most successful example (Chasseaud, 1974; Zera and Nagasawa, 1980). This prodrug is enzymatically deacetylated *in vivo* to yield cysteine, which is a biosynthetic precursor of glutathione. Midodrine is a glycinamide prodrug that yields desglymidodrine, an α agonist, following *in vivo* exposure (McClellan *et al.*, 1998; Cruz, 2000).

Promoetiy-assisted Cleavage

A second method for increasing the hydrolysis rate of N-acyl prodrugs is to design promoieties that undergo an intramolecular cyclization reaction to release

the parent amine (**8**) and the cyclized promoiety (**9**). A general diagram for this approach is shown in Scheme 2.



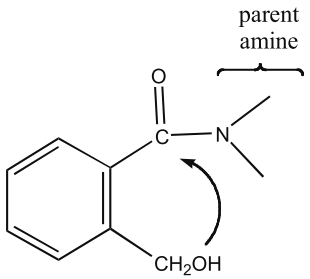
Scheme 2.

Specially designed acyl groups that participate in such a reaction have been described. One of the first applications of this strategy was with 2-hydroxymethylbenzamide conjugates (**10**), which undergo cyclization in aqueous solution to release phthalide and the free amine (Belke *et al.*, 1971; Okuyama *et al.*, 1973; Chiong *et al.*,1975).

Although the intramolecular catalysis was shown to increase the hydrolysis rate, it was still quite slow. Subsequent studies have demonstrated that substitutions of the ring and methylene hydrogens of **10** lead to derivatives with hydrolysis rates 10,000-fold faster than those of underivatized analogs (Fife and Benjamin, 1974; Chiong *et al.*, 1975). Replacement of the primary hydroxyl group in **10** with a primary amine has also been shown to lead to prodrugs with rapid intramolecular aminolysis to give the parent amine and phthalimidine (Fife and DeMark, 1976). Other structural variations on this basic strategy have been investigated, many of which were able to display significant improvements in ring cyclization rates (Cain, 1976; Nielsen and Bundgaard, 1986).

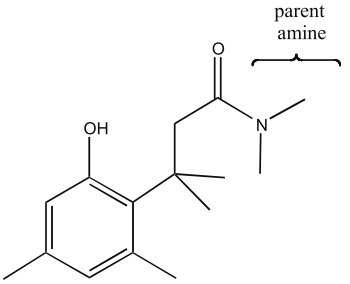
A noteworthy example was described by Amsberry and Borchardt (1990) who have shown a dramatic enhancement in the lactonization rate of hydroxyamides by incorporating a "trimethyl lock" series of substitutions (**11**), which led to a half-life of 65 s at pH 7.5 for release of the parent amine.

The authors subsequently described a double prodrug strategy that involved esters on the phenol groups of **11** (Amsberry *et al.*, 1991). In doing so the authors



10

Structure 10.



11

Structure 11.

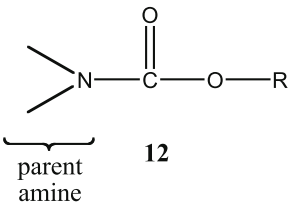
demonstrated that the rate-determining step in the formation of the parent drug was now controlled by esterase activity. This approach is attractive in that the prodrugs are quite stabile at neutral pH conditions but rapidly release parent amine after *in vivo* exposure to esterases. This same methodology has been used to link amine-containing drugs to polyethylene glycol for potential applications in targeting drugs to tumors, relying on the enhanced permeation and retention effect of polymeric molecules (Greenwald *et al.*, 1999).

In a similar approach to the trimethly lock system, Wang *et al.* (1998) have shown that lactonization of O-hydroxy-cis-cinnamic amides to coumarins is fast and can be considered to be a useful prodrug strategy for derivatizing amines

A number of other triggering mechanisms to initiate the lactonization steps in prodrug release have been described. For reviews see Shan *et al.* (1997) and Testa and Mayer (1998). Because of the high reductive capacity of many tumor cells, potential tumor specific prodrugs have been designed that rely upon reduction as a triggering mechanism for bioconversion. Amsberry and Borchardt (1991) also developed a double prodrug concept that requires quinine reduction to trigger the timethyl lock lactonization reaction. Similarly, Sykes *et al.* (1999) have investigated a series of model prodrugs that require nitro-to-hydroxylamine reduction, which can then undergo fast intramolecular cyclization to release the parent amine.

Carbamates

The carbamate functionality has been utilized in many prodrug strategies designed for amines (**12**). As will be discussed, all carbamate-type derivatives are at least double prodrugs, some are considered to be higher order (*i.e*, triple, etc.).



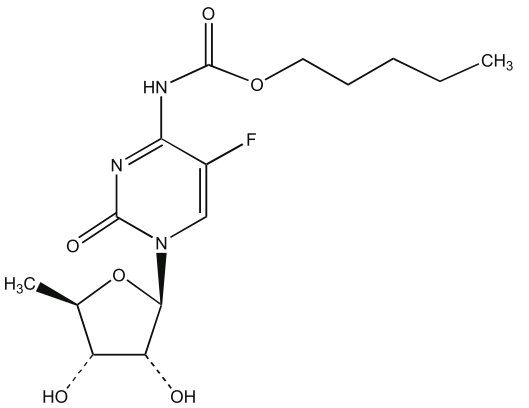
12

Structure 12.

Simple carbamic acids of amines (**12**, R = H) are too unstable for use as prodrugs as they quickly break down to generate carbon dioxide and the parent amine. Esters of carbamic acids are therefore employed in prodrug strategies. In such cases, the rate-determining step in bioreversion is the removal of the alcohol (R in **12**). The hydrolysis of the carbamic acid esters *in vivo* is generally thought to be catalyzed by esterases as there does not appear to be a carbamate-specific enzyme in mammals. Cholinesterases have been shown to specifically hydrolyze some carbamates; however, their rates for doing so are quite slow and could not account for the relatively fast *in vivo* hydrolysis (Wilson *et al.*, 1961).

When R in **12** is a simple hydrocarbon, the resultant prodrugs are quite stable and typically are not very effective (Kupchan and Isenberg, 1967). A notable exception to this generalization is capecitabine (**13**), the commercially available prodrug of 5-fluorouracil (5-FU), an anticancer agent (Tsukamoto *et al.*, 2001).

This prodrug was designed to improve the oral bioavailability and specificity of 5-FU to tumor cells. The ester in **13** is readily removed by liver carboxylesterase following oral absorption and the remaining transformations to 5-FU are catalyzed by cytidine deaminase and thymidine phosphorylase. The latter enzyme is highly enriched in tumors, thus providing selective release of 5-FU in cancer cells. This prodrug is currently approved as a first line of therapy for colorectal and breast cancers, and is also approved for use in combination with other anticancer drugs (Walko and Lindley, 2005).



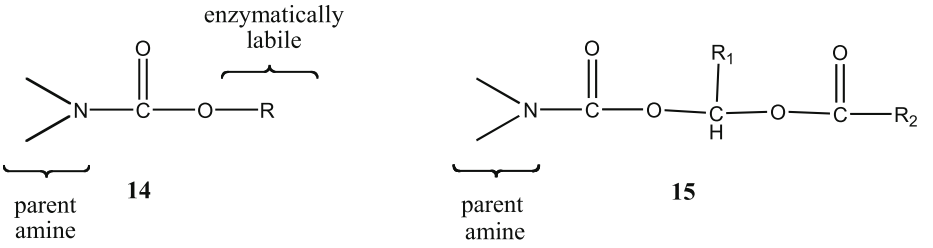
13

Structure 13.

Double Prodrugs

Considering the inherent stability of most simple carbamic acid alkyl esters, a variety of double prodrug strategies have been designed that take on the general structure **14**. R groups are designed to be enzymatically labile and will, in turn, release the carbamic acid that readily decomposes to give the parent amine.

Theoretically, such prodrugs could then have good chemical stability and *in vivo* lability. Accordingly, (acyl-oxy)alkylcarbamates (**15**) have been explored as bioreversible prodrugs of amines. Some of the pioneering work in this area was

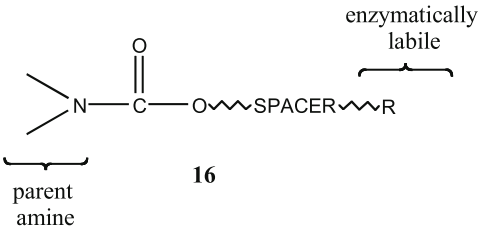


Structures 14 and 15.

done by Alexander and coworkers (Alexander *et al.*, 1988; Gogate *et al.*, 1987, Gogate and Repta, 1987). The results from these studies indicated that prodrugs had good chemical stability (shelf life of greater than 3 years at physiological pH) and are substrates for plasma esterases. It is important to note that this strategy may be limited to secondary amines since primary amines were shown to be subject to an intramolecular O to N-acyl migration in solution.

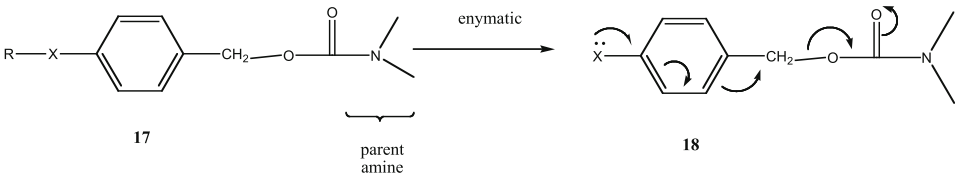
Higher Order Prodrugs

A problem inherent in simple double prodrugs obtained by directly attaching an enzymatically labile group to the carbamic acid group in **14** is that the ability of the enzyme to interact with its substrate may be compromised if the parent amine is bulky. In such circumstances the addition of a spacer to physically separate the enzymatically labile group from the drug was expected to normalize hydrolysis rates. The prototypic structure of this strategy is illustrated in **16**.



Structure 16.

An example of this prodrug approach utilizing an electronic cascade type spacer was originally proposed by Carl *et al.* (1981). The elimination mechanism is illustrated in Scheme 3.



Scheme 3.

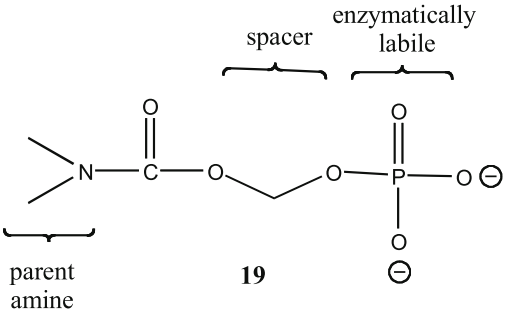
Model prodrugs (**17**, X = O, R = N-Boc-Lys) were designed to be substrates for proteolytic cleavage by trypsin to generate **18**. The available lone pair electrons associated with X on **18** can resonate to stabilize the development of the positive charge on the benzylic carbon and, therefore, favor the solvolytic cleavage of the benzyl-carbamate bond. The authors stated that the potential drawback to this strategy lies in the generation of the iminoquinone methide byproduct, which may present toxicity issues.

A number of prodrug strategies have been developed with variations in the triggering event that catalyzes the release of the parent amine. Senter *et al.* (1990) described a related prodrug strategy to target mitomycin C to tumor cells that have higher reductive capacity. In their approach, X in **17** represents sulfur, which exists in a disulfide linkage. After reduction of the disulfide, the elimination cascade is initiated and free amine is released as previously described. Interestingly, the prodrugs were approximately 50-fold more potent than mitomycin itself, indicating that either the prodrug showed enhanced permeation into cancer cells or, possibly, the formation of the thioquinone methide may have contributed to the toxicity. Mauger *et al.* (1994) synthesized prodrugs of anticancer agents in which X was NO_2 in **17** . These prodrugs were used in antibody-directed enzyme prodrug therapy (ADPET) with a nitroreductase enzyme. A variety of similar strategies using this self-immolating spacer strategy have been described and are covered in more detail elsewhere in this book. de Groot *et al.* (1999) utilized this technique to target cancer cells by designing prodrugs where the R in **17** represents the peptide substrates of plasmin, an enzyme shown to have higher activity at the surface of cancer cells.

Linking polyethylene glycol to this promoiety has also been investigated in an attempt to take advantage of the enhanced permeation and retention phenomenon that has been shown to promote tumor specificity to macromolecules (Greenwald *et al.*, 1999).

The use of multiple electronic cascade spacer groups in prodrugs has also been investigated (de Groot *et al.*, 2001). This approach was shown to increase the rate of enzyme hydrolysis by up to 10 times for bulky amine-containing drugs such as paclitaxel. As expected, the benefit of multiple spacers was less pronounced for the smaller and less bulky anthracycline class of anticancer agents.

Safadi *et al.* (1993) evaluated the utility of oxymethyloxycarbonyl spacer group to link aromatic and aliphatic amines to a phosphate moiety (**19**).



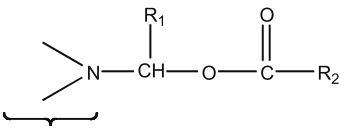
19

Structure 19.

These prodrugs were engineered to improve the aqueous solubility of amines; they have also been applied to hindered alcohols. The bioconversion mechanism involves an initial rate-determining dephosphorylation step followed by fast release of the spacer group (formaldehyde and carbon dioxide) to generate the parent amine. The prodrugs were rapidly hydrolyzed in the presence of alkaline phosphatase to the corresponding amine. The prodrugs were also susceptible to chemical hydrolysis that was attributed to the intramolecular hydrolytic catalysis by the neighboring phosphate group on the carbamate carbonyl functionality. Prodrugs derived from an aliphatic amine were chemically more stable than those derived from an aromatic amine.

N-acyloxyalkyl Derivatives

The general structure of N-acyloxyalkyl derivatives is illustrated in **20**. Most often, this type of promoiety has found its application in improving the delivery of drugs containing acidic NH groups (*i.e.*, imides, sulfonamides, amides, carbamates, ureas, etc.), which are discussed in detail in the chapter in this book by Guarino and Stella. One reason for this is that more basic amine groups derivatized with this functionality tend to be chemically quite unstable (Sloan and Koch, 1983). It is thought that the stability of these analogs is inversely proportional to the ability of the lone pair of electrons on the amine to stabilize the positive charge that builts up on the alpha carbon during solvolysis. As will be discussed in the next section, derivatization of tertiary amines with this group is possible.



20

Structure 20.

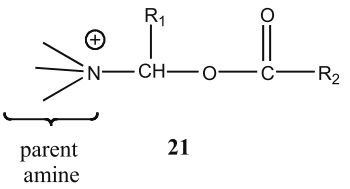
Quaternary Ammonium Derivatives

The majority of prodrug strategies discussed in this chapter will inevitably cause a reduction in the pKa value of the parent amine. This can be expected to result in reduced water solubility and enhanced membrane permeability. Derivatization of tertiary amines is different in that it results in the creation of a quaternary ammonium compound that is ionized regardless of solution pH and therefore would be expected to have improved water solubility. As a result, the majority of the strategies discussed in this section are intended to improve aqueous solubility of parent tertiary amines.

Most successful prodrug strategies described here can be classified as double prodrugs that require enzymatic hydrolysis to trigger initiation of a fast chemical

degradation of a spacer group to produce the parent tertiary amine. This is because simple alkylations of tertiary amines, albeit effective in improving water solubility (Nielsen *et al.*, 2005), typically result in quaternary ammonium salts that are very stable *in vivo* and fail to hydrolyze.

As previously mentioned, N-acyloxyalkyl-type prodrug derivatives of most primary and secondary amines (**20**) are very unstable. However, when applied to tertiary amines, the resultant quaternary analogs (**21**) are typically stable and can be enzymatically hydrolyzed *in vivo*.



21

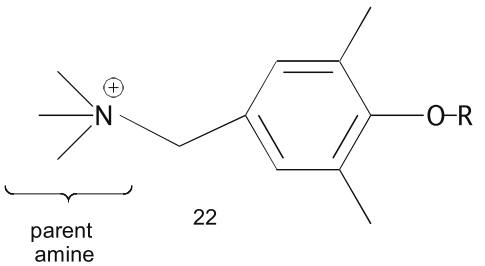
Structure 21.

Vinogradova *et al.* (1980) investigated a variety of N-(alkyloxymethyl) and N-(acyloxymethyl) derivatives on a number of different tertiary amine-containing drugs. Their work illustrated that both prodrug types were chemically stable; however, only N-(acyloxymethyl) prodrugs were able to release parent tertiary amine after intravenous administration in rats.

A more recent application of the N-(acyloxyalkyl) prodrug strategy was employed to a platelet-activating factor antagonist in order to improve its water solubility (Davidsen *et al.*, 1994; Albert *et al.*, 1996). The prodrugs displayed significantly enhanced water solubility, and their buffer and plasma stability could be adjusted through variations in the promoiety (R_1 and R_2 in **21**). The acetoxymethyl promoiety was subsequently selected for Phase I clinical evaluations in man, where rapid release of the parent amine was observed (Albert *et al.*, 1997).

Ichikawa *et al.* (2001) also applied this derivatization strategy to the triazole ring of a water-insoluble antifungal agent. Nine different prodrugs with variations in the acyl portion (R_2) of **21** were synthesized. The promoiety with R_1 = H and R_2 = CH_3 was ultimately selected for *in vivo* evaluations in rats, and the prodrug half-life was shown to be approximately 6 min after intravenous administration.

Bogardus and Higuchi (1982) studied the kinetics and mechanism of hydrolysis of a labile quaternary ammonium prodrug that has a general structure depicted in **22**. When R is a methyl or acyl group, the prodrugs were chemically very stable. When R is H, the prodrug is relatively unstable and undergoes



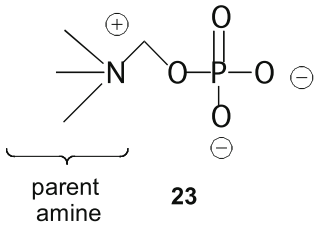
22

Structure 22.

unimolecular dissociation to generate quinone methide and the parent tertiary amine. The authors speculated that when R is an acyl group the breakdown *in vivo* would be controlled by esterase activity.

The previously described quaternary derivatives have been shown to behave as prodrugs and to lead to improved solubility; however, they may experience somewhat limited interest because of the association between quaternary compounds and *in vivo* toxicity (Cooper, 1988; Dimmock *et al.*, 1994; Sanders *et al.*,1995).

To overcome the perceived *in vivo* toxicity of quaternary ammonium prodrugs, a novel prodrug approach using N-phosphonooxymethyl promoeities (**23**) was applied to tertiary amines with poor aqueous solubility. Three model drugs—loxapine, cinnarizine, and amiodarone—were selected as model compounds to examine the utility of the N-phosphonooxymethyl prodrug concept. This prodrug strategy incorporated a negatively charged phosphate group in the promoiety as a means of overcoming the potential toxcicity limitation associated with quaternary ammonium prodrugs (Krise *et al.*, 1999a). The net charge of the molecule can vary from anionic (at physiological pH) to zwitterionic (at pH values near 3), thus masking the quaternary center. The rate-determining step in bioconversion involves enzyme-catalyzed dephosphorylation followed by a rapid breakdown of the hydroxymethyl intermediate to yield one mole of formaldehyde and the parent tertiary amine.



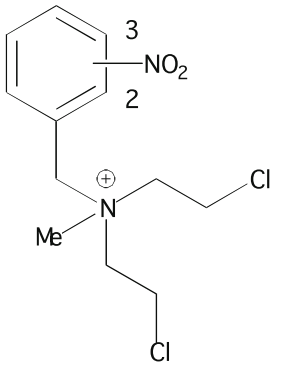
23

Structure 23.

The prodrugs were shown to be chemically stable and water soluble and to rapidly convert to the parent tertirary amine with no apparent toxicity after intravenous administration in dogs and rats (Krise *et al.*, 1999a,b).

Tercel *et al.* (1993) have proposed a prodrug strategy for tertiary amines with the intent of improving selectivity of cancer drugs using a bioreductive prodrug strategy. The nitrogen mustard anti-cancer agent mechlorethamine was quaternized using a nitro-reductive electronic cascade elimination strategy (**24**).

The nitrobenzyl promoiety was designed to undergo selective bioconversion in hypoxic cancer cells. One-electron reduction of the nitroaromatic portion causes the release of the reactive aliphatic mustard. The quaternary nature of the prodrug is considered attractive because it essentially deactivates the cytotoxicity of the mustard as well as increasing its water solubility. More recently, this group has evaluated additional prodrugs of mechlorethamine with modifications to the nitroheterocyclic ring (*i.e.*, nitroimidazole and nitropyrrole) to optimize reduction pathways in order to activate prodrug release via radiotherapy (Tercel *et al.*, 2001).



24

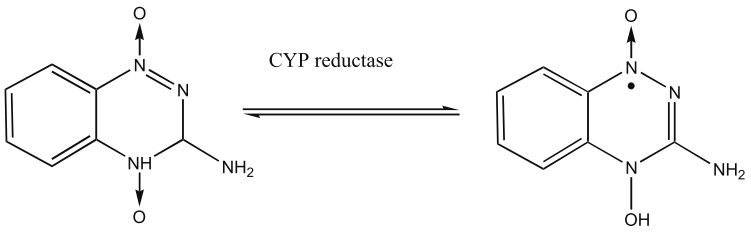
Structure 24.

Soft Quaternary Drugs

Bodor (1984) has coined a term "soft drug" to signify a transiently modified molecule that is essentially designed to behave as an active drug, but contains a readily biodegradable chemical appendage that, when cleaved through metabolism, renders the molecule inactive, facilitating a predictable clearance and, thus, improved safety. An important distinction between prodrugs and soft drugs is that the latter are designed to be active themselves whereas, by definition, prodrugs should have no activity. Soft quaternary drugs do, however, behave like prodrugs in that they are both designed to undergo a conversion event after systemic exposure. Examples of soft drug approaches relevant to prodrugs of tertiary amines can be found in applications with antimicrobials, anticholinergics and antitumor agents (Bodor and Kaminski, 1980; Bodor *et al.*, 1980a,b). Generally, the described strategies have employed N-(acyloxyalkyl) groups as the biodegradable moiety (see **21**).

N-Oxides of Amines

N-oxides represent a class of prodrugs that capitalize on the hypoxia associated with many cancer cells. Numerous N-oxide and related bioreductive prodrug strategies have been described (for a review on this topic see Denny (2004)). Two clinically promising prodrugs in this category will be highlighted



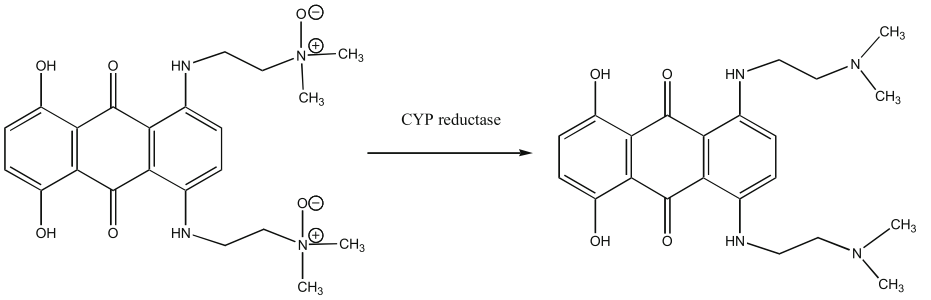
25 **26**

Structures 25 and 26.

here. Tirapazamine (**25**) is currently in clinical trials and, if successful, may be the first tumor-activated prodrug to reach the market.

This prodrug undergoes one-electron reduction (Scheme 4), which is thought to be carried out by NADPH cytochrome P450 reductase (Riley and Workman, 1992), to produce the cytotoxic nitroxide (**26**). This agent is believed to generate high concentrations of radicals in the vicinity of DNA, which results in double strand breaks (Brown, 1999).

The anticancer agent AQ4N (**27**) is an example of an aliphatic N-oxide that is reduced selectively in tumor cells to produce the parent tertiary amine (**28**, see Scheme 5) (Raleigh *et al.*, 1998). The tertiary amine is a strong binder of DNA and inhibitor of DNA topoisomerase II (Smith *et al.*, 1997).



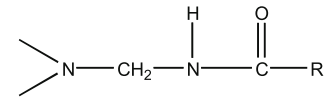
27 **28**

Scheme 5.

N-Mannich Bases

The N-Mannich reaction can be considered a prodrug strategy for derivatizing amines or amides (**29**). This section will focus on the use of the strategy for derivatizing amines since the application to amides is highlighted elsewhere in this book.

The principal reason for making N-Mannich bases of amines has been to improve membrane permeation. N-Mannich bases typically have better membrane permeation relative to parent amines because of the lowering of the amine pKa values that occur through this derivatization reaction. The pKa values of amines have repeatedly been shown to decrease by approximately 3 units after derivatization (Johansen and Bundgaard, 1980). Prodrugs, therefore, will be less ionized at physiological pH values than will the parent amines. Log D octanol-buffer partition coefficients (at pH 7.4) have been shown to increase over 100-fold



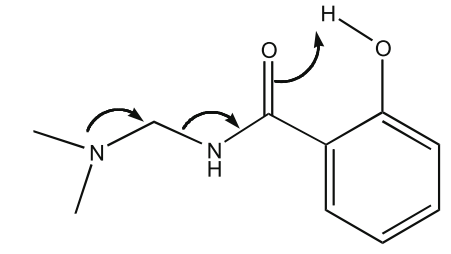
parent amine **29**

Structure 29.

for weak bases with pKa values above neutrality. The majority of this increase can be attributed to the change in ionization state at physiological pH rather than to the lipohilic contribution of the amide promoiety. In addition to increasing lipohilicity, N-Mannich bases have also been used to limit pre-systemic metabolic inactivation by N-acetylation (Bundgaard and Johansen, 1981).

Variations in the amide functionality (R in **29**) used in the N-Mannich reaction can significantly influence the bioreversion rates of resultant prodrugs. Bundgaard (1985) investigated a series of 24 N-Mannich prodrugs at pH 7.4 at 37°C and found their half-lives to vary between 0.06 and 1,400 min. Generalized characteristics that facilitate hydrolysis include availability of the lone pair of electrons associated with the amine and the propensity of the carbonyl to accept a proton. Interestingly, it was also found that having a proton donor group (*i.e.*, salicylamide **30**) in close proximity to the amide carbonyl dramatically increases the hydrolysis rate. As will be discussed, a number of N-Mannich prodrugs utilize this group in the promoiety because of the fast hydrolysis.

Cogan and coworkers (2004) have made several N-Mannich base derivatives of anthracycline drugs. Several prodrugs with variations in the amide region (R in **29**) were synthesized and evaluated. After initial characterizations, the authors focused their efforts on the salicylamide derivative (**30**) because of its rapid bioconversion properties. Interestingly, the authors reported that the reason for making these prodrugs was to take advantage of release of formaldehyde in the vicinity of DNA in cancer cells, which has been shown to promote covalent coupling of the drug to DNA (Taatjes *et al.*, 1996). The authors also investigated the effect of esterification of the phenol group of the salicylamide N-Mannich prodrugs in a double prodrug strategy aimed at improving the chemical stability of the resultant prodrug for purification and storage purposes. The hydrolysis of the prodrugs by esterases was shown to occur readily after *in vivo* administration. The same group has also recently investigated phenolic esters of **30** that are recognized by the androgen receptor in an effort to promote the specific localization of the prodrugs to cancer cells (Cogan and Koch, 2003).

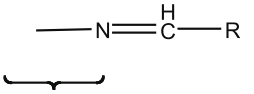


30

Structure 30.

Schiff bases

Amine prodrugs that are considered to be Schiff bases take on the general structure shown in **31**.



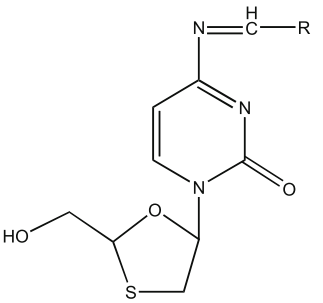
31

Structure 31.

In many regards, Schiff base prodrugs are similar to N-Mannich prodrugs because both derivatives result in substantial reductions in the pKa of the parent amine and are useful for improving membrane permeability. Some of the early applications of this approach were undertaken to improve the blood-brain barrier permeability of γ-aminobutric acid (Worms *et al.*, 1982). This approach was also applied to the histamine derivative (R)-α-methylhistamine, a potent H3-receptor agonist, which suffers from poor bioavailability and CNS delivery. Krause *et al.* (1996) have investigated a variety of azomethine prodrugs of this drug. Of the Schiff bases synthesized, the alkyl imines were found to be too unstable for isolation whereas diaryl and benzaryl imines were sufficiently stable and permitted increased bioavailability and CNS delivery *in vivo* in mice.

More recent examples include prodrugs of antiviral nucleoside type drugs. Many prodrugs originating from the free exocyclic amine of various pyrimidine and purine nucleic bases have been prepared and evaluated in an attempt to increase lipophilicity (see Anastasi *et al.*, 2003, for a review).

Formamidine-type prodrugs represent a type of Schiff base prodrug that has received considerable attention. A representative structure of these prodrugs is shown in **32**, where the parent drug is 3TC.



32

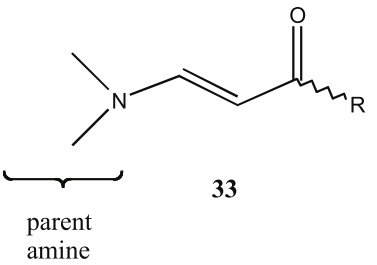
Structure 32.

Prodrugs with a lipophilic R group in **32** were shown to increase membrane permeability and water solubility. The improvements in solubility are thought to

occur through the loss of intermolecular hydrogen-bonding potential associated with the parent amine. It was shown that electron-donating R groups tended in have increased hydrolysis rates as opposed to electron-withdrawing groups, which stabilize the Schiff base (Anastasi *et al.*, 2004).

Enaminones

The general structure of enaminone type prodrugs is shown in **33**. Related enamine prodrugs have not found much application primarily because of their high degree of chemical instability in aqueous solutions. Enaminones, however, have been shown to have improved chemical stability due to the intramolecular hydrogen bond between the parent amine and the γ carbonyl. This can also be due to the extended conjugation of the vinylic double bond with the carbonyl group.



33

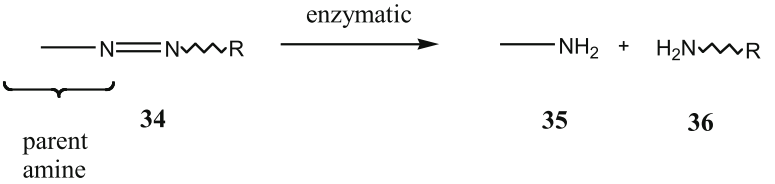
Structure 33.

This class of prodrugs has been used to reduce the nucleophilicity of parent amines. For example, Jensen *et al.* (1980) have synthesized an acetylacetone prodrug of cycloserine in order to prevent a nucleophilic dimerization reaction that was shown to occur in both the solid and concentrated aqueous solutions.

Murakami *et al.* (1981) evaluated enaminone prodrugs of a variety of β-lactam antibiotics in an effort to enhance their membrane permeability. The authors concluded that these prodrugs were more lipophilic than the parent compounds, but the prodrugs improved the bioavailability of the drugs only after rectal administration. Oral administration did not lead to increased bioavailability, presumably due to stability problems with these prodrugs in the acidic gastric environment. This finding is consistent with the work of Naringrekar and Stella (1990), who studied the mechanism of hydrolysis of enaminones as prodrugs for amines. They came to the conclusion that the concept of emaminone prodrugs intended to improve oral delivery may be unrealistic. If such prodrugs are designed to have adequate chemical stability at acidic pH values (similar to what is encountered in the GI tract), then hydrolysis at pH 7.4 would be too slow. Similarly, if these prodrugs were designed to have fast hydrolysis at pH 7.4, they would be far too unstable in acidic conditions and thus fail to be attractive prodrugs for amines.

Azo compounds

The formation of azo compounds (**34**) has been a widely utilized prodrug strategy for targeting drugs to the colon. The enzyme responsible for reducing the azo bond and releasing the parent amine (**35**) and the amine promoiety (**36**) is azoreductase. A variety of prodrugs have been developed that foster the delivery of 5-amino salicylic acid (5-ASA) to the colon. Examples include sulfasalazine, ipsalazine, balsalazine, and olsalazine (see Sinha and Kumria, 2001; Chourasia and Jain, 2003). The prodrugs are quite chemically stable and are poorly absorbed from the small intestine. When these prodrugs reach the colon, they are cleaved by azoreductase, an enzyme specifically produced by the colon microflora.

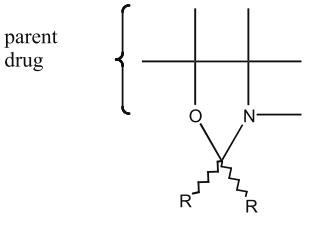


Scheme 6.

More recently polymers and dendrimers have been used as prodrug-type carriers that release 5-ASA in the colon (Wiwattanapatapee *et al.*, 2003). One advantage of the polymer linkage is that the large size of the prodrugs would preclude any absorption in the small intestine. Some polymers were designed with mucoadhesive properties (Kopecek *et al.*, 1992). Such bioadhesive polymers have also been coupled to 9-aminocamptothecin through an azo linkage (Sakuma *et al.*, 2001).

Oxazolidines

The general structure of oxazolidine prodrugs is depicted in **37**. The application of this prodrug approach is limited to primary or secondary amines with a β-alcohol group present. This strategy has received considerable attention since several marketed drugs, including selected sympathomimetics and β-blockers, contain this arrangement of functional groups.



37

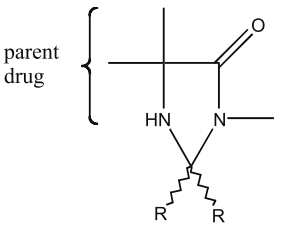
Structure 37.

The creation of prodrugs involves a cyclic condensation of the β-aminoalochol with an aldehyde or ketone. The resultant prodrugs have lower pKa associated with the amine functionality and, therefore, are considered useful in increasing the membrane permeation of drugs. The hydrolysis of a number of oxazolidine prodrugs derived from (–)-ephedrine and (+)-pseudoephedrine have been studied (Bundgaard and Johansen, 1982; Johansen and Bundgaard, 1983). Resultant prodrugs have been shown to be more lipophilic than parent drugs and to experience fast chemical hydrolysis to release the parent drug and aldehyde or ketone at physiological pH. The hydrolysis rates have been shown to decrease with increasing basicity of the oxazolidine moiety. Increasing the steric bulkiness of substituents of the aldehyde linker was also associated with decreased hydrolysis rates. Generally, prodrugs were shown to have a 3-4 h half-life at pH 1, which decreased to approximately 5 min at pH 7.4. The fast hydrolysis at neutral pH may limit the usefulness of this strategy in many applications.

More recently, Fenick and coworkers (1997) evaluated prodrugs of daunorubicin and doxorubicin where the aminoalcohol was condensed with formaldehyde. Like the previously described N-Mannich prodrugs of anthracyclines, these prodrugs were made in an attempt to take advantage of formaldehyde release, which can promote covalent attachment of these drugs to DNA. The prodrugs showed promising behavior in cultured cancer cells; however, the hydrolysis rate was extremely rapid and thus compromised the ability to formulate and deliver these prodrugs.

4- Imidazolidinones

A concept similar to the oxazolidine strategy is also applicable to drugs containing the α aminoamide moiety. The general structure of 4-imidazolidinone-type prodrugs is shown in **38**. These prodrugs can also be regarded as cyclic N-Mannich base derivatives.



38

Structure 38.

Most of the work in this area has been concentrated on improving the delivery of peptide type drugs in which this functional group arrangement is almost always present. However, this technique has also been successfully employed to make the prodrug hetacillin through the condensation of ampicillin with acetone (Tsuji and

Yamana, 1974). The prodrug was useful in stopping the concentration dependent intermolecular aminolysis problem associated with this ampicillin.

A more recent application of this technique was demonstrated by condensing prilocaine with either formaldehyde or acetaldehyde (Larsen *et al.*, 2003). These prodrugs were synthesized to make a lipophilic prodrug that could provide sustained delivery from an oil vehicle following subcutaneous administrations.

Conclusion

In summary, this review, although not comprehensive, has attempted to highlight and assess examples of prodrug approaches applied to various aliphatic and aromatic amines. Over the past 25 years, nearly 1000 papers that pertain to prodrugs of amines have been published. Obviously, this review has referenced only a fraction of this body of work. It is somewhat disappointing to realize how few commercially available prodrugs have arisen from these efforts. However, it is important to give credit to those systematic evaluations of prodrug strategies that were done to understand, on a mechanistic level, why prodrugs did or did not work. It is only with this foundation that successful strategies have evolved. Likewise, development of prodrugs of the future will certainly rely on the lessons learned from the work done to date, even though the intended applications may be considerably different. We hope that this chapter will further encourage and guide research into the use of prodrugs for improved delivery, targeting, and pharmacokinetic profiles of amine-containing drugs.

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3.4

Prodrugs of Amides, Imides and Other NH-acidic Compounds

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Table of Contents

List of Abbreviations

ED_{50} .....Median effective dose
GIT .....Gastrointestinal tract
IM .....Intramuscular
IV .....Intravenous
MDCK-wt .....Madin-Darby canine kidney-wild type
SIBLM.....Simulated Intestinal Bile Salt-Lecithin Mixture
$t_{1/2}$ .....Half-life
t_{90} .....Time for 10% degradation
TD_{50} .....Median toxic dose

Keywords

Prodrugs, Acyloxyalkyl, Phosphoryloxyalkyl, Hydroxyalkyl, N-Mannich Base, Sulfenamide, Amide, Imide, Sulfonamide, NH-acid, N,O-Acyl Transfer, 1,4-Imidazolidinone, 5-Oxazolidinone, Redox Prodrugs, N-Dealkylation

Introduction

The term 'NH-acidic compound,' in the context of this chapter, refers to amides, carbamates, ureas, imides, and sulfonamides (Figure 1). However, the above term will not include amines, as they are discussed in a previous chapter. This chapter reviews various prodrug strategies for modifying the physical, chemical, and biochemical properties of NH-acidic compounds. Synthetic methodologies are not covered, as this information is readily available from the primary references cited.

Amide	R_1 = C(O)R	R_2 = R
Carbamate	R_1 = C(O)OR	R_2 = R
Urea	R_1 = C(O)NRR'	R_2 = R
Imide	R_1 = C(O)R	R_2 = C(O)R
Sulfonamide	R_1 = $S(O)_2R$	R_2 = R

R = alkyl, aryl or hydrogen

Figure 1. Various types of NH-acids.

This chapter categorizes the prodrug literature by design approach and attempts to summarize the general physicochemical properties of each prodrug design type. Most of the included design approaches have been previously reviewed (Pitman, 1981; Bundgaard, 1982, 1983, 1985a,b, 1989, 1992a,b; Ferres, 1983; Sloan, 1989; Oliyai, 1996). Assuming that most readers will already be somewhat familiar with the more established design approaches for NH-acids, the first design section of this chapter (Sulfenamide Prodrugs) summarizes a recent discovery from our own work, which demonstrates the use of sulfenamides as prodrugs for NH-acids (Guarino *et al.*, 2003; Guarino, 2004). The more established prodrug technologies are then described in the subsequent sections of this chapter.

General Definitions

As stated above, the term 'NH-acidic compound' will include amides, carbamates, ureas, imides, and sulfonamides as well as other N-H bond-containing compounds having a pKa value in the range of the former listed compounds. To help define things further, the phrase 'imide-like' will describe an NH-acid where the derivatizable nitrogen is between two adjacent carbonyl functionalities, while the phrase 'amide-like' will describe an NH-acid where the derivatizable nitrogen is adjacent to just one carbonyl functionality. Some NH-acids, such as the hydantoins, can fit into either the 'amide-like' or 'imide-like' categories, depending on which nitrogen is the derivatization target (Figure 2); on the other hand, compounds such as sulfonamides do not fit into either classification and therefore are given their own category. Finally, for convenience, a simple amide structure will generically represent NH-acids in figures that show

general prodrug structures or in schemes showing basic bioreconversion processes; however, the reader should recognize the equal validity of inserting an 'imide-like' compound, or other NH-acid, in its place.

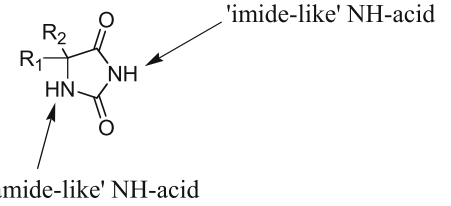
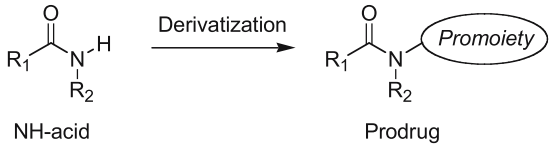


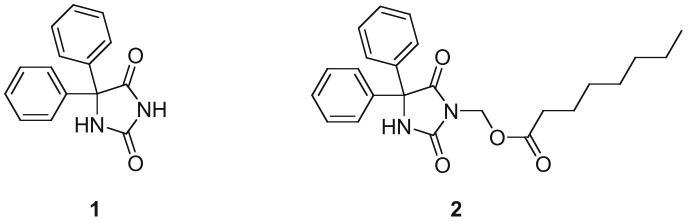
Figure 2. The 'imide-like' and 'amide-like' NH-acids of a generic hydantoin.

General Advantages of Derivatizing NH-Acidic Compounds

The removable proton of an NH-acidic functionality allows the attachment of a promoiety, thereby creating a prodrug (Scheme 1). By removing this proton, one hydrogen bond donor group is eliminated, which can have a dramatic effect on properties such as crystal lattice energy, solubility, dissolution rate, and permeability (Lipinski *et al.*, 1997, 2001). For high melting, poorly soluble compounds, the removal of the potential for intermolecular hydrogen bonding in the solid state (crystal lattice weakening) can often increase the aqueous and lipid solubilities/dissolution rates simultaneously. Phenytoin (**1**), for example, is a poorly soluble NH-acid whose inferior pharmaceutical properties arise mainly from its very stable crystal lattice structure. Stella *et al.* (1998; 1999) showed that a phenytoin prodrug (**2**), synthesized by the replacement of the imide-type NH proton of **1** with a octanoyloxymethyl promoiety, possessed enhancements in aqueous dissolution rate in SIBLM (simulated contents of the GIT), lipid solubility



Scheme 1. The derivatization of a generic NH-acid to form a prodrug.

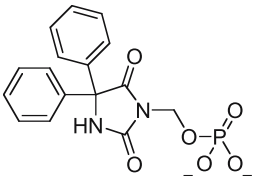


1 **2**

Structures 1-2.

(solubility in cyclohexane), and oral bioavailability in adult male beagle dogs, especially in the fed state. These types of potential drug delivery enhancements are further discussed in the section N-Acyloxyalkyl Prodrugs.

In addition to the potential advantages from the removed hydrogen bond donor, the characteristics of a carefully chosen promoiety can further enhance the delivery properties of the parent compound. For instance, the addition of an ionizable group, such as a phosphate moiety, can dramatically increase the aqueous solubility of a drug. One example of this approach is fosphenytoin (**3**), a water-soluble phosphoryloxymethyl prodrug of phenytoin (discussed further in N-Acyloxyalkyl Prodrugs).



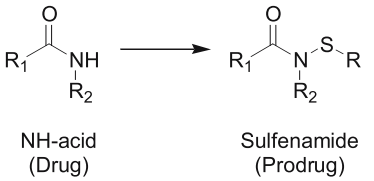
3

Structure 3.

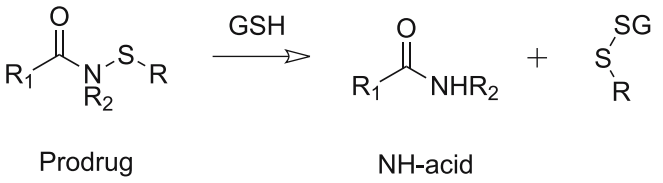
Sulfenamide Prodrugs (Recent Discovery)

General information on sulfenamides and their historical uses exists in a number of published reviews (Kharasch *et al.*, 1946; Davis, 1973; Craine and Raban, 1989; Koval, 1990, 1996). A sulfenamide is defined by a single covalent bond between a nitrogen atom and a bivalent sulfur atom. As shown in Scheme 2, the promoiety (S-R) is attached to the NH-acid via a sulfenamide bond to form the prodrug, and the bioreconversion (Scheme 3) is expected to occur from a nucleophilic substitution reaction by endogenous nucleophiles (glutathione, cysteine, etc).

The aqueous stability, with respect to pH, of sulfenamides derived from amide-like NH-acids was characterized by examining a benzamide-cysteine prodrug (**4**) (Figure 3) and a carbamazepine-cysteine ethyl ester prodrug (**5**) (Figure 4) (Guarino, 2004). The degradation of both **4** and **5** could be described by pseudo-first-order kinetics throughout the entire pH range studied (pH 4.0-7.0). Prodrugs **4** and **5** possessed good aqueous stability with maximum half-lives of 6.3 years (pH 6.0, 25°C) and 180 days (pH 4.0, 25°C), respectively; however, while **5** degraded predominantly by N-S bond cleavage at pH 4.0, the dominant

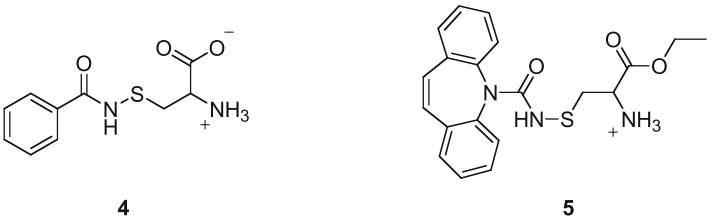


Scheme 2. The derivatization of a generic NH-acid to form a generic sulfenamide prodrug.



Scheme 3. The bioreconversion of a generic sulfenamide prodrug to form a generic NH-acid following nucleophilic attack by glutathione.

degradation pathway for this prodrug at the higher pH values consisted of base-catalyzed ethyl ester hydrolysis in the promoiety rather than N-S bond cleavage. Therefore, the pH-rate profile generated for **5**, which shows the effects of pH on the overall aqueous stability, does not represent the N-S bond stability of **5** at the higher pH values.



4 **5**

Structures 4-5.

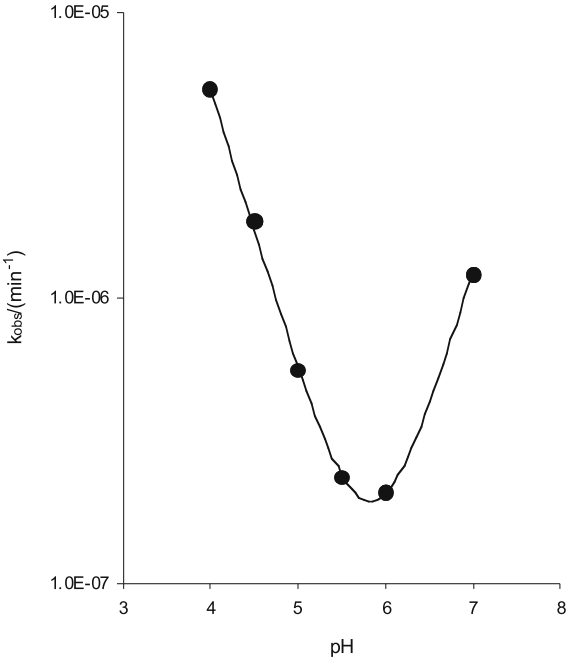


Figure 3. The 25°C projected pH-rate profile of **4** (from elevated temperature studies) at 35 mM buffer concentration and ionic strength of 0.15. The solid line represents a mathematical fit of the data. For details of the kinetic analysis, refer to (Guarino, 2004).

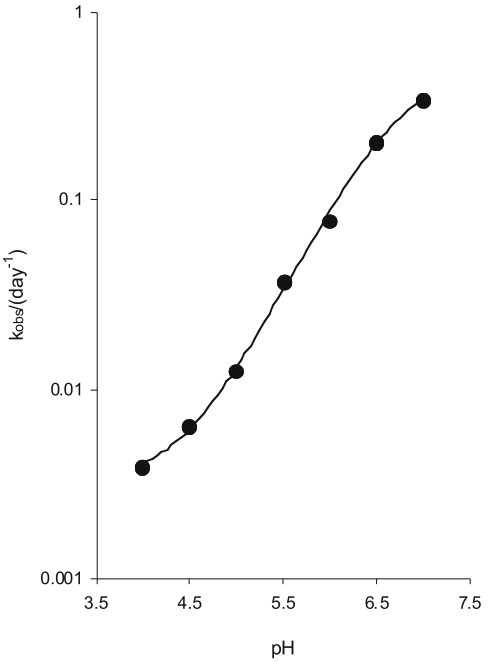
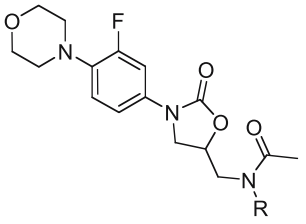


Figure 4. The 25°C projected pH-rate profile of **5** (from elevated temperature studies) at 35 mM buffer concentration and ionic strength of 0.15. The solid line represents a mathematical fit of the data. For details of the kinetic analysis, refer to (Guarino, 2004).

Overall, both **4** and **5** possessed greater aqueous solubility than did their parent compounds. However, due to its predominant zwitterionic nature throughout the pH range studied, **4** possessed a very slow dissolution rate (qualitative observation). Compound **5**, on the other hand, exhibited rapid dissolution, presumably due to the removal of the zwitterionic character from the cysteine promoiety through esterification of the carboxylic acid. Finally, **4** showed fast and quantitative reconversion to benzamide in buffered-solution (pH 6.0, 25°C) containing cysteine as a thiol-based nucleophile, which is supporting evidence that the desired bioreconversion for these sulfenamide prodrugs would occur *in vivo*.

For further proof of concept of the sulfenamide technology for amide-like NH-acids, Guarino and coworkers (Guarino *et al.*, 2003; Guarino, 2004) synthesized and studied a series of linezolid-sulfenamide derivatives (**6-10**)



Linezolid R = H
6 R = SC_6H_5
7 R = $SCH_2CH_2C(O)OCH_2CH_3$
8 R = $SCH_2CH_2C(O)OC(CH_3)_3$
9 R = $SCH_2CH_2NHC(O)OC(CH_3)_3$
10 R = $SCH_2CHC(O)OC(CH_3)_3$
$NHC(O)OC(CH_3)_3$

Structures 6-10.

(Zyvox® is the trade name for linezolid). The linezolid-thiopropionoic acid ethyl ester prodrug (**7**) showed particular promise in its relatively good synthetic access, its instantaneous, quantitative reconversion in dog whole blood, and its dramatic enhancement in the delivery of linezolid across an MDCK-wt cell monolayer in the apical-to-basolateral direction. Despite the obvious complexity shown in the biphasic appearance of linezolid from **7** (Figure 5), which will require further study to confidently elucidate, **7** clearly enhances the apical-to-basolateral delivery of linezolid across an MDCK-wt cell monolayer by approximately eightfold during the first 30 min.

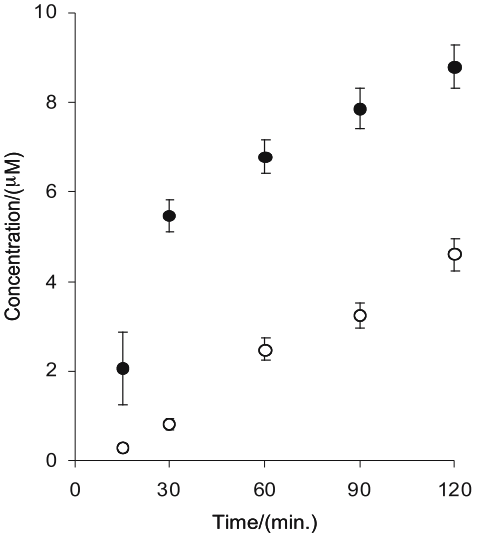
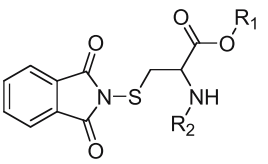


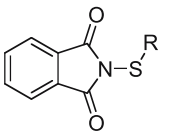
Figure 5. Appearance of linezolid in the receiver compartment (basolateral side) after placing a 40 μM concentration of either linezolid (○) or **7** (●) on the apical side of an MDCK-wt cell monolayer at time zero. For further details, refer to (Guarino, 2004).

Transitioning to the imide-based sulfenamides, eight sulfenylated phthalimides (**11-18**) were either synthesized or obtained commercially, and their aqueous stabilities were examined with respect to pH (Guarino, 2004). Unfortunately, the degradation of these phthalimde-based sulfenamides appeared to occur by a combination of N-S bond cleavage to release phthalimide and hydrolysis of the phthalimide ring of the sulfenamide to form the corresponding phthalamic acid-based sulfenamide. Through mathematical modeling of the convoluted kinetic profiles, the rate constant describing N-S bond cleavage was extracted and the maximum half-lives, corresponding to degradation of the N-S bond, for six of the eight phthalimide-based sulfenamides (**11-16**) were estimated to be in the range of 1 to 10 days (pH 4.0, 25°C), depending on the actual compound. These values are in stark contrast to the maximum half-lives found for the amide-based sulfenamides (**4** and **5**), suggesting that there may be greater promise in applying this technology toward amide-type NH-acids. However, it is important to note that two of the eight phthalimide-based sulfenamides (**17** and

18) showed no evidence of N-S bond cleavage within the time frame of the study (~1 week), suggesting that, with careful promoiety selection, it still may be possible to design stable sulfenamide prodrugs of imide-based NH-acids. For instance, it would be informative, before totally discounting this technology for imide-based NH-acids, to study the aqueous stability of a series of sulfenamides generated from a simple acyclic imide so that the N-S bond stability can be directly characterized in the absence of any complicating ring-opening side reactions. It would be especially interesting to check the aqueous stability of two sulfenamide derivatives synthesized from a simple acyclic imide using the same two promoieties found in **17** and **18** to see if there is any special stability offered by these promoieties. Regarding reconversion in the presence of thiols, **11** showed instantaneous quantitative reconversion in buffered-solution (pH 4.0, 25°C) containing cysteine, which provides support that the desired bioreconversion would also take place *in vivo* for sulfenamide prodrugs of imide-type NH-acids.



11 $R_1 = CH_2CH_3$, $R_2 = C(O)OCH_2C_6H_5$
12 $R_1 = H$, $R_2 = C(O)OCH_2C_6H_5$
13 $R_1 = CH_2CH_3$, $R_2 = H$
14 $R_1 = H$, $R_2 = H$



15 $R = C(S)N(CH_3)_2$
16 $R = CH_3$
17 $R = C_6H_5$
18 $R = C_6H_{11}$

Structures 11-18.

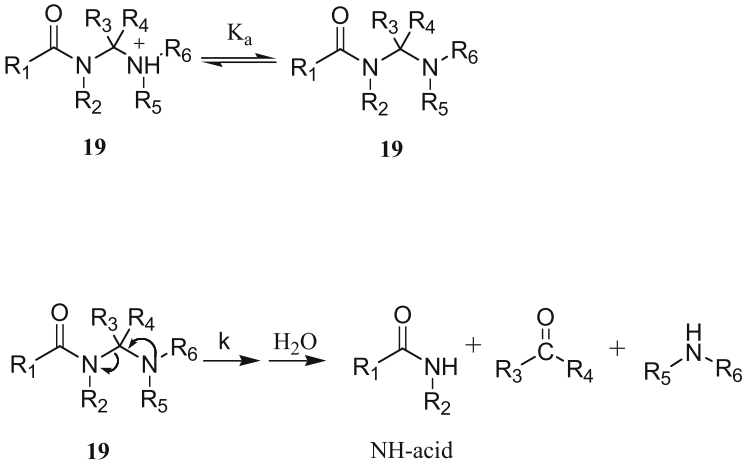
In conclusion, the work of Guarino and coworkers (Guarino *et al.*, 2003; Guarino, 2004) showed that sulfenylation of NH-acids produces sulfenamide derivatives that behave as prodrugs *in vitro*. Firstly, the prodrugs reconvert quickly and quantitatively to release the NH-acid in the presence of thiols. Secondly, sufficient aqueous stability exists, especially for the amide-based sulfenamides. Thirdly, the sulfenamide prodrugs enhanced various physicochemical properties related to drug delivery such as permeability, aqueous solubility, and dissolution rate; moreover, the sulfenamide prodrug technology allows great design freedom to achieve these physicochemical enhancements because the R-group of the promoiety (S-R) can possess either hydrophilic or lipophilic characteristics. The sulfenamide prodrug technology appears to be most promising for the weaker NH-acids, such as simple amides, carbamates, and ureas, since sulfenamide derivatives from these types of functionalities have shown good aqueous stability while maintaining fast bioreconversion rates.

To our knowledge, no one to date has utilized sulfenamide chemistry to design prodrugs for NH-acids (Guarino *et al.*, 2003). Due to the significant void in the prodrug literature of a general approach to making stable, but rapidly

bioreversible, derivatives of weaker NH-acids, sulfenamide prodrugs should favorably complement the existing NH-acid prodrug technologies, without redundancy.

N-Mannich Base Prodrugs

Although not the first known accounts, the bulk of the systematic research establishing N-Mannich bases as potential prodrugs of NH-acidic compounds occurred in the late 1970s to mid 1980s. Scheme 4 displays the structure and bioreconversion of a generic N-Mannich base prodrug (**19**). As shown in Scheme 4, the parent NH-acid, an aldehyde (formaldehyde is the most commonly used) and an amine are produced from the bioreconversion reaction, thereby making N-Mannich base derivatives an equally legitimate prodrug approach for both NH-acids and amines.



Scheme 4. The bioreconversion of a generic N-Mannich base prodrug.

Bundgaard and Johansen (1980a,c; Johansen and Bundgaard, 1981a) conducted the first thorough examination of N-Mannich bases as potential prodrugs of NH-acids. They studied the aqueous degradation kinetics of a series of N-Mannich base prodrugs derived from different types of NH-acids (imides, amides, thioamides, ureas, etc.). Summarizing their results, N-Mannich base prodrugs were found to give sigmoidal-shaped pH-rate profiles (Figure 6), have greater chemical stability in their protonated state, and degrade to quantitatively release the parent drug by pseudo-first-order kinetics that were insensitive to the presence of buffer species, ionic strength, or plasma enzymes. The absence of catalysis by buffers and plasma enzymes supports the proposed unimolecular degradation mechanism for N-Mannich bases occurring through a rate-determining N-C bond cleavage (Scheme 4). An alternative degradation mechanism involving formation of an N-hydroxymethyl intermediate was ruled out since the analogous N-hydroxymethyl derivatives possessed greater stability than the N-Mannich bases. Although the relationship of aqueous stability to pH

for N-Mannich bases typically follows a sigmoidal-shaped profile (Figure 6), deviations from the expected shape can result if there are additional ionizable groups or other reactive sites in the molecule (Vej-Hansen and Bundgaard, 1979; Johansen and Bundgaard, 1980a; Bundgaard and Johansen, 1981b).

From their studies, Bundgaard and Johansen (1980a; 1980c) developed the first correlations between structure and aqueous stability for N-Mannich bases, which others would later expand upon. Generally, the stability of the deprotonated form decreased with increased steric bulk in the amine substituents, increased amine basicity, and increased NH-acid acidity. Although steric factors in the amine substituents did not affect the stability of the protonated form, increased NH-acid acidity and decreased amine basicity did result in a very slight reduction in aqueous stability of the protonated species. Most N-Mannich base prodrugs from imide-type NH-acids (phenytoin, barbital, etc.) degrade too rapidly for quantitative kinetic analysis, due to the high acidity (relatively speaking) of the parent compounds. To achieve N-Mannich bases from 'imide-type' NH-acids with enough stability for kinetic characterization, poorly basic aromatic amines, such as p-aminobenzoic acid, must be used (Bundgaard and Johansen, 1981b). Also of note, upon N-amidomethylation to form an N-Mannich base, the pKa of the amine drops anywhere from 2 to 4 units, depending

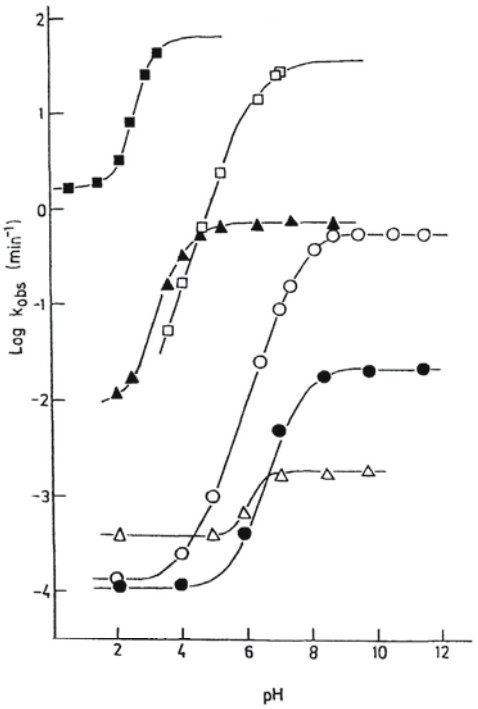
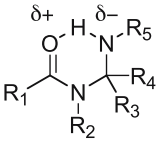


Figure 6. The pH-rate profiles for the aqueous degradation of various N-Mannich base prodrugs at 37°C. ■, N-(Morpholinomethyl)-*p*-toluenesulphonamide; □, N-(piperidinomethyl)trichloroacetamide; ▲, N-(morpholinomethyl)trichloroacetamide; ○, N-(diethylaminomethyl)benzamide; ●, N-(isobutylaminomethyl)benzamide; △, N-(benzylaminomethyl)benzamide. Reproduced from (Bundgaard and Johansen, 1980a).

on the acidity of the NH-acid. The prodrug designer must plan for this pKa drop since the N-Mannich base pKa (not the original amine pKa) determines the ionization that will directly influence the physicochemical properties of the prodrug.

In a follow-up study, Johansen and Bundgaard (1980b) investigated the ability of N-Mannich bases to enhance the solubility, dissolution rate, and partitioning behavior of NH-acids. N-Mannich bases synthesized from secondary amines showed good solubility enhancements; however, the N-Mannich bases from primary amines showed only small increases in solubility at best, and even, in some cases, decreased solubility relative to the parent NH-acid. The authors proposed that for N-Mannich base prodrugs from primary amines, intramolecular hydrogen bonding between the amine proton and the carbonyl oxygen (**20**) hindered solvation of the polar groups of the molecule. Intermolecular hydrogen bonding in the crystal lattice, on the other hand, was ruled out due to the similar melting points found for the two types of N-Mannich bases.

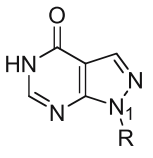


20

Structure 20.

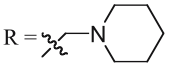
In another study, the dissolution rate for allopurinol was improved using N-Mannich base derivatization (Bundgaard and Johansen, 1981a). Due to the acidity of allopurinol (pKa 10.5), the aqueous stability of the N-Mannich base prodrugs from piperidine (**21**), morpholine (**22**), and dimethylamine (**23**) could not be quantified. However, despite its poor aqueous stability, **21** still displayed an increased dissolution rate in 0.1 M HCl over allopurinol by a factor of 45. In a later study, Bundgaard and Johansen (1980b) determined that the dissolution rates for N-Mannich base salts were even greater than their free base forms; moreover, the type of counterion used to make the salt (organic versus halide) affected the resulting solubility and dissolution rate properties, although not in a predictable manner (Johansen and Bundgaard, 1980b).

In the area of transdermal delivery, Sloan *et al.* (1984; 1988) showed the utility of a series of N-Mannich base prodrugs for enhancing permeation of 5-

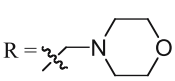


Allopurinol R = H

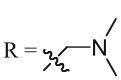
21 R =



22 R =



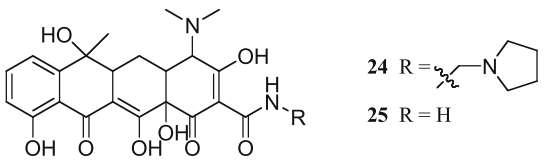
23 R =



Structures 21-23.

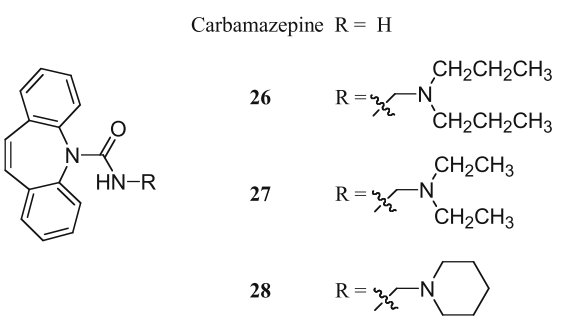
fluorouracil and theophylline (very acidic NH-acids) through hairless mouse skin. These N-Mannich base prodrugs showed improved permeation in this model and performed better than the N-acyloxyalkyl prodrugs also studied. Furthermore, it is important to note that the poor aqueous stability, arising from the high relative acidity of the parent NH-acids, is not necessarily a concern for dermal or transdermal delivery as long as the prodrugs are protected from water exposure in a non-aqueous vehicle.

Rolitetracycline (**24**), an example of a marketed N-Mannich base prodrug, is a water-soluble prodrug of tetracycline (**25**) developed for parenteral use. Bundgaard and associates (Vej-Hansen and Bundgaard, 1979; Johansen and Bundgaard, 1981a) found that the $t_{1/2}$ of **24** was 43 min at 35°C in pH 7.4 buffer, as well as in 25% plasma. As expected, the reconversion reaction produced tetracycline, formaldehyde, and pyrrolidine in the pH range of 1.5 to 9. Due to its limited aqueous stability, **24** is formulated as a dry mixture (drug and excipients) that is reconstituted with water before administration. The t_{90} of **24** following reconstitution with water was 1.6 h at 21°C and 11 h at 4°C.



Structures 24-25.

To improve the delivery properties of carbamazepine (amide-type weak NH-acid), Bundgaard *et al.* (1982) conducted a physicochemical and pharmacokinetic study of three prepared carbamazepine N-Mannich base prodrugs incorporating dipropylamine (**26**), diethylamine (**27**), or piperidine (**28**). Prodrug **26** (as the HCl salt) displayed enhanced solubility and dissolution properties (> 10,000-fold increase) relative to carbamazepine, and showed fast reconversion at pH 7.4 ($t_{1/2}$ = 7 min at 37°C). Following intramuscular injection, the carbamazepine blood levels obtained from **26** were superior to the blood levels from a molar equivalent carbamazepine suspension (Figure 7); however, unexpectedly, the reverse was found with oral dosing.



Structures 26-28.

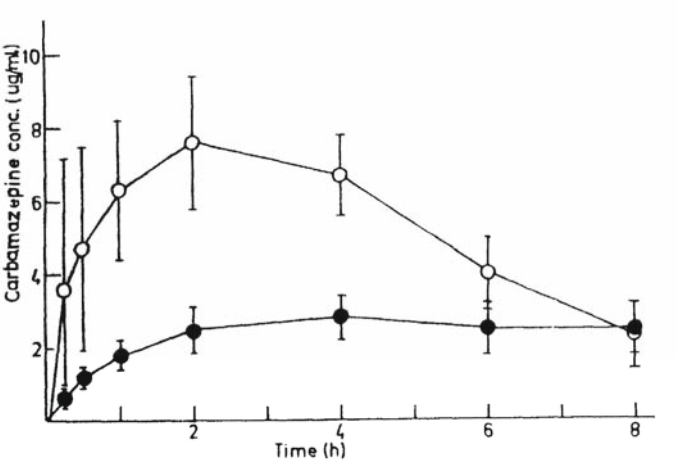
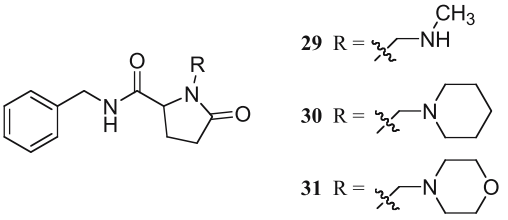


Figure 7. Mean carbamazepine plasma levels (±S.D.) versus time plots obtained after intramuscular administration of carbamazepine (●) and **26** (○) to rats at a dose of 100 mg/kg carbamazepine equivalents. Reproduced from (Bundgaard *et al.*, 1982).

Finally, N-Mannich bases have been employed to protect peptides from degradation by enzymes such as pyroglutamyl aminopeptidase (Bundgaard and Møss, 1989) and α-chymotrypsin (Kahns and Bundgaard, 1991a). In studies with pyroglutamyl aminopeptidase, the methylamine- (**29**), piperidine- (**30**), and morpholine-derived (**31**) N-Mannich base prodrugs of L-pyroglutamyl benzylamide provided 100% protection for the parent compound. It was also hypothesized that N-Mannich base prodrugs could enhance the lipophilicity of peptides, thereby potentially increasing their permeability.

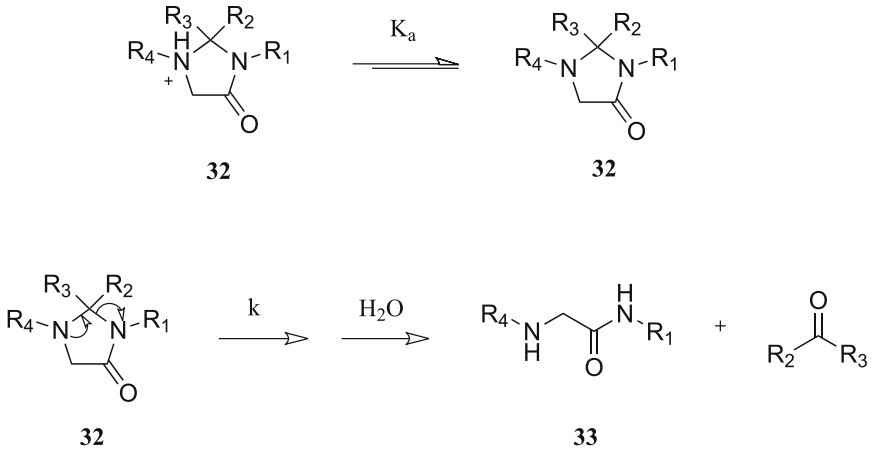


Structures 29-31.

To conclude, the most common problem with N-Mannich base prodrugs is their aqueous stability. Ideally, an N-Mannich base prodrug would exhibit a large differential in aqueous stability between its protonated and deprotonated forms, permitting possible formulation at an acidic pH (protonated form) and an appropriate reconversion rate in the neutral to slightly basic pH of the blood or small intestine (deprotonated form). Of course, solid dosage formulations and/or other non-aqueous vehicles, if appropriate for the intended treatment, offer additional possibilities for developing N-Mannich base prodrugs with unfavorable aqueous stability characteristics. Finally, within the N-Mannich base approach, design flexibility exists in the choice of amine basicity, amine substituent bulk, and salt selection.

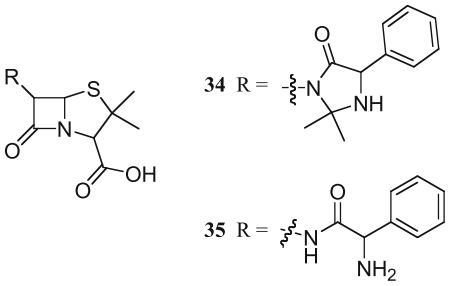
Imidazolidinone Prodrugs (Cyclic N-Mannich Base Prodrugs)

The 1,4-imidazolidinone structure (**32**) can serve as a cyclic N-Mannich base prodrug of an acyclic α-aminoamide moiety (**33**) (a moiety commonly found in peptides with a free N-terminal amine). Being cyclic N-Mannich bases, the bioreconversion of 1,4-imidazolidinones (Scheme 5) generally follows the same mechanism as the acyclic N-Mannich bases described in the previous section; furthermore, their pH-rate profiles typically have a sigmoidal shape. A one-carbon spacer, originating synthetically from an aldehyde or ketone, connects the amide and amine of **33** to form **32**. Finally, the pKa of the amine group typically drops approximately 4 units upon derivatization into a 1,4-imidazolidinone ring.

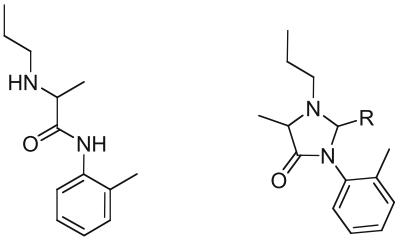


Scheme 5. The bioreconversion of a generic 1,4-Imidazolidinone prodrug.

Hetacillin (**34**) is a 1,4-imidazolidinone prodrug (using an acetone-based linker) of ampicillin (**35**), a peptide-like antibiotic, which suffers from polymerization at higher concentrations (Schwartz and Hayton, 1972). This polymerization occurs from intermolecular nucleophilic attack by the free amine from one ampicillin molecule on the beta-lactam ring of another. By incorporating the amine of **35** into a 1,4-imidazolidinone to form **34**, the stability improved six-fold. This stabilization arises primarily from the equilibrium existing between **34** and **35**. Prodrug **34** reconverts *in vivo* with a half-life of 11 min; furthermore, **34** gave a slightly enhanced oral bioavailability of **35** (38%) compared to **34** *per se* (29%) (Jusko and Lewis, 1973). Finally, Klixbüll and Bundgaard (1985) characterized a series of additional 1,4-imidazolidinone prodrugs of **35** whose spacers were derived from various aldehydes and ketones. They found that at pH 7.45 and 37°C, the 1,4-imidazolidinone derivatives from aldehydes and ketones degraded much faster ($t_{1/2}$ = 4-31 min) than the analogous prodrug from formaldehyde ($t_{1/2}$ = 29 h). Also studying the stability effects of the different spacers, Larsen *et al.* (2003) examined two 1,4-imidazolidinone prodrugs of prilocaine (**36**) using a formaldehyde-based (**37**) and an acetaldehyde-based



Structures 34-35.



36 **37** R = H

38 R = CH_3

Structures 36-38.

(**38**) spacer. Not surprisingly, **38** degraded faster ($t_{1/2}$ = 1.4 h at pH 7.4 and 37°C) than **37** ($t_{1/2}$ = 6.9 h at pH 7.4 and 60°C). Furthermore, **37** showed enhanced lipophilicity as displayed in its Viscoleo® (light vegetable oil)/aqueous buffer (pH 7.4) partition coefficient value (log P_{app} 1.21) relative to **36** (log P_{app} 0.8).

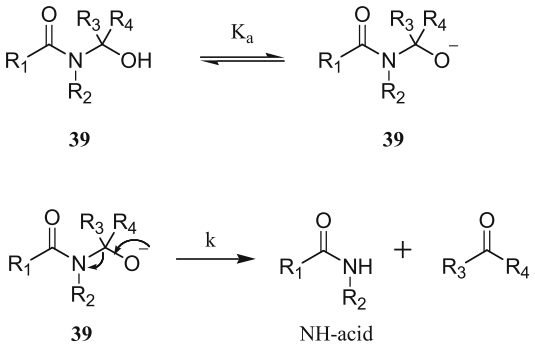
For peptides, 1,4-Imidizolidinone prodrugs have shown promise in protecting against proteolysis and increasing lipophilicity. For instance, Bundgaard and associates (Rasmussen and Bundgaard, 1991; Bak *et al.*, 1999) stabilized Leu-enkephalin and Met-enkephalin against proteolysis by aminopeptidase N and angiotensin-converting enzyme by derivatizing the N-terminus into a 1,4-imidazolidinone ring using ketone-based linkers. In a study of a 1,4-imidazolidinone series of model dipeptides, Klixbüll and Bundgaard (1984) documented the potential for enhancing peptide lipophilicity and revealed the destabilizing steric effects of bulky neighboring amino acid residues on the hydrolytic stability of the 1,4-imidazolidinone ring. However, these 1,4-imidazolidinone peptide derivatives showed relatively slow reconversion half-lives, with values from 0.9-530 h (pH 7.4, 37°C) for the 1,4-imidazolidinone model dipeptide prodrugs (Klixbüll and Bundgaard, 1984) and 3.1-18.8 h (80% human plasma, 37°C) for the 1,4-imidazolidinone Leu-enkephalin and Met-enkephalin prodrugs (Rasmussen and Bundgaard, 1991; Bak *et al.*, 1999), which might significantly hinder their ability to perform as prodrugs.

In conclusion, 1,4-imidazolidinone prodrugs typically degrade in the same manner as acyclic N-Mannich bases. Due to the significant pKa drop for the amine upon derivatization, the removal of an NH hydrogen bond donor from the amide and the possible addition of lipophilic substituents via the carbonyl-based

linker, 1,4-imidazolidinone prodrugs show some promise in increasing the lipophilicity of peptides and other parent compounds containing an α-aminoamide moiety. However, the slow reconversion (pH 7.4, 37°C) seen for most of the 1,4-imidazolidinone prodrugs limits the utility of this prodrug design.

N-Hydroxyalkyl Prodrugs

Scheme 6 displays the structure and bioreconversion of a generic N-hydroxyalkyl prodrug (**39**). As seen in Scheme 6, the hydroxylate form of the N-hydroxyalkyl prodrug undergoes a unimolecular degradation involving the formation of an aldehyde and the rate-determining N-C bond cleavage, resulting in an NH-acid anion, which quickly gains a proton from the surrounding solvent.



Scheme 6. The bioreconversion of a generic N-hydroxyalkyl prodrug.

Similar to their initial N-Mannich base investigation, Johansen and Bundgaard (1979; Bundgaard and Johansen, 1980d, 1981a) synthesized and physicochemically characterized N-hydroxymethyl prodrugs of a series of NH-acidic compounds (amides, thioamides, carbamates, imides, and hydantoins). The aqueous degradation of the prodrugs showed an apparent first-order dependence on hydroxide ion concentration from slightly acidic solution up to ~ pH 10 (slope of ~1 in pH-rate profile) and displayed no sensitivity to buffer catalysis, plasma enzyme catalysis, or ionic strength effects; however, the pH-rate profile slope begins to level off from a value of 1 to 0 as the pH approaches and passes beyond the hydroxyl pKa value of the N-hydroxymethyl promoiety. Furthermore, even though the pH-rate profiles for N-hydroxymethyl prodrugs typically display a slope of 1 for hydrolysis in slightly acidic to basic solutions, dominant acid-catalyzed hydrolysis can exist for these derivatives in very acidic solutions (pH < 3) (Tenn *et al.*, 2001).

The pKa of the NH-acid has the largest influence on the prodrug degradation kinetics with the more acidic parent compounds giving the least stable prodrugs (Johansen and Bundgaard, 1979; Bundgaard and Johansen, 1980d) (Figure 8). For N-hydroxymethyl prodrugs, a reconversion half-life (pH 7.4, 37°C) of less than an hour requires that the pKa (measured at 20-25°C) of the parent NH-acid be less than ~13. The N-hydroxyalkyl technology can be extended to slightly weaker

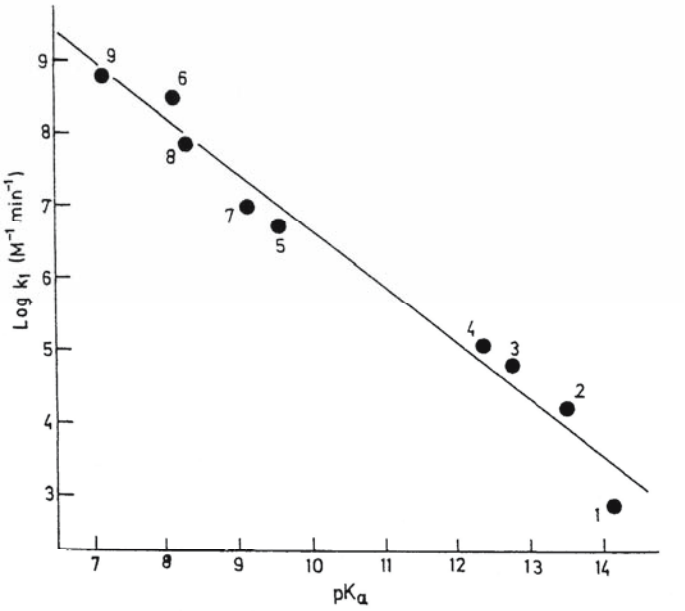
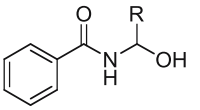


Figure 8. Plot of the logarithm of the apparent hydroxide ion catalytic rate constants (k_1) for decomposition of various N-hydroxymethyl derivatives at 37 °C against pK_a of the parent compounds. 1, Chloroacetamide; 2, dichloroacetamide; 3, thiobenzamide; 4, trichloroacetamide; 5, succinimide; 6, 5-chloro-2-benzoxazolinone (chlorzoxazone); 7, 5,5-dimethylhydantoin; 8, phenytoin; 9, nitrofurantoin. Reproduced from (Bundgaard and Johansen, 1980d).

NH-acids by using aldehydes instead of formaldehyde in the synthesis (Bundgaard and Johansen, 1984; Bundgaard and Buur, 1987). For example, at pH 7.4 and 37°C, N-hydroxymethyl benzamide, **40**, has a half-life of 160 h, while N-hydroxybenzyl benzamide, **41**, (from benzaldehyde) has a half-life of only 6.5 min (Bundgaard and Johansen, 1984).



40 R = H

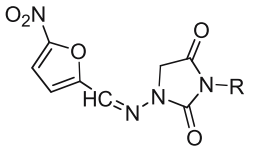
41 R = C_6H_5

Structures 40-41.

Regarding aqueous solubility, studies have generally found that N-hydroxymethyl derivatives can successfully enhance the solubility of their parent NH-acids by roughly three-to-fivefold (22-25°C) (Johansen and Bundgaard, 1979; Bundgaard and Johansen, 1980d; Bansal *et al.*, 1981). Consistent with their enhanced solubilities, N-hydroxymethyl prodrugs have also shown superior dissolution rates relative to their parent compounds (Johansen and Bundgaard, 1980b; Bansal *et al.*, 1981). The solubility and dissolution rate improvements were attributed to a combination of crystal lattice weakening and the increased

solvation potential of the CH_2OH promoiety relative to the original NH group in the parent compound.

Sorel and Roseboom (1979) characterized the stability and pharmacokinetics of hydroxymethylnitrofurantoin (**42**), a hydroxymethyl prodrug of the antibacterial nitrofurantoin (**43**), used in treating urinary tract infections. A half-life of 5 s at pH 7.4 for conversion of **42** to **43** was estimated by extrapolation from the pH-rate profile of **42**. From the pharmacokinetic studies, only **43** was found to be excreted in the urine following oral dosing of **42**. Combining the *in vitro* stability and *in vivo* urinary excretion data, it was clear that **42** acted as a prodrug of **43**. Furthermore, from a previous study, Spencer and Michels (1964) found that **42** had an approximately four-times greater aqueous solubility than **43**.

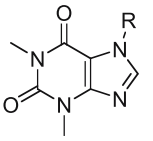


42 R = CH_2OH

43 R = H

Structures 42-43.

Other researchers have attempted to enhance the transdermal delivery of NH-acids via N-hydroxymethyl prodrugs. For instance, N_7-hydroxymethyl theophylline (**44**) showed enhanced aqueous and lipid solubility over theophylline (**45**), which led to an improved delivery of the prodrug across hairless mouse skin (Sloan and Bodor, 1982; Kerr *et al.*, 1998). However, a combination of the long lag time (4.3 h) from the application of **44** (donor side) to the appearance of **45** in the receiver compartment with the poor aqueous stability of **44** suggests that N-hydroxymethylation is probably a poor prodrug design choice for enhancing theophylline transdermal delivery.



44 R = CH_2OH

45 R = H

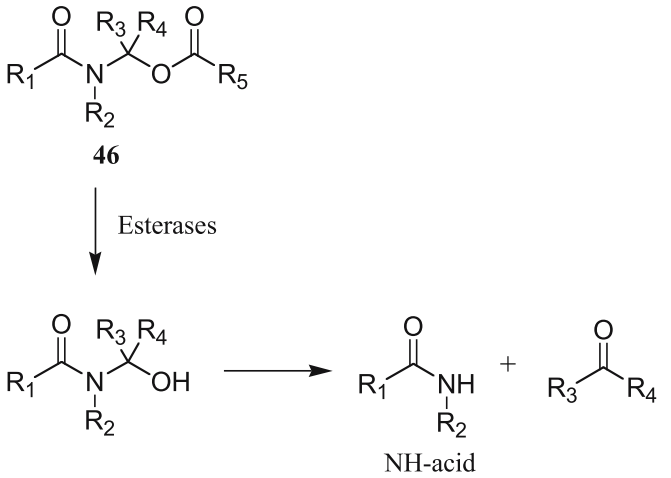
Structures 44-45.

Finally, the N-hydroxyalkyl prodrug technology has been used to stabilize susceptible peptide bonds toward proteolytic enzymes, such as carboxypeptidase A, pyroglutamyl aminopeptidase, and α-chymotrypsin. For instance, N-hydroxymethylation of a series of model N-terminal-protected dipeptides stabilized the amide bonds from proteolysis by carboxypeptidase A (Bundgaard and Rasmussen, 1991a; Friis *et al.*, 1996). In the same manner, N-hydroxymethylation successfully stabilized a L-pyroglutamyl benzylamide model compound against proteolysis by pyroglutamyl aminopeptidase (Møss and Bundgaard, 1992a). However, attempts to avoid proteolysis by α-chymotrypsin by designing N-hydroxymethyl prodrugs resulted in stabilization of some peptides (Kahns *et al.*, 1993) and destabilization of others (Kahns and Bundgaard, 1991a).

In conclusion, the existing literature clearly shows that N-hydroxyalkyl derivatives can successfully act as prodrugs for NH-acids. However, potential problems with slow reconversion rates generally limit the practical utility of this technology to fairly acidic NH-acids (pKa <13). Furthermore, this technology offers very little design flexibility. In fact, arguably the most important outcome from the derivatization of an NH-acid into an N-hydroxyalkyl prodrug is the resulting introduction of a terminal hydroxyl group, which is relatively easy to acylate. Acylation of an N-hydroxyalkyl prodrug produces an N-acyloxyalkyl prodrug, which is the subject of the next section.

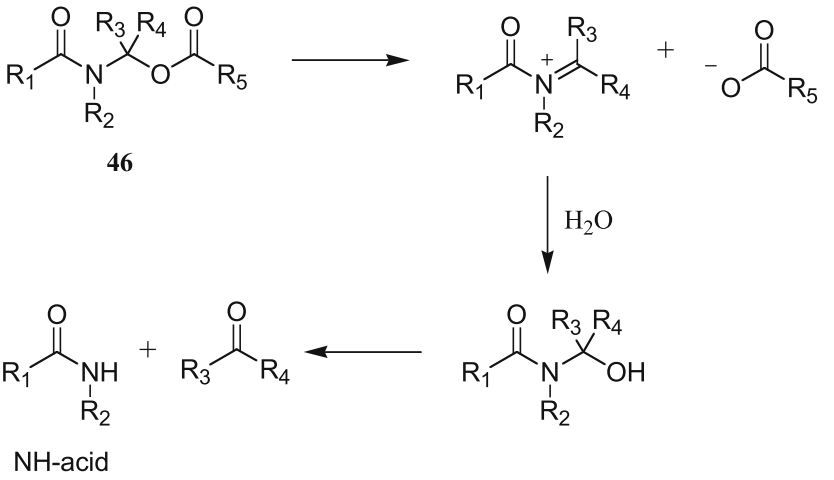
N-Acyloxyalkyl Prodrugs

Scheme 7 displays the structure and bioreconversion of a generic N-acyloxyalkyl prodrug (**46**). As seen in Scheme 7, esterase-mediated hydrolysis of the acyl moiety creates an N-hydroxyalkyl intermediate, which subsequently undergoes unimolecular degradation to release the NH-acid. Although the promoiety of **46** includes a terminal carbon-based ester, the promoiety design of N-acyloxymethyl prodrugs are not limited to terminal carbon-based ester functionalities. Non-carbon based esters have successfully been used, such as phosphate esters to make N-phosphoryloxyalkyl prodrugs, and will be classified in this section as a subgroup of N-acyloxyalkyl prodrugs.



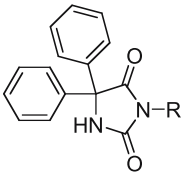
Scheme 7. The bioreconversion of a generic N-acyloxyalkyl prodrug.

In aqueous solution, absent of hydrolases, the degradation of N-acyloxyalkyl prodrugs can follow either the basic process shown in Scheme 7 or an alternative degradation process. The alternative degradation route begins with an elimination reaction producing a carboxylate and an N-acylimine, followed by water addition to the N-acylimine to give an N-hydroxyalkyl compound, which further degrades to produce the N-H acidic parent compound and an aldehyde



Scheme 8. An alternative degradation mechanism for N-acyloxyalkyl prodrugs.

(Scheme 8). Typically, N-acyloxyalkyl prodrugs of imide-type NH-acids degrade in aqueous solution through ester hydrolysis according to Scheme 7. These types of N-acyloxyalkyl prodrugs have drastically improved stability over their N-hydroxyalkyl analogues. For example, N-acetyloxymethyl phenytoin (**47**) is over 5 times more stable in aqueous solution (pH 7.5-12.5; 37°C) than N-hydroxymethyl phenytoin (**48**) (Bundgaard and Johansen, 1980d). However, many of the N-acyloxyalkyl prodrugs from amide-type NH-acids, as well as some sulfonamides, degrade according to Scheme 8, which results in an unexpected poor aqueous stability for these compounds in the slightly acidic to neutral pH range (Bundgaard and Nielsen, 1987; Bundgaard *et al.*, 1988; Bundgaard and Rasmussen, 1991b; Iley *et al.*, 1991; Calheiros *et al.*, 1995; Lopes *et al.*, 1999, 2000).



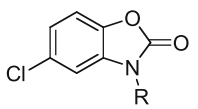
Phenytoin R = H

47 R = $CH_2OC(O)CH_3$

48 R = CH_2OH

Structures 47-48.

One of the more popular acyl moieties used in designing N-acyloxyalkyl prodrugs have been the amino acid esters. For instance, using N,N-dimethyl glycine as the acyl moiety, Johansen and Bundgaard (1981b) synthesized an N-acyloxymethyl prodrug of chlorzoxazone (**49**), which possessed enhanced solubility and dissolution rate properties, and degraded in 50% human plasma at

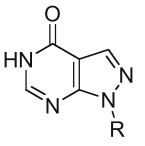


Chlorzoxazone R = H

49 R = $CH_2OC(O)CH_2N(CH_3)_2$

Structure 49.

37°C with a half-life of 14 min. In similar work, Bundgaard and associates (Bundgaard and Falch, 1985b,c; Bundgaard *et al.*, 1985) synthesized a series of N-acyloxymethyl prodrugs of allopurinol, the most promising of which used amino acid esters (**50**-**56**) as their acyl group. These amino acid ester prodrugs possessed increased lipophilicity and water solubility, making them potential candidates for rectal delivery. Of particular note, **51**, **52**, and **53** displayed very promising rectal bioavailability in rabbits (57%, 74% and 94%, respectively) relative to allopurinol (3%). Finally, similar to the allopurinol prodrugs, an N,N-dimethyl glycine prodrug of theophylline (**57**) displayed enhanced lipophilicity and water solubility relative to its parent compound, theophylline (Sloan and Bodor, 1982).



Allopurinol R = H

50 R = $CH_2OC(O)CH_2N(CH_3)_2$

51 R = $CH_2OC(O)CH_2N(CH_2CH_3)_2$

52 R = $CH_2OC(O)CH_2N(CH_2CH_2CH_3)_2$

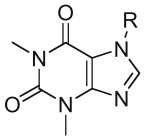
53 R = $CH_2OC(O)CH(CH_3)N(CH_2CH_3)_2$

54 R = $CH_2OC(O)CH(C_6H_5)NH_2$

55 R = $CH_2OC(O)CH(CH_2C_6H_5)NH_2$

56 R = $CH_2OC(O)CH(CH_2CH_2(CH_3)_2)NH_2$

Structures 50-56.

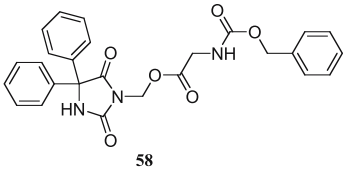


Theophylline R = H

57 R = $CH_2OC(O)CH_2N(CH_3)_2$

Structure 57.

Amino acid ester promoieties have also shown promise for improving the safety, or protective index, of phenytoin (Scriba and Lambert, 1999) as well as its aqueous solubility (Bosch *et al.*, 1999). Scriba and Lambert (1999) synthesized N-benzyloxycarbonyl protected amino acid esters of which the glycine derivative (**58**) displayed the most potential. Prodrug **58** rapidly reconverted in 5% rat plasma at 20°C ($t_{1/2}$ = 1 min) while possessing fair aqueous stability at pH 7.4 and 20°C ($t_{1/2}$ = 8.6 days); furthermore, this prodrug enhanced the protective index (TD_{50}/ED_{50}) of phenytoin from 6.6 to 11.2.

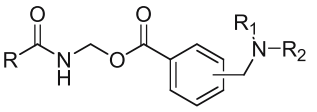


58

Structure 58.

However, although promising for enhancing solubility and permeability, N-acyloxyalkyl prodrugs using amino acid ester acyl moieties tend to have aqueous stability problems in the pH range ideal for their aqueous formulation (pH 3-5), making them inadequate prodrugs for ready-to-use parenteral formulations. The poor aqueous stability can stem from activation of the acyl group toward hydrolysis

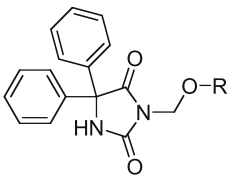
by the electron-withdrawing protonated amine and intramolecular catalytic assistance to hydrolysis by the amine. Bundgaard *et al.* (1989) alleviated the stability problems due to the amine by designing a phenylmethylene spacer between the acyl carbonyl and the amine (**59**). The phenyl structure introduces a constraint against intramolecular catalysis by the amine, while the methylene spacer prevents a significant pKa drop for the amine, which would diminish the pH range where the amine, in its protonated form, could enhance solubility.



59

Structure 59.

Yamaoka *et al.* (1983) successfully synthesized a homologous series of N-acyloxymethyl phenytoin prodrugs (acetyl through decanoyl) (**60**-**69**) with lowered melting points and higher solubilities in organic solvents, allowing the prodrugs to be dissolved in lipid vehicles for oral delivery. The 3-pentanoyloxymethyl phenytoin prodrug (**63**), which was the most promising prodrug of the series, possessed a half-life of 1.9 min in human plasma and 0.82 min in rat liver homogenate at 37°C. When dosed orally from a lipid vehicle (tributyrin), the prodrug gave superior blood levels of phenytoin compared to those of sodium phenytoin dosed orally as an aqueous solution.



60 R = $C(O)CH_3$
61 R = $C(O)C_2H_5$
62 R = $C(O)C_3H_7$-n
63 R = $C(O)C_4H_9$-n
64 R = $C(O)C_4H_9$-t
65 R = $C(O)C_5H_{11}$-n
66 R = $C(O)C_6H_{13}$-n
67 R = $C(O)C_7H_{15}$-n
68 R = $C(O)C_8H_{17}$-n
69 R = $C(O)C_9H_{19}$-n

Structures 60-69.

Buur *et al.* (1985) also investigated the use of simple alkyl-based acyl moieties in making N_1-, N_3-, and N_1,N_3-acyloxyalkyl prodrugs of 5-fluorouracil. These prodrugs all had enhanced lipophilicity without appreciable loss in aqueous solubility; moreover, some of the prodrugs (**70**-**72**) showed enhancements in both aqueous and lipid solubility. The N_1-acyloxymethyl derivatives hydrolyzed faster than did the N_3-derivatives in aqueous solution; however, in the presence of enzymes (plasma and rat liver homogenate), the reverse was true. In a related study, Buur *et al.* (1986) compared the physicochemical properties of N_3-ethoxy- (**73**) and N_3-phenoxycarbonyloxymethyl (**74**) prodrugs to N_1-ethoxy- (**75**) and N_1-phenoxycarbonyloxymethyl (**76**) prodrugs of 5-fluorouracil and found that **74** held the most promise due its fair aqueous stability ($t_{1/2}$ = 16.3 h at 37°C) and rapid reconversion rate in 80% human plasma ($t_{1/2}$ = 10 min at 37°C).

5-Fluorouracil $R_1 = H$
$R_3 = H$

70 $R_1 = CH_2OC(O)CH_3$
$R_3 = H$

71 $R_1 = CH_2OC(O)C_2H_5$
$R_3 = H$

72 $R_1 = H$
$R_3 = CH_2OC(O)CH_3$

73 $R_1 = H$
$R_3 = CH_2OC(O)OC_2H_5$

74 $R_1 = H$
$R_3 = CH_2OC(O)OC_6H_5$

75 $R_1 = CH_2OC(O)OC_2H_5$
$R_3 = H$

76 $R_1 = CH_2OC(O)OC_6H_5$
$R_3 = H$

Structures 70-76.

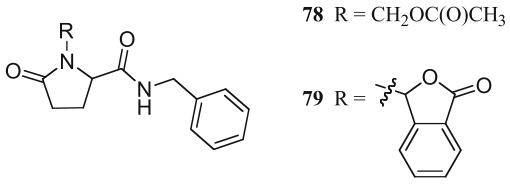
Buur and Bundgaard (1987) also showed that, while 5-fluorouracil has no bioavailability following rectal administration, 1-butyryloxymethyl-5-fluorouracil (**77**), due to its improved partition coefficient, gave a bioavailability for 5-fluorouracil of 62% when administered rectally in rabbits. Møllgaard *et al.* (1982) also showed that **77** enhanced the *in vitro* permeation of 5-fluorouracil across human skin by a factor of 5. Esterases metabolized the prodrug upon passage through the skin, which resulted in 5-fluorouracil being the only compound appearing in the receiver compartment. Sloan and associates (Taylor and Sloan, 1998; Roberts and Sloan, 2003; Sloan *et al.*, 2003) compared the transdermal and dermal delivery enhancements of both N_1-alkylcarbonyloxymethyl and N_3-alkylcarbonyloxymethyl prodrugs of 5-fluorouracil using hairless mouse skin. Of these generally lipophilic prodrugs, the ones with better aqueous solubility gave the greatest delivery enhancements. Furthermore, the N_1-derivatives displayed the best dermal delivery while the N_3-derivatives gave the best transdermal delivery.

5-Fluorouracil $R_1 = H$

77 $R_1 = CH_2OC(O)C_3H_7$

Structure 77.

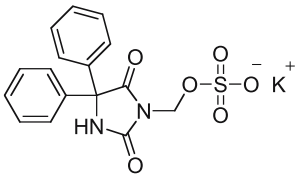
Researchers have investigated, with mixed results, the use of N-acyloxyalkylation to protect peptides against proteolysis and enhance their lipophilicity. On the positive side, Møss and Bundgaard (1992a) successfully protected L-pyroglutamyl benzylamide, a model pyroglutamyl peptide, against pyroglutamyl aminopeptidase proteolysis by designing acetoxymethyl (**78**) and phthalidyl (**79**) N-acyloxyalkyl prodrugs, which quantitatively reconverted in 80% plasma (pH 7.4, 37°C) to give the model peptide with half-lives of 2.3 h and 8.4 h, respectively. However, neither Møss and Bundgaard (1992b) nor Kahns and Bundgaard (1991a) found the same proteolytic protection from all N-acyloxyalkyl prodrugs.



Structures 78-79.

For example, an N-phthalidyl prodrug of N-benyloxycarbonyl-glycyl-L-prolin-eamide (Z-Gly-ProNH_2) showed greater instability to prolyl endopeptidase compared to Z-Gly-ProNH_2 *per se* (Møss and Bundgaard, 1992b); similarly, N-phthalidylation of the C-terminal amide group in N-Ac-Phe-NH_2 resulted in an N-acyloxyalkyl prodrug that degraded faster by α-chymotrypsin than did the parent compound (Kahns and Bundgaard, 1991a).

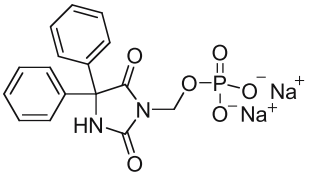
As mentioned in the beginning of this section, the N-acyloxyalkyl prodrug technology has been expanded to include non-carbon-based terminal esters. Williams *et al.* (1983) investigated the use of a sulfate ester of the N-hydroxymethyl derivative (N-Sulfuryloxymethyl prodrug) of phenytoin (**80**) as an alternative to a typical N-acyloxyalkyl derivative. Sulfatases were expected to initiate the *in vivo* reconversion of **80** followed by N-dehydroxymethylation to give phenytoin. However, **80** rapidly cleared ($t_{1/2}$ = 34 min) from the body and phenytoin never reached a detectable limit in the blood. The combination of a slow *in vivo* reconversion and a fast elimination half-life made **80** a poor prodrug choice for phenytoin.



80

Structure 80.

Unlike sulfate esters, phosphate esters have shown promise in the prodrug literature. By examining a series of water-soluble N-acyloxymethyl prodrugs of phenytoin, Varia, Stella, and others (Varia and Stella, 1984a,b; Varia *et al.*, 1984a,b) discovered phosphoryloxymethyl phenytoin (**81**; fosphenytoin) as a stable, water-soluble prodrug that eventually reached commercial success (Cerebyx®) as a parenteral substitute for sodium phenytoin. Fosphenytoin contains a phosphate ester as its 'acyl' moiety, which hydrolyzes *in vivo* in the presence of phosphatases. Fosphenytoin displayed very good chemical stability in pH 7.4 phosphate buffer (25°C) with a half-life of 18.6 years. Due to quantitative reconversion, phenytoin blood levels from **81** matched those from sodium phenytoin following intravenous dosing in rats (Varia and Stella, 1984b) (Figure 9) and humans (Stella, 1996) (Figure 10); furthermore, following intramuscular administration, **81** gave higher phenytoin blood levels than did sodium phenytoin (Varia and Stella, 1984b). However unlike sodium phenytoin, **81** did not exhibit tissue damage following



Structure 81. **81**

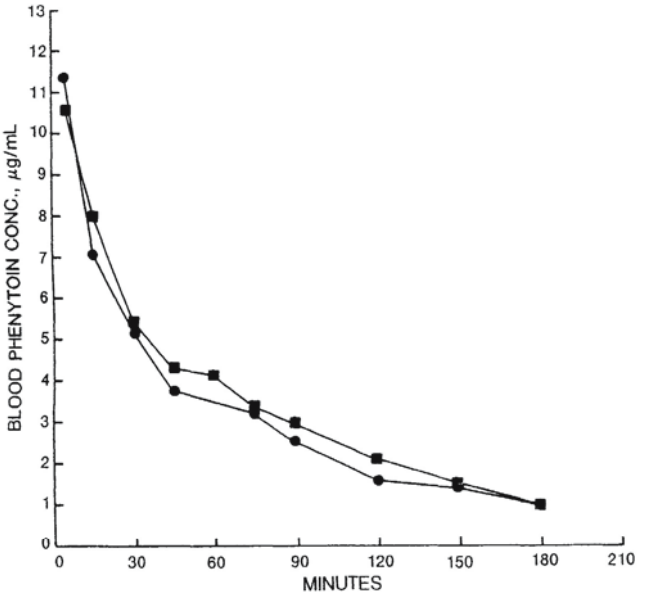


Figure 9. Plots of blood phenytoin concentrations versus time after intravenous administration of 10 mg/kg (phenytoin equivalent) doses of sodium phenytoin (●) and fosphenytoin (■) to the same rat. Reproduced from (Varia and Stella, 1984b).

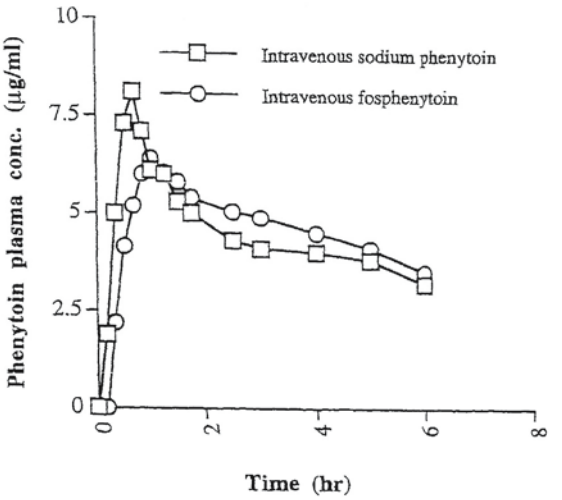


Figure 10. Phenytoin plasma concentration (mean, 12 subjects) versus time (first 6h) curves after intravenous infusion administration over 30 min of equimolar doses (250 mg phenytoin) of sodium phenytoin (□) and fosphenytoin (○). Reproduced from (Stella, 1996).

intramuscular or subcutaneous administration (Stella, 1996). More information about **81** exists in the fosphenytoin monograph in this book as well as in a review by Stella (1996).

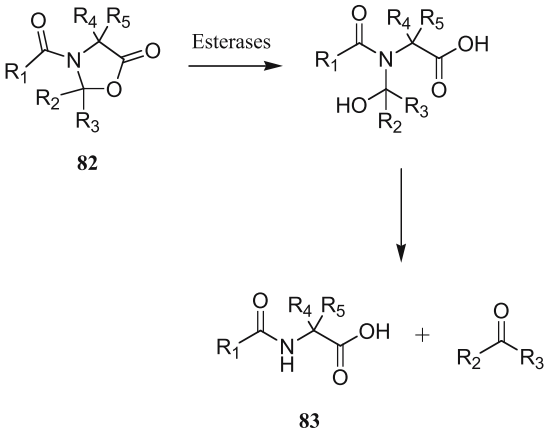
Scriba and associates (Scriba, 1993a,b; Scriba *et al.*, 1995a,b,c; Scriba and Lambert, 1997) synthesized a series of phenytoin-lipid N-acyloxymethyl prodrugs. The prodrugs reconverted to give phenytoin by lipolysis of the glyceride portion (by lipases) followed by enzymatic hydrolysis of the acyloxymethyl portion. The prodrugs successfully improved the oral bioavailability of phenytoin in rats by a factor of 2 to 4 relative to phenytoin *per se*; moreover, the anticonvulsant effect of phenytoin occurred sooner from the prodrugs, as a result of the improved phenytoin delivery. From similar studies, polyunsaturated fatty acid-based N-acyloxyalkyl derivatives also showed potential as prodrugs of theophylline (Burke *et al.*, 1997; Redden *et al.*, 1998, 1999), as well as phenytoin (Redden *et al.*, 1999), for enhancing the intestinal, dermal, or blood-brain barrier permeation of their parent compounds.

In conclusion, N-acyloxyalkyl prodrugs show the most promise for the imide-type NH-acids due to their enhanced aqueous stability. However, for amide-type NH-acids, this technology has not shown the same promise, mainly because (1) N-acyloxyalkyl prodrugs from amides typically degrade by an alternative elimination-addition mechanism (Scheme 8), which dramatically compromises their aqueous stability and (2) the N-hydroxyalkyl intermediates (especially the N-hydroxymethyl derivatives) tend to be too stable, resulting in long half-life values for *in vivo* reconversion. To insure rapid reconversion *in vivo*, while still possessing good formulation stability, the chosen acyl moiety should be a good substrate for enzyme-mediated hydrolysis. Besides improving stability, the addition of the acyl moiety also provides greater flexibility in prodrug design toward enhancing either lipophilicity or hydrophilicity, relative to the N-hydroxyalkyl technique.

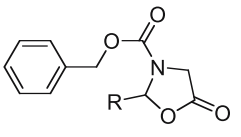
Oxazolidinone Prodrugs (Cyclic N-Acyloxyalkyl Prodrugs)

The 5-oxazolidinone structure (**82**) can serve as a cyclic N-acyloxyalkyl prodrug of an acyclic α-amidocarboxy moiety (**83**). Because they are cyclic N-acyloxyalkyl derivatives, the bioreconversion of 5-oxazolidinones (Scheme 9) generally follows the same mechanism as that of the acyclic N-acyloxyalkyl prodrugs seen in the previous section. A one-carbon spacer, originating synthetically from an aldehyde or ketone, connects the nitrogen and the carboxylic acid oxygen of **83** to form **82**.

Although the 5-oxazolidinones undergo the same basic degradation reaction as linear N-acyloxyalkyl prodrugs, they typically possess greater reactivity compared to their linear analogs (Buur and Bundgaard, 1988). With everything else being equal, the 5-oxazolidinones derived from aldehydes or ketones possess greater reactivity than their formaldehyde-based analogs. For example, between the formaldehyde- (**84**), acetaldehyde- (**85**), and benzaldehyde- based (**86**) 5-oxazolidinone derivatives of N-benzyloxycarbonyl glycine studied by Buur and



Scheme 9. The bioreconversion of a generic 5-Oxazolidinone prodrug.



84 R = H

85 R = CH_3

86 R = C_6H_5

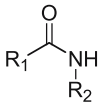
Structures 84-86.

Bundgaard (1988), only the benzaldehyde derivative produced a sufficiently reactive N-hydroxyalkyl intermediate such that lactone hydrolysis became the rate-determining step in neutral aqueous solution. In plasma, however, since esterases catalyze only the first step (lactone hydrolysis) and N-dehydroxyalkylation depends solely on pH (refer to N-hydroxyalkyl section), the *in vivo* degradation rate for a 5-oxazolidinone typically depends on the stability of the N-hydroxyalkyl intermediate at pH 7.4.

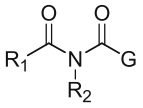
In conclusion, due to the esterification of the carboxylic acid and the removal of the amide N-H proton (hydrogen bond donor), the conversion of an acyclic α-amidocarboxy moiety into a 5-oxazolidinone can increase the lipophilicity of the parent compound. This prodrug strategy has particular promise for peptides and N-acylated amino acids, which can typically benefit from increases in lipophilicity for successful drug delivery; in addition, the alkylation of the amide can protect the peptide from enzymatic proteolysis. However, to ensure reasonably fast degradation of the N-hydroxyalkyl intermediate, the 5-oxazolidinone synthesis should involve aldehydes and ketones instead of formaldehyde.

N-Acyl Prodrugs

Three basic design subcategories exist within the general N-acyl category-the N-alkylcarbonyl or N-arylcarbonyl derivatives (amide formation) (**87**), the N-alkoxycarbonyl derivatives (carbamate formation) (**88**), and the N-carbamoyl derivatives (urea formation) (**89**). Scheme 10 shows the desired bioreconversion pathway for a generic N-acyl prodrug. Unfortunately, hydrolysis at the other



NH-acid

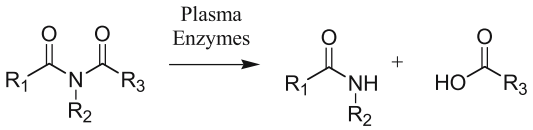


87 G = R_3

88 G = OR_3

89 G = NR_3R_4

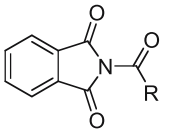
Structures 87-89.



Scheme 10. The bioreconversion of a generic N-acyl prodrug.

carbonyl can also occur, leading to non-productive degradation of the N-acyl prodrug.

To better understand the governing factors for the different possible degradation pathways of N-acyl derivatives, Stella and Higuchi (1973a), using phthalimide as a model imide compound, investigated the hydrolytic decomposition of an N-acyl phthalimide series (**90**-**96**) to determine whether the electronic properties of the N-acyl moiety affected the resulting degradation pathway in a rational and predictable manner. They concluded that N-acyl phthalimides with electron-donating acyl moieties (**90**-**94**) would degrade predominantly to form ring-opened N-acyl phthalamic acid derivatives, while those with electron-withdrawing acyl moieties (**95** and **96**) would degrade primarily to phthalimide. Therefore, as the electron-withdrawing capacity of the acyl moiety increases, the reactivity of the acyl carbonyl rises faster than that of the phthalimide ring carbonyls, eventually resulting in the predominant hydrolysis at the acyl carbonyl producing phthalimide (the desired reaction), despite the statistical tendency to attack either of the two identical ring carbonyls to form N-acyl phthalamic acid.

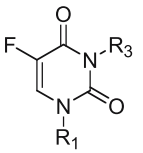


90 R = CH_3
91 R = OC_2H_5
92 R = C_2H_5
93 R = Morpholino
94 R = $N(CH_3)_2$
95 R = CH_2Cl
96 R = CH_2Br

Structures 90-96.

Following the study by Stella and Higuchi (1973a), the large majority of the investigations into N-acyl prodrugs used the imide-type 5-fluorouracil as the parent NH-acid. Buur and Bundgaard (1984a,b; 1985; 1986a,b; 1987) examined

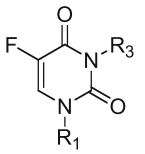
the properties of simple N-acyl (alkyl- and arylcarbonyl), N-alkoxycarbonyl, and N-carbamoyl prodrugs of 5-fluorouracil (very acidic NH-acid). For the simple N-acyl category, Buur and Bundgaard (1984b) examined a series of N_1-acyl (**97**), N_3-acyl (**98-101**) and N_1,N_3-acyl (**102-105**) prodrugs of 5-fluorouracil. While the N_1-acyl derivatives displayed very poor aqueous stability (Buur and Bundgaard, 1984b; Beall *et al.*, 1993), the N_3-acyl prodrugs possessed more promising characteristics with respect to their aqueous stability ($t_{1/2}$ ~ 0.5-5 days at pH 4, 37°C; $t_{1/2}$ ~ 40 min to 2 days at pH 7.4, 37°C), human plasma stability (4.6-110 min. in 80% plasma, 37°C), and, in the cases of **98** and **99**, simultaneously enhanced aqueous solubility and lipophilicity. Finally, **99** gave a 93% bioavailability of 5-fluorouracil when administered rectally in rabbit compared to 0% bioavailability from 5-fluorouracil *per se* (Buur and Bundgaard, 1987). However, despite the very poor aqueous stability of N_1-acyl prodrugs, they can still show utility if protected from aqueous environments. For instance, Jolimaître *et al.* (2003) achieved an *in vitro* sustained release of 5-fluorouracil into buffered solution from N_1-alkylcarbonyl prodrugs in silicone oil. This type of non-aqueous formulation could potentially produce a sustained intraocular delivery of 5-fluorouracil to treat proliferative vitreoretinopathy from an N_1-acyl prodrug with poor aqueous stability.



5-FU	R_1 = H	R_3 = H
97	R_1 = $C(O)CH_3$	R_3 = H
98	R_1 = H	R_3 = $C(O)CH_3$
99	R_1 = H	R_3 = $C(O)C_2H_5$
100	R_1 = H	R_3 = $C(O)C_3H_7$
101	R_1 = H	R_3 = $C(O)C_6H_5$
102	R_1 = $C(O)CH_3$	R_3 = $C(O)CH_3$
103	R_1 = $C(O)C_3H_7$	R_3 = $C(O)C_3H_7$
104	R_1 = $C(O)C_6H_5$	R_3 = $C(O)C_6H_5$
105	R_1 = $C(O)CH_3$	R_3 = $C(O)C_2H_5$

Structures 97-105.

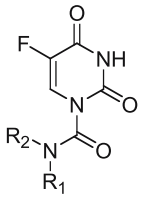
For the N-alkoxycarbonyl category, Buur and Bundgaard (1984a) characterized the physicochemical properties for a series of N_3-alkoxy carbonyl and N_1,N_3-alkoxycarbonyl prodrugs of 5-fluorouracil (**106-110**). Of the series, only **108** had both decent aqueous stability ($t_{1/2}$ of 80 h at pH 7.4 and 37°C) and a fairly fast reconversion rate in rat liver homogenate ($t_{1/2}$ of 20 min. at 37°C). In addition, Buur and Bundgaard (1986a; 1987) studied a series of N_1-alkoxycarbonyl prodrugs of 5-fluorouracil (**111-117**), which displayed less aqueous stability ($t_{1/2}$ from 18 to 550 min at pH 7.4 and 37°C) and faster plasma reconversion ($t_{1/2}$ from 0.5 to 3.1 min). Showing notable promise, **114** gave 100% and 58% bioavailability of 5-fluorouracil following rectal and oral administration in rabbits, respectively. Also of note, Steffansen *et al.* (1996) used N_1-alkoxycarbonyl prodrugs dissolved in silicone oil 1000 to produce a sustained release of 5-fluorouracil into buffered solution, suggesting a potential way to achieve sustained intraocular delivery of 5-fluorouracil to treat proliferative vitreoretinopathy.



106	$R_1 = C(O)OC_6H_5$	$R_3 = C(O)OC_6H_5$
107	$R_1 = C(O)OCH_2C_6H_5$	$R_3 = C(O)OCH_2C_6H_5$
108	$R_1 = H$	$R_3 = C(O)OC_6H_5$
109	$R_1 = H$	$R_3 = C(O)OCH_2C_6H_5$
110	$R_1 = H$	$R_3 = C(O)OC_2H_5$
111	$R_1 = C(O)OCH_3$	$R_3 = H$
112	$R_1 = C(O)OC_2H_5$	$R_3 = H$
113	$R_1 = C(O)OCH_2CH_2Cl$	$R_3 = H$
114	$R_1 = C(O)OC_4H_9$	$R_3 = H$
115	$R_1 = C(O)OCH_2CH(CH_3)_2$	$R_3 = H$
116	$R_1 = C(O)OC_6H_5$	$R_3 = H$
117	$R_1 = C(O)OCH_2C_6H_5$	$R_3 = H$

Structures 106-117.

Finally for the N-carbamoyl category, Buur and Bundgaard (1985) characterized the physicochemical properties for a series of N_1-carbamoyl prodrugs of 5-fluorouracil (**118-122**). Although, as a group, these derivatives possessed poor aqueous stability, the alkyl-based derivatives (**118-120**) displayed significantly better stability ($t_{1/2}$ = 8-11 min. at pH 7.4 and 37°C) than the one aryl-based derivative (**121**) ($t_{1/2}$ = 5 s at pH 7.4 and 37°C). Surprisingly however, the degradation rate for **118-120** actually slowed in 80% human plasma (two-to-sevenfold half-life increase), possibly due to non-catalytic plasma protein binding or the formation of protective inclusion aggregates with lipoproteins. All the derivatives (**118-122**) possessed a greater lipophilicity and a lower aqueous solubility relative to 5-fluorouracil; furthermore, **118-121**, which contain an N-H proton in the promoiety, displayed greater lipophilicity than **122**, presumably due to intramolecular hydrogen bonding between the carbamoyl N-H proton and the nearest carbonyl oxygen of 5-fluorouracil. Finally, Ozaki *et al.* (1997; 1998) synthesized N_1-carbamoyl prodrugs of 5-fluorouracil with enhanced antitumor activities following oral administration in mice, showing the potential promise in this technology.

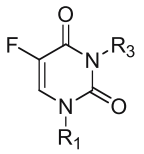


118	$R_1 = H$	$R_2 = CH_3$
119	$R_1 = H$	$R_2 = C_2H_5$
120	$R_1 = H$	$R_2 = C_4H_9$
121	$R_1 = H$	$R_2 = C_6H_5$
122	$R_1 = CH_3$	$R_2 = CH_3$

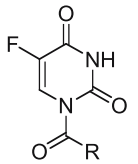
Structures 118-122.

As well as displaying the potential for solubility and lipophilicity enhancements, N-acylation has also shown promise in enhancing the transport of NH-acids into or across various epithelial boundaries. For instance, to enhance the transdermal delivery of 5-fluorouracil, Sloan and associates conducted transport studies of a homologous series of N_1-alkylcarbonyl (**123-128**) (Beall and Sloan, 1996), N_3-alkylcarbonyl (**129-132**) (Beall and Sloan, 2001), N_1N_3-bisalkylcarbonyl (**133-136**) (Beall and Sloan, 2002), N_1-alkoxycarbonyl (**137-142**) (Beall *et al.*, 1994), and N_1-carbamoyl (**143-147**) (Sloan *et al.*, 1993) prodrugs across hairless

mouse skin. All the prodrugs enhanced the transdermal delivery of 5-fluorouracil, but to different extents. With the exception of **137**, the N_1-alkyloxycarbonyl prodrugs (**137-142**) provided the greatest transport of 5-fluorouracil across hairless mouse skin, while the N_1-alkylcarbonyl prodrugs (**123-128**) produced the highest dermal-to-transdermal delivery ratio, probably due to their lower hydrolytic stability (higher percentage of reconversion within the skin) (Beall *et al.*, 1994, 1996, 1997; Beall and Sloan, 1996). Since all of these derivatives possessed sufficient lipophilicity for transport, a positive linear correlation existed between permeability and aqueous solubility within each category of N-acyl prodrugs (Sloan *et al.*, 1993; Beall *et al.*, 1994; Beall and Sloan, 1996, 2001, 2002). Using guinea-pig skin instead of hairless mouse skin, Chikhale *et al.* (1994) also successfully enhanced the transdermal delivery of 5-fluorouracil by N-acylation. Finally, from their *in vitro* Caco-2 cell transport experiments, Buur *et al.* (1996) showed the potential of lipophilic N-acyl prodrugs to enhance the permeability of 5-fluorouracil across the gut epithelium.



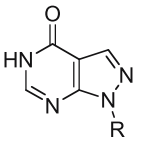
123 $R_1 = C(O)CH_3$ $R_3 = H$
124 $R_1 = C(O)C_2H_5$ $R_3 = H$
125 $R_1 = C(O)C_3H_7$ $R_3 = H$
126 $R_1 = C(O)C_4H_9$ $R_3 = H$
127 $R_1 = C(O)C_5H_{11}$ $R_3 = H$
128 $R_1 = C(O)C_7H_{15}$ $R_3 = H$
129 $R_1 = H$ $R_3 = C(O)CH_3$
130 $R_1 = H$ $R_3 = C(O)C_2H_5$
131 $R_1 = H$ $R_3 = C(O)C_3H_7$
132 $R_1 = H$ $R_3 = C(O)C_4H_9$
133 $R_1 = C(O)CH_3$ $R_3 = C(O)CH_3$
134 $R_1 = C(O)C_2H_5$ $R_3 = C(O)C_2H_5$
135 $R_1 = C(O)C_3H_7$ $R_3 = C(O)C_3H_7$
136 $R_1 = C(O)C_4H_9$ $R_3 = C(O)C_4H_9$



137 $R = OCH_3$
138 $R = OC_2H_5$
139 $R = OC_3H_7$
140 $R = OC_4H_9$
141 $R = OC_6H_{13}$
142 $R = OC_8H_{17}$
143 $R = NHCH_3$
144 $R = NHC_2H_5$
145 $R = NHC_3H_7$
146 $R = NHC_4H_9$
147 $R = NHC_8H_{17}$

Structures 123-147.

For the acidic NH-acid allopurinol, Bundgaard and Falch (1985a; 1985d) found that both alkyl- and aryl-based N_1-acyl prodrugs (**148-151**) displayed quantitative reconversion ($t_{1/2}$ = 2.5-6 min; 80% plasma, 37°C), enhanced lipophilicity (log P from -0.35 to 1.20 octanol-water partition coefficient at 22°C) and, in some cases (**148**), even slightly enhanced water solubility (S = 0.75 mg/mL at 22°C) relative to allopurinol (log P -0.55; S = 0.5 mg/mL); moreover, **148-151** enabled a 10 to 15% increase in allopurinol bioavailability from rectal administration in fatty acid suppositories (Bundgaard and Falch, 1985a; Bundgaard *et al.*, 1985). Despite the enhancements possible from **148-151** for rectal delivery, they showed inferior performance compared to the analogous N-acyloxyalkyl allopurinol prodrugs. In addition to the above studies, Bundgaard and Falch

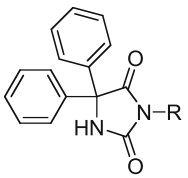


Allopurinol R = H
148 R = $C(O)CH_3$
149 R = $C(O)CH_2CH_3$
150 R = $C(O)CH_2CH_2CH_3$
151 R = $C(O)C_6H_5$
152 R = $C(O)CH_2N(CH_3)_2$
153 R = $C(O)CH_2CH_2N(CH_3)_2$

Structures 148-153.

(1985a,d) investigated some amine-containing N-acyl prodrugs (**152**, **153**), which suffered from inadequate stability ($t_{1/2}$ = 0.7 - 7.3 min at pH 4.0 and 37°C) due to intramolecular catalysis by the amine, for parenteral delivery.

To enhance the oral bioavailability of phenytoin, an imide-type NH-acid, Tanino *et al.* (1998) and Ogiso *et al.* (1998) synthesized some N_3-acyl prodrugs (methylcarbonyl (**154**), ethoxycarbonyl (**155**), and isopropoxycarbonyl (**156**)), which produced enhanced phenytoin blood levels in rats relative to phenytoin *per se*. From the solubility data in bile salt solution and bile salt-oleic acid mixed micelles, the authors hypothesized that the superior bioavailability of the prodrugs primarily came from enhanced dissolution rates.

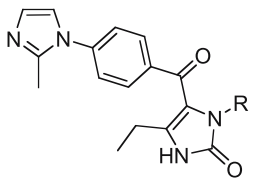


Phenytoin R = H
154 R = $C(O)CH_3$
155 R = $C(O)OCH_2CH_3$
156 R = $C(O)OCH(CH_3)_2$

Structures 154-156.

To complete the N-acyl approaches for the more acidic NH-acids, Shaw *et al.* (1992) studied a series of N-acyl prodrugs (**158**-**162**) for an NH-acidic cardiotonic agent (**157**) (pKa = 10.6). Showing particular promise, **159** possessed fair aqueous stability ($t_{1/2}$ = 25 h; pH 7.4, 37°C), good plasma reconversion ($t_{1/2}$ = 10 min in human plasma), and enhanced lipophilicity (13-fold increase over parent compound) while maintaining decent aqueous solubility (only a twofold decrease from the parent compound). These enhancements resulted in an oral bioavailability in dogs of greater than 75% from **159** compared to less than 20% from **157** *per se*.

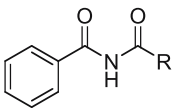
Transitioning to the less acidic NH-acids, Bundgaard and associates (Bundgaard *et al.*, 1986; Kahns and Bundgaard, 1991b,c), using benzamide, N-methyl benzamide, and salicylamide as model amides, determined that



157 R = H
158 R = $C(O)CH_3$
159 R = $C(O)C_6H_5$
160 R = $C(O)CH_2CH_3$
161 R = $C(O)CH(CH_3)_2$
162 R = $C(O)CH_2C_6H_5$

Structures 157-162.

N-acylation held the most promise, compared to N-alkoxycarbonylation and N-carbamylation, for designing prodrugs of simple amides. However, significant problems existed even with the most promising N-acyl derivatives (**163-168**). For example, in 80% human plasma, all but one of the N-acylated derivatives (**164-168**) displayed slow degradation ($t_{1/2}$ from 2.5 to 9.2 h) and non-quantitative reconversion due to hydrolysis at the alternate carbonyl, suggesting the possibility of inadequate bioreconversion properties for these prodrugs *in vivo*. The exception was **163**, which, although possessing limited aqueous stability ($t_{1/2}$ = 1.1 h at pH 7.4 and 37°C), showed quantitative reconversion to benzamide in 80% human plasma with a reasonable half-life of 15 min.

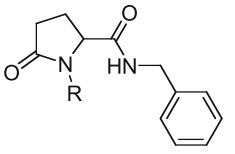


163 R = H
164 R = CH_3
165 R = C_2H_5
166 R = C_3H_7
167 R = C_4H_9
168 R = C_6H_5

Structures 163-168.

Attempts to stabilize peptides against proteolysis using N-acyl prodrugs have found mixed results. Bundgaard and Møss (1989; Møss and Bundgaard, 1992a) showed that simple N-acyl prodrugs (**170-174**) of L-pyroglutamyl benzylamide (**169**) provided protection from pyroglutamyl aminopeptidase-mediated cleavage. However, all of the prodrugs, except **173**, lacked quantitative reconversion in 80% human plasma due to hydrolysis occurring at both available carbonyls. Out of the series, only **173** showed fast plasma reconversion ($t_{1/2}$ = 8 min.); unfortunately, **173** also had very limited aqueous stability ($t_{1/2}$ = 14 min.; pH 7.4, 37°C). Using a model peptide (Z-Gly-ProNH_2), Møss and Bundgaard (1992b) showed that enzymatic cleavage in rabbit gut homogenate of the C-terminal prolineamide residue by prolyl endopeptidase could be slowed by a factor of 1.5-6 by N-acylating the C-terminal prolineamide residue; however, the N-acyl derivatives showed fairly long half-lives in 80% human plasma ($t_{1/2}$ = 14-42 h.; 37°C), suggesting that the *in vivo* reconversion kinetics for these prodrugs may be too slow for practical use. And lastly, attempts to protect a C-terminal amide of a model peptide through N-acetylation (N-Ac-Phe-NH-$COCH_3$) actually resulted in an accelerated degradation of the C-terminal amide by α-chymotrypsin (Kahns and Bundgaard, 1991a).

Larsen and Bundgaard (1987) studied N-acyl derivatives of sulfonamides as possible prodrugs. They examined a series of N-acyl, N-alkoxycarbonyl, and N-carbamoyl prodrugs of a primary model sulfonamide as well as one N-acetyl

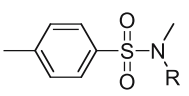


169 R = H
170 R = $C(O)CH_3$
171 R = $C(O)C_2H_5$
172 R = $C(O)C_3H_7$
173 R = $C(O)CH_2Cl$
174 R = $C(O)C_6H_5$

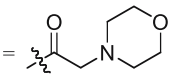
Structures 169-174.

prodrug of a secondary sulfonamide (**175**). Of the series, **175** showed the most promise in having decent aqueous stability ($t_{1/2}$ = 102 h at pH 7.4 and 37°C) and adequate reversibility in 80% human plasma ($t_{1/2}$ = 29 min at pH 7.4 and 37°C). Larsen *et al.* (1988) took a further look into N-acyl prodrugs of secondary sulfonamides by investigating the physicochemical effects of adding an ionizable amine group to the acyl moiety (N,N-diethylaminoacetyl, **176**, and morpholinoacetyl, **177**). Although **176** and **177**, as fumarate salts, provided dramatic enhancements in water solubility relative to **175** (~50-fold increase), a price was paid in decreased aqueous stability half-lives (3.2 - 49 h; pH 7.4, 37°C) and increased human plasma reversion half-lives (1.0 - 6.8 h at pH 7.4 and 37°C) compared to 175 ($t_{1/2}$ = 102 h and 29 min, respectively). The N-acyl prodrug, Parecoxib (**179**), has achieved clinical success as a water-soluble N-acyl prodrug of the primary sulfonamide COX-2 inhibitor, Valdecoxib (**178**) (Talley *et al.*, 2000). The N-acylation of **178**, using a propanoyl moiety, lowers the pKa of its sulfonamide NH proton, enabling development of a sodium salt parenteral formulation of the prodrug, **179**. Finally, Almansa *et al.* (2004) synthesized a N-phosphorylated prodrug (**180**) of cimicoxib (**181**). Prodrug **180** had an aqueous solubility at room temperature greater than 100 mg/mL (pH 7) and rapidly converted to give **181** following IV administration in dogs.

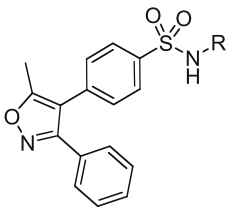
In conclusion, N-acyl derivatives of NH-acids can act as prodrugs to temporarily enhance physicochemical properties. The problems with this design approach mainly arise from the potential for multiple degradation pathways and



175 R = $C(O)CH_3$
176 R = $C(O)CH_2N(C_2H_5)_2$



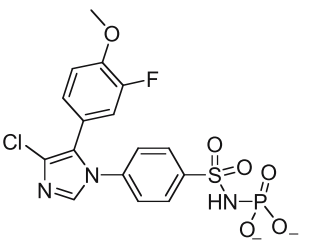
Structures 175-177.

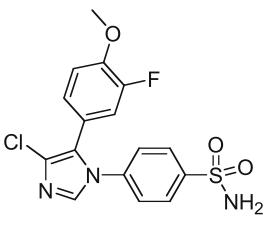


178 R = H

179 R = $C(O)C_2H_5$

Structures 178-179.





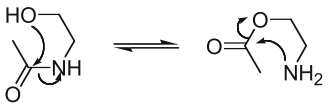
180 **181**

Structures 180-181.

the common finding of small differentials between aqueous stability and plasma stability. However, some promise appears to exist, particularly for very acidic imide- and amide-type NH-acids as well as sulfonamides.

N,O Acyl Transfer Prodrugs

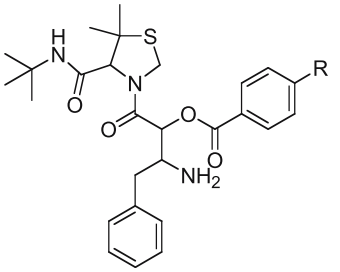
If an NH-acid contains a β-hydroxyamide moiety (182), then an intramolecular N,O acyl transfer reaction can occur through a 5-membered ring to form a rearranged derivative containing an ester and a free amine (**183**), as shown in Scheme 11. A pH-dominated equilibrium exists between the **182** and **183** isomers, where **183** predominates at acidic pH values and **182** predominates at neutral pH. This pH-dependent equilibrium makes **183** a potential prodrug of **182** that should reconvert in the neutral to slightly alkaline regions of the body. Prodrug **183** should have increased aqueous solubility over **182** due to the ionizable amine moiety, which can significantly simplify the formulation requirements for **183** compared to **182** *per se*.



182 **183**

Scheme 11. The bioreconversion of a generic N,O acyl transfer prodrug.

Steric (Oliyai and Stella, 1995; Kiso *et al.*, 1999; Hamada *et al.*, 2003) and electronic (Oliyai and Stella, 1995; Hamada *et al.*, 2003) effects significantly affect the prodrug reconversion speed (O,N acyl transfer), with the speed decreasing from bulky substituents around the amine and/or the O-acyl reaction centers (steric effect), electron-withdrawing groups around the amine (electronic effect), and electron-donating groups around the O-acyl (electronic effect). To study the electronic effect separately from steric influences, Hamada *et al.* (2003) examined a series of para-substituted O-benzoyl prodrugs (**184-188**) of HIV protease inhibitors and constructed a Hammet Plot showing a linear free energy relationship between the O,N acyl transfer rate constants and the acid-dissociation equilibrium constants for the corresponding para-substituted benzoic acids. The



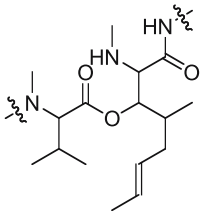
184 R = H
185 R = NO_2
186 R = Cl
187 R = CH_3
188 R = OCH_3

Structures 184-188.

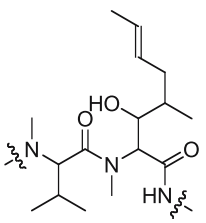
para substituents, ranging from an electron-withdrawing nitro group (**185**) to an electron-donating methoxy group (**187**), dramatically affected the reconversion rate ($t_{1/2}$ from 2.6-67 min).

Cyclosporin A, an immunosuppressive drug, can undergo N,O acyl transfer in acidic solution to form isocyclosporin A. Bundgaard and Friis (1992) investigated isocyclosporin A (**189**) as a potential water-soluble N,O acyl transfer prodrug of cyclosporin A (**190**). However, the fairly slow reconversion of isocyclosporin A in 80% human plasma ($t_{1/2}$ = 12.1 h; 37°C) might limit the actual usefulness of this technology for enhancing the delivery properties of cyclosporin A. Oliyai and Stella (1992) also examined the acyl transfer reaction between **189** and **190** by conducting a kinetic study in both aqueous and non-aqueous solution; they determined that plasma protein binding did not affect the conversion kinetics.

Aspartyl protease inhibitors, such as renin inhibitors and HIV protease inhibitors, commonly possess high lipophilicity necessary for their activity, which typically results in low aqueous solubility. Hurley *et al.* (1993) and Kiso and associates (Kiso *et al.*, 1999; Hamada *et al.*, 2002, 2004) synthesized N,O acyl transfer prodrugs of different aspartyl protease inhibitors that were 1000- to 8000-fold more soluble than their parent compounds and had fast reconversion times ($t_{1/2} \sim$ 0.5-8 min; pH 7.4 and 37°C); furthermore, Hurley *et al.* (1993) found good aqueous stability for their prodrug, as an HCl salt, in distilled water at room temperature ($t_{1/2}$ > 96 h).



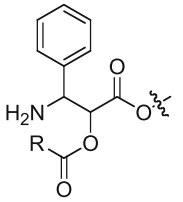
189
(Partial Structure)



190
(Partial Structure)

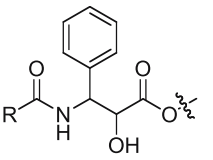
Structures 189-190.

Paclitaxel (**191**), an anti-cancer drug containing a β-hydroxyamide moiety, suffers from poor solubility and must be formulated using an irritating detergent (Cremophor EL). In response to this problem, Hayashi *et al.* (2003) synthesized an N,O acyl transfer prodrug (**192**) of paclitaxel, which displayed enhanced water solubility as an HCl salt (1800-fold increase). Prodrug **192** reconverted to paclitaxel quantitatively with a half-life of 15 min (pH 7.4, 37°C), but showed no degradation for 6 h in aqueous solution at pH 2 (37°C); moreover, the HCl salt of **192** showed no degradation in the solid state for a month when stored at 4°C. Finally, non-productive enzyme-mediated hydrolysis of the O-benzoyl group in **192** did not occur in the presence of porcine liver esterase, suggesting promise for quantitative *in vivo* prodrug reconversion. In a similar study, Skwarczynski *et al.* (2003) synthesized a water-soluble N,O acyl transfer prodrug (**194**) of canadensol



Prodrug (Partial Structure)

192 R = C_6H_5
194 R = $CH(CH_3)_2$



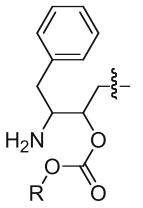
Drug (Partial Structure)

191 R = C_6H_5
193 R = $CH(CH_3)_2$

Structures 191-194.

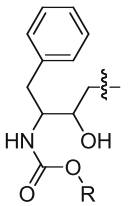
(**193**) (a taxoid derivative) that enhanced the aqueous solubility (pH 7.4) by a factor of 10. Prodrug **194** showed no degradation during 6 h at pH 2 (37°C) while displaying fast reconversion at pH 7.4 ($t_{1/2}$ = 4.3 min; 37°C).

Kazmierski *et al.* (2003) investigated the utility of N,O alkoxycarbonyl transfer prodrugs (**195-197**) for Amprenavir-based HIV protease inhibitors (**198-200**) containing a β-hydroxycarbamate moiety. However, the unsymmetrical dicarbonate prodrugs showed a lack of quantitative reconversion upon intramolecular aminolysis. More specifically, in 50/50 THF/phosphate buffer (pH 7.0 or 8.0), the prodrugs rearranged to form both the parent compounds and a non-productive 2-oxazolidinone side product (**201**).



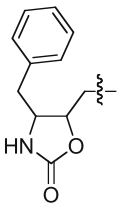
Prodrug
(Partial Structure)

195 R = CH_3
196 R = Allyl
197 R =



Drug
(Partial Structure)

198 R = CH_3
199 R = Allyl
200 R =



201
(Partial Structure)

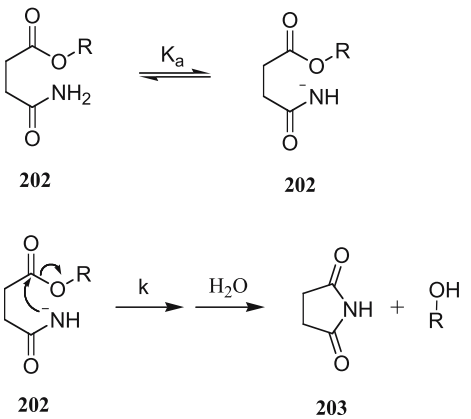
Structures 195-201.

In conclusion, N,O acyl transfer reactions can successfully create bioreversible prodrugs of NH-acids containing a β-hydroxyamide moiety. The O-acyl prodrugs typically possess greater solubility due to the ionizable amine. Furthermore, this design lessens toxicity concerns since a promoiety does not release into the body during the reconversion process. However, the utility of this approach exists only for NH-acids containing a β-hydroxyamide moiety; moreover, for fast

reconversion, the amine and ester groups in the O-acyl prodrug should have freedom from steric and electronic constraints, which are intrinsic to the original drug structure and, therefore, not subject to modification. Finally, for quantitative reconversion, the O,N acyl transfer reconversion reaction must happen significantly faster than the enzyme-mediated hydrolysis of the O-acyl moiety.

Acyclic Prodrugs of Cyclic Drugs

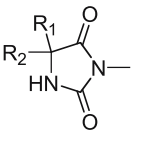
A number of drugs such as, but not limited to, hydantoins and barbituric acids, possess 5-, 6-, or 7-membered rings containing NH-acid functionalities. By hydrolyzing the NH-acid functionality, the ring can open, giving an acyclic structure with a terminal carboxylic acid and either a terminal amine (lactam parent compound) or amide-like functionality (cyclic imide-like parent compound). Esterification of the terminal carboxylic acid will yield an acyclic prodrug that possesses enhanced physicochemical properties and quick intramolecular reconversion under physiological conditions. Scheme 12 shows the bioreconversion of an acyclic prodrug (**202**) of a cyclic succinimide structure (**203**). The reconversion kinetics display base catalysis, giving pH rate profiles with a slope of ~1 in slightly acidic to basic solutions; furthermore, consistent with the proposed intramolecular mechanism, the kinetics show no sensitivity to buffer concentration or the presence of esterases. The reconversion rate depends on flexibility of the acyclic structure, nucleophilicity of the amide-like or amine terminus, and electrophilicity of the ester carbonyl carbon. Stella and Higuchi (1973c) specifically examined the flexibility factor of the acyclic structure on the cyclization rate in their study of various C_2-substituted hydantoic acids.

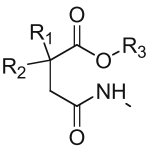


Scheme 12. The bioreconversion of a acyclic prodrug of succinimide.

Hydantoins possess a 5-membered ring structure containing an imide-like and amide-like NH-acid. Stella and Higuchi (1973b) synthesized acyclic prodrugs of 3-methylphenytoin (**204**) and mephenytoin (**205**) by hydrolyzing the hydantoin ring structures followed by esterification of the resulting terminal carboxylic acids

to either a simple alkyl ester (**206-207**) or a β-N,N-diethylaminoethyl ester (**208-209**). Prodrugs **208** and **209** showed improved aqueous solubility, at pH values below the amine pKa, and fairly quick reconversion rates ($t_{1/2}$ of 6.8 and 16.9 min, respectively; pH 7.4, 37°C); however, only fair aqueous stability existed for these prodrugs ($t_{1/2}$ of 10 and 28.5 h, respectively; pH 6.0, 25°C), suggesting the need for a reconstitutable parenteral formulation if given intraveneously. On the other hand, **206** and **207** had slower reconversion kinetics ($t_{1/2}$ of 57.3 and 73.0 min, respectively) at pH 7.4 and 37°C, making them less promising as prodrugs.



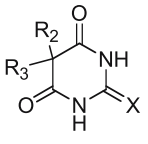


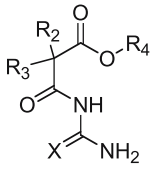
204 $R_1 = C_6H_5$ $R_2 = C_6H_5$
205 $R_1 = C_6H_5$ $R_2 = C_2H_5$

206 $R_1 = C_6H_5$ $R_2 = C_6H_5$ $R_3 = C_2H_5$
207 $R_1 = C_6H_5$ $R_2 = C_2H_5$ $R_3 = CH_3$
208 $R_1 = C_6H_5$ $R_2 = C_6H_5$ $R_3 = C_2H_4N(C_2H_5)_2$
209 $R_1 = C_6H_5$ $R_2 = C_2H_5$ $R_3 = C_2H_4N(C_2H_5)_2$

Structures 204-209.

Barbituric acids (**210**) consist of a 6-membered ring containing imide-like NH-acid functionalities. Bundgaard and associates (Bundgaard *et al.*, 1978, 1979a) studied a series of ring-opened acyclic prodrugs of different barbituric acids to establish an understanding of the electronic and steric effects of different substituents on the reconversion kinetics. The reconversion half-lives ranged from 8 to 395 min (pH 7.4, 37°C), with methyl 2,2-diallylmalonurate (**211**) reconverting the fastest and isopropyl 2,2-diethylmalonurate (**212**) reconverting the slowest (Bundgaard *et al.*, 1979a); however, **211** also displayed very limited aqueous stability ($t_{1/2}$ = 45 min; pH 7.4, 24°C). All of the acyclic prodrugs displayed increased lipophilicity over that of their barbituric acid parent compounds. In a subsequent study, Bundgaard *et al.* (1980) synthesized an acyclic prodrug of a barbituric acid with dramatically enhanced water solubility (75% w/v at 23°C as the HCl salt) using a β-N,N-dimethylaminoethyl ester (**213**). At pH 7.4 and 37°C, **213**





210 X = O
216 $R_2,R_3 = C_2H_5$ X = S

211 $R_2,R_3 = CH_2{=}CHCH_2$ $R_4 = CH_3$ X = O
212 $R_2,R_3 = C_2H_5$ $R_4 = CH(CH_3)_2$ X = O
213 $R_2,R_3 = C_2H_5$ $R_4 = C_2H_4N(CH_3)_2$ X = O
214 $R_2,R_3 = C_2H_5$ $R_4 = CH_3$ X = O
215 $R_2,R_3 = C_2H_5$ $R_4 = CH_3$ X = S

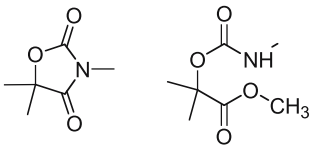
Structures 210-216.

reconverted quickly ($t_{1/2}$ = 2.8 min) relative to the simple methyl ester analog (**214**) ($t_{1/2}$ = 21 min) (Bundgaard *et al.*, 1978); furthermore, **213** showed a t_{90} at pH 4 and 23°C of 63 h, making it suitable for a reconstituted injectable formulation. Finally, Bundgaard *et al.* (1979b) studied methyl diethylthiomalonurate (**215**), an acyclic prodrug of thiobarbital (**216**) with enhanced lipophilicity, and found that it reconverted 42 times faster ($t_{1/2}$ = 0.5 min) than its oxy acyclic analog (**214**) ($t_{1/2}$ = 21 min) (Bundgaard *et al.*, 1978) at pH 7.4 and 37°C.

Bundgaard and Larsen (1979b) further showed the utility for acyclic prodrugs with their methyl ester acyclic prodrug (**218**) of trimethodione (**217**). Prodrug **218** possessed enhanced lipophilicity (log P = 0.41) over trimethodione (log P = 0.07) and reconverted quickly ($t_{1/2}$ = 0.8 min) at pH 7.4 and 37°C. However, Bundgaard and Larsen (1979a) did not witness the same quick reconversion for the methyl ester acyclic prodrugs (**221**-**223**) of phensuximide (**219**) and glutethimide (**220**), which displayed half-lives of 2 h (**221**-**222**) and 17 h (**223**), respectively, at pH 7.4 and 37°C.

Finally, Venuti *et al.* (1988) designed a series of esterified acyclic prodrugs of lixazinone that showed reconversion half-lives at pH 7.4 and 37ºC ranging from 2 to 347 min. Unfortunately, even the most promising of the prodrug series showed 3 to 10 times less potency than lixazinone, following intravenous and i.d. administration, respectively.

In conclusion, esters of ring-opened derivatives can serve as prodrugs for cyclic NH-acids. The steric and electronic properties in the termini and backbone of the prodrug influence the cyclization speed of the prodrugs. Finally, esterification of the terminal acid allows flexibility in prodrug design; for example, the



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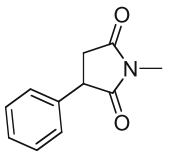
218



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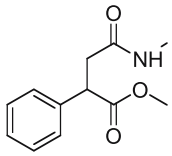

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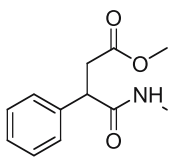

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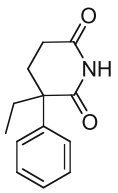

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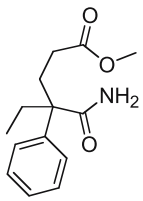


223

Structures 217-223.

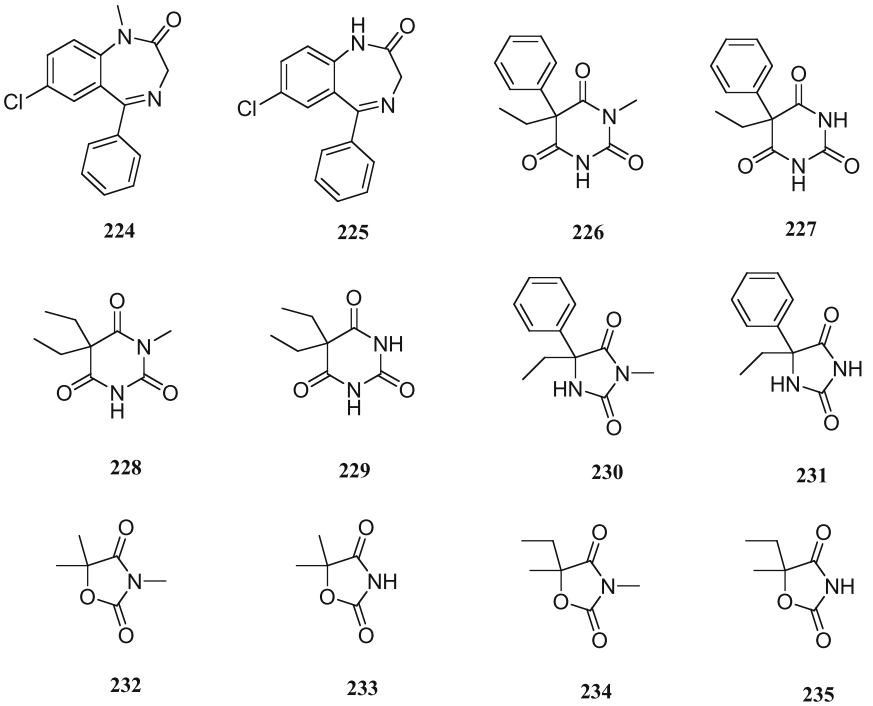
acyclic prodrugs can be designed to enhance either lipophilicity or aqueous solubility, depending on the properties of the ester chosen.

Redox Prodrugs

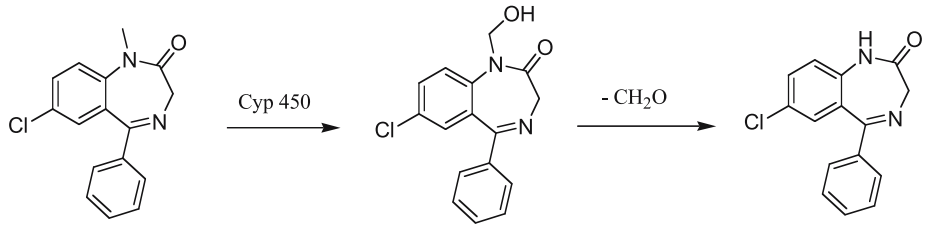
Unlike the previous sections of this chapter, the objective of this final section is to be illustrative, not comprehensive, as a full treatment for this topic could easily require a chapter unto itself. Historically, there has been significantly less effort given to the rational design of redox prodrugs. By including a few illustrative examples, it is hoped that this section will spark more interest and encourage researchers to further explore and develop the area of redox prodrugs.

A redox prodrug is defined, for the purposes of this section, as a prodrug designed to undergo an enzymatically mediated redox reaction to form the drug. The redox reaction can cause either a reduction or an oxidation of the prodrug, depending on the structure of the prodrug. The compounds included as examples in this section are not prodrugs in the truest sense of the word since they possess intrinsic therapeutic activity. However, much can still be learned from their metabolic transformations and applied to the design of true redox prodrugs of NH-acids.

One typical redox reaction that can occur *in vivo* is N-demethylation to form an NH-acid. Some examples of this reaction are diazepam (**224**) to desmethyldiazepam (**225**), mephobarbital (**226**) to phenobarbital (**227**), metharbital (**228**) to barbital (**229**), methoin (**230**) to 5-ethyl-5-phenylhydantoin (**231**), trimethadione

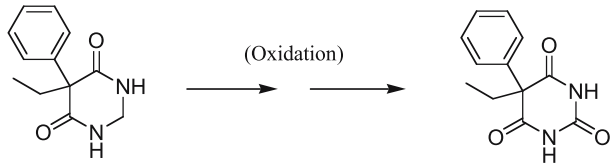


Structures 224-235.



224 **225**

Scheme 13. The bioconversion of diazepam to desmethyldiazepam.



236 **227**

Scheme 14. The bioconversion of primidone to phenobarbital.

(**232**) to 5,5-dimethyl-2,4-oxazolidinedione (**233**), and paramethadione (**234**) to 5-ethyl-5-methyl-2,4-oxazolidinedione (**235**) (Butler and Waddell, 1958; de Silva *et al.*, 1966; Eadie, 1991). As shown in Scheme 13 for diazepam, these N-demethylation conversion reactions occur by initial oxidation of the methyl group to form a hydroxymethyl derivative, followed by loss of formaldehyde to give the NH-acid (in this case, desmethyldiazepam). When thinking of the above examples in the context of prodrugs, it is interesting to point out what Butler and Waddell (1958) wrote as part of the concluding sentence in their article concerning the N-demethylation metabolism of various N-methylated anticonvulsant drugs: ". . . the question is raised whether for practical purposes the clinical use of one of these methylated drugs amounts only to a devious means of administering the corresponding non-methylated compound."

Another oxidation reaction that could potentially have utility for delivering NH-acids is oxidation of a methylene group to a carbonyl that is adjacent to an NH-group, such as that found in primidone (**236**). As shown in Scheme 14, **236** is oxidized to phenobarbital (**227**) *in vivo* by conversion of its methylene group to a carbonyl (Eadie, 1991; El-Masri and Portier, 1998). Therefore, phenobarbital can be formed either by N-demethylation of mephobarbital (**226**), as discussed in the previous paragraph, or by oxidation of the cyclic methylene group of primidone (**236**) to a carbonyl.

In summary, the above examples illustrate the potential for exploiting metabolic redox processes to convert redox prodrugs into their parent NH-acids. As mentioned before, the area of redox prodrugs is not nearly as developed as that of the more traditional prodrug approaches that comprise the bulk of this chapter. However, hopefully, as metabolic processes continue to be better understood, more researchers will be encouraged to explore the potential of redox prodrugs.

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3.5

Prodrugs of Benzamidines

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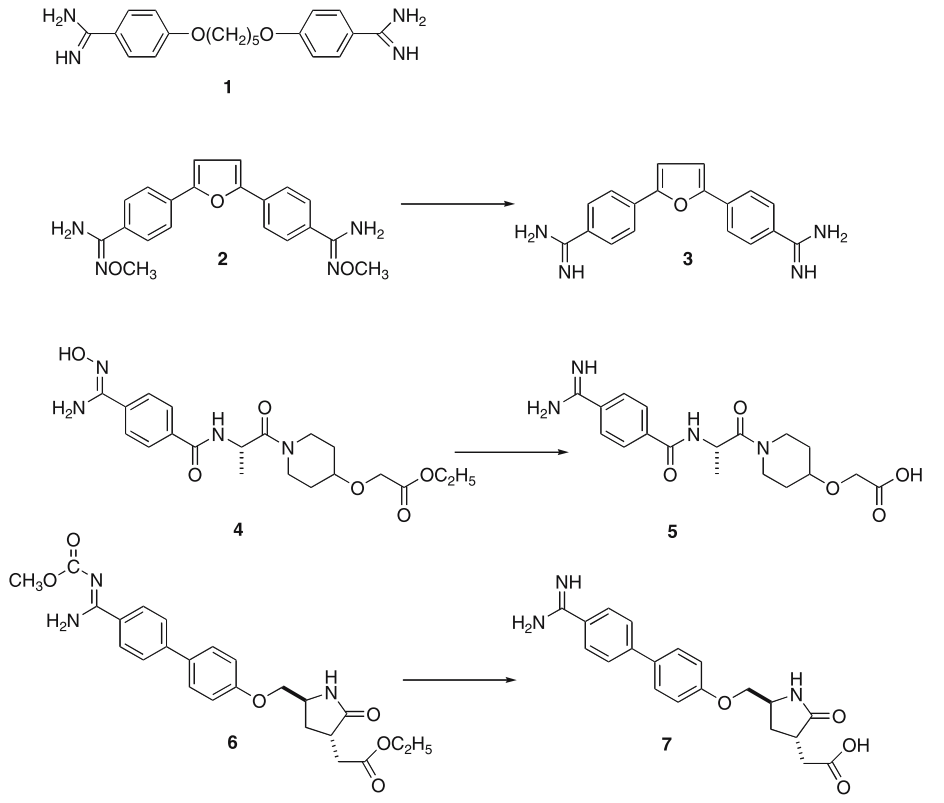
Table of Contents

List of Abbreviations

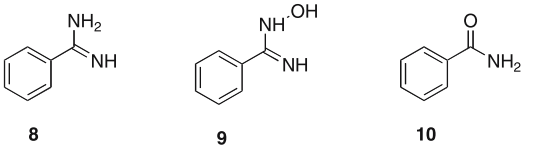
ADP.....adenosine diphosphate
i.p. .....intraperitoneally
i.v. .....intravenously
nm.....nanometer
NO.....nitrous oxide
p450.....cytochrome p450
p.o. .....orally
PCP.....*Pneumocystis carnii* pneumonia

Introduction

Benzamidines figure prominently as pharmacophores in several classes of drugs. These include anti-infectives such as anti-parasitic (Clement and Raether, 1985), anti-bacterial (Lamb and White, 1939), and anti-malarial (Leban *et al.*, 2004) agents, alphaIIb-betaIIIa integrin antagonists (Weller *et al.*, 1996), and inhibitors of the proteases factor VIIa (Parlow *et al.*, 2003) and factor Xa (Pinto *et al.*, 2001). Several have demonstrated utility in human studies; among these, pentamidine (**1**) is a prototype of a class of DNA minor grove binding agents that are effective for the treatment of African trypanosomiasis and leishmanisis as well as the opportunistic infection *pneumocystis carnii* pneumonia prevalent among AIDS patients. Pentamidine, which is administered by aerosol, was introduced to the United States market for *p. carnii* in 1989. Furamidine (**2**) is currently undergoing clinical trials as an orally active prodrug of the corresponding bis-amidine (**3**) for the same indication. Among others, sibrafiban (**4**) (Cases *et al.*,



Structures 1-7.



Structures 8-10.

1999; Dooley and Goa, 1999) and lefradafiban (**6**) (Muller *et al.*, 1997) are orally active prodrugs for the benzamidines **5** and **7**, which are clinically efficacious as platelet aggregation inhibitors.

Benzamidines are strongly basic; the parent, benzamidine (**8**), has a pKa of 11.6 (Albert *et al.*, 1948). Although it is possible to modulate the basicity of benzamidines somewhat by substitution on the aromatic ring, the resulting molecules remain strongly basic and fully protonated at physiologic pH. Membrane permeation is generally poor and, thus, considerable effort has been devoted to the discovery of suitable prodrugs with improved physical chemical properties to enable the oral delivery of benzamidines. Considerable effort has also been expended to find isosteric replacements for benzamidines, and the literature associated with each target class should be consulted to seek examples.

The most common approach has been to prepare the corresponding hydroxyamidines. For a review see (Clement, 2002). For example, the pKa of N-hydroxybenzamidine (**9**) is only 4.8–5.0 (Albert *et al.*, 1948; Pearse and Pflaum, 1959). Other approaches, all intended to reduce the basicity of the parent molecule to facilitate membrane transport have been less commonly applied and include formation of alkoxybenzamidines, acyl benzamidines, and alkoxycarbonyl benzamidines.

N-Hydroxybenzamidines

Metabolism

Hydroxybenzamidines are efficiently reduced to the corresponding benzamidines by liver microsomes and are subject to reduction by the microsomal fractions from other tissues as well (Clement, 2002). Oxidation of benzamidine (**8**) (Clement *et al.*, 1988a,c) and pentamidine (**1**) by liver microsomes has also been observed; however, the reverse reaction predominates *in vivo* (Berger *et al.*, 1990; Clement *et al.*, 1992; Clement and Jung, 1994). Components capable of efficiently carrying out the reduction of N-hydroxybenzamidine (**9**) have been reconstituted from pig liver and consist of cytochrome b5, NADH cytochrome b5 reductase, a member of the p450 family and phosphatidylcholine (Clement *et al.*, 1997). The specific p450 enzyme involved is not one of the major oxidative metabolism enzymes but a member of the 2D family (Clement, 2002). This system is also capable of reducing other oxygenated amines, including N-hydroxyguanidines and N-hydroxyamidinohydrazones, although they are considerably poorer substrates than benzamidines.

Although **9**, the simplest member of the N-hydroxybenzamidine family, has been shown to cause DNA single strand breaks in rat hepatocytes and DNA amplification in SV-40 transformed hamster cells (Clement *et al.*, 1988b), neither the N-mono- nor the N,N'-di-hydroxylated analogues of pentamadine (**1**) are active in the Ames test, perhaps due to their efficient reduction to the parent amidine (Berger *et al.*, 1990).

Other possible fates of N-hydroxybenzamidines include phase 2 metabolism to conjugates subject to rapid elimination and further oxidation to form the corresponding amide and nitrogen oxides. Clement *et al.* (2001) examined the fate of N-hydroxybenzamidine **9** in a pig hepatocyte culture and have shown that in addition to the expected reduction product **8**, glucuronidation and, to a lesser extent, sulfation products were formed. Both were inactive in Ames tests employing the TA98 and TA100 strains.

Rat liver microsomes catalyze the reduction of hydroxybenzamidine (**9**) to the corresponding amide **10**. The reaction requires oxygen and NADPH. The basal rates are low, but are accelerated 10-fold by induction of the 3A family cytochrome p450 enzymes by pretreatment of the rats with dexamethasone. It is inhibited by troleandomycin, further supporting the role of the 3A family of p450 enzymes (Andronik-Lion *et al.*, 1992; Jousserandot *et al.*, 1995; Mansuy *et al.*, 1995). A limited number of examples indicate that N-hydroxyguanidines are significantly better substrates for this oxidation than N-hydroxybenzamidines (Mansuy and Boucher, 2002). Comparison of the rates of oxidation of 4-chloro-N-hydroxybenzamidine, N-hydroxybenzamidine, and 4-hexyloxy-N-hydroxybenzamidine suggests that the presence of the electron-donating ether substitution increases the rate of oxidation 2–3-fold (Jousserandot *et al.*, 1995).

Thus, although N-hydroxybenzamidines are generally efficiently reduced *in vivo* to the corresponding benzamidines, other pathways are possible and may be competitive, depending on the particular substrate and host species.

Bis-N-hydroxybenzamidines

The anti-trypanocidal activity of bis-benzamidines and bis-N-hydroxybenzamidines in mice was first reported by Lamb and White (1939). Pentamidine (**1**) was later introduced to the US market for the prevention and treatment of *pneumocystis carnii* pneumonia (PCP), but is limited to administration by aerosol or intravenous injection. Off-label indications include Leishmaniasis and Trypanosomiasis African (African sleeping sickness). The bis-hydroxyamidine analogue of pentamidine was also reported to be active, but less potent than the parent drug in mice and hamsters infected with parasites (Clement and Raether, 1985) and inactive *in vitro*.

A systematic study of pentamidine and a series of pairs of homologous bis-benzamidines with the corresponding bis-(N-hydroxybenzamidines) by the oral and intravenous routes in a rat model of infection by *Pneumocystis carnii* is summarized in Table 1 (Hall *et al.*, 1998). All of the unsubstituted benzamidines reported in the table were active by the intravenous route, indicating that within this series the parent molecules were effective anti-*Pneumocystic* agents, but only one of them, the 2,2'-dimethoxy derivative **16**, had any appreciable oral activity. The corresponding bis-N-hydroxybenzamidines **11**, **13**, **15**, and **17** were active by both the oral and intravenous routes and were as effective as the parent molecules **1**, **12**, **14** and **16**. Compound **11** is converted by the 9000 × g supernatants from rat liver, kidney, lung or heart to a mixture of the bis-benzamidine **1** and the

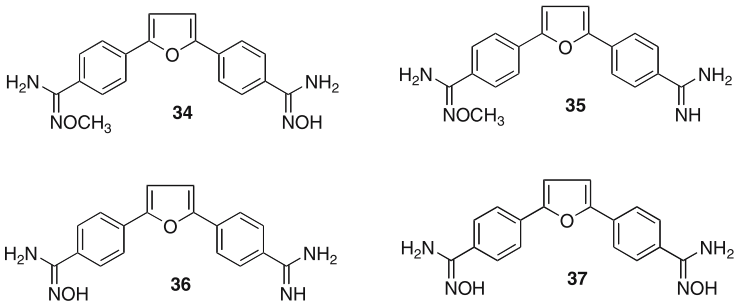
mono-N-hydroxybenzamidine **18**. Compounds **11**, **13**, **15** and **17** were converted by rat hepatocytes to mixtures of benzamidines and mono-hydroxybenzamidines. The 2,8-bis-(hydroxyamidino)dibenzofuran **31** was converted to the bis-amidine **30** by hepatocytes less efficiently than was the bis-N-hydroxypentamidine **11** and, although orally active, was less effective *in vivo* after intravenous administration than **30** (Wang *et al.*, 1999).

In contrast, the mono-N-hydroxybenzamidines **18** and **19** were inactive orally, but were active intravenously, suggesting that the presence of a single unmodified amidine in a molecule is incompatible with oral absorption. Other bis-N-hydroxybenzamidines **21**, **23**, **25**, **27** and **29** were not active by either route. These molecules did undergo reduction in cell-free rat liver microsome preparations, verifying that they are capable of conversion to the active species; however, only minimal conversion was observed when they were incubated with rat hepatocytes. It appears that they suffer from poor cellular uptake or that, once reduced, the parent molecule is trapped within the cell, preventing its release into the medium. The structural features responsible for this failure are not obvious, although the increase in the number of hydrogen bond donors and the polar surface area associated with these species is suspected to contribute.

Bis-N-alkoxyamidines

In related work, a small series of bis-(N-alkoxy-2,5-benzamidino)-substituted furan derivatives derived from **3** was investigated in a similar manner for treating rats infected with *Pneumocystis carnii* (Boykin *et al.*, 1996). The data from this study are summarized in Table 2. The parent bis-benzamidine **3** was more potent than pentamidine in this model, and both the N-bis-hydroxy (**32**) and particularly the N-bis-methoxy (**2**) analogues were highly effective after oral administration. The latter compound is being studied clinically as an orally active treatment for *Pneumocystis carnii*. Surprisingly, the homologous ethoxy derivative **33** was devoid of activity by either route in this study and, in subsequent studies, offered only weak protection in a mouse model of Trypanosomiasis (Ansede *et al.*, 2004).

In Caco-2 cells, the bis-amidine **3** was poorly able to permeate (permeability coefficient, Papp = 3.8×10^{-7} cm/s) and was sensitive to Ca^{2+} concentration in the media, suggesting transport via the paracellular route (Zhou *et al.* 2002a). The bis-methoxyamidine **2** (Papp 6.04×10^{-5} cm/s) has good permeation and was somewhat more able to permeate than was the ethoxy homolog **33** (Papp 0.59×10^{-5} cm/s); higher homologues were only minimally able to permeate (Ansede *et al.*, 2004). Preliminary metabolism studies of **2** indicate stepwise formation of the parent via sequential O-dealkylation and reduction steps involving the formation of **34–37** (Zhou *et al.*, 2002b; Anbazhagan *et al.*, 2003). Tellingly, studies in human microsomes indicate that **33** ($t_{1/2}$ 35 min.) is metabolized significantly more slowly than the methoxy derivative **2** ($t_{1/2}$ 4.2 min.) and that the predominant pathway is hydroxylation on the beta-carbon of the ethoxy moiety rather than dealkylation (Ansede *et al.*, 2004). Thus, in this series, only the methoxy- derivative **2** is a suitable prodrug for the amidine **3**.



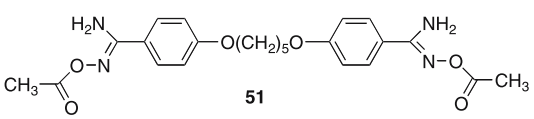
Structures 34-37.

Additional examples of N-alkoxybenzamidines from a related aza-series are available. Data on duration of survival of mice after infection with Trypanosome b. rhodesiense in comparison with pentamidine, **2**, and **3** are summarized in Table 3 (Ismail *et al.*, 2003). The bis-benzamidines derivatives **38**, **42**, **45**, and **48** were highly effective *in vitro* against trypanosome *b. rhodesiense* whereas, as expected, the candidate prodrug N-hydroxy and N-alkoxy analogues **39–41**, **43–44**, **46–47**, **49–50** were >1000-fold less potent (data not shown). *In vivo*, all of the benzamidines offered protection from infection when administered i.p., and the corresponding prodrugs, with the exception of the bis-hydroxyamidine **46** and **49–50**, had oral activity. In general, the bis-(N-alkoxyamidines), including bis-(ethoxyamidine) **41**, were more effective than the corresponding bis-(hydroxyamidines).

To summarize the data in Tables 1-3, a variety of bis-benzamidino derivatives are active as anti-parasitic and anti-infective agents, but are generally not orally bioavailable. The corresponding bis-(N-hydroxybenzamidines) are reliably reduced to the parent bis-benzamidines in cell-free systems and some, but not all, are efficiently reduced by hepatocytes as well. In several cases, bis-(N-alkoxybenzamidines) seem to serve as better prodrugs for benzamidines than the corresponding bis-(N-hydroxybenzamidines), perhaps because of their enhanced lipophilicity and reduced number of hydrogen bond donors, facilitating cellular uptake. Unfortunately, the authors of these papers did not report the pharmacokinetic properties of these molecules, so conclusions must be drawn from outcomes of experimental infections in mice and rats.

Bis-(N-acetoxybenzamidines)

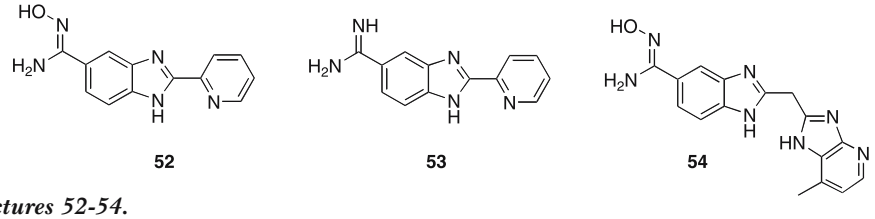
A German patent reports on the favorable oral activity of the bis-(N-acetoxybenzamidino) pentamidine analog **51** in a rat model of *Pneumocystis carnii* infection in comparison with the same dose of Pentamidine given intramuscularly (Clement, 1995). The details of the prodrug conversion were not elucidated.



Structure 51.

N-Hydroxybenzamidines as Protease Inhibitors and Integrin Antagonists

A number of serine protease inhibitors related to trypsin including thrombin, factor Xa, and Factor VIIa have a requirement for a benzamidine or benzamidine mimic to bind to the P1 site, which recognizes an arginine in the native substrate. Caco-2 permeability of prototype Factor Xa inhibitors showed that the apparent permeability (Papp) of the prodrug N-hydroxyamidinobenzimidazole **52** was 232 $\times$ 10^{-4} cm/s in the apical to basolateral (A to B) direction versus 3.4 $\times$ 10^{-4} cm/s for the parent amidinobenzimidazole **53**. Furthermore, the permeability of **52** in the basolateral to apical (B to A) direction was only 112 $\times$ 10^{-4} cm/s whereas other analogues were poorly transported in the A to B direction (**54**, for example, had a Papp in the A to B direction of 2 $\times$ 10^{-4} cm/s and a Papp in the B to A direction of 40 $\times$ 10^{-4} cm/s) and were substrates for efflux enzymes (Schipper *et al.*, 2001). These findings highlight the need for each candidate prodrug to be carefully profiled in relevant cellular and *in vivo* models.



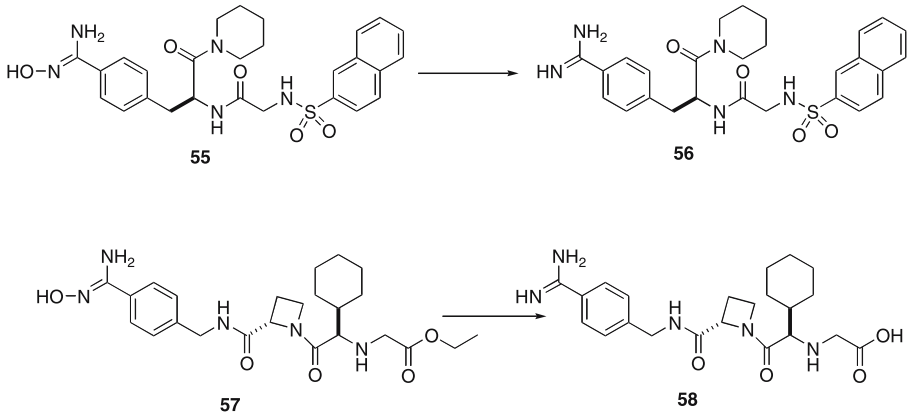
52 **53** **54**

Structures 52-54.

An early study with the N-hydroxybenzamidine **55** showed that it was approximately 100-fold less potent as a thrombin inhibitor than the corresponding benzamidine **56** and was converted to **56** by 9000g rat liver homogenates. Intraportal administration of **55** to either rats or rabbits led to the detection of both **55** and **56** in a 1:1–3 ratio in the bile after 4 h (Hauptmann *et al.*, 1988).

The conversion of the double prodrug ximelagatran (H37695) (**57**) of the thrombin inhibitor melagatran (H31968) (**58**) has been studied in more detail in animals and humans. Caco-2 permeation by the active thrombin inhibitor **58** is only 3 $\pm$ 1 $\times$ 10^{-4} cm/s versus 240 $\pm$ 70 $\times$ 10^{-4} cm/s for the double prodrug, suggesting that the prodrug would have superior oral bioavailability (Sorbera *et al.*, 2001). *In vitro* studies show that various systems, including human and pig liver and kidney microsomal fractions as well as mitochondrial fractions, are capable of converting **57** to **58**. Although differing fractions are relatively more efficient for ester hydrolysis or hydroxyamidine reduction, both reactions are generally fast and the individual intermediates were only seen transiently (Clement and Lopian, 2003). These findings have been extended to human studies. In a phase I study, healthy males were treated with **57** orally or intravenously. In both cases, conversion to the active thrombin inhibitor **58** was rapid, although minor amounts of **57** as well as the two possible intermediate metabolites were detected. The bioavailability of **58** in man is only 5.8 $\pm$ 2.3% whereas the bioavailability of **58** following oral dosing of **57** in man was approximately 20% (Erickson *et al.*, 2000;

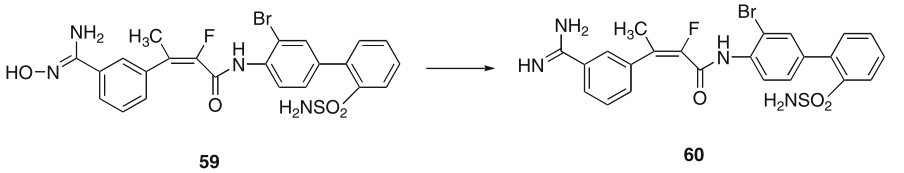
Gustafsson *et al.*, 2001; Sorbera *et al.*, 2001); thus, the use of the prodrug strategy improved bioavailability in man by 2.5–3-fold.



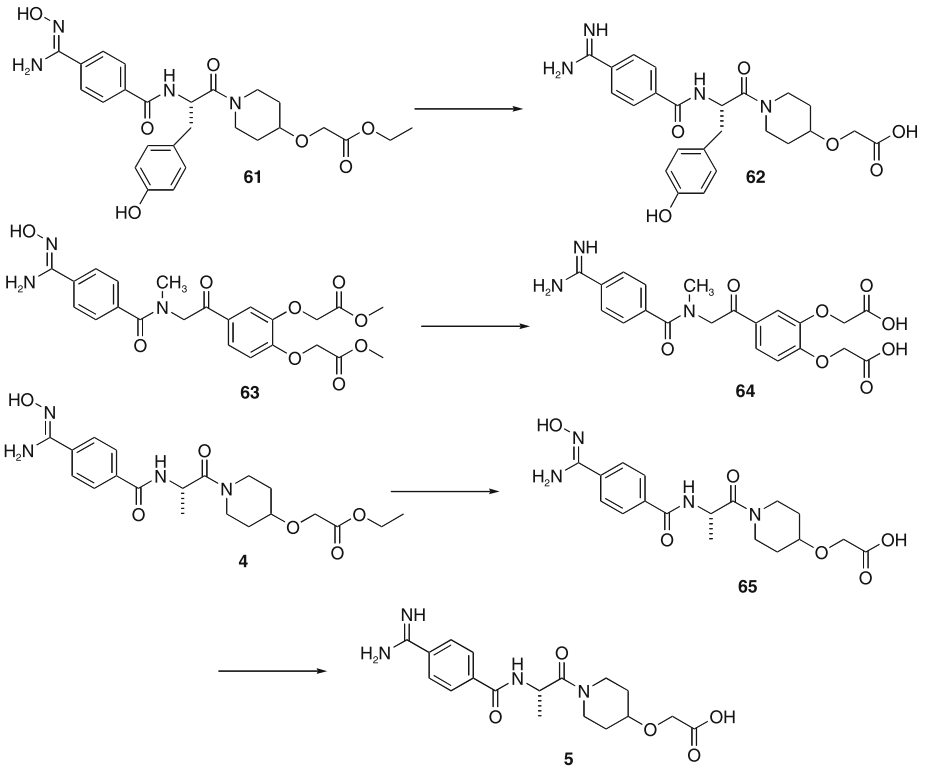
Structures 55-58.

N-Hydroxyamidines have also been employed in an effort to improve the bioavailability of a series of factor Xa inhibitors based on the potent acrylic amide derivative **60**. Selected from a small series of halogen-substituted analogues, the hydroxybenzamidine **59** had good oral bioavailability in the rat (52%), but the plasma level of unchanged drug exceeded that of the active moiety and conversion amounted to only about 20% (Song *et al.*, 2003).

Closely related structures have been investigated as fibrinogen GPIIb-IIIa receptor antagonists. The benzamidines **62**, **64**, and **5** are each potent antagonists with low oral bioavailability in mice. The oral bioavailability of **62** in rats is about 1%, and masking the carboxylic acid or benzamidine individually did not improve oral absorption. Compound **62** (lamifiban) has also been studied in humans as a intravenously administered platelet aggregation inhibitor for the treatment of acute coronary syndrome (Dooley and Goa, 1999; Harrington, 2002). Masking both functional groups of these molecules simultaneously in the form of the N-hydroxybenzamide ethyl esters **61**, **63**, and **4** led to several-fold improvement in oral exposure in mice (Weller *et al.*, 1996). Compound **4** (sibrafiban) was studied in more depth and has been tested in humans. In rats, ester hydrolysis to give **65** is faster than N-hydroxybenzamidine reduction; after oral dosing with **4**, peak plasma levels of **65** occur early, and it is quickly eliminated by conversion to **5** ($t_{1/2}$ = 0.35 h) whereas **5** has a relatively long half-life. Pharmacokinetic parameters of **4** in rat, dog, and rhesus monkey are shown in Table 4; they indicate relatively good exposure of **5** in all three species. In man, doses of 5–15 mg led to peak



Structures 59-60.



Structures 61-65.

plasma concentrations of the active metabolite at 6 h, and bid dosing afforded continuous inhibition of platelet aggregation (Merlos and Graul, 1998).

In another set of examples, summarized in Table 5, a series of potent spirocyclic GPIIb-IIIa antagonists were similarly protected as bis-prodrugs using a strategy identical to that employed for **4**. Compounds were initially profiled in rat, and those having the best exposure were further tested in dogs and cynomologous monkeys. Like other classes of drug candidates, actual exposures among these compounds are subtly dependent on structure and not easily predicted. The data in Table 6, summarizing the pharmacokinetic data for the active metabolites of **66–68**, indicate that these compounds, particularly **67**, have good exposure, half-life, and clearance in both species (Mehrotra *et al.*, 2004).

N-Alkoxycarbonyloxybenzamidines and N-Acyloxybenzamidines

Due to potential unfavorable membrane transport properties imparted by the presence of a hydroxy group in the N-hydroxybenzamidines, attempts to mask it by acylation have been made. In addition to the example of a bis-(N-acetoxy)benzamidine cited earlier, acyloxy derivatives of N-hydroxybenzamidines of interest as platelet aggregation inhibitors were also investigated. The acyl derivatives **73–75** were considered as potentially improved prodrugs for the GPIIa-IIIb receptor antagonists **62**, **64**, and **5**. Based on an assessment of their

oral antiplatelet activity in mice, the triple prodrugs **73** and **74** were comparable to the corresponding N-hydroxy analogues **61** and **63**. The butoxycarbonyloxy derivative **75** was highly effective in the mouse model and was evaluated further. Bioavailability of the active metabolite **5** after oral administration of **75** to rats, dogs, and rhesus monkeys was 9.8, 10.1, and 4%, respectively, and comparison with the data in Table 4 indicates that this modification is clearly inferior as a prodrug to the simpler N-hydroxybenzamidine ethyl ester **4** (Weller *et al.*, 1996).

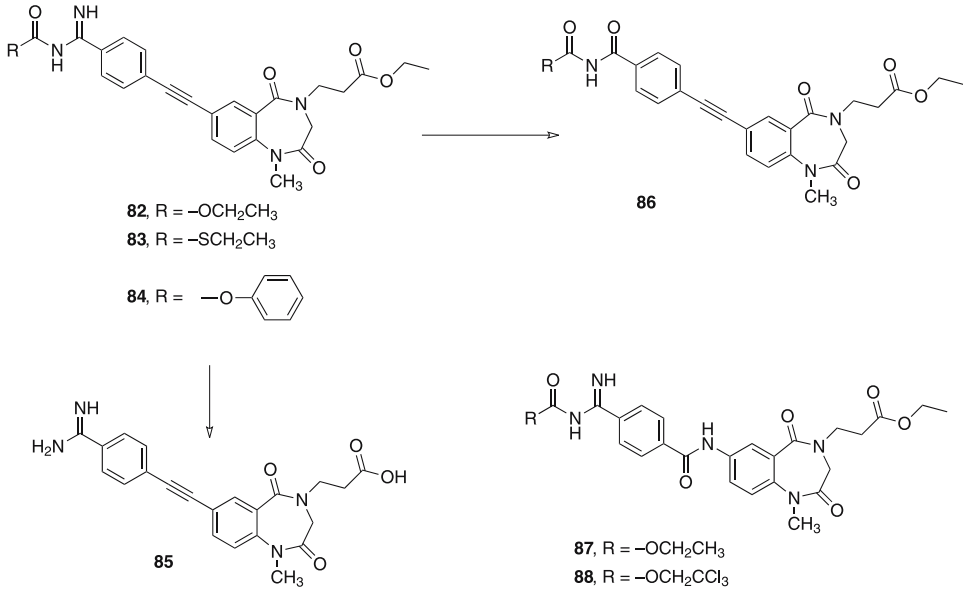
The benzamidine monoester **76**, whose structure is shown in Table 7, is also of interest as a potent GPIIa-IIIb receptor antagonist. In order to improve on its limited oral bioavailability, the N-hydroxybenzamidine **77** and a number of acyloxybenzamidines **78–81** were examined in an *ex vivo* guinea pig model of ADP-induced platelet aggregation. None of these candidate prodrugs had appreciable intrinsic anti-platelet activity; however, the methoxycarbonyloxy (**78**), the methylthiocarbonyloxy (**79**), and the ethylcarbamyloxy (**81**) derivatives all showed good *ex vivo* inhibition of platelet aggregation after oral administration to guinea pigs. The time course of activity for these three compounds is shown in Table 8. From consideration of its relatively rapid onset and appreciable activity 24 h after dosing, **78** was considered to be the most interesting for further investigation. In the guinea pig, the oral bioavailability of the parent molecule was improved from 2.8 to 5.3% by administration of the prodrug **78** (Kitamura, 2001).

N-Acyl- and N-Alkoxycarbonylbenzamidines

Genentech workers have examined the physical properties and chemical stability of the alkoxycarbonyl derivatives **82–84** in gastrointestinal fluids and rat plasma. Typical of this class, the pKa of **82** is 5.6, consistent with a high percentage of the neutral species under physiological conditions. The ethyl carbamate **82** is relatively stable under basic conditions; however, rate constants for hydrolysis of analogues with better leaving groups like the alkylthiocarbonyl and the phenoxycarbonyl derivatives **83** and **84**, respectively, are 25- and 95-fold higher, respectively, at pH 9. These compounds also undergo acid-catalyzed degradation to the imide **86**, particularly at pH <6, suggesting a limitation of incorporating better leaving groups into alkoxycarbonyl prodrugs of amidines. The authors of this study estimate that during gut transit (6.5 hr) chemical hydrolysis of **82** (R = ethoxy) would amount to 5%, whereas that of **84**, R = (phenoxy) would amount to 30%. These compounds are subject to enzymatic degradation as well. Exposure of **82** and **84** to gavage fluid obtained from rabbit jejunum led to complete conversion of the latter compound in < 1 min; this was in contrast to **82**, which is estimated to have a half-life of 20 min. Interpretation of these studies must be done with care since they are double prodrugs and ester hydrolysis occurs in parallel with N-carbamoyl degradation.

Compound **82** was stable in rat plasma and, after i.v. administration to rats, showed only 5% bioconversion to **85** over 30 min whereas **84** underwent complete

conversion in this time frame. Despite the differences in stability, oral bioavailability of **85** after oral administration of either **82** or **84** in rats was only about 5% (Shahrokh *et al.*, 1998). Oral bioavailability of the related ethoxycarbonyl analog **87** in dogs was also 5%, while that of the more labile trichloroethoxycarbonyl derivative **88** was 29% at 5 mg/kg but fell to 11% at 14 mg/kg (Blackburn *et al.*, 1998).



Structures 82-88.

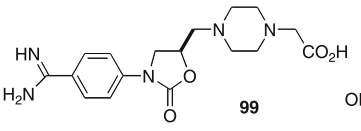
Rahmathullah *et al.* (1999) have reported on the oral and intravenous activity of a series of carbamate analogues of the bis(benzamidine) **3** in rats infected with *Pneumocystis carnii*. The data, which are summarized in Table 9, indicate that all of the tested carbamates except the N-alkylthiocarbonyl derivative **91** were active when administered intravenously, suggesting that they are all effectively converted *in vivo*. The phenoxycarbonyl analogues **94–96** had somewhat better oral efficacy than simple methoxycarbonyl- (**89**) or benzyloxycarbonyl- (**92**) substituted species after oral administration. The related acetal **97** and the carbonate **98** were also active by the intravenous route, but had only limited oral activity. For those compounds with poor oral activity, the problem is likely stability in gastric or intestinal fluids or, possibly, poor absorption.

Alkoxycarbonyl derivatives can serve as useful prodrugs for benzamidines in man. The methoxycarbonyl methyl ester **6** (lefradafiban, BIBU104) is efficiently converted to the active platelet aggregation inhibitor **7** (fradafiban, BIBU 52) after oral administration as determined by monitoring the plasma concentrations of **7** and by *ex vivo* platelet aggregation studies. Doses of 10–150 mg of **6** give plasma drug levels corresponding to intravenous doses of 1–15 mg of **7**.

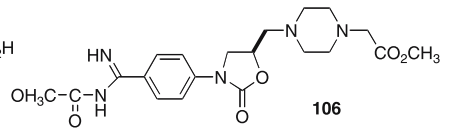
The benzamidine **99** (EMD 76334) is a very potent GPIIb-IIIa antagonist active after i.v. administration in guinea pig and monkey models of *ex vivo*

collagen-induced platelet aggregation; it was tested clinically by the intravenous route. Neither **99** nor its N-methoxycarbonyl- or N-benzoyl- mono-prodrugs had appreciable oral bioavailability, and the series of bis-prodrugs shown in Table 10 were prepared. Compounds were compared on the basis of their ability to inhibit collagen-induced platelet aggregation *ex vivo* in both species after oral dosing. Both N-aryl carbonyl and N-alkoxycarbonyl derivatives were orally active in the guinea pig, but only the N-alkoxycarbonyl substituted compounds, particularly **106** (EMD 122347), were efficacious in both species (Gante *et al.*, 1996).

The potent lipophilic leukotriene B4 receptor antagonist **112** (BIIL 284, amelubant) is absorbed and rapidly hydrolyzed to its active metabolite **113** (BIIL 315) and its corresponding biologically active glucuronide (BIIL 260) after oral dosing in mice, guinea pigs, and monkeys. It is active in several animal models of LTB4-induced inflammation with an oral dose of 0.3 mg/kg providing blockade of monkey LTB4 receptors lasting 24 h (Birke *et al.* 2001). In a clinical pharmacology study, rheumatoid arthritis patients were treated with doses of 25 or 150 mg of **112**, given once a day for 14 days. Maximum plasma exposure to the active metabolite **113** occurred 4 h after dosing and was dose-proportional. Complete inhibition of LTB4-induced expression of the integrin CD11b/18 (Mac-1) was achieved 2 h after the 150 mg and 4 h after the 25 mg dose (Alten *et al.*, 2004).

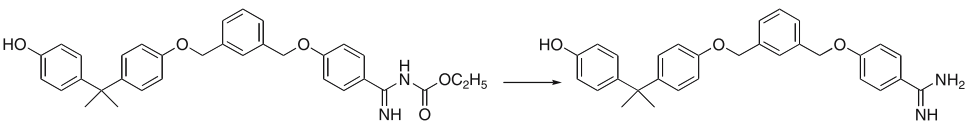


99



106

Structures 99, 106.



112 **113**

Structures 99, 106.

Oxadiazolidinones, Thiadiazolidinones, and 5-Membered Heterocycles Derived from Hydroxybenzamidine

The report that benzamidine **115** was observed as a metabolite of the angiotensin II receptor antagonist **114** in humans (Kohara *et al.*, 1995; 1996) prompted Kitamura *et al.* (2001) to explore the oxadiazolidinones, thiazolidinones, and other 5-membered hetereocyclic rings derived from hydroxybenzamidine **77** (shown in Table 7) as potential prodrugs for the potent integrin Iib-IIIa antagonist **76**. Incubation of the oxadiazoldinone **116** or the corresponding thiadiazolidinone **118** with guinea pig liver homogenate for 1 hour led to formation of 15 and 39%, respectively, of the benzamidine **76**; however,

there was no conversion when **116** was incubated with guinea pig plasma or small intestine homogenate.

Based on the finding that metabolic conversion to the desired benzamidine was feasible, **116** was evaluated for oral activity in guinea pigs using an *ex vivo* platelet aggregation assay to determine parent drug activity in plasma. Pharmacokinetic data indicate that C_{max} for **76** occurred at 2 h and was not detectable 8 h after dosing with **116**. A series of analogues was prepared as summarized in Table 11, which describes the *ex vivo* ADP-induced platelet aggregation inhibition observed after oral administration of candidate prodrugs of **76** to guinea pigs. A 0.3 mg/kg oral dose of **76** inhibited ADP induced platelet aggregation by a maximum of 65% 2 h after dosing, and inhibition fell precipitously thereafter. On the other hand, oral administration of the same dose of **116** led to a maximal 91% inhibition of ADP-induced platelet aggregation, and inhibition was still 38% after 24 h. While the authors did not report pharmacokinetic data to support conversion of **116** *in vivo*, they presume the observed effects are due to conversion to the active parent **76** since the unchanged oxadiazolidinone **116** itself is inactive as an inhibitor of aggregation.

Since the investigators sought compounds with a more rapid onset, they further investigated the thiadiazolidinone **118** (Table 7) as well as the other 5-membered heterocyclic derivatives shown in Table 11. The most efficacious as determined by percent inhibition of platelet aggregation 2 h post dosing was the thioxadiazolindone **120**. Both the 5-trifluoromethyl- and 5-cyanooxadizoles **123** and **125** were as effective as **116** in this setting. Interestingly, they were markedly superior to the 5-methyl- (**121**), 5-phenyl- (**122**), or 5-methoxy- (**126**) analogues, suggesting that the presence of an electron-withdrawing group facilitated conversion to the active metabolite. As a note of caution, the corresponding methoxycarbonyl derivative **78** (Table 7) was somewhat more effective in the *ex vivo* platelet aggregation assay and had a bioavailability of only 5.3%. While further validation in the context of particular benzamidines of interest would be necessary, oxazolidinones, oxathiadiazolones, and oxadiazoles should be considered as potentially useful prodrugs.

Conclusion

Interest in finding effective prodrug strategies for the oral delivery of benzamidine-containing drug molecules for the treatment of a variety of conditions remains high. Several approaches, all aimed at masking the basicity of the amidino moiety, have proven clinically successful although none produce high oral bioavailability (>50%). As with other prodrug approaches, each candidate compound must be investigated carefully for stability, conversion, side reactions, and species selectivity.

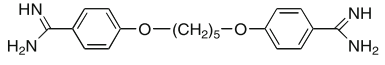
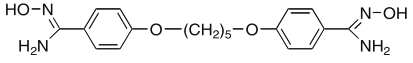
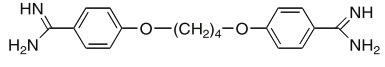
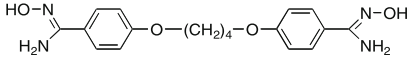
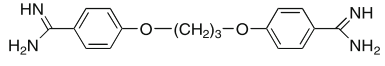
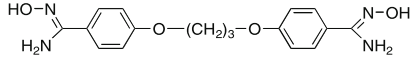
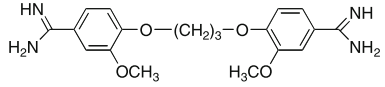
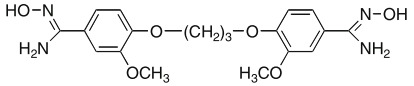
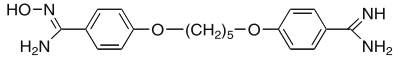
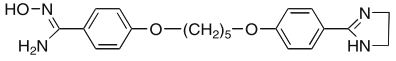
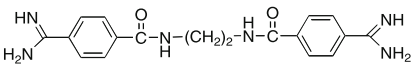
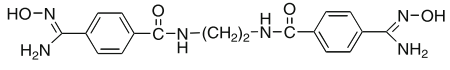
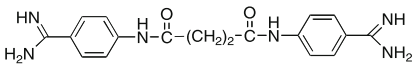
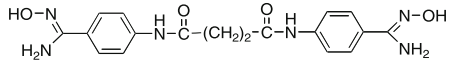
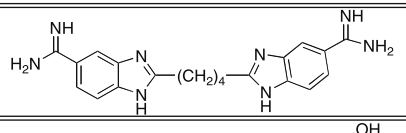
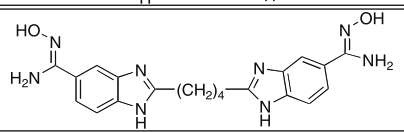
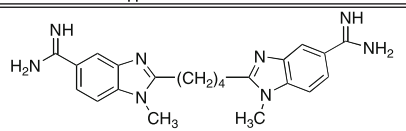
Compd	Structure	Oral Dosing[b]		IV Dosing[c]	
		Dose μmol/kg	% Saline[d]	Dose μmol/kg	% Saline[e]
1		33	133±40	22	2.0±0.37[f]
11		33	17.3±11.5 [f]	22	0.11±0.02[f]
12		33	46.0±18.6	24.6	[0.5][g]
13		33	1.4±0.6 [f]	22	0.09±0.05[f]
14		33	43.5±16.8	24.5	[0.5] [g]
15		33	2.3±1.0 [f]	22	0.02±0.01[f]
16		33	8.5±1.5 [f]	11.2	[0.6][g]
17		33	3.7±3.1 [f]	22	0.02±0.01[f]

Table 1. Anti-Pneumocystis Activities of bis-N-hdyroxyamidines and the corresponding amidines[a].

Compd	Structure	Oral Dosing[b]		IV Dosing[c]	
		Dose μmol/kg	% Saline[d]	Dose μmol/kg	% Saline[e]
18		33	86±40	22	0.65±0.40[f]
19		57.8	84.0±14.6	10	16.8±6.8[f]
20		Not done		22	6.5±1.8[f]
21		33	103±27.4	22	81.7±13
22		Not done		22	1.7±0.9[f]
23		33	182±87	22	153±40
24		Not done		18	0.27±0.1[f]
25		45.3	107±29	22	330±160
26		33	112±60	11	0.11±0.09[f]

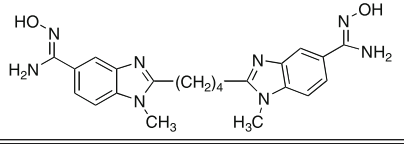
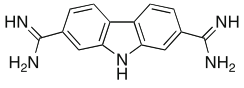
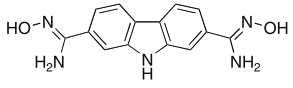
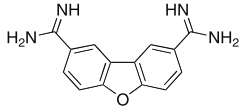
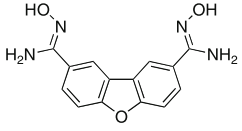
Compd	Structure	Oral Dosing[b]		IV Dosing[c]	
		Dose μmol/kg	% Saline[d]	Dose μmol/kg	% Saline[e]
27		33	166±32	22	103±48
28		71	77±21	26.2	0.13±0.04 [f]
29		33	127±68	11	48±25
30[h]		Not done		10 22	7.2±3.8 0.10±0.02
31[h]		33	45.1±21.6	10	15.76±14.6

Table 1 (continued). Anti-Pneumocystis Activities of bis-N-hdyroxyamidines and the corresponding amidines[a].

[a]Data except for compounds 30 and 31 are reproduced with permission from Hall *et al.*, 1998. [b.] Compounds were administered by gavage to groups of 6 rats once daily for 14 days. One rat treated with 13, one with 15 and one with 25 died during the experiment. [c.] Compounds were administered via the tail vein to groups of 6 rats daily for 14 days. Three rats dosed with 17 and three dosed with 25 died during the experiment. [d.] Lung cyst counts were reported as a percentage of those found in saline treated control animals. Cysts/gram of lung were 37.7×10^6 for the oral saline control (n = 6). [e.] Lung cyst counts were reported as a percentage of those found in saline-treated control animals. Cysts/gram of lung were 44.7×10^6 for the oral saline control (n = 65) and 0.9×10^6 for the i.v. pentamidine group. [f.] $p < 0.05$ from control. [g.] Numbers in square brackets are histological scores determined from stained lung sections. Scores range from 0.5 to 4.0 (Tidwell *et al.*, 1990). h. Data reproduced with permission from Wang *et al.*, 1999.

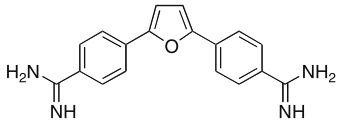
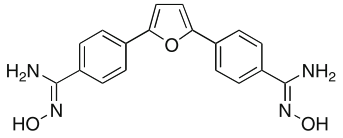
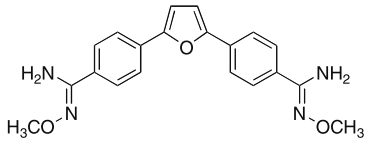
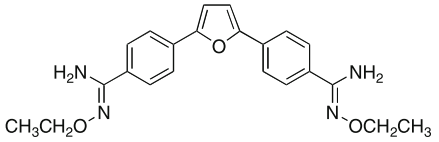
Compd	Structure	Oral Dosing		IV Dosing	
		Dose μmol/kg	% Saline[b]	Dose μmol/kg	% Saline[b]
1	Saline Control Pentamidine	Not done		- 22	100±9.5 3.9±1.5
3		66 39.8	25.2±9.8 42.3±12.6	26.6 13.3 2.7 0.3 0.03	Toxic 0.79±0.34 7.3±3.6 6.9±3.4 26.3±5.0
32[c]		33 11 5.5	3.1±1.3 197±64 127±48	22 11 5.5 2.7 0.3	0.67±0.46 1.4±0.6 42±34 54±27 203-56
2[c]		33 22 11 5.5	1.8±0.5 12.0±11.5 35±21 264±73	22	14.3±5.1
33[c]		33	122±48	22	105±50

Table 2. Anti-Pneumocystis Activities of 2,5-Bis-benzamidofuran Derivatives[a].

[a.] Data reproduced with permission from Boykin et al., 1996. [b.] Immunocompromised rats were treated either by oral gavage or intravenously with the doses indicated and at the end of the experiment lung cyst counts were reported as a percentage of those found in saline treated control animals. [c.] Maleate salt.

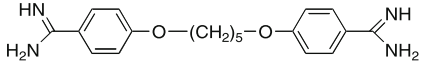
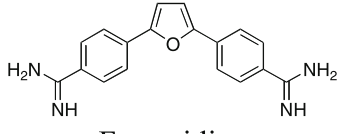
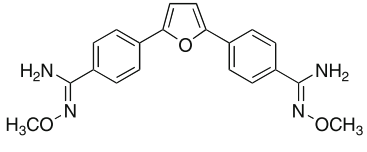
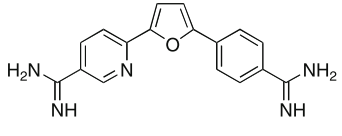
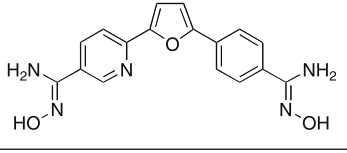
Compd	Structure	Route	Dose (mg/kg)	Cures[c]	Survival (days)[d]
	Untreated				7-8
1	 Pentamidine	i.p.	20	0/4	40.8
3	 Furamidine	i.p.	20	0/4	52.5
2		p.o.	50	2/5	60
38		i.p.	20	4/4	60
39		p.o.	100	0/4	54

Table 3. Anti-Trypanosomal Activity of Aza-2,5-Bis-benzamidofuran Derivatives[a, b].

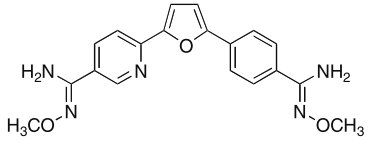
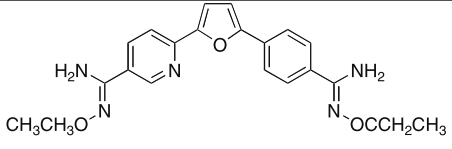
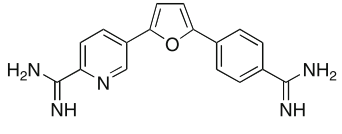
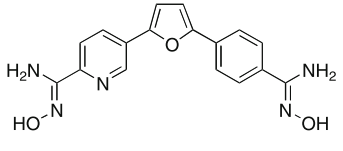
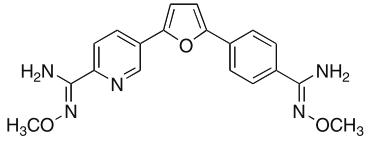
Compd	Structure	Route	Dose (mg/kg)	Cures[c]	Survival (days)[d]
	Untreated				7-8
40		p.o.	75	4/4	60
			5	4/4	60
41		p.o.	100	4/4	60
42		i.p.	20	2/4	56.5
43		p.o.	75	3/4	52.8
44		p.o.	75	4/4	60
			5	1/4	34.3

Table 3 (continued). Anti-Trypanosomal Activity of Aza-2,5-Bis-benzamidofuran Derivatives[a, b].

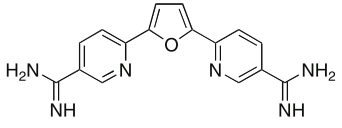
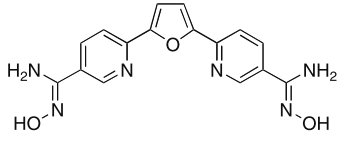
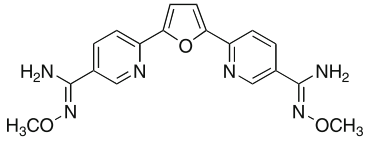
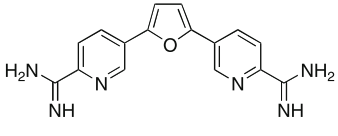
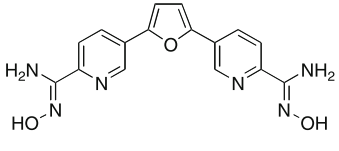
Compd	Structure	Route	Dose (mg/kg)	Cures[c]	Survival (days)[d]
	Untreated				7-8
45		i.p.	20	4/4	60
46		p.o.	75	0/4	18
47		p.o.	50	4/4	60
48		i.p.	20	3/4	59
49		p.o.	50	1/4	30

Table 3 (continued). Anti-Trypanosomal Activity of Aza-2,5-Bis-benzamidofuran Derivatives[a, b].

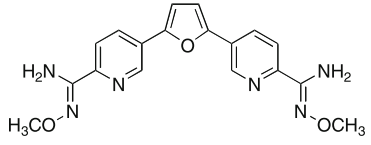
Compd	Structure	Route	Dose (mg/kg)	Cures[c]	Survival (days)[d]
	Untreated				7-8
50		p.o.	100	0/4	21.8

Table 3 (continued). Anti-Trypanosomal Activity of Aza-2,5-Bis-benzamidofuran Derivatives[a, b].

[a.] Data reproduced with permission from Ismail et al., 2003.

[b.] Groups of four mice were infected intraperitoneally with 2 × 10^5 blood stream forms of Trypanosome *b. rhodesiense* STIB 900. On days 3, 4, 5 and 6 post infection, they were treated with test drugs either i.p. for the amidines or orally for the candidate prodrugs, generally at the highest tolerated doses. Parasitemia was checked daily for the first 14 days and 2 × weekly thereafter until day 60.

[c.] Number of mice surviving to day 60 parasite free.

[d.] Average days of survival post infection.

Species	Dose(mg/kg)	$T_{1/2}$ (hr)	F (%)
Rat	4	4.1±1.7	26±5
Dog	2	11.1±1.1	25±6
Rhesus Monkey	1	5.1±1.5	33±6

Table 4. Pharmacokinetics of 5 after Oral Administration of 4 in Rat, Dog and Rhesus Monkey[a]

[a]Data reproduced with permission from Weller *et al.*, 1996.

	66		67		68	
	Dog	Cyno	Dog	Cyno	Dog	Cyno
F %	39	19	73	32	23	31
T1/2 Hr	10.3	8.9	9.0	14.2	10.3	7.5
Vss (L/kg)	5.8	5.0	4.1	6.3	5.1	1.7
Cl (ml/min/kg)	6.4	6.5	8.4	5.1	3.3	2.7

Table 6. Pharmacokinetic Properties of Parent Drugs After Oral Administration of 66–68 to Dog and Cynomologous Monkey.

[a]Data reproduced with permission from Mehrota *et al.*, 2004

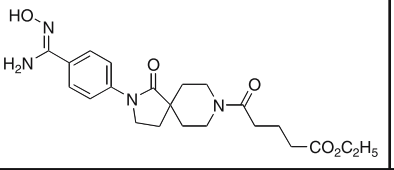
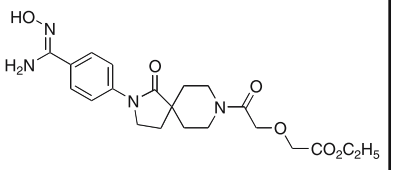
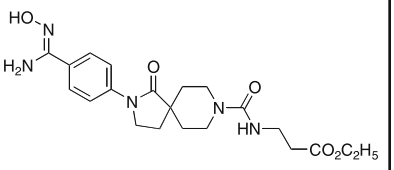
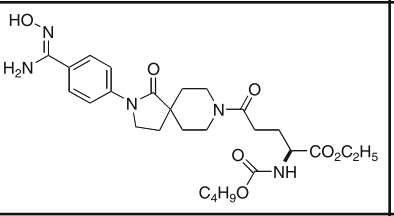
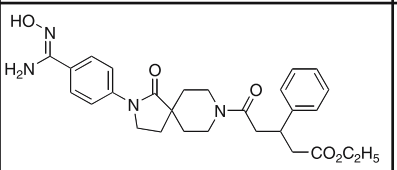
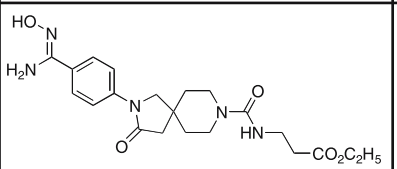
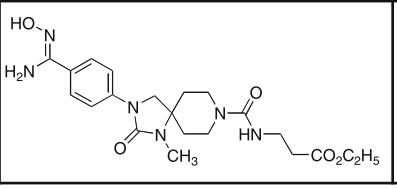
Compd	Structure	Polar Surface Area (Å²)[b]	clog P[c]	% F (po)	T1/2 (Hours, po)
66		125.5	3.81±0.68	36	1.14
67		134.8	3.79±0.79	36	1.57
68		137.6	1.44±0.69	22.3	1.42
69		163.9	5.47±0.79	6.4	
70		125.5	5.32±0.72	2.0	
71		137.6	15.2±0.69	14	
72		138.3	0.53±0.80	2.60	

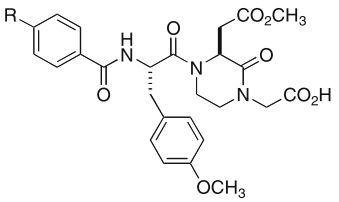
Table 5. Bioavailability of Parent GPIIa-IIIb Antagonists After Oral Dosing of Prodrugs to Rats[a].

[a]Data reproduced with permission from Mehrota *et al.*, 2004

[b]Compounds were dosed at 1 mg/kg

[c]Calculated using fragment method (Ertl *et al.* 2000)

[d]Calculated using ACD/LogP version 4.56 (Advanced Chemical Development, Inc.)



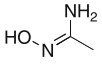
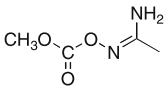
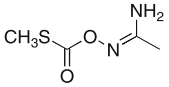
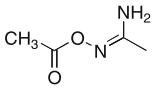
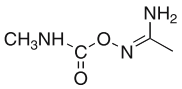
Cmpd	R	*In vitro* Platelet Aggregation (IC50 50, nM)[b]	*Ex vivo* Platelet Aggregation in Guinea Pigs[c]		
			Dose (mg/kg)	Maximum % Inhibition	Time of Maximum Inhibition (h)
76		13	0.3	65	2
77		1,900	0.3	73	8
78		2,000	0.3	96	4-8[d]
79		320	0.3	89	4-8[d]
80		300	0.3	62	4
81		270	0.3	92	8

Table 7. Inhibition of ADP Induced Platelet Aggregation in Guinea Pigs After Oral Administration of Candidate Prodrugs of the Amidine 76[a].

[a]Data reproduced with permission from Kitamura et al., 2001.

[b]Inhibition of ADP-induced aggregation of guinea pig platelet rich plasma. The candidate prodrugs have virtually no intrinsic activity.

[c]Compounds were given p.o. and blood was collected at 2, 4, 8 and 24 hrs for determination of ADP induced platelet aggregation.

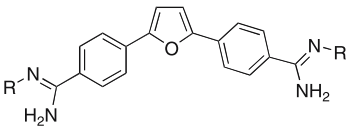
[d]Inhibition high at both 4 and 8 h.

Time After Dose (h))	Compound[b]		
	78	**79**	**81**
1	48	37	20
2	66	69	62
4	82	89	62
8	96	87	92
24	31	10	15

Table 8. Percent of Ex-vivo Platelet Aggregation in the Guinea Pig After Oral Administration of Prodrugs of 76[a].

[a]Data reproduced with permission from Kitamura *et al.*, 2001.

[b]Structures are in Table 7.



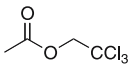
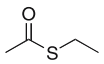
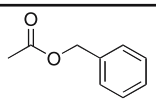
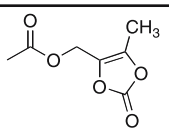
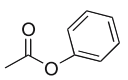
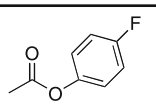
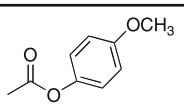
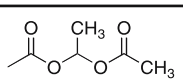
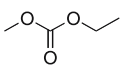
Compd	R	Oral Dosing[b]		IV Dosing[c]	
		Dose mmol/kg	% Saline[d]	Dose mmol/kg	% Saline[d]
1	Pentamidine			22	3.1±0.9
3	H	39.8	44.5±13.3	13.3	0.83±0.36
89		33	49.7±20.5	22	6.9±6.0
90		33	8.6±9.1	22	1.9±1.8
91		33	19.5±14.2	22	83.0±43.6
92		33	18.1±9.2	11	0.03±0.02
93		33	18.7±11.9	22	0.02±0.01
94		33	5.7±5.2	22	3.6±1.8
95		33	2.2±0.3	22	0.02±0.01
96		33	2.1±2.1	22	0.02±0.01
97		33	57.2±10.2	11	1.2±1.0
98		33	96.9±48.5	34.7	1.7±0.6

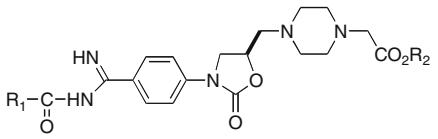
Table 9. Anti-Pneumocystis Activities of bis-(N-Alkoxycarbonylamidines)[a].

[a]Data reproduced with permission from Rahmathullah *et al.*, 1999.

[b]Oral dosing by gavage to immunosuppressed rats infected with *P. carinii*.

[c]Intravenous dosing to immunosuppressed rats infected with *P. carinii*.

[d]Percent of cytes/g of lung tissue relative to pooled control animals. Control = 100%.



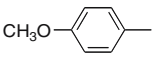
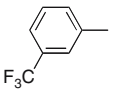
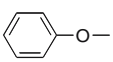
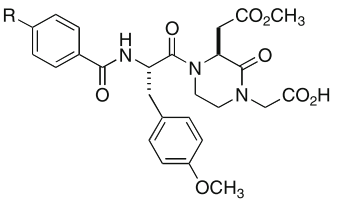
Compd	R1	R2	Stereo-chem	Guinea Pig ED_{50} (mg/kg)		Cyno. MK ED_{50} (mg/kg)	
				iv	po	iv	po
100		CH_3CH_2–	R	0.09	0.32	0.13	1.30
101		$(CH_3)_3C$–	R	0.4	1.0		>1
102		CH_3CH_2–	R		<1		>1
103		CH_3CH_2–	R		<1		>1
104		CH_3CH_2–	RS		<1		>1
105	CH_3O–	CH_3–	R		0.1		0.8
106	CH_3O–	CH_3CH_2–	R		0.2	0.07`	0.5
107	CH_3O–	$(CH_3)_3C$–	RS		>1		
108	CH_3CH_2O–	CH_3CH_2–	R		0.17		0.7
109	$CH_3(CH_2)_3O$–	CH_3CH_2–	R		<1		>1
110	$(CH_3)_2CHO$–	CH_3CH_2–	R		0.4		1.3
111		CH_3CH_2–	RS		0.2		

Table 10. Ex vivo Inhibition of Collagen Induced Platelet Aggregation in the Guinea Pig and Cynomologus Monkey by Double Prodrugs of 99[a].

[a]Data reproduced with permission from Gante *et al.*, 1996.



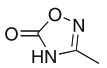
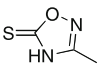
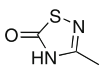
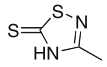
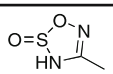
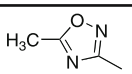
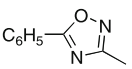
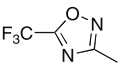
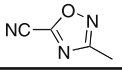
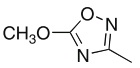
Compd	R	*In vitro* Platelet Aggregation	*Ex vivo* Platelet Aggregation in Guinea Pigs[c]		
		(IC50 50, nM)[b]	Dose (mg/kg)	Maximum % Inhibition	Time of Maximum Inhibition (h)
76		13	0.3	65	2
116		>30,000	0.3	91	8
117		30,000	0.3	33	8
118		>30,000	1.0	25	8
119		>30,000	0.3	<20	–
120		4,400	0.3	88	2
121		16.000	0.3	45	8
122		>30,000	0.3	<10	–
123		17,000	0.3	84	8
124		11,000	0.3	60	8
125		20,000	0.3	91	8
126		9,000	0.3	<20	–

Table 11. Inhibition of ADP Induced Platelet Aggregation in Guinea Pigs After Oral Administration of Candidate Prodrugs of the Amidine 76[a].

[a]Data reproduced with permission from Kitamura et al., 2001.

[b]Inhibition of ADP induced aggregation of guinea pig platelet rich plasma. The candidate prodrugs have virtually no intrinsic activity.

[c]Compounds were given p.o. and blood was collected at 2, 4, 8 and 24 hrs for determination of ADP-induced platelet aggregation.

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3.6

Prodrugs of Phosphonates, Phosphinates, and Phosphates

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Introduction

The aim of this chapter is to review the recent progress in the design and development of prodrugs of phosphonate, phosphinate, and phosphate functional groups to improve their physicochemical properties, membrane permeability, oral bioavailability, and drug targeting. Phosphonates, phosphinates, and phosphates are prominently represented as pharmacophores in various classes of biological agents. These include antiviral and anticancer nucleotides, inhibitors of biosynthesis of cholesterol, angiotensin-converting enzyme inhibitors, and bisphosphonates for the treatment of osteoporosis. It is generally well recognized that the therapeutic potential of drugs containing a phosphonate, phosphonate, or phosphate functional group is limited by their inadequate membrane permeation and oral absorption. Phosphonate, phosphinate, and phosphate groups carry one or two negative charges at physiological pH values making them very polar (Figure 1). This high polarity is the basis for many deficiencies in terms of drug delivery. Specifically, ionized species do not readily undergo passive diffusion across cellular membranes. Because of their high polarity, these agents often exhibit a low volume of distribution and, therefore, tend to be subject to efficient renal clearance as well as possibly biliary excretion.

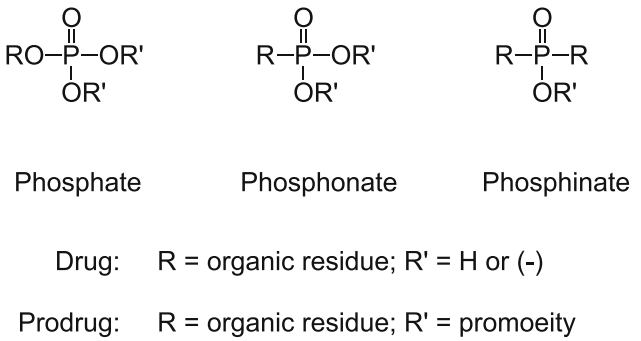


Figure 1. General structure of phosphate, phosphonate, and phosphinate drugs. R represents an organic residue while R' represents H or anionic charge for the parent drug or promoiety for prodrug.

Phosphates, particularly those of primary alcohols and phenols, are known to be excellent substrates for many phosphorylases present in the body. The rapid dephosphorylation results in a short duration of action. However, recent studies have shown that, when delivered into cells through a prodrug strategy, a phosphate-containing drug may survive long enough to provide a sufficient biological effect. For example, prodrugs of nucleoside monophosphate antiviral agents can be effective in terms of delivering a nucleoside monophosphate into cells, if the rate of phosphorylation of the nucleoside monophosphate to the corresponding di- and triphosphate is greater than the rate of dephosphorylation

of the nucleoside monophosphate to the parent nucleoside. However, no prodrugs of phosphates have reached the market, and the utility of this prodrug concept has not been established thus far. On the other hand, phosphinates and phosphonates have the advantage of being chemically and enzymatically stable. As a result, there are many examples of commercially successful prodrugs of phosphinates and phosphonates. Examples of these prodrugs are described throughout this chapter and have also been discussed in other reviews and chapters.

Key Issues in Designing a Prodrug for a Drug Molecule Carrying a Phosphinate, Phosphonate, or Phosphate Group

Medicinal chemists have designed a variety of promoieties to mask ionizable phosphinate, phosphonate, or phosphate groups. This generally involves derivatization of the phosphorus-coupled oxygen(s) to form neutral ester(s), which will decrease the polarity by increasing the lipophilicity of the drug molecule. Thus, such derivatization strategies can be expected to alter cell and tissue distribution/elimination patterns of the parent drug. Once the prodrug gets into the systemic circulation or target tissues, the ester prodrug moiety may be cleaved through an enzymatic and/or chemical process to release the corresponding free acid of phosphinate, phosphonate, or phosphate to achieve the desirable biological effect.

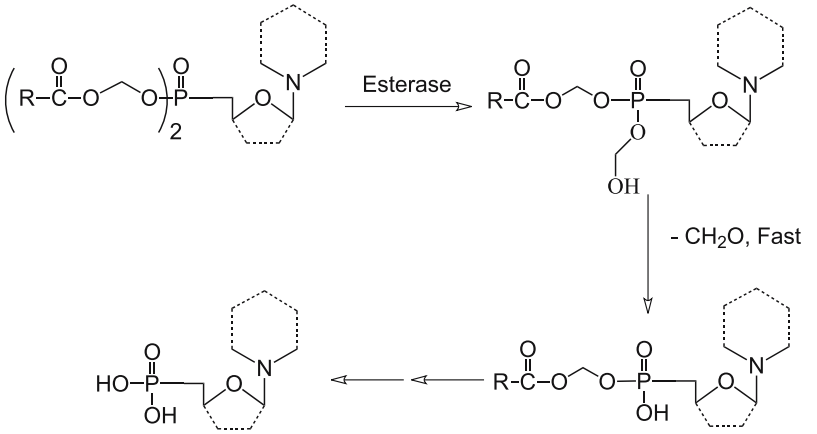
The strategies used in the design of a prodrug for a drug molecule carrying a phosphinate, phosphonate, or phosphate group are very similar to the strategies used in the design of a prodrug for a molecule carrying a carboxyl group. In fact, many prodrug moieties for phosphinate, phosphonate, or phosphate were initially developed for carboxylic acids. Therefore, many basic considerations are the same. Generally speaking, a successful prodrug should have:

- sufficient chemical stability to allow for formulation development using a suitable dosage form.
- sufficient chemical stability and enzymatic stability to survive in the gastrointestinal (GI) tract.
- sufficient aqueous solubility and permeability to allow for rapid dissolution and absorption.
- rapid conversion to the parent drug at the site of action.
- degradation byproducts that are not toxic.
- and, finally, a cost-effective and commercially viable synthesis.

However, several additional issues must be considered when designing a prodrug for phosphinates, phosphonates, or phosphates due to their unique structural features, and this makes the design of such prodrugs more complicated and challenging than the design of prodrugs for carboxylic acids.

Simple, unhindered alkyl esters of carboxylic acids can be rapidly converted to the corresponding carboxylic acids. However, conversion of a neutral alkyl ester of phosphinate, phosphonate, or phosphate is very slow in most tissues. As

a result, other undesirable metabolic or clearance mechanisms may become important. A common strategy for increasing the rate of hydrolysis of these prodrugs is to use a spacer group to separate the neutral ester of a phosphinate, phosphonate, or phosphate in such a way that a cascade reaction provides an effective cleavage via a triggering mechanism. For example, in the acyloxyalkyl prodrug moiety shown in scheme 1, the triggering mechanism is enabled by coupling a carboxylic ester through an acetal group to the phosphonate. When the carboxylic ester is cleaved by an enzyme, such as carboxyesterase, the hydroxyl alkyl group derived from the alkylene spacer group will fall off rapidly as an aldehyde through hydrolysis to release the phosphonate. To date, various combinations of the spacer groups and triggering mechanisms have been developed. These combinations will be described in detail in the following sections.

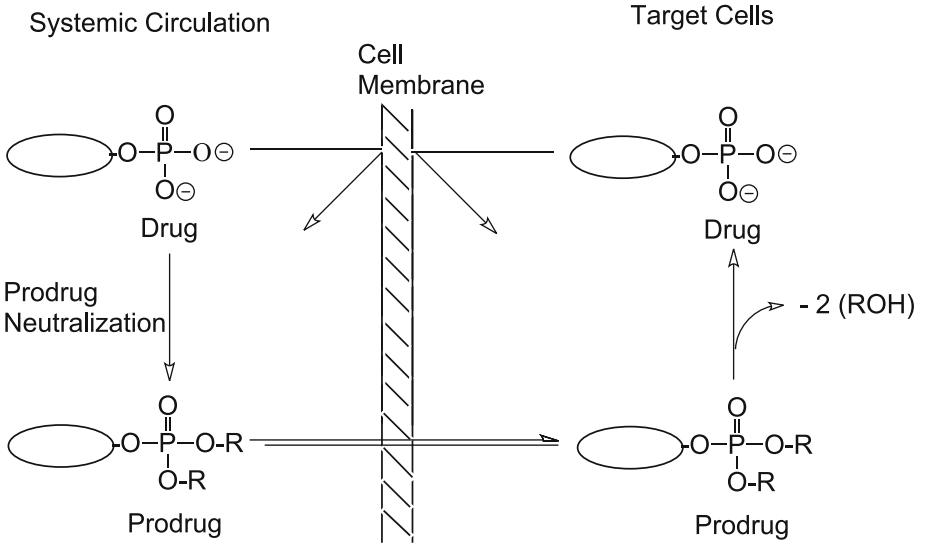


Scheme 1. General mechanism for bioreversion of acyloxymethyl phosphonate prodrugs. The acyl group is cleaved through esterase activity to yield a hydroxymethyl analog. This analog quickly decomposes to formaldehyde and a monoester prodrug. The second group is subsequently cleaved by the same mechanism but possibly by a different enzyme.

Both phosphonate and phosphate groups carry two negative charges at nearly all physiological pH values. Since monoesters of these groups are still considered to be quite polar, most successful strategies employ two moieties to mask the negative charges. In one approach, the two prodrug moieties are identical; for example, a phosphonate group can be masked with two identical acyloxyalkyl groups (Scheme 1). The first acyloxyalkyl group is cleaved by an esterase-like enzyme to release one phosphonic acid group. The phosphonate monoester formed carries one negative charge and is usually a poor substrate for a common esterase. Therefore, cleavage of the second acyloxyalkyl group generally requires a different enzyme, such as a phosphodiesterase. If the cleavage of the second acyloxyalkyl group is slow, the intermediate phosphonate monoester may

accumulate in tissues and the total efficiency of delivering a phosphonate diacid will be low. In a different approach, a special structural arrangement is designed, so that when the first prodrug moiety is cleaved by an enzyme, the second prodrug moiety will fall off rapidly by itself through a simple process such as hydrolysis. In this case, only one enzyme is needed to trigger release of both moieties, and the efficiency of delivering a phosphonate diacid may be high. However, if the two prodrug moieties are different, the phosphorus atom is a chiral center and the two prodrug isomers may have different metabolic behaviors. Specific examples of the two approaches will be described in detail in the following sections.

Drug molecules containing a phosphinate, phosphonate, or phosphate group are very polar and have low membrane permeability. However, once these agents are delivered into the cells, the low membrane permeability may become an advantage if the target is located intracellularly. The low membrane permeability will significantly reduce the passive diffusion of the active polar drug out of the cells, resulting in a long intracellular half-life and accumulation of the active drug inside cells. As shown in Scheme 2, the optimal scenario would be for the prodrug to have complete enzymatic and chemical stability during the absorption process and in blood but readily revert to the active polar drug once the prodrug has permeated the targeted cells. In other words, by utilizing the low membrane permeability of phosphinate, phosphonate, or phosphate, it would be possible to achieve cell-targeted delivery through a prodrug approach and avoid systemic clearance by hiding the active polar drug in target cells. As a result, the dose and frequency of administration of a drug could be reduced and potential toxicity due to systemic exposure of the active polar drug minimized. Specific examples of this prodrug approach will also be described in detail in the following sections.



Scheme 2. The scheme illustrates the potential advantages of prodrugs over the parent phosphonate for intracellular targeting.

Assay Systems to Evaluate the Phosphonate, Phosphinate, or Phosphate Prodrugs

The most reliable predictors of ultimate human bioavailability of drugs have been comparisons of bioavailabilities in several different animal species. Since animal pharmacokinetic studies are laborious and expensive, it is highly desirable to develop *in vitro* experiments that will predict the results of the animal studies. By using *in vitro* experiments, it is possible to screen a large number of prodrugs to find those showing the most promise for further *in vivo* studies. In addition to the screening process, the *in vitro* experiments may also be used to deconvolute the complicated process of absorption, distribution, metabolism, and excretion of a prodrug.

A variety of *in vitro* experiments have been used for the evaluation of potential prodrugs. The simplest is chemical stability and solubility in aqueous buffers over a wide pH range. Prodrugs must have a minimum chemical stability to meet the requirements of formulation and shelf-life and to survive the conditions of the GI tract. Prodrugs must exhibit a minimum amount of aqueous solubility for oral absorption, ideally over 1 mg/mL. The log of the octanol-water partition coefficient (logP) is also useful as a measure of the lipophilicity of a prodrug and its ability to cross biological membranes. Prodrugs with logP values in the range of +0.5 to ~+2.5 usually exhibit appreciable permeation *in vitro* and *in vivo*.

Biochemical data should also be collected to predict the metabolism and transport of prodrugs. Various tissue fluids and homogenates can be used to approximate the conditions that would be encountered by a prodrug during and after absorption. Animal and human tissues can be used in an attempt to calibrate an animal model for preliminary *in vivo* data. Tissue fluids and homogenates can be, for example, include intestinal wash, intestinal homogenate, liver homogenates, and plasma. For a prodrug designed for intracellular delivery, effective conversion of the prodrug in target cells can be evaluated using cells or tissue homogenates. For example, for a prodrug targeting liver disease, liver homogenate or fresh hepatocytes can be used to evaluate the conversion of the prodrug to the corresponding active parent drug. In addition, a Caco-2 (human colon adencarcinoma) cell monolayer diffusion apparatus can be used to screen prodrugs for intestinal permeation. Prodrugs with permeation coefficient values greater than 10×10^{-6} cm/s are generally considered good candidates for having acceptable permeation in animal models.

Specific Examples

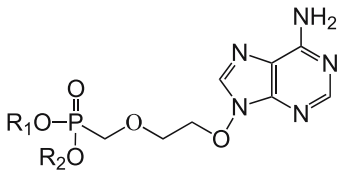
Phosphonate Prodrugs

The search for phosphonate prodrugs has been primarily inspired by the advent of acyclic and cyclic nucleoside phosphonates. This class of nucleoside analogs possesses broad-spectrum antiviral activity with a great degree of *in vivo*

and *in vitro* selectivity (De Clercq *et al.*, 1987). Due to the chemical nature of the P-C bond, phosphonates are chemically and enzymatically stable. The design of phosphonate prodrugs has been commercially rewarding leading to the discovery and development of a number of new drugs for the treatment of hepatitis B and acquired immunodeficiency syndrome (Abrams *et al.*, 1994). Specific examples of phosphonate prodrugs are listed below:

Simple alkyl, benzyl, aryl and haloalkyl esters

Serafinowska and co-workers (1995) have synthesized a series of dialkyl and dibenzyl ester prodrugs of 9-[2-(phosphonomethoxy)ethoxy]adenine (Figure 2) in an effort to improve its poor bioavailability.

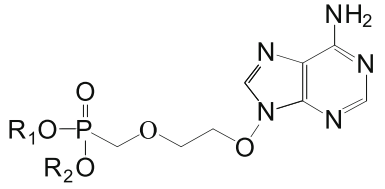


9-[2-(Phosphonomethoxy)ethoxy]adenine (R_1, R_2 = H)

Figure 2. Structure of 9-[2-(phosphonomethoxy)ethoxy]adenine.

Simple alkyl diesters of 9-[2-(phosphonomethoxy)ethoxy]adenine showed reasonable aqueous solubility (~10 mg/mL) and good chemical stability, but were not efficiently converted to 9-[2-(phosphonomethoxy)ethoxy]adenine in rat or human tissue samples (Serafinowska *et al.*, 1995). After oral administration to mice, the investigators monitored blood concentrations of the diester, monoester, and free acid (**1**-**5**, Table 1). In all cases, the diester was well absorbed. However, subsequent conversion to the monoester and free acid was dependent upon the nature of the alkyl ester. Short chain diesters were predominantly detected unchanged in the blood after oral administration. As the alkyl size increased, the prodrugs tended to break down more efficiently to the monoester; however, the monoesters tended to build up in the blood and failed to convert to the parent phosphonic acid. However refer to section 4.1.5 for an interesting example of a long chain fatty acid mono ester that undergoes intracellular conversion. Table 1 also contains oral bioavailability data for a number of diaryl prodrugs of 9-[2-(phosphonomethoxy)ethoxy]adenine (**22**-**30**). The most promising diaryl prodrug was the di-phenyl derivative **23** (**23** is a hydrochloride salt of **22**), which gave 50% oral bioavailability of the parent phosphonic acid (Serafinowska *et al.*, 1995).

Starrett *et al.* (1994) explored the effectiveness of dialkyl ester prodrugs to improve the bioavailability of 9-[2-(phosphonomethoxy)ethyl]adenine and



			total AUC 15-180 min (μM)			
Number	**R1**	**R2**	**1**	**monoester**	**diester**	**bioavailability of 1 (%)[a]**
1	H	H	-	-	-	2
2	Me	Me	0	9	39	0
3	Et	Et	4	35	9	8
4	i-Pr	i-Pr	5	29	10	10
5	n-Bu	n-Bu	0	69	2	0
6	$Me_3CCO_2CH_2$	$Me_3CCO_2CH_2$	15	0	0	30
7	$Me_3CCO_2CH_2$	Me	0	9	0	0
8	$Me_3CCO_2CH_2$	Et	0	40	0	0
9	$Me_3CCO_2CH_2$	i-Pr	0	8	0	0
10	$Me_3CCO_2CH_2$	n-Bu	0	11	0	0
11	$Me_2C(CH_2Cl)CO_2CH_2$	$Me_2C(CH_2Cl)CO_2CH_2$	0	0	0	0

Table 1. Concentrations of 9-[2-(phosphonomethoxy)ethoxy]adenine (1) and its mono- and dialkyl esters in the blood following oral administration of esters to mice. Reproduced with permission.

			total AUC 15-180 min (μM)			
Number	**R1**	**R2**	**1**	**monoester**	**diester**	**bioavailability of 1 (%)[a]**
12	$Me_2CHCO_2CH(Me)$	$Me_2CHCO_2CH(Me)$	12	0	0	24
13	Me3CCO$_2$CH(Me)	$Me_3CCO_2CH(Me)$	37	0	0	74
14	$C_6H_5CH_2O$	$C_6H_5CH_2O$	2	18	0	4
15	4-Br$C_6H_4CH_2O$	4-Br$C_6H_4CH_2O$	1	3	9	2
16	4-ClC$_6$H4CH$_2$O	4-Cl$C_6H_4CH_2O$	4	12	31	8
17	4-$Me_2CHCO_2C_6H_4CH_2O$	4-$Me_2CHCO_2C_6H_4CH_2O$	0.5	0	0	1
18	4-AcOC$_6$H4CH$_2$	4-AcO$C_6H_4CH_2$	4	0	0	8
19	Br$(CH_2)_2$O	Br$(CH_2)_2$O	4	0	0	8
20	Cl_2CHCH_2O	Cl_2CHCH_2O	3	13	15	6
21	EtO$(CH_2)_2$O	EtO$(CH_2)_2$O	0	22	13	0
22	C_6H_5O	C_6H_5O	13	1	0	26
23b	C_6H_5O	C_6H_5O	25	0	0	50
24	4-BrC_6H_4O	4-BrC_6H_4O	3	0	0	6
25	2-MeC_6H_4O	2-MeC_6H_4O	11	0	0	22
26	2-AcOC_6H_4O	2-AcOC_6H_4O	5	16	0	10

Table 1 (continued). Concentrations of 9-[2-(phosphonomethoxy)ethoxy]adenine (1) and its mono- and dialkyl esters in the blood following oral administration of esters to mice. Reproduced with permission.

			total AUC 15-180 min (μM)			
Number	**R1**	**R2**	**1**	**monoester**	**diester**	**bioavailability of 1 (%)[a]**
27	4-AcOC$_6$H4O	4-AcOC$_6$H$_4$O	4	5	0	8
28	4-AcOCH$_2$C$_6$H$_4$O	4-AcOCH$_2$C$_6$H$_4$O	0	1	0	0
29	4-MeOC$_6$H$_4$O	4-MeOC$_6$H$_4$O	5	14	0	10
30	4-Me$_3$CCO$_2$C$_6$H$_4$O	4-Me$_3$CCO$_2$C$_6$H$_4$O	0	0	0	0

Table 1. Concentrations of 9-[2-(phosphonomethoxy)ethoxy]adenine (1) and its mono- and dialkyl esters in the blood following oral administration of esters to mice. Reproduced with permission.

[a]The bioavailability of 1 after oral administration of prodrugs was calculated from the equation: % bioavailability = (AUC 1)/(iv AUC 1) $\times$ 100, where iv AUC 1 = 50 μM h.

[b]23 is the hydrochloride salt of **22**

obtained similar results. The acyclic nucleotide analog, also known as adefovir or PMEA (structure shown in Scheme 4), is an antiviral agent active against both human immunodeficiency virus (HIV) and hepatitis B virus (HBV). Shaw and Cundy (1993) studied in detail the *in vitro* and *in vivo* properties of a series of dialkyl and diaryl prodrugs of adefovir. They found that the diaryl esters of adefovir were chemically less stable at high pH than the dialkyl esters, but were adequately stable at physiological pH and stable to rat intestinal washings. Two diaryl prodrugs, the diphenyl ester and the bis(*o*-ethoxyphenyl) ester of adefovir, were rapidly transported in the Caco-2 membrane permeation assay and converted to adefovir in liver homogenates. Thus, the two prodrugs seemed to be promising candidates with the potential for good absorption followed by hepatic conversion to adefovir. When the two prodrugs were evaluated in a male Sprague-Dawley rat model (Shaw *et al.*, 1997a), the diphenyl ester was moderately absorbed as the prodrug (22%) but poorly converted to adefovir (2.8%), while the bis(o-ethoxyphenyl) ester was well absorbed (51% total) and efficiently converted to adefovir with a total oral availability of 44.3%) as adefovir. In addition to adefovir, several metabolites were found in the *in vivo* experiment, the most notable of which was adenyl-9-acetic acid. Similarly, in cynomolgus monkeys, both ester prodrugs were well absorbed and efficiently converted to adefovir.

(S)-1-(3-Hydroxy-2-phosphonylmethoxypropyl)cytosine (cidofovir or HPMPC shown in Figure 3) is an acyclic nucleotide analog approved for the treatment of cytomegalovirus (CMV) retinitis in patients with AIDS using IV infusion (Hitchcock *et al.*, 1996). Cyclic HPMPC [(S)-1-(2-hydroxy-2-oxo-1,4,2-dioxaphosphorinan-5-yl)methyl)cytosine] is a cyclic analog of cidofovir, which exhibits similar *in vitro* and *in vivo* antiviral activity to cidofovir, but is 10–40-fold less nephrotoxic in rats, guinea pig, and monkeys (Bischofberger *et al.*, 1994). *In vitro* metabolism studies have revealed that cyclic HPMPC is stable in plasma, liver, and intestinal homogenates (Mendel *et al.*, 1997). Inside intact cells, however, it is rapidly converted to cidofovir by cCMP phosphodiesterase (Mendel *et al.*, 1997). Cyclic HPMPC can therefore be regarded as an "intracellular prodrug" of cidofovir (Figure 3). The intracellular generation of cidofovir from cyclic HPMPC minimizes systemic exposure of cidofovir and reduces the potential for nephrotoxicity (Hitchcock *et al.*, 1995).

Like cidofovir, cyclic HPMPC is charged at physiological pH and has poor permeation across the human intestinal mucosa (Cundy *et al.*, 1996). However, cyclic HPMPC carries only one ionizable oxygen atom requiring masking via a prodrug approach to increase permeation. As shown Figure 4, a series of aryl ester prodrugs of cyclic HPMPC was synthesized and evaluated for their physiochemical and biological properties (Oliyai *et al.*, 1999). The rationale for using a salicylate ester promoiety is to separate the prodrug triggering mechanism from the nucleotide and phosphonate triester group to ensure a rapid and effective conversion to cyclic HPMPC by utilizing the intramolecular catalysis of salicylic acid (Scheme 3). Under suitable conditions, it is possible to selectively form either the axial or the equatorial isomer at the phosphorus center. The axial isomer is usually more stable than the equatorial isomer toward chemical and enzymatic

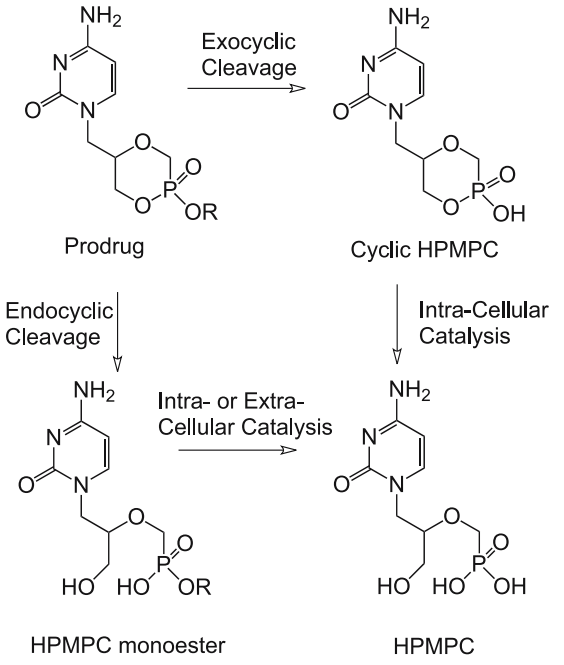
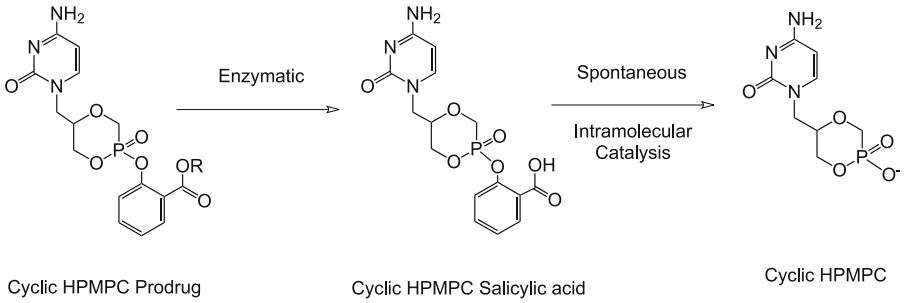


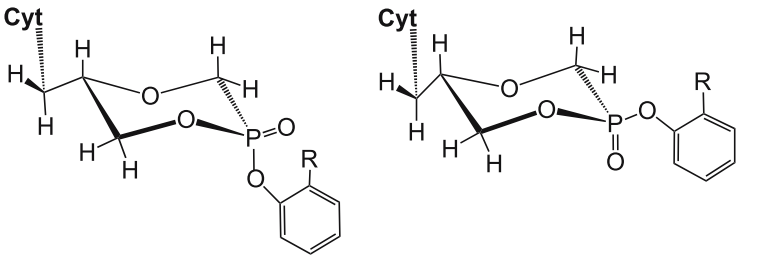
Figure 3. Metabolic and hydrolytic degradation pathways involving P-O bond cleavage for aryl ester prodrugs of cyclic HPMPC.

hydrolysis (Oliyai *et al.*, 1999). Scheme 3 shows the conversion of salicylate prodrug of cyclic HPMPC to cyclic HPMPC in the presence of porcine liver carboxyesterases (PLCE). The axial isomer of butylsalicylyl cyclic HPMPC (Figure 4, 2ax) had oral bioavailability of 46.3% as measured by cyclic HPMPC when dosed orally in beagle dogs at 8 mg/kg (Oliyai *et al.*, 2001).

Sekiya *et al.* (2002) reported a bis(2,2,2-trifluoroethyl) ester of 2-amino-6-(4-methoxyphenylthio)-9-[2-(phosphonomethoxy)ethyl] purine, MCC-478 (shown in Figure 5). The prodrug showed good antiviral activity (EC_{50} = 0.05 μM) against hepatitis B virus and low cytotoxicity (CC_{50} > 1000 μM) *in vitro* in HuH-6 cells. When the prodrug was dosed orally in mice at 100 mg/kg, the corresponding monoester (Figure 5) was detected as the main metabolite in the plasma and liver.



Scheme 3. Conversion scheme for salicylate ester prodrugs of cyclic HPMPC.



Cyclic HPMPC Prodrugs	R
Ethylsalicylyl (**1**)	$COOCH_2CH_3$
Butylsalicylyl (**2**)	$COO(CH_2)_3CH_3$
Pentylsalicylyl (**3**)	$COO(CH_2)_4CH_3$
Hexylsalicylyl (**4**)	$COO(CH_2)_5CH_3$
Isoethylhexylsalicylyl (**5**)	$COOCH_2CH(C_2H_5)(CH_2)_3CH_3$
Dodecylsalicylyl (**6**)	$COO(CH_2)_9CH_3$
Cyclohexylsalicylyl (**7**)	$COOC_6H_{11}$
Phenethylsalicylyl (**8**)	$COOCH_2CH_2C_6H_5$
Phenylsalicylyl (**9**)	$COOC_6H_5$
Phenyl (**10**)	H
o-Ethoxyphenyl (**11**)	OC_2H_5
o-Valerylphenyl (**12**)	$C(O)(CH_2)_3CH_3$
o-Pentylphenyl (**13**)	$(CH_2)_4CH_3$

Figure 4. Isomers of aryl ester prodrugs of cyclic HPMPC.

The authors claimed that the monoester is also active against HBV (EC_{50} = 0.07 μM).

In general, the results on alkyl, haloalkyl, benzyl, and aryl prodrugs of phosphonates suggest that the ability of these prodrugs to be hydrolyzed from the

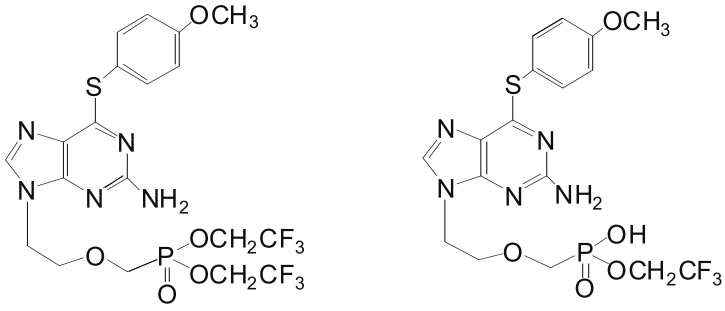


Figure 5. Structures of MCC-478 and its monoester metabolite.

diester to the monoester prodrug and ultimately to the parent phosphonic acid does not rely solely on their chemical lability. For example, prodrugs with good leaving groups are not always the most efficient prodrugs. The mechanism of conversion of the diester prodrug to the monoester prodrug is not well understood, and the *in vivo* rate of conversion of diester prodrugs to the corresponding monoester is generally too rapid to be explained solely by chemical hydrolysis. On the other hand, no one, to our knowledge, has identified a true phosphotriesterase in mammalian systems. One could speculate that either non-specific enzymes or micro environmental pH changes are responsible for the conversion. Conversion of the monoester prodrug to the parent phosphonic acid drug can be explained by the presence of phosphodiesterases (Kelly and Butler, 1977; Kelly *et al.*, 1975). Phosphonate monoesters with systemic variation of their leaving groups were tested as substrates for 5'-nucleotide phosphodiesterase leading to the conclusion that aliphatic and benzyl esters were generally poor substrates in contrast to aryl ester derivatives which were shown to be good substrates for this diesterase. It was suggested that the geometry of the aryl group may be more important than its inductive nature.

Acyloxyalkyl and alkoxycarbonyloxyalkyl esters

One approach to overcoming the hydrolytic resistance observed with many of the prodrugs previously reviewed would be to utilize an enzymatically labile spacer group prodrug moiety. Acyloxyalkyl prodrugs could serve as neutral lipophilic prodrugs. In theory, these prodrugs could transverse cell membranes by passive diffusion and revert, intracellularly, to the parent diacid after cleavage of the acyl group by esterases and rapid elimination of the spacer group by releasing an aldehyde. The most common spacer group is a methylene group, which has no chirality issue and is converted to formaldehyde.

Investigators have utilized this strategy and evaluated acyloxymethyl ester prodrugs for a variety of antiviral agents (Iyer *et al.*, 1989; McGuigan *et al.*, 1992b; Starrett *et al.*, 1992). Starrett and co-workers synthesized the bis(pivaloyloxymethyl) prodrug of the antiviral agent adefovir to increase antiviral activity. Starrett's studies showed the prodrug to have substantially increased anti-herpes simplex virus activity *in vitro* compared with adefovir. The bis(pivaloyloxymethyl) prodrugs of adefovir, 9-[2-(phosphonomethoxy)-propyl]adenine (PMPA), and 9-[2-(phosphonomethoxy)propyl]diaminopurine (PMPDAP) were also synthesized. The bis(pivaloyloxymethyl) derivatives were biologically more active than the unmodified analogs and showed enhanced antiviral activities *in vitro* (Srinivas *et al.*, 1993), most probably due to increased permeation of the prodrugs compared to the parent compound. To gain an understanding of the metabolism of this prodrug system, the [^{3}H]-bis(pivaloyloxymethyl) derivative of adefovir was incubated in an *in vitro* cell culture system. It was observed that [^{3}H]-bis(pivaloyloxymethyl) adefovir was rapidly hydrolyzed to the parent compound (adefovir) within cells. It was also found, however, that the diester prodrug releases a substantial quantity of the mono(pivaloyloxymethyl) adefovir extracellularly,

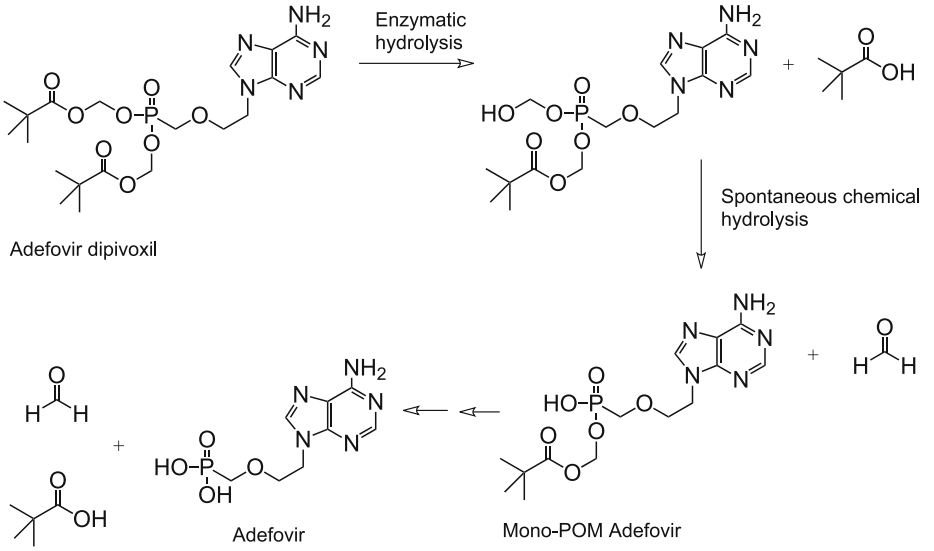
which could be a limitation of these prodrugs since the mono(pivaloyloxymethyl) species would not likely penetrate biological membranes.

In addition to seeking increased antiviral activity, investigators have also evaluated acyloxyalkyl ester prodrugs of adefovir and 9-[2-(phosphonomethoxy)ethoxy]adenine in order to increase their poor oral bioavailabilities (Starrett *et al.*, 1994; Serafinowska *et al.*, 1995). Compounds **6**-**13** (Table 1) showed dramatically increased oral bioavailability for some acyloxy alkyl prodrugs. The bis(pivaloyloxymethyl) prodrug (**6**) had an oral bioavailability of 30% and introduction of a methyl substitution at the alpha carbon of **6** to give **13** led to an oral bioavailability of 74%.

Based upon an *in vitro* stability and transport screen, bis(pivaloyloxymethyl) adefovir was selected as a potential oral prodrug for further *in vivo* animal studies. The bioavailability of bis(pivaloyloxymethyl)adefovir utilizing three different formulations was subsequently evaluated in monkeys (Cundy *et al.*, 1994). The three formulations (hydroxypropyl-β-cyclodextrin, PEG400, and aqueous suspension) were used to explore any dissolution limitations with the poorly water-soluble prodrug. The prodrug was shown to deliver an acceptable amount (≈ 24% bioavailability on average) of adefovir by the oral route using any of these formulations. The choice of formulation, therefore, was not limited by the low aqueous solubility of the prodrug, although the bioavailability may have been limited by the hydrolytic instability of the prodrug in the GI tract. An oral tablet formulation was developed for bis(pivaloyloxymethyl)adefovir (adefovir dipivoxil) and was evaluated in human clinical studies for the treatment of hepatitis B virus (HBV) infections (10 mg once daily). Adefovir dipivoxil showed about 30% bioavailability of adefovir in human (Hepsera® Package Insert), which is similar to the results obtained early from dogs and monkeys. The efficacy and safety of adefovir dipivoxil has been established in the treatment of hepatitis B in various human clinical studies (Hadziyannis *et al.*, 2003). In 2002, the FDA granted market approval for the oral tablet formulation of adefovir dipivoxil under the name of Hepsera® for the treatment of hepatitis B infections.

Among the acyloxyalkyl ester prodrugs evaluated for adefovir, pivaloyloxymethyl and 1-pivaloyloxyethyl groups provided the highest oral bioavailability as the prodrug moiety. The bioconversion of pivaloyloxymethyl and 1-pivaloyloxyethyl ester prodrugs leads to the formation of pivalic acid and an aldehyde (Scheme 4). The major clinical toxicological concern resulting from the pivalic acid released by the prodrug is related to the impact of pivalic acid on carnitine homeostasis. Therefore, extensive studies have been carried out to search for a new prodrug moiety to deliver an acyclic nucleotide analog suitable for the treatment of HIV because the disease requires relatively high doses and chronic treatment.

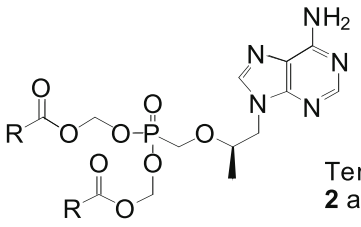
A series of novel prodrugs were designed to overcome the pharmacokinetic limitations of 9-[2-(phosphonomethoxy)propyl]adenine (tenofovir or PMPA) (Arimilli *et al.*, 1997; Shaw *et al.*, 1997b). Tenofovir (structure shown in Scheme 5) is an acyclic nucleotide analog that is active against both HIV and HBV. These prodrugs were engineered to mask the polar phosphonic acid functionality using



Scheme 4. Structures of adefovir dipivoxil, adefovir, and bioconversion pathway.

a novel oxycarbonyloxymethyl linker to permit passive diffusion in the intestinal tract. The resulting alkyl methyl carbonate prodrugs of tenofovir were synthesized and evaluated both *in vitro* and *in vivo*. The bioconversion of alkyl methyl carbonate prodrugs of tenofovir to tenofovir is mediated by nonspecific carboxylesterases (Shaw *et al.*, 1997b). The bioconversion mechanism involves rapid enzymatic hydrolysis of the bis-ester followed by spontaneous decomposition of the carbonic acid monomethyl phosphonate ester. The corresponding mono-ester undergoes a similar degradation, leading to the rapid formation of tenofovir. The conversion process is rapid, as demonstrated in preclinical and clinical studies. Table 2 shows the results obtained from the *in vitro* and *in vivo* studies. While the bis[(pivaloyloxy)methyl] prodrug of tenofovir (compound **2** in Table 2) was also evaluated and demonstrated high oral bioavailability in fasted beagle dogs, this prodrug was not selected for clinical development (Shaw *et al.*, 1997b). The amount of pivalic acid that would be released from the breakdown of the bis[(pivaloyloxy)methyl] tenofovir was a concern, considering the requisite high dose of the prodrug. All alkyl methyl carbonate prodrugs of tenofovir showed high antiviral activity in cell culture assays, except for **5d**, which contains a sterically hindered t-butyl group. It is clear that the carbonate prodrugs of tenofovir can be effectively converted to tenofovir by intracellular enzymes. However, the carbamate prodrugs of PMPA (**5h** and **5i**) showed poor antiviral activities, suggesting that the carbamate group is a poor prodrug moiety, probably due to its high stability.

These alkyl methyl carbonate prodrugs of tenofovir showed a reasonable oral bioavailability, varying from 16–30% in dogs, calculated based on the plasma



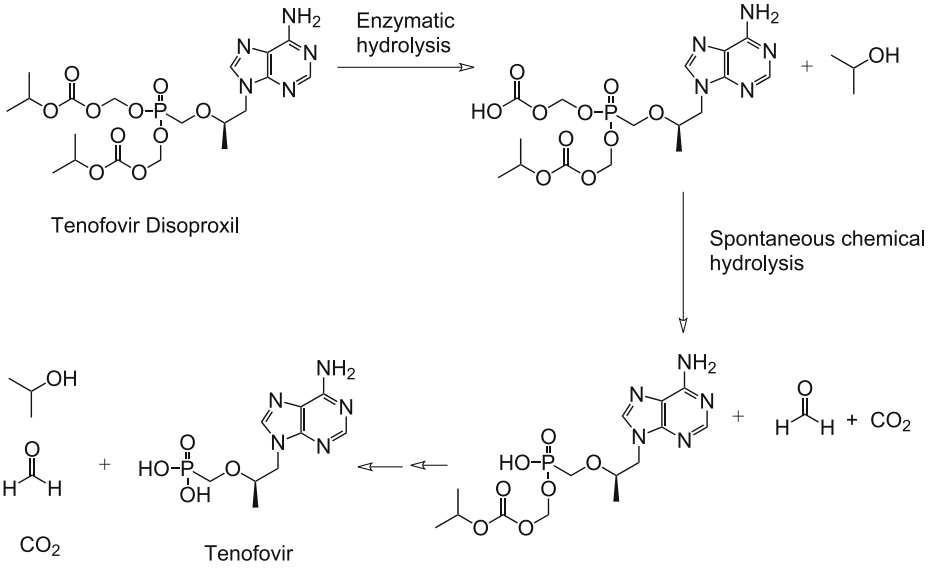
Tenofovir Prodrugs
2 and **5a - i**

	EC_{50} -(μM)	CC_{50} (μM)	T $^{1}/_{2}$ (min) in dog tissues		% F as tenofovir in dogs
			Intestinal homogenate	Plasma	
2, R = *t*-Bu	0.5	250	10.4	35.5	37.8
5a, R = OEt	0.002	40	23.3	16.6	24.5
5b, R = O(*n*-Bu)	ND	ND	< 5	< 5	18.0
5c, R = O(*i*-Bu)	< 0.001	30	15	< 5	20.8
5d, R = O(*t*-Bu)	0.2	10	26.6	21.2	30.7
5e, R = O(neopentyl)	< 0.001	3	< 5	< 5	16.0
5f, R = O(*i*-Pr)	0.003	50	52.6	20.5	30.1
5g, R = O(3-pentyl)	< 0.001	40	30	15	28.8
5h, R = NH(*t*-Bu)	> 10	10	ND[a]	ND	ND
5i, R = N(*n*-Pr)$_2$	3	10	ND	ND	ND

Table 2. *In vitro* anti-HIV-1 activity (EC_{50}) and cytotoxicity (CC_{50}) and metabolic stabilities of tenofovir prodrugs and oral bioavailability (% F) as tenofovir in dogs.
[a]ND = not determined

exposure of tenofovir (Shaw *et al.*, 1997b). The diisopropyl carbonate ester (**5f** or tenofovir disoproxil) was selected for clinical evaluation based its chemical stability, aqueous solubility, intestinal homogenate stability, and bioavailability following oral dosing in dogs. The bioconversion of tenofovir disoproxil to tenofovir leads to the formation of two equivalents each of isopropyl alcohol, carbon dioxide, and formaldehyde (Scheme 5). In contrast to the pivaloyloxymethyl-containing prodrug, the chronic administration of tenofovir disoproxil has no impact on carnitine homeostasis.

Interestingly, in preclinical studies in dogs, the intracellular levels of tenofovir in peripheral blood mononuclear cells (PBMCs) were fivefold greater after oral administration of tenofovir disoproxil than following an equivalent subcutaneous dose of tenofovir. Correspondingly, in human clinical trials, the change in HIV viral load was threefold greater after oral administration of tenofovir disoproxil



Scheme 5. Structure of tenofovir disoproxil, tenofovir, and bioconversion pathway.

fumarate than after an equivalent exposure of intravenously administered tenofovir (Cundy, 1999). The "enhanced" anti-HIV activity observed in patients receiving the oral prodrug relative to the intravenously administered parent drug may be attributable to an increase in the intracellular concentration of tenofovir, which is likely the result of better intracellular distribution of the oral prodrug. A tablet formulation was developed containing 300 mg of tenofovir disoproxil fumarate. Based on the results from the human clinical studies, the oral tablet formulation of tenofovir disoproxil fumarate (300 mg once daily) was approved by the FDA in 2001 for the chronic treatment of patients infected with HIV under the name of Viread® (Viread® Package Insert).

Bisphosphonates, characterized by having a P-C-P backbone, as shown in Figure 6, are effective inhibitors of bone resorption and used in the treatment of various bone diseases, including osteoporosis. Due to the high polarity of the two phosphonate groups, this class of drugs usually has low oral bioavailability. However, the dosage of bisphosphonates for the treatment of osteoporosis is usually low and infrequent. For example, the mean oral bioavailability of alendronate sodium is about 0.6 % (Fosama® Package Insert). The recommended dosage of alendronate sodium is one 70 mg tablet once weekly or one 10 mg tablet once daily. The mean oral bioavailability of ibandronate sodium is about 0.6 % (Boniva® Package Insert). The recommended dosage of ibandronate sodium is one 150 mg tablet once monthly or one 2.5 mg tablet once daily. To date, only limited efforts have been made to develop prodrugs for bisphosphonates (Vepsalainen, 2002). Niemi *et al.* (2000) reported a series of simple alkyl ester prodrugs and acyloxymethyl ester prodrugs of etidronic acid and acetyletidronic acid. The *in vitro* evaluation of the prodrugs showed that the trisubstituted pivaloyloxymethyl ester of acetyletidronate has adequate water-solubility and

lipophilicity (Log P = 0.6 at pH 7.4). The prodrug is relatively stable in 80% human serum ($T_{1/2}$ = 4.8 h) and is rapidly metabolized in 10% rabbit liver homogenate ($T_{1/2}$ = 2.7 min). However, no *in vivo* data are available for prodrugs of bisphosphonates.

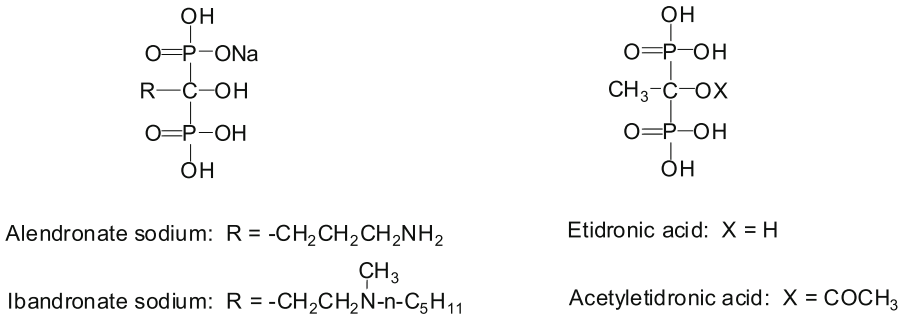
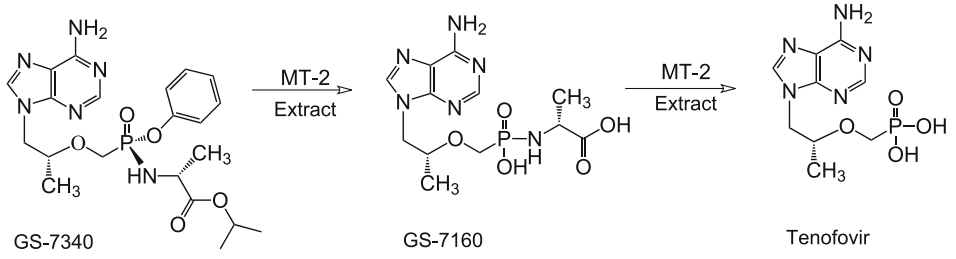


Figure 6. Structures of bis-phosphonates.

Phosphonamidates

Phosphonamidates and phosphoramidates have been explored as prodrug modifications because of the potential for the P-N bond cleavage by chemical and enzymatic hydrolysis. Synthesis and evaluation of a series of novel phosphonamidates of adefovir and tenofovir were reported (Scheme 6) (Ballatore *et al.*, 2001; Eisenberg *et al.*, 2001; Lee *et al.*, 2005). The most advanced lead compound of the new class of prodrugs is GS-7340, an isopropylalaninyl monoamidate, phenyl monoester prodrug of tenofovir. This prodrug was designed to circulate systemically as the prodrug and undergo selective conversion to tenofovir inside cells. GS-7340 demonstrates extremely potent *in vitro* activity and selective targeting to lymphoreticular tissues and PBMCs *in vitro* and *in vivo*.



Scheme 6. Metabolic conversion of GS-7340 to tenofovir.

GS-7340 was synthesized from tenofovir and L-isopropyl alanine ester in a non-stereospecific synthesis, resulting in the formation of equal amounts of two stereoisomers at phosphorus. These two diastereomers, GS-7339 and GS-7340 with R and S configurations at phosphorus (Lee *et al.*, 2005) , respectively, were subsequently separated by chromatography. To assess the ability of these prodrugs

				$T_{1/2}$ (min)	
	EC50 (μM)	**CC_{50} (μM)**	**Selectivity Index**	**MT-2 Cell Extract**	**Human Plasma**
Tenofovir	5	6,000	1,250	NA[a]	NA
Tenofovir DF	0.05	50	1,000	70.7	0.41
GS-7171 (diastereomeric mixture)	0.01	95	9,500	ND[a]	ND
GS-7339 (R diastereomer)	0.06	> 100	> 1,700	> 1000	231
GS-7340 (S diastereomer)	0.005	40	8,000	28.3	90
GS-7485 (D-ala)	10	ND	ND	> 700	> 200

Table 3. In vitro anti-HIV-1 activity (EC50), cytotoxicity (CC50) and in vitro metabolic stabilities of tenofovir and tenofovir prodrugs.

[a]NA = not applicable; ND = not determined

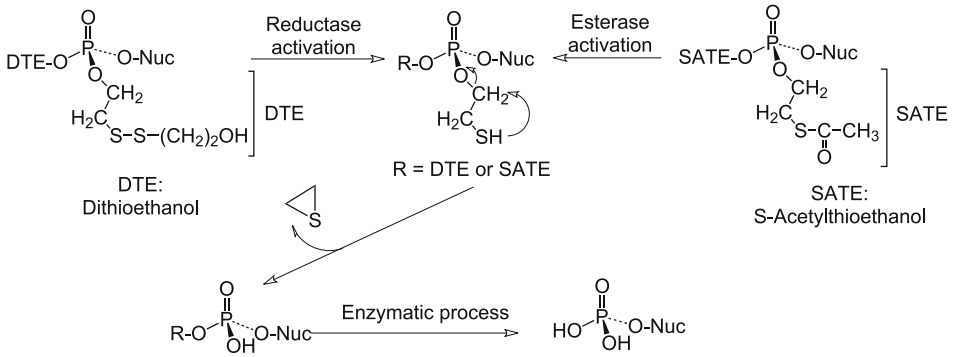
to cross the cellular membrane and undergo intracellular metabolism to tenofovir, their *in vitro* activities were measured against HIV-1 in MT-2 cells. The antiviral activities for the diastereomeric mixture (GS-7171), the individual diastereomers (GS-7339 and GS-7340), and the diastereomic mixture of D-alaninyl analog (GS-7485) are shown in Table 3. Compared to tenofovir, the individual isomers, GS-7339 and GS-7340 were 83- and 1000-fold more active, respectively, whereas the D-isopropyl alaninyl analog (GS-7485) and the metabolite (GS-7160) showed activity similar to that of tenofovir. The enhanced activities of the L-alaninyl prodrugs as compared to tenofovir are a result of greater cellular permeation and rapid conversion to tenofovir inside the MT-2 cells. The dramatically reduced activity (1000-fold) of the D-alaninyl analog (GS-7485) compared to the L-alaninyl analog (GS-7171) demonstrates a strong metabolic preference inside the MT-2 cells for the L-amino acid. The 12-fold greater activity for GS-7340 compared to GS 7339 further suggests that intracellular metabolism is also sensitive to stereochemistry at phosphorus.

Table 3 also lists the *in vitro* half-lives of the monoamidate prodrugs and tenofovir disoproxil fumarate (tenofovir DF) in MT-2 cell extract and human plasma. GS-7340 was metabolized threefold faster in MT-2 cell extract than in plasma. As shown in Scheme 6, the putative first step in the conversion of the GS-7340 to tenofovir is the hydrolysis of the amino acid ester that is sensitive to stereochemistry at both the amino acid and at phosphorus. Thus in MT-2 cells,

the primary metabolite observed is the alaninyl amidate GS-7160, which is slowly hydrolyzed to tenofovir. The oral bioavailability of GS-7340 is greater than 70% in dogs at the 20 mg/kg dose. This was calculated by comparing the plasma levels of GS-7340 after oral administration to those after IV bolus administration of GS-7340.

S-Acetylthioethanol (SATE) and dithiodiethanol (DTE) esters

An effective prodrug of a phosphonate requires a spacer group to separate the neutral phosphonate and the triggering mechanism. In the acyloxymethyl and alkoxycarbonyloxymethyl prodrug moieties in adefovir dipivoxil and tenofovir disoproxil, respectively, the spacer group is a methylene group or formaldehyde. In the phosphonamidate prodrug moiety, the spacer group is an amino acid, such as alanine. As another example of the spacer group, S-acetylthioethanol (SATE) or dithiodiethanol (DTE) has been used to deliver a nucleotide or nucleoside monophosphate (Scheme 7) (Puech *et al.*, 1993; Lefebvre *et al.*, 1995).



Scheme 7. Mechanism of bioconversion of SATE and DTE nucleoside monophosphate prodrugs.

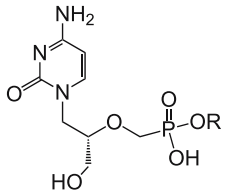
The rationale for using SATE or DTE lies in the ability of the spacer group to preferentially form an unstable 2-thioethyl intermediate once inside the cell by the action of a carboxyesterase or a reductase. The 2-thioethyl moiety collapses to episulfide and the nucleotide. This strategy has been applied to adefovir, ddUMP, and AZTMP (Benzaria *et al.*, 1996; Peyrottes *et al.*, 2004). The half-lives of the ddUMP prodrugs were evaluated in culture medium and in CEM cell extract. The rate of prodrug decomposition was >50 times faster in the cell extract than in the culture medium for both the bis(SATE) and bis(DTE) ddUMP prodrugs. *In vitro* experiments with adefovir, ddUMP, and AZTMP using thymidine kinase (TK)-deficient cell lines showed that these prodrugs may provide an efficient means for the delivery of phosphonate or nucleoside monophosphate intracellularly. However, the clinical usage as well as long-term safety profile of this prodrug strategy have not been established (Jones and Bischofberger, 1995).

Quinone methides

As a different approach to improve the phosphonate diester as a substrate for esterases by using a spacer group to distance the acyl group from the phosphonate center, Mitchell *et al.* (1992) investigated the metabolic activation of the 4-acyloxybenzyl group. They prepared the bis(4-acetoxybenzyl) ester of methylphosphonate, a model compound, and a series of bis(4-acyloxybenzyl) prodrugs of phosphonoacetate, an antiviral drug. The authors demonstrated that both the chemical and enzymatic hydrolysis of the mono(4-acyloxybenzyl) esters and the enzymatic hydrolysis of the bis(4-acyloxybenzyl) esters proceed through hydrolysis of the acyl group to produce the unstable 4-hydroxybenzyl esters. The resulting 4-hydroxy-substituent promotes cleavage of the benzyl-oxygen bond to release the phosphonic acid. This prodrug approach was also applied to AZTMP in a separate report (Thomson *et al.*, 1993). The authors synthesized a series of mono- and bis(4-acyloxybenzyl) esters of AZTMP in which the acyl substitution was Me, Et, *i*-Pr, and *t*-Bu. In the presence of porcine liver carboxyesterase, the prodrugs were readily decomposed to the 5'-monophosphate of AZT; however, the anti-HIV activities were at best comparable to that of AZT. The clinical usefulness of 4-acyloxybenzyl prodrugs is limited by the potential formation of quinone methide intermediates, which are known to be toxic.

Long-chain alkyloxyalkyl monoesters

Hostetler and co-workers synthesized a series of monoester prodrugs of cidofovir (CDV) containing a long-chain alkyloxyalkyl group (Figure 7) (Ciesla *et al.*, 2003; Quenelle *et al.*, 2004). These prodrugs of cidofovir are designed to cross cell membranes by phospholipid uptake pathways, passive diffusion, and membrane flippase activity. Once the prodrug has reached the intracellular compartment of the target cell, cidofovir is cleaved from the prodrug by the hydrolytic action of phospholipase C on the P-O-alkyl bond. Uptake by these routes is more efficient than passive diffusion of cidofovir itself and leads to a more rapid accumulation of cidofovir in target cells. Another purpose of the prodrug



R =	Compounds
H	Cidofovir
$CH_3(CH_2)_{15}O(CH_2)_3$	Hexadecyloxypropyl cidofovir, HDP-CDV
$CH_3(CH_2)_{17}O(CH_2)_2$	Octadecyloxyethyl cidofovir, ODE-CDV
$CH_3(CH_2)_7CH{=}CH(CH_2)_8O(CH_2)_2$	Oleyoxyethyl cidofovir, OLE-CDV
$CH_3(CH_2)_7CH{=}CH(CH_2)_8O(CH_2)_3$	Oleyoxypropyl cidofovir, OLP-CDV

Figure 7. Structures of cidofovir and cidofovir ether lipid esters.

design is that the long-chain alkyloxyalkyl monoester is designed to mimic lysophosphatidylcholine (LPC) and thus use the natural LPC uptake pathway in the small intestine to achieve high oral availability.

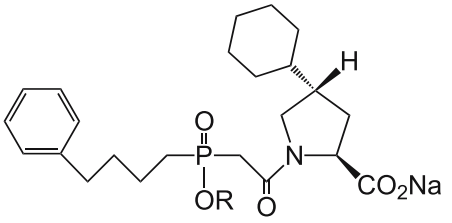
By using these prodrugs, antiviral effects of cidofovir can be significantly improved in various *in vitro* antiviral assays. For example, HDP-cidofovir and ODE-cidofovir are 58- and 231-fold more active, respectively, against vaccinia virus and 75- and 149-fold more active, respectively, against cowpox virus than parent cidofovir in human foreskin fibroblast cells. These results clearly demonstrate that the monoester prodrug provides a very effective way to deliver cidofovir into cells. In a study using ^{14}C-labeled cidofovir and HDP-cidofovir, the prodrug was able to generate much higher levels of intracellular cidofovir and, consequently, higher levels of intracellular cidofovir diphosphate, the active antiviral agent, in human lung fibroblast cells (Ciesla *et al.*, 2003).

In experiments to assess the oral bioavailability of the monoester prodrugs, a dose of ^{14}C-labeled HDP-cidofovir (10 mg/kg) was administered to mice by intraperitoneal and oral routes. The concentration of HDP-cidofovir in plasma after oral administration reached a peak of 3.1 μM at 3 h. Comparison of the oral and parenteral areas under the curve for a 72-h time period ($AUC_{0\text{-}72h}$) indicated that the oral bioavailability of HDP-cidofovir was 88%. In comparison, the oral bioavailability of cidofovir in rats was less than 3%. By using the lipid trafficking pathway, the prodrug may facilitate the distribution of the drug in target organs. In fact, after oral administration of HDP-cidofovir and ODE-cidofovir in mice, the concentrations of cidofovir in the liver, spleen, and lung are considerably higher than after parenteral administration of cidofovir. However, the concentration in the kidney, the site of cidofovir toxicity, is considerably lower than the concentration after intraperitoneal administration of cidofovir. Due to their high oral bioavailability, oral HDP-cidofovir and ODE-cidofovir are very effective in the treatment of cowpox and vaccina virus infections as well as murine cytomegalovirus infections in mice (Painter and Hostetler, 2004).

Phosphinate Prodrugs

Phosphinate carries one negative charge and requires only one prodrug moiety to mask the negative charge. The prodrug strategies developed for sterically hindered carboxylic acids such as acyloxyalkyl are very suitable for this purpose. Several prodrugs of phosphinate-containing drug molecules have been reported. One of them, fosinopril, has been used in clinical practice as oral antihypertensive agent for many years.

Rational design of angiotensin-converting enzyme (ACE) inhibitors has led to the development of SQ 27,519 or fosinoprilat, a phosphinic acid-containing drug (Figure 8). The phosphinic acid group was found to interact with zinc present in the binding site of ACE (Thorsett *et al.*, 1982). The poor oral activity of fosinoprilat (< 5 % bioavailability) made the parent compound unsuitable for development as an oral antihypertensive agent.



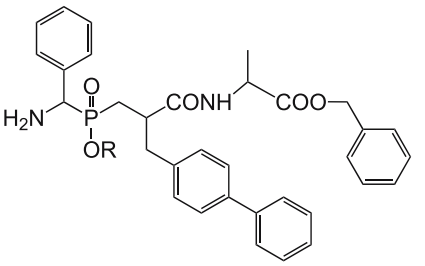
Fosinoprilat R = H

Fosinopril R = -CH(CH(CH$_3$)$_2$)OCOC$_2$H$_5$

Figure 8. Structures of fosinoprilat (SQ 27,519) and its acyloxyalkyl prodrug, fosinopril.

In an attempt to overcome the lack of bioavailability, an acyloxyalkyl prodrug, fosinopril, was designed (Figure 8). The disposition of fosinopril was extensively studied and fosinopril was found to be primarily metabolized by intestinal and liver esterases to yield fosinoprilat after oral absorption (Morrison *et al.*, 1990). This metabolism was shown to occur either during or soon after absorption. The acyloxyalkyl ester imparts a lipophilic nature to the drug and allows enhanced passive diffusion across membranes, which results in an oral bioavailability of 32–36% in humans. Ranadive *et al.* (1992) performed studies to establish a relationship between lipophilicity and oral bioavailability for several ACE inhibitors and reported octanol-water distribution coefficients at several pH values for both fosinopril and fosinoprilat. As expected, the ester prodrug has distribution coefficients that are orders of magnitude greater than that of the parent drug. The greater lipophilicity, as indicated by the increased distribution coefficients, correlated well with the bioavailability data on these compounds. The correlation between lipophilicity and bioavailability generally holds true for compounds that predominantly utilize passive diffusion and have sufficient solubility in the GI tract. Friedman and Amidon (1989) subsequently confirmed the mechanism of GI absorption of fosinopril to be predominantly passive in nature. Fosinopril is currently approved as an oral antihypertensive agent and has been extensively evaluated for its clinical effectiveness and safety.

Prodrugs of phosphinic dual inhibitors of the enkephalin-degrading zinc-containing metallopeptidases, neutral endopeptidase (NEP) and aminopeptidase N (APN), have been synthesized (Chen *et al.*, 2001). As in the case of fosinoprilat,



(1) R = CH_3CH_2Ph
(2) R = $CH_3(CH_2)_2SCOCH_3$
(3) R = $CH_3(CH_2)_2SCOtBu$
(4) R = $CH_3(CH_2)_2SCO$-Ph
(5) R = $CH_3CH_2OCOtBu$
(6) R = $CH_3CH(i\text{-}pr)OCOPh$

Figure 9. Structures of Dual NEP/APN inhibitor and selected phosphinate prodrugs.

a phosphinic acid group is required for the inhibitory activity through the interaction with zinc present in the binding sites of both NEP and APN. However, due to high polarity and low brain penetration, the parent compounds induced only a minimal analgesic effect (approximately 25% of a maximal effect) when dosed at 100 mg/kg in animals through IV administration. A series of prodrugs shown in Figure 9 that induced long-lasting antinociceptive response in the hot plate test in mice with a ceiling effect ranging from 25 to 42% of analgesia has been synthesized.

Phosphate Prodrugs

Most of the studies on phosphate prodrugs have been carried out on nucleoside analog monophosphates. Purine and pyrimidine nucleoside analogs have found great utility in treatment of neoplastic and viral diseases, (Neil *et al.*, 1970; Cooney *et al.*, 1986). A recent review has discussed the progress of phosphate prodrugs (Freeman and Ross, 1997). These nucleoside drugs rely on viral or/and host kinase-mediated (*i.e.*, thymidine kinase) activation step(s) to produce the phosphorylated nucleosides necessary to display biological activity, that is, the nucleosides are themselves prodrugs. Unfortunately, dependency on the kinase-mediated activation can lead to the development of resistance (Reichard *et al.*, 1962; Uchida and Kreis, 1969). The first step in the phosphorylation of a nucleoside to the monophosphate is known to be highly specific and often causes the development of resistance (Balzarini *et al.*, 1987; Johnson and Fridland, 1989). Therefore, it can be argued that one approach to circumventing the resistance development problem is to administer the monophosphate-containing nucleoside drug. This strategy has two flaws. (1) The highly polar monophosphate has limited passive absorption properties and, therefore, the membrane permeation is low. (2) Rapid *in vivo* dephosphorylation of the monophosphate is observed with this class of drugs (Schrecker and Goldin, 1968; LePage *et al.*, 1975). Prodrug strategies have been investigated to overcome this liability as discussed in this portion of the chapter on phosphorus-coupled oxygen ester prodrugs of nucleotides, namely, nucleoside analog monophosphates.

Simple alkyl, benzyl, aryl and haloalkyl esters

Rosowsky *et al.* (1982) have examined several mono-5'-(alkyl phosphate) esters of araC (Figure 10, **1**-**5**) in an effort to deliver araCMP to cancer cells. The cytotoxicity of the proposed prodrugs toward cultured L1210 leukemia and B16 melanoma cells appeared to obey an inverse structure-activity relationship with respect to alkyl chain length. The *n*-butyl and *n*-hexyl prodrugs were approximately half as active as the ethyl prodrug. The structure-activity relationship seemed to plateau as chain length became longer, as exemplified by *n*-octyl and *n*-$C_{16}H_{33}$ esters having nearly the same ID_{50} values. Similarly, Mullah and coworkers (1992) produced 5'-O methyl and 5'-O phenyl monoesters of 2',3'-didehydro-2',3'-dideoxyadenosine and 2',3'-didehydro-2',3'-dideoxycytosine, which displayed *in*

vitro results similar to those of the parent nucleosides. When incubated in a serum-containing medium, the phenyl prodrug produced parent nucleoside along with nucleoside monophosphate; the methyl prodrug was not evaluated in this manner.

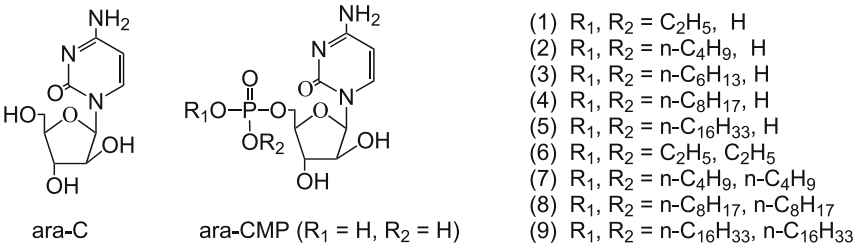
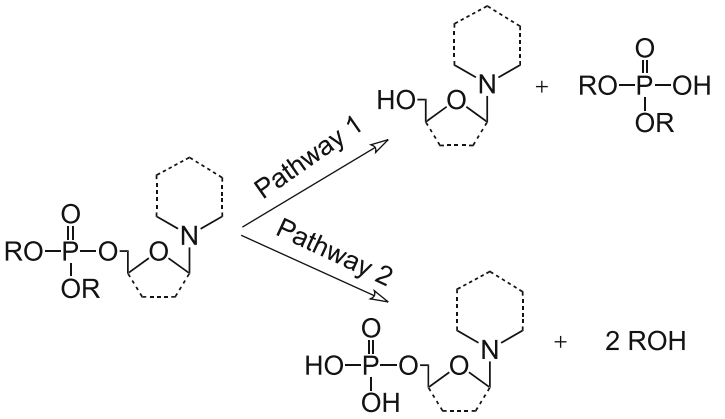


Figure 10. Structures of araC, araCMP, and araCMP alkyl prodrugs. Structures (1)-(4) are mono-alkyl prodrugs while (5)-(9) are dialkyl prodrugs.

Investigators have also synthesized diesters of phosphates and evaluated their effectiveness as prodrugs in *in vitro* and *in vivo* tests. The results were not consistent with the observation with the monoesters. For example, Colin *et al.* (1989) showed a clear relationship between inhibition of thymidine incorporation by mammalian epithelial cells and lipophilicity (increased alkyl chain length) of diester prodrugs of araCMP utilizing a series of protecting groups similar to those studied by Rosowsky *et al.* (1982) (Figure 10, **6**-**9**). The *in vitro* activity was lowest for the ethyl ester and highest for the hexyl ester. Similar work performed on araAMP gave a correlation between an *in vitro* inhibition of DNA synthesis and lipophilicity; however, no anti-viral activity at concentrations up to 100 μg/mL was detected against a range of viruses (McGuigan *et al.*, 1989a,b). AZTMP alkyl esters were also employed to improve membrane permeability (McGuigan *et al.*, 1990a). *In vitro*, the diester prodrugs showed a complete lack of inhibition of HIV, which was attributed to the high stability of the simple alkyl esters with little or no conversion to the active 5'- phosphate.

By what mechanism do these alkyl esters of monophosphates exert their potential biological effect? The prodrugs could act either as a depot for the nucleoside in which the P-O-nucleoside link would be broken to provide the nucleoside (pathway 1, Scheme 8) or as an intracellular source of the nucleoside monophosphate by cleavage of the P-O-alkyl bond (pathway 2, Scheme 8). The first possibility (pathway 1), although potentially useful, would fail to overcome the resistance seen with many of these drugs, *i.e.*, it still delivers the nucleoside rather than its monophosphate.

In an effort to increase the lability of the phosphate-promoiety bond (pathway 2, Scheme 8), several haloalkyl prodrug esters have been made and evaluated (McGuigan *et al.*, 1993; Naesens *et al.*, 1994). The halo-substitution was thought to favor the formation of nucleoside monophosphates and increase biological



Scheme 8. Illustration of the possible decomposition pathways for a generic nucleotide prodrug. Pathway 1 represents cleavage of the P-O-nucleoside link and results in nucleoside release. Pathway 2 represents cleavage of the P-O-alkyl linkage and results in accumulation of nucleotide inside the cell.

activity. Bis(haloethyl) phosphate derivatives of AZT and 2', 3'-dideoxycytidine (ddC) (Figure 11) have been synthesized and evaluated (McGuigan *et al.*, 1990b). However, the *in vitro* anti-HIV activities of these prodrugs were lower than those of the parent nucleosides. For ddCMP, the trichloroethyl moiety was found to be more efficacious than the trifluoroethyl moiety, while for AZTMP the mono- and di-haloalkyl prodrugs and AZTMP were almost equally effective. When this strategy was subsequently applied to araAMP and araCMP (McGuigan *et al.*, 1992a), *in vitro* experiments showed that the trichloro-containing prodrugs were consistently more active than the trifluoro-containing prodrugs. For the araCMP prodrug, the activity was found to be greater than that of the parent nucleoside. These observations were not consistent with the expected lability of the prodrug esters, as one would expect the trifluoro analogs to be the most labile. The observed trend in activity, however, was found to correlate with lipophilicity as exemplified by the larger octanol/water partition coefficient for the trichloro-containing prodrug.

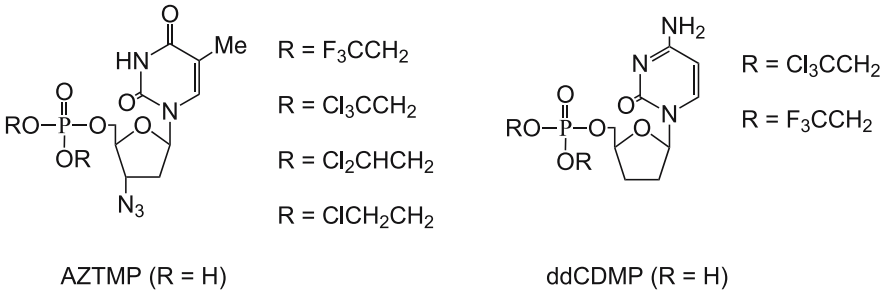


Figure 11. Structures of AZT monophosphate and ddC monophosphate along with their corresponding haloalkyl prodrugs.

Acyloxyalkyl and alkoxycarbonyloxymethyl esters

Sastry *et al.* (1992) synthesized the bis(pivaloyloxymethyl) prodrug of 2',3'-dideoxuridine 5'-monophosphate (ddUMP). Metabolism studies in two human T cell lines led to the formation of 5'-mono-, di-, and tri-phosphates of dideoxyuridine (ddU) after exposure to bis(pivaloyloxymethyl) ddUMP. In contrast, these phosphorylated metabolites were not observed in cells treated with ddU or ddUMP alone, suggesting that this prodrug was able to release ddUMP intracellularly. Freed *et al.* (1989) utilized the same prodrug strategy for 5-flouro-deoxyuridine monophosphate (5dUMP) and obtained results consistent with intracellular delivery of the monophosphate.

In a more complete mechanistic study involving the hydrolysis of bis(pivaloyloxymethyl)AZTMP, investigators were able to gain a further understanding of the decomposition pathways (Pompon *et al.*, 1994). One interesting observation was that the removal of the two pivaloyloxymethyl groups was controlled by different enzymes. The authors speculated that hydrolysis of mono(pivaloyloxymethyl)AZTMP to AZTMP was catalyzed primarily by phosphodiesterases, whereas the bis(pivaloyloxymethyl) ester hydrolysis to its mono(pivaloyloxymethyl) ester was catalyzed by esterases.

cyclo*Sal-pronucleotides*

Meier and coworkers (Meier, 2002; Meier *et al.*, 1999) proposed a new class of prodrugs, *cyclo*Sal-pronucleotides (Figure 12), to deliver nucleoside analog monophosphates. A possible conversion mechanism for the prodrugs is shown in Scheme 9. The prodrugs are stable in acidic media. Under neutral and basic conditions, however, the prodrugs undergo a chemical hydrolysis to generate

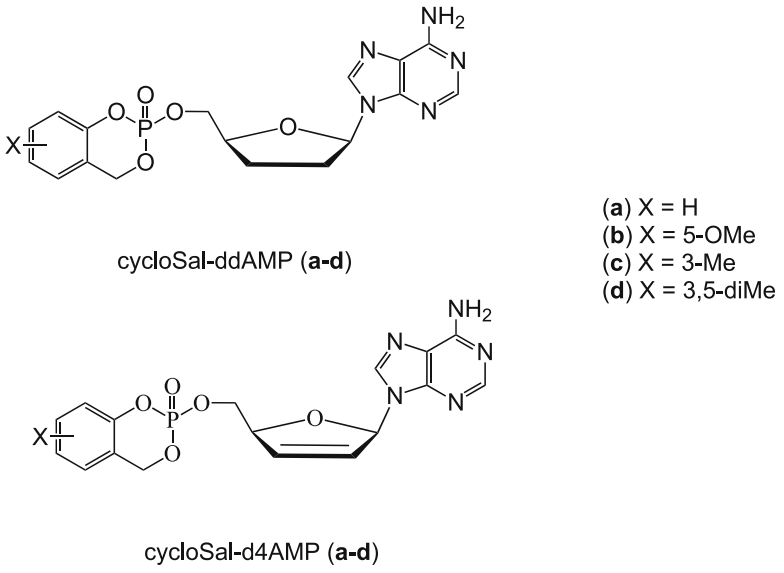
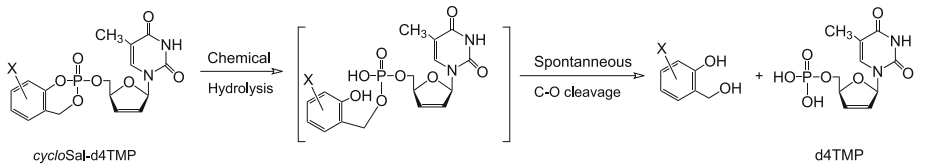


Figure 12. Structures of *cyclo*Sal-ddAMP and *cyclo*Sal-d4AMP phosphotriester prodrugs.



Scheme 9. Chemical hydrolysis pathway of *cyclo*Sal phosphotriesters.

nucleoside analog monophosphates and salicyl alcohol. No enzyme was found to be involved in the conversion process. Therefore, this is a class of prodrugs that depend mainly on chemical hydrolysis for activation.

Table 4 shows the hydrolysis rate at pH 7.3 and the anti-HIV activity in CEM cells for a series of *cyclo*Sal-d4TMP prodrugs carrying different substituent groups on the salicylalcohol unit. The data listed in the table are very consistent with the cascade-reaction mechanism and confirm the design of a delivery mechanism independent of enzymatic activation. For example, the prodrug carrying a nitro group had a very fast chemical hydrolysis rate at neutral pH and showed anti-HIV activity very close to that of the parent nucleoside, d4T. The EC_{50} was low in thymidine kinase (TK)-competent cells but high in TK-deficient cells. In other words, this prodrug may deliver only the parent nucleoside into the cells due to its poor chemical stability. However, for the prodrugs carrying a weak electron-

	Hydrolysis at 37°C ($T^1/_2$ in hour)			EC50 (μM)			CC50 (μM)
X	pH 6.9	pH 7.3	pH 8.9	HIV-1 in CEM/TK^+	HIV-2 in CEM/TK^+	HIV-2 in CEM/TK^-	
5-NO_2	4.1	0.15	0.06	0.29	0.40	40.0	75
5-Cl	6.4	0.7	0.3	0.42	1.40	2.67	49
5-H	24.5	4.5	1.1	0.28	0.10	0.50	47
5-Me	28.3	8.0	1.3	0.18	0.34	0.18	38
3,5-diMe	98.2	32	3.4	0.09	0.17	0.08	21
3-Me	68.5	24	1.5	0.057	0.07	0.048	26
3-*i*-Pr	ND[a]	28	ND	0.047	0.08	0.065	18
3-*t*-Bu	ND	96	ND	0.18	0.15	0.18	49
d4T	NA[a]	NA	NA	0.25	0.15	50	56

Table 4. *In vitro* hydrolysis, antiviral activity (EC_{50}), and cytotoxicity (CC_{50}) of *cyclo*Sal-d4TMP prodrugs

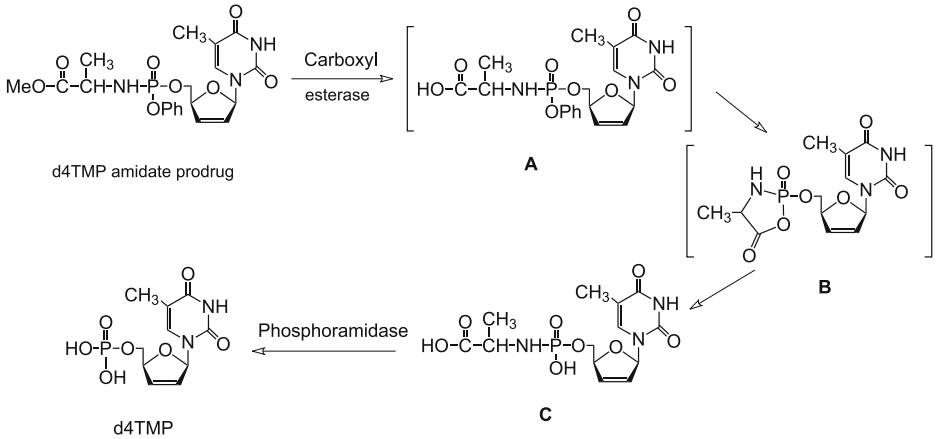
NA[a]=not applicable, ND=not determined

donating group, such as a methyl group, the EC_{50} was lower than that of d4T in TK-competent cells. Importantly, the prodrugs showed similar anti-HIV activity in both TK-competent cells and TK-deficient cells, suggesting that the TK phosphorylation step is bypassed and the prodrugs deliver d4TMP into the cells. The prodrug carrying a *t*-butyl group is highly stable against chemical hydrolysis and less active in both TK-competent cells and TK-deficient cells.

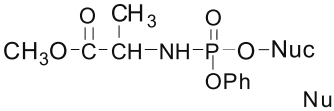
The *cyclo*Sal-strategy has further been applied successfully to adenosine analogs such as ddA and d4A. The main purpose of the approach is to bypass activation steps involving several enzymes, such as adenosine deaminase. In fact, the prodrugs are much more potent (10–50-fold) than ddA and d4A, respectively, against HIV in the cell culture assay. It seems that using these prodrugs significantly improves the efficiency of intracellular activation of adenosine analogs. A *cyclo*Sal-prodrug was also synthesized for the monophosphate of acyclovir (ACV), an important antiviral agent against herpes viruses. The prodrug showed high activities against herpes virus (both Type 1 and Type 2), varicell-zoster virus, and cytomegalovirus in both TK-competent cells and TK-deficient cells, while ACV was active only in TK-competent cells. The phosphorus atom in the *cyclo*Sal-prodrugs is a newly formed chiral center. Therefore, the prodrugs are usually obtained as a roughly 1:1 diastereomeric mixture. The two isomers can be separated by HPLC using an RP-C18 column. Usually, the R_P isomer is 5-10 fold more active than the S_P isomer.

Phosphoramidates

McGuigan *et al.* (1996), Balzarini *et al.* (1993), and Cahard *et al.* (2004) synthesized a series of aryl monoester aminoacid ester monophosphoramidate prodrugs for various cyclic and acyclic nucleoside analog monophosphates. According to a putative mechanism (Scheme 10) proposed for the prodrugs, the conversion process is initiated by cellular carboxyl esterases and progresses through three intermediates (A, B, and C) to generate the corresponding nucleoside analog monophosphate inside cells.



Scheme 10. Mechanistic conversion of d4TMP amidate prodrug to d4TMP.



Nuc = d4T, d4A, ddA, 3TC, AZT

	EC_{50} (μM)			CC_{50} (μM)
Nuc/Nuc prodrug	HIV-1 in CEM/TK$^+$	HIV-2 in CEM/TK$^+$	HIV-2 in CEM/TK$^-$	CEM/TK$^+$
d4T	0.16	0.27	25	≥100
d4T MP prodrug	0.085	0.10	0.075	>100
d4A	20	20	ND[a]	91
d4A MP prodrug	0.006	0.018	ND	3.8
ddA	4	8	ND	>250
ddA MP prodrug	0.016	0.035	ND	6.5
3TC	0.01	0.02	ND	>250
3TC MP prodrug	2.5	3.0	ND	>250
AZT	0.005	0.008	>100	>100
AZT MP prodrug	0.055	0.070	12	172

Table 5. *In vitro* antiviral activity (EC_{50}) and cytotoxicity (CC_{50}) of nucleoside analog monophosphoramidate prodrugs.
[a]ND = not determined

For most nucleoside analogs, intermediate C and the nucleoside analog monophosphate are predominant species observed after treatment with cell extracts. Experimental results also suggest the presence of a phosphoramidase catalyzing the hydrolysis of intermediate C to the nucleoside analog monophosphate. However, identification of the enzyme has not yet been achieved. The involvement of intermediates A and B was supported by the following two observations. First, when β-alanine is used in the phosphoramidate prodrug, the conversion process is stopped at intermediate A, indicating that the 5-membered cyclic intermediate B is preferable. Second, when the aryl group was replaced with an alkyl group, the process was also stopped at intermediate A. Therefore, a good leaving group like phenol is essential for the completion of the process. Extensive SAR studies have been carried out on d4T prodrugs. In general, alanine is by far the best amino acid unit in the phosphoramidate prodrug, and L-amino acids are more biologically active than D-amino acids.

Benzyl and phenyl alanine esters were converted faster and these prodrugs are more active compared to methyl and ethyl alanine esters. The use of t-butyl esters significantly reduced activity. For the substituent on the phenyl group, lipophilic substituents with mildly electron-withdrawing character are preferred.

As with other nucleoside analog monophosphate prodrugs, the key issue for phosphoramidate prodrugs is whether the prodrug is able to effectively deliver a monophosphate into cells to achieve the goal of a 'kinase bypass.' Although the prodrug approach can be applied to a wide range of nucleoside analogs, the prodrug will improve the biological effect of a parent nucleoside analog only when the nucleoside analog has poor monophosphorylation in target tissues. For example, as shown in Table 5, the methylalaninyl monoamidate phenyl monoester prodrug of d4T showed a 5–10-fold increase in antiviral activity against HIV in various cell lines compared to parent d4T; this improved activity was maintained in TK-deficient cells. The same prodrugs of d4A and ddA are about 3000- and 300-fold more active against HIV than are d4A and ddA, respectively. However, the same prodrug applied to 3TC and AZT showed no advantage over the parent drugs. In fact, it is known that monophosphorylation of AZT is very fast in cells. The prodrug approach failed for acyclovir, where the phosphoramidate was significantly less active than the parent against HSV-2. Therefore, in addition to the original idea of utilizing the 'kinase bypass' concept to overcome the resistance problem, delivery of a nucleoside analog monophosphate into cells through a prodrug may also significantly improve the biological activity of a nucleoside analog that has poor biological activity due to insufficient monophosphorylation. Thus, successful use of the 'kinase bypass' concept depends not only the structure of the nucleoside analog but also on the cell type of the target tissue.

HepDirect technology

A series of cytochrome P_{450} 3A-activated prodrugs (HepDirect Prodrugs) were synthesized and evaluated for the potential of delivering a phosphate or phosphonate-containing drug into the liver (Figure 13) (Erion *et al.*, 2004). The parent drug can be either an antiviral agent, such as adefovir and 3TC, or an anticancer agent, such as araA and araC. Different from most of the nucleoside and nucleotide prodrugs, which are activated by esterases, the HepDirect prodrugs are designed to undergo an oxidative cleavage reaction catalyzed by cytochrome P_{450} (CYP) isoenzymes expressed predominantly in the liver. The oxidation-based conversion process is shown in Scheme 11. Various cytochrome P_{450} isoenzymes are able to catalyze the oxidative cleavage reaction. However, cytochrome P_{450} 3A provides the highest activity. Studies also showed that in order to achieve fast oxidative cleavage and high-efficiency conversion of the prodrug, an *m*-chlorophenyl group is preferred as the substituent group on the dioxaphosphorane ring and the cis configuration of the two substituent groups on the ring is important (see Figure 13).

As shown in Table 6, when incubated with freshly isolated rat hepatocytes, these prodrugs are able to deliver much higher levels of nucleotide diphosphate

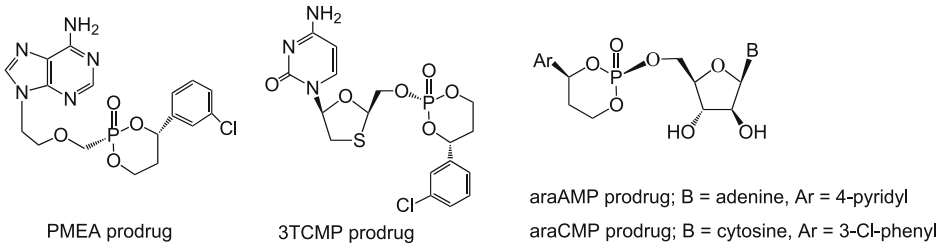
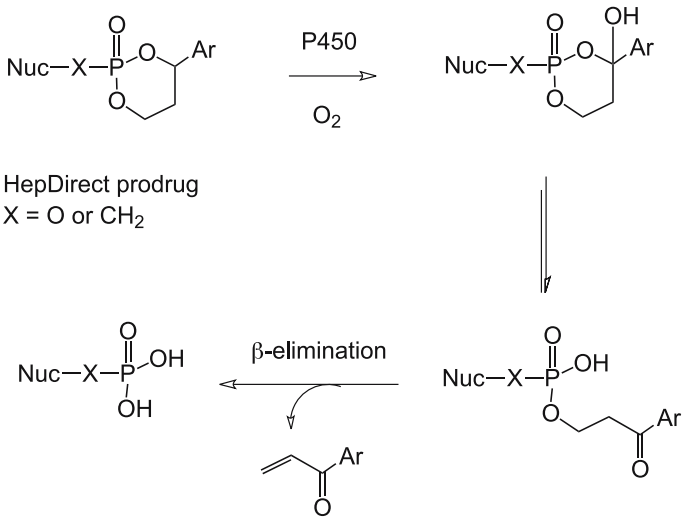


Figure 13. Structures of selected HepDirect prodrugs.



Scheme 11. HepDirect prodrug bioconversion mechanism.

Nuc/Nuc prodrug	C_{max} (pmol/mg)	$AUC_{0\text{-}4h}$ (pmol-h/mg)
Adefovir	41 ± 6	87 ± 12
Adefovir prodrug	379 ± 131	756 ± 291
3TC	6 ± 0	19 ± 0.3
3TCMP prodrug	201 ± 35	648 ± 88
araA	379 ± 17	486 ± 3
araAMP prodrug	93 ± 13	122 ± 11
araC	< 2	< 5
araCMP prodrug	175 ± 64	398 ± 112

Table 6. Activation of HepDirct prodrugs of nucleotide analog and nucleoside analog monophosphates in rat hepatocytes[a].

[a]Maximum concentration and total exposure of triphosphate after the parent drug or prodrug was incubated with fresh rat hepatocytes at 100 μM for 4 hours.

for adefovir and nucleoside triphosphate for 3TC, araA, and araC compared to the corresponding parent drugs. Importantly, the adefovir diphosphate is the species providing the biological activity, while the triphosphate is the active species for 3TC, araA, and araC. In addition, when the 3TC prodrug was dosed in rats through intravenous injection at 30 mg/kg, the liver level of 3TC triphosphate was about tenfold higher than the liver level obtained after intravenous injection of 3TC at 230 mg/kg. At the same time, the plasma level of 3TC triphosphate was about 50-fold lower than the plasma level obtained after intravenous injection of 3TC. These results demonstrate that this prodrug technology is very efficient in delivering a fully phosphorylated active species into cells for a nucleotide or nucleoside monophosphate-based drug in both *in vitro* and *in vivo* experiments. For the nucleoside monophosphate-based drugs, such as 3TCMP, araCMP, and araAMP, rapid phosphorylation of the monophosphate in target cells may prevent dephosphorylation of the monophosphate and result in accumulation of the desired corresponding nucleoside triphosphate in target cells.

Conclusions

In summary, this review, although not comprehensive, attempts to highlight and assess examples of prodrug approaches applied to phosphonate, phosphinate, and phosphate functional groups. A number of examples of clinically and commercially successful prodrugs of phosphonate and phosphinate functional group-containing drugs are cited. Issues relating to chemical design, stability, metabolism, and pharmacokinetics profile are discussed. We hope that this chapter will further stimulate more extensive research into the use of prodrugs for improved delivery, targeting, and pharmacokinetic profiling of phosphonate, phosphinate, and phosphate functional group-containing drugs.

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3.7

Functional Group Approaches to Prodrugs: Functional Groups in Peptides

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Table of Contents

Abbreviations

AP .....apical
BL.....basolateral
Bop-Cl .....bis(2-oxo-3-oxazolidinyl)phosphinic chloride
CNS.....central nervous system
CPA .....carboxypeptidase A
DADLE .....Tyr-D-Ala-Gly-Phe-D-Leu-OH
DCC.....1,3-dicyclohexylcarbodiimide
DCM.....dichloromethane
DEA .....diethylamine
DIEA .....*N*,*N*-diisopropylethylamine
DMAP 4-*N*,*N*-dimethylaminopyridine
DMF.....*N*,*N*-dimethylformamide
EDC .....1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide
HBSS.....Hank's balanced salt solution
HBTU ....O-benzotriazoyl-*N*,*N*-tetramethyluronium hexafluorophosphate
HOBt 1-hydroxybenzotriazole
LAH.....lithium aluminum hydride
LDA .....lithium diisopropylamide
NMM .....*N*-methylmorpholine
NMR.....nuclear magnetic resonance
P_{app} .....apparent permeability coefficient
PCC .....pyridinium chlorochromate
PLE.....porcine liver esterase
TBDMS .....*t*-butyldimethylsilyl
TBDMS-Cl.....*t*-butyldimethylsilyl chloride
TEA.....triethylamine
TFA.....trifluoroacetic acid

Introduction

There is no doubt that peptides play critical roles in various biological processes. Naturally, a large number of biologically active peptides have been discovered, many of which are clinically used pharmaceutical agents. However, there are intrinsic physicochemical and pharmaceutical properties associated with peptides that hinder their development as oral- and CNS-active pharmaceutical agents. These properties include high polarity and hydrogen-bonding potential, and the presence of charged functional groups, all of which are significant contributing factors to the generally poor permeation properties of peptides across membrane barriers. In addition, peptides typically undergo rapid metabolism, which leads to short half-lives *in vivo* (<30 min). Consequently, peptides are generally considered poor candidates for development as orally and CNS-active pharmaceutical agents. Although the fundamental stability issues can sometimes be addressed with structural modifications and the introduction of D-amino acids, the poor membrane permeation is generally intrinsic to the peptide structural features. One way to overcome this problem is the prodrug approach, *i.e.*, to temporarily and bioreversibly mask those functional groups responsible for the undesirable physicochemical and pharmaceutical properties of peptides (Audus *et al.*, 1995; Borchardt, 1995; Artursson and Borchardt, 1997; Gangwar *et al.*, 1997b; Pauletti *et al.*, 1997a; Shan *et al.*, 1997; Wang *et al.*, 1999c).

The specific functional groups that contribute to the poor permeation properties of a peptide include amino, carboxyl, and guanidino, which are charged and of high polarity, as well as hydroxyl and amido groups that have high polarity and hydrogen-bonding potential. It has been well-demonstrated that a decrease in hydrogen-bond potential by alkylation of the amido nitrogen significantly facilitates the transport of peptides (Burton *et al.*, 1992, 1996; Conradi *et al.*, 1992; Ho *et al.*, 1993; Kim *et al.*, 1993; Chikhale *et al.*, 1994). However, there are no good approaches to bioreversibly alkylate and de-alkylated amido nitrogen and, consequently, such an approach cannot be readily adapted for prodrug preparation. Therefore, prodrug derivatization of peptides has been focused on bioreversibly derivatizing carboxyl, amino, guanidino, and hydroxyl groups. The derivatization of such individual functional groups on small molecules through ester and amide formation and *N*-oxidation is discussed in other sections of this book. This section will discuss only those approaches unique to peptides and peptide mimetics. It should be noted that this section does not strive to be comprehensive in covering every article that has appeared in the literature. There are many review papers that serve that purpose (Oliyai and Stella, 1993; Oliyai, 1996; Gangwar *et al.*, 1997b; Shan *et al.*, 1997; Wang *et al.*, 1999c; Borchardt and Wang, 2000). Instead, this section will focus on the strategies and problems of various unique approaches using selected examples.

One of the unique approaches to the preparation of prodrugs for peptides is the cyclic prodrug concept by Borchardt and co-workers. In this approach, an amino and a carboxyl group are masked with a single prodrug moiety. The advantages of the cyclic prodrug strategy include (1) the masking of two ionizable functional groups using one prodrug moiety, (2) the easy regeneration of both

functional groups using a single chemical or enzymatic reaction, (3) the promotion of intramolecular hydrogen bond formation that reduces the intermolecular hydrogen-bond potential and helps to improve membrane permeability, (4) the stabilization of the peptides toward exo-peptidase-mediated hydrolysis through the masking of the terminal amino and carboxyl groups, and (5) the possibility of restricting the conformational flexibility of the molecule, leading to a more compact structure with improved permeability.

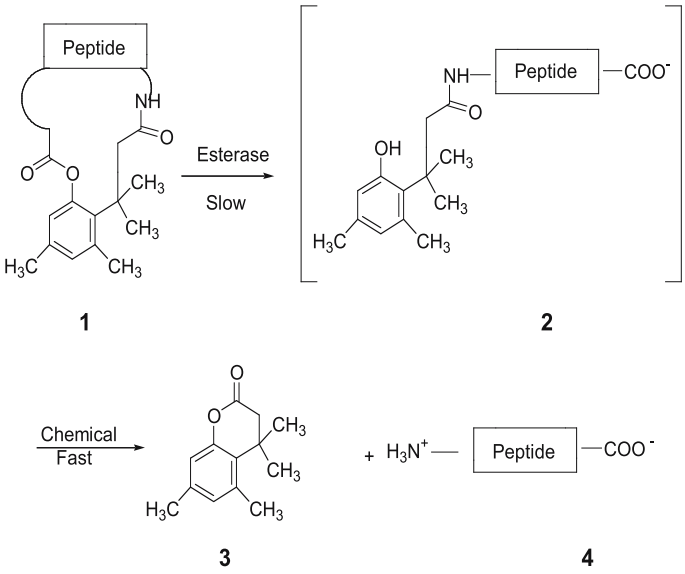
This cyclic prodrug concept was first demonstrated with a "trimethyl lock"-facilitated lactonization of phenylpropionic acid and an acyloxyalkoxyl system. In a similar fashion, Wang and co-workers have developed a coumarin-based cyclic prodrug system for peptides and peptidomimetics. The use of a coumarin prodrug moiety has one unique benefit, *i.e.*, the toxicity profile of the end product of the prodrug moiety, coumarin, is well understood (Fentem, 1992; National Toxicology Program, 1993; Pelkonen, 1997), which helps to eliminate one major uncertainty in prodrug development. This section will give some examples of each cyclic prodrug system in discussing the issues involved. Two other unique prodrug approaches for peptides are the utilization of the N-O acyl migration derivatization and the oxazolidinone approach. These will also be discussed using a few selected examples.

Cyclic Prodrugs of Peptides

Phenylpropionic acid and acyloxyalkoxy systems

Borchardt and co-workers (1996) developed the cyclic prodrug concept with two specific systems—a phenylpropionic acid system and an acyloxyalkoxy system. In the first, the design takes advantage of the facile lactonization of the phenylpropionic acid facilitated by a "trimethyl lock" system (**Scheme 1**) (Wang *et al.*, 1997). In this system, the presence of a "trimethyl lock" is known to significantly facilitate the lactonization reaction of *o*-hydroxyphenylpropionic acid and its derivatives on the order of 10^5–10^7 (Milstein and Cohen, 1970, 1972; Borchardt and Cohen, 1972; Hillery and Cohen, 1983). In this design, an amino and a carboxyl group of a peptide can be masked simultaneously through amide and ester bond formation with the respective carboxyl and hydroxyl groups of the "trimethy lock" system. The masking of the two polar and ionizable functional groups results in a significant modification of the physicochemical properties of the peptides.

The initial proof of principle studies with this prodrug strategy were carried out with a hexapeptide segment of delta-sleep-inducing peptide, DSIP (H-Trp-Ala-gly-gly-Asp-Ala-OH) (Wang *et al.*, 1997). This cyclic prodrug of the model peptide showed significantly improved physicochemical properties and membrane permeability, presumably due to the masking of the two ionizable polar functional groups (Pauletti *et al.*, 1997b). Specifically, the apparent permeability coefficient (P_{app}) of the prodrug, using the Caco-2 cell culture model (Borchardt,



Scheme 1. A phenylpropionic acid-based cyclic prodrug system.

1995), was 12.1 × 10^{-8} cm/s. In contrast, the Papp value of the linear hexapeptide was less than 0.17 × 10^{-8} cm/s. In an *in vitro* study, the original peptide can be regenerated through esterase-mediated hydrolysis, as designed, using porcine liver esterase as a model enzyme. The half-life was about 1795 min. When 90% human blood plasma was used, the half-life was significantly shorter at about 508 min. The prodrug was also shown to significantly stabilize the peptide against protease-mediated degradation. For example, in 90% human plasma, the linear peptide was degraded very quickly with $t_{1/2}$ = 3.7 min, whereas the cyclic prodrug withstood the degradation with $t_{1/2}$ = 1729 min in the presence of paraxon, a known esterase inhibitor (Pauletti *et al.*, 1997b). **Table 1** lists some of the properties of the prodrugs in comparison with those of the parent drugs.

The same prodrug approach was later applied to the preparation of prodrugs of other biologically important peptides. Two such examples are the opioid peptides [Leu^5]-enkephalin and DADLE (Wang *et al.*, 1996b, 1999a). These two peptide were chosen because of their biological significance and the fact that the same structural features (*e.g.*, free *N*-terminal amino and C-terminal carboxyl groups) that are critical to their affinity and specificity for the opioid receptors also bestow these molecules with undesirable physicochemical properties (*e.g.*, charge, hydrophilicity, high hydrogen-bonding potential) that limit their permeation via the transcellular route. Again, both prodrugs showed markedly improved membrane permeation and stability (Gudmundsson *et al.*, 1999c). For example, based on the estimated P_{app} values in a Caco-2 cell culture model, the prodrug of [Leu^5]-enkephalin was approximately 1677-fold more able to permeate than was the opioid peptide itself. Similarly, the cyclic prodrug of DADLE was approximately 77-fold more able to permeate the Caco-2 monolayers than was DADLE. It should be noted that the improvement in membrane permeability as shown does

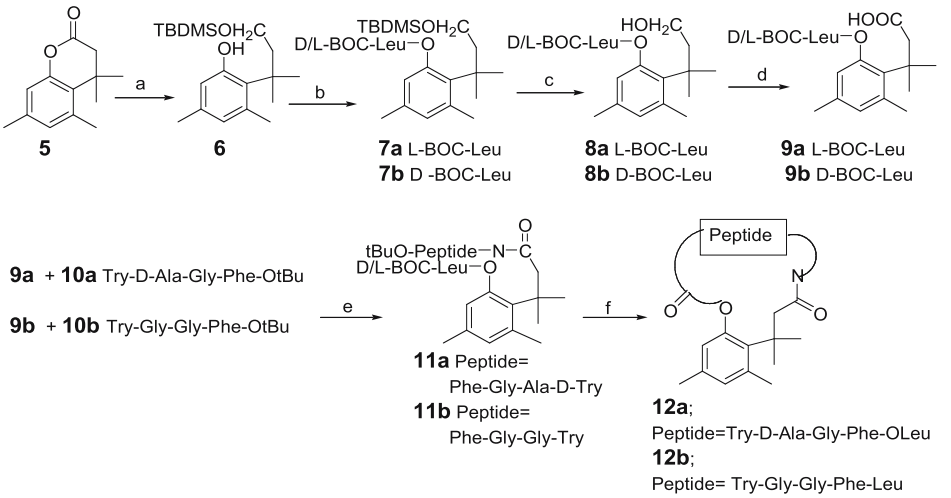
Peptide	Hydro-phobicity [log k'1AM]	Half-life in HBSS (min)	Half-life in 90% Human Plasma (min)	Caco-2 Permeability (cm/s)	Perm-eability Improve-ment
DSIP			3.7	0.17×10^{-8}	
DSIP prodrug		1795 ± 289	508 ± 24	12.1×10^{-8}	71-fold
Leu-enkephalin	0.17			$<0.0031 \times 10^{-6}$	
Leu-enkephalin prodrug	3.32	91 ± 1	79 ± 1	$5.20 \pm 0.08 \times 10^{-6}$	1677-fold
DADLE	0.43			$0.078 \pm 0.007 \times 10^{-6}$	
DADLE prodrug	3.32	90 ± 2	79 ± 1.5	5.98 ±0.51 x 10–6	77-fold

Table 1. Transport, physicochemical, and permeability properties of the phenylpropionic acid prodrugs of peptides.

not necessarily reflect the improvement of the intrinsic permeability alone. Specifically, the higher magnitude of improvement of the [Leu^5]-enkephalin prodrug was partially due to the much improved stability of the prodrug compared to the parent peptide. In contrast, DADLE is metabolically stable and, therefore, the magnitude of improvement in membrane permeability was largely due to the changes in the "intrinsic" permeability itself. Characterization of the membrane interaction potentials (logk'_{1AM}) of the cyclic prodrugs showed that the cyclic prodrugs had a significantly higher tendency to partition into the artificial membrane than did the opioid peptides (**Table 1**). A good correlation was observed between the membrane interaction potentials and the actual cell permeation characteristics of the phenylpropionic acid-based prodrugs; these were sufficiently favorable to allow these prodrugs to traverse the monolayer via the transcellular pathway.

The synthesis of these prodrugs represents a significant challenge, and the unique strategies used are worthy of discussion; we will use the synthesis of the [Leu]-enkephalin and DADLE prodrugs as examples (Wang *et al.*, 1999a). Due to the facile lactonization of compounds analogous to **2**, the carboxyl group has to be preserved in a latent form and "unmasked" only after the ester bond formation between the phenol hydroxyl and peptide carboxyl groups. Therefore, the synthesis starts with the reduction of the lactone (**5**) with lithium aluminum hydride to give the corresponding diol that is then protected as the silyl ether (TBDMS) to give **6**. The protected diol **6** is then converted to the corresponding

esters **7a** and **7b**. The deprotection of the hydroxyl group followed by oxidation gives carboxylic acids **9a** and **9b**, which are coupled to the corresponding linear tetrapeptides to give **11**. Simultaneous deprotection of the Boc and *t*-butyl groups with TFA followed by cyclization using Bop-Cl in the presence of TEA gives the final cyclic prodrugs in about 10% overall yields (**Scheme 2**) (Wang *et al.*, 1999a).



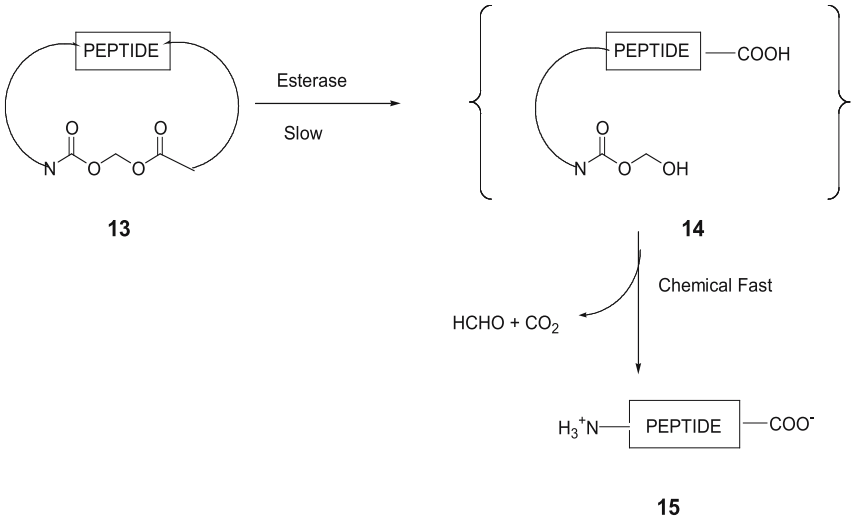
a) i. LAH/THF, ii. TBDMSCl, DCM; b) i. t-BuOK/THF, ii. D-Boc-Leu-OpNP or L-Boc-Leu-OpNP; c) H_2O-THF-HOAC (1:1:3); d) i. PCC, ii. $KMnO_4$; e) EDC/HOBT/NMM/DCM; f) i.75% TFA/DCM Phenol, ii. Bop-Cl/TEA/DCM/DMF.

Scheme 2. Synthesis of phenylpropionic acid-based cyclic prodrugs of DADLE (12a) and [Leu]5-enkephalin (12b).

The second esterase-sensitive cyclic prodrug strategy uses an acyloxyalkoxy prodrug moiety as a linker, which takes advantage of the ready collapse of the carbamate intermediate (**14**) generated from the esterase-catalyzed hydrolysis of the first ester group (**Scheme 3**) (Gangwar *et al.*, 1997a).

The initial demonstration of feasibility was also carried out with a hexapeptide segment of DSIP (Gangwar *et al.*, 1997a). The cyclic prodrug was 76-fold more able to permeate than was the linear hexapeptide when tested using the Caco-2 cell culture model (Pauletti *et al.*, 1996). As designed, the cyclic prodrug is more stable and quantitatively converted to the parent drug in the presence of an esterase (**Table 2**).

The acyloxyalkoxy linker was further examined by preparing the cyclic prodrugs of two opioid peptides, [Leu^5]-enkephalin (Tyr-Gly-Gly-Phe-Leu-OH) and DADLE (Tyr-D-Ala-Gly-Phe-D-Leu-OH) (Bak *et al.*, 1999b). As expected, the acyloxyalkoxy cyclic prodrugs of DADLE and [Leu^5]-enkephalin were readily converted to the parent peptides in the presence of porcine liver esterase, and



Scheme 3. An acyloxyalkoxy-based cyclic prodrug system.

Peptide	Hydro-phobicity [log k'$_{1AM}$]	Half-life in HBSS (min)	Half-life in 90% Human Plasma (min)	Caco-2 Permeability (cm/s)	Perm-eability Improve-ment
DSIP			3.7	0.17×10^{-8}	
DSIP prodrug		206 ± 11	100 ± 4	1.30×10^{-7}	76
Leu-enkephalin	0.17	-		$<0.31 \times 10^{-8}$	
Leu-enkephalin prodrug	1.36	444 ± 26	77.8 ± 0.5	$1.80 \pm 0.89 \times 10^{-8}$	6-fold
DADLE	0.43	-		$7.80 \pm 0.71 \times 10^{-8}$	
DADLE prodrug	1.63	442 ± 11	215 ± 23	$1.86 \pm 1.19 \times 10^{-8}$	4-fold decrease

Table 2. Transport, physicochemical, and permeability properties of the acyloxyalkoxy prodrugs of peptides.

were significantly more lipophilic than the parent peptides (**Table 2**). Specifically, the partitioning experiments showed the membrane interaction potential as measured using an HPLC approach (log k'_{1AM}) for the cyclic prodrug of DADLE to be 1.63, while for DADLE it was 0.43. It is worth noting that the membrane

permeation of the acyloxyalkoxy-based cyclic prodrug of DADLE across Caco-2 cell monolayers in the apical (AP) to basolateral (BL) direction was approximately four times lower than that of DADLE itself, in spite of having a membrane interaction potential permeation (log k'_{IAM}) approximately four times higher. One possible reason is that the acyloxyalkoxy-based prodrugs are substrates for P-glycoprotein due to changes in their conformation upon conversion from a linear peptide to a cyclic prodrug. These apically polarized efflux systems are present in the transcelluar pathway and are known to restrict the permeation of hydrophobic molecules, including peptides (e.g., cyclosporin). The permeation of this prodrug in the BL-AP direction is 52-fold higher than in the AP-BL direction; this gives a good indication of its substrate property for P-glycoprotein. Thus, the P-glycoprotein substrate properties of the acyloxyalkoxy-based prodrugs reduce their ability to permeate across the Caco-2 cell membrane in the AP-to-BL direction (Bak *et al.*, 1999b; Gudmundsson *et al.*, 1999a).

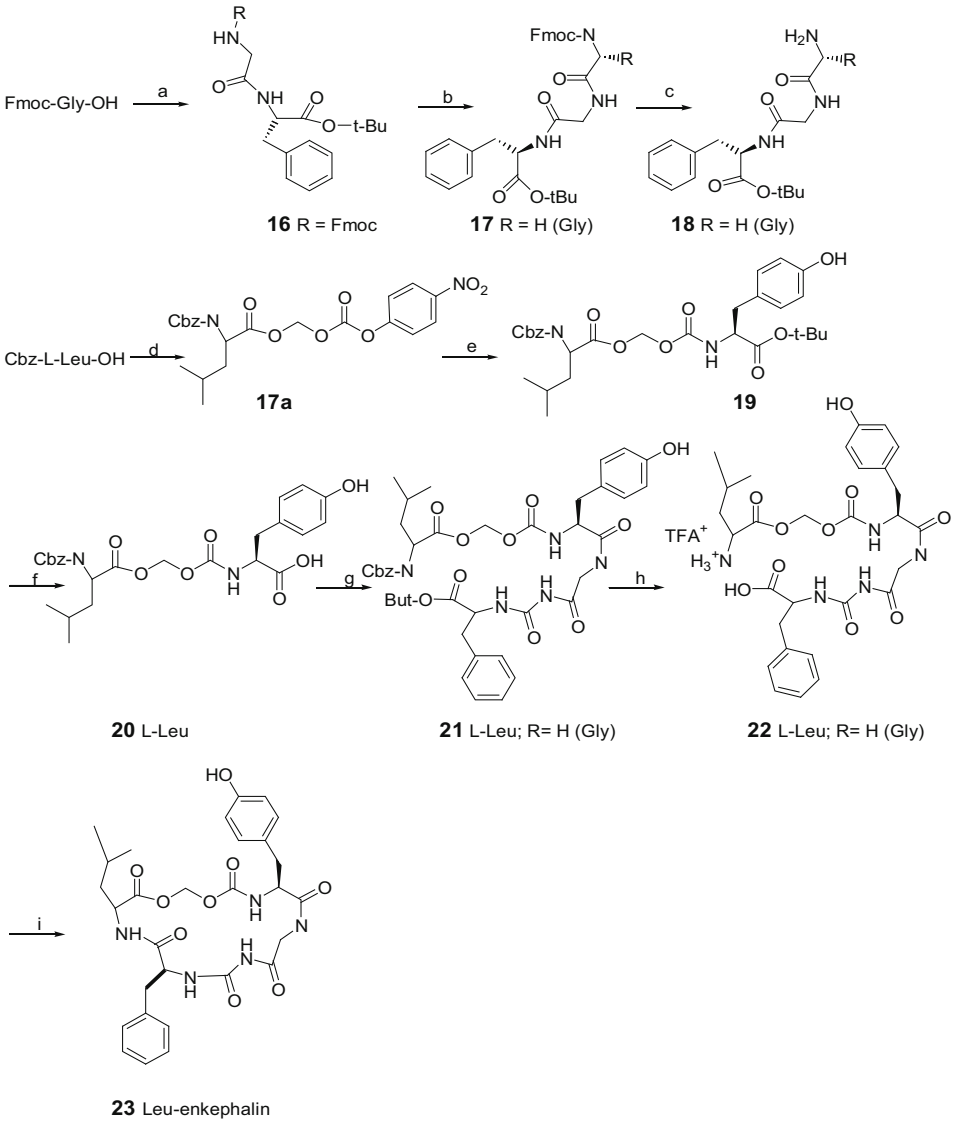
The synthesis of these prodrugs is not a trivial issue, and much work has been done to search for the optimal approaches. Due to the facile collapse of the hemiacetal intermediate (**14**), direct acylation of the hemiacetal intermediate is not possible for the preparation of these prodrugs. Instead, the synthesis requires converging two fragments, **18** and **20**, for each cyclic peptide (**Scheme 4**). Using [Leu]-enkephalin as an example, the synthesis of its prodrug, **18**, starts with the coupling of Fmoc-Gly-OH and Phe-OtBu to give compound **16**. After deprotection, the coupling of the second amino acid (Fmoc-Gly-OH) gives compound **17**. Repetition of the deprotection followed by coupling with **20** yields product **21**. The deprotection of the Cbz- group by hydrogenation and the *t*-butyl ester group with TFA followed by cyclization using HBTU in DMF then gives the desired cyclic prodrug **23** in 40% yield (Bak *et al.*, 1999b).

Coumarinic acid-based prodrugs

Based on the cyclic prodrug concept developed by Borchardt and co-workers, the Wang laboratory developed a coumarin-based prodrug system for amines and peptides. This system takes advantage of the ready lactonization of coumarinic acid and its derivatives due to the presence of a *cis* double bond (**Scheme 5**) (Wang *et al.*, 1996b). Although the coumarin-based prodrug system is similar to the phenylpropionic acid system in terms of the release mechanism, the chemistry of the release reactions, and the release rate, it has many unique features. First, the final product after the drug is released is coumarin, which has been found in extensive studies to be non-toxic (Fentem and Fry, 1993; Fentem *et al.*, 1992; National Toxicology Program, 1993; Van Iersel *et al.*, 1994; Pelkonen *et al.*, 1997). Second, the starting material for the synthesis of the coumarin-based prodrugs is inexpensive and readily available. Third, the release rate of the coumarin-based prodrug system can be further manipulated by the introduction of additional substituents on the aromatic ring or the acyl group.

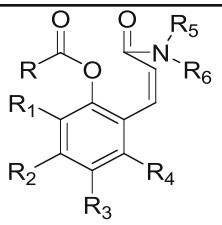
Structural effect on the release kinetics

In developing the coumarin-based prodrug system, the Wang group first examined the structural effect on the release rates through the synthesis and evaluation of a series of coumarinic acid-based prodrugs. **Table 3** shows the release rates, in phosphate buffer at pH 7.4 in the presence of PLE, of analogs



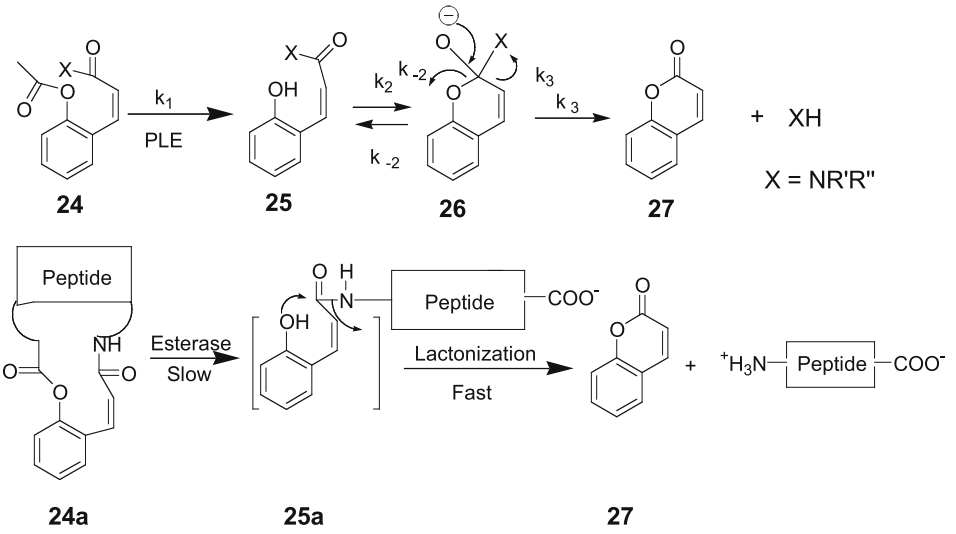
a)EDC, HOBT, NMM, HCl, Phe-OtBu, DCM; b) Fmoc-Gly-OH, EDC, HOBT, NMM, DCM; c) Di- ethylamine; d) 1-iodomethyl-*p*-nitrophenylcarbonate,DMF, 25°C; e) tyrosine *t*-butyl ester NMM, HOBT, DMF, 25°C; f) TFA, DCM; g) 18, EDC,HOBT, NMM; h) i H_2, Pd-C, ii. TFA-DCM; i) HBTU, DIEA, DMF

Scheme 4. Synthesis of the acyloxyalkoxy-based cyclic prodrug of [Leu^5]- enkephalin (23).



Compound	R	R_1	R_2	R_3	R_4	R_5	R_6	k_{obs} (10^4) (s^{-1})	$t_{1/2}$ (min)
28	Me	H	H	H	H	H	$PhCH_{2}$-	4.34	15.95
29	Et	H	H	H	H	H	$PhCH_2$	3.90	17.88
30	i-Pr	H	H	H	H	H	$PhCH_2$	3.49	19.88
31	t-Bu	H	H	H	H	H	$PhCH_2$	3.43	20.32
32	Me	H	H	H	H	H	Pr-	5.56	12.5
33	Me	H	H	H	H	H	Cyclohexyl-	1.37	50.73
34	Me	H	H	H	H	H	p-MeO-Ph	0.37	1.63
35	Me	H	H	H	H	Me	$PhCH_{2}$-	2.1	32.53
36	Me	H	H	H	H	Et-	Et-	0.37	188
37	Me	Me	H	H	Me	Me	$PhCH_{2}$-	14.4	4.8
38	Me	Me	H	H	Me	Et	Et	6.1	11.4
39	Me	H	Me	H	Me	Me	$PhCH_{2}$-	11	6.3
40	Me	H	Me	H	Me	Et	Et	3.7	18.8
41	Me	Me	Me	H	H	Me	$PhCH_{2}$-	21.8	3.2
42	Me	Me	Me	H	H	Et	Et	5.8	11.9
43	Me	Me	H	Me	H	Me	$PhCH_{2}$-	8.2	8.5
44	Me	Me	H	Me	H	Et	Et	1.5	46.4
45	Me	Me	H	H	H	Me	$PhCH_{2}$-	9.4	7.4
46	Me	Me	H	H	H	Et	Et	2.3	30.6
47	Me	H	Me	Me	Me	Me	$PhCH_{2}$-	7.3	9.5
48	Me	H	Me	Me	Me	Et	Et	1.5	45.1
49	Me	H	H	Me	H	Me	$PhCH_{2}$-	2.6	26.4
50	Me	H	H	Me	H	Et	Et	0.34	204
51	Me	H	Me	H	H	Me	$PhCH_{2}$-	2.2	32.1
52	Me	H	Me	H	H	Et	Et	0.32	212
53	Me	H	OCH_3	H	H	Me	$PhCH_{2}$-	2	34.5
54	Me	H	OCH_3	H	H	Et	Et	0.35	197

Table 3. Substituent effect on the release rates of coumarin-based prodrugs.



Scheme 5. Facile cyclization of coumarinic acid and its analogs.

with varying substituents on the acyl group (R), the phenyl ring (R_1-R_4) and the amine part (R_5-R_6).

Briefly, variations of the steric bulkiness of the acyl group (R) have only a minor effect on the overall half-lives of prodrugs **28**-**31**, which suggests that the first step (k_1), the esterase-catalyzed hydrolysis of phenol ester, is not the rate-limiting step (**Scheme 5**) (Wang *et al.*, 1998). On the other hand, the structural features of the amine moiety showed much greater effect on the release rates. Specifically, factors that can stabilize a developing negative charge on the nitrogen during the collapse of tetrahedral intermediate **26** (**Scheme 5**) tend to facilitate the lactonization, while steric hindrance tends to hinder the lactonization (Wang *et al.*, 1998). Such results indicate that it is the collapse of the tetrahedral intermediate that is the rate-limiting step in this cyclization reaction.

With respect to the steric effect on the phenyl ring, it was found that the methyl substituent ortho to the phenol hydroxyl group (R_1) or the alkyl side chain (R_4) could enhance the rate of lactonization, presumably by forcing the phenol hydroxyl group and the carboxyl group closer to each other or by restricting the freedom of rotation. The electronic effect of substituents at the R_2 and R_3 positions did not play an important role in influencing the overall half-lives (Liao and Wang, 1999; Liao *et al.*, 2000).

Coumarin-based prodrugs of opioid peptides

The coumarinic acid-based cyclic prodrug strategy was used for the preparation of the prodrugs of opioid peptides [Leu^5]-enkephalin and DADLE (Wang *et al.*, 1996b, 1998; Gudmundsson *et al.*, 1999c). As expected, the coumarinic acid-based cyclic prodrugs of the opioid peptides were shown to slowly degrade to the parent peptides in stoichiometric amounts in the presence of an

esterase. In 90% human plasma and rat liver homogenate, the rates of disappearance of the cyclic prodrugs were significantly faster than in HBSS, pH 7.4. For example, the half-life of the DADLE prodrug is about 7.2 min in rat liver homogenates and 375 min in HBSS. This enhanced rate of prodrug conversion in liver homogenates can be inhibited significantly by paraoxon, a known inhibitor of serine esterase. Such results indicate that the bioconversion is largely an esterase-mediated process. When applied to the AP side of a Caco-2 cell monolayer, the coumarinic acid prodrug of [Leu^5]-enkephalin exhibited significantly higher stability against peptidase-mediated hydrolysis than did the parent peptide. The prodrug of DADLE displayed similar stability. The permeation of the [Leu^5]-enkephalin cyclic prodrug through Caco-2 monolayers was 665-fold higher than that of the parent peptide, while the cyclic DADLE prodrug was approximately 31-fold more able to permeate than its original peptide (Gudmundsson *et al.*, 1999c). The membrane interaction potentials for these

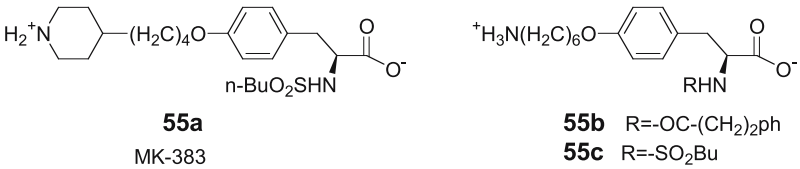
Peptide	**Hydrophobicity [log k'$_{IAM}$]**	**Half-life in HBSS (min)**	**Half-life in 90% Human Plasma (min)**	**Caco-2 Permeability (cm/s)**	**Permeability Improvement**
Leu-enkephalin	0.17			$<0.0031 \times 10^{-6}$	
Leu-enkephalin prodrug	2.70	299	108	2.06×10^{-6}	665-fold
DADLE	0.43			0.078×10^{-6}	
DADLE					
prodrug	2.91	375	113	2.42×10^{-6}	31-fold
55b				3.88×10^{-7}	
55b					
prodrug		301 ± 12	57 ± 2	1.90×10^{-6}	5-fold
55c				3.94×10^{-7}	
55c					
prodrug		630 ± 14	91 ± 1	2.42×10^{-6}	6-fold

Table 4. Transport, physicochemical, and permeation properties of coumarin-based prodrugs.

compounds determined using a chromatographic method correlate very well with their permeabilities. These results are listed in **Table 4**.

Application to peptide mimetics

The coumarin-based prodrug strategy has also been used for the preparation of cyclic prodrugs of peptidomimetics such as RGD (Arg-Gly-Asp) analogs, MK-383 (**55a**) and **55b** and **55c** (**Figure 1**). These cyclic prodrugs, as expected, released the parent drugs upon incubation with porcine liver esterase (**Scheme 5**) (Wang *et al.*, 1999b). The transport characteristics of cyclic prodrugs of the RGD analogs also showed significant improvement over those of the corresponding parent drugs, when examined using the Caco-2 cell culture model (**Table 4**) (Camenisch *et al.*, 1998). The apparent membrane permeabilities of RGD analogs 55b and 55c were determined to be 3.88×10^{-7} cm/s and 3.94×10^{-7}, respectively (Camenisch *et al.*, 1998). The coumarinic acid-based prodrugs of **55b** and **55c**, on the other hand, exhibited apparent membrane permeabilities that were approximately five- (1.90×10^{-6} cm/s) and sixfold (2.42×10^{-6} cm/s) higher than those of the corresponding RGD analogs, respectively. It should be noted that the magnitude of the improvement with the peptidomimetics was less than that of observed with peptides such as [Leu]-enkephalin and DADLE (Gudmundsson *et al.*, 1999c). One possible explanation for this difference is that intramolecular hydrogen bond formation in the cyclic prodrug of DADLE could have helped to improve membrane permeation (Gudmundsson *et al.*, 1999a) while similar intramolecular hydrogen bonds can not be formed with the cyclic prodrugs (**55b** and **55c**) of the RGD peptide mimetics because of the lack of regularly spaced amide bonds.



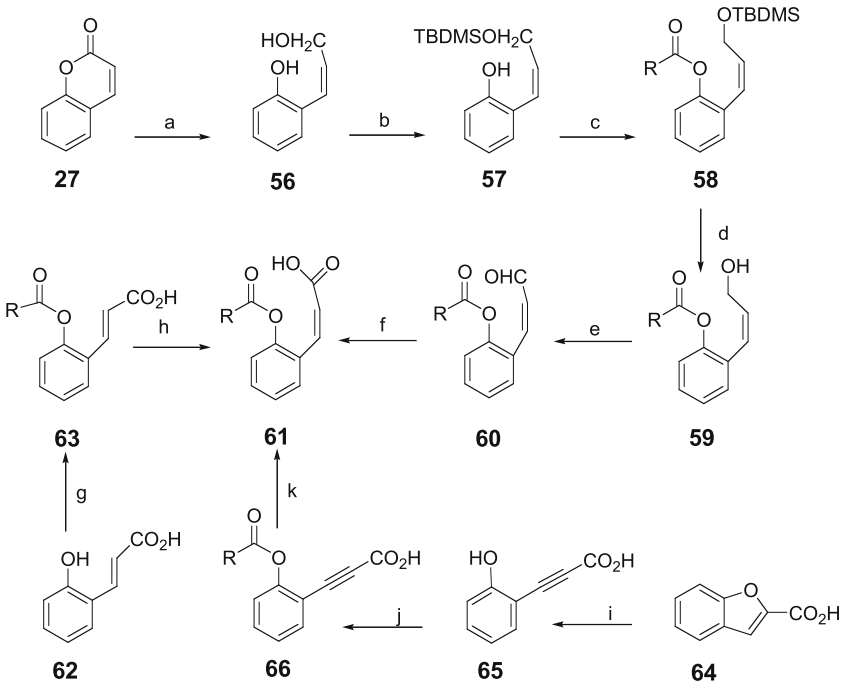
55a MK-383

55b R=-OC-$(CH_2)_2$ph

55c R=-SO_2Bu

Figure 1. Coumarin-based cyclic prodrugs of RGD analogs.

The synthesis of the coumarin-based prodrugs takes an approach similar to that of the phenylpropionic acid-based system. Therefore, the key is the synthesis of the acylated cis-coumarinic acid **61** (**Scheme 6**). Because of the facile lactonization and short half-lives of coumarinic acid and its derivatives **25** (**Scheme 5**), direct acylation of the phenol hydroxyl group of coumarinic acid is not feasible. The Wang group has developed three synthetic approaches to **61** (Wang *et al.*, 1996a, 1998; Zheng *et al.*, 1999). The first method is somewhat lengthy and starts with the reductive opening of the lactone ring of coumarin **27** (**Scheme 6**). The two hydroxyl groups can then be differentiated by the selective



a) LAH/ 0 ºC, 44%; b) TBDMS-C1/DMAP/CH_2Cl_2, 83%; c) RCOOH/DCC/HOBt, 76-100%; d) H_2O-THF-HOAc (1:1:3), 100%; e) MnO_2, 51-94%; f) H_2O_2, $NaClO_2$, 10 ºC, 96-100%; g) $(RCO)_2O$/Et3N/DMAP or RCOOH/DCC/HOBt/DMAP; h) UV, MeOH; i) LDA/THF, -78 ºC, 89%; j) Ac_2O/THF, TEA (87%) or Boc-Leu-OH, $(CH_3)_3COCl$, TEA, -5ºC, 80%; k) benzylamine, EDC, HOBt, DMAP, CH_2Cl_2, 80%

Scheme 6. Three synthetic approaches to the key intermediate, 61, for the synthesis of coumarin-based prodrugs.

protection of the allyl hydroxyl group with a sterically hindered silyl group, TBDMS. After acylation of phenol hydroxyl group and deprotection of TBDMS, oxidation of the primary hydroxyl group would give the desired acid **61** (Wang *et al.*, 1996a, 1998). The second approach starts from o-hydroxyl-trans-cinnamic acid (**62**); first the phenol hydroxyl group is acylated, followed by a photoisomerization from the *trans* to the *cis* form to give **61**. The third method is based on the hydrogenation of an alkyne group to give the desired cis double bond. Specifically, compound **65** can be acylated first before catalytic hydrogenation using Lindlar catalyst to give the desired acid **61** (Zheng *et al.*, 1999).

Conformational effect of the cyclic prodrugs on their permeability.

Although the primary reason for the physicochemical property changes of a peptide upon formation of a cyclic prodrug in the above-mentioned cases was thought to be the masking of two ionizable functional groups; conceivably, other

factors such as conformation, formation of intramolecular hydrogen bonds, etc. could also affect the outcome. To understand how the solution structures of the acyloxyalkoxy-based, phenylpropionic acid-based, and coumarinic acid-based cyclic prodrugs could be a factor, conformational studies using spectroscopic techniques (2D-NMR, CD) and molecular modeling were performed.

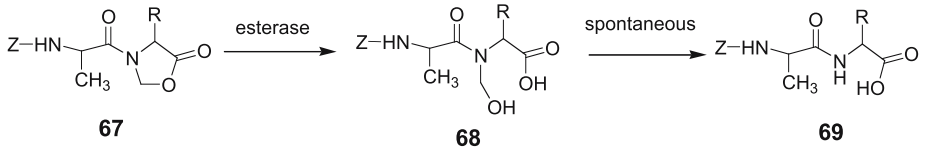
These studies indicated that the coumarinic acid-based cyclic prodrugs have compact and rigid secondary structures composed of beta-turns that are partially stabilized by the formation of intramolecular hydrogen bonds. The existence of these well-defined secondary structures in the cyclic prodrugs, particularly with the formation of intramolecular hydrogen bonds, correlated well with the ability of the prodrugs to partition into an artificial membrane and to permeate the Caco-2 cell monolayers (Gangwar *et al.*, 1996; Gudmundsson *et al.*, 1999a). Phenylpropionic acid cyclic prodrugs also exhibited more defined rigid and compact secondary structures, composed of beta turns, than did the parent peptides. The formation of intramolecular hydrogen bonds, as well as the incorporation of the lipophilic phenylpropionic acid promoiety into the peptide sequence, may partially account for the improved ability of the prodrugs to partition into an artificial membrane (Gudmundsson *et al.*, 1999a).

The solution conformations of acyloxyalkoxy-based prodrugs are different from those of the coumarin-based and the phenylpropionic acid-based systems. While the phenylpropionic acid-based and coumarin-based prodrugs exist in well-defined solution conformations with significant secondary structures (*e.g.*, beta-turns), the acyloxyalkoxy-based prodrugs have two major conformers that exist in equilibrium as determined by NMR. These conformers arise from the cis-trans isomerization of the carbamate junction between the promoiety and the peptides. It is possible that the unique solution conformations of the acyloxyalkoxy-based prodrugs allow them to interact with P-glycoprotein, which is the reason for their enhanced permeation in the BL-to-AP direction in Caco-2 experiments (Bak *et al.*, 1999a). Alternatively, the acyloxyalkoxy linker itself cannot be ruled out at this time as the structural feature recognized by P-glycoprotein.

An Oxazolidinone Approach

Aiming at developing a strategy to improve the stability of peptides toward carboxypeptidases, Friis and coworkers (1996) used an oxazolidinone group as a bioreversible mask of the terminal carboxyl group of a peptide. The prodrug **67** can release the parent peptide drug through a two-step process (**Scheme 7**): 1) enzymatic or chemical hydrolysis of the ester to give an *N*-hydroxymethyl derivative **68** and 2) the spontaneous chemical degradation to release the peptide **69**. The work is based on similar earlier work by Bundgaard in studying the effect of *N*-hydroxyalkylation on the stability of peptides (Buur and Bundgaard, 1988; Bundgaard and Rasmussen, 1991a,b). Two Z-protected dipeptides (Z = benzyloxycarbonyl) (Z-Ala-Gly-COOH and Z-Ala-Ala-COOH) were used as model peptides to study the stability and carboxypeptidase lability of the peptide

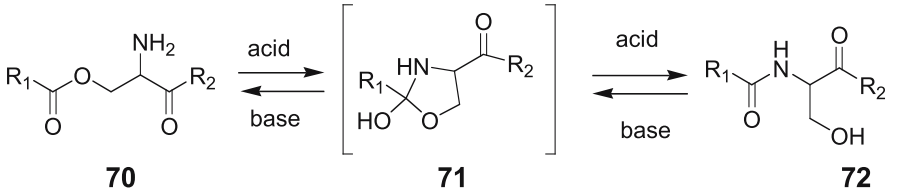
prodrugs. It was found that the prodrug **67** degraded quantitatively to the parent dipeptides in aqueous buffer solution at pH 7.4 and in 80% human plasma via the formation of the α-hydroxymethyl intermediate **68**. The prodrug **67** resisted carboxypeptidase A (CPA)-mediated hydrolysis. For example, Z-Ala-Ala-COOH has a half-life of about 47 min in the presence of CPA (25 μ/mL). However, its corresponding prodrug **67** has a half-life of about 11.6 h under identical conditions, which is about the same as the half-life of the peptide prodrug in the absence of CPA. Such a prodrug derivatization could also help to lower the polarity of a peptide and, therefore, increase its passive permeation through biological membranes. This prodrug system has the potential to be used for improving the absorption of peptides because of decreased enzymatic (carboxypeptidase) degradation and enhanced membrane permeability. However, more work needs to be done in studying the impact of this derivatization on the membrane permeability in order to assess the true potential of this prodrug system.



Scheme 7. An oxazolidinone approach to peptide prodrug derivatization.

N,O-intramolecular Acyl Migration-based Prodrug

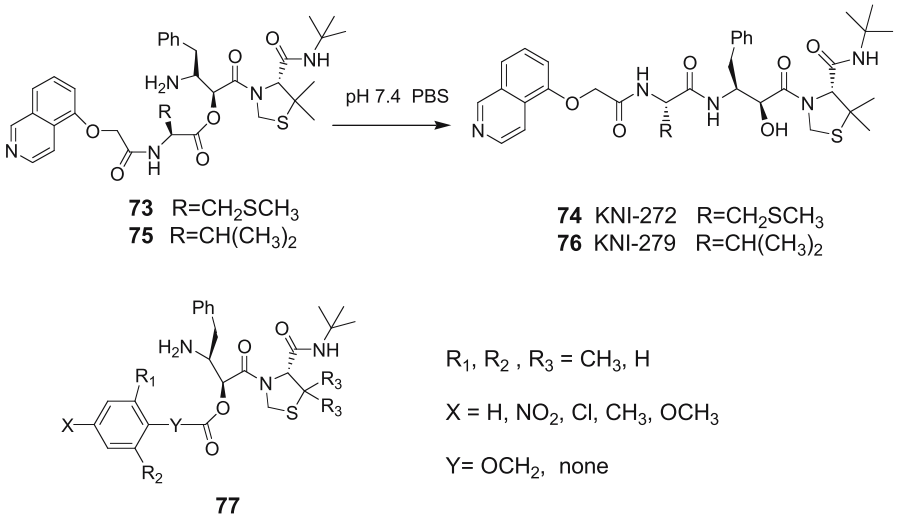
To increase the aqueous solubility of peptides, an *N,O*-intramolecular acyl migration reaction can be used as another approach for prodrug preparation for peptides containing a β-hydroxy group (**Scheme 8**). These kinds of prodrugs can undergo acid-catalyzed *N,O*-acyl transfer to form the corresponding *O*-peptide prodrug, which has a protonated amine group that can impart more desirable water solubility properties to the molecule. Under physiological conditions, the *O*-peptide prodrug undergoes non-enzymatic conversion to form the parent polypeptide (**72**, **Scheme 8**). The rate and the extent of *N*-peptide formation are the principal determinants to the applicability of *O,N*-acyl prodrug of *N*-peptides in a particular application (Oliyai and Stella, 1992; Oliyai *et al.*, 1994, 1995).



Scheme 8. *N,O* intramolecular acyl migration for the regeneration of the parent peptide drug.

Hurley *et al.* (1993) and Oliya and Stella (1995) have separately shown that *O*-peptide prodrugs derived from primary amines are readily and quantitatively converted to the parent *N*-peptides ($t_{1/2}$ is about 1 min at 37 °C and pH 7.4). For example, under simulated physiological conditions, the *O*-peptide prodrug of cyclosporin A (CsA), isocyclosporin A (isoCsA), showed quantitative conversion to the parent polypeptide (CsA) (Oliyai and Stella, 1992; Oliyai *et al.*, 1994, 1995). However, the rate of conversion was too slow for isoCsA to be considered as a prodrug of CsA. This may be attributed to the fact that the *O*-peptide prodrug of CsA contains a secondary amine functionality, which is sterically hindered.

In recent years, this approach has been used for preparing prodrugs of HIV protease inhibitors such as KNI-272 and its analogues that possess the allophenylnorstatin (Apns) residue (Hamada *et al.*, 2002, 2003). Because the structures in these compounds have both adjacent alpha-hydroxyl and beta-amino groups and an acyl group connected to hydroxyl group, the *N*→*O* intramolecular acyl migration can be triggered through the formation of an energetically favorable five-member ring intermediate. Two specific examples are prodrugs **73** and **75**, which showed over 4000-fold improvement in water solubility (>300 mg/mL) in comparison with the parent compounds, **74** (KNI-272) and **76** (KNI-279) (**Scheme 9**). These prodrugs were stable as the HCl salt and in strongly acidic solutions corresponding to gastric juice (pH 2.0) and can be converted to the parent compounds promptly in an aqueous environment from slightly acidic to basic pH (Hamada *et al.*, 2002, 2003).



Scheme 9. Examples of *N,O* intramolecular acyl migration prodrugs.

The relationship between the structural features of the O-acyl moiety and the migration rate has been studied for analogs of **77**. The substituent (X) on the phenyl ring had a very significant effect on the migration rates. With the

introduction of an electron-withdrawing group, such as a nitro or chloro, an accelerated migration rate was observed, while with the introduction of an electron-donating group, such as a methyl or methoxy group, the migration rate decreased. The Hammett plot for the migration rate constants of the prodrugs gave a linear free energy relationship ($\rho = 1.31$, $r = 0.9982$) against the standard reference reaction, ionization of para-substituted benzoic acid. This suggests that the migration reaction proceeds under a single mechanism with the same rate-limiting step, and the electronic properties of the substituents play a very important role in determining the migration rate of these prodrugs (Hamada *et al.*, 2004). In addition to the electronic effect, the steric features of the substituents may also play a role in affecting the reaction rate. When linker Y is a flexible methylene or oxymethylene group, the migration rate is higher than if the phenyl ring is directly connected to the carbonyl carbon (Hamada *et al.*, 2004). R_1 and R_2 groups also influence the migration rate of the acyl group. The presence of two methyl groups at the R_1 and R_2 positions lowered the migration rate compared with that of the compound in which only one of the R groups is a methyl group. The R_3, on the other hand, is too far from the migration center to have any steric effect on the migration rate (Hamada *et al.*, 2004).

Conclusions

In the post-genomic era, one can expect to see the discovery of more and more biologically active peptides. However, the development of peptide-based pharmaceutical agents, especially for oral applications, has been hampered by the generally poor permeability and stability of peptides. Prodrug strategies represent a unique approach to solving such problems. We hope that this chapter has shown, in general terms, the kind of strategies available for peptide prodrug preparation and will stimulate more research in the development of new methods in this area.

Acknowledgments

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3.8

Macromolecular Prodrugs of Small Molecules

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Table of Contents

Abbreviations

ADEPT .....Antibody-directed enzyme prodrug therapy
ADH .....Adipic dihydrazide
Ara-C .....Cytarabine
AUC .....Area under the curve
AZT .....Zidovudine
C6D .....Carboxy-n-pentyl-dextran
C6D-ED C6D-ethylenediamine
CDI .....N,N'-carbonyldiimidazole
CM-dextran .....Carboxymethyl dextran
Dach-PT(chlorato).....*Cis*-Dichloro(cyclohexane-*trans*-l-1,2-diamine)platinum(II)
DCC .....N,N'-dicyclohexyl carbodiimide
DCM-dextran .....Dicarboxymethyl dextran
EDC .....N-ethyl-N'-(dimethylaminopropyl)carbodiimide)
EEDQ N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline
EPR .....Enhanced permeability and retention
HA .....Hyaluronan
HPMA .....N-(2-hydroxypropyl)methacrylamide
HP(SEC) .....High-performance size-exclusion chromatography
MMC-D .....Mitomycin C-dextran conjugate
MTX .....Methotrexate
NHS .....N-hydroxysuccinimido-hemisuccinate
OX-Dextran .....Fully oxidized dextran
PAA .....Poly-L-aspartic acid
Pas .....Plasminogen activator
PDEPT .....Polymer-directed enzyme prodrug therapy
PDM .....N,N-di-(2-chloroethyl)-4-phenylene diamine mustard
PGE_1 .....Prostaglandin E_1
PHEA .....Poly[α,β-(N-2-hydroxyethyl-DL-aspartamide)]
PHEG .....Poly[N^5-(2-hydroxyethyl)-L-glutamine]
PHPA .....Poly[α,β,-(N-3-hydroxypropyl-DL-aspartamide)]
PLCAI .....Poly-(L-lysine citramide imide)
PLGA .....Poly-L-glutamic acid

PLGA-HZ.....PLGA-hydrazide
POX-Dextran .....Partially oxidized dextrans

Keywords

Macromolecular, Prodrugs, Polymer, EPR, Polymer Drugs, Polymer Therapeutics

Introduction

In this chapter the term "macromolecular prodrug" will refer to a type of therapeutic agent in which multiple pharmacologically active small molecules (drugs) are covalently, yet reversibly, linked to a macromolecular polymer (promoiety). The versatile nature of these polymers allows them to address several problems. First, the increased time needed for polymer-drug cleavage imparts an inherent sustained release capability to macromolecular prodrugs, providing increased plasma half-life and more consistent administration; this may decrease the spikes in blood levels that contribute to toxicity in many pharmaceutical agents (Larsen and Johansen, 1989). Second, the preferential degradation of certain polymers in the colon has led some researchers to use this strategy for colon targeted delivery (Larsen *et al.*, 1989; McLeod *et al.*,1993). Third, the high aqueous solubility of many of these polymers may help solve solubility problems associated with a parent drug. Fourth, tumor targeting with macromolecular prodrugs is a promising area due to the enhanced permeability and retention (EPR) phenomena of macromolecules in tumors (Maeda and Matsumura, 1989).

Several other chapters in this book address these rationales from different vantage points: controlled release, altered drug clearance and targeting, the lymph system, colon, and tumor. These specific uses will be addressed in their respective sections in this volume; the purpose of this chapter is to provide a survey of the various macromolecular promoieties used, the drug functional groups to which they may be bound, and the spacers used to attach such drugs to the macromolecules. This chapter is not intended to provide a comprehensive review of all the myriad examples of macromolecular prodrugs in the literature, but does seek to highlight representative examples, summarized in Table 1. When available, *in vivo* data will be provided, but it is not always available in this field of research.

Enhanced Permeability and Retention Effect (EPR)

The enhanced permeability and retention effect is a very important concept in macromolecule targeting to tumors. The EPR effect refers to the increased ability of macromolecules to permeate tumors, due to their leaky vasculature and their increased residence time, due to the decreased lymphatic drainage of tumors compared to normal tissue. This effect allows macromolecules and any associated anti-cancer drug molecules to accumulate in tumors.

The mechanism of enhanced permeability and retention is proposed by Maeda and Matsumura (1989). Enhanced permeability may be explained by two phenomenon. First, tumor tissues are highly vascularized, thus increasing blood flow to the tumor. Second, tumor vasculature is highly porous due to the architectural incompleteness of the tumor vessels; this allows macromolecules with molecular weights over 50 kDa to leak into the interstitial space of the tumors. Molecules of this size are not able to penetrate the vessels of normal tissues. Further, the authors go on to describe the root of increased retention as the

Polymer	Polymer Functional Group	Spacer	Drug Functional Group	Drug	Reference
Poly-α,β-aspartic acid	COOH	Direct Amide	1°NH	Procaine, (Histamine), Isoniazid	Giammona *et al.*, 1989
Poly-L-aspartic acid	COOH	Direct Ester	1°OH	Doxorubicin	Pratesi *et al.*, 1985
Poly-(L-lysine citramide imide)	COOH	Direct Amide	2°NH	Norfloxacin	Gac *et al.*, 2000
Poly-L-lysine	NH	Direct Amide	COOH	Methotrexate	Ryser and Shen, 1978
Poly-L-glutamic acid	COOH	Direct Ester	OH	Paclitaxel	Singer *et al.*, 2003
Poly-L-glutamic acid	COOH	Direct Ester	2°OH	Paclitaxel	Li *et al.*, 1998
Poly-L-glutamic acid	COOH	Ethyl	COOH	Prostaglandin E_1	Hashida *et al.*, 1999
Poly-L-glutamic acid	COOH	N-N	Ketone	Prostaglandin E_1	Hashida *et al.*, 1999
Poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)]	1°OH	Direct Ester	COOH	Gemfibrozil	Lovrek *et al.*, 2000
Poly[α,β-(*N*-3-hydroxypropyl-DL-aspartamide)]	1°OH	Direct Ester	COOH	Gemfibrozil	Lovrek *et al.*, 2000
Poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)]	1°OH	Succinate (ethyl)	1°OH	Zidovudine (AZT)	Giammona *et al.*, 1998
Poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)]	1°OH	Peptide: (Val-Leu-Lys)	NH	Cytarabine (Ara-C)	Cavallaro *et al.*, 2001
Poly[N^5-(2-hydroxyethyl)-L-glutamine]	1°OH	Peptide: (Gly, Gly-Leu-Phe, or Gly-Phe-Ala-Leu)	NH	N,N-di-(2-chloroethyl)-4-phenylene diamine mustard (PDM)	Soyez and Schact, 1997
Dextran	OH	Direct Ester	COOH	Naproxen	Harboe *et al.*, 1988
Dextran	OH	Direct Ester	COOH	Diclofenac, Ibuprofen, Ketoprofen, Fenoprofen	Larsen and Johansen, 1989

Table 1. Examples of macromolecular prodrugs

Polymer	Polymer Functional Group	Spacer	Drug Functional Group	Drug	Reference
Dextran	OH	Succinate (ethyl), Glutarate (propyl), Maleinate (ethylene)	1°OH	Metronidazole	Larsen *et al.*, 1988a
Dextran	OH	Succinate (ethyl), Glutarate (propyl)	1°OH	Dexamethasone, Methylprednisolone	McLeod *et al.*, 1993
Dextran	OH	Pentyl	2°NH	Mitomycin C	Kojima *et al.*, 1980
Dextran	OH	Alkyl	OH	Tacrolimus	Yura *et al.*, 1998
Dextran, Inulin, Poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)]	OH	Succinate (ethyl)	1°OH	Metronidazole	Vermeersch *et al.*, 1985
Partially Oxidized Dextran	Aldehyde	R-NH-R	1°NH	Naproxen	Azori *et al.*, 1986
Carboxymethyl Dextran	COOH	Peptide (poly-Gly, etc)	1°NH	CPT analogue	Tsujihara *et al.*, 1998
Carboxymethyl Dextran, Oxidized Dextran	COOH	Direct Ester	Dihydroxy	cis-Dichloro(cyclohexane-*trans*-l-1,2-diamine)platinum(II) (Dach-PT(chlorato)) dihydroxy analogues	Ohya *et al.*, 1996
Dicarboxymethyl Dextran	COOH	Direct Ester	Cl	Cisplatin	Ohya *et al.*, 2001
Hyaluronan	COOH	Adipic dihydrazide-succinate	2°OH	Paclitaxel	Luo and Prestwich, 1999
Hyaluronan	COOH	Adipic dihydrazide-succinate	2°NH	Doxorubicin	Luo *et al.*, 2002
Hyaluronan	COOH	Adipic dihydrazide-(succinate)	COOH (OH)	Ibuprofen, (Hydrocortisone)	Pouyani and Prestwich, 1994

Table 1. Examples of macromolecular prodrugs (continued)

Polymer	Polymer Functional Group	Spacer	Drug Functional Group	Drug	Reference
Hyaluronan	COOH	Adipic dihydrazide	2°NH	Daunomycin	Morales *et al.*, 1998
Hyaluronan	COOH	Direct Amide	NH	Mitomycin C	Akima *et al.*, 1996
Hyaluronan	COOH	Direct Amide	NH	Daunomycin and Adriamycin	Cera *et al.*, 1988
Hyaluronan	COOH	Direct Ester	1°OH	Cortisone and hydrocortisone	DellaValle and Romeo, 1989
Hyaluronan	1°OH	Direct Ester	COOH	Butyrate	Coradini *et al.*, 1999
N-(2-hydroxypropyl)methacrylmide (HPMA)	Amide	Peptide	2°OH	Paclitaxel	Meerum Terwogt *et al.*, 2001
HPMA	Amide	Gly-Phe-Leu-Gly	2°NH	Doxorubicin	Seymour *et al.*, 2002
HPMA	Amide	Gly-Phe-Leu-Gly	Cl	Cisplatin	Gianasi *et al.*, 2002
HPMA	Amide	Gly-Phe-Leu-Gly	2°NH	Doxorubicin	Vasey *et al.*, 1999
HPMA	Amide	NH-Cephalosporin C-COOH	2°NH	Doxorubicin	Satchi-Fainaro *et al.*, 2003
Hydrazide derivatives of carboxymethyl dextran, polyglutamate, alginic acid, and carboxymethyl cellulose	Hydrazide	Hydrazone	Ketone	Daunorubicin	Hurwitz *et al.*, 1980

Table 1. Examples of macromolecular prodrugs (continued)

immature development of the lymphatic system in solid tumors. The removal of macromolecules from the interstitial space of normal tissues is accomplished through lymphatic drainage; however, in tumors this system is not fully capable, thus allowing macromolecules to reside for longer periods in the interstitium. Macromolecular prodrugs of anticancer agents are designed to exploit this increased residence time in the tumor.

Therefore, one sees that the EPR effect will lead the drug to the doorstep of the target, the interstitium outside the cancer cell. Takakura and Hashida (1995) add that another well-known phenomenon, the increased endocytic activity of

tumor cells, will even open the door, providing a direct mechanism for the internalization of macromolecular prodrugs.

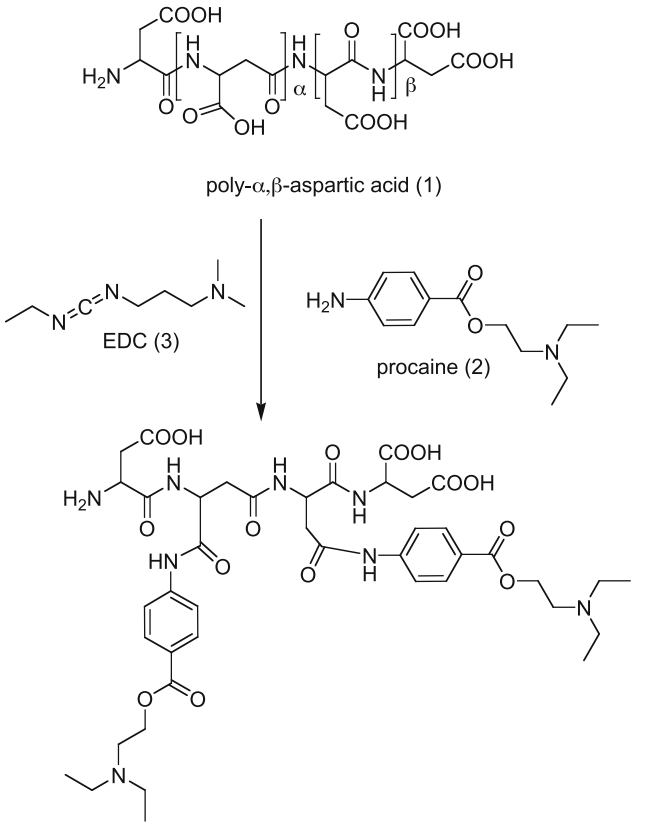
Linkage Importance

In one of the earliest reviews addressing polymeric therapeutics, Ringsdorf (1975) addressed many of the same issues that are still being studied today—depot effect, targeting, solubility, and the importance of linkage technology. The design of the spacer group, which forms the linkage between drug and macromolecules, often has the largest effect on drug release in particular environments. In a review on linkage technology Soyez *et al.* (1996) highlight many key factors to consider. The vast majority of spacer groups are susceptible to hydrolysis, whether passive or enzyme-catalyzed; thus, one must consider the pH as well as enzymes present in target cells and tissues since they will vary depending upon the target. For example, macromolecules are subject to endocytosis and may be incorporated into lysosomes that are generally acidic (pH 4.5-5.5) and contain a wide array of hydrolytic enzymes, which vary between cell types (Krinick and Kopecek, 1991).

Poly-amino Acids

Poly-amino acids are polymers consisting, usually, of a single repeating amino acid residue, such as poly-L-lysine. Often, the amino acid is chemically modified to alter its physiochemical properties or to increase its ease of linkage to a drug or targeting moiety. This class of macromolecular promoiety is a very popular area of research for several reasons; the multiple functional groups available in the amino acids comprising these polymers provide flexibility in coupling strategies, they are generally relatively nontoxic, and they are usually readily metabolized (Soyez *et al.*, 1996).

An example of an unmodified poly-amino acid is poly-α,β-aspartic acid (**1**). Giammona *et al.* (1989) have directly coupled; procaine (**2**), histamine, and isoniazid to this macromolecular promoiety. The authors were able to control the relative amounts of α and β-peptide bonds in the polymer by controlling the pH at which the polymers were condensed. They found that polymers with a high degree of α-peptide bonds contained a high amount of α-helical structure, and lower solubility, compared to polymers with a larger amount of β-peptide bonds. The authors did not state the exact proportion of α:β in the polymers used as drug carriers, nor did they compare the relative amount of drug bound to an α or β fragment, or any potential difference in drug release between the two. These are all drugs with primary amines and they were attached directly to the carboxylic acid of this promoiety via amide coupling with the reagent EDC (N-ethyl-N'-(dimethylaminopropyl)carbodiimide)) (**3**). Scheme 1 illustrates this reaction with procaine as a model drug. This carbodiimide coupling reaction is often used in ester and amide bond formation in the drug-polymer linkages that will be discussed in this chapter. A detailed explanation of the mechanism can be found in the text by Jones (2000) or in most synthetic organic chemistry texts. This direct

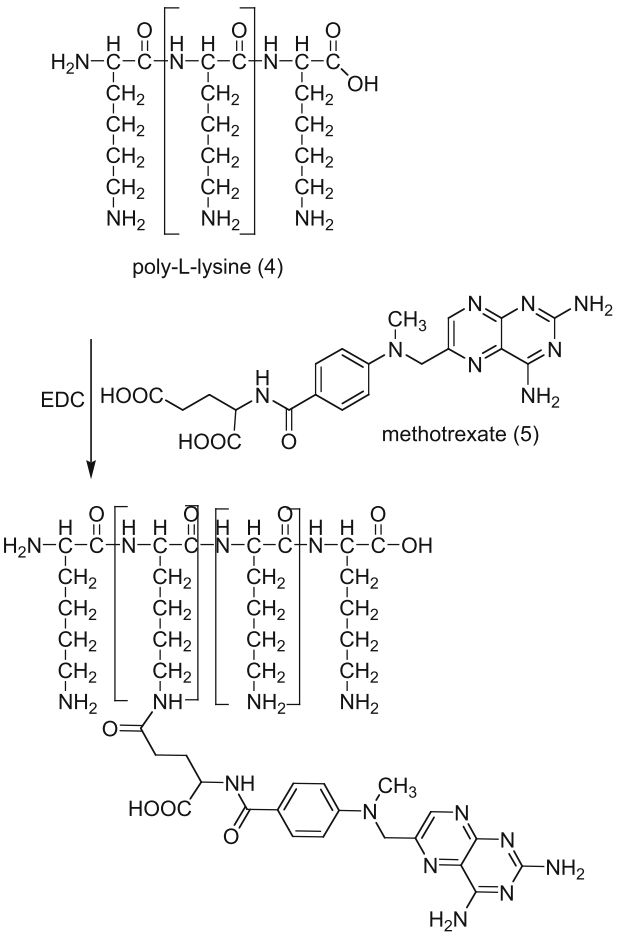


Scheme 1. Coupling of a drug with an amine group to a polymer with carboxylic acids by utilizing a coupling reagent, in this case, EDC. A common method of direct drug attachment.

attachment strategy is a simple method, yet it has several drawbacks. Steric hindrance of the release mechanism may decrease drug release from the macromolecular prodrug, crosslinking of the polymer may occur depending upon the presence of other functional groups and finally, it is not universally applicable. It is also important to note that amide bonds are generally more resistant to hydrolytic cleavage than are ester bonds.

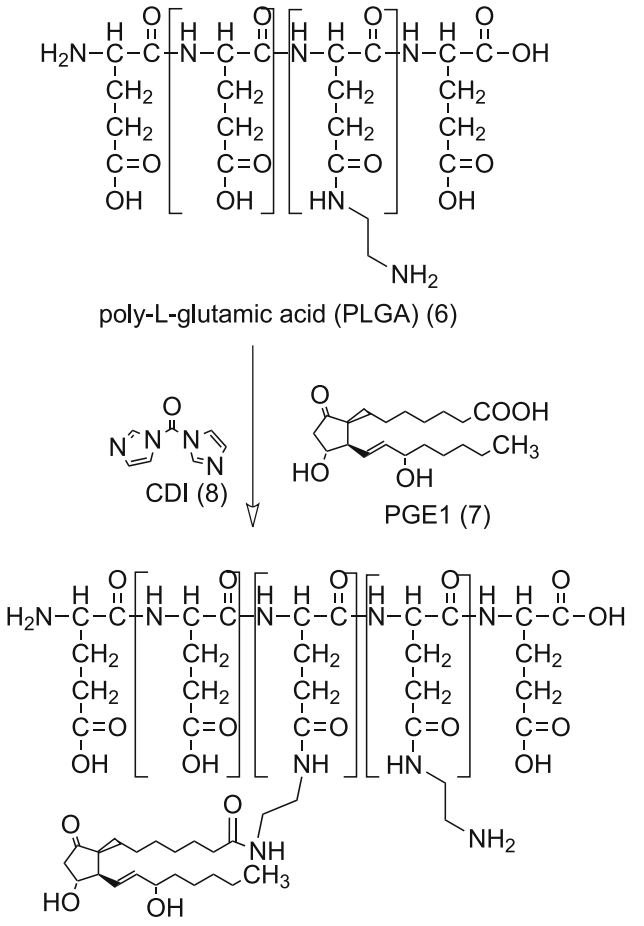
Ryser and Shen (1978) provide another example of this coupling reaction utilizing poly-L-lysine (**4**) and the anticancer drug methotrexate (MTX) (**5**). However, in this reaction the polymer contains the amine and the drug the carboxylic acid (Scheme 2). In this example an increase in drug uptake in cultured cells was observed, and it was possible to demonstrate significantly slower cell growth in a MTX-resistant cancer cell model upon exposure to the prodrug when compared to free MTX.

Poly-L-glutamic acid (PLGA) (**6**) is a commonly used pharmaceutical polymer applied to the macromolecular prodrug area. PLGA has been used with a direct ester linkage to paclitaxel (Li *et al.*, 1998). Hashida *et al.* (1999) linked prostaglandin E_1 (PGE_1) (**7**) to PLGA using two different methods. First, ethylene-



Scheme 2. Coupling of a drug with a carboxylic acid to a polymer with amines via a coupling reagent.

diamine spacer arms were attached to carboxylic acids of PLGA (**6**) via the EDC (**3**) coupling reaction described above. The carboxylic acid of PGE_1 was then activated for coupling with the reagent N,N'-carbonyldiimidazole (CDI) (**3**) and was coupled to the free end of the ethylenediamine spacer, forming an amide bond (Scheme 3). Using such a spacer was hypothesized to allow less steric hindrance at the linkage and provide easier drug release. However, the opposite proved to be true; no significant release of drug was observed from this prodrug. The second method utilized a hydrazide spacer formed by reacting poly(γ-benzyl glutamate) with hydrazine monohydrate to yield PLGA-hydrazide (PLGA-HZ) (**9**). When PLGA-HZ was reacted in an ethanol/water buffer with the PGE_1 ketone, the hydrazide reacted with the ketone, creating the new imine bond linkage (Scheme 4) (Hashida *et al.*, 1999). The authors further conjugated galactose residues to the PLGA to target the polymeric prodrug to hepatocytes since hepatocytes are capable of receptor-mediated glactose endocytosis. The radiolabeled drug did,

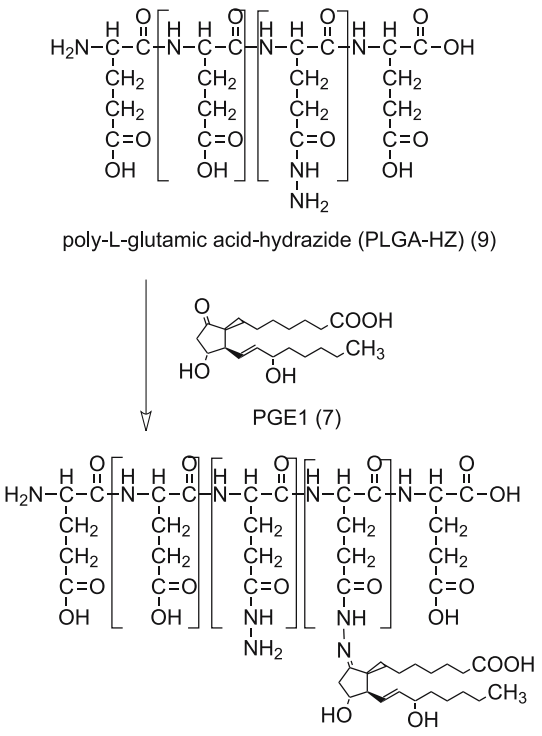


Scheme 3. Coupling of PGE_1 carboxylic acid to PLGA through ethylenediamine spacer arms.

indeed, accumulate in the liver, the target organ; in a separate experiment, drug release in liver homogenate was demonstrated for the hydrazide derivative (Hashida *et al.*, 1999).

A particular problem with most macromolecular prodrugs is characterization. In the above examples, no experiments were reported that attempted to address whether or not the products were crosslinked, intrachain or interchain. Based on the synthetic procedures, especially the method that incorporated the ethylenediamine spacer, one would suspect crosslinking would be likely; however, no data addressing this issue were presented.

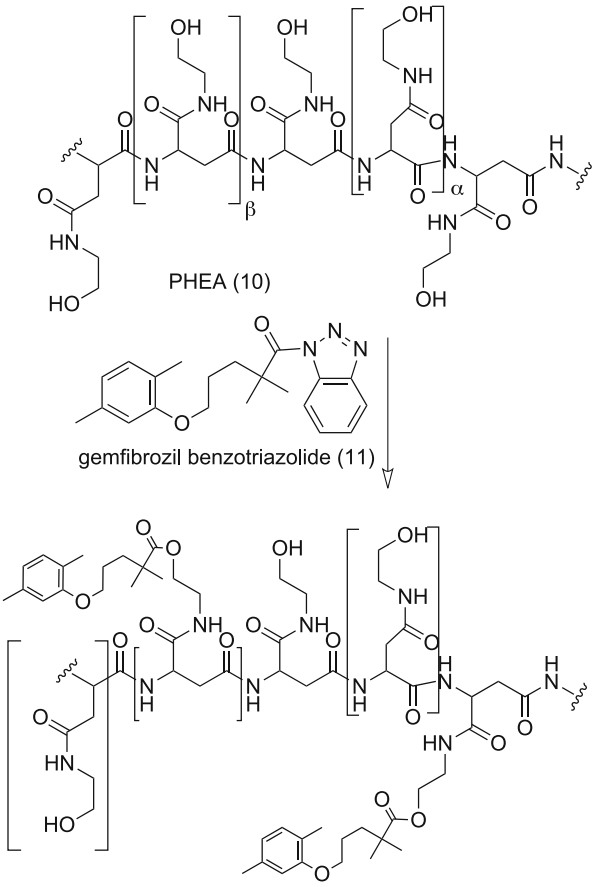
A poly-L-aspartic acid (PAA) conjugate was used by Pratesi *et al.* (1985) in an attempt to decrease the toxicity of doxorubicin. Coupling was accomplished by synthesizing a 14-bromo-doxorubicin derivative by substitution of the primary alcohol group in the original molecule. Nucleophilic displacement of the bromine by the PAA carboxylic acid then formed a direct ester linkage. Thus, after hydrolysis, doxorubicin would be released. The authors observed decreased



Scheme 4. Coupling of PGE_1 ketone to PLGA-HZ.

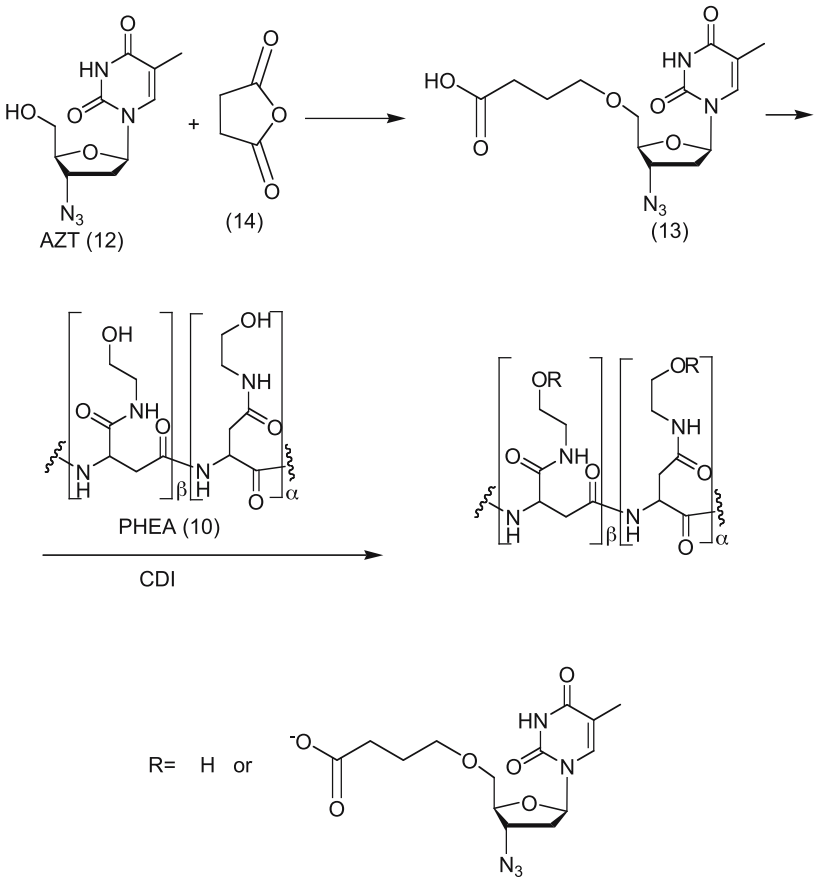
toxicity compared to free doxorubicin, measured by a 2.5-fold increase in LD_{25} in C3H/He mice. The prodrug achieved comparable efficacy in four tumor models: macrophage tumor J774 in female BALB/c mice, Lewis lung carcinoma in BDF1 (C57BL × DBA/2) mice, murine reticulum cell sarcoma M5076/73A (M5) in female C57BL mice, and mammary adenocarcinoma in female C3H/He mice with efficacy measured by an increase in life span.

The above examples utilized polymers constructed of natural amino acids. In addition, many poly-amino acids used as macromolecular promoieties contain synthetic amino acids with modified functionalities, usually for the purpose of modifying solubility or expanding drug loading capabilities. Lovrek *et al.* (2000) demonstrated two examples: poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)] (PHEA) and poly[α,β-(*N*-3-hydroxypropyl-DL-aspartamide)] (PHPA), each containing a synthetic primary alcohol that is exploited to load drug. In this example, gemfibrozil was conjugated to the polymers via the gemfibrozil benzotriazolide (**11**) to yield an ester (Scheme 5). The conjugates were soluble compounds with solubility decreasing as drug loading increased between 10–50%. In this case, the authors determined that polymer solubility depended on the presence of free primary alcohols, which were also utilized for drug loading. It is a common finding that the drug loading functionality is also responsible for polymer solubility, whether that functionality is an alcohol, carboxylic acid, amine, etc.



Scheme 5. Gemfibrozil conjugated to PHEA via the gemfibrozil-benzotriazolide, yielding a direct ester linkage.

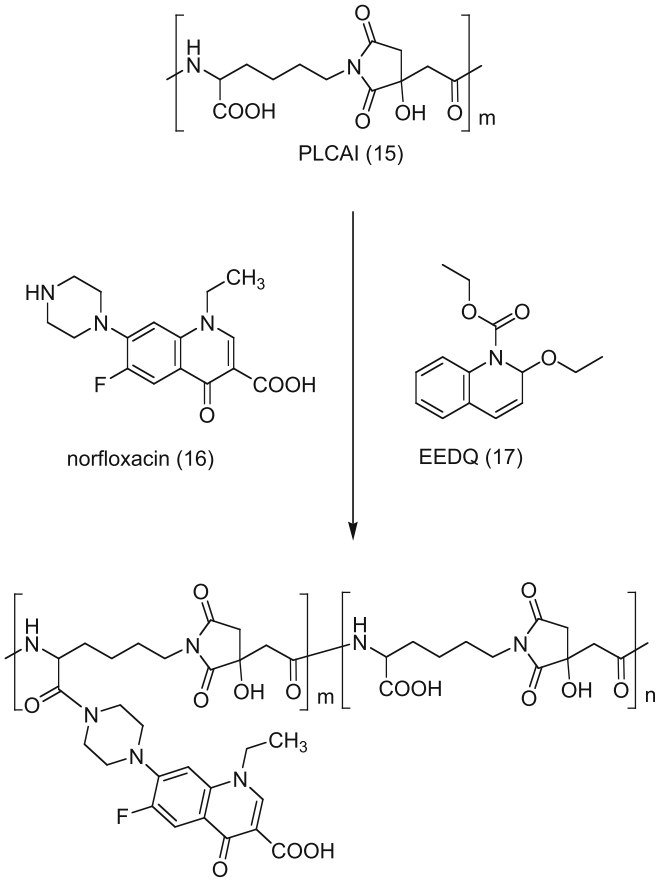
PHEA was also used by Giammona *et al.* (1998) who have conjugated the antiretroviral drug zidovudine (AZT) to PHEA (**10**). In this case, the polymer and the drug have the same functional group, a primary alcohol, and they were linked via a succinate spacer. Use of a spacer with hydrolyzable linkages at both ends is very common in macromolecular prodrugs. Here, it was accomplished by synthesizing 5'-O-succinylAZT (**13**) from AZT (**12**) and succinic anhydride (**14**) and coupling this product to PHEA with the coupling reagent CDI (**8**) (Scheme 6). Complicating this type of prodrug is the possibility that two hydrolytic products may be formed—AZT (**12**), when the drug side of the linker hydrolyzes first, and 5'-O-succinylAZT (**13**) when the polymer side of the linker hydrolyzes first. If the amount of unwanted linker-drug conjugate formed *in vivo* is high, toxicology and bioconversion studies will be necessary. In this case, both products were seen, although the quantity of AZT (**12**) was higher than that of 5'-O-succinylAZT (**13**). The ratio of the two products varied when hydrolysis was studied in numerous aqueous buffer solutions as well as in plasma, illustrating the complexity of this problem.



Scheme 6. Coupling of AZT to PHEA through a succinate spacer.

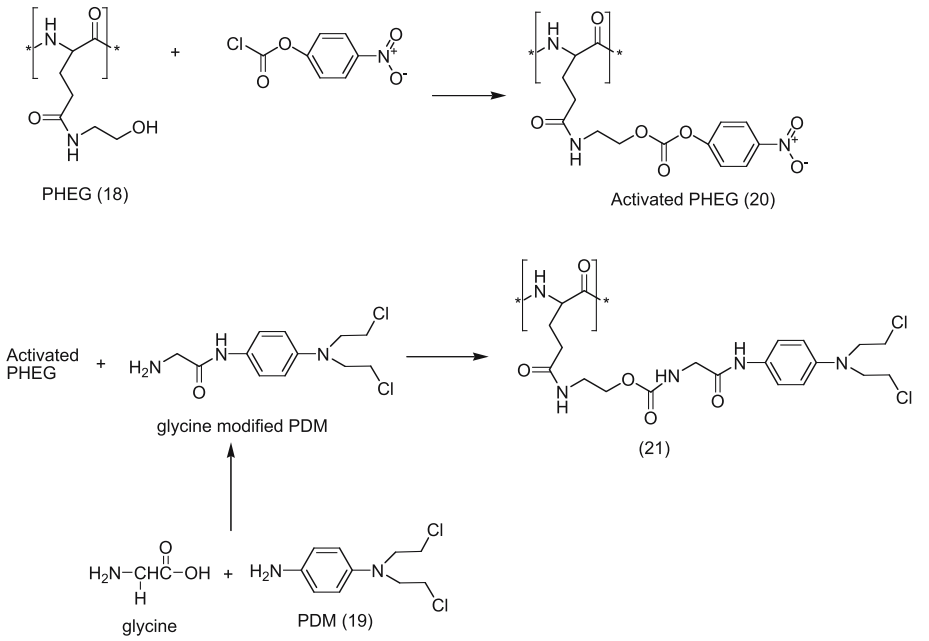
Gac *et al.* (2000) used poly-(L-lysine citramide imide) (PLCAI) (**15**) to deliver norfloxacin (**16**) (Scheme 7). Carboxylic acids of PLCAI were coupled to the piperazine secondary amine of norfloxacin (**16**) using N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (**17**) as a coupling reagent. Further, mannosyl residues were coupled to the polymer to target the mannose-specific lectin present on macrophage outer membranes. This system was studied with an *in vivo* competition assay utilizing glucose oxidase, an enzyme whose clearance is known to be dependent on mannose-specific lectin-mediated macrophage endocytosis. Upon intravenous administration of glucose oxidase and the various prodrugs to Swiss mice, it was determined that the mannose-bearing prodrugs suppressed clearance of glucose oxidase. This indicates a strong interaction with the targeted endocytosis system. In this example, about 1.2% of the total drug load was trapped as the free drug in the polymer matrix and was not actually conjugated drug, illustrating another difficulty in preparing and characterizing macromolecular prodrugs.

Soyez and Schacht (1997) demonstrated another common linkage technique, the use of peptide spacers. The authors utilized the polymer poly[N^5-(2-hydrox-



Scheme 7. Norfloxacin coupled to Poly-(L-lysine citramide imide).

yethyl)-L-glutamine] (PHEG) (**18**) to deliver N,N-di-(2-chloroethyl)-4-phenylene diamine mustard (PDM) (**19**), an anticancer alkylating agent. The aniline amine of PDM was first coupled to a peptide spacer (Gly, Gly-Leu-Phe, or Gly-Phe-Ala-Leu) via an amide bond. The primary alcohols of PHEG (**18**) were then activated by forming the 4-nitrophenyl carbonates (**20**). Finally, the terminal amine group of the peptide spacer was reacted with the activated PHEG (**20**), forming a carbamate bond (**21**) (Scheme 8). In a separate paper, the group reported that the Gly-Phe-Ala-Leu was a good substrate for collagenase IV, which is overexpressed by some tumor types (Soyez *et al.*, 1999). The following cell types were incubated in media with and without bacterial collagenase IV as well as the prodrugs: P388 murine lymphoblastic leukemia, MCF7 human breast carcinoma (wild type and anthracycline drug-resistant type), C26 murine colorectal carcinoma, and A-375M human melanoma. The prodrugs proved to be more cytotoxic when in the presence of collagenase IV. Because collagenase IV is overexpressed by some tumors, it is hypothesized that the spacer could play a crucial role in preferential drug release to those tumors; however, the *in vivo* data necessary to definitively prove this concept were not available.



Scheme 8. Coupling of PDM to PHEG utilizing a peptide spacer.

This case also provides an example of a clear alteration in the activity of the drug by attachment to a polymer. PDM (**19**), like all nitrogen mustards, is active as the aziridinium intermediate; the formation of this intermediate is facilitated by electron-donating groups, like the original amine, and suppressed by electron-withdrawing groups such as the amide in the conjugate. The anticipated decrease in toxicity of the conjugated macromolecular prodrug was observed (Soyez *et al.*, 1999).

In a similar example, Cavallaro *et al.* (2001) used related chemistry to link cytarabine (Ara-C), an anticancer drug, to PHEA (**10**) through a Val-Leu-Lys spacer that was designed to be a good substrate for plasminogen activator (PAS) enzymes. These enzymes are overexpressed in and around tumors, thus it is presumed that drug release would be targeted to the site of action.

Singer *et al.* (2003) described the use of a poly-amino acid macromolecular promoiety used with paclitaxel in clinical trials in an attempt to broaden the therapeutic window via passively increasing the delivery of drug to tumor tissue utilizing the EPR phenomena. The carboxylic acid of PLGA (6) was attached to the 2' alcohol of paclitaxel, forming a direct ester linkage via carbodiimide coupling. The PLGAs used had molecular weights of 20–35 kDa and were approximately 9% substituted with paclitaxel, making the polymers 20% paclitaxel by weight. This macromolecular prodrug entered Phase II clinical studies for the treatment of ovarian cancer, non-small-cell lung cancer, and colon cancer. In the Phase I studies patients were dosed via a 30-min intravenous infusion every 21 days at doses of up to 266 mg/m^2. Information from these studies suggests that a

higher maximum tolerated dose will be achieved using this technology; further, a decrease in certain adverse effects, most notably alopecia and neuropathy, was observed. The early success of this prodrug has led to the initiation of three Phase III trials in patients with non-small cell lung carcinoma (Singer *et al.*, 2005).

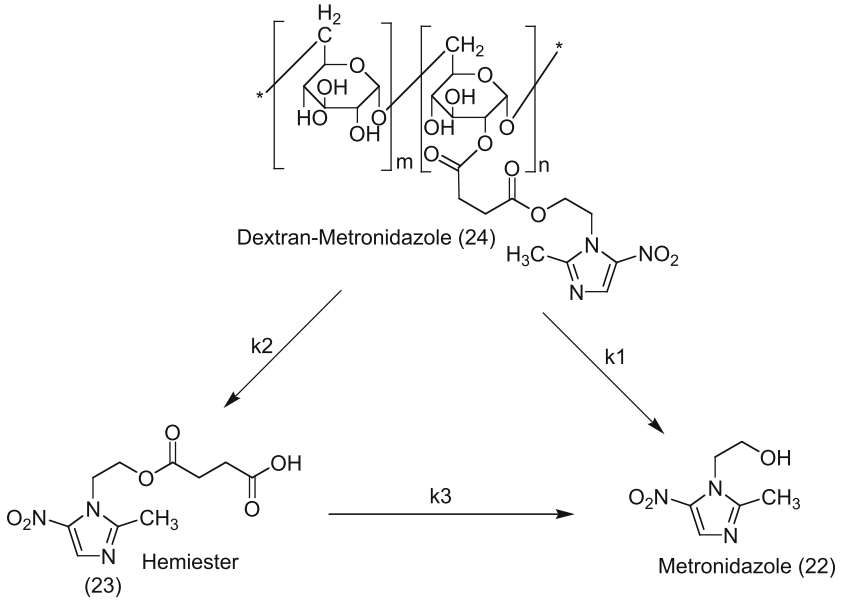
Drugs have commonly been linked to antibodies directed to a target cell antigen. This model is known as antibody-directed enzyme prodrug therapy (ADEPT); it and its variants are discussed in another chapter in this book. However, many of the same spacer technologies discussed above apply to this area.

Polysaccharides

One of the most studied polysaccharides in the macromolecular prodrug area is dextran. Larsen (1989) has written an excellent review on this topic (updated in a chapter in this book) in which he describes some of the properties of dextrans that make them potentially useful macromolecular promoieties. First, dextrans have been used clinically for many years to maintain blood volume and to treat other circulatory diseases. Thus, their clinical use and low occurrence of adverse side effects are well understood. Second, dextrans have a large number of hydroxyl groups, which may be utilized for drug attachment, whether that be direct attachment or via a spacer group. Third, the high hydroxyl content provides for high solubility even at relatively high levels of drug conjugation.

Vermeersch *et al.* (1985) utilized a method of drug conjugation discussed above (for PHEA and AZT) employing succinic anhydride (**14**). In this example, metronidazole (**22**) was coupled to polymers containing multiple hydroxyl groups. The primary alcohol of metronidazole (**22**) was reacted with succinic anhydride (**14**) to form the metronidazole monosuccinate ester (**23**). This product was then coupled to the hydroxyl-containing polymers dextran, inulin (another polysaccharide), and PHEA using the coupling agents N,N'-dicyclohexyl carbodiimide (DCC) (**35**) or CDI. The CDI (**8**) reaction proved more efficient, affording yields of 80% and up to 13 substitutions per 100 dextrose units.

Subsequently, Larsen *et al.* (1988a) applied this synthetic approach and synthesized the metronidazole monosuccinate (**23**) as well as the corresponding monoglutarate and monomaleate. These hemiesters were then coupled to dextran as described above. Using high-performance size-exclusion chromatography (HP(SEC)), the authors were able to follow the rate of disappearance of the dextran metronidazole conjugates (**24**) as well as the appearance of the metronidazole hemiesters (**23**) and metronidazole itself (**22**). With this technique and the previously measured hydrolysis rates of the individual hemiesters (Larsen *et al.*, 1988b), they were able to model the kinetics of the regeneration of metronidazole and the corresponding monoester derivative from the dextran-metronidazole conjugates (**24**) (Scheme 9). In aqueous buffer at pH 7.4 half-lives for the succinate, glutarate and maleate esters were 32.1, 50.6, and 1.5 h, respectively. These half-lives were determined by following the disappearance of the dextran conjugate using (HP(SEC)) and, thus, represent the appearance of both the metronidazole hemiesters and metronidazole itself. It was determined



Scheme 9. Release of metronidazole from metronidazole-dextran conjugates. Succinate spacer is illustrated in this example.

that drug regeneration in plasma proceeded no faster than regeneration in buffered solution, suggesting that enzymatic catalysis was not involved in the presence of plasma. Since some evidence of enzyme catalysis was seen in the hydrolysis of the hemiesters to the metronidazole, it was postulated that the steric hindrance of the dextran macromolecule made enzyme access to the esters impossible (Larsen *et al.*, 1988a).

Harboe *et al.* (1988) directly coupled the carboxylic acid of naproxen to dextran using DCC (**35**) as a coupling agent; these conjugates were hypothesized to create a depot effect, where the drug would be localized and have controlled release properties. This same technique was used by Larsen and Johansen (1989) to attach the acids diclofenac, ibuprofen, ketoprofen, and fenoprofen to dextran. Again, the stabilities of the various conjugates were similar in aqueous buffer, synovial fluid, and human plasma, pointing to the inability of plasma enzymes to cleave these bonds, presumably due to steric hindrance. However, it was observed that rates of drug appearance from the polymers did increase in liver homogenates. This was postulated to be due to the fact that the liver contains dextranases that decrease the molecular weight of the dextran macromolecules, thus decreasing the steric hindrance.

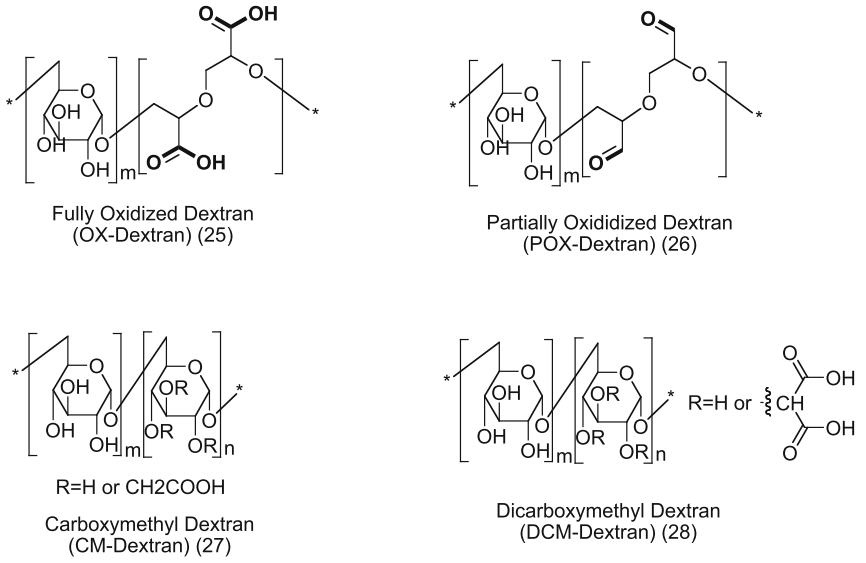
In an attempt to exploit this phenomenon, Harboe *et al.* (1989a) administered the naproxen-dextran conjugates orally to pigs. They found that 90.7% of the naproxen dose entered the systemic circulation; the T_{max} of the conjugates was extended almost eightfold and the C_{max} was nearly doubled over the orally dosed free naproxen, suggesting that these conjugates have the potential to confer extended release characteristics to drugs. Since it is reasonable to presume that

bound drug could not enter the circulation directly, and since the hydrolytic release of naproxen as well as release in plasma and liver homogenate proceeded with half-lives over 150 h, the observed bioavailability pointed toward simultaneous dextranase and esterase activity in the pig gastrointestinal tract. It was also observed that naproxen-dextran conjugates of lower molecular weight yielded increased plasma naproxen levels in shorter time periods (Harboe *et al.*, 1989b), further supporting dextranase involvement. Larsen *et al.* (1989) then determined that the site of drug liberation in pigs was the cecum, and especially the colon; this was due to the presence of dextranases secreted from bacteria in the colon.

McLeod *et al.* (1993) have explored this colon targeting ability further by conjugating the glucocorticoids dexamethasone and methylprednisolone to dextran. These drugs are indicated in the treatment of colitis, thus targeting them to the colon is of importance to both increase efficacy and decrease systemic toxicity. These drugs contain primary alcohols, and the hemiesters of these alcohols were synthesized and coupled to dextran as described previously. Similar to the directly linked anti-inflammatory-dextran conjugates described above, these conjugates were also found to release drug preferentially in rat cecum and colon contents rather than upper gastrointestinal tract (McLeod *et al.*, 1994a). The practicality of this approach was further supported by evidence that these orally administered dextran-drug conjugates, in rats, decreased systemic blood levels of glucocorticoids while increasing levels in the cecum and colon tissues, the intended sites of action (McLeod *et al.*, 1994b).

Others looked to these dextran succinate conjugates to target methylprednisolone to the liver as an immunosuppressive agent. While it was shown that drug release in the enzyme-rich environment of the liver was no greater than in aqueous buffer or plasma (Mehvar *et al.*, 2000), it is also known that dextrans accumulate in the liver (Mehvar *et al.*, 1994). Macromolecule accumulation in the liver is explained physiologically by the large fenestrae in the endothelial cells of the liver sinusoids that allow passage of larger particles. Most other tissues have capillary walls with smaller pores. Thus, it was postulated that drug targeting to the liver could still be successful based upon preferential prodrug residence in the liver, rather than preferential prodrug conversion. This hypothesis proved to hold true as free methylprednisolone accumulated in liver tissue at threefold greater concentration when dextran-methylprednisolone succinate was administered intravenously to rats compared to dosing of free methylprednisolone (Zhang and Mehvar, 2001). This increased level of drug in liver tissue resulted in an increased immunosuppressive effect *in vivo* as measured by a decrease in spleen lymphocyte proliferation in a rat model (Mehvar and Hoganson, 2000).

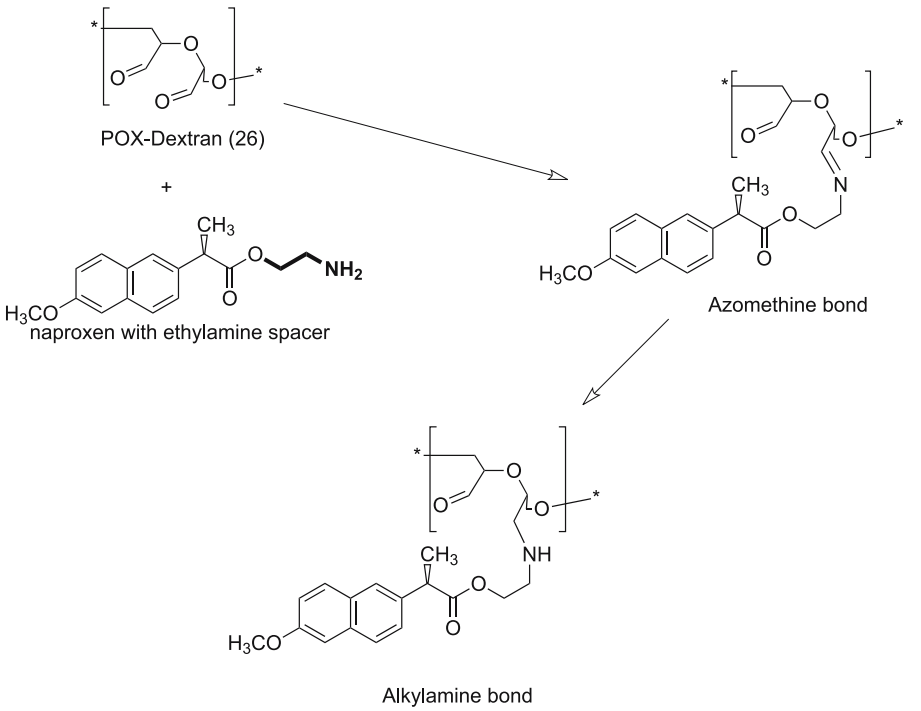
Modified dextrans are also used as macromolecular promoieties. Some examples of modified dextrans are those that contain carboxylic acids such as fully oxidized dextran (OX-dextran) (**25**) (Scheme 10), partially oxidized dextrans (POX-dextran) (**26**), and carboxymethyl dextran (CM-dextran) (**27**). The synthesis of these polymers is described by Ohya *et al.* (1996). Azori *et al.* (1986) utilized POX-dextran (**26**) to couple drugs with primary amines, or linkers with primary



Scheme 10. Modified dextrans.

amines, to dextran via an azomethine bond that was subsequently converted into an alkylamine bond (Scheme 11). Carboxymethyl dextran (**27**) is also commonly used as a macromolecular promoiety. This synthetic side arm provides dextran with carboxylic acid functionalities increasing both its solubility and its drug attachment options. Tsujihara *et al.* (1998) utilized CM-dextran in synthesizing a macromolecular prodrug of a camptothecin analog that contained a primary amine. This amine was then coupled to the C-terminal end of an N-terminal Boc-protected peptide, generally a poly-Gly, via the coupling agent EDC (**3**); the deprotected N-terminus was then coupled to the CM-dextran (**27**), again with EDC. Drug was released by lysosomal enzymatic cleavage. It was also determined that longer chains released drug faster, and that inserting a phenylalanine into the linker also sped up cleavage (Harada *et al.*, 2000). Studies in nude mice bearing multiple types (MX-1, LX-1, HT-29, WiDr, and St-4) of human tumor xenografts showed an increase in efficacy, as measured by tumor growth, over the free drug after i.v. injection (Okuno *et al.*, 2000). Pharmacokinetic data showed that the camptothecin analog circulated at higher concentrations for longer periods of time when coupled to CM-dextran; however, actual half-lives were not reported. Further, drug was shown to accumulate in the tumor, spleen, liver, and lymph nodes when delivered as the polymer conjugate relative to free drug. Tumor accumulation was presumed to be due to EPR effects.

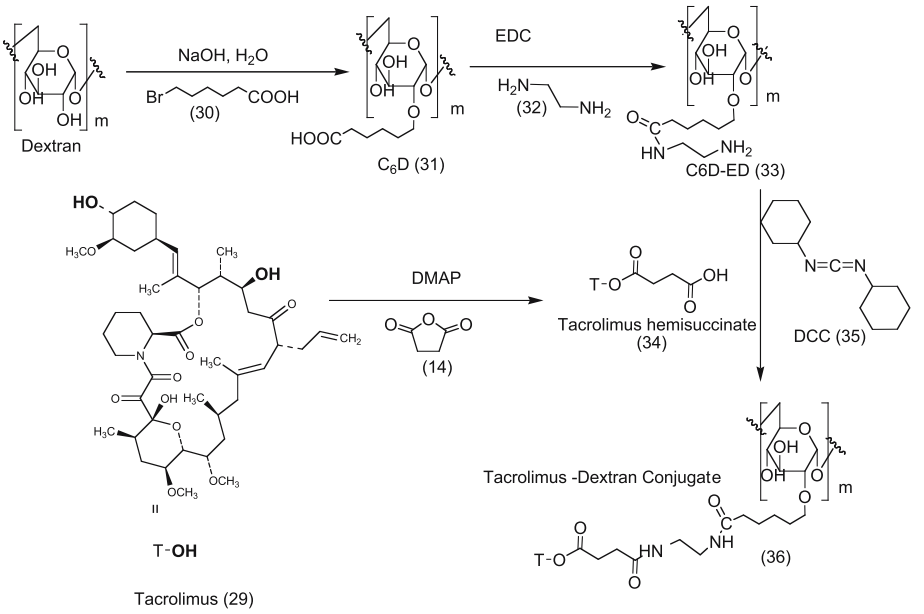
Ohya *et al.* (1996) utilized CM-dextran (**27**) and OX-dextran (**25**) as promoieties by attaching dihydroxy analogs of the anticancer agent *cis*-dichloro(cyclohexane-*trans*-l-1,2-diamine)platinum(II) (Dach-PT(chlorato)) via ester bonds. Release rates of the active compound, as well as cytotoxicity, were largely dependent on the solubility of the prodrugs, which varied with degree of substitution. In general, the CM-dextran prodrugs released drug at a faster rate



Scheme 11. Coupling of drugs with primary amines to POX-dextran via an alkylamine bond.

in pH 7.4 phosphate buffer; compared to the other conjugates; however, they also had a slower rate of dissolution, complicating some of the drug release studies. Dicarboxymethyl dextran (DCM-dextran) (**28**) (Scheme 10) is a related modified dextran that was used to deliver cisplatin in an analogous reaction (Ohya *et al.*, 2001). In this example, the workers also incorporated a targeting moiety, galactose, into the polymer as a galactose cluster, targeting hepatoma cells. The authors demonstrated an increase in binding to RCA_{120} lectin, receptors with known affinity for β-D-galactose and β-D–*N*-acetylgalactosamine, and an increase in cytotoxicity to HepG2 human hepatoma cells. These studies showed that the targeting moieties increased affinity for hepatocytes. However, that does not necessarily demonstrate that these prodrugs will accumulate in hepatocytes *in vivo*.

The anticancer drug mitomycin C was conjugated to dextran through an ε-aminocaproic acid spacer group (Kojima *et al.*, 1980). Hydroxyls of dextran were activated with cyanogen bromide and then coupled to the amine of ε-aminocaproic acid, forming an isourea bond and giving the polymer a cationic character. The resulting carboxylic acids, now attached via a spacer to dextran, were then coupled to the secondary amine of mitomycin C with the reagent EDC (**3**) (Kojima *et al.*, 1980). These mitomycin C-dextran conjugates (MMC-D) were investigated for lymphatic-specific delivery in rats after intramuscular injection. Takakura *et al.* (1992) demonstrated that macromolecules over 20 kDa are



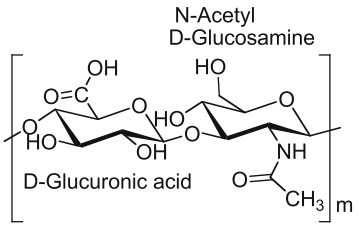
Scheme 12. Coupling of tacrolimus to dextran.

transported mainly by the lymph system after intramuscular injection. MMC-Ds of high molecular weight (500 kDa) showed good delivery to lymph nodes, up to 41.6% of the total dose, and suppressed metastasis to the lymph system after animals were inoculated with a leukemia cell line (Takakura *et al.*, 1984). In these same conjugates, workers also observed drug accumulation in tumors using a rabbit *in situ* vascular perfusion model (Atsumi *et al.*, 1987) as well as a tissue-isolated tumor preparation with a single-pass vascular perfusion apparatus (Imoto *et al.*, 1992). The accumulation presumably resulted from EPR effects (ADEPT chapter).

This same group also used dextran as a macromolecular promoiety with the immunosuppressant drug tacrolimus (**29**). The synthetic route provides a very interesting example of coupling a drug via its hydroxyl group to a polymer that also contains hydroxyl groups, while avoiding polymer crosslinking (Scheme 12). Dextran was first treated with 6-bromohexanoic acid (**30**) in basic aqueous solution to yield carboxy-n-pentyl-dextran (C6D) (**31**); this carboxylic acid-containing dextran was then treated with ethylenediamine (**32**) in the presence of EDC (**3**) to selectively yield C6D-ethylenediamine (C6D-ED) (**33**) (Yura *et al.*, 1998). A secondary alcohol of tacrolimus (**29**) was then reacted with succinic anhydride (**14**) to yield the hemisuccinate (**34**). Finally, the free carboxylic acid of tacrolimus-hemisuccinate (**34**) and the primary amine of C6D-ED (**33**) were coupled via dicyclohexylcarbodiimide (DCC) (**35**) to yield the tacrolimus-dextran conjugate (**36**) (Yura *et al.*, 1998). After i.v. administration in rats, the conjugates circulated with a terminal half-life 18 times greater than free tacrolimus; in an aqueous solution buffered to pH 7.4, free drug was released with a half-life of 150 h (Yura

et al., 1999). The result is that tacrolimus may potentially be dosed at longer intervals and with decreased variability in blood levels, providing a safer mode of administration.

Another polysaccharide that has been under investigation as a macromolecular promoiety for several years is hyaluronan (HA) (**37**). HA is a negatively charged polysaccharide of high molecular weight consisting of alternating units of D-glucuronic acid and N-acetyl D-glucosamine (Scheme 13). HA is found throughout the extracellular space of all human tissue, and is completely biocompatible (Laurent and Fraser, 1992). A further advantage of HA is that it has been demonstrated to undergo CD44 receptor-mediated endocytosis. This receptor is overexpressed in many cancer cell types (Hua *et al.*, 1993), meaning that HA has built in targeting ability.

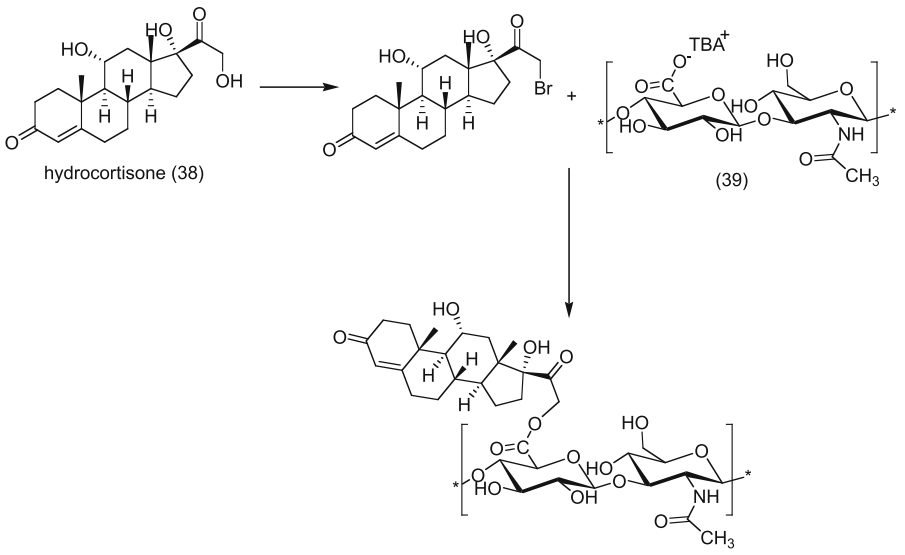


Hyaluronan (HA) (37)

Scheme 13. Hyaluronan (HA).

Akima *et al.* (1996) directly coupled the amines of mitomycin C to the carboxylic acids of hyaluronan using EDC (**3**). They were able to demonstrate specific drug delivery to the lymph system as well as efficacy in C3H/He male mice with xenograft MH-134 ascites tumors. This technique was also used by Cera *et al.* (1988) to attach the anticancer drugs daunomycin and adriamycin to HA. Coradini *et al.* (1999) directly coupled butyrate, investigated as an anticancer compound, to the primary alcohol of HA using the butyrate anhydride. Activity of the compounds was measured by inhibition of proliferation of the MCF7 breast cancer cell line. The efficacy of the prodrugs depended on the degree of substitution; those with too little or too much loading were less effective than free butyrate. The authors also found that the molecular weight of the polymer was not a factor in its efficacy in these *in vitro* studies.

DellaValle and Romeo (1989) directly attached the corticosteroids cortisone and hydrocortisone (**38**) to hyaluronic acid via ester links by first converting the drugs' primary alcohols to bromides and then reacting them with the tetrabutylammonium salt of HA (**39**) (Scheme 14). As with some of the previously discussed dextran conjugates, while chemical hydrolysis and, specifically, hydroxide ion catalysis was observed, these macromolecular prodrugs were not suitable substrates for esterases. At pH 8.0 and 25°C, the first-order rate constant for the hydrolysis of these prodrugs was around 9×10^3 h^{-1}, and they were not affected by

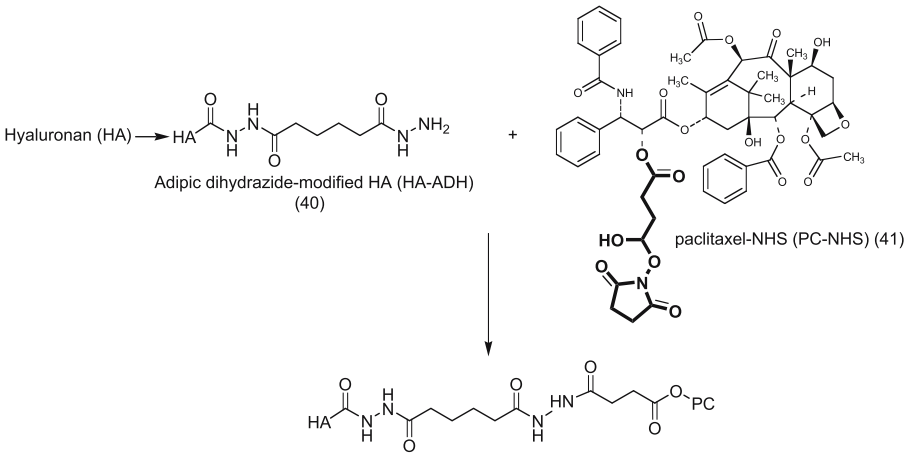


Scheme 14. Coupling of drugs with primary alcohols to HA.

the presence of porcine liver esterase (Rajewski *et al.*, 1992). Unlike the scenario with dextran, the rate of enzymatic hydrolysis was not increased even in the presence of hyaluronan-degrading enzymes. These prodrugs were also formulated into microspheres and the release of drug was compared to drug release from microspheres in which drug was simply physically incorporated (Benedetti *et al.*, 1990). Release of the physically incorporated drug was nearly complete in 10 min, while release from the prodrugs occurred as a zero-order process over more than 100 h (Benedetti *et al.*, 1990).

Luo and Prestwich (1999) have used a novel adipic dihydrazide-succinate linkage system (**40**) to couple HA to paclitaxel. This linkage was attached to the 2' alcohol of paclitaxel via the paclitaxel-N-hydroxysuccinimido-hemisuccinate (paclitaxel-NHS) (**41**) (Scheme 15). This same strategy has been employed to synthesize various hyaluronan derivatives, including cross-linked hyaluronan (Vercruysse and Prestwich, 1998). The authors were able to demonstrate selective cytotoxicity toward cell lines that overexpress HA receptors. A similar strategy was employed to link doxorubicin to HA (Luo *et al.*, 2002). However, in this example, the secondary amine of doxorubicin was used to make the doxorubicin-NHS, which was then coupled to HA-ADH (**40**). This versatile synthetic technique was also used to attach hydrocortisone via a primary alcohol, ibuprofen via a carboxylic acid (Pouyani and Prestwich, 1994), and daunomycin (Morales *et al.*, 1998) to HA.

Hurwitz *et al.* (1980) employed hydrazide derivatives of carboxymethyl dextran, polyglutamate, alginic acid, and carboxymethyl cellulose. These derivatives were then reacted with the ketone-containing drug daunorubicin to yield respective polymer-daunorubicin prodrugs with a hydrazone linkage. The prodrugs were tested against free daunorubicin for *in vivo* activity in mice

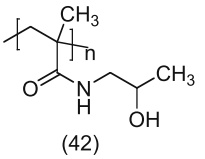


Scheme 15. Attachment of paclitaxel to HA utilizing adipic dihydrazide linkage technique.

implanted intraperitoneally with Yac lymphoma cells. All mice were treated with a single dose 2 days after implantation of lymphoma cells. The mice treated with prodrugs had an increased survival rate; however, the dosage regimen may not be a fair comparison, considering that the prodrugs likely act as a controlled-release device.

Synthetic Polymers

Thus far, some of the most clinically successful macromolecular analogs have been prodrugs based on the promoiety N-(2-hydroxypropyl)methacrylamide (HPMA) (**42**) (Scheme 16). Some of these prodrugs have entered phase I/II clinical studies. A sampling of these prodrugs is described below.

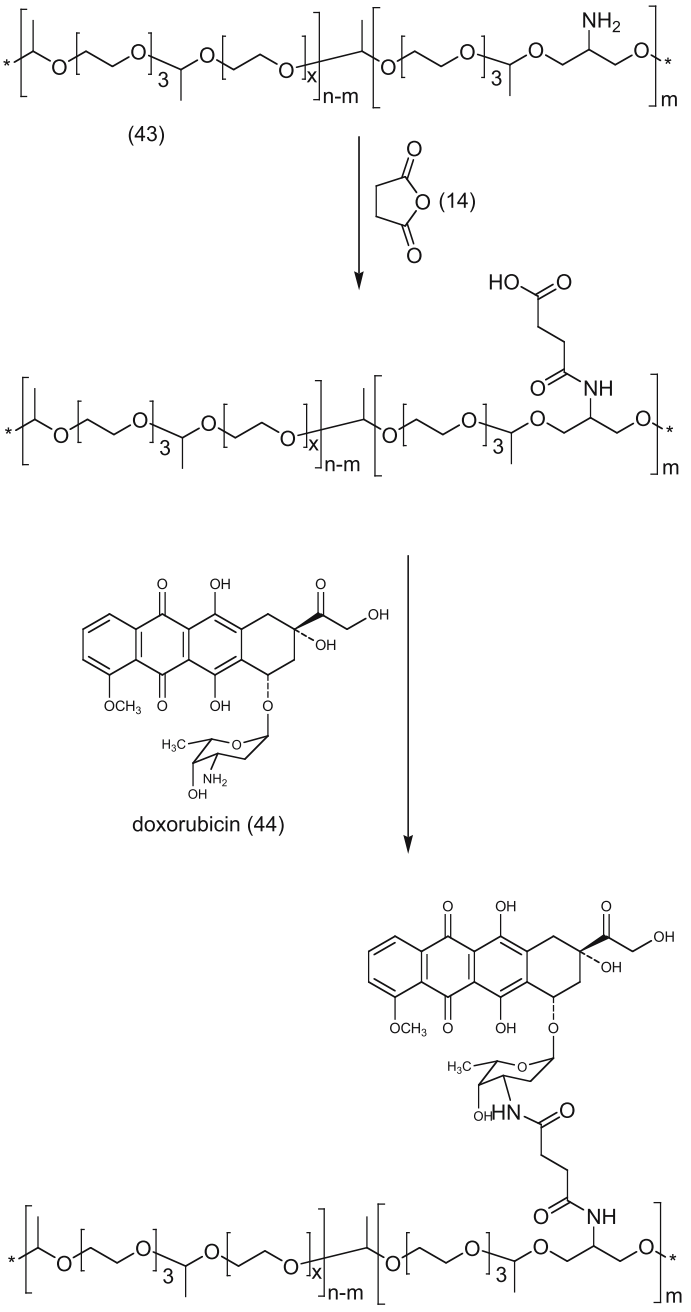


Scheme 16. N-(2-hydroxypropyl)methacrylamide, HPMA

Vasey *et al.* (1999) conducted a phase I study on an HPMA-doxorubicin prodrug that utilized a Gly-Phe-Leu-Gly peptide spacer linked via amide bonds on either side. The authors concluded that some adverse effects were lessened and that no polymer-related adverse effects were seen. The HPMA promoiety with a Gly-Phe-Leu-Gly spacer was also used by Gianasi *et al.* (2002) to form macromolecular prodrugs of cisplatin. Seymour *et al.* (2002) studied a very similar system, also in a phase I study that incorporated galactosamine units on the polymer to target the drug to the liver. Using ^{123}I-labeled drug and full-body gamma-camera

imaging, the authors determined that the targeting moiety increased delivery to the liver over the non-targeting version.

Meerum Terwogt *et al.* (2001) described the behavior of an HPMA-paclitaxel drug in a phase I study. In this example, the polymer was bound to drug via an amide-linked Phe-Leu spacer with an ester bond linking paclitaxel to the spacer.



Scheme 17. Conjugation of doxorubicin to amino-pendent polyacetals through a succinate spacer

They found that this prodrug had very low toxicity in 12 human subjects. Further, the prodrug was able to maintain blood levels of free paclitaxel in a therapeutic range for 9.5–21.2 h. This is a considerable improvement over the blood levels obtained upon dosing of paclitaxel in the current Cremophor EL/ethanol formulation. Moreover, treatment with the prodrug achieved partial remission in one patient and stable disease in two others. However, the study was halted when a severe, irreversible neurotoxicity in a rat model was observed.

Satchi-Fainaro *et al.* (2003) studied an interesting linkage system developed under the polymer-directed enzyme prodrug therapy (PDEPT) concept. This approach uses macromolecular prodrugs to deliver drugs to a tumor together with a polymer-enzyme complex that is also targeted to the tumor to achieve local release of the free drug. In this example doxorubicin was coupled to HPMA through a cephalosporin spacer. An HPMA-β-lactamase polymer is then used to cleave the cephalosporin linker, releasing doxorubicin. In male C57BL/6J mice inoculated with B16F10 melanoma cells, the PDEPT combination achieved a statistically significant greater mean survival time compared to either free doxorubicin or the HPMA-doxorubicin alone.

Tomlinson *et al.* (2003) developed a new acid-sensitive polymer delivery system, amino-pendent polyacetals (**43**). These polymers were designed to selectively degrade rapidly inside of cells, in low pH environments such as endosomal and lysosomal compartments, while degrading slowly only at the neutral pH encountered in blood. These polymers were conjugated to doxorubicin (**44**) as a model drug through a succinate spacer (Scheme 17). Initial studies in C57/B black mice injected with B16F10 tumor cells showed that these prodrugs have increased plasma half-lives as well as increased tumor accumulation compared to HPMA analogs.

Conclusion

To date, marketed examples of macromolecular prodrugs of the type discussed above remain elusive. Regulatory hurdles such as reproducibility and analytical challenges have hindered development. When macromolecules are used just as pharmaceutical excipients, receiving regulatory approval for their use requires establishment of their usefulness and safety. When drugs are covalently attached to the polymer, the macromolecular entity becomes a New Chemical Entity or NCE. As an NCE the macromolecular prodrug becomes subject to more strict regulatory controls. Reproducibility of the manufacture of the materials, their analytical characterization, and patient-to-patient variation in release and overall exposure must be considered. This need for tight controls has probably contributed to the lack of commercial success of macromolecular prodrugs to the present time. Nevertheless, this strategy provides the opportunity to address many pharmaceutical issues—pharmacokinetics, targeting, solubility, etc.—in a manner flexible enough to accommodate a broad range of compounds. Only time will tell if macromolecular prodrugs will have commercial success.

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3.9

Miscellaneous Functional Groups

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Table of Contents

Introduction

This section describes recent examples of prodrugs of functional groups not commonly associated with prodrug strategies, including nitrogen-containing heteroaromatic rings, thiols, nucleic acids, and enediynes. The prodrugs for several addional functional groups that are not covered in this book were reviewed in the book "Design of Prodrugs," edited by Bundgaard (1985); examples include derivatives of CH acidic compounds, imine bisulphite addition products, and glycosidic products.

Prodrugs of Nitrogen-containing Heteroaromatic Rings

Many otherwise desirable drug candidates are limited by their poor water solubility; this problem is particularly acute for the development of injectable drugs when the compounds are highly lipophilic and lack functional groups suitable for prodrug formation. For candidates incorporating nitrogen-containing heteroaromatic rings, quaternary ammonium prodrugs provide an opportunity to address this problem.

Bogardus and Higuchi (1982) reported model studies of the quaternary ammonium salt of pyridine, the N-(4-acetoxy-3,5-dimethylbenzyl)pyridinium salt (**2**), as a possible prodrug of pyridine. This prodrug rapidly generates pyridine by hydrolysis of the acetate group to give a *para*-hydroxybenzylpyridinium species **1**, followed by rapid fragmentation. Since the hydrolysis of **1** in the presence of imidazole at pH 12.7 yielded N-(4-hydroxy-3,5-dimethylbenzyl)imidazole (**5**), the formation of a reactive quinone methide intermediate (**3**) that was trapped by imidazole was suggested (Figure 1).

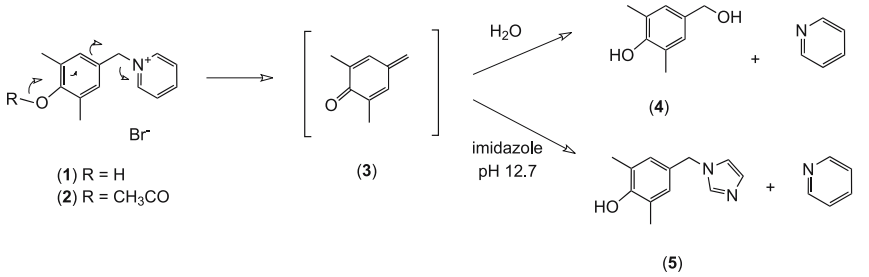


Figure 1.

Ohwada *et al.* (2002) applied this concept to the solubilization of poorly soluble azole antifungal agents such as BMS-207147 (7). Thus, the highly water-soluble (49 mg/mL) N-(4-sarcosyloxy-3,5-dimethylbenzyl)-triazolium salt derivative **6** is rapidly converted to the active drug (**7**) in human plasma ($T_{1/2}$ <2 min) and shows potent antifungal activity *in vivo* by both oral and intravenous administration (Figure 2). A general concern with this approach is that generation of the reactive and potentially toxic quinone methide intermediate (**3**) may limit the utility of this type of prodrug for clinical use.

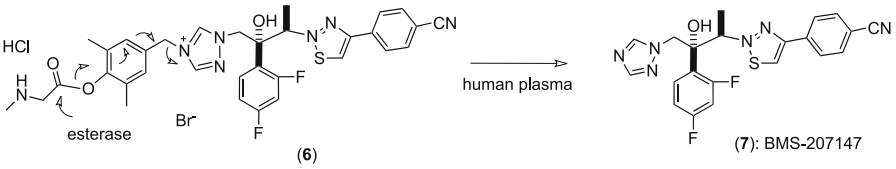


Figure 2.

Several attempts to circumvent formation of reactive leaving groups using this strategy through employment of various acyloxymethyl quarternary ammonium prodrugs are summarized in Table 1. Davidsen *et al.* (1994) reported the N-(acyloxymethyl)pyridinium salts, **9** (ABT-299) and **10** as water-soluble prodrugs of platelet activating factor antagonist, A85783 (**8**). Compound **9** is active in platelet activator challenge assays after *i.v.* and oral administration in mice, rats, and guinea pigs. After pharmacokinetic studies in rats indicated that it was rapidly converted to **8** (Albert *et al.*, 1996), it was taken into human trials.

In a similar approach, Ichikawa *et al.* (2001) reported prodrugs of the poorly soluble antifungal triazole **11** (TAK-456) and the selection of the N-acyloxymethy moiety **12** (TAK-467) for development. In a further extension of this approach, a patent application described the N-phosphonooxymethyl quartenary triazolium salt prodrug (**13**) of the antifungal agent **7** Golik *et al.*, (2001). All of these examples represent attempts to create soluble prodrugs of highly lipophilic, insoluble drugs for *i.v.* injection. Data for water solubility and bioconversion of these compounds in human plasma are summarized in Table 1.

As part of a further refinement of this approach, Ohwada *et al.* (2003) seeking safer leaving groups, designed the more complex triazolium derivative (**14**) that generates the previously described antifungal agent **7**. Conversion proceeds by hydrolysis of the ester moiety by human plasma esterases followed by intramolecular cyclization of the resulting alcohol and successive fragmentation as shown in Figure 3. In this case, the cleaved fragment is the cyclic carbamate **15**, which would not be expected to raise safety concerns. This prodrug, **14**, is highly water soluble (>100 mg/mL) and is rapidly converted to 7 in human plasma ($T_{1/2}$ < 1 minute) *in vitro*. The rapid conversion was due to conformational restriction of the promoiety so that the hydroxyl group of the intermediate resulting from ester hydrolysis is poised for intramolecular attack of the carbonyl, facilitating intra-molecular cyclization. Interestingly, the related prolyl ester **16** has a half-life of 126 min in human plasma as opposed to less than 2 min for **14**, indicating the need for careful ester screening when adopting this approach. This effective conversion of **14** to **7** was also confirmed *in vivo*. Thus, **14** had good efficacy in systemic mycosis models in mice after both *p.o.* and *i.v.* administration (Ohwada *et al.*, 2003). Schmitt-Hoffmann *et al.* (2003, 2004a,b) further reported that **14** showed a good pharmacokinetic profile after oral administration to humans. After a single oral administration of 100 mg of **14**, high plasma concentrations of active drug **7** (C_{max} = 1447 ng/mL; AUC= 36978 ng*h/mL; $T_{1/2}$= 63.1 h) were observed. The concentration of the prodrug **14** was below detection limits.

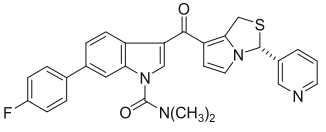
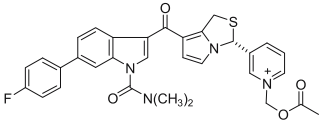
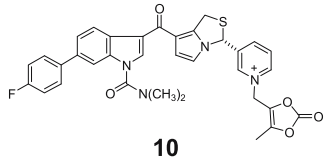
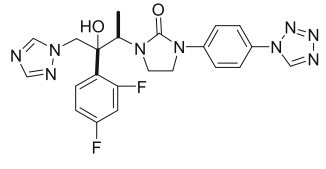
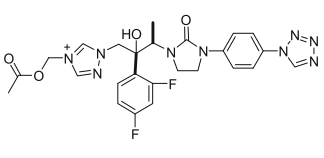
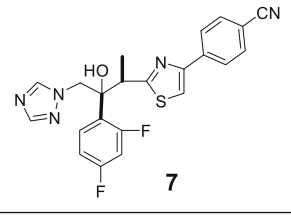
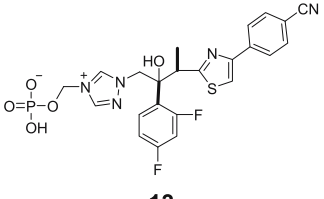
Structure of parent molecule	Structure of prodrug	Utility	Literature Reference	Water solubility	Stability in water	Stability in human plasma
 **8** (A-85783)	 **9** (ABT299)	Platelet activating factor antagonist	Davidsen *et al.* (1994)	>20 mg/mL (pH3)	T1/2 = 96min (pH7 buffer)	1 min
	 **10**			>20 mg/mL (pH4)	T1/2 = 246 min (pH7 buffer)	2.8 min
 **11** (TAK-456)	 **12** (TAK-467)	Systemic antifungal agent	Ichikawa *et al.* (2001)	10 mg/mL	1.8% remained in 5% aqueous. glucose after 1 day	6 min
 **7**	 **13**	systemic antifungal agent	Golik *et al.* (2001)			

Table 1. Prodrugs of N-heteroaromatic compounds.

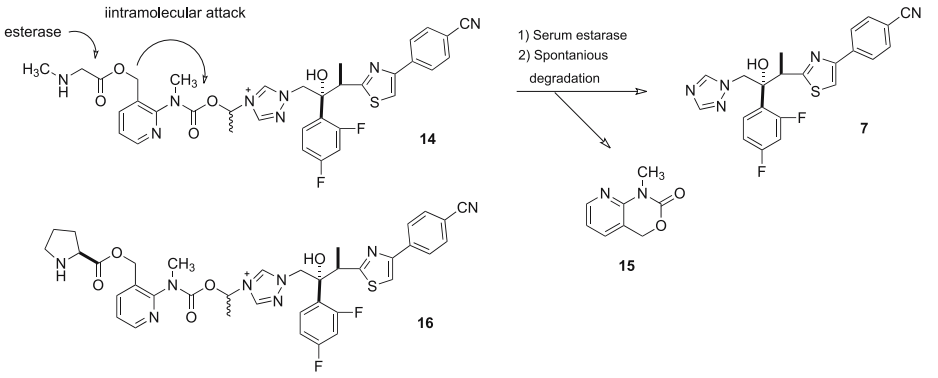


Figure 3.

Another strategy for generating prodrugs of nitrogen-containing heteroaromatics is formation of quaternary ammonium N-glycosides. Arison *et al.* (2002) reported in a patent application the quaternary ammonium N-glycoside salt **17** as a water-soluble prodrug of the $GABA_A$ receptor ligand **18** for the therapy of disorders of the central nervous system such as anxiety and convulsions. Although the authors report **17** can be converted to **18** by the action of glucuronidase *in vivo* after oral administration (Figure 4), there was no conversion data.

In conclusion, formation of appropriately reactive quaternary ammonium salts of nitrogen containing heteroaromatic rings is a strategy that may have general applicability for the conversion of poorly soluble drugs to more water-soluble derivatives suitable for injection and, in selected cases, oral administration. Since there is a wide latitude for the selection of the acyl moieties, it should be possible to customize the rate of release of the parent.

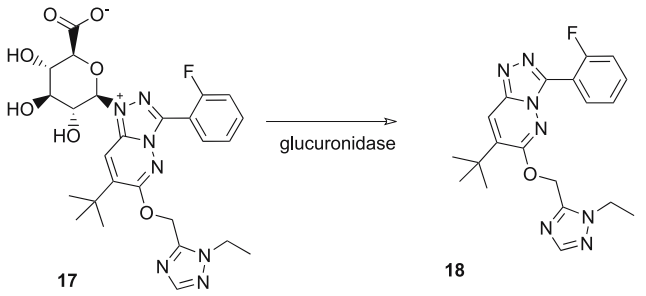


Figure 4.

Prodrugs of Thiols

Thiols are highly reactive and can be oxidized to various products, including covalently bound disulfides. Blood plasma contains endogenous thiols including peptides and proteins that may form disulfide conjugates with drugs containng thiols; this formation can be a strategy for prolonging the effective lifetime of the drugs. Due to the inherent chemical instability of thiol-containing drugs, it is

generally necessary to form chemically stable prodrugs to achieve delivery and sustained exposure. A large number of thiol prodrugs are reported. They are classified into four groups: thioesters, oxothiazolines, thiophosphates, and disulfides. Several recent examples are listed in Table 2.

Strategies include formation of simple thioesters. An example is **20** (MDL 100240), a thioester prodrug of the dual angiotensin-converting enzyme/neutral endopeptidase inhibitor **19** (MDL100173) (Rossi, 2003). This compound appears to undergo rapid hydrolysis to the active thiol **19**, which in turn is conjugated to plasma thiols, resulting in a terminal elimination half-life of 35.7 h in dogs. However, in humans, a single oral dose of 50 mg was inadequate to provide 90% inhibition of angiotensin converting enzyme for 24 h. Pharmacokinetic modeling indicated that multiple doses would be required to achieve clinically useful inhibition (Pfister *et al.*, 2004).

Thiols have been promoted as anti-oxidants and cytoprotectants for use in combination with radiation therapy. An example, the oxothiazolidine prodrug of cysteine **22** (OTC) was proposed to relieve cells of oxidative stress by repleting cellular glutathione stores and was proposed for treatment of HIV infection (Pace *et al.*, 1995). The thioposphate ester **23** (Amifostine) is a prodrug for the cytoprotective agent aminopropylaminoethanethiol **24** (WR-2721). After *i.v.* injection, **23** undergoes rapid hydrolysis to **24** which in turn distributes rapidly to cells. It is used clinically as a cytoprotective agent during chemotherapy (Koukourakis, 2002).

Examples of disulfide prodrugs are shown in Table 2 and include a single report of captopril-SS-PEG (26) (Vincentella, 1996) as well as the mytomycin C derivatives **28** and **29** (Vyas *et al.*, 1985; Kono *et al.*, 1989; Kohn and Wang, 1996; Wang and Kohn, 1999). The design of mitomycin prodrugs was based on the observations that *in vivo* antitumor activity depends on the enzymatic reduction of the quinone moiety and resistance of tumor cells to mitomycin is frequently related to deficient reductase activity. Thus, prodrugs **28** and **29**, which could be converted to the active form by a non-enzymatic bioreductive process were developed. He *et al.* (1994) proposed the mechanism of activation for the disulfide prodrugs, shown in Figure 5, that includes (i) thiol (*e.g.*, glutathione) initiated disulfide exchange to give **30**, (ii) generation of cyclic intermediate **31**, and (iii) the formal intramolecular transfer of electrons from the disulfide group to the quinone moiety to produce **33** (Figure 5). Further steps in the activation process include loss of methanol to give **34**, followed by further loss of the carbamate or by aziridine ring opening to provide reactive nucleophile traps **35** and **36**. Both prodrugs exhibit improved cytotoxic activity and safety profile compared with mitomycin C (Xu *et al.*, 1992; Dirix et al, 1995; Yen and Au, 1997) Data on the pharmacokinetics and antitumor activity of **29** in mouse tumor models have been summarized (Bradner *et al.*, 1990; Robinson *et al.*, 1996).

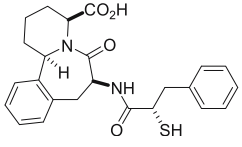
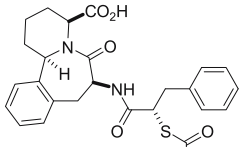
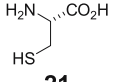
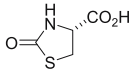
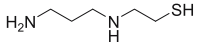
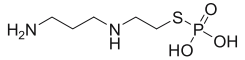
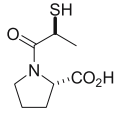
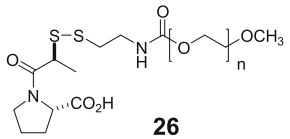
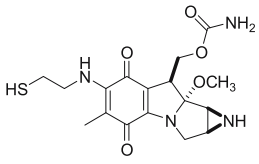
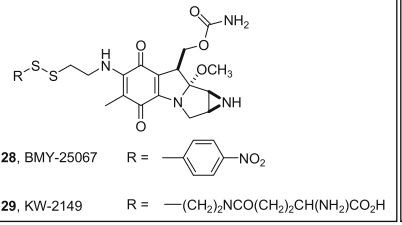
Type of Prodrug	Parent molecule	Prodrug	Indication	Literature
Thioester (R-S-Ac)	 **19** (MDL 100173)	 **20** (MDL100240)	Orally active dual angiotensin-converting enzyme (ACE)/neutral endopeptidase (NEP) inhibitor for cardiovascular conditions.	Rossi *et al.* (2003)
2-Oxothiazolidine (R-S-CO-N-R')	 **21**	 **22** (OTC)	Glutathione-repleting agent for treatment of HIV infection	Pace and Leaf (1995)
Thiophosphate (R-S-PO3H2)	 **23** (WR-1065)	 **24**	Broad-spectrum cytoprotective agent	Koukourakis (2002)
Disulfide (R-S-S-PEG)	 **25** (Captopril)	 **26**	Antihypertentive	Vincentellia *et al.* (1996)
Disulfide (R-S-S-R')	 **27**	 **28**, BMY-25067 R = **29**, KW-2149 R = $-(CH_2)_2NCO(CH_2)_2CH(NH_2)CO_2H$	Anticancer	Vyas et al, (1985) Kono et al. (1998) He et al. (1994) Kohn and Wang (1996); Wang and Kohn (1999)

Table 2. Prodrugs of Thiols

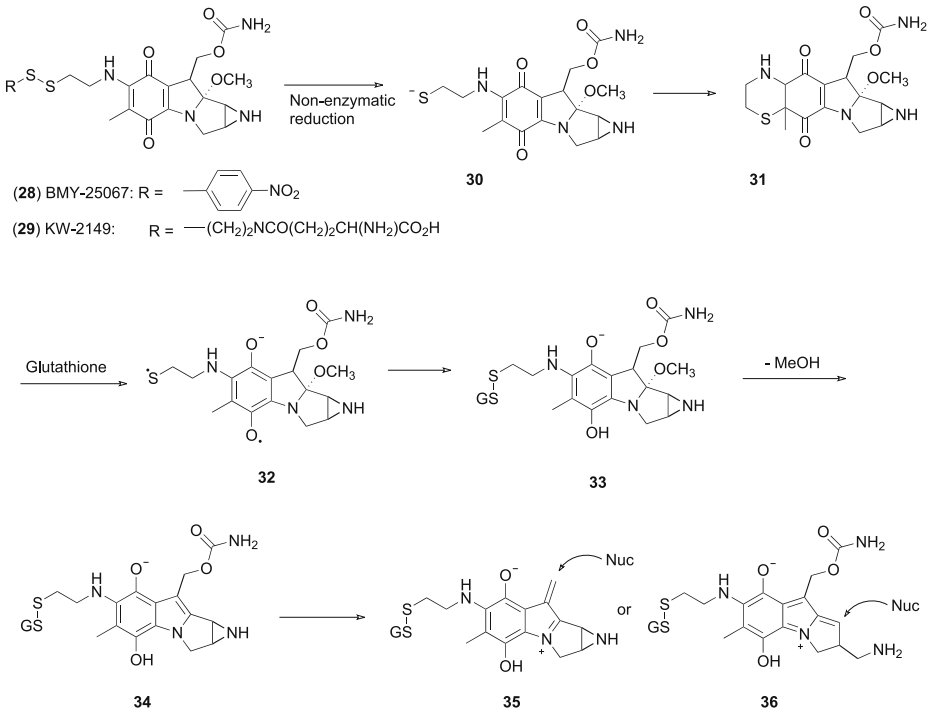


Figure 5.

Tumor-selective Prodrugs of Pyrimidines

Ishitsuka *et al.* (1980) discovered that oral 5'-deoxy-5-fluorouridine (5'-DFUR, **37**) acts as a prodrug of anticancer agent 5-fluorouracil **38** (5-FU). Thymidine phosphorylase is highly expressed in various human tumors compared with adjacent normal tissues and serves to enzymatically release **38**, thus achieving tumor-selective delivery. While compound **37** is widely used as a cancer therapeutic in Japan, further improvements in the tumor-selective delivery of 5-FU were sought.

To this end, Shimma *et al.* (2000) developed capecitabine (**40**), a cytidine derivative, as a prodrug of 5'-DFUR (**37**). Cytidine deaminase can convert cytidine derivatives to uridines. This enzyme is highly expressed in the liver and various solid tumors, but has very low expression in bone marrow progenitor cells. Thus, prodrug conversion should occur selectively in the target tissues resulting in lower myelotoxicity and higher tumor selectivity. Capecitabine itself is inactive, but is sequentially converted to 5'-DFCR (**41**) by human liver carboxylesterase, then to 5'-DFUR (**37**) by cytidine deaminase, which is located in both liver and tumors, and finally to active 5-FU (**38**) by thymidine phosphorylase. A detailed description of the prodrug design of **40** is provided in the Case Histories.

An additional example of a cytidine-type prodrug is a prodrug of 5-vinyluracil (**42**) that is an inhibitor of dihydropyrimidine dehydrogenase (DPD), an enzyme that metabolizes 5-FU to inactive 5,6-dihydro-5-fluorouracil (**39**). Antitumor activity of 5-FU is limited against tumors that overexpress dihydropyrimidine

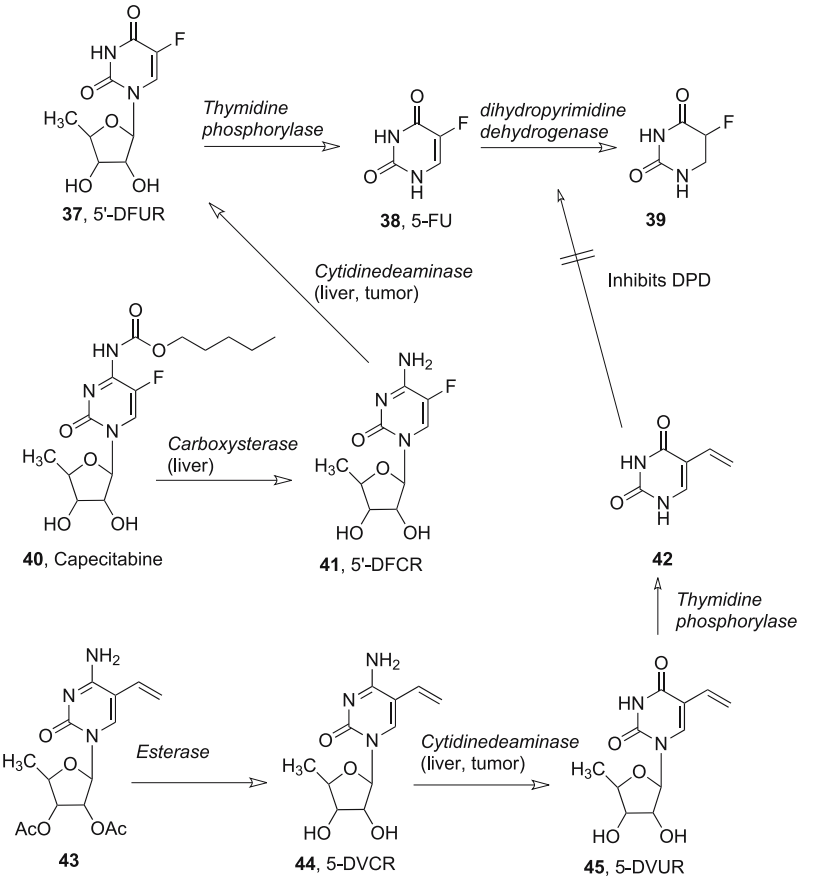


Figure 6.

dehydrogenase, including liver and non-small cell lung carcinomas. To enhance the antitumor activity of the 5-FU prodrugs **37** and **40** while minimizing toxicity, Hattori *et al.* (2003) developed a tumor-selective dihydropyrimidine dehydrogenase inhibitor **43**, a prodrug of 5-vinyluracil (**42**), by applying the same prodrug strategy previously employed for 5'-DFUR and capecitabine.

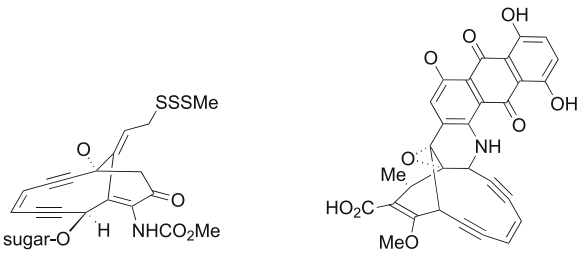
Thus, the prodrug **43** is sequentially converted to 5'-deoxy-5-vinylcytidine **44** by esterases that are present in serum and liver, then to 5'-deoxy-5-vinyuridine **45** by cytidine deaminase present in liver and tumors and, finally, to 5-vinyluracil **42**, an active DPD inhibitor, by thymidine phosphorylase, which is also localized in liver and tumors. Tumor-selective delivery of DPD inhibitor **42** was confirmed after oral administration of the prodrug **43** to mice bearing HT-3 human cervical cancer xenografts. Co-administration of oral **43** and **37** (capecitabine) significantly increased the concentration of 5-FU in tumors without increased toxicity in the same xenograft model, thus enhancing the efficacy of **37**.

One of the difficulties in a multi-step prodrug design requiring sequential activation by a series of enzymes is that each intermediate has to be a good substrate for the corresponding activation enzyme. This must be carefully evaluated during the optimization study of prodrugs on a case-by-case basis,

paying particular attention to differences in substrate specificity among enzymes derived from different species used to evaluate overall conversion. Where possible, human enzymes should be evaluated. The principle of multiple-step prodrug offers the opportunity of achieving a high degree of tumor or tissue selective active drug delivery if one is able to take advantage of an understanding of the tissue distribution of target enzymes.

Prodrugs of Enediynes

Calichemicin (**46**) and dynemicin (**47**) are known to be potent antitumor antibiotics that contain a conjugated enediyne as a common structure; this has been summarized in a number of reviews (Borders and Doyle, 1994; Doyle and Kadow, 1994; Grissom et al, 1996; Lhermitte and Grieerson, 1996a,b; Meunier, 1996). Nucleophilic cleavage of the trisulfide moiety in **46** or reduction of the quinone system in **47** triggers the thermal cyclization of the enediyne system through a Bergman cyclization (Bergman, 1973) and forms reactive 1,4-benzenoid diradical intermediates such as **48**, which cause DNA damage. In this respect, **46** and **47** may be considered as natural prodrugs for reactive enediynes (Maier, 1995). A proposed mechanism for this reactive intermediate formation is shown in Figure 8.



46, Calicheamicin **47**, Dynemicin A

Figure 7.

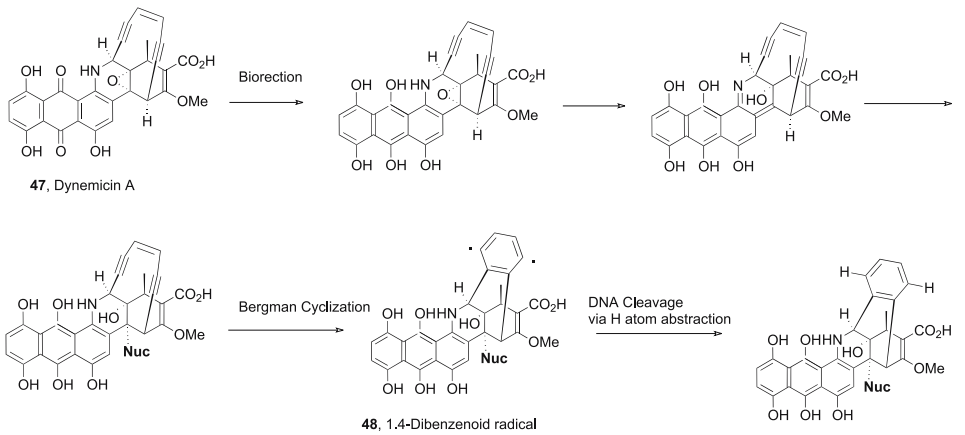


Figure 8. Proposed mechanism of action of Dynemicin (Shair, *et al.*, 1996)

Prodrug aproaches for bioactivation have been applied to develop alternative methods for the generation of simpler reactive enediynes. Hay *et al.* (1995) reported that 4-nitrobenzylcarbamate **49** exhibited a 90-fold increase in cytotoxicity against UV4 cells in the presence of *E. coli* nitroreductase and cofactor NAD(P)H compared to the compound alone, suggesting its potential as a prodrug for the amine **50** (Hay *et al.*, 1995) (Figure 9). The reason that **50** undergoes Bergman cyclization while **49** does not can be explained by the same mechanism as described in Figure 8. Elimination of the carbamate group facilitates the epoxide ring opening, enabling the two acetylene groups to approach one another and thus allowing the Bergman cyclization to proceed.

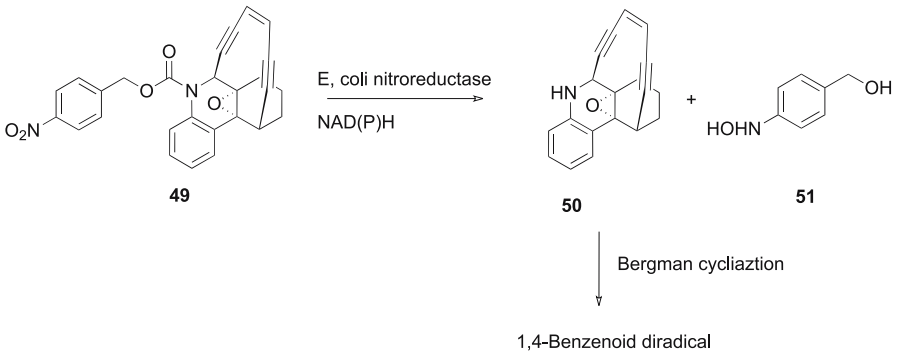


Figure 9.

Shair *et al.* (1996) synthesized the quinone imine **52** as a truncated version of dynemicin A in which epoxide solvolysis of **53** is triggered after bioreduction of **52**, thereby initiating a cascade that leads to formation of cytotoxic intermediates. Compound **52** exhibited 7- to 43-fold greater potency *in vitro* against several cancer cell lines (HL-60, MT-2, MT-4, 833K and SK-Br-3) than did mitomycin C. It was also superior to mitomycin C in an *in vivo* screen; in a B2D6F1 mouse Lewis Lung Adenocarcinoma model, 10-day treatment with a 0.5 mg/kg of **52** resulted in a 50% reduction in tumor mass. Thus, a quinoneimine system turned out to be useful as a prodrug for dynemicin A type Bergman benzenoid diradical (Figure 10). In each of the above examples, the strategy was to effect a chemical transformation rendering the expoxide labile, thus facilitating its opening and, through the resulting relief of conformational strain, triggering the Bergman cyclization.

In a related effort, Dai *et al.* (2002) reported on a novel class of enediyne prodrugs characterized by the esters **54** (R = H) and **55** (R = OMe) that was found

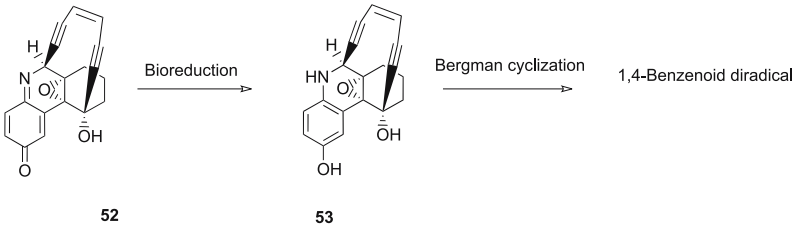


Figure 10.

to exhibit almost the same level of DNA cleavage activity as the potent enediyne **56**. The authors suggested that **56** might dissociate in aqueous media to the allylic cation **57**, which was then trapped by H_2O preferentially at the γ-position to furnish **58**; **58** could, in turn, cyclize to a 1,4-benzenoid diradical according to known enediyne chemistry (Figure 11). Both compounds **54** and **55** exhibited cytotoxicity against a P388 cancer cell line with IC_{50} values of 7.7 μM for **54** and 23 μM for **55**. The 1- and 2- analogs of **54** substituted with naphthyl instead of phenyl are even more promising compounds, with IC_{50} values of 2.4 and 2.8 μM, respectively.

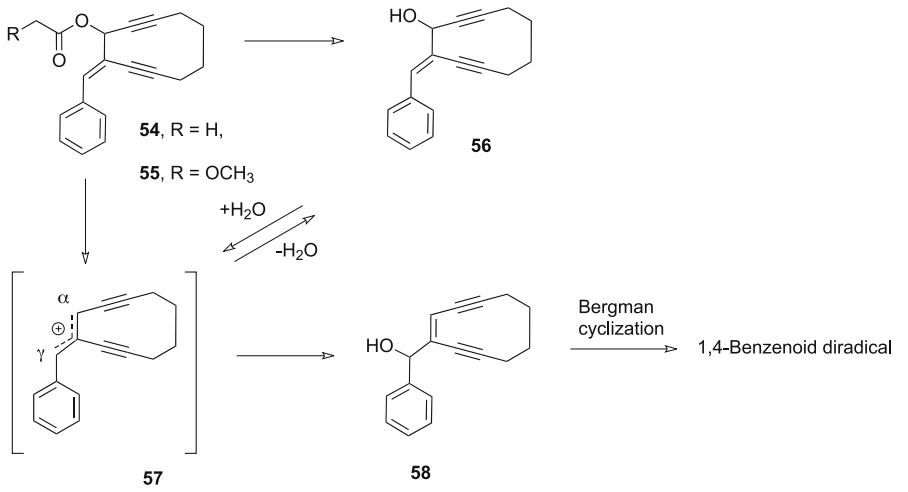


Figure 11.

Conclusion

Many candidate drug molecules are limited by poor solubility or distribution to the target tissue and lack functional groups traditionally associated with the prodrug strategies reviewed in other chapters in this book. As the examples summarized above demonstrate, creative, viable approaches to non-traditional prodrugs may be feasible, limited only by the imagination and resolve of the medcinal chemist.

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Part 4.1

Prodrugs – Preclinical and Clinical Considerations

4.1.1

Prodrugs: Absorption, Distribution, Metabolism, Excretion (ADME) Issues

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Table of Contents

Abbreviations

ADEPT .....antibody-directed enzyme prodrug therapy
ADME .....absorption, distribution, metabolism, excretion
AIDS .....acquired immune deficient syndrome
CNS .....central nervous system
CPA .....cyclophosphamide
CPT-11 .....irinotecan
CYP .....cytochrome P450
ddI .....dideoxy inosine
5-FU 5-fluorouracil
5'-DFUR .....5'-deozy-5-fluorouridine
GDEPT .....gene-directed enzyme prodrug therapy
HIV .....human immuno deficiency virus
P-gp .....p-glycoprotein
PK/PD .....pharmacokinetic/pharmacodynamic
UGT .....UDP-glucuronosyl transferase
VDEPT .....virus-directed enzyme prodrug therapy

Key Words

ADME, Pharmacokinetics, Pharmacodynamics, Toxicity, Prodrugs, Oral absorption, Tumor-specific delivery, Solubility, Nucleoside prodrugs, Oxazaphosphorines, CYP

Introduction

Over the past 20 years, there has been a growing recognition that the discovery of effective therapeutic agents involves designing compounds that possess appropriate "pharmaceutical" or "drug-like" properties in addition to high affinity for their biological targets. The pharmaceutical or drug-like properties include solubility, permeation across barriers such as the intestinal epithelium or blood-brain barrier, and metabolic and excretory clearance. Appropriate balance of these properties enables drug molecules to attain and maintain sufficient systemic and/or target concentrations to exert therapeutic effects through optimum absorption, distribution, metabolism, and excretion (ADME) processes. The ADME processes, in conjunction with the biological properties, define therapeutic profiles of drug molecules (Thakker, 2006). A drug that is poorly absorbed, rapidly metabolized, or rapidly excreted via the renal or hepatic route will not attain its full therapeutic potential. Such a drug will require higher doses to achieve sufficiently high systemic or target concentrations for efficacy, which may not be practical in some cases or may cause adverse effects in others. Thus, good pharmaceutical or drug-like properties are often defined as physicochemical properties of drug candidates that enable a drug candidate to navigate through the physical, biochemical, and physiological barriers posed by the ADME processes. The pharmaceutical properties of a drug candidate are optimized by *de novo* designing appropriate physicochemical attributes into the molecule or via formulation of the drug candidate with agents that can improve certain aspects of the physicochemical properties. An additional approach that has been used to impart good pharmaceutical properties or to correct specific sub-optimal properties is that of designing a prodrug that transiently modifies physicochemical properties of a drug candidate to overcome a shortcoming.

Since the concept of prodrug was originally proposed (Albert, 1958; Harper, 1959), there have been numerous examples of the use of prodrugs to improve therapeutic profiles of drug molecules. There are several excellent recent reviews on prodrugs and prodrug design (Beaumont *et al.*, 2003; Denny, 2004; Ettmayer *et al.*, 2004; Rooseboom *et al.*, 2004; Testa, 2004), including those contained in the prodrug theme issue of *Advanced Drug Delivery* edited by Stella (1996a). In this chapter we will examine the ADME issues associated with prodrug design and performance that are not often addressed in the prodrug literature.

The discussion in this chapter is framed by a provocative assertion that, except when drug targeting is the objective, prodrug design is driven almost exclusively by the perceived need to change certain physicochemical properties (*i.e.*, lipophilicity, solubility) and by considerations regarding the ease of prodrug synthesis. In contrast, factors that might influence the pharmacokinetic behavior of the prodrug and the active drug relative to each other, *i.e.*, distribution, metabolic stability, renal or biliary excretion, and protein binding, are rarely considered strategically in the design of prodrugs. Another factor that is not often considered strategically is the choice of the target enzyme, *i.e.*, the enzyme that converts the prodrug to the active drug. The enzymology, expression levels, tissue distribution, and inter-species conservation of structure-function of the target

enzyme may play a critical role in the success or failure of a prodrug strategy; and, yet, selection of the target enzyme often occurs by default as a result of other considerations. Another critical question that is rarely addressed is whether a particular prodrug will be a viable candidate for preclinical and clinical drug development when the scope of ADME and toxicological evaluation is significantly increased by the necessity to evaluate pharmacokinetic/pharmacodynamic (PK/PD) or toxicological properties of the prodrug and/or promoiety. It is not surprising, therefore, that while there are hundreds of reports on the design of prodrugs which show proof of concept, relatively few prodrugs have reached the market.

It is reasonable to propose that physicochemical properties of the prodrug, the active drug and, in some cases, the promoiety should be considered in the context of their ADME and toxicological behavior. Further, the catalytic properties, distribution, and expression level of the target enzymes should be considered in the design of a prodrug in order to achieve greater success in developing prodrug-based therapeutic agents. With this as a context some of the more commonly pursued objectives for prodrug design will be considered; these include improving oral absorption, improving CNS penetration across the blood-brain barrier, targeting to tumors, and improving aqueous solubility.

Prodrugs for Improved Oral Bioavailability

General Considerations

When a drug exhibits poor oral bioavailability, one needs to consider the underlying factors that may have contributed to this problem. These factors include poor solubility in the intestinal fluid, poor permeation across the cell membrane, efflux across the apical membrane, and rapid pre-systemic elimination via either metabolism in the intestinal epithelium or liver, or biliary excretion in the liver (Houin and Woodley, 2002; Stella, 2006b; Thakker, 2006). Many successful prodrugs have been designed for improving oral bioavailability of drugs that exhibit poor permeability across the cell membrane, some of which are represented in Figure 1; however, there are only a few examples of prodrugs designed to circumvent rapid pre-systemic elimination.

To improve absorption of a poorly permeable drug, the most commonly used prodrug strategy is to increase passive diffusion across the intestinal epithelium by increasing the lipophilicity of the drug (Beaumont *et al.*, 2003, Ettmayer *et al.*, 2004; Stella, 2006a). The polarity of the drug is reduced by masking hydrophilic non-ionizable groups or ionizable groups (Stella, 2006a); this is frequently accomplished by preparing ester prodrugs (Beaumont *et al.*, 2003; Ettmayer *et al.*, 2004; Stella, 2006a). Ideally, if the prodrug is converted to the active drug as it is traversing the epithelial layer, and if the conversion to the active drug is complete in the intestinal tissue, then little or no systemic exposure to the prodrug will occur. This is highly desirable because systemic exposure to the prodrug, which is

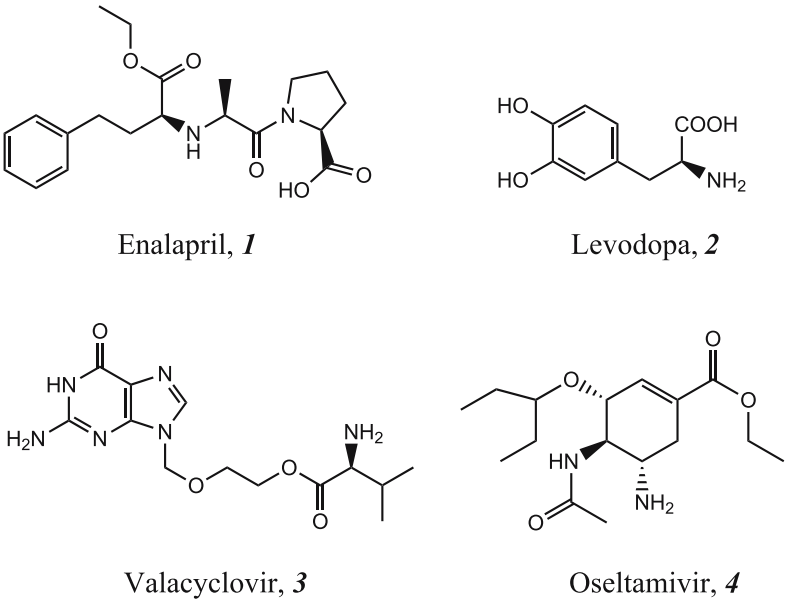


Figure 1. Selected examples of marketed prodrugs.

a distinct chemical entity with its own pharmacological and toxicological profile, is avoided or minimized (Figure 2). This can also be accomplished if the prodrug undergoes exhaustive first-pass metabolism to the active drug in the liver; however, in this scenario, the liver is likely to be exposed to high concentrations of the prodrug as well as the active drug. Rarely does one find such an idealized situation. Instead, the conversion of the prodrug to the active drug occurs at multiple sites (*e.g.*, intestine, liver, lung, kidney) after it has entered the systemic circulation, and the rate of the conversion of the prodrug to active drug is such that systemic exposure to both the prodrug and the active drug occurs to varying extents (Figure 2). Such a profile of a prodrug requires measuring not only the active drug concentrations but also the concentrations of the prodrug and, in some cases, the promoiety in preclinical and clinical studies.

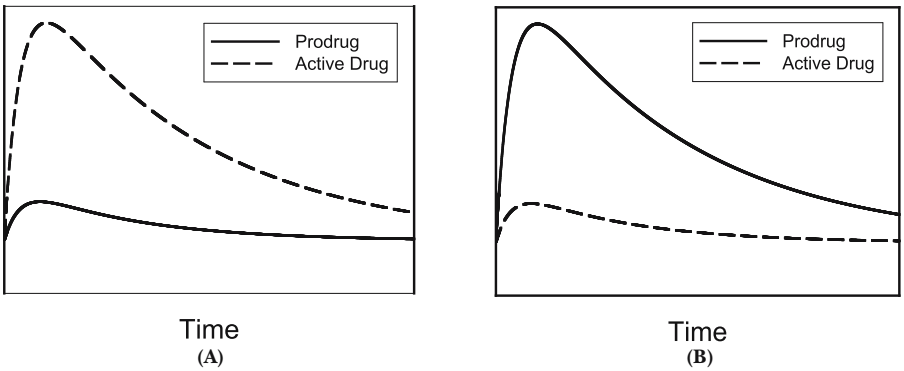


Figure 2. Desired plasma concentration (y-axis) - time profiles for a prodrug and corresponding active drug when the prodrug is designed to (A) improve oral absorption or (B) achieve tissue-specific or organ-specific delivery.

Ester Prodrugs: Carboxyesterase Multiplicity, Tissue Distribution, and Species Differences

Ester prodrugs constitute a large percentage (almost 50%) of marketed prodrugs (Ettmayer *et al.*, 2005). Examples of several marketed prodrugs are shown in Table 1. Esterifying a carboxyl group or alcohol functionality is the most widely used prodrug design approach to mask a charge or to increase the lipophilicity of a poorly absorbed drug (Beaumont *et al.*, 2003). Besides the simplicity of chemical manipulation, the wide distribution as well as broad and overlapping substrate selectivity of esterases has contributed to the widespread use of ester prodrugs.

To achieve the desired clinical objectives with respect to where and how rapidly the prodrug needs to be converted back to active drug, one has to consider the expression pattern of the target enzyme, its selectivity, and inter-species differences in expression levels and structure-function. However, a review of the literature suggests that these factors are rarely considered in the design of a prodrug with the exception of those that are intended for targeting a specific tissue (*e.g.*, tumor) or organ (*e.g.*, brain). As an example, we will look at esterases, the most widely utilized enzyme target for prodrug design. Carboxyesterases (EC 3.1.1.1), acetylcholinessterase (EC 3.1.1.7), butyrylcholinesterase (EC 3.1.1.8), and cholesterol esterase (EC 3.1.1.13) all play a role in the hydrolysis of ester prodrugs. Carboxyesterases are among the most ubiquitous proteins (Hosokawa *et al.*, 1987; Satoh and Hosokawa, 1995), and over 300 nucleotide sequences for genes encoding carboxyesterases have been listed (Redinbo and Potter, 2005). The enzymes in this class have been named based on their substrate selectivity as well as their tissue expression patterns. However, overlapping substrate selectivity and tissue expression pattern of many carboxyesterases makes it extremely difficult to assess which enzymes are responsible for the conversion of an ester prodrug to an active drug in preclinical species and in humans (Satoh and Hosokawa, 1995). At least four distinct carboxyesterases have been identified in humans; these are hCE1 (expressed in liver, macrophages, and lung epithelia), hiCE (expressed in intestine, liver, kidney, heart, and skeletal muscle), hCE2 (expressed in liver), and hCE3 (expressed selectively in the brain) (reviewed in Redinbo and Potter, 2005; Xie *et al.*, 2002). hCE1 appears to hydrolyze esters containing smaller sized alcohol functionalities whereas hiCE hydrolyzes esters containing larger alcohol functionalities (Redinbo and Potter, 2005). Based on sequence homology, Satoh and Hosokawa (1998) proposed to classify human carboxyesterases into four families—CES1, CES2, CES3, and CES4. These enzymes have differential sensitivity to inhibition by phenylmethylsulfonyl fluoride and paraoxon (Xie *et al.*, 2002). Similarly, rat liver contains four isozymes of carboxyesterases, hydrolase A (HA), B (HB), S (HS), and E, and mouse liver contains three isozymes M-LK, ME, and MS (Xie *et al.*, 2002). The hydrolytic activity of liver enzymes from several species toward four different substrates is shown in Table 2 (Satoh and Hosakawa, 1995). These data clearly show that these esterases metabolize the four substrates at vastly different rates and that liver enzymes from different species hydrolyze each substrate with a wide range of activity.

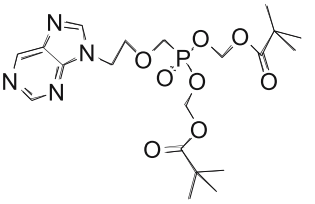
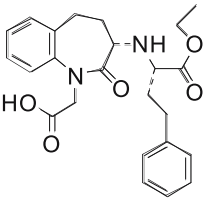
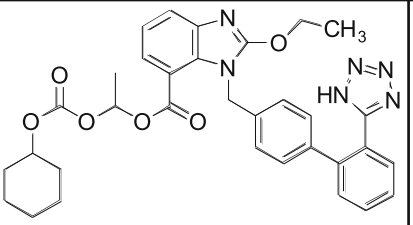
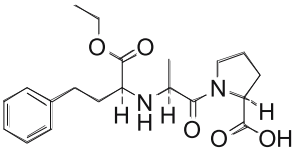
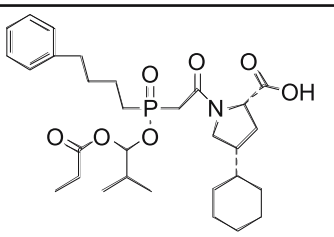
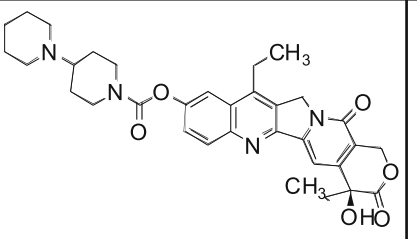
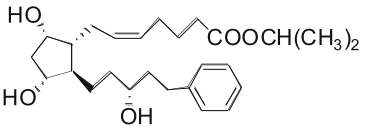
Prodrug	Brand Name	Chemical Structure	Indication
Adefovir dipivoxyl	Hepsera®		Hepatitis B
Benazepril	Lotensin®		Hypertension
Candesartan cilexetil	Atacand®		Hypertension
Enalapril	Vasotec®		Hypertension
Fosinopril	Monopril®		Hypertension
Irinotecan	Camptosar®		Metastatic carcinoma of colon/rectum
Latanoprost	Xalatan®		Glaucoma

Table 1. Marketed Ester Prodrugs.

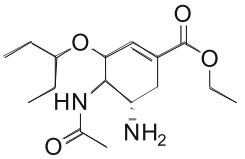
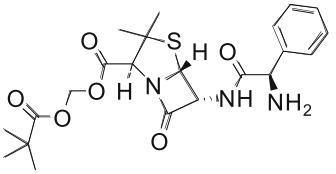
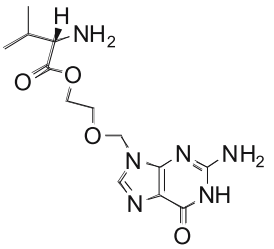
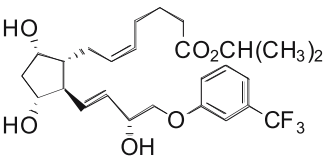
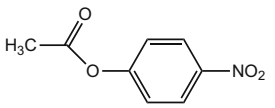
Prodrug	Brand Name	Chemical Structure	Indication
Oseltamivir	Tamiflu®		Upper respiratory Systemic Circulation
Pivampicillin			Bacterial infection
Valaciclovir	Valtrex®		Genital Herpes
Travoprost	Travatan®		Glaucoma

Table 1 (continued). Marketed Ester Prodrugs.

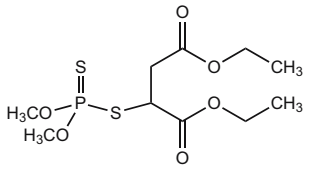
Despite extensive use of esterases as the target for prodrug design, it is difficult to identify a study in which tissue distribution and selectivity of esterases was considered prospectively in the design and development of ester prodrugs. In fact, with few exceptions (*e.g.*, Vere Hodge *et al.*, 1989, Morrison *et al.*, 1990), the knowledge about the tissue or organ where predominant hydrolysis occurs is incomplete, and knowledge about the role of specific esterases in the hydrolysis of ester prodrugs is virtually absent. Vere Hodge *et al.* (1989) showed that hydrolysis of one of the two ester groups in famciclovir (Famvir®, **9**) is hydrolyzed by the esterases present in intestinal epithelium and the other ester group is hydrolyzed by liver esterases. Morrison *et al.* (1990) showed that the rank order of hydrolytic activity toward fosinopril (**10**), determined using tissue homogenates *in vitro*, was liver/kidney > small intestine > lung >> blood. However, upon oral administration to dogs, 75% of the absorbed fosinopril was hydrolyzed by the intestine and the remaining 25% by the liver. These results show that *in vitro* assessment of the hydrolytic rates may be a useful tool to assess likely *in vivo* sites where ester prodrugs would be converted to the active drug in a given species; however, it does not always accurately predict the relative rates of *in vivo* conversion of a prodrug at specific sites because of the confounding physiological processes. The literature

Species	Specific Activity (nmol/mg/min)			
	PNPA, *5*	**MALA,** *6*	**BUTA,** *7*	**ISOC,** *8*
Rat	1.93	0.075	0.12	0.002
Mouse	8.52	0.014	0.01	0.003
Hamster	14.9	0.026	0.58	0.008
Guinea pig	19.0	0.041	0.04	0.029
Rabbit	10.3	0.060	1.08	0.006
Pig	10.9	0.029	1.62	0.004
Cow	8.77	*	1.11	0.002
Dog	2.42	0.027	1.06	0.002
Monkey	4.37	0.043	<0.005	0.006
Human	1.95	0.116	0.13	0.001

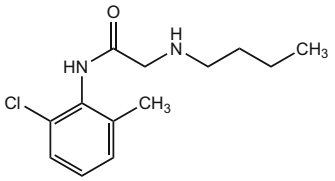
Table 2. Activities of Hepatic Carboxyesterases from Different Species[1].
[1]Adapted from Satoh and Hosokawa (1995). PNPA, p-nitrophenyl acetate; MALA, malathione; BUTA, butanilicaine; ISOC, isocarboxazid. * <0.002 nmol/mg/min



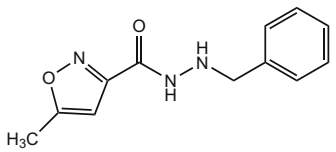
PNPA, *5*



MALA, ***6***



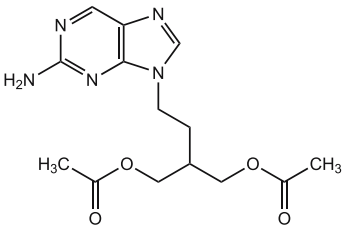
BUTA, *7*



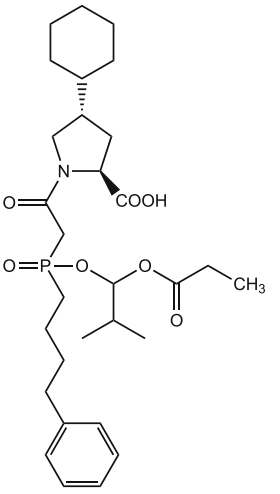
ISOC, ***8***

Structures 5-8.

is full of reports in which biological conversion of a series of ester prodrugs to the active drugs is evaluated using rat and human plasma or blood, or sometimes using liver and/or intestinal homogenates in addition to plasma. The intent of these studies is to assess the relative merits of the prodrugs being tested. However,



Famciclovir, ***9***



Fosinopril, ***10***

Structures 9-10.

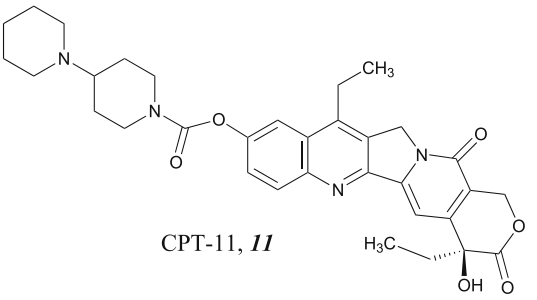
when such studies are conducted without performing any exploratory work to elucidate the selectivity of different esterases toward the target compounds, they do not yield meaningful results; in fact, the results are often misleading. Such studies should be interpreted with extreme caution because of the differences in distribution pattern, selectivity, and activity of multiple carboxyesterases across different species. In contrast, examples of well-conceived *in vitro/in vivo* studies can also be found in the literature (Mamidi *et al.*, 2002; Liederer *et al.*, 2005) in which the esterase selectivity is carefully investigated using relevant tissue sources and/or selective esterase inhibitors and *in vivo* studies are performed to assess the ability of the *in vitro* assays to predict the *in vivo* behavior of several prodrug candidates. For example, Mamidi *et al.* (2002) showed that among acetyl, propionyl, and butyryl esters of the COX-2 inhibitor celecoxib, the propionyl and butyryl esters were hydrolyzed much more rapidly than the acetyl derivative by rat liver and intestinal tissue homogenates, but no such discrimination was exhibited by rat plasma. *In vivo* studies in the rat showed that following oral administration of the propionyl and butyryl esters the celecoxib $AUC_{0-\infty}$ values were almost equivalent (134 and 132 μg.h/mL, respectively) and much greater than following oral administration of the acetyl ester (26 μg.h/mL). These type of results provide a clear guide as to which *in vitro* system should be used to optimize ADME properties of prodrugs for ultimate selection as lead development candidates. Obviously, in this example, one would want to assess the similarities and differences in the selectivity of esterases in the rat vs. human to determine if rat is an appropriate species to optimize ADME of celecoxib ester prodrugs.

Irinotecan: Could the Toxicity Have Been Avoided by Proper Choice of Carboxyesterase as a Target Enzyme for Re-conversion to SN-38?

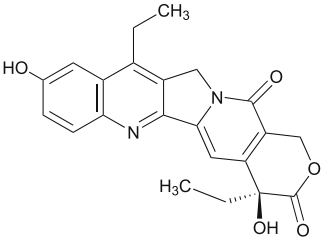
As an example of the importance of esterase selectivity on the disposition and pharmacodynamics properties of the prodrug/drug combination, the esterase selectivity and toxicity profile of an anticancer agent irinotecan (CPT-11, **11**) is discussed here. Irinotecan is used in the treatment of colon cancer. It is an ester prodrug designed to improve the solubility of the camptothecin analog SN-38 (**12**). Irinotecan (**11**) is hydrolyzed predominantly to **12** by the carboxyesterase hiCE (Humerickhouse *et al.*, 2000), although hepatic hCE-2 is also implicated in the conversion of **11** to **12** (Mathijssen *et al.*, 2001, and references therein). While the prodrug was designed to improve the solubility of **11** for parenteral administration, the solubility profile of **12** makes it a viable candidate for oral administration despite its anticipated first-pass metabolism by esterases in the intestine and liver. The dose-limiting toxicity of irinotican is delayed diarrhea (Abigerges *et al.*, 1994), which can be attributed to hydrolysis of **11** to **12** due to catalytic activity of hiCE in the intestinal epithelium (Khanna *et al.*, 2000; Wadkins *et al.*, 2004). Thus, hiCE is required to convert irinotican into the active drug, but in this case it also contributes to the toxicity of the prodrug caused by this conversion. The question that one must ask is if this toxicity of irinotecan could have been designed out by choosing an ester that was a poor substrate for hiCE. It has also been suggested that biliary excretion of **12** and its glucuronide metabolite **12-G**, which is hydrolyzed by UDP-glucuronosyl transferase 1A1 and 1A7 (UGT1A1/7) back to **12** in the intestine, is responsible for the generation of high concentrations of **12** in the intestine and, thus, the toxicity (Mathijssen *et al.*, 2001, and references therein). However, biliary excretion of **11** (3–22% of dose) far exceeds that of **12** (0.1– 3% of dose) or **12-G** (0.6 to 1.1% of dose) (Lokiec *et al.*, 1995); thus, the role of hiCE in generating localized high concentrations of **12** in the intestine cannot be underestimated.

Interestingly, it has been shown that rabbit liver carboxyesterase can hydrolyze **11** to **12** much more efficiently than can human liver carboxyesterases (Danks *et al.*, 1999). Thus, expression of rabbit liver carboxyesterase in Rh30 rhabdomyosarcoma human tumor cells, grown as xenogaphs in immune-deprived mice, sensitized the tumor to irinotecan much more than did expression of human liver carboxyesterase in the same cells. Rabbit liver carboxyesterase is being considered for enzyme/prodrug therapy with irinotecan (Danks *et al.*, 1999). Interspecies differences in carboxyesterase activity, responsible for conversion of **11** to **12**, have an important implication in the use of the xenograph model to assess efficacy of the prodrug against various human tumors. It appears that this model over-predicts the efficacy of irinotican, in part due to higher carboxyesterase activity in the rabbit compared to humans.

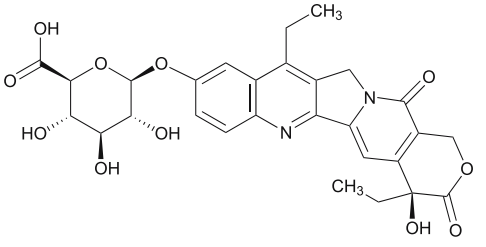
Clearly, there is increasing awareness about the importance of understanding the selectivity of carboxyesterases in the reconversion of prodrugs. This is evident from several recent publications on the selectivity of esterases toward hydrolysis of ester prodrugs of nucleoside drugs such as 5'-deoxy-5-fluorocytidine, floxuruidine, and gemcitabine (Vig *et al.*, 2003; Landowski *et al.*, 2005; Song *et al.*,



CPT-11, ***11***



SN-38, ***12***



SN-38 12-glucuronide, ***12-G***

Structures 11-12-G.

2005). Attempts are also underway to identify genetic factors responsible for inter-individual variation in the pharmacodynamics response or toxicity of a prodrug. Thus, Charasson *et al.* (2004) identified 11 single nucleotide polymorphisms in the *hCE2* gene; however, none of these polymorphisms resulted in an altered amino acid sequence and, consequently, catalytic activity of the enzyme toward reconversion of **11** into **12**.

Sub-cellular Localization of Carboxyesterases: A Factor Rarely Considered for Ester Prodrug Design

The carboxyesterases present in the intestinal epithelium and hepatocytes are predominantly localized in the endoplasmic reticulum. Hence, hydrolysis of the ester prodrugs by these enzymes would yield the active drugs in the intracellular space of the enterocytes and hepatocytes. To achieve good oral bioavailability, a drug needs to exit the enterocytes across the basolateral membrane of enterocytes and across the sinusoidal membrane of hepatocytes (Figure 3). Considering the fact that drugs regenerated by hydrolysis of the ester prodrugs are likely to be hydrophilic or charged molecules, their exit from enterocytes or hepatocytes may

require a transporter-mediated mechanism. In the absence of such a mechanism, the ester prodrug may not yield significant enhancement in the oral absorption of the hydrophilic or charged drug molecules. Several absorptive and efflux transporters in the sinusoidal membrane of hepatocytes have been identified over the past decade (Chandra and Brouwer, 2004); however, our knowledge about the efflux transporters in the basolateral membrane of enterocytes is somewhat limited. In evaluating ester prodrugs for their ability to improve oral bioavailability of a drug, the ability of the released drug to exit across the basolateral membrane of the enterocytes and/or across the sinusoidal membrane of the hepatocytes is often not considered. These prodrugs are frequently evaluated by examining their permeation across Caco-2 cell monolayers or other *in vitro* models for intestinal epithelium. If attention is not paid to mass balance in these experiments, the conclusions derived solely based on the permeability values of ester prodrugs could be misleading, as such results would not take into account any drug that is formed inside the cells due to hydrolysis that cannot traverse the basolateral membrane into the receiver chamber. Furthermore, such studies do not consider the possibility that a significant amount of the prodrug may be hydrolyzed in the hepatocytes and that free drug might not be able to exit the hepatocytes across the sinusoidal membrane.

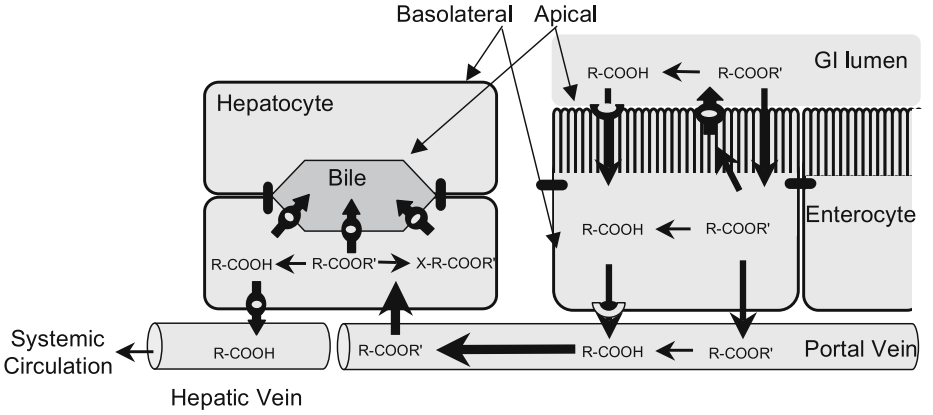


Figure 3. Intracellular hydrolysis of an ester prodrug in enterocytes or hepatocytes.
The illustration shows that free carboxylic acid (active drug) formed in enterocytes may not be able to cross the basolateral membrane of enterocytes to enter into the portal circulation or cross the basolateral membrane of hepatocytes to enter into the systemic circulation unless it is a substrate for a transporter(s) present in these membranes. Also, the prodrug or the drug could be a substrate for efflux transporters in the apical membranes of enterocytes and/or hepatocytes, and thus could be pumped into the intestinal lumen and bile canaliculi, respectively. Also shown is the likely metabolic route that would not lead to the generation of the active drug; while this "non-productive" metabolism is only shown in hepatocytes, it could also occur in enterocytes.

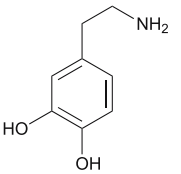
Apical/Canalicular Efflux Transporters and Metabolism as Barriers to Oral Bioavailability of Prodrugs

Prodrugs that are designed to increase oral bioavailability are typically (but not always) more lipophilic than the corresponding drug molecules. However, the chemical change that imparts lipophilicity can also make the prodrug susceptible to efflux across the apical membrane of enterocytes (Borchardt, 1999) and/or the canalicular membrane of hepatocytes via one or more efflux transporters. As an example, irinotecan (**11**), an ester prodrug of SN-38 (**12**), is much more rapidly excreted in the bile (3–22% of the dose) than is **12** (0.1–0.9% of dose) (Lokiec *et al.*, 1995) as discussed in the previous section on esterase selectivity. Studies performed in cell culture models of intestinal epithelium (*e.g.*, Caco-2 cells, MDR-MDCK cells) can readily assess whether a compound is a substrate for an efflux transporter (Polli and Serabjit-Singh, 2004; Polli *et al.*, 2001) and if an efflux transporter such as P-glycoprotein (P-gp) is attenuating absorptive transport of a compound (Troutman and Thakker, 2003). Prodrugs should not be evaluated only for apical efflux in the intestinal epithelium but also for rapid biliary excretion via canalicular efflux transporters. An *in vitro* model B-CLEAR®, which could prove to be very useful in identifying and designing out rapid canalicular efflux in hepatocytes, has been developed to assess the efflux of drug molecules across the canalicular membrane of the hepatocytes (Zamek-Gliszczynski and Brouwer, 2004),

Prodrugs for CNS Delivery across the Blood-brain Barrier

Delivery of drugs to targets in the CNS requires that the drug molecules can effectively cross the blood-brain barrier, which involves the highly specialized endothelial lining of the capillaries supplying blood to the brain tissues. As is the case for the intestinal epithelium, the presence of tight junctions, apically directed efflux transporters, and drug-metabolizing enzymes makes it difficult for many drug molecules to traverse the blood-brain barrier. Thus, hydrophilic drugs cannot permeate the blood-brain barrier because of their inability to partition into the endothelial cell membrane and/or to traverse the restricted intercellular space. In contrast, lipophilic drugs sometimes cannot cross the blood-brain barrier because they are subject to metabolism or apically directed efflux. Almost all prodrug strategies employed for CNS drug delivery are designed to improve the permeation of hydrophilic drugs across the blood-brain barrier. Designing prodrugs for CNS or other site-specific delivery requires more sophisticated approaches than derivatizing the hydrophilic drug to increase lipophilicity. As articulated in several reviews (Stella and Himmelstein, 1985; Boddy *et al.*, 1989; Anderson, 1996), site-specific delivery via a prodrug requires that the prodrug is not only able to cross the blood-brain barrier but is selectively converted to the active drug at the target site (*e.g.*, CNS) and that the active drug is retained at the target site for a prolonged period.

Prodrugs designed to improve CNS delivery of drugs across the blood-brain barrier (Bodor, 1987; Brewster and Bodor, 1992; Anderson, 1996) should not be converted to active drugs by first-pass metabolism in the intestine and/or in the liver; instead, the prodrug should persist in the blood/plasma compartment so that it can cross the blood-brain barrier and deliver the drug into the CNS compartment (see Figure 2). Ideally, conversion of the prodrug to the active drug should occur selectively in brain tissue. However, even if the prodrug is metabolized to the active drug and other metabolites systemically, sufficiently high CNS concentrations of the active drug can be attained if the systemic clearance of the prodrug is relatively low and if active drug generated in the CNS tissue is not readily diffused out of the brain or cleared rapidly by the brain enzymes. CNS delivery of dopamine (**13**) by the prodrug levodopa (**4**) provides an excellent example of a prodrug strategy for brain delivery of a drug in which the conversion of the prodrug to the drug (by dopa decarboxylase) is not brain-specific, but the effective brain delivery of the drug is achieved because the drug is trapped in the brain once it is formed there. In fact, levodopa (**4**) is administered with carbidopa, a dopa decarboxylase inhibitor, to diminish systemic decarboxylation of the prodrug. Levodopa crosses the blood-brain barrier by virtue of its substrate activity for the large neutral amino acid transporters present in the blood-brain barrier (Wade and Katzman, 1975). With such a profile, the active drug concentration can build up in the CNS compartment over a period of time. The key factor for success in CNS delivery of a drug via this approach is that the drug is not able to diffuse out readily into the systemic circulation. This concept of trapping the drug in the target tissue has been nicely discussed with the help of simulation of pharmacokinetic behavior of the prodrug and the drug under different scenarios by Stella and Himmelstein (1985).



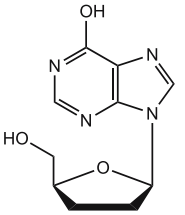
Dopamine, ***13***

Structure 13.

Anderson *et al.* (1992), in their studies with CNS delivery of dideoxyinosine (ddI) (**14**) prodrugs, have suitably illustrated the concept of selective conversion and retention. The dideoxy nucleoside, **14**, is one of many agents approved for the treatment of human immunodeficiency virus (HIV) infection, and its CNS delivery is sought to treat AIDS dementia resulting from the CNS infection of HIV virus. The CNS penetration of ddI is very poor, presumably due to its hydrophilic nature; hence, a series of 5'-esters and 6-halo derivatives were evaluated as prodrugs to improve CNS penetration of **14** (reviewed in Anderson, 1996).

Simulations of the steady-state brain concentration of **14** upon intravenous infusion of **14** or its prodrugs showed that, at a given log P value of a prodrug, greater enhancement in the CNS concentration of the drug was achieved as the bioconversion rate of the prodrug in the brain tissue relative to plasma (or blood) increased (Figure 4). Interestingly, the *in vivo* studies in rats showed that 5'-esters, even 5'-butyrate or pivalate with octanol/water partition ratios of 4.0 and 4.5, respectively, did not afford very much enhancement in the CNS concentration of **14** upon intravenous infusion because hydrolytic rates were considerably slower in brain tissue vs. plasma. In contrast, 6-chloro and 6-bromo didoxypurine, which are converted to **14** by adenosine deaminase, provided ten- and fourfold enhancement, respectively, in the CNS concentration of **14**, consistent with almost 14-fold greater rate of conversion to ddI in rat brain tissue vs. blood (Anderson, 1996).

An important aspect of the prodrug design for CNS delivery that is perhaps implicit but often not emphasized sufficiently is that the bioconversion of the



ddI, ***14***

Structure 14.

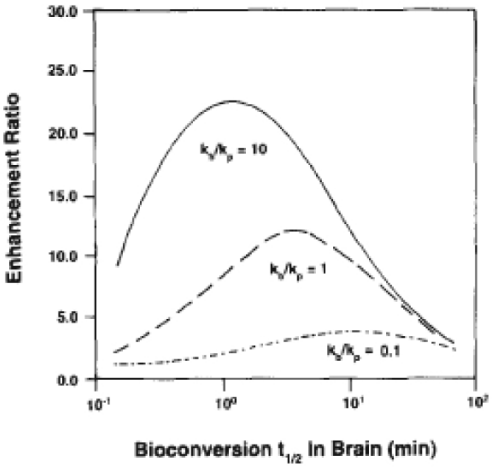
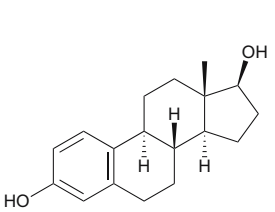
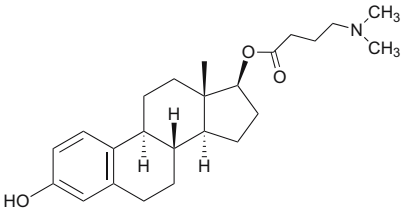


Figure 4. Enhancement of ddI (14) brain delivery by a prodrug as a function of prodrug half-life in the brain tissue. The illustration shows simulations of the enhancement ratio (steady-state brain concentration of 14 obtained from a prodrug to that obtained by infusion of 14) versus the prodrug half-life in the brain tissue. This relationship is illustrated at three different ratios of (prodrug to drug) conversion rates by the brain tissue versus plasma. (adapted from Anderson, 1996).

prodrug should not occur prematurely in the gastrointestinal tract, liver, or plasma/blood, and a large percentage of the administered dose of the prodrug (not the drug) should reach the systemic circulation and blood-brain barrier (see Figure 2). For this reason, esters are likely to be a poor choice for a prodrugs strategy to deliver a drug into CNS, tumors, or other tissues because of their rapid hydrolysis in intestine, liver, and plasma/blood. However, it has been shown that ester prodrugs, when administered nasally, not only avoid first-pass metabolism in the gastrointestinal tract and liver but also escape dilution and hydrolysis in the blood. With the finding that estrogen intake may delay the onset and decrease the risk of Alzheimer's disease, Xu *et al.* (1998) and Al-Ghananeem *et al.* (2002) have sought a prodrug approach to achieve CNS delivery of estradiol (**15**). Oral administration of estradiol in the rat resulted in extensive first-pass metabolism in the gastrointestinal tract and liver (Bawarshi-Nassar *et al.*, 1989); hence, the intranasal route was considered based on prior reports that nasally administered drugs can achieve high CNS concentrations (Hussain, 1998). Because estradiol does not have sufficiently high aqueous solubility for nasal formulation, ester prodrugs containing alkylamine functionalites (*e.g.*, **16**) were prepared and tested for CNS delivery of estradiol via nasal administration of the prodrugs. The rat cerebrospinal fluid concentrations of estradiol following nasal administration of N,N-dimethylaminobutyric esters (at either the 3- or the 17-postion) were five- to ninefold higher than following intravenous administration.



Estradiol, ***15***



Estradiol 17-N,N-dimethylaminobutyate, ***16***

Structures 15-16.

Tumor-specific Delivery of Cytotoxic Drugs

Prodrugs have found an important role in cancer chemotherapy because of the need to reduce systemic toxicity of anticancer drugs, many of which are potent cytotoxic agents. For prodrugs used to target tumors (*e.g.*, **17–20** in Figure 5), the major consideration is to minimize systemic exposure of the highly cytotoxic anticancer agents while achieving high exposures of the tumor tissue to the drug (Sinhababu and Thakker, 1996; Denny, 2004; Rooseboom *et al.*, 2004; Riddick *et al.*, 2005) (Figure 2). Thus, it is highly desirable (i) that the prodrug is not significantly converted to the active drug in any of the non-tumor tissues but is converted to the active drug selectively in the tumor tissue and (ii) that the drug is retained in the tumor tissue without significant leakage (diffusion) into the

systemic circulation. Many prodrug strategies employed in cancer chemotherapy include selective conversion to the active drug in the tumor mass; however, examples of prodrugs can be found (nucleosides and oxazaphosphorines) that are not necessarily converted to the active drug in the tumor tissue. Various approaches for tumor-targeting of anticancer drugs via prodrugs have been reviewed previously (Sinhababu and Thakker, 1996; Denny, 2004; Ettmayer *et al.*, 2004; Rooseboom *et al.*, 2004; Riddick *et al.*, 2005) and will not be revisited here. Instead, we will address the ADME and toxicity issues associated with the use of specific enzyme targets for prodrug design. Because anticancer agents are necessarily cytotoxic, the threshold for selectivity to regenerate the active drug in the tumor tissue and its retention within the tumor mass is greater than tissue targeting of other classes of therapeutic agents. However, one finds several examples of prodrugs that are far from selective in delivering the anticancer agents to tumors. This may be a reflection of the philosophy in the cancer chemotherapy that any approach that provides even a small advantage over the use of highly cytotoxic agents should be incorporated into the cancer chemotherapy regimen.

Prodrug design for tumor targeting relies on enzyme activity that is greater in the tumor tissues than in the corresponding normal tissues. Rooseboom *et al.* (2004) have published an expansive review of the endogenous enzymes targeted for designing prodrugs as anticancer agents. Alternatively, prodrugs are designed to exploit the unique environment found in the tumor tissue, most notably hypoxia. Doxorubicin (**17**), mitomycin C (**18**), and tirapazamine (**19**) (Figure 5) are excellent examples of prodrugs that are activated in the hypoxic environment of solid tumors. Recently, more elaborate prodrug strategies have been designed in which the target enzyme that converts the prodrug to the active drug is artificially delivered to the tumor site. This is accomplished by covalently linking it to tumor-specific antibodies (antibody-directed enzyme prodrug therapy (ADEPT)) or by delivering the gene that encodes for the prodrug-activating enzyme via viral vectors (virus-directed enzyme prodrug therapy (VDEPT)) or non-viral vectors (gene-directed enzyme prodrug therapy (GDEPT)) (reviewed in Denny, 2004; Rooseboom *et al.*, 2004). In these approaches, exogenous enzymes can be

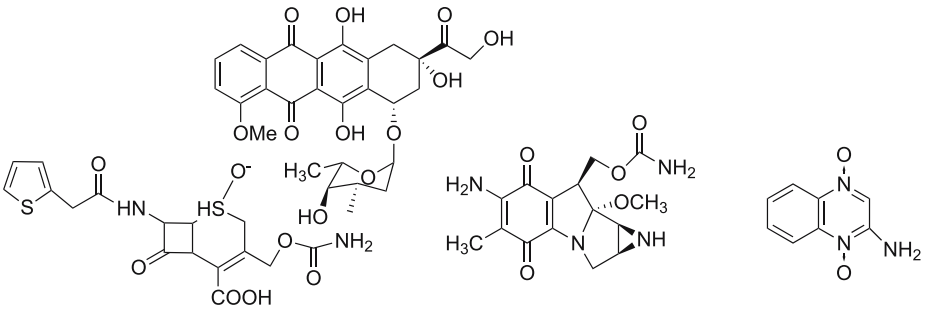


Figure 5. Examples of tumor-activated prodrugs.

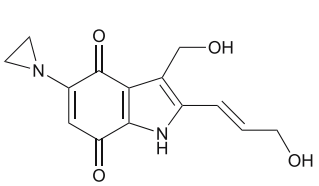
delivered to the tumor tissue to achieve absolute tumor-selectivity because these enzymes are not expressed in any other tissues. In the VDEPT and GDEPT approaches, the gene encoding the prodrug-activating enzyme must be responsive to tumor-specific promoters to ensure tumor-specific expression of the enzyme.

While many of the prodrug strategies for tumor targeting appear to be conceptually quite attractive, there are only a few examples of marketed prodrugs that can truly be considered tumor-activated prodrugs. There are several reasons for this rather limited success of tumor-activated prodrugs. Perhaps the most important factor that contributes to the failure of tumor-activated prodrug strategy for cancer chemotherapy is the heterogeneity of cells within any solid tumor. Thus, not all the cells in a tumor are likely to have high expression of a prodrug-activating enzyme; similarly, not all cells within a tumor are in a hypoxic environment. This implies that, for a tumor-specific prodrug strategy to succeed, the active drug produced by a fraction of the tumor cells should not only be effective in killing the cells in which it is produced but also kill other cells in the tumor mass by diffusing through the tumor via the so called "bystander effect." Of course, this requires that the active drug, once produced within a cell, is able to diffuse out of the cell and through several layers of cells within the tumor mass. On the other hand, the active drug should not diffuse out of the tumor tissue and enter the systemic circulation because this would lead to the failure of the targeting strategy and cause systemic toxicity. Such a balance is difficult to achieve, which explains why there are not many successes as measured by marketed therapeutic agents to exemplify tumor-activated prodrugs. Many of the more elaborate approaches such as ADEPT, VDEPT, and GDEPT have not yet produced marketed anticancer therapy because they also suffer from the same challenge of producing an optimum "bystander effect" of the released active drug. In addition, these approaches depend on delivering large molecules such as antibodies or genes to cells inside the tumor tissue, which is a formidable task in itself.

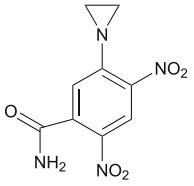
Prodrugs based on Reductive Activation

The rationale for the design of prodrugs that can be converted to the active drug via reductive metabolism is based on the observation that cells within solid tumors are typically in hypoxic environment. Thus functionalities that can be readily reduced, *i.e.*, quinone, quinonemethide, nitro group, and nitroxide radical, are often incorporated into the design of prodrugs that are selectively activated to anticancer drugs in the tumor tissue. Enzymatic reduction of these functionalities can occur under normoxic conditions in many tissues, but the reduced functionality is rapidly re-oxidized in the presence of oxygen. However, in the hypoxic environment of solid tumors, reduction of these functionalities is not readily reversed, leading to the generation of sufficiently high concentrations of reactive radical intermediates that can covalently modify nucleic acids. Several classes of bioreductive prodrugs have been designed, and many are in various stages of clinical development (Stratford *et al.*, 2003). Examples of prodrugs that are activated by reductive metabolism include quinones (**18**, EO-9 (**21**)), aromatic

nitro compounds (CB1954 (**22**)), and N-oxides (**19**). These prodrugs are reduced by cytochrome P450 (CYP) reductase, CYP, DT-diaphorase, xanthine oxidase, or nitric oxide synthase. Prodrugs based on bioreductive activation have been reviewed previously (Sinhababu and Thakker, 1996; Denny, 2004; Rooseboom *et al.*, 2004). The two enzymes that are most often attributed to the bioreductive activation of prodrugs designed for cancer chemotherapy, DT-diaphorase and CYP reductase, are widely distributed. Thus, prodrugs activated by these enzymes suffer from significant adverse effects. Interestingly, DT-diaphorase is overexpressed in many tumors (Workman *et al.*, 1989; Schlager and Powis, 1990); however, some have questioned its role in the bioactivation of antitumor drugs (cf. Riley and Workman, 1992; Rooseboom *et al.*, 2004). Two-electron reduction catalyzed by this enzyme is likely to generate stable hydroquinones rather than the more reactive radical intermediates. The expression of CYP reductase in the tumor tissue is often lower than in the normal tissues (Forker *et al.*, 1996), thus making it a less attractive target for tumor-specific delivery of anticancer agents via bioreductive prodrugs. These observations suggest that the distribution and activity profile of the two most important bioreductive enzymes is not particularly ideal for tumor-selective activation of prodrugs. Yet, prodrugs such as **18** that are believed to be activated by this mechanism (Tomasz, 1995; Sinhababu and Thakker, 1996; Hargrave *et al.*, 2000; Rooseboom *et al.*, 2004) are successful cancer chemotherapeutic agents. Mitomycin C (**18**) increases cure rate in head and neck cancer patients when used in conjunction with radiotherapy (Fischer, 1993; Haffty *et al.*, 1993). Thus, it is likely that the hypoxia in the solid tumor provides sufficient driving force for the reductive metabolism of such prodrugs to overcome less than optimum enzyme distribution. Selection of agents based on the reductive enzymes involved in their bioactivation, the expression pattern of the enzymes in the target tumor, and the hypoxic fraction of tumor biopsies may lead to more effective treatment regimens. It is important to recognize that prodrugs designed to be activated via the bioreductive mechanism are likely to cause toxicity to organs, such as the liver, that contain hypoxic tissues.



EO-9, ***21***



CB1954, ***22***

Structures 21-22.

Nucleosides as Prodrugs of Anticancer Agents

Nucleoside analogs have been used as anticancer agents for more than two decades; the most prominent example of these is cytosine arabinoside (Ara-C) (**23**)

structure (Hadfield and Sartorelli, 1984; Wipf and Li, 1994; Anantham, 1995). Nucleoside analogs have also been extensively used as antiviral agents (*e.g.*, acyclovir). The nucleoside agents are prodrugs because these agents work via interruption of the nucleic acid synthesis of tumor cells by mimicking naturally occurring nucleotides after they are converted to their corresponding 5'-nucleotides by kinases and other phosphorylating enzymes. Unfortunately, numerous enzymes are involved in the biosynthesis of nucleotides from the corresponding nucleoside analogs, and in their metabolism; these enzymes are widely distributed throughout the body. Hence, only a small percentage of the administered nucleoside prodrug is likely to reach the tumor tissue, thus requiring administration of large doses. It is impossible to restrict bioactivation of these prodrugs within the tumor tissue and, thus, systemic or tissue-specific toxicity is inevitable. The prodrug 5-fluorouracil (5-FU) (**24**) is a pyrimidine base analog whose antitumor (cytotoxic) activity is attributed predominantly to the metabolically produced nucleotide 5-fluoro-2'-deoxyuridine- 5'-monophosphate, which is a potent inhibitor of thymidylate synthase (Sinhababu and Thakker, 1996, and references therein). The clinical utility of 5-FU is limited by severe toxicity to bone marrow and gastrointestinal tract and rapid clearance of the drug (Hadfield and Sartorelli, 1984). Interestingly, new prodrugs of **24**, a prodrug in its own right, have been designed as exemplified by ftorafur (**25**) (Hadfield and Sartorelli, 1984) or 5'-deoxy-5-fluorouridine (5'-DFUR, **26**) (Armstrong and Diasio, 1980; Patterson *et al.*,1995). 5'-DFUR (**26**) is clinically used for treatment of breast, colorectal, and gastric cancers. The conversion of **26** to **24** is mediated by thymidine phosphorylase, which is expressed at significantly higher levels in several tumors (references in Rooseboom *et al.*, 2004). It is assumed that **24** will be formed in greater amounts in the tumor tissue than in normal tissues and, thus, it will exhibit lower toxicity than **24**. However, the use of **26** is also limited by gastrointestinal toxicity. This should not be surprising because **24** is not likely to be captured and trapped in tumors effectively. Yet another prodrug, capecitabine (**20**), has been designed that is converted to **26** by initial carboxyesterase-catalyzed hydrolysis to 5'-deoxy-5-fluorocytidine (**27**) and its subsequent enzymatic conversion to **26** by cytidine deaminase. Then, **26** is further converted to **24** by thymidine phosphorylase, which, in turn, is converted to 5-fluoro-2'-deoxy-uridine-5'-monophosphate (**28**) to exert its antitumor activity. This series of metabolic conversions is delineated here (Figure 6) to emphasize a pattern often seen in the design of nucleoside analog-based anticancer agents. As toxicity liabilities of a prodrug are identified, newer prodrugs are designed that are further removed from the active agent in comparison to the original prodrug. In some cases prodrugs are designed that are three to five enzymatic steps removed from the active drug. While one cannot question the improvement observed in the clinical profile of these prodrugs over the previously used prodrug, clearly one must question how rational such an approach is in improving anticancer therapy via the prodrug design. It is practically impossible to predict or control intra- or inter-patient variability in the metabolic transformations leading to the formation of the active agent when two to five enzymatic steps are required for the formation of the active

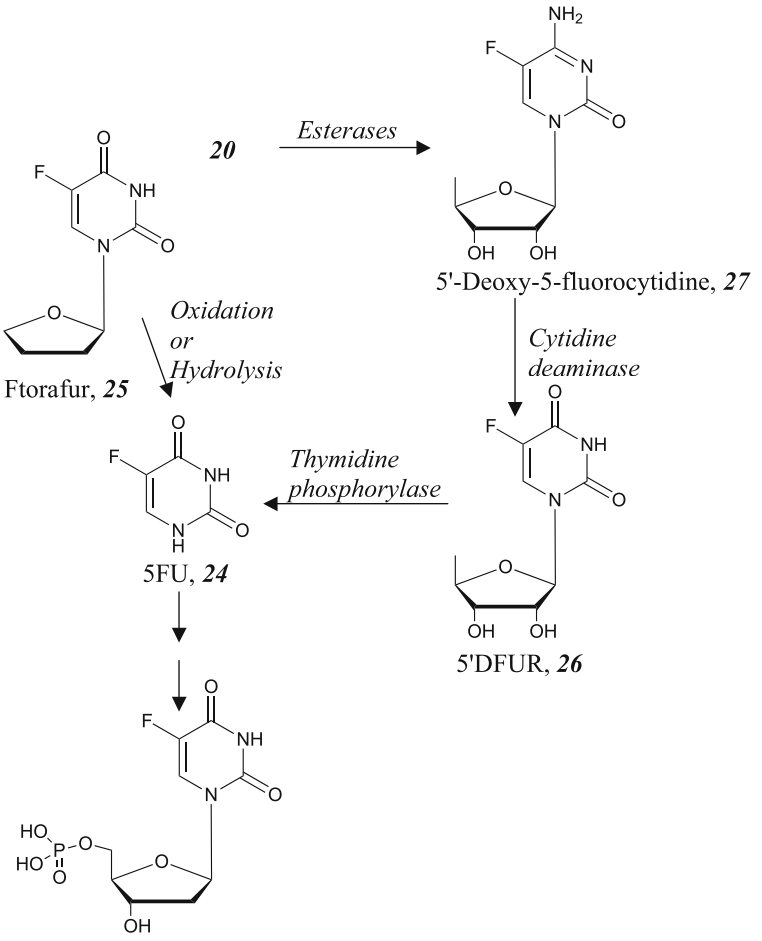
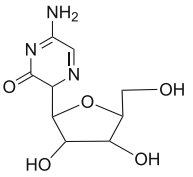


Figure 6. Formation of 5FU (24) from two different prodrugs, Capecitabine (20) and Ftorafur (25), and its activation to biologically active agent 5-fluoro-2'deoxy-5'-monophosphate (28). While 24 is a prodrug in its own right, additional prodrugs that served as its metabolic precursors have been designed to improve its toxicity profile.



Ara-C, ***23***

Structure 23.

agent. When one considers that the prodrug, the intermediates, and the active agent may each be subject to multiple metabolic and excretory clearance mechanisms, the complexity of the system becomes so enormous that it is difficult to accept the premise that the success of such agents is entirely due to rational design of the prodrug.

Oxazaphosphorines as Prodrugs of Anticancer Agents

Cyclophosphamide (CPA, **29**), ifosfamide (**30**), and trofosfamide (**31**) are members of an oxazaphosphorine class of prodrugs that are bioactivated to alkylating anticancer agents by CYP enzymes (Sinhababu and Thakker, 1996; Boddy and Yule, 2000, Rooseboom *et al.*, 2004). CPA (**29**), the most frequently used alkylating anticancer agent (Kwon, 1999), was introduced in 1960 as an anticancer agent and was designed to be activated by phosphoramidase to bis(chloroethyl)amine (Struck, 1995; Sinhababu and Thakker, 1996). Thus, **29** may be one of the first prodrugs designed to improve the therapeutic index of a highly toxic therapeutic agent; however, its bioactivation was later found not to be as originally intended. CPA (**29**) is oxidized by hepatic CYP enzymes to 4-hydroxy-CPA (**32**), which is sequentially converted to aldophosphamide (**33**) and then to pharmacologically active phosphoramide mustard (**34**) plus acrolein (**35**) (Figure 7); **35** is believed to cause haemorrhagic cystitis. Alternatively, the aldehyde group in aldophosphamide is oxidized by aldehyde dehydrogenase to form inactive carboxyphosphamide (**36**). CYP-catalyzed oxidative de-chloroethylation of CPA leads to the release of chloroacetaldehyde (**37**), which is considered to be responsible for the neurotoxicity of CPA (Boddy and Yule, 2000, and references therein). The other members of this class of prodrugs, ifosfamide (**30**) and trofosfamide (**31**), differ from **29** in the structure of the phosphoramide mustard functionality (Rooseboom *et al.*, 2004) and are also activated via initial 4-hydroxylation followed by spontaneous ring opening and release of the corresponding phodphoramide mustards.

In vitro studies suggest that the pivotal 4-hydroxylation of CPA that ultimately leads to the release of the anticancer agent is predominantly carried out by CYP2B6 with a smaller contribution from CYP2A6, CYP2C9, and CYP3A4 (Chang *et al.*, 1993; Roy *et al.*, 1999). In contrast, the N-dechloroethylation reaction leading to the formation of neurotoxic chloroacetaldyhyde is predominantly carried out by CYP3A4 (Bohnestengel *et al.*, 1996; Ren *et al.*, 1997). Interestingly, the 4-hydroxylation leading to metabolic activation of ifosfamide is catalyzed predominantly by CYP3A4 rather than CYP2B6 (Chang *et al.*, 1993; Roy *et al.*, 1999). All of these CYP enzymes are expressed at the highest level in the liver. It is reasonable to expect that the cytotoxic but pharmacologically active metabolite phospharamide mustard (**34**) and its precursor (**32**), as well as **35** and **37**, the metabolites responsible for CPA toxicity, are produced primarily in the liver and, to a lesser extent, in tumor and other tissues. Thus, it is not surprising that cancer chemotherapy involving CPA and other members of this class is associated with significant systemic toxicity.

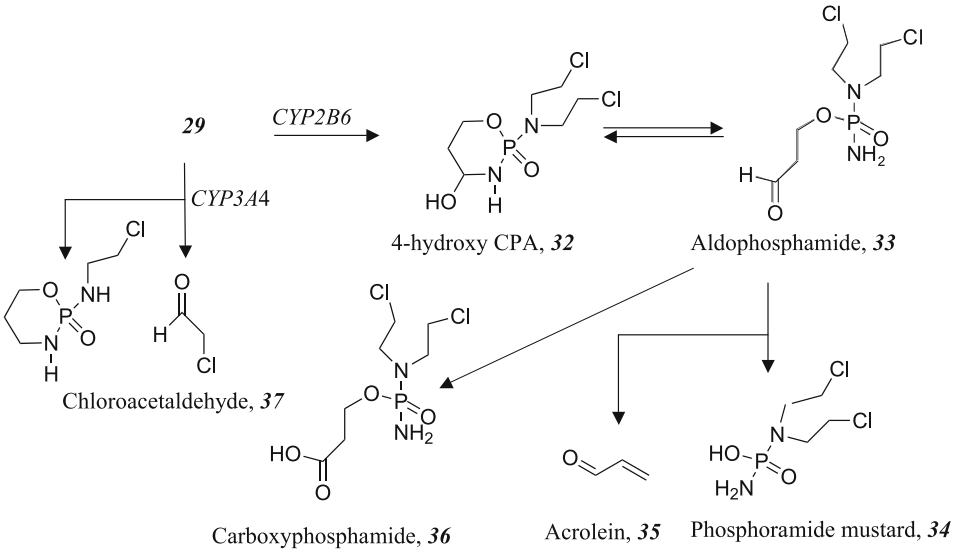


Figure 7. Activation of CPA (29) by CYP enzymes to the anticancer agent phosphoramide mustard (34) and to toxic metabolites acrolein (35) and chloroacetaldehyde (37). The reaction scheme is based on information reviewed in Boddy and Yule (2000), Rooseboom et al. (2004), and Sinhababu and Thakker (1996).

Pharmacokinetic studies revealed a high degree of inter-patient variability in the exposure to the parent drug and other pharmacokinetic parameters (Boddy and Yule, 2000 and references therein). A similar variation in the exposure to the active and toxic metabolites is expected. This is likely a consequence of the fact that CPA is predominantly activated and cleared by CYP-mediated metabolism. The CYP enzymes involved in the metabolism of CPA exhibit wide inter-individual variations, are subject to inhibition by co-administered drugs due to their broad substrate selectivity, and are inducible by co-administered drugs, all contributing to the variability in the pharmacokinetics of CPA and its metabolites (see section Cytochrome P450 (CYP) Enzymes as a Target for Prodrug Design). CPA (**29**) metabolism is also subject to auto-induction, resulting in increased metabolic clearance and shorter half-life following repeated administration at 24-h intervals (Boddy and Yule, 2000, and references therein). CPA (**29**) exhibits dose-dependent metabolism such that the relative formation of the inactive metabolite increases and formation of the active metabolite decreases at higher doses. Thus, efficacy of this agent may not increase proportionately to the increase in the dose. A large number of drug interactions have been observed between **29** and a variety of compounds such as allopurinol, chloramphenicol, chlorpromazine, fluconazole, predenisolone, and thiotepa (Boddy and Yule, 2000 and references therein); most of these interactions are presumably caused by inhibition of CYP-mediated metabolic activation and deactivation of **29**. Compounds such as dexamethasone and phenytoin induce metabolism of **29**.

Directed Enzyme Prodrug Therapy

Several approaches have been developed to achieve selective activation of prodrugs in tumors that involve delivering an exogenous enzyme to the tumor tissue and systemically administering non-toxic prodrugs that would be selectively converted to the active drug by the exogenously administered enzyme in the tumor tissue. The driving force behind this approach is the assumption that, by selecting a target enzyme for prodrug design that is not present in the mammalian tissues (*e.g.*,viral or bacterial enzyme) and delivering it to the tumor tissue, one can achieve ultimate selectivity in that the prodrug would not be converted to the active drug in any normal tissues. The target enzyme is delivered to the tumor tissue as a conjugate with tumor-specific antibody by the technique known as ADEPT. Alternatively, the gene encoding for the prodrug-activating exogenous enzyme is packaged in a viral vector (VDEPT) or non-viral vector (GDEPT) and delivered to the tumor tissue where the target enzyme would be expressed for selective conversion of prodrugs designed to be activated by this enzyme. These approaches for tumor-selective activation of prodrugs have been reviewed previously (Sinhababu and Thakker, 1996; Denny and Wilson, 1998; Napier *et al.*, 2000; Ettmayer *et al.*, 2004; Rooseboom *et al.*, 2004; Riddick *et al.*, 2005).

Each of these approaches for delivering the prodrug-activating exogenous enzyme to the tumor tissue faces formidable challenges that should not be underestimated. In ADEPT, immunogenicity of the enzyme-antibody conjugate may severely limit the ability of the complex to reach the target tissue. Even if it does reach the target, its diffusion into the tissue through the intercellular matrix is extremely slow due to its large size. Finally, in many cases, the prodrug activation needs to occur inside the cell and, if so, the enzyme-antibody complex needs to enter the cell once it reaches the tumor tissue. The rates at which the enzyme-antibody complex can be internalized are often too slow to achieve sufficiently high intracellular activity of the target enzyme. GDEPT and VDEPT approaches do not face many of the problems encountered in the ADEPT approach because both these methods focus on delivering the gene that encodes the exogenous target enzyme rather than the protein. However, approaches involving viral and non-viral gene delivery to the target tissue are not without difficulties. Efficient and effective target-specific delivery upon systemic administration of genes by either of these tactics is an objective that is far from accomplished. Our knowledge of ADME behavior of large proteins and genes is rudimentary and, thus, any prodrug approach that relies on efficient and selective delivery of these types of molecules to tumor tissues should be considered experimental at this stage.

Drug-induced Resistance to Chemotherapy

Induction of resistance to therapy in response to treatment of cancer with chemotherapeutic agents is a well-recognized phenomenon. Several underlying cellular mechanisms that lead to the development of the resistance have been postulated; these include increased efflux, reduced drug activation or increased

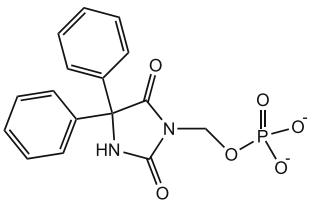
inactivation, increased repair of drug-induced damage of nucleic acid targets, and alteration in apoptopic response (Moscow *et al.*, 2003, Riddick *et al.*, 2005). Among these, some mechanisms, such as increased efflux by upregulation of efflux transporters and increased phase II metabolism to detoxify drugs/prodrugs, induce resistance via modulating the cellular disposition/pharmacokinetics of chemotherapeutic agents. These mechanisms are often considered in explaining the failure of cancer chemotherapeutic agents. It is important to consider whether prodrugs designed to enhance the quality of anticancer chemotherapy become targets for such mechanisms.

Prodrugs for Improved Solubility of the Drug

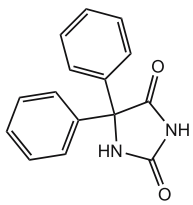
Poor aqueous solubility can often prevent a drug from achieving its full therapeutic potential. For orally administered drugs, poor solubility in the intestinal contents would result in poor absorption across the intestinal epithelium because a sufficiently high concentration gradient between the apical and serosal sides of the intestinal epithelium to drive absorptive transport is not achieved. There are numerous formulation approaches to improve the solubility and, thus, absorption of orally administered drugs (Yalkowsky, 1981). Prodrugs are also used to improve aqueous solubility and oral absorption of poorly soluble drugs by incorporating ionizable groups into the prodrug design (Fleisher *et al.*, 1996). However, incorporation of ionizable functional groups into the prodrug to improve aqueous solubility creates a molecule that is likely to poorly permeate the cellular membrane of intestinal epithelial cells. To address this, targeting the prodrug reconversion sites at the intestinal mucosal membrane is sought in the design of prodrugs to improve oral absorption (Fleisher *et al.*, 1996). Thus, high solubility of the prodrug will achieve high concentration at the interface of intestinal fluid and epithelial cell membrane. Rapid conversion of the polar prodrug by an enzyme at the brush border can accomplish the release of the lipophilic drug at the interface, leading to its rapid partitioning into the apical membrane. Clearly, the enzymes targeted for such a prodrug strategy must be present in the apical cell membrane of the enterocytes in such a way that their active sites are accessible to the substrate present in the intestinal fluid; alkaline phosphatase is an example of an enzyme that is highly expressed in the intestinal brush border membrane. An alternative approach involves designing prodrugs that are substrates for absorptive transporters present in the apical membrane of the intestinal epithelial cells. For example, amino acid or peptide derivatives of the insoluble drugs can facilitate absorption via the amino acid transporters or the peptide transporter pepT1. Valacyclovir (**3**) (Beauchamp *et al.*, 1992; de Miranda and Burnette, 1994) and famciclovir (**9**) (Vere Hodge *et al.*, 1989) are excellent examples of marketed prodrugs that are absorbed across the intestinal epithelium via the pepT1 transporter (Ganapathy *et al.*, 1998; Han *et al.*, 1998). Interestingly, these are amino acid esters of nucleoside analogs and, therefore, one would expect them to be substrates for an amino acid transporters; however, they turned out to be substrates for pepT1 (Ganapathy *et al.*, 1998; Han *et al.*, 1998). It is important

to recognize that absorption of prodrugs that are substrates for absorptive transporters in the intestinal epithelium is likely to be dose-dependent (saturable) and subject to interactions with co-administered drugs as well as food. Furthermore, there is a higher probability of inter-subject variability in the absorption of such prodrugs.

Prodrugs are also designed to improve aqueous solubility for parenteral administration. The first successful application of a prodrug strategy to improve aqueous solubility of a poorly soluble drug was demonstrated by Stella and co-workers (Stella, 1996b) by designing fosphenytoin (**38**), a phosphate ester of the sparingly soluble weakly acidic drug phenytoin (**39**). Fosphenytoin is rapidly hydrolyzed by alkaline phosphatases in the liver and intestine, and the hydroymethyl intermediate is then spontaneously decomposed to generate phenytoin (Stella, 1996b, and references therein). Thus, plasma concentration vs. time profiles of phenytoin after intravenous infusion of phenytoin or equivalent dose of fosphenytoin are virtually superimposable (Stella, 1996b). For a prodrug designed to improve aqueous solubility of a drug, this represents an ideal scenario because exposure to the prodrug is minimized. In the case of **38**, the release of formaldehyde as a consequence of the degradation of the hydroxymethyl intermediate may raise safety concerns; the broad issues of the exposure to formaldehyde released from phosphomethyl or aceotoxymethyl prodrugs need to be addressed (Rooseboom *et al.*, 2004, and references therein).



Fosphenytoin, ***38***

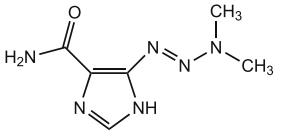


Phentoin, ***39***

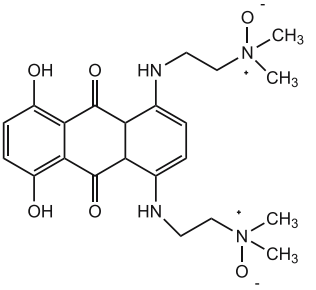
Structures 38-39.

Cytochrome P450 (CYP) Enzymes as a Target for Prodrug Design

CPA (**29**) is perhaps the best known prodrug that is activated to the active drug by CYP enzymes, although it is worth noting that this prodrug was not designed to be activated by CYP enzymes. For an enzyme family that is perhaps the most important class of drug metabolizing enzymes, CYP enzymes appear to be rarely used as target enzymes for activation of prodrugs. Some examples of prodrugs that are activated by CYP enzymes (Roseboom *et al.*, 2004) are ftorafur (**25**) (prodrug of **24**), dacarbazine (**40**) (a prodrug that is activated by CYP enzymes leading to the release of aminoimidazole carboxamide, nitrogen, and CH_3^+), oxazaphosphorines (**29**, **30**, **31**) (prodrugs that are activated by CYP enzymes to oxazaphosphorine mustards), and AQ4N (**41**) (a prodrug that is activated by CYP



Dacarbazine, ***40***



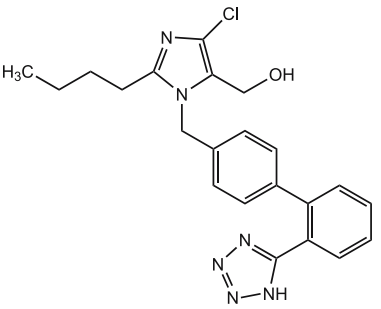
AQ4N, ***41***

Structures 40-41.

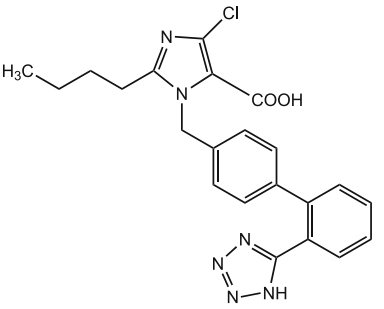
enzymes by reduction of N-oxide functionalities). Interestingly, tumor-selective CYP gene delivery has been conceived as an approach to improve the therapeutic efficacy of **29** (Riddick *et al.*, 2005). This approach is designed to achieve high levels of expression of CYP2B6, the CYP enzyme predominantly responsible for activation of **29** to the active drug with anti-tumor activity, in the tumor tissue by selective delivery of CYP2B6 gene to the tumor by several alternative approaches (Riddick *et al.*, 2005, and references therein). This approach has yielded promising clinical outcomes in recent phase I/II gene therapy trials, in which treatment involving **30** cells expressing CYP2B1 and **29** retroviral delivery of CYP2B6 gene, yielded positive clinical outcomes in pancreatic carcinoma patients (Lohr *et al.*, 2002) and breast cancer patients (Riddick *et al.*, 2005), respectively.

Losartan: A Case Study for Drug-drug Interactions and Genetic Polymorphism Associated with a CYP-activated Prodrug

The antihypertensive drug losartan (**42**) is oxidized by CYP enzymes to the highly potent antgiotensin II antagonist EXP3174 (**43**) that is responsible for most of losartan's pharmacological activity (Lo *et al.*, 1995, and references therein). Losartan is not a prodrug in the strict sense of the word because it is an angiotensin II antagonist with its own pharmacological activity profile. However, examination of pharmacokinetic/pharmacodynamic profiles of **42** and **43** provides excellent insights into the problems associated with prodrugs that are activated by CYP enzymes. Conversion of **42** to **43** is mediated by CYP3A4 and CYP2C9. The oral bioavailability of **42** is approximately 32%, with most of the remaining dose being metabolized in the liver during absorption (first-pass metabolism). Approximately 14% of the dose is converted to **43**. Thus, there is significant systemic exposure to both the parent drug **42** and the active metabolite **43**. In fact, the plasma concentration-time profiles show that the metabolite **43** is present at higher concentration than the parent drug after the first one or two hours of dosing, despite the fact that only 14% of the dose is converted to **43**. This is because the clearance of the metabolite is approximately 1/10th that of the parent. While the parent drug **42** is mainly eliminated via non-renal mechanisms, **43** is eliminated by both renal and non-renal mechanisms.



Losartan, ***42***



EXP3174, ***43***

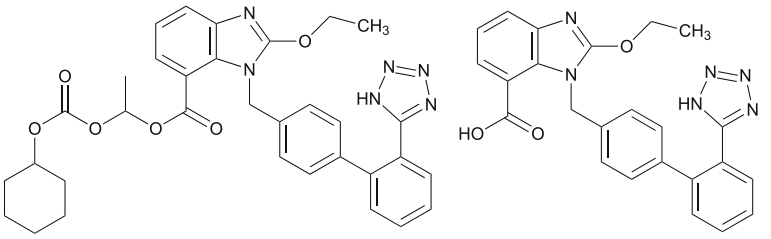
Structures 42-43.

These results highlight the fact that the parent drug and its metabolite may have distinctly different pharmacokinetic profiles, including metabolic fates and elimination routes. For a prodrug that is activated via a CYP enzyme, incomplete conversion to the active drug, as is seen with **42**, should be anticipated because of relative low turnover of substrates by CYP enzymes, for example, in comparison to esterases. This would require that analysis and ADME studies of the prodrug and the active drug be conducted during the preclinical and clinical development of such a prodrug.

Another important consequence of CYP-mediated activation of a prodrug is the high probability of drug-drug interactions because of likely inhibition or induction of CYP enzymes by a large number of drugs and xenobiotics. Losartan (**42**) again provides an excellent case study. Through *in vitro* studies, it has been shown that **42** is oxidatively metabolized predominantly by CYP2C9 and to a lesser extent by CYP3A4 (Sterns *et al.*, 1995; Yasar *et al.*, 2001). Inter-individual variability of CYP2C9 is as high as tenfold, primarily due to two single nucleotide polymorphisms (SNPs), CYP2C9*2 and CYP2C9*3, each resulting in one amino acid substitution. The CYP2C9*2 and CYP2C9*3 enzymes exhibit reduced maximum velocity and intrinsic clearance compared to the native enzyme CYP2C9*1 toward several substrates. The CYP2C9*1/*2 (8–12.5%) and CYP2C9*1/*3 (3–8.5%) genotype occur at a significant frequency in Caucasian populations (Miners and Birkett, 1998). In a clinical study conducted in healthy volunteers to examine interactions between losartan (**42**) and phenytoin (**39**) (Fischer *et al.*, 2002), there were two CYP2C9*1/*2 genotypes out of a total of 16 subjects. Interestingly, baseline AUC (0–24 h) for **42** was threefold higher in the two CYP2C9*1/*2 subjects compared to the mean AUC (0–24 h) in the remaining 14 CYP2C9*1/*1 subjects. Co-administration with phenytoin caused mean AUC (0–2 h) for **42** to increase by 29% in CYP2C9*1/*1 subjects, perhaps due to inhibition of CYP2C9-mediated metabolism of **42**. In contrast, mean AUC (0–24 h) for **42** decreased in the two CYP2C9*1/*2 subjects. The cause for the decreased concentration of **42** in CYP2C9*1/*2 subjects was speculated to be induction of the enzyme; however, this hypothesis was not confirmed by further *in vivo* or *in vitro* experiments.

Tirkkonen and Laine (2004) have investigated the frequency of potential drug-drug interactions involving three prodrugs, losartan, codeine, and tramadol, that are converted to the active drug by CYP enzymes. Losartan is converted to the active drug by CYP2C9 whereas codeine and tramadol are converted to the active drug by CYP2D6. The drug-drug interaction potential was assessed by determining the frequency of co-administration of these prodrugs with known CYP2C9 or CYP2D6 substrates/inhibitors. The results showed that co-administration of losartan and a CYP2C9 inhibitor was found in 19.4% of all losartan treatment periods, and co-administration of a CYP2D6 inhibitor with codeine was found in 21.3% of codeine treatment periods and 20.3% of tramadol treatment periods. Overall, 1999 patients (19%) were prescribed co-administration of medications that could lead to drug-drug interactions over the 6-year study period. These results highlight drug-drug interactions as a major issue with prodrugs that depend on activation by CYP enzymes.

These examples underscore the complexity in the fate of a drug that is predominantly metabolized by one or more CYP enzymes. Thus, a prodrug that is designed to be activated by CYP-mediated metabolism would likely have a fairly complex pharmacokinetic/pharmacodynamic profile of activation to the active drug, and clearance of both the prodrug and the active drug. Such a prodrug would necessarily have a more involved and complex drug development program. Interestingly, another successful angiotensin II receptor antagonist candesartan cilexetil (**44**) is an ester prodrug in which the pharmacologically critical carboxyl functionality is unmasked by ester hydrolysis rather than CYP-mediated oxidative metabolism (Burnier, 2001 and references therein). The ester prodrug **44** is completely and rapidly converted to the active drug (**45**) during gastrointestinal absorption (Burnier, 2001), thus yielding a much simpler pharmacokinetic/pharmacodynamic profile than that of losartan.



Candesartan cilexetil, ***44*** Candesartan, ***45***

Structures 44-45.

The case history of losartan (**42**) has highlighted the potential difficulties associated with prodrugs that are activated by CYP enzymes. Thus, the reasons for relatively limited use of CYP as target enzymes for prodrug activation are many; these include variable expression among individuals based on genetic, physiological, and environmental factors, wide distribution in the body that does not allow organ/tissue-specific activation of prodrugs, rapid first-pass metabolism of prodrugs in the liver (even when it is not desired), relatively low turnover numbers

for most substrates resulting in slow release of the active drug, and inhibition or induction of CYP enzymes by a large number of xenobiotics that can lead to drug interactions.

Conclusion

Since the inception of the concept in late 1950s separately by Albert and Harper, the simple concept has mushroomed into a major area of research effort both in academic laboratories and in pharmaceutical and biotechnology companies. A large number of papers published each year report novel prodrugs or new applications of existing prodrug approaches for improving drug delivery. The prodrug design ranges from a simple esterification of alcohol or carboxylic acid functionality to a highly complex system involving genes encoding exogenous enzymes engineered with tissue-specific promoters and packaged in viral delivery systems. The prodrug literature has been examined in several excellent reviews including the most recent reviews cited in this chapter. The major purpose of this review is to examine the prodrug field from the vantage point of a drug metabolism scientist/pharmacokineticist and ask these questions: Are most prodrugs designed with a good understanding of the ADME properties of the prodrug, the active drug, and, in some instances, the promoiety? Is the selection of target enzymes; *i.e.*, the enzyme that would reconvert the prodrug to the active drug, carefully considered in designing most prodrugs so that the conversion of the prodrug to the active drug occurs in the right compartment and at the right rate? Are complexities associated with the target enzyme, *i.e.*, multiplicity, intra- and inter-subject variability, inter-species differences, potential for adverse effects or failure of therapy due to drug-drug interactions or genetic polymorphism, considered when prodrugs are designed? Are appropriate *in vitro* and *in vivo* systems used for assessment and screening of prodrugs with due consideration of the target-enzyme related issues? Unfortunately, the answers to most of these questions would be "no" or "maybe." The reason why it is important to raise these questions is very simple. Prodrugs should be and are taking their rightful place as an integral component of drug discovery and development strategy and are no longer considered an ad hoc troubleshooting tool. If one accepts this premise, then it necessarily follows that prodrug design is not an academic exercise and that prodrugs should be designed to yield a successful therapeutic agent with acceptable efficacy and adverse effect profile and viable means to deliver it to the patient.

Several classes of prodrugs were chosen for discussion in this chapter to aid in the discussion of above issues. There are prodrugs on the market representing some categories of prodrugs discussed here, clearly indicating that these prodrug strategies can produce useful therapeutic agents. However, it should be apparent from the discussion of several important classes of prodrugs that, despite published examples of highly sophisticated prodrug design, this field is in its infancy with respect to rationally designed prodrugs that can meet the challenges of drug development hurdles to produce therapeutically useful agents.

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4.1.2

Formulation Challenges of Prodrugs

Robert G. Strickley and Reza Oliyai

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Introduction

This chapter aims to review and highlight some examples of challenges and limitations of formulating prodrugs for clinical and commercial development. The reader should note that the existing literature on the formulation and pharmaceutical development of prodrugs is somewhat limited as pharmaceutical companies tend to not publish pharmaceutical development data. While the prodrug strategy has been used to overcome various drug delivery issues, it tends to present various formulation challenges. These challenges arise from various physicochemical properties of the prodrug as compared to the parent drug such as:

- Lack of chemical stability, often the drug-promoiety linker
- Potential for the formation of degradation by-products that are reactive intermediates resulting in secondary degradation pathways
- Aqueous solubility
- Aqueous solubility of degradation products
- Disruption of solid state crystallinity
- Polymorphism

The choice of formulation for a given prodrug is governed by multiple factors including the intended route of administration, aqueous solubility, chemical stability, permeability, and the clinically desired release profile. The potential formulation and pharmaceutical development issues must be considered and carefully evaluated in the design and selection of prodrugs for clinical and commercial development. Chemical stability of a prodrug in some cases limits the usefulness of a specific class of molecules. By their nature, many prodrugs tend to be more chemically unstable than the parent drug. Therefore, prodrugs must possess adequate chemically stability to allow for development of commercially viable dosage form. For example, Safadi and co-workers described the development of phophoryloxymethyl carbonate as novel water soluble prodrugs for hindered alcohols. The lack of adequate chemical stability limited the commercial and clinical potential of this prodrug concept (Safadi *et al.*, 1993). Chemical stability and formulation issues were also considered in the selection of a tenofovir prodrug for clinical and commercial development (Arimilli *et al.*, 1997; Shaw *et al.*, 1997).

The limitation in shelf-life due to chemical stability can be due to the loss of intact prodrug, which is normally limited to 5–10% over a two year period, or to the growth of an undesired or toxic degradation product whose acceptable concentration is established on a case-by-case basis. The primary decomposition pathway typically involves the chemical degradation of the prodrug at the promoiety functional group. The most common chemical reaction of a prodrug is hydrolysis, but other reactions also occur such as cyclization and dimerization. Methods to minimize the primary degradation of prodrugs include minimizing the amount of water in the dosage form, modifying the formulation composition to create a stable environment surrounding the prodrug, use of a non-aqueous

solution formulation, lyophilization for a parenteral formulation, minimize the fraction of prodrug solubilized in a suspension, and reduced storage temperature.

Secondary decomposition pathways may also occur as the result of formation of reactive intermediates such as formaldehyde or mix anhydrides. This may involve the chemical reaction of a degradation by-product(s), which is formed during primary degradation, with the intact prodrug or degradation products, (Yuan *et al.*, 2000). Alternatively, the reactive intermediates such as formaldehyde can react with gelatin capsule shells leading to cross-linking of the capsule shell and retardation of the *in vitro* dissolution profile (Digenis *et al.*, 1994).

Typically, the chemical stability of the prodrug as a function of solution pH (*i.e.*, a pH-rate profile) is studied to determine the solution pH of maximum stability, and these solution studies can guide the solid-state formulation development. However, the formulation scientist must be aware that solid-state degradation kinetics may or may not be predicted by the solution pH-rate profile, because chemical reactions in solid-state are more complex than in a homogenous solution. Although the effect of solution pH on drug stability is well documented with accepted theoretical models, the effect of solid-state 'pH' is not well documented or understood. The definition of solid-state 'pH', in a lyophile or in a solid-state formulation is unclear and there is no methodology to directly measure solid-state pH (there is no solid-state pH electrode). Formulation scientists are well aware that adding an acidic or a basic excipient into a solid-state formulation can very well affect chemical stability. However, formulation development using pH modifying excipients is largely empirical, and guided by actual solid-state stability studies as a function of temperature, humidity and water content (*i.e.*, levels of desiccation).

Solid-state chemical stability is a complex manifestation of many factors including not only easily measurable macroscopic factors such as water content, temperature and relative amounts of prodrug and excipients, but also non-measurable microscopic factors such as microenvironment solid-state 'pH', favorably oriented nearest neighbors and reactive functional groups, contact points between solid prodrug and excipient, molecular mobility of water, prodrug and other reactive molecules, and the inherent non-homogeneity of a solid-matrix on the molecular scale. Published studies on solid-state stability of prodrugs (Badawy *et al.*, 1999; Badawy *et al.*, 1999; Gu *et al.*, 1990; Strickley *et al.*, 1989; Yuan *et al.*, 2000) clearly show that bimolecular chemical reactions can indeed occur in solids even when limited water is available and that there really is a solid-state 'pH' effect.

In addition, oral prodrugs should have favorable solubility and dissolution profile in the gastrointestinal tract to facilitate oral absorption. Solubility can also be a challenge if the prodrug is formulated in a liquid dosage form since the prodrug and any degradation products must be soluble over the lifetime of the product prior to administration. Physical stability challenges in prodrug formulations include maintaining solubility of the prodrug and all of the degradation products in liquid formulations, and integrity of the capsule shell if used in either a solid or liquid oral formulation due to generation of a reactive

degradation product(s). For example, fosamprenavir, a water-soluble phosphate ester prodrug, was developed to increase the aqueous solubility of amprenavir. Amprenavir has an aqueous solubility of only 0.04 mg/mL and is formulated for adult use in soft gelatin capsules (SGC) containing 150 mg of drug along with d-alpha tocopheryl polyethylene glycol (TPGS), polyethylene glycol 400 (PEG 400), and propylene glycol. The aqueous solubility of fosamprenavir (calcium salt) is said to be > 0.3 mg/mL and it is formulated in a tablet dosage form equivalent to 600 mg of amprenavir. Thus, dosing becomes more convenient at two tablets twice-a-day and blood levels of amprenavir are comparable to those from the less convenient amprenavir soft gelatin capsules formulation.

Oral Formulations

Most prodrugs for oral administration are designed for improved oral bioavailability over the active moiety by increasing solubility and/or permeability. Oral prodrugs can also be designed to decrease gastric irritation, or to target release of the active in the colon. Normally the preferred oral formulation is a solid dosage form such as a tablet or capsule. The challenges in developing a tablet or capsule include optimizing not only the formulation by proper selection and proportions of excipients, but also the manufacturing process such as a wet or a dry granulation, as well as the packaging configuration such as desiccation to minimized water content and to maximize solid-state stability.

Solid-state Stability: Effect of Water

Any molecule that is susceptible to hydrolysis in aqueous solution is also susceptible to hydrolysis in the solid-state. The rate of hydrolysis in the solid-state is usually, but not always, slower than in aqueous solution under similar conditions. The factors that affect the rate of solid-state hydrolysis include the amount of water, the activity and distribution of the water molecules, the availability of water molecules from the environment (*i.e.*, humidity), the location of water molecules relative to the reactants reactive functional group(s), the temperature, and pH (solid-state 'pH' is discussed later). The challenges for the formulation scientist in developing a prodrug that is susceptible to hydrolysis is to either minimize the content and availability of water, and/or to create a stabilizing solid-state microenvironment surrounding and contacting the prodrug particles. Also, to minimize the degradation of a water sensitive prodrug it may be advantageous to manufacture using a water-free process such as either a dry granulation using roller compaction or a wet granulation using as the granulation media a non-aqueous solvent such as an alcohol.

To minimize the content and availability of water in order to improve solid-state chemical stability some prodrugs tablets are packaged with a desiccant to limit the rate of water-assisted degradation. For example, tenofovir disoproxil fumarate (Viread® and Truvada™) and adefovir dipivoxil (Hepsera®) are susceptible to hydrolysis and are packaged with a desiccant to ensure a two-year

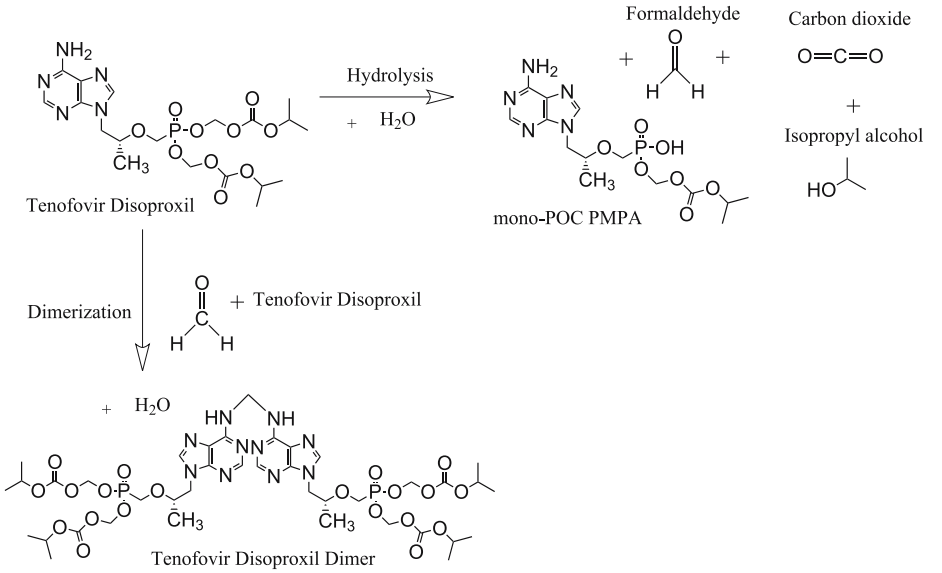


Figure 1. Degradation scheme for tenofovir disoproxil (Yuan *et al.*, 2001).

shelf-life at room temperature. The hydrolysis of both tenofovir disoproxil and adefovir dipivoxil is rather complex. The hydrolysis of tenofovir disoproxil generates a molecule of isopropyl alcohol, carbon dioxide and formaldehyde that can also further react with another molecule of tenofovir disoproxil to form a formaldehyde-mediated covalent dimer (Yuan *et al.*, 2001) (Figure 1). The hydrolysis of adefovir dipivoxil generates a molecule of pivalic acid and formaldehyde that can also further react with another molecule of adefovir

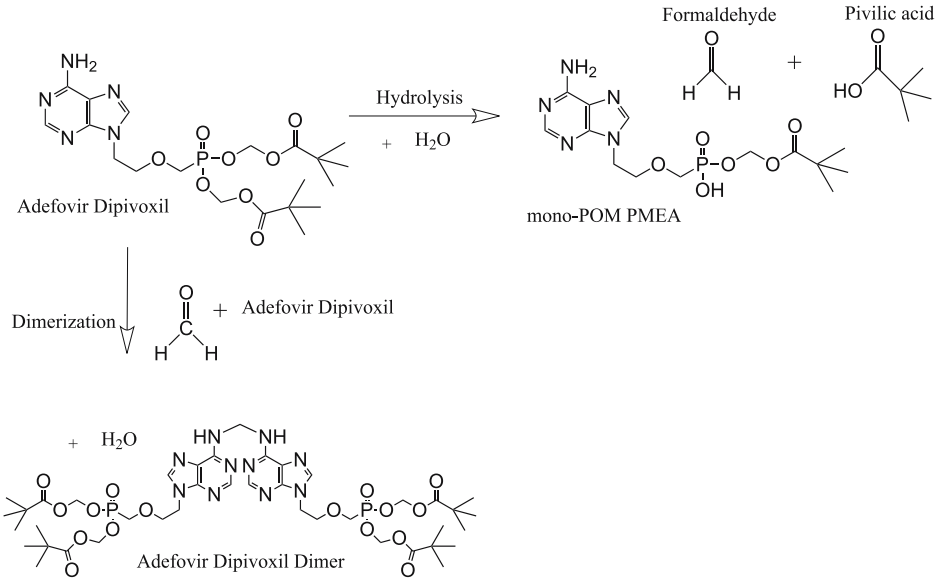


Figure 2. Degradation scheme for adefovir dipivoxil (Yuan *et al.*, 2000).

dipivoxil to form a formaldehyde-mediated covalent dimer (Yuan *et al.*, 2000) (Figure 2).

The ethyl ester prodrugs benazepril hydrochloride (Lotensin®, Lotensin HCT® and Lotrel®) and enalapril (Vaseretic®) are also packaged with a desiccant. Enalapril is susceptible to both ester hydrolysis and intramolecular cyclization to a diketopiperazine (Figure 3).

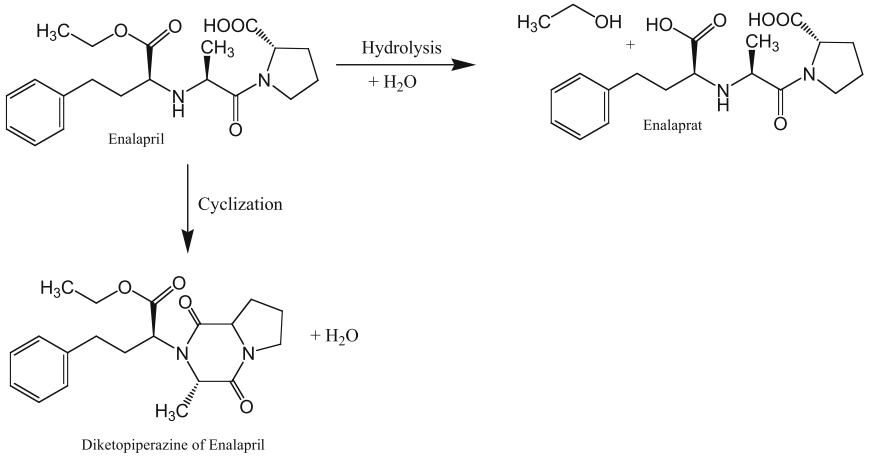


Figure 3. Degradation scheme for enalapril, which is packaged with a desiccant to limit the rate of ester hydrolysis and intramolecular cyclization.

Solid-state Stability: Effect of 'pH'

The solid-solid 'pH' may be conceptualized as the relative amounts of ionized and un-ionized species, and has been shown to play a critical role in the solid-state stability of many compounds. The microenvironment surrounding a solid particle can be influenced by pH-modifying excipients, and creating a uniform solid-state microenvironmental 'pH' can be challenging. The two examples that follow of solid-state 'pH' (DMP 754 and Moexipril) have in common the importance of identifying both a quantitative formulation and a manufacturing process that evenly distributes all of the ingredients, provide a temporary aqueous medium for proton transfer, and ultimately creates a solid-state microenvironment in which the prodrug is chemically stabilized.

The control of the microenvironment 'pH' assisted in optimizing the solid-state stability of DMP 754, which is a methyl ester prodrug of a platelet IIb/IIIa glycoprotein receptor antagonist (Badawy *et al.*, 1999; Badawy *et al.*, 1999). In the solid-state DMP 754 is susceptible to both ester hydrolysis to form the carboxylic acid, and also amidine hydrolysis to form the amide (Figure 4). In solid-state, significant degradation of DMP 754 occurred in the presence of different excipients, but anhydrous lactose caused the lowest rate of degradation, and the rate was proportional to the excipient/drug ratio. In solution the only observed route of degradation is ester hydrolysis and the maximum stability was in pH 4

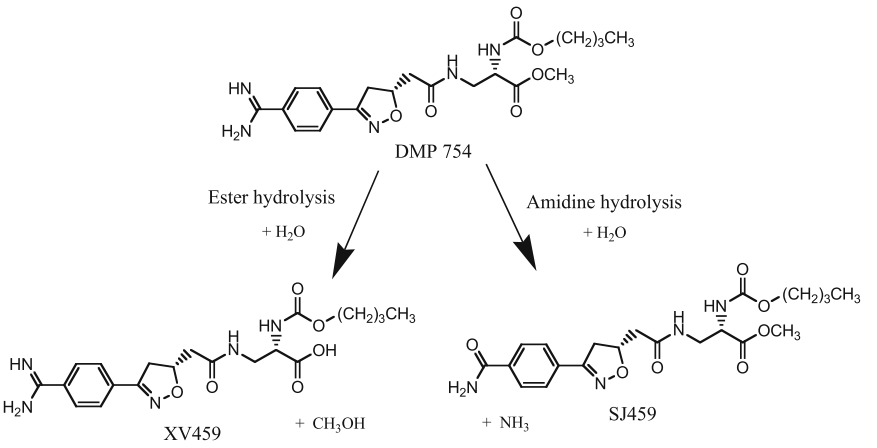


Figure 4. Degradation scheme for DMP 754, whose overall solid-state stability in the presence of lactose was minimized by controlling the micro-environment pH to 4 by evenly distributing disodium citrate as the granulation solution during wet granulation. (Badawy *et al.*, 1999; Badawy *et al.*, 1999).

buffer solutions. DMP 754 is the acetate salt and a saturated aqueous solution has a pH of 6.8, and a saturated solution of lactose has a pH of 6. Therefore, the formulation scientists reasoned that a lactose/DMP 754 solid-state microenvironment 'pH' would be near the pH range of 6–6.8, and that solid-state stabilization could be achieved by lowering the solid-state microenvironment pH to 4, which is the pH of maximum stability in aqueous solution. After carefully studying many acidic excipients the rates of solid-state ester hydrolysis and amidine hydrolysis were minimized when using acids that had saturated solution pH values of 4.6 and < 2.5, respectively. The pH-modifying excipient agent chosen was disodium citrate since it was able to reduce the rate of both ester and amidine hydrolysis, and its saturated solution pH is 4.6.

The manufacturing process also had an affect on the solid-state stability of DMP 754 (Badawy *et al.*, 1999). The formulation with disodium citrate was more stable after a wet granulation than after a dry blend or a dry granulation.. The authors state "the higher stability of capsules manufactured by wet granulation may be explained by the more uniform distribution of citrate in this formulation." and in this case the disodium citrate was added in the granulation solution and "was found to be more diffuse in the wet granulation samples compared to localized distribution in the dry granulation" (Badawy *et al.*, 1999; Ennis *et al.*, 1997) . Interestingly, the dry blends (97.17% anhydrous lactose, 2.5% disodium citrate, and 0.33% DMP 754) were less stable as compressed tablets compared to the bulk powder or to powder-filled capsules, and the authors attribute this to an increase in the number of contact points between lactose and DMP 754. However, after the wet granulation, compression did not result in destabilization as did the dry blends. The authors also demonstrate that ester and amidine hydrolysis are affected differently by the varying manufacturing processes, in that the rate of

ester hydrolysis was increased but amidine hydrolysis decreased dry granulation compared to a dry blend. The authors also examined the effect of manufacturing process on the water content and hygroscopicity, and found that the wet granulated material had higher water content and was more hygroscopic than the dry granulated material. However, the authors concluded that "the microenvironment pH control in the case of the wet granulation formulation was probably able to compensate for the increased hygroscopicity, resulting in a more stable dosage form than the less hygroscopic dry granulation formulation, which lacked effective pH control" (Badawy *et al.*, 1999).

Moexipril (formerly RS-10085) is an ethyl ester prodrug of the active carboxylic diacid moexiprilat (formerly RS-10029), which is an ACE inhibitor, and is marketed as a tablet formulation as Univasc® and in a fixed dose combination with hydroclorothiazide as Uniteric®. Moexipril is a chirally pure dipeptide analog with an internal secondary amine and a terminal carboxylic acid and a terminal ethyl ester. The degradation pathways include epimerization, unimolecular intramolecular cyclization to a diketopiperazine, and bimolecular ester hydrolysis (Figure 5) (Gu and Strickley, 1987; Gu *et al.*, 1990; Strickley *et al.*, 1989). The degradation kinetics (Figure 6) and product distribution (Figure 7) are pH-dependent in both solution and in the solid-state. The formulation challenges with moexipril include: 1) identifying a manufacturing process that results in chemically stable tablets; 2) minimizing water content to stabilize the ethyl ester; and 3) to choose the proper solid-state 'pH' to stabilize against intramolecular cyclization.

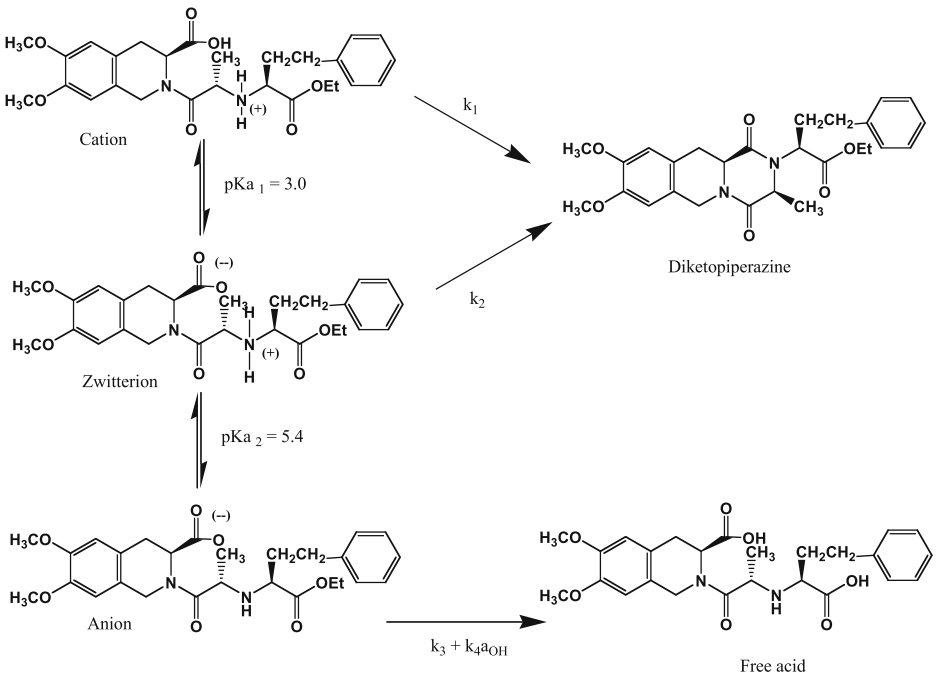


Figure 5. Degradation scheme for RS-10085 (Moexipril) via ester hydrolysis to a free acid, and via intramolecular cyclization to a diketopiperazine. (Strickley *et al.*, 1989).

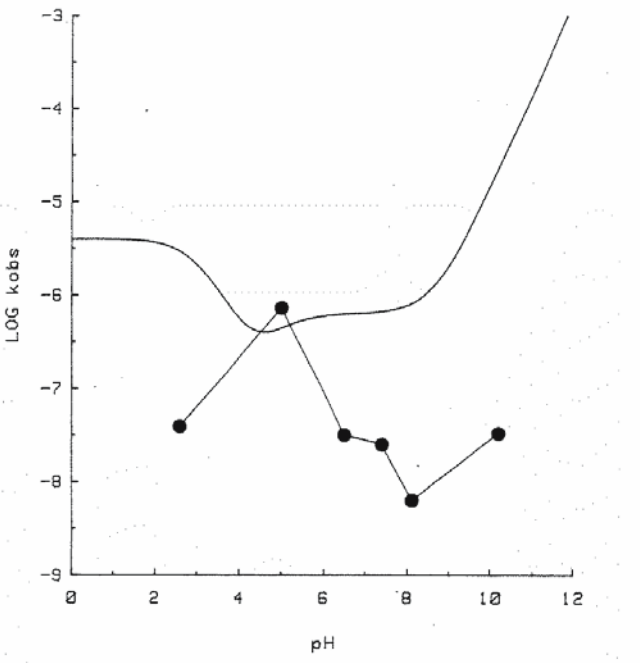


Figure 6. pH-rate profiles of RS-10085 (Moexipril) in solid-state (line segment with data points) and solution (solid curve) at 50°C. In solution the maximum stability was at pH 4.5, but in solid-state was 'pH' 8.0 (Strickley *et al.*, 1989).

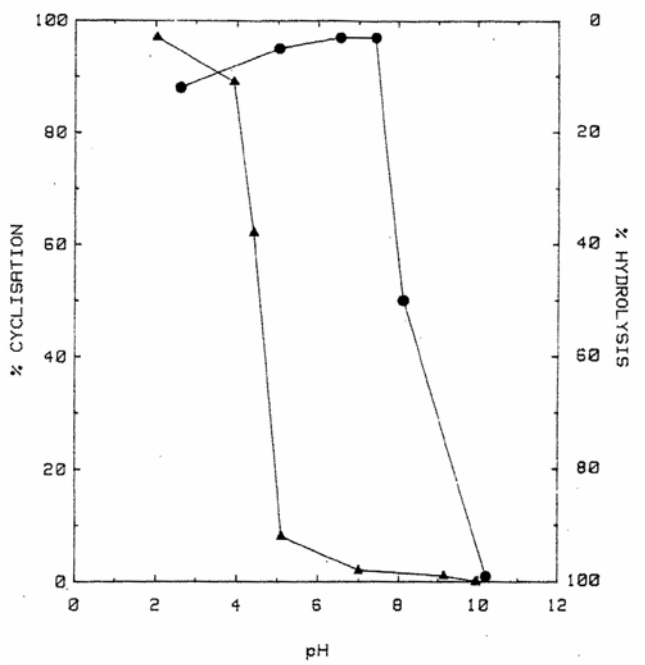


Figure 7. Product distribution profiles of RS-10085 (Moexipril) in solid-state (circles) and solution (triangles) at 50°C (Strickley *et al.*, 1989)

				Degradation products	
Excipient	Wet or Dry[a]	% Relative Humidity	% Remaining moexipril	% Cyclization (preferred at lower pH)	% Hydrolysis (preferred at higher pH)
Ascorbic acid	Dry	50	91.2	8.6	0
	Wet	50	76.2	18.7	4.4
	Dry	Desiccated	98.0	2.1	0
	Wet	Desiccated	98.7	~ 1	0
Sodium bicarbonate	Dry	50	61.2	24.9	4.9
	Wet	50	98.6	0.5	0.4
	Dry	Desiccated	70.9	26.2	0
	Wet	Desiccated	99.0	2.2	0.8
Sodium carbonate	Dry	50	25.8	70.1	2.4
	Wet	50	97.9	< 1	2.2
	Dry	Desiccated	78.2	21.7	0
	Wet	Desiccated	99.1	0.4	0.4
Calcium carbonate	Dry	50	42.2	43.2	5.7
	Wet	50	91.3	4.8	3.9
	Dry	Desiccated	91.2	14.3	0
	Wet	Desiccated	91.1	7.6	1.4

Table 1. Stability results of wet granulated and dry powder mixes of (5/45/50) moexipril/lactose/excipients after exposure to 60°C and 50% relative humidity or desiccated for 13 days. (Gu *et al*., 1990).

[a]Wet granulation or a dry powder mix.

It was shown that in the presence of slightly basic excipients (sodium bicarbonate, sodium carbonate, and calcium carbonate), moexipril had rapid solid-state degradation when manufactured via a dry blend process, but was much more chemically stable when manufactured via a wet granulation process (Gu *et al.*, 1990), Table 1. The authors concluded that 'via wet granulation, alkalizing agents were found to be effective in stabilizing moexipril hydrochloride in the solid state. Supported by the product distribution profile, the stabilization is postulated to result from the neutralization of the acidic drug by basic excipients at the outer surface of the granulated material" (Gu *et al.*, 1990). The pH-dependent chemical stability of moexipril is aqueous solution is quite different from that in solid-state, such that in solution maximum stability is observed at pH 4.5, but in the solid-

state 'pH' 4.5 is the least stable and maximum stability is observed in 'pH' 8 solid-state (lyophilized powders), Figure 6. This suggests that during the wet granulation process in the presence of slightly basic excipients the solid-state microenvironment 'pH' shifted to a region where moexipril is more chemically stable.

An example of a prodrug that is formulated in the solid-state with a pH-modifying excipient is temozolomide, which is stable in acidic solution (pH <5) but labile at pH > 7, and is formulated as a solid with tartaric acid then filled into hard gelatin capsules. Presumably tartaric acid is added to the temozolomide formulation as a stabilizing agent.

These five examples on solid-state stability adefovir dipivoxil, tenofovir disoproxil fumarate, enalapril, moexipril and DMP 754 illustrate the importance of detailed preformulation characterization to assist in the rational and scientific approach to solid-state formulation development of a prodrug that has potential chemically stability issues.

Oral Solutions

Oral solutions are commonly used as commercially available pediatric formulations. Oral solutions are also commonly used in preclinical *in vivo* pharmacokinetic studies for oral dosing via gavage. The formulation challenges for oral solutions for pediatric use include taste, preservative efficacy, drug solubility, and chemical stability (Ansel *et al.*, 1999) The formulation challenges for oral solutions for preclinical *in vivo* pharmacokinetic are similar to those for parenterals (see below) and include solubility, short-term chemical stability and *in vivo* compatibility.

The only prodrug commercially available as an oral solution is prednisolone phosphate sodium, which is a water soluble phosphate ester prodrug of prednisolone and is available in at least two oral solution formulations for pediatric administration (Physician's Desk Reference, 2005). Pediapred® contains 1 mg/mL equivalent of prednisolone in an aqueous solution composed of dibasic sodium phosphate, EDTA, methylparaben, sodium biphosphate, sorbitol, and raspberry flavor. Orapred® contains 3 mg/mL equivalent of prednisolone in an aqueous solution composed of ethyl alcohol 2%, fructose, glycerin, monoammonium glycyrrhizinate, povidone, sodium benzoate, sorbitol, and grape flavor. The oral solutions produce a 14% higher peak plasma levels of prednisolone, which also occur 20% faster than the peak observed with tablets. The recommended storage condition for Orapred® oral solution is refrigeration at 2–8°C, and for Pediapred® oral solution is 4–25°C but may also be refrigerated.

Liquid Filled Capsules

The formulation challenges for a prodrug in a liquid filled capsule include chemical stability, and solubility of the prodrug in order to dissolve the dose in the limited volume of one capsule, ~ 0.7 cm^3 per capsule. Solvents include simple

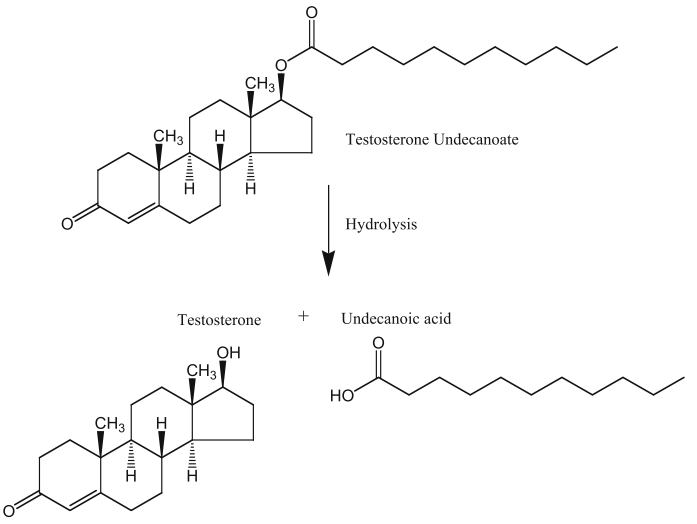


Figure 8. Testosterone undecanoate is an ester prodrug of testosterone and is solubilized in oleic acid in 40 mg Restandol 40 mg soft gelatin capsules. The capsules are stored refrigerated by the wholesaler, but at room temperature by the patient.

organic solvents to complex self-emulsifying systems (Strickley, 2004). Only a few prodrugs are commercially available in liquid filled capsules, and one can only surmise that the reason is limited solubility and/or chemical stability. In two of the three examples that follow, the prodrug is an oily liquid that prevented the development of a solid oral dosage form, and thus a solubilized formulation was developed.

Testosterone is used in hormone replacement therapy and for oral delivery the undecanoate ester prodrug is employed, while in intramuscular parenteral applications various testosterone ester prodrugs (*i.e.*, cypionate, and enanthate) or testosterone itself is used and dissolved in sesame oil (Delatestryl®) or arachris oil (Sustanon). Testosterone is inactive orally due to first-pass liver metabolism, but testosterone undecanoate is able to by-pass the liver via the lymphatic system and is thus the orally active form of testosterone for hormone replacement therapy (**http://emc.medicines.org.uk**). Testosterone undecanoate is solubilized in oleic acid in Restandol 40 mg soft gelatin capsules. The oral dose of testosterone undecanoate is 40–160 mg HSE once daily, which is 1–4 Restandol 40 mg capsules. The testosterone undecanoate capsules are stored refrigerated by the wholesaler, but at room temperature by the patient. These required storage conditions are presumably due to limited chemical stability of testosterone undecanoate in the solubilized formulation at room temperature during extended storage. Restandol has been available in the United Kingdom since 1981.

Indometacin farnesil is an ester prodrug of indometacin, and is used as an anti-inflammatory and analgesic agent. Indometacin farnesil is an oily liquid that is practically insoluble in water, and is solubilized to a viscous liquid in a mixture

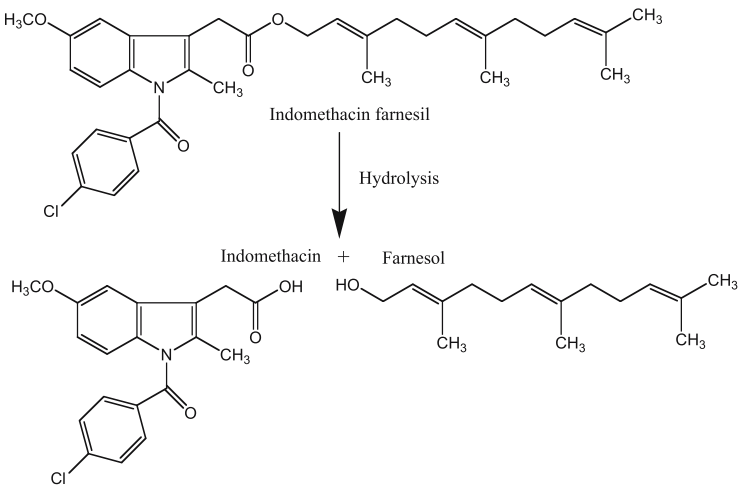


Figure 9. Indometacin farnesil is an ester prodrug of the active moiety indometacin, and is solubilized to a viscous liquid in a mixture of cremophor RH 60, hydrogenated oil, and glyceryl monooleate in 200 mg Infree® soft gelatin capsules.

of cremophor RH 60, hydrogenated oil, and glyceryl monooleate in 200 mg Infree® soft gelatin capsules (**http://www.e-search.ne.jp/~jpr/jpr_db/eindex.html**). The oral bioavailability of indometacin farnesil is decreased when administered to fasted patients, but is well absorbed following an ordinary meal with 10 grams of fat. The dose of indometacin farnesil is 200 mg, which is one capsule, twice-a-day. The capsules are to be stored at room temperature. Indometacin farnesil has been available in the Japan since 1991.

Tocopherol nicotinate is the nicotine ester of α-tocopherol, which is used in the treatment of hypertension, hyperlipidemia and peripheral circulatory disturbances. Tocopherol nicotinate is an orange-yellow liquid or solid that is practically insoluble in water. Tocopherol nicotinate is solubilized to a viscous suspension or semisolid in a mixture of medium-chain triglycerides, glycol esters of fatty acids, along with aspartic acid in 200 mg Juvela®N soft gelatin capsules (**http://www.e-search.ne.jp/~jpr/jpr_db/eindex.html)**. The oral bioavailability of unchanged tocopherol nicotinate increased 30-fold after a meal compared to fasting. The dose of tocopherol nicotinate is 200 mg, which is one capsule, three times daily. The capsules are to be stored at room temperature. Tocopherol nicotinate has been available in Japan since 1984.

Parenteral Formulations

Parenteral formulation challenges of prodrugs include solubility, chemical stability, and physical stability. The goal is to obtain the desired concentration of the prodrug in a pharmaceutically acceptable parenteral formulation in which the prodrug has acceptable stability, and the formulation has acceptable physical stability. The definition of acceptable stability depends on the stage of preclinical,

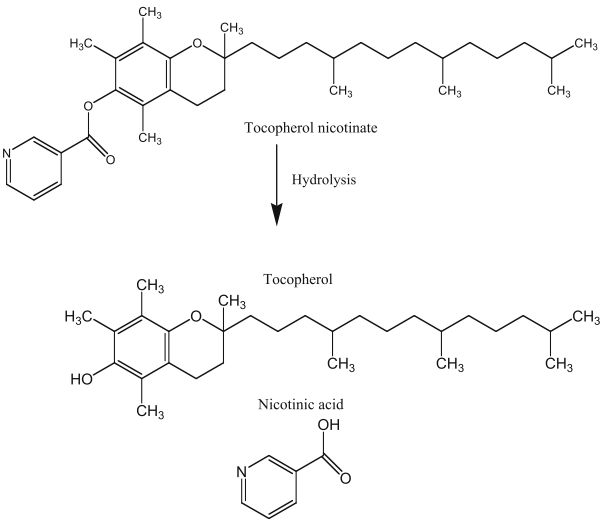


Figure 10. Tocopherol nicotinate is the nicotine ester of α-tocopherol, and is solubilized to a viscous suspension or semisolid in a mixture of medium-chain triglycerides, glycol esters of fatty acids, along with aspartic acid in 200 mg Juvela®N soft gelatin capsules.

clinical or commercial development and is typically a shelf-life (5–10% degradation) of days, months or years, respectively.

Prodrugs that are specifically intended for parenteral administration are rationally designed chemically for a desired physiochemical property such as improved water-solubility for rapid-release upon injection, decreased water-solubility for extended release after subcutaneous or intramuscular injection, increased lipid solubility for formulation optimization, or increased chemical stability. Table 2 is a listing of selected commercially available prodrugs for parenteral administration, which are available in many different formulation types: ready-to-use aqueous solutions, ready-to-use non-aqueous solutions, concentrated solutions for dilution, ready-to-use suspensions, and lyophilized powders for reconstitution (Strickley, 1999).

Solubility Enhancement

Many techniques and excipients are used to enhance solubility of drugs for parenteral administration via injection. Excipients include acids/bases, water-soluble organic solvents, surfactants, phospholipids, complexing agents, water insoluble solvents such as long-chain triglycerides (Strickley, 2004) and tocopherol derivatives, (Constantinides *et al.*, 2004).

The combination of pH adjustment and cosolvent is a common method to achieve a proper balance between solubility enhancement and chemical stability. For example, moexipril has maximum chemical stability in aqueous solution at pH 4.5 with a shelf-life of 2 months, but can be formulated to achieve a two year shelf-life in a concentrated solution of 90% ethanol and 10% aqueous buffer pH 8.0, which is diluted prior to injection (Gu and Strickley, 1990).

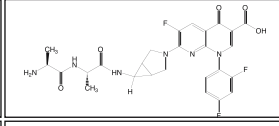
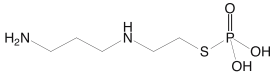
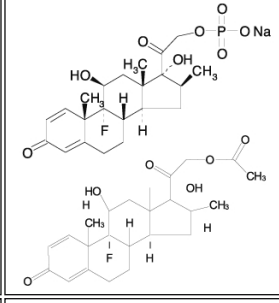
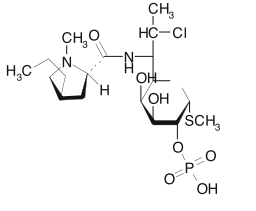
Drug Name/ Marketed Name	Structure	Formulation	Type of prodrug	Preadministration preparation	Route of Administration
Alatrofloxacin mesylate/ Trovan		**Solution** 5 mg/mL pH 3.4–4.3	Amide	Dilute to 1–2 mg/mL with 5% dextrose	IV infusion over 60 min
Amifostine/ Ethyol		**Lyophilized powder** 500 mg	Phosphorylated thiol	Reconstitute with saline to 50 mg/mL (stable at room temperature for 5 h). May be further diluted to 5 mg/mL with saline.	IV infusion over 15–30 min
Betamethasone Phosphate sodium and Betamethasone Acetate Celestone soluspan		**Suspension** Betamethasone Phosphate sodium 3 mg/mL, Betamethasone Acetate 3 mg/mL, Sodium phosphate dibasic 7.1 mg/mL Sodium phosphate monobasic 3.4 mg/mL, EDTA 0.1 mg/mL, Benzalkonium chloride 0.2 mg/mL pH 6.8–7.2	Water soluble phosphate ester and Water insoluble acetate ester	None	IM
Clindamycin Phosphate/ Cleocin phosphate		**1) Solution** 150 mg/mL, EDTA 0.5 mg/mL, Benzyl alcohol 9.4 mg/mL pH = 5–6. **2)** Ready to use solution 0.5–18 mg/mL Dextrose 5%, EDTA 0.04 mg/mL. pH 5.0–7.5 under nitrogen	Water soluble phosphate ester	Dilute concentrated solution with saline or lactated Ringer's to = 18 mg/mL.	IV infusion at 30 mg/h

Table 2. Selected List of Prodrugs for Parenteral Administration (Strickley, 1999).

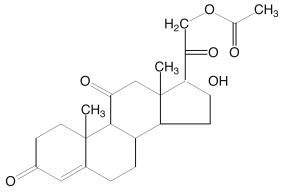
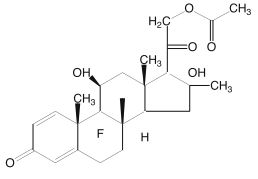
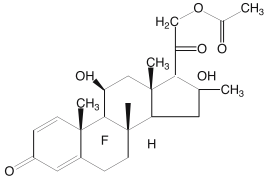
Drug Name/ Marketed Name	Structure	Formulation	Type of prodrug	Preadministration preparation	Route of Administration
Cortisone Acetate/ Cortone		**Suspension** 50 mg/mL, Sodium carboxymethylcellulose 5 mg/mL, TWEEN 80 at 4 mg/mL, Sodium chloride 9 mg/mL, Benzyl alcohol 9 mg/mL	Water insoluble acetate ester	None	IM only
Dexamethasone Acetate/ Decadron-LA		**Suspension** 8 mg/mL TWEEN 80 at 0.75 mg/mL, Sodium chloride 6.7 mg/mL, Creatinine 5 mg/mL, EDTA 0.5 mg/mL, Benzyl alcohol 9 mg/mL, Sodium bisulfite 1 mg/mL pH 5.0–7.5	Water insoluble acetate ester	Gentle swirl contents to resuspend settled particles.	IM/ Intralesional/ Intra-articular/ Soft tissue
Dexamethasone Acetate/ Dalalone D.P.		**Suspension** 16 mg/mL Sodium carboxymethylcellulose 5 mg/mL TWEEN 80 at 0.75 mg/mL Sodium chloride 6.7 mg/mL, Creatinine 5 mg/mL, EDTA 0.5 mg/mL, Benzyl alcohol 9 mg/mL, Sodium bisulfite 1 mg/mL pH 5.0–7.5	Water insoluble acetate ester	Gentle swirl contents to resuspend settled particles.	IM// Intra-articular/ Soft tissue (Not intralesional)

Table 2 (continued). Selected List of Prodrugs for Parenteral Administration (Strickley, 1999).

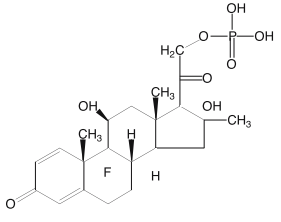
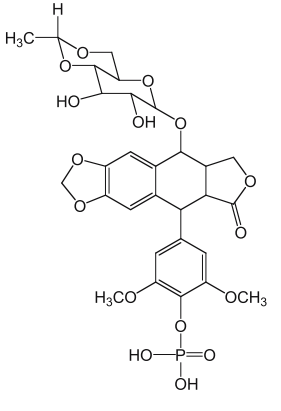
Drug Name/ Marketed Name	Structure	Formulation	Type of prodrug	Preadministration preparation	Route of Administration
Dexamethasone Phosphate sodium/ Decadron		**Solution** 4 and 24 mg/mL w/wo Lidocaine 10 mg/mL, Creatinine 8 mg/mL, Sodium citrate 10 mg/mL, Methylparaben 1.5 mg/mL, Propylparaben 0.2 mg/mL, Sodium bisulfite 1 mg/mL pH 5.0–7.5 under nitrogen	Water soluble phosphate ester	For IV infusion dilute with saline or dextrose 5%.	IV bolus/ IV infusion/ IM/ Intralesional/ Intra-articular/ Soft tissue
Etoposide Phosphate/ Etopophos		**Lyophilized powder** 100–1000 mg, Sodium citrate 32–327 mg/mL, Dextran 40 at 300–3000 mg	Water soluble phosphate ester	Reconstitute with WFI, saline or dextrose 5% to 10–20 mg/mL which is further diluted with saline or dextrose 5% to 0.2–0.4 mg/mL.	IV infusion over 30–60 min

Table 2 (continued). Selected List of Prodrugs for Parenteral Administration (Strickley, 1999).

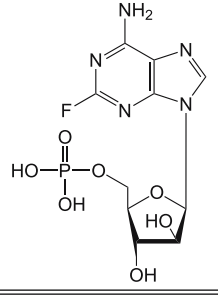
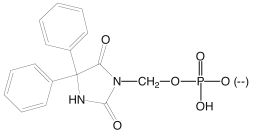
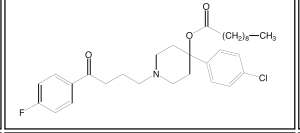
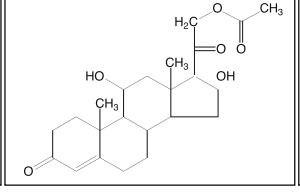
Drug Name/ Marketed Name	Structure	Formulation	Type of prodrug	Preadministration preparation	Route of Administration
Fludarabine Phosphate		**Lyophilized powder** 50 mg, Mannitol 50 mg pH 7–8	Water soluble phosphate ester	Reconstitute with 2 mL WFI to 25 mg/mL then further diluted with 100–125 mL saline or dextrose 5% to ~ 0.5 mg/mL.	IV infusion over 30 min
Fos-phenytoin/ Cerebyx	 Calcium salt	**Solution** 75 mg/mL Tromethamine, pH = 8.6–9.0	Water soluble hydroxy-methyl phosphate ester	None for IM. For IV infusion dilute with saline or dextrose 5% to 1.5– 25 mg PE/mL.	IM/ IV infusion at = 150 PE/minute
Haloperidol Decanoate/ Haldol decanoate		**Non-aqueous solution** 50–100 mg/mL in **Sesame Oil** Benzyl alcohol 1.2%	Water insoluble deconate ester	None	IM
Hydrocortisone Acetate/ Hydrocortone Acetate		**Suspension** 50 mg/mL TWEEN 80 at 4 mg/mL, Sodium carboxymethylcellulose 5 mg/mL, Sodium chloride 9 mg/mL, Benzyl alcohol 9 mg/mL	Water insoluble acetate ester	None	IM/ Intralesional/ Intra-articular

Table 2 (continued). Selected List of Prodrugs for Parenteral Administration (Strickley, 1999).

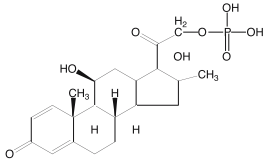
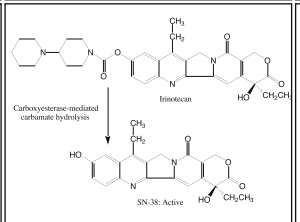
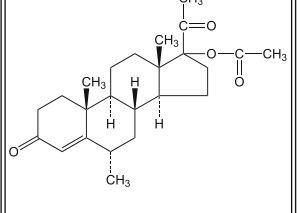
Drug Name/ Marketed Name	Structure	Formulation	Type of prodrug	Preadministration preparation	Route of Administration
Hydrocortisone Phosphate sodium/ Hydrocortone Phosphate		**Solution** 50 mg/mL Creatinine 8 mg/mL, Sodium citrate 10 mg/mL, Sodium bisulfite 3.2 mg/mL, Methylparaben 1.5 mg/mL, Propylparaben 0.2 mg/mL, pH 7.5–8.5	Water soluble phosphate ester	None or dilute with saline or dextrose 5%.	SC/ IM/ IV bolus/ IV infusion
Irinotecan HCl/ Camptosar		**Solution** 20 mg/mL, Sorbitol 45 mg/mL, Lactic acid 0.9 mg/mL pH 3.0–3.8	Water soluble carbamate	Dilute with dextrose 5% or saline to 0.12–1.1 mg/mL.	IV infusion over 90 min
Medroxy-proges-terone Acetate/ Depo-Provera		**Suspension** 150–400 mg/mL, PEG 3350: 20–29 mg/mL, TWEEN 80 at 2.4 mg/mL, Sodium chloride: 8.7 mg/mL, Methylparaben: 1.4 mg/mL, Propylparaben: 0.15 mg/mL	Water insoluble acetate ester	None	IM once every 3 months

Table 2 (continued). Selected List of Prodrugs for Parenteral Administration (Strickley, 1999).

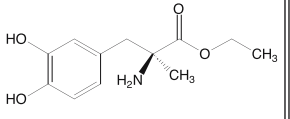
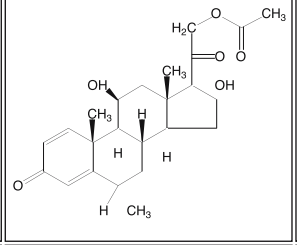
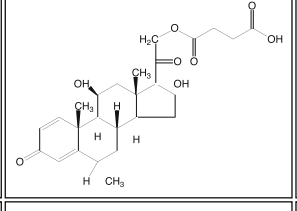
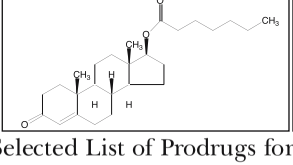
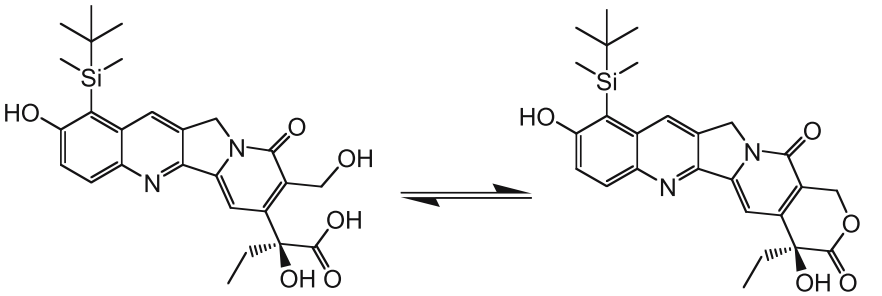
Drug Name/ Marketed Name	Structure	Formulation	Type of prodrug	Preadministration preparation	Route of Administration
Methyldopate HCl/ Aldomet Ester HCl		**Solution** 50 mg/mL Citric acid 5 mg/mL, Sodium bisulfite 3.2 mg/mL, onothioglycerol 2 mg/mL, EDTA 0.5 mg/mL, Methylparaben 1.5 mg/mL, ropylparaben 0.2 mg/mL pH 3–4.2	Ethyl ester	Dilute with dextrose 5% to 10 mg/mL.	IV infusion over 30–60 min
Methyl-prednisolone Acetate/ Depo-Medrol		**Suspension** 20–80 mg/mL PEG 3350 3%, TWEEN 80 at 2 mg/mL, Sodium phosphates 2 mg/mL, Benzyl alcohol 9 mg/mL, Sodium chloride (isotonic), pH 3.5–7.0	Water insoluble acetate ester	None	IM/ Intrasynovial/ Soft tissue or Intralesional
Methy-prednisolone Succinate sodium / Solu-Medrol		**Lyophilized powder** 40–2000 mg Sodium phosphates 18 mg/mL w/wo Lactose 25 mg/mL, Benzyl alcohol 9 mg/mL pH 7–8	Water soluble succinate ester	Reconstitute with WFI to 40–65 mg/mL. For IV infusion further dilute with saline or dextrose 5%.	IM/ IV bolus/ IV infusion
Testosterone Enanthate/ Delatestryl		**Non-aqueous solution** 200 mg/mL **Sesame oil,** Chlorobutanol 5 mg/mL	Water insoluble heptanate ester	None	IM

Table 2 (continued). Selected List of Prodrugs for Parenteral Administration (Strickley, 1999).

An interesting example of a prodrug that was formulated in a novel parenteral formulation is DB-67 (Figure 11), which is an experimental cancer drug, that can be isolated as either the inactive ring-opened carboxylate or the active water-insoluble lactone form. The parenteral formulation utilized complexation, pH adjustment, supersaturation and in-situ generation of the active lactone (Xiang and Anderson, 2002). Stable supersaturated solutions were obtained by mixing a concentrated alkaline aqueous solution of the DB-67 carboxylate with an acidified solution containing 22.2% (w/v) of the complexing agent sulfobutylether-β-cyclodextrin. Ring-closure yielded supersaturated solutions that could be lyophilized and reconstituted to clear, stable, supersaturated solutions.



DB-67 Carboxylate-form (favored at high pH) DB-67 Lactone-form (favored at low pH)

Figure 11. DB-67 is the inactive ring-opened carboxylate form of the active water-insoluble lactone. The parenteral formulation utilized complexation, pH adjustment, supersaturation and in-situ generation of the active lactone (Xiang and Anderson, 2002).

Water-soluble Prodrugs

The parenteral formulations of water soluble prodrugs are either solutions (ready-to-use, or concentrates) or lyophilized powders. The solution formulations are either ready to use solutions, or solutions to be diluted prior to administration. The solution formulations are usually fairly simple formulations and the ready-to-use formulations are made isotonic by sodium chloride, dextrose, sorbitol, or the prodrug itself. The solutions are usually buffered to the pH range of 3–9 with tromethamine, sodium citrate or lactic acid. The ready-to-use solution formulations can be administered as is by either IV bolus, subcutaneous, or intramuscular injection, or they can be diluted with intravenous fluids prior to infusion.

Water-soluble Prodrugs – Solubility Considerations

The shelf-life of a water soluble prodrug can often be limited by the solubility of a water insoluble degradation product. The shelf-life of the commercially available intramuscular and intravenous solution formulation of fosphenytoin,

Cerebyx®, is limited by this solubility criteria due to the formation of the water insoluble phenytoin, a primary degradation product, and the recommended storage condition is refrigerated at 2–8°C. However, the shelf-life limitation of fosphenytoin can be switched from solubility criteria to stability criteria, which is 0.5% production of phenytoin, and the storage temperature increased to 25°C by increasing the solubility of phenytoin. The 25°C shelf-life of fosphenytoin in 80 mg/mL solutions at pH 7.4 and 8.0 is limited to 0.9 and 2.0 years by phenytoin solubility, but upon addition of 60 mM of the complexing agent Captisol® ($(SBE)_{7M}$-β-CD) that forms a water soluble complex with phenytoin, the shelf-life becomes limited by stability criteria and is increased to 4.7 and 8.4 years, respectively (Narisawa and Stella, 1998) (Figure 12).

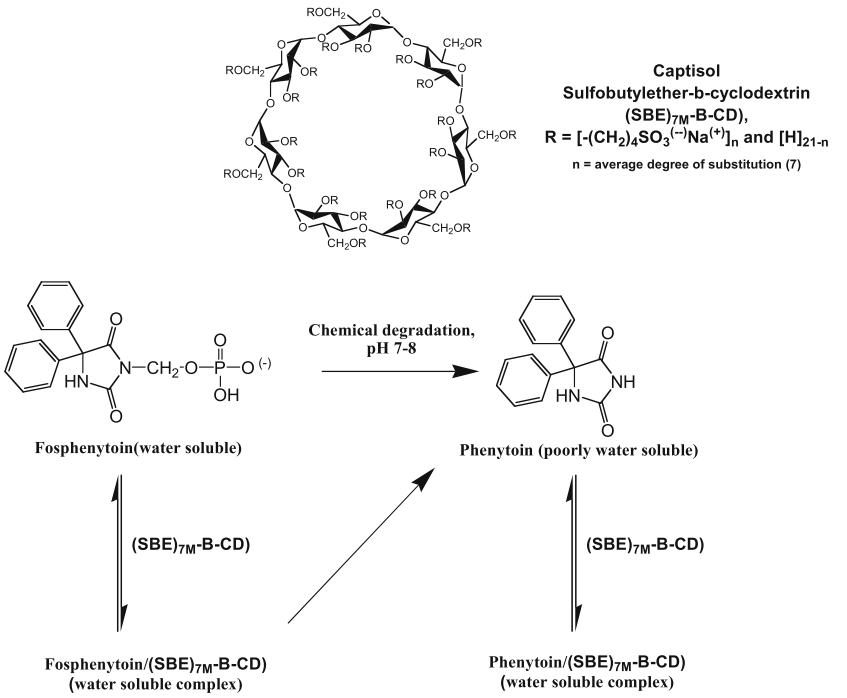


Figure 12. Fosphenytoin degrades to the porly water soluble phenytoin, which can precipitate thus limiting the shelf-life of the parenteral formulation. Solubilization of phenytoin by forming a water soluble complex with Captisol® ($(SBE)_{7M}$-β-CD) increases the shelf-life (Narisawa and Stella, 1998).

Water-soluble Prodrugs – Stability Considerations

Irinotecan is an antineoplastic agent and is a water-soluble prodrug of the lipophilic metabolite SN-38 (Figure 13). The prodrug converts to the active via carboxylesterase-mediated cleavage of the carbamate bond between the camptothecin moiety and the dipiperidino side chain. A formulation challenge for

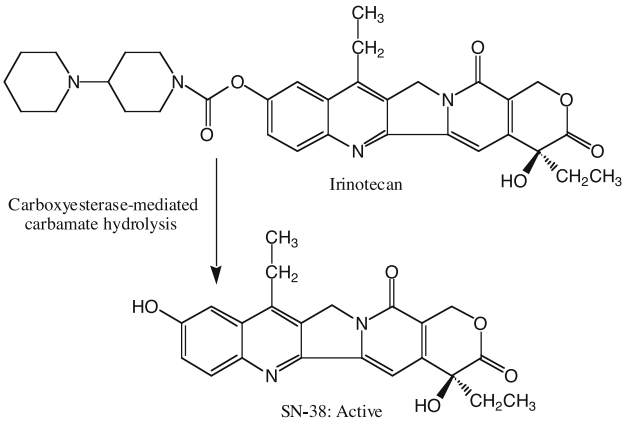


Figure 13. Irinotecan is a water-soluble prodrug of the lipophilic metabolite SN-38 and converts to the active via carboxylesterase-mediated cleavage of the carbamate bond between the camptothecin moiety and the dipiperidino side chain. The commercial parenteral product Camptosar® is a sterile isotonic (4.5% sorbitol) aqueous solution at pH 3.0 by lactic acid.

irinotecan is to prevent the pH-induced ring opening of the lactone to the less cell permeable carboxylate-form, and the commercial product Camptosar® is a sterile isotonic (4.5% sorbitol) solution at pH 3.0 by lactic acid.

Some water soluble prodrugs have inherent chemical stability issues and are formulated as lyophilized powders such as amifostine, etoposide phosphate, and fludarabine phosphate (Table 2).

Lipid-soluble Prodrugs

The prodrug approach can also been used to increase the lipid solubility of water-insoluble drugs for formulation optimization or improved pharmacokinetic properties (Versluis *et al.*, 1998). The formulation optimization idea and practice is to create a better drug delivery system for a water-insoluble drug that is difficult to formulate and/or deliver with standard techniques. The formulation challenges in this prodrug approach include identifying a suitable lipid-based formulation in which the prodrug is both soluble and chemically stable as well as physically stable (*i.e.*, drug leakage, or particle size increases of an emulsion) in order to ensure long term storage by minimizing chemical degradation and ensuring solubilization of the prodrug and any degradation products.

The antimicrotuble agent paclitaxel is widely used to treat various carcinomas, but is water insoluble and the commercially available generic parenteral formulation is a mixture of Cremophor EL and ethanol that is diluted with infusion fluids just prior to intravenous infusion, but the relatively high amounts of Cremophor EL delivered can cause clinical complications. Thus much research efforts have been devoted to discovery water-soluble (Lee *et al.*, 2002; Rose *et al.*,

1997; Wrasidlo *et al.*, 2002) or lipid-soluble prodrugs (Lundberg *et al.*, 2003; Stevens *et al.*, 2004) in order to better deliver paclitaxel.

One of the lipid-soluble prodrug of paclitaxel is paclitaxel oleate (Figure 14), which was formulated in an oil-in-water lipid emulsion (Lundberg *et al.*, 2003). The secondary alcohol on the side-chain of paclitaxel was esterified with oleic acid, which both reduced the water solubility from 12 μM for paclitaxel to 0.034 μM for the oleate ester, as well as increased the octanol/water partition coefficient, $K_{o/w}$, from 311 for paclitaxel to 8074 for the oleate ester. The emulsion is made by dispersing 120 nM paclitaxel oleate, 5.6 uM triolein (a triglyceride: the trioleate ester of glycerol), 6.4 μM egg phosphatidylcholine, 1.5 μM polysorbate 80, and 80–0.36 μM PEG 2000 modified phosphatidylethanolamine. This formulation is lyophilized, then reconstituted with PBS, heated to 45°C, vortexed, sonicated then filtered. The recovery of paclitaxel oleate was ~ 98%. A preclinical pharmacokinetic study in rabbits showed higher and more prolonged plasma concentrations as well as an altered distribution in the plasma protein pools when administered as the oleate prodrug compared to free paclitaxel formulated in Cremophor EL and ethanol.

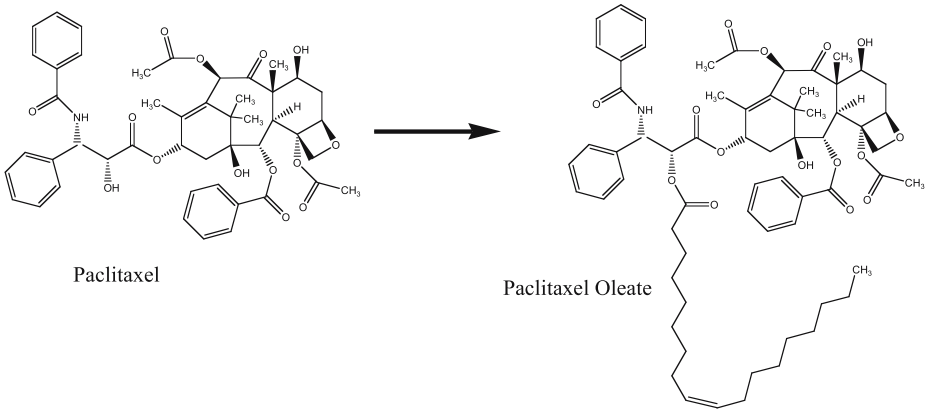


Figure 14. Paclitaxel oleate is a lipid-soluble prodrug of Paclitaxel and was formulated in an oil-in-water lipid emulsion (Lundberg *et al.*, 2003).

Lipid-soluble Prodrugs – Extended Release Solutions

Sesame oil formulations of oil soluble drugs provide a sustained release depot at the injection site that releases drug by diffusion-like uptake of oil. For example, the prodrugs haloperidol deconate and testosterone enanthate are formulated in 100% sesame oil and administered intramuscularly once-a-month. The formulation challenges include identifying a suitable oil with the proper prodrug solubility based upon the desired dose and the volume injected. Further challenges with an oil formulation include oxidation of the oil during storage, and also the presence of trace amounts of peroxides in the oil that can react with oxidizable function groups in the prodrug. Fortunately super-refined oils are commercially available, which minimize any issues with oxidation.

Water-insoluble Prodrugs – Extended Release Suspensions

Suspension formulations of water-insoluble prodrugs provide a sustained release in which the rate of release is determined by the rate of *in vivo* dissolution of the prodrug. Suspension formulations are normally aqueous-based and contain water-insoluble prodrugs that are lipophilic esters of alcohols. For example, hydrocortisone acetate and dexamethasone acetate are acetate esters of their alcohol-containing parent drug, and are administered intramuscularly, intralesionally or intra-articularly once every 1–3 weeks. The contraceptive medroxyprogesterone acetate is administered intramuscularly once every 13 weeks. Aqueous based suspensions typically contain TWEEN 80 at ~ 0.75–4 mg/mL (0.4%) along with a suspending agent such as sodium carboxymethylcellulose at ~ 5 mg/mL. The formulation challenges include chemical stability of the prodrug, which is determined by the total concentration of prodrug, the fraction of prodrug in the aqueous phase (*i.e.*, the solubility of the prodrug in the aqueous phase), and the rate of degradation in the solution phase. Other formulation challenges for a suspension include physical stability of the suspension in regards to particle settling, particle agglomeration, and the "syringability" meaning the ability to accurately withdraw the suspension into a syringe from the vial using the appropriate gauge of needle for injection.

Conclusion

The examples of formulation challenges for prodrugs in this chapter illustrate the importance of detailed preformulation characterization to assist in the rational and scientific approach to solid-state and solution formulation development of a prodrug that has potential chemical stability and/or solubility issues. Experimentally identifying the route(s) of degradation, the effect of pH, the effect of water content, and the effect of the manufacturing process on the chemical stability of the prodrug are quite useful in developing a stable dosage form and packaging configuration. In developing a liquid formulation of a prodrug, the chosen solvent system must be able to solubilize the prodrug and any degradation products that can be generated during manufacturing and storage. The concepts of microenvironment solid-state 'pH', molecular mobility, favorably oriented nearest neighbors, contact points between solid prodrug and excipient are all useful for the formulation scientist to keep in mind when interpreting observations and developing stable prodrugs dosage forms.

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4.2.1

Safety Assessment of Prodrugs

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Introduction

An important aspect of drug development is the overall safety assessment process. Safety assessment considers the range of potential risks that patients may encounter, through detailed studies of preclinical species. This process guides the course of clinical development and serves as an important consideration in the ultimate benefit/risk evaluation by the sponsor and international regulatory agencies. Safety assessment is increasingly vast in scale and scope. Thus, for the purposes of this chapter, no attempt will be made to teach the process of safety assessment. The purpose of this chapter is to familiarize the reader with the various aspects of prodrugs requiring special consideration during this critical part of drug development.

The process of safety assessment can be viewed in many ways. Often, the overall process is described as a series of steps designed to support important clinical or regulatory milestones. However, for the purposes of this chapter, safety assessment will be described in the context of the work needed to be done to justify registration of a new molecular entity (NME). In order, these steps can be described as 1) risk identification, 2) risk evaluation, 3) safety assessment, 4) benefit/risk analysis, and 5) registration.

This chapter will focus primarily on the special issues prodrugs present in the overall process of safety assessment. It should be recognized at the outset that prodrugs present more facets to be considered than do conventional small molecules. Thus, safety assessment will be more complex, with more factors that need to be considered. It is also likely that these extra considerations will add greater scope, and, hence, effort and cost, to the safety assessment process. However, some facets of prodrugs will facilitate safety assessment, and these opportunities should be recognized and seized when possible. Finally, and most importantly, it must be remembered that those facets of a prodrug that add complexity are also those that will likely make the difference between a successful drug and one that otherwise would fail. Given the high cost of drug development and the unfortunately low rate of drug success, an appropriately designed prodrug will often well warrant the added costs of complexity.

Risk Identification

The purpose of risk identification is to consider and define all manner of adverse effects that could possibly occur as a result of exposure to the NME. For the most part, this evaluation primarily considers risks to the patient. However, exposure during manufacturing, packaging, prescribing, and medical administration also needs to be considered. The risk assessment for these functions is somewhat different than for patient safety and, therefore, will not be addressed in this chapter.

It must be emphasized that risk identification should be considered separately from risk evaluation. During the course of risk identification, it is often necessary to administer very high multiples of the intended clinical dose. This is done to

allow a consideration of all possible adverse effects that might be encountered, regardless of exposure. The subsequent process of risk evaluation and safety assessment will begin to parse out those effects most likely to be encountered in the course of medical treatment.

Exposure to Drug Substance(s)

Attainment of high exposures in preclinical test species frequently is problematic in drug development. Most often, dissolution–rate-limited absorption is the basis for low exposures. Inability to attain adequate exposure in safety assessment studies can greatly limit the ability to conduct a robust assessment of risk in a facile manner. Consequently, the solubility limitations of a prospective drug present additional complications to the ordinary development plan that may add both cost and delay. In this regard, prodrugs can often greatly facilitate risk identification.

Prodrugs that increase aqueous solubility can dramatically increase systemic exposure for an otherwise solubility-challenged drug (Figure 1). In the case presented, substantially higher drug levels were achieved after administration of the prodrug. The high exposures achieved, along with the nicely linear dose dependency of exposure, allowed easy identification of adverse effects at high doses as well as clear no effect levels.

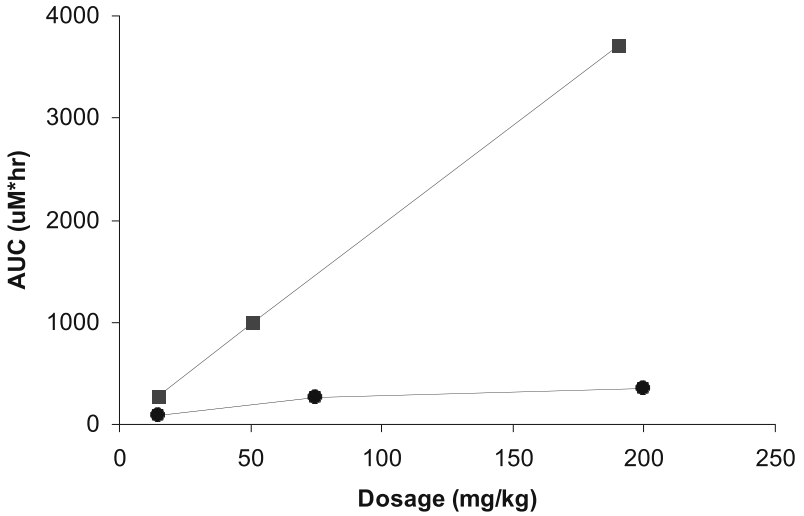

Figure 1. Overcoming solubility-limited absorption via prodrugs. Male rats were dosed with various doses of either compound "A" (circles) or its phosphoamidate prodrug (squares). Plasma concentrations of compound "A" were measured over time, and the integrated area under the curve was calculated. The exposure from prodrug was increased tenfold at the highest dose tested and remained linear over the dose range.

It should be noted that the same properties that allow substantially greater systemic drug exposure can also, indirectly, present some surprises. In many cases, a prodrug may be a follow-on to a previously studied molecule. In these cases, it is not unusual to observe an unremarkable initial course of safety assessment. In large part, the lack of observations may be ascribed to relatively low exposure due to absorption limitations. Subsequent study of equimolar doses of prodrug can lead to a startling array of adverse effects that had not been observed previously. However, risk evaluation incorporating toxicokinetics can reveal attainment of understandably high exposure levels.

Prodrugs that enable facile membrane permeation to an otherwise membrane impermeant drug will often allow adequate systemic exposures after oral administration. It should not be overlooked that the conduct of safety assessment studies with oral administration are substantially easier than a comparable program using parenteral administration.

Prodrugs designed to provide a 'handle' for active uptake processes may or may not facilitate exposure increases in safety assessment studies. In cases where transport allows uptake of an otherwise impermeant molecule, exposure is obviously improved. Theoretically, capacity limitations of the active uptake system may not allow sufficient drug transport during the absorption window to provide substantive exposure multiples. A more likely problem is possible interspecies differences in transporter characteristics. The consequence of these differences would be vastly different exposures to drug for a given dose between species. For these reasons it is important to consider these factors prior to formal safety assessment.

Species Selection

Selection of appropriate preclinical species for risk assessment must not be underestimated. An additional set of considerations is imposed by the study of prodrugs. It is difficult to put a priority to the properties to be considered during species selection. Occasionally, the decision is made simple by one or more properties of a preclinical species causing the species to be completely inappropriate for safety assessment. Frequently, though, there are a number of pros and cons that must be sorted through before judgment is rendered. Arguably, the most important point to consider is whether the NME can interact in the intended pharmacological manner in the preclinical species being considered. Lack of pharmacological response with a novel target can greatly compromise the ability to consider the risks of therapeutic treatment. There are also some very real practical considerations that must be borne in mind in this regard. For example, substantial historical datasets of clinical chemistry parameters and histopathological lesion incidence are readily available for commonly used preclinical species (mice, rats, monkeys, dogs). While some lesser used species may provide the best pharmacological match with the target of interest, it may be quite difficult to conduct a robust risk assessment.

ADME Considerations

For the purposes of this chapter, properties typically thought of as ADME (absorption, distribution, metabolism, excretion) properties will be evaluated in the most detail. In practice, ADME properties are also frequently a critical consideration in species selection. As already described, prodrugs are often designed to impart important absorption attributes. However, most prodrugs are designed to liberate drug through enzymatic processes. For the purposes of risk identification, it is important to know whether the preclinical species have the ability to liberate drug in a manner similar to humans. This is not always the case and, in instances where meaningful interspecies differences in prodrug cleavage rates are unavoidable, safety assessment will become commensurately more complicated. Fortunately, these properties are often easily studied across species *in vitro*, enabling an appropriately informed choice of species prior to first administration to humans. More discussion of this point will be made in other sections of this chapter.

Prodrugs may also be designed to impart properties affecting distribution. In some cases, extreme pharmacologic or cytotoxic potency is targeted to a specific tissue type through affinity-directed prodrugs. The pharmacological 'payload' is then either actively transported into the tissue and enzymatically liberated therein or simply liberated by tissue-localized enzymatic activity. Regardless, uninformed or unavoidable selection of preclinical species with markedly different key enzymological properties than humans can greatly complicate safety assessment.

Metabolism is particularly important for prodrugs as it is an essential step in separating drug from prodrug. Beyond this, interspecies differences in the metabolism of the drug itself are inevitably an important factor in species selection. However, this topic is outside the scope of this chapter, and the reader is referred to separate reviews on this topic (Caldwell, 1992; Cashman *et al.*, 1996). Easily overlooked is the potential for the prodrug to alter the metabolic properties of the drug itself. Ordinarily, this is not a critical factor, as cleavage occurs presystemically before substantive metabolism of the drug can occur, or the cleavage rate is very fast relative to the rate of the parent drug's metabolism. In some instances the physical properties incurred by design of the prodrug may inadvertently alter the metabolism of the drug itself. An interesting case in point is simvastatin (Zocor®, Merck & Co.), a commercially marketed HMGCoA reductase inhibitor (Vickers *et al.*, 1990). The active pharmacophore among commercially available statins is the 'open' hydroxy acid (Figure 2). Simvastatin is marketed as the lactone form of this hydroxy acid. The lactone and hydroxy acid of statins co-exist in chemical equilibrium. The hydroxy acid of most statins predominates at physiological pH. After oral administration of simvastatin, the lactone is highly extracted by the liver and rapidly hydrolyzed by enzymatic and hydrolytic mechanisms. While this hydrolytic step is reasonably fast, the nature of the relatively lipophilic lactone also predisposes it to relatively rapid metabolism of the lactone ring by cytochromes P-450. This is an aspect of statin metabolism that is generally not seen with the other statins administered as hydroxy acids. It is also interesting to note that glucuronication of the acid itself can further lead to ring closure back to

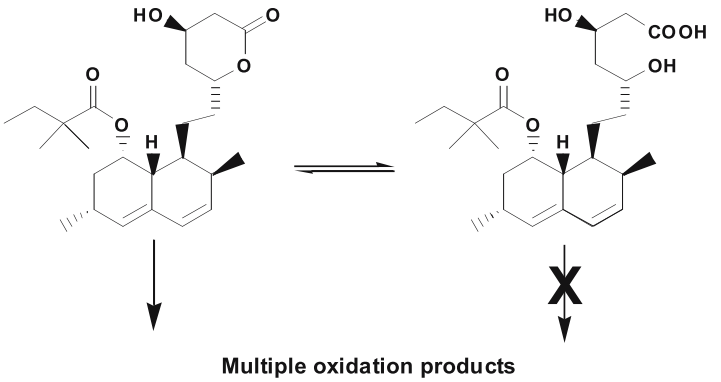

Figure 2. The structure of the lactone simvastatin and its respective hydroxy acid. While the lactone is readily metabolized to multiple oxidation products, the acid is largely not affected by cytochromes P450.

the lactone (Prueksaritanont *et al.*, 2002). These observations point out the unintended complexities often incurred by 'simply' creating a prodrug to ameliorate another aspect of drug developability.

Risk Evaluation

While risk identification is an essential part of drug development, by itself it does nothing to help evaluate benefit/risk. Risk evaluation and the closely related process of safety assessment begin to put the identified risks into perspective. Specifically, risk evaluation sorts through the range of identified risks to determine whether the observed changes generally would pose a hazard to human health. Additional information, typically exposure margins, is considered to evaluate whether the changes pose a high risk to health or are of a low probability of causing adverse effects in patients. Once again, the subject of pharmaceutical risk evaluation is sufficiently broad that no attempt will be made to describe it in any detail. However, those aspects of risk evaluation particularly impacted in the course of prodrug development will be covered.

Histopathological Assessment

Understandably, it might be said that during risk evaluation 'the lesion comes first.' Tissue lesions, clinical changes, or other effects to the preclinical species are considered carefully to help understand human risk. There is a very broad range of considerations given here. For example, are the changes life threatening or relatively benign? Are the effects commonly seen in rodents, for example, and known to be a relatively low risk in humans? Do the effects arise through a series of events likely to occur in all species, including humans, or do understandable differences in biochemistry and physiology exist between the preclinical species and humans that make these effects unlikely to be a cause of concern? Generally

speaking, these types of considerations are mostly related to the drug itself and would be seen whether the drug or prodrug had been administered. On the other hand, in some instances the changes may be entirely related to the prodrug itself, or the situation may exist where it is difficult to attribute the changes to one particular entity. For these reasons, it is particularly important to give special consideration to the aforementioned aspects of risk identification as well as studies designed to take into account special attributes of the prodrug in question. If the data are not collected in a conscientious fashion, it can be very difficult to understand what they mean.

Qualitative Differences from the Prodrug Itself

It is easy to envisage many scenarios whereby the prodrug may elicit effects not attributable to the drug itself. The marked difference in physicochemical properties between the drug and its respective prodrug could easily lead to changes attributable to local irritating effects. This is particularly true when the prodrug takes on amphiphilic character leading to 'detergent-like' effects on exposed tissues. In these instances, the effects are usually localized at the site of application and can be characterized histopathologically as being "irritating" in nature. Depending on the dose, route, rate of administration, and the stability of the prodrug itself, lesions may become more systemic. For example, red blood cell hemolysis may occur as a consequence of high local levels of surfactant activity during intravenous administration. While these effects are usually readily recognized, systemic organ effects may become evident (hemosiderin deposits) and will also need to be considered.

Exposure Assessment

Without question, exposure assessment is absolutely essential for adequate risk evaluation. This is even more important in the development of prodrugs, where evaluation of the risks from drug versus prodrug may need to be distinguished. For this reason, strategies must include what compounds to measure (referred to as 'analytes'), when they will be measured, where they will be measured, and how the measurements will be done.

By definition, a prodrug is a molecule designed to be converted to the active moiety in the anticipated manner. At various times after prodrug administration, potential exists for exposure primarily to the prodrug itself, a combination of prodrug and drug and, finally, the drug alone. An accurate risk evaluation therefore requires documentation of the various drug-related entities present in the course of safety assessment studies. The collection and analysis of this type of exposure information during safety studies is termed toxicokinetics.

The first step toward collection of toxicokinetic data is definition of the toxicokinetic and bioanalytical strategy. This strategy defines what types of information will be collected, when they will be collected, how they will be collected, and how they will be integrated into the overall risk evaluation scheme. Development of prodrugs entails a particular range of factors that must be considered. It could be

argued that, with regard to toxicokinetics, prodrug development essentially means the assessment of two quite distinct entities.

Analyte Selection

At a minimum, one will almost certainly be conducting analyses for both the prodrug and the liberated drug. In some situations, it may be prudent to also analyze the part of the prodrug liberated during conversion to drug. In many cases, this byproduct represents an ordinary physiological constituent and the contribution from the prodrug to the systemic pool is physiologically irrelevant. Clearly, this may not always be the case and, if the liberated moiety is unique, its place in risk evaluation should be considered carefully. In some instances, the physical properties of the prodrug may also give rise to a unique set of prodrug-derived metabolites separate from those of the parent compound itself. It may be possible to discern this type of situation preclinically from *in vitro* data, but conceivably this may not become apparent until later in the course of development (during animal and human radiolabeled metabolism studies). Since metabolites always need to be considered during risk evaluation, prodrug metabolites may also need to be considered in the course of safety assessment.

Analytical Method Development

The degree of difficulty for bioanalytical method development and analysis of prodrugs should not be underestimated. Prodrugs are designed to confer dramatically different chemical and physical aspects to the parent drug. These differences become readily apparent bioanalytically. To generalize, the differences between a hydrophilic, charged species and a more lipophilic neutral species confer marked differences to virtually all aspects of a bioanalytical method. Consequently, sample extraction, chromatography, ionization, fragmentation, and detector response may be quite different between the two species. This imposes considerable extra work and complexity in development, validation, and routine implementation of bioanalytical methods.

Sampling Considerations

Two other aspects of toxicokinetics need special consideration during prodrug safety assessment, and these two aspects are somewhat inter-related. Many prodrugs are designed to release drug via hydrolytic mechanisms. Naturally, the hydrolytic process is ordinarily optimized toward the hydrolytic abilities of humans. However, hydrolytic capacity can vary widely among species (Buchwald, 2001; Buchwald and Bodor, 2002). Generally speaking, rodent species used for safety assessment can have very high levels of esterases. The bioanalytical consequence of this is the need to pay particular attention to prodrug stability during sample collection, storage, and analysis. Preserving prodrug stability during sample collection can be particularly problematic, as many different means

of hydrolytic enzyme inhibition must be surveyed to find a practical, robust means to ensure prodrug stability *ex vivo*.

A proper safety assessment for prodrugs will demonstrate that the potential toxicity of both parent drug and prodrug has been assessed in preclinical species. Toxicokinetic studies are designed toward this end for risk evaluation. Because of interspecies differences in hydrolytic capacity, it may become necessary to collect specially stabilized samples very shortly after prodrug administration. Often, prodrug may be entirely converted to drug within seconds to minutes even after high dosages. The impact of this consideration is the need for carefully coordinated dosing/bleeding schedules, extra collection time points, and extra sample analyses. Considering the high doses that may be administered, it will not be unusual to see large differences in prodrug and drug concentrations. These large concentration differences, which can be on the order of four to five orders of magnitude, put extra demands on bioanalytical dynamic range and may require separate analytical methods, special dilution schemes, or substantial reanalysis to get all analytes in range.

Section Summary

Risk evaluation is the heart of safety assessment. It is the means by which actual risks to patients are assessed. Prodrugs present a special set of considerations during risk evaluation:

- Prodrugs may produce changes inherently different from those of the drug itself.
- Toxicokinetics facilitates relating effects versus administered dose, relative risk at clinical doses, and the contributions of drug or prodrug to the changes observed.
- Analyte selection and analytical method development for prodrugs can be complex.
- The analysis of prodrugs requires particular attention to sampling strategies.

Safety Assessment

It may already be apparent that the phrase “safety assessment” may take on many meanings in drug development. Often, it is the name of the organization that conducts this work in pharmaceutical companies. In this section the term will be discussed in two ways. First, safety assessment will be described as a goal, plan, or process taken toward the objective of drug development and registration. Second, safety assessment can be an ongoing action conducted by the sponsor and regulatory agencies to ensure ethical conduct of human clinical trials.

Any part of drug development as large, complex, and critical as safety assessment must have a defined overall objective and a strategically sound plan. For any drug, an overarching goal is to assess the safety of a drug taken in a particular therapeutic regimen. Thus, the safety assessment of a new prodrug

must consider the patient population and its demographics, as well as the route of administration, duration of treatment, and any other special considerations. A plan is then developed comprising studies of various designs that allows enrollment of volunteers or patients at appropriate stages of the clinical development plan. Each study is designed to answer the question, "Can this type of person be exposed to this agent in this way?" The sponsor is obliged to demonstrate ample evidence that this can be done responsibly. Prodrugs impose added complexity on this process, as one must not only complete the overall assessment but also demonstrate that preclinical species were exposed not to just one entity (parent drug) but potentially to two or more molecular species (prodrug and liberated fragments). As touched upon earlier, the designed chemical lability of prodrugs and their physical diversity from the parent drug impart special challenges that may need to be faced repeatedly in the process. For example, if it is known or reasonable to assume that prodrug circulates to some degree in patients, it may become necessary to consider this not only in ordinary preclinical toxicity studies but also in genotoxicity and reproductive toxicity studies. In turn, the nature of these studies may require the sponsor to consider the particular nature of the prodrug in the context of the study. If a prodrug is actively transported into a particular cell type, is it feasible to test genotoxicity in bacteria or other cell lines that may not allow facile transfer? How might administration of a prodrug limit or alter exposure to the fetus? Proper planning, conduct, and interpretation of studies with these complexities may often require input from scientists of various disciplines. The complexities associated with prodrugs virtually assure that inadequate consideration of all potential issues will lead to a compromised study outcome.

The development of most drugs is usually evolutionary. Even before development is formally commenced, one can see that dose, treatment duration, and clinical setting will change over time. It is rarely the case that development proceeds as initially envisioned; unanticipated complications or extra demands are frequently added throughout the process. Therefore, it is prudent to continually reconsider how newly acquired information and/or changes in clinical plan may impact the assessment of patient safety. In the author's experience, these changes can occur literally at any moment throughout development. However, there are particular development mileposts where one is most likely to acquire data that may dictate a need to reassess patient safety.

Results from Initial Human Trials

Recognizing that all preclinical work done is simply a surrogate for expected human response, results from initial studies in human subjects can sometimes lead to findings requiring reorientation of assumptions. One might find that the anticipated rapid cleavage of prodrug is actually much slower than expected. As a consequence, one may need to reevaluate whether the available safety dataset still provides an appropriate level of assurance for patient safety. If not, the clinical plan may need to be modified. Alternatively, additional safety studies may need to

be conducted to provide the proper assurances for patient protection. Along these lines, human biotransformation studies may also indicate unexpected metabolic transformations not predicted by preclinical study of the prodrug. In extreme cases, it may become apparent that the development path is sufficiently complicated to render further development untenable.

Toxicology Study Outcomes

Significant adverse toxicity findings invariably impact development plans. The same might be said of studies that have otherwise favorable outcomes but are too compromised to support the development plan. In both cases, prodrugs in some ways may pose a higher risk of surprises here than a parent compound itself. First, the design of any study with a prodrug is predicated on reasonable anticipation of prodrug processing. For myriad reasons this may not transpire. Second, it is not unusual to resort to development of a prodrug when the pharmaceutical properties of the parent molecule preclude marketability. In these instances, it is common to assume that the prodrug will be safe, 'just like the parent,' and that prodrug development will be a reasonably straightforward affair. However, poorly designed or characterized prodrugs will inevitably show their flaws. Even the most well designed and studied prodrugs may display unexpected interactions. Finally, if the most astute assumptions of a well-designed prodrug are predicated on results from uneventful yet poorly conducted and characterized safety studies of the parent drug, disappointment may prevail. Even under the best of circumstances one may discover toxicity findings at drug concentrations not achievable by the previously studied parent. In these instances, delays or at least heightened anxieties may arise before the adverse findings are properly put into perspective.

Risk/Benefit Analysis

The decision to continue development or register a drug is ultimately decided by evaluation of benefits to patient against the risks posed to them. For the most part, this judgment is largely subjective. No matter how subjective the judgment, the quality of the decision will be limited by the quality of the data and its interpretation. Obviously, this applies to prodrugs no differently than to the respective parent drug itself. However, the added complexities posed by prodrugs will inevitably require greater effort during risk evaluation to yield a perspective of adequate quality for proper risk/benefit decision making. On the other hand, risk/benefit judgment may be completely straightforward in those instances where previous experience with the parent drug gave a clear risk/benefit analysis. Its very reasonable to assume that equivalent data acquired from a prodrug should yield the same favorable risk/benefit decision achieved with the parent. In fact, this is certainly an impetus for the development of many prodrugs. It is important to bear in mind that regulatory expectations may have changed significantly since the original parent drug was studied. Therefore, it may be prudent to consider whether the same dataset collected previously would still be viewed favorably by current standards of drug development.

Registration

Arguably, the most important aspect of drug registration is the ultimate risk/benefit decision. However, given the importance and impact of such a decision, regulatory agencies impose a relatively high level of stringency on the documentation of studies conducted toward safety assessment. The US Federal Drug Administration requires that studies providing data submitted to support overall safety assessment be conducted under the provisions of Good Laboratory Practices (GLP). While GLP do not ensure proper study design or data evaluation, they do provide substantial assurance that submitted data accurately describe how studies were conducted and the validity of the data used for decision making. The scope of impact from GLP is substantial. For the most part, increased levels of documentation are required. The increased documentation ranges from greater laboratory record-keeping to separate work streams conducted to document what might ordinarily be assumed. Again, this effort may not be qualitatively different for a prodrug than for the parent drug itself, but the complexities arising from prodrugs may require extra effort. First, it may be necessary to redocument under GLP conditions critical pieces of information previously attained from the parent drug under non-GLP conditions. Second, as mentioned in prior sections, some obvious aspects of prodrugs, such as stability, may require added levels of laboratory work and record-keeping to provide proper assurance that the reported studies are accurately represented. Third, the unique physical properties of prodrugs may predispose them to be more difficult to work with and, by extension, more likely to fail tests conducted under stringent conditions.

Summary

The decision to develop any drug requires substantial commitment and resources. Prodrugs present opportunities to enable success where this may not otherwise be possible. However, the extra dimensions offered by prodrugs will almost inevitably require additional consideration and effort. The variety of possible prodrugs and their unique characteristics demand careful evaluation on a case-by-case basis. With these special considerations in mind, appropriate planning for safety assessment may begin. While the fundamental process of safety assessment for a prodrug is no different than that for any other molecule, there will continue to be an extra level of added thought needed to collect and interpret the data required for a sound assessment of safety.

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4.2.2

Toxicological Issues with Pivalate Prodrugs

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Introduction

The incorporation of pivalate (trimethylacetic acid) through an ester linkage to a bioactive molecule is an effective and established strategy for engineering a drug with improved systemic delivery of the bioactive molecule (Beaumont *et al.*, 2003). Compared with the native molecule, the pivalate-containing prodrug will have higher lipophilicity and, thus, will cross lipid biomembranes more readily. The ester is then hydrolyzed to form the desired bioactive molecule and pivalate. Hydrolysis is catalyzed by a spectrum of enzymes found in the blood and several organs, including the intestine and liver (Cook *et al.*, 1995; Takahashi *et al.*, 1995; Beaumont *et al.*, 2003).

The handling of pivalate in humans poses unique toxicological considerations distinct from those associated with the bioactive molecule whose delivery it facilitates. Pivalate is metabolized by enzyme systems and pathways intended to oxidize the broad spectrum of organic acids generated during intermediary metabolism. However, pivalate is not an effective substrate for some of these pathways and cannot be completely oxidized to CO_2 and water. The accumulation of pivalate derivatives has the potential to disrupt cellular metabolism, with clinically relevant consequences. Understanding the mechanisms underlying the changes in metabolism after pivalate exposure allows definition of clinical conditions and therapeutic regimens where the use of pivalate prodrugs will be associated with no significant risk of adverse events and of other situations where such risks will be predictably high.

Fate of Pivalate in Mammals

Organic acids are metabolized through a variety of catabolic and anabolic reactions. The majority of oxidative pathways require that the organic acid first be activated through formation of a thioester with coenzyme A (CoASH), yielding the corresponding acyl-coenzyme A (acyl-CoA). The thioester results in acidification of the α-carbon of the organic acid, increasing its reactivity, and also provides a structural recognition site for various enzymes (Potter, 1957). Acyl-CoA formation is catalyzed by a family of enzymes, the acyl-CoA synthetases, found ubiquitously in cells and various organelles (Aas, 1971; Scholte and Groot, 1975). Acyl-CoA formation is an ATP-requiring reaction.

Incubation of rat hepatocytes with pivalate results in pivaloyl-CoA formation (Ruff and Brass, 1991). Indirect evidence of pivaloyl-CoA formation has also been obtained using rat heart cells (Diep *et al.*, 1995b), and the reaction presumably occurs in all mammalian cells with broad-spectrum acyl-CoA synthetase activities. In general, acyl-CoAs turn over rapidly in cells, as they serve as substrates for other reactions. However, pivaloyl-CoA cannot be further oxidized in mammalian cells and, thus, can accumulate when pivalate is present. Incubation of rat hepatocytes with 1 mM pivalate results in 50–80% of the total coenzyme A pool being sequestered as pivaloyl-CoA (Ruff and Brass, 1991).

Cellular mechanisms exist to protect cells from harmful acyl-CoA accretion (see below). Several acyl-CoA hydrolases have been described that generate the free organic acid and CoASH from the acyl-CoA (Bronfman and Leighton, 1984; Kuramochi *et al.*, 2002). Because these enzymes have high K_m values, they do not interfere with normal metabolism. The acyl-CoA hydrolase reactions may also have limited turnover rates, allowing high concentrations of the substrate organic acid to overwhelm the removal system. Rather than being released through hydrolysis, the acyl group can be transferred from coenzyme A to another molecule via an acyltransferase reaction. Potential acceptor molecules include free amino groups, glucuronic acid, and carnitine. Formation of a hydrophilic conjugate allows urinary excretion of the organic acid that cannot be metabolized. In the case of pivaloyl-CoA, glucuronic acid appears to be the major acceptor in monkey with large amounts of pivaloyl glucuronide appearing in the urine (Vickers *et al.*, 1985). In contrast, in man pivaloylcarnitine is the dominant form of pivalate appearing in the urine (Vickers *et al.*, 1985) and accounts for almost all pivalate elimination (Konishi and Hashimoto, 1992; Brass *et al.*, 2003).

Impact of Pivalate on Cellular Metabolism

Accumulation of a specific acyl-CoA can have profound adverse consequences on overall cellular metabolic homeostasis (Brass, 1994). A large fraction of the total cellular coenzyme A pool can be trapped as the acyl-CoA, limiting CoASH availability for other reactions. More significantly, specific acyl-CoAs can function as inhibitors of important metabolic pathways. This inhibition may be mimicking physiological regulatory mechanisms. For example, acetyl-CoA is a physiological inhibitor of the enzyme pyruvate dehydrogenase (Bremer, 1969). High concentrations of acetyl-CoA normally reflect enhanced generation of acetyl-CoA at rates exceeding the capacity of the Krebs cycle to utilize this important intermediate. As a result, pyruvate dehydrogenase is inhibited, and acetyl-CoA production from glucose is slowed. However, propionyl-CoA is also an inhibitor of pyruvate dehydrogenase (Bremer, 1969). Propionyl-CoA content is normally very low in cells and, thus, does not play a role in regulation of pyruvate dehydrogenase under physiologic conditions. In contrast, in genetic diseases in which propionyl-CoA metabolism is impaired or when large amounts of propionate are added to model systems, propionyl-CoA does accumulate (Brass and Beyerinck, 1988) and results in inappropriate inhibition of pyruvate oxidation (Patel *et al.*, 1983; Brass *et al.*, 1986).

Despite the accumulation of significant concentrations of pivaloyl-CoA, high concentrations of pivalate have minimal affects on cellular glucose and fatty acid oxidation in model systems (Ruff and Brass, 1991; Ji and Tremblay, 1993). Addition of 5 mM pivalate to rat hepatocytes resulted in a 34% inhibition of pyruvate oxidation and had no affect on palmitate oxidation (Ruff and Brass, 1991). This compares with a 60% inhibition of pyruvate oxidation (Brass *et al.*, 1986) and a 30% inhibition of palmitate oxidation (Ruff and Brass, 1991) with addition of 5 mM propionate. As the degree of CoASH depletion is similar when

hepatocytes are incubated with pivalate or propionate, the lesser degree of metabolic disruption seen with pivalate suggests that pivaloyl-CoA itself is less toxic than propionyl-CoA.

Effect of Pivalate on Carnitine Homeostasis

Overview of carnitine homeostasis in humans

The major clinical toxicological concerns resulting from the pivalate released by prodrugs are related to the impact of pivalate on carnitine homeostasis. Carnitine is found ubiquitously in mammalian cells and has important roles in intermediary metabolism (Bremer, 1983). Transfer of an acyl moiety from acyl-CoA to carnitine results in formation of acylcarnitines in reactions catalyzed by carnitine acyltransferases. The formation of the carnitine ester from the coenzyme thioester conserves the energy of the thioester bond, and the carnitine acyltransferase reaction is readily reversible. Thus, tissues contain both carnitine and a spectrum of acylcarnitines, the sum of which yields the total carnitine content. Carnitine is an obligate cofactor for mitochondrial fatty acid oxidation, as acylcarnitines function to move activated fatty acids to the mitochondrial matrix for oxidation (Bremer, 1983).

The cellular carnitine pool is much larger than the coenzyme A pool. For example, skeletal muscle contains approximately 30 nmol total coenzyme A per g wet weight of tissue (Friolet *et al.*, 1994) as compared with 4000 nmol total carnitine per g wet weight of tissue (Hiatt *et al.*, 1989). This disparity allows the carnitine pool to buffer the coenzyme A pool from transient increases in acyl-CoA concentrations and makes the carnitine pool more refractory to depletion.

Tissue carnitine stores are derived from both dietary sources and endogenous biosynthesis. In healthy humans, biosynthetic capacity is sufficient to meet all requirements for carnitine. The final steps of carnitine biosynthesis occur in the liver and kidney (Hoppel and Davis, 1986). Carnitine is carried to tissues from either the intestine (dietary sources) or the biosynthetic organs to other tissues. Tissues vary widely in their total carnitine content. For example, in humans the plasma total carnitine concentration is approximately 50 μM, while skeletal muscle contains approximately 4000 μmoles per kg wet weight (Hiatt *et al.*, 1989). Specific transport systems maintain the concentration gradients between tissue and plasma (Kerner and Hoppel, 1998; Lahjouji *et al.*, 2001; Tein, 2003). Tissues also vary in the kinetics of interaction with the plasma. The liver carnitine pool turns over relatively rapidly, while the skeletal muscle pool equilibrates with plasma only slowly (Brooks and McIntosh, 1975; Rebouche and Engel, 1984). As a result, plasma concentrations may change over short periods of time and be a poor predictor of tissue carnitine status. A 70 kg man has approximately 20 g of total carnitine, with >95% of this total in skeletal muscle (Brass, 1995).

No irreversible metabolism of carnitine occurs in humans. Carnitine is eliminated via the urine as carnitine and acylcarnitines. The renal tubule contains

a saturable reabsorption transporter and, under physiologic conditions, the majority of the filtered carnitine is reabsorbed (Engel *et al.*, 1981).

Cellular Pivaloylcarnitine Generation

Pivaloyl-CoA is a substrate for purified carnitine acetyltransferase, and carnitine pivaloyltransferase activity is present in rat liver (Ruff and Brass, 1991). Pivaloylcarnitine production has also been demonstrated in rat heart cells (Diep *et al.*, 1995b). The specific carnitine acyltransferase responsible for pivaloylcarnitine formation in human tissues has not been identified. After *in vivo* administration to rats, pivaloylcarnitine is found in numerous tissues (Diep *et al.*, 1995a).

The formation of pivaloylcarnitine decreases cellular carnitine content as the carnitine pool is redistributed from carnitine toward pivaloylcarnitine and pivaloylcarnitine leaves the cell for elimination. This depletion of carnitine may affect cellular metabolism independent of the effects of CoASH depletion or potential actions of pivaloyl-CoA. As noted above, *in vitro* pivalate did not acutely inhibit fatty acid oxidation by rat hepatocytes (Ruff and Brass, 1991). However, the magnitude of carnitine depletion was not measured in these studies. Rat liver has limited carnitine pivaloyltransferase activity (Diep *et al.*, 1995a) and, thus, these *in vitro* experiments may have been too acute to allow for significant carnitine depletion. In the case of pivalate prodrug ingestion, the potential for ongoing carnitine depletion is present during the entire dosing interval.

Pivalate and carnitine homeostasis: animal models

Administration of sodium pivalate to rats results in increased urinary total carnitine excretion and decreased plasma total carnitine concentrations after only a few days of treatment (Figure 1) (Bianchi, 1991). The increased urinary carnitine excretion was in the form of acylcarnitines, presumably pivaloylcarnitine. The loss of pivaloylcarnitine can be considered obligatory as cells cannot metabolize pivaloyl-CoA and, thus, pivalate cannot be eliminated through any other mechanism. In the baseline, steady-state situation, daily urinary total carnitine losses equal the sum of new carnitine biosynthesis and carnitine absorbed from dietary sources. Thus, in the absence of increased carnitine intake or biosynthesis, the increased total carnitine losses after pivalate prodrug administration represent depletion of body carnitine stores.

In contrast, skeletal muscle total carnitine content is unaffected during the first 4 days of pivalate treatment in rats (Figure 1) (Bianchi, 1991). This is consistent with the plasma and skeletal muscle representing discrete compartments for carnitine. Further, while urinary carnitine losses increased approximately fivefold with pivalate treatment, the large reserves of the skeletal muscle meant that little net change occurred in this compartment. In contrast, after 156 days of pivalate treatment, muscle total carnitine content was approximately 40% of control values (Bianchi, 1991). Liver total carnitine content initially fell at a rate intermediate to that seen in plasma and skeletal muscle, stabilized,

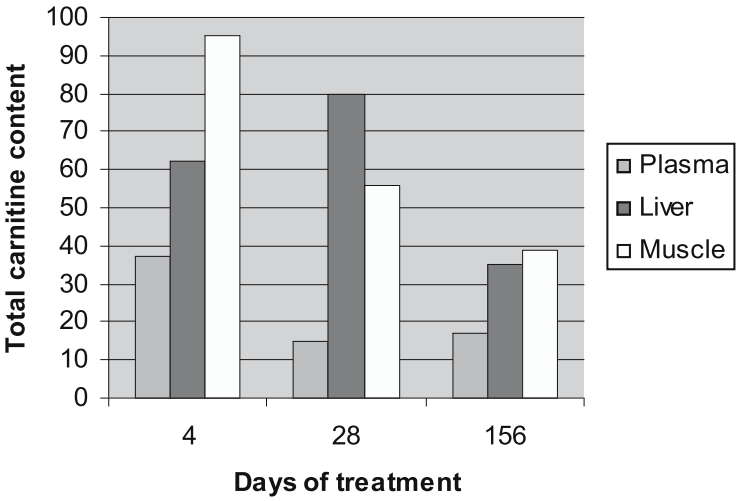

Figure 1. Effect of pivalate treatment on total carnitine concentrations in rat. Rats received drinking water containing either 20 mmol/L sodium pivalate or 20 mmol/L sodium bicarbonate (control) for 56 days. At the times indicated plasma and tissues were collected and analyzed for total carnitine content. Values for pivalate-treated animals are shown expressed as percent of control and represent the mean of 6 rats per group. Note the rapid decrease in plasma total carnitine concentrations and the slower decrease in skeletal muscle. Data adapted from Bianchi and Davis (1991).

and fell to less than 40% of control by 156 days of treatment (Figure 1). These observations emphasize the progressive nature of carnitine depletion during pivalate prodrug treatment and, thus, the importance of time and the cumulative pivalate dose in defining the impact on tissue carnitine content.

Diep and colleagues (Diep *et al.*, 1992) treated rats with the pivalate prodrug pivampicillin for 24 days and confirmed that pivalate treatment affected carnitine homeostasis in rats. Consistent with the differential turnover rates of tissues (Brooks and McIntosh, 1975), plasma and liver carnitine concentrations were most dramatically affected, while heart and skeletal muscle carnitine contents were decreased by only 30–35%.

Despite the dramatic changes in tissue carnitine content in these experiments, functional or metabolic changes have been difficult to identify. Animals treated with pivalate for eight weeks ate normally and gained weight at rates similar to control rats (Bianchi, 1991). More importantly, when rats treated with pivalate were fasted, they did not develop hypoglycemia, suggesting that hepatic metabolic homeostasis was largely preserved despite the large decrease in hepatic carnitine content (Bianchi, 1991). The ability of the pivalate-treated liver to maintain normal glucose production was confirmed by Nakajima and colleagues (Nakajima *et al.*, 1996). Surprisingly, concentrations of circulating β-hydroxybutyrate were increased with fasting of pivalate-treated rates as compared with controls (Bianchi, 1991). The increased β-hydroxybutyrate concentrations may in part reflect decreased peripheral β-hydroxybutyrate clearance (Bianchi *et al.*, 1996).

Nonetheless, the ability to mount a brisk ketogenic response, which requires hepatic fatty acid oxidation, was apparently preserved despite pivalate-induced carnitine depletion. Hepatic triglyceride accumulation has been reported in rats treated with pivalate (Bianchi *et al.*, 1996), suggesting a degree of impaired fatty acid oxidation.

Work by Broderick and colleagues in the rat model further illustrates the interrelationship between intensity of pivalate exposure, degree of carnitine depletion and organ function. Consistent with earlier reports, 2 weeks of pivalate treatment was associated with a mild (24%) reduction in myocardial carnitine content (Broderick *et al.*, 2001). This treatment was associated with no changes in cardiac function, except for impaired recovery from no flow ischemia. In contrast, 28 weeks of pivalate treatment decreased heart carnitine content by 60%, impaired myocardial fatty acid oxidation and decreased cardiac work (Broderick *et al.*, 1995).

Rat models suggest that compensatory mechanisms may lessen the impact on overall carnitine homeostasis resulting from the pivaloylcarnitine losses. Rats have decreased urinary carnitine clearance over time when treated with pivalate (Bianchi, 1991). This carnitine conserving mechanism may be similar to the decreased urinary carnitine clearance observed in the rat with fasting (Brass and Hoppel, 1978). Nakajima and colleagues (Nakajima *et al.*, 1999) suggested that hepatic carnitine uptake and biosynthesis might both be increased in pivalate-treated rats. However, whole animal carnitine biosynthetic rates were not directly measured, limiting the interpretation of these data.

Pivalate and carnitine homeostasis: studies in humans

Qualitatively, the response to pivalate prodrug administration in humans is analogous to the response to pivalate administration in animals as summarized in Figure 2. Administration of pivalate prodrugs to humans is associated with an increase in urinary total carnitine excretion secondary to losses of large amounts of pivaloylcarnitine in the urine (Vickers *et al.*, 1985; Melegh *et al.*, 1987; Holme *et al.*, 1989; Konishi and Hashimoto, 1992; Pap *et al.*, 1999). These changes are observed within 24 hours of drug administration.

Plasma carnitine concentration also falls rapidly after pivalate prodrug administration in humans (Melegh *et al.*, 1997; Brass *et al.*, 2003). In contrast to urinary total carnitine excretion, urinary excretion of unesterified carnitine is not increased (Melegh *et al.*, 1997; Brass *et al.*, 2003). Thus, the decrease in plasma carnitine concentration must reflect a complex redistribution of the carnitine pool as cells utilize carnitine for pivaloylcarnitine formation. An interesting hypothesis is that carnitine is moved into cells through an exchange transporter (Sartorelli *et al.*, 1985; Siliprandi *et al.*, 1987) as the pivaloylcarnitine moves out of cells and through the plasma to the urine.

The changes in plasma and urinary carnitines after pivalate prodrug administration can be understood through the use of a balance study in which carnitine losses are quantified and related to the steady state condition prior to prodrug dosing. In a recent study, subjects were dosed with one of two dosing regimens of

REACTION	PROCESS
Pivalate prodrug → Pivalate + active molecule	Hydrolysis
Pivalate + CoASH + ATP → Pivaloyl-CoA + AMP	Acyl-CoA Synthetase
Pivaloyl-CoA + carnitine ↔ Pivaloylcarnitine + CoASH	Carnitine acyltransferase
$Pivaloylcarnitine_{tissue}$ → $Pivaloylcarnitine_{plasma}$	Plasma membrane transport
$Pivaloylcarntine_{plasma}$ → $Pivaloylcarnitine_{urine}$	Urinary excretion

Figure 2. Generation and fate of pivalate after pivalate prodrug ingestion in man. Pivalate is generated by hydrolysis of the prodrug. This reaction takes place in the intestine, liver, and plasma, as well as in other tissues. Pivalate is then activated to pivaloyl-CoA at the expense of ATP in reactions catalyzed by acyl-CoA synthetases found in tissues. The pivaloyl moiety can then be reversibly transferred to carnitine by acyltransferases located in most tissues. The pivaloylcarnitine can then be transported from tissue to plasma for excretion in the urine.

cefditoren pivoxil, and blood and urine samples were analyzed for carnitine and specific acylcarnitines (Figure 3) (Brass *et al.*, 2003). Large amounts of pivaloylcarnitine were detected in the urine on each day of cefditoren pivoxil dosing. Urinary carnitine excretion decreased but, because of the large amounts of urinary pivaloylcarnitine, urinary total carnitine excretion was markedly increased. For example, 14 days of cefditoren pivoxil dosing (490 mg twice daily) increased total urinary carnitine from 0.46 mmol/day to 1.3 mmol/day. Almost all of the 1.3 mmol/day of urinary total carnitine during cefditoren pivoxil dosing was pivaloylcarnitine. The decrease in excretion of carnitine and carnitine derivatives other than pivaloylcarnitine limited the total carnitine losses during dosing. That is, the approximately 1.3 mmol/day of pivaloylcarnitine excreted was not simply added to the baseline total carnitine excretion of 0.46 mmol/day. The decreased carnitine excretion largely reflected the decrease in plasma carnitine concentrations, but a change in renal carnitine handling was also suggested (Brass *et al.*, 2003). Small amounts (<30 μmol/day) of unconjugated pivalate were detected in the urine.

Cefditoren pivoxil dosing was associated with a rapid decrease in plasma carnitine and acetylcarnitine concentrations and the appearance of pivaloylcarnitine in the plasma (Figure 3). Plasma carnitine and pivaloylcarnitine concentrations tended to plateau after 10 days of prodrug dosing. For example, in males, plasma carnitine concentrations fell from approximately 45 μM predosing to 11 μM after 14 days of cefditoren pivoxil dosing (490 mg twice daily). Pivaloylcarnitine concentrations went from undetectable to approximately 7.4 μM in the same patients over the same interval. The decrement in plasma carnitine

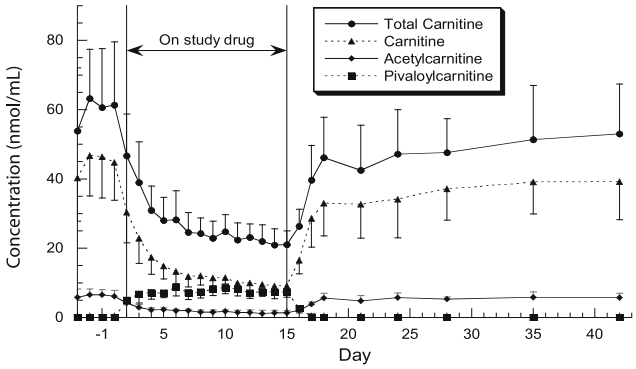

Figure 3 – Panel A

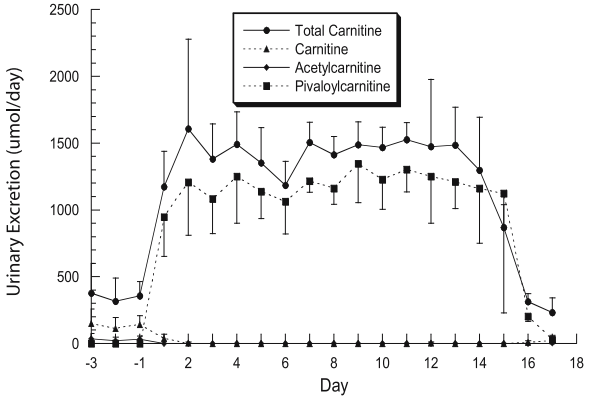

Figure 3 – Panel B

Figure 3. Effect of cefditoren pivoxil administration on carnitine homeostasis. After a three-day stabilization period, seven healthy male patients were given 400 mg cefditoren as cefditoren pivoxil twice daily for 14 days. Twenty-four hour urine collections and blood samples were obtained daily. Samples were analyzed for total carnitine, carnitine, acetylcarnitine and pivaloylcarnitine. Plasma concentrations (panel A) and urinary excretion rates (panel B) are expressed as means with standard deviations. Similar data were obtained in female patients. From Brass et al. (2003).

concentrations was dose dependent, and the nadir plasma carnitine concentration was lower in females than in males.

Based on the daily urine collections, mass balance approaches could be used to understand the fate of the ingested pivalate and the impact on total body carnitine stores (Table 1) (Brass *et al.*, 2003). During 10 days of treatment with cefditoren pivoxil (245 mg BID), patients ingested a total of 7.9 mmol of pivalate. During the 10 days of treatment and two days post-treatment, men receiving this dose excreted a total of approximately 6.5 mmol of pivaloylcarnitine. Analysis of urine for total pivalate excretion (pivalate and all pivalate conjugates) confirmed

that pivaloylcarnitine accounted for greater than 95% of pivalate excretion. Pivaloylcarnitine recovery in the urine represents approximately 80% of the ingested pivalate prodrug on a molar basis. The absolute bioavailability of cefditoren is less than 20%. The higher systemic delivery of pivalate is best explained by hydrolysis of prodrug within the intestine with subsequent absorption of pivalate but not cefditoren. The difference between pivalate ingestion and pivaloylcarnitine excretion probably represents cefditoren pivoxil that is neither absorbed nor hydrolyzed within the gastrointestinal tract.

Pivalate balance				
	Total ingestion as prodrug	Pivaloylcarnitine excretion[a]	recovery	
Males	7.9 mmoles	6.50 mmoles	82%	
Females	7.9 mmoles	6.63 mmoles	84%	
Total carnitine balance				
	Baseline excretion/day	Projected excretion without prodrug over 10 days	Observed excretion over 10 days	Net losses
Males	0.41 mmoles	4.1 mmoles	8.5 mmoles	4.4 mmoles
Females	0.30 mmoles	3.0 mmoles	7.7 mmoles	4.7 mmoles

Table 1. Balance studies following ingestion of 245 mg cefditoren pivoxil twice daily for 10 days in healthy subjects based on urinary excretion patterns. Data are mean values with $n = 8$ for males and $n = 7$ for females. Data from Brass *et al.* (2003).

[a] = total of 10-day treatment period and 3 days post treatment.

At baseline these same male patients excreted an average of 0.41 mmol/day of total carnitine (Table 1) (Brass *et al.*, 2003). During the 10-day treatment period, total carnitine excretion was 8.5 mmoles, reflecting net excess carnitine losses of 4.4 mmoles. These losses are less than the molar ingestion of pivalate prodrug (7.9 mmoles) and pivaloylcarnitine excretion (6.5 mmoles). This lower than expected impact of pivalate prodrug ingestion reflects the less than 100% bioavailability of pivalate when taken as the prodrug, and the decreased excretion of carnitines other than pivaloylcarnitine discussed above. Thus, the molar dose of pivalate represents a worst case for the magnitude of net carnitine losses, and actual losses may be substantially less than this amount. Higher doses of cefditoren pivoxil administered for longer durations yielded qualitatively similar

results, but with higher net carnitine losses, as would be expected (Brass *et al.*, 2003).

The net carnitine losses of 4.4 mmol after the 10-day treatment regimen can be analyzed in the context of total body carnitine homeostasis. The plasma and extracellular fluid compartment have a total carnitine concentration of 0.05 mM, and consist of approximately 15 L, for a total of 0.75 mmol of total carnitine. Thus, the increased losses of carnitine are dramatic on the scale of the plasma and extracellular fluid compartment, and this is reflected in the dramatic fall in plasma carnitine concentrations. In contrast, the total body carnitine pool, largely in skeletal muscle, is approximately 120 mmol (Brass, 1995). In this context, the loss of 4.4 mmol is small (3.6%) and is unlikely to have clinical consequences or be detected when assessing muscle carnitine content. This reasoning makes clear that the impact of prodrug ingestion on carnitine homeostasis will be determined by the amount of pivalate that reaches the circulation per dose and the duration of therapy.

Pivalate and carnitine homeostasis: clinical experience in humans

Other clinical research and clinical experience with pivalate prodrugs confirms and reinforces the tenets established in the balance study detailed above. As discussed above, pivalate prodrug administration is associated with increased urinary total carnitine excretion and decreases in plasma carnitine concentrations (Melegh *et al.*, 1997; Brass *et al.*, 2003). In contrast, 12 days of treatment with pivmecillinam (1200 mg/day) was associated with no significant change in muscle carnitine content, despite decreases in plasma carnitine concentrations to an average of 27% of pretreatment values (Abrahamsson *et al.*, 1994). This is consistent with the slower turnover of the muscle carnitine pool and the small quantitative impact of the net carnitine losses on the scale of the muscle carnitine stores. In a separate study in which treatment was carried out for 7 to 8 weeks, muscle total carnitine content fell by 55% (Abrahamsson *et al.*, 1995).

Muscle carnitine stores clearly can be decreased to a clinically significant extent if pivalate prodrug treatment is continued for extended periods of time. Holme and colleagues (Holme *et al.*, 1989) reported two patients who had been maintained on pivalate prodrugs for an extended period (one for 22 months, one for 30 months). In these patients muscle carnitine content was decreased to only 10% of reference range values. Similar results were subsequently reported in a series of 17 children treated with pivalate prodrugs for a period of at least one year. Muscle carnitine content was severely depressed in those patients in whom it was measured (Holme *et al.*, 1992).

Despite the wide global use of pivalate prodrugs and the inevitable changes in carnitine homeostasis with their use, few adverse clinical sequelae have been reported from either clinical or research settings. In their initial report, Holme *et al.* (1989) suggested that prolonged pivalate prodrug use in children might be associated with hypoglycemia or systemic symptoms such as tiredness and nausea. Similar symptoms were frequently seen in a subsequently studied group of 17

children treated with pivalate prodrugs (Holme *et al.*, 1992). The symptoms were reported to improve with discontinuation of the prodrug or with carnitine supplementation.

Fasting has been employed as a metabolic stress test after pivalate prodrug exposure. These assessments have documented individual cases of fasting-induced hypoglycemia and blunted ketosis, consistent with effects of severe hepatic carnitine deficiency (Holme *et al.*, 1992; Diep *et al.*, 1993). These abnormalities reverse after discontinuation of pivalate exposure (Diep *et al.*, 1993). Abrahamsson used serial echocardiogram to assess cardiac status during 7 to 8 weeks of pivmecillinam treatment (Abrahamsson *et al.*, 1995). Left ventricular mass decreased by 10% with treatment, but no ventricular functional abnormalities were identified.

Baseline urine carnitine losses reflect biosynthetic and dietary intake rates. Thus, if a significant depletion of body carnitine stores has occurred due to long-term pivalate prodrug exposure, the 0.3–0.5 μmoles of carnitine added to the system normally each day will require months to reestablish the pretreatment steady state. The slow kinetics of the replenishment of carnitine stores was confirmed by Diep *et al.* (1993) when patients were followed after pivalate prodrug discontinuation. Plasma carnitine concentrations did not return to the normal range for 4 to 12 months.

Thus, short treatment (less than or equal to two weeks) appears to produce no change in muscle carnitine stores and no clinical adverse effects. While significant changes in carnitine content in other tissue compartments cannot be excluded, no clinical observations suggest that this occurs. In contrast, long-term treatment with high doses of pivalate prodrugs can clearly cause decreases in body carnitine stores and may be associated with adverse metabolic and functional effects. Therapy with carnitine during treatment or after discontinuation can normalize plasma carnitine concentrations (Melegh *et al.*, 1997). However, the clinical relevance of this correction of a laboratory value is unclear. Thus, carnitine supplementation should be considered only in those situations where pivalate prodrug treatment is required for extended periods of time or in clinical conditions of high risk for carnitine depletion (see below).

Pivalate and carnitine homeostasis: considerations in special clinical populations

The loss of carnitine secondary to pivalate prodrug ingestion will be dependent on the amount of drug ingested and the amount of pivalate reaching the circulation. The impact of the resultant carnitine loss will be accentuated under conditions of smaller carnitine stores at the time of therapy or an increased demand for carnitine to support cellular function.

The presence of an inherited disorder affecting carnitine homeostasis would in principle dramatically increase an individual's risk from pivalate prodrug ingestion. Inherited primary carnitine deficiency results from an inherited defect in one of the transmembrane carnitine transport proteins. This transport defect

results in an increased loss of urinary carnitine secondary to impaired renal tubular reabsorption, an inability to move carnitine from plasma into tissue, or a combination of these functional deficits (Di Donato *et al.*, 1984; Treem *et al.*, 1988; Shapira *et al.*, 1993). As a result, tissue carnitine content and, hence, carnitine stores, are extremely low (Treem *et al.*, 1988; Shapira *et al.*, 1993). Under these conditions, any increase in urinary carnitine losses might have adverse clinical consequences. Primary carnitine deficiency is extremely rare and presents early in life; these patients will be under the care of a specialist once the diagnosis is established. Carnitine supplementation has a therapeutic benefit in these patients and might protect against inadvertent exposure to pivalate prodrugs. However, in some cases muscle carnitine content remains extremely low despite carnitine supplementation (Treem *et al.*, 1988) and generation of pivaloylcarnitine might still have deleterious effects on muscle metabolism. Thus, pivalate prodrugs should be considered contraindicated in the very rare patient with carnitine deficiency.

Acyl-CoAs will accumulate in cells if an inherited enzyme defect prevents their metabolism. Under these conditions, carnitine is required to remove the acyl group as the acylcarnitine and reestablish CoASH availability. The acyl moiety that cannot otherwise be metabolized is excreted in the urine as the acylcarnitine, increasing carnitine losses. As the endogenous acyl-CoA production is ongoing, supraphysiologic amounts of carnitine are required to sustain acyl group removal. These conditions, which in many ways are analogous to long-term pivalate prodrug treatment, have been termed states of carnitine insufficiency (Chalmers *et al.*, 1983). Most patients with a clinically significant inherited metabolic disease will be under medical care and receiving carnitine therapy. However, some patients may have a milder condition that remains untreated, and they would be at theoretical risk for decompensation if carnitine losses were accelerated by pivalate prodrug administration. This scenario is entirely theoretical, and no cases following this pattern have been reported.

Most adult medications, including the currently available pivalate prodrugs, are used at fixed doses and are not adjusted for the patient's body weight. As the amount of carnitine lost will be dependent on the amount of pivalate in the circulation, the percentage of the body carnitine pool lost will depend on the total size of the pool. Carnitine stores are primarily in muscle and, thus, the total carnitine pool size is related to muscle mass, or lean body mass. Therefore, it is clear that a small adult will lose a higher percentage of his/her total body carnitine than will a larger person. As experience detailed above suggests that in an otherwise healthy individual at least 60–80% of the carnitine pool must be lost before clinical sequelae develop, a twofold difference in carnitine stores due to body weight below the norm is unlikely to make a clinically significant impact. That is, the 10% carnitine losses projected with a dosing regimen in a 70 kg person would lead to a 20% loss if the same regimen were administered to a 35 kg person. This large numerical difference is unlikely to be of clinical importance.

In contrast to adults, children are typically dosed on a per body weight basis. Thus, the pivalate load from prodrug administration will scale proportionally with

the patient's weight and, hence, carnitine stores. However, this reassuring situation will be lost if carnitine content per muscle mass is decreased. While it had been reported that muscle carnitine content per muscle weight is normal in full-term infants (Shenai and Borum, 1984), recent data suggest that muscle carnitine content does not reach adult levels until age 12 months (Angsten *et al.*, 2003). This may result in baseline carnitine contents of only 30% of adult levels; thus, caution should be exercised in the use of carnitine prodrugs in children less than a year of age. Premature infants may have dramatically decreased muscle carnitine content (Shenai and Borum, 1984; Angsten *et al.*, 2003), and this should be considered before a pivalate prodrug is used in this population.

With aging, muscle carnitine content in adults is minimally affected (Opalka *et al.*, 2001) or unchanged (Cederblad *et al.*, 1976). Thus, in the absence of other conditions, the elderly do not pose a special problem with respect to pivalate prodrug use. While a number of diseases may be associated with altered carnitine metabolism, reflecting underlying metabolic derangements, most conditions do not affect muscle carnitine stores. Specifically, carnitine content per gram of skeletal muscle has been reported to be unaffected by diabetes (Cederblad *et al.*, 1977), alcohol abuse (de Sousa *et al.*, 1988), protein-calorie malnutrition (Wennberg *et al.*, 1992), and liver disease (Wennberg *et al.*, 1992). Long-term hemodialysis for treatment of end-stage renal disease is associated with decreased muscle carnitine content, with values falling to 50% of healthy controls in some patients (Moorthy *et al.*, 1983; Hiatt *et al.*, 1992). End-stage renal disease may also be a condition of carnitine insufficiency due to underlying metabolic abnormalities (Brass *et al.*, 2001) and, if so, would necessitate higher carnitine availability to maintain tissue function. Based on the reduced stores of individuals, a prodrug regimen that decreases body stores to 90% of control levels in a healthy subject may result in carnitine stores of 40% of control levels in a hemodialysis patient. This is unlikely to be of clinical consequence, and no adverse experience has been reported with pivalate prodrugs in hemodialysis patients.

Maternal carnitine may be a significant source of carnitine for the developing fetus. The placenta contains a high-affinity carnitine transport system that may be important in maternal-fetal carnitine homeostasis (Wu *et al.*, 1999). Maternal plasma carnitine concentrations fall normally during pregnancy and reach concentrations less than 20 μM (Cederblad *et al.*, 1985). Neonatal carnitine plasma concentrations are higher than maternal carnitine concentrations, but fall in the days following birth (Novak *et al.*, 1981). This suggests that placental transport is important in maintaining fetal carnitine concentrations. In animal models, maternal pivalate exposure during pregnancy can result in carnitine deficiency in the offspring (Davis, 1995; Ricciolini *et al.*, 2001). No studies have established the effects of pivalate exposure during pregnancy on either maternal or neonatal carnitine status in humans. An analysis of 414 pregnancies in which the women took pivmecillinam demonstrated no increase in the frequency of low birth weight infants, neonatal hypoglycemia or any other adverse outcome (Larsen *et al.*, 2001). No details were available on the dose and duration of

pivmecillinam exposure in these women. Thus, despite this encouraging clinical experience and the high-affinity of the placental transporter for carnitine, the use of pivalate prodrugs during pregnancy, if required, should be limited to short duration and low absolute pivalate exposure.

Valproate (2-propylpentanoate) is an anticonvulsant drug. As an organic acid, valproate can form both valproyl-CoA and valproylcarnitine (Becker and Harris, 1983; Millington *et al.*, 1985; Ponchaut and Veitch, 1993). Additionally, oxidative metabolites of valproate also form coenzyme A and carnitine esters and have been implicated in the rare hepatotoxicity of valproate (Triggs *et al.*, 1990; Ponchaut and Veitch, 1993; Krahenbuhl *et al.*, 1995). It has been proposed that treatment with high dose carnitine ameliorates valproate hepatotoxicity (Bohan *et al.*, 2001). Thus, a potential adverse drug interaction between valproate and pivalate prodrugs has been suggested. Valproate therapy does not result in the massive increases in urinary total carnitine excretion observed with pivalate prodrugs and, thus, is unlikely to cause systemic carnitine depletion despite decreases in plasma carnitine concentrations (Stadler *et al.*, 1999). The severe hepatotoxicity associated with valproate therapy is rare and appears to be an idiosyncratic reaction (Bryant and Dreifuss, 1996). The status of the carnitine pool has not been shown to be a risk factor for valproate hepatotoxicity, and no cases of hepatotoxicity precipitated by pivalate prodrugs have been reported in valproate users. A case of possible hyperammonemic encephalopathy has been reported in a 72-year-old woman taking valproate and pivmecillinam (Lokrantz *et al.*, 2004). The patient developed symptoms after two weeks of pivmecillinam treatment. There was no other evidence of hepatic dysfunction. This is the only case report suggesting a pivalate prodrug-valproate interaction, and thus the interaction remains largely theoretical.

Current Clinical Status and the Future of Pivalate Prodrugs

Pivalate prodrugs are used throughout the world. It has been estimated that over one billion treatment days with pivalate prodrugs have been accrued (Brass, 2002). Despite this wide use, reports of possible adverse events secondary to pivalate-induced carnitine deficiency remain extremely rare. This is likely due to the limited magnitude of the pivalate exposure when these drugs are used as recommended. Table 2 details the maximal daily pivalate exposure associated with each of the therapeutically used pivalate prodrugs at their recommended doses. On a mole-for-mole basis, this represents the worst-case scenario for net carnitine losses. As discussed above, this worst-case scenario is unlikely due to the bioavailability of the pivalate commonly being less than 100% and to decreases in carnitine elimination that offset the pivaloylcarnitine losses. The pivalate exposure can be compared with the average adult rate of carnitine biosynthesis plus dietary intake of approximately 0.3– 0.5 mmol/day (Brass, 2002; Brass *et al.*, 2003).

Drug	Dose	Daily pivalate load*	Duration of therapy	Clinical impact
Adefovir dipivoxil	10 mg/day	0.04 mmoles	Up to 48 weeks	Minimal – Note A
Cefditoren pivoxil	400 mg BID	1.3 mmoles	Up to 14 days	Minimal – Note B
Cefetamet pivoxil HCl	500 mg BID	1.8 mmoles	Short term	Minimal – Note B
Pivampicillin HCl	1050 mg BID	4.2 mmoles	Up to 14 days	Risk of significant losses – Note C
Pivmecillinam	200 mg Q8H	1.3 mmoles	Up to 3 days	Minimal – Note B

Table 2. Clinically used pivalate prodrugs* Calculated based on 100% of administered pivalate released and reaching systemic circulation

BID = Twice daily

Q8H = Every eight hours

Notes:

A - daily carnitine losses small compared to body pool.

B – potential for clinically significant carnitine losses if taken for longer than label indicates or if decreased carnitine stores.

C – approximately 3.5% of stores lost per day, or 49% over 14 days. Percent losses increased if initial stores less than 20 g estimated for 70 kg healthy male, or if treatment extended.

Adefovir dipivoxil is unique among pivalate prodrugs used clinically in that it is approved for long-term treatment. However, the daily pivalate load is only 0.04 mmol/day, which is small compared with the normal daily total carnitine production rate and the total body carnitine pool (120 mmol). Thus, significant carnitine depletion with adefovir dipivoxil use is unlikely.

In contrast, each of the pivalate-containing antibiotics is used in daily doses that may substantially impact total carnitine excretion (Table 2). Cefditoren pivoxil, cefetamet pivoxil, and pivmecillinam may also be associated with net carnitine losses of 1–2 mmol/day using the worst-case estimates. In the case of cefditoren pivoxil, the actual losses are approximately 30–50% of the worst-case estimate (Table 1). Nonetheless, even when the worst-case estimates are used, the average 70 kg person would lose less than 2% of their body carnitine pool per day, or less than 25% after two weeks. Loses of this magnitude are likely to be tolerated, and leave a safety margin for patients with lower carnitine reserves (see above). Pivampicillin used in maximal doses may lead to carnitine losses twice

those observed with the other antibiotics. While still unlikely to result in clinically significant carnitine deficiency, the safety margin may be less than with other pivalate-containing antibiotics. In all cases, duration of use beyond the 14-day course indicated in most approved labels would be associated with increasing risk of clinically important carnitine deficiency.

Regulatory authorities have mandated that information about pivalate prodrug antibiotics shall incorporate language addressing potential toxicity related to carnitine deficiency. Thus, language indicating that the drugs are contraindicated in carnitine deficiency is common (Brass, 2002). Individual countries have included warnings for use in hemodialysis patients, diabetics, premature infants, during pregnancy, or with concomitant valproate therapy. As discussed above, these warnings may not always reflect true clinical risk. Importantly, as serum carnitine concentrations are a poor reflection of tissue carnitine content, measurements of serum carnitine prior to or during pivalate prodrug therapy are unlikely to identify patients at clinical risk from carnitine deficiency. Clinicians should be aware that patients with low carnitine stores (genetic disease, premature infants, perhaps children less than one year of age, and adults with very low muscle mass) may be at risk of carnitine deficiency if pivalate prodrugs drugs are used at usual doses and, in all cases, if the drugs are used at greater than label doses or for prolonged periods of time.

Pivalate prodrugs pose unique toxicology problems compared to other esterified prodrugs where the released acid can be completely oxidized by mammalian cells. Nonetheless, the properties of pivalate may give it unique advantages in prodrug design (Takahashi *et al.*, 1995; Beaumont *et al.*, 2003). Use of a pivalate prodrug will inevitably result in perturbed carnitine homeostasis. The magnitude and potential clinical relevance of the alteration in carnitine metabolism can be predicted based on the cumulative pivalate exposure from the therapeutic regimen of the prodrug. Low exposures compared to the size of the endogenous carnitine pool are extremely well tolerated in most clinical situations and need not be considered an absolute contraindication to the use of pivalate esters. Careful consideration of these factors should be given prior to the decision to commit to a pivalate-based prodrug strategy, and clinicians must be aware of the alterations in carnitine homeostasis associated with pivalate prodrugs that are used in clinical practice.

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Part 5

Case Studies

5.1

Case Study: Adefovir Dipivoxil: An Oral Prodrug of Adefovir

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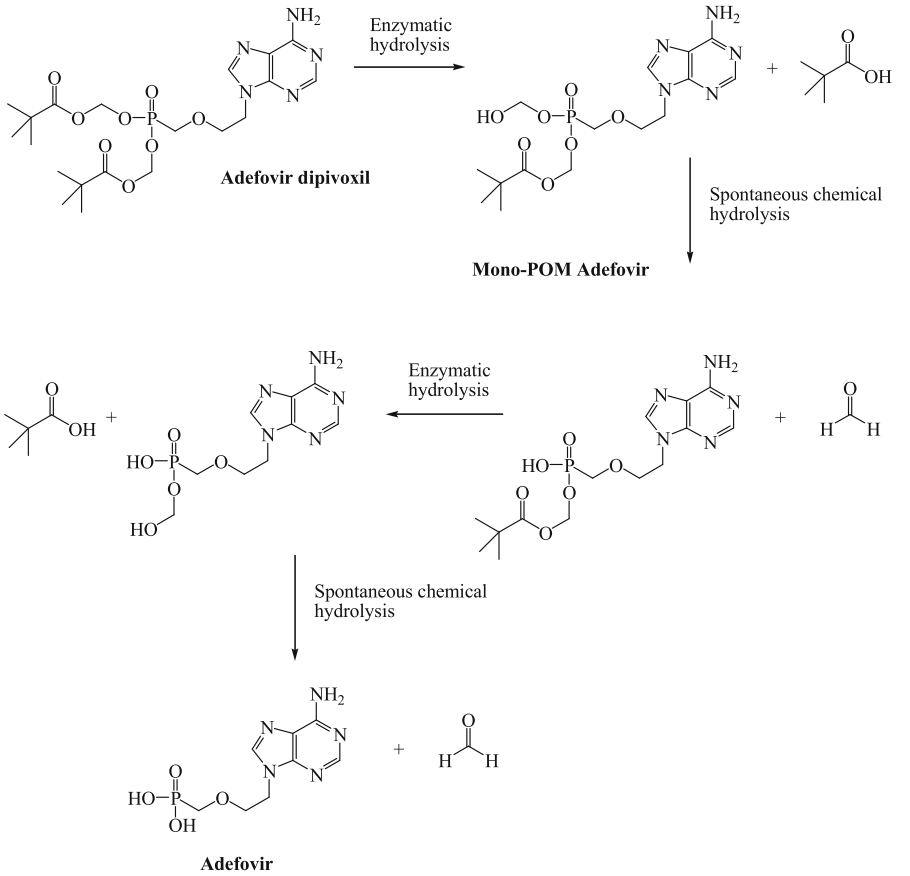
Table of Contents

Name:

9-[2-[[bis[(pivaloyloxy)methoxy]-phosphinyl]methoxy]ethyl]adenine, Hepsera®

Structures and Bioconversion Pathway



Scheme 1.

Rationale for Adefovir Dipivoxil

The potential antiviral effect of adefovir (Scheme I), an acyclic nucleoside phosphonate analog of 2'-deoxyadenosine monophosphate, was first studied by Holý and De Clercq over 15 years ago (De Clercq *et al.*, 1986, 1987; Holý *et al.*, 1999, 2002). In this class of compounds, the ribose phosphate group is replaced with the isopolar phosphonomethyl ether functionality. Adefovir is recognized by host kinases and is phosphorylated *in situ* to the virologically active adefovir diphosphate (Qaqish *et al.*, 2003). Adefovir diphosphate inhibits hepatitis B virus (HBV) DNA polymerase (reverse transcriptase) in addition to other viral DNA polymerases. This inhibition results in DNA chain termination and impairment

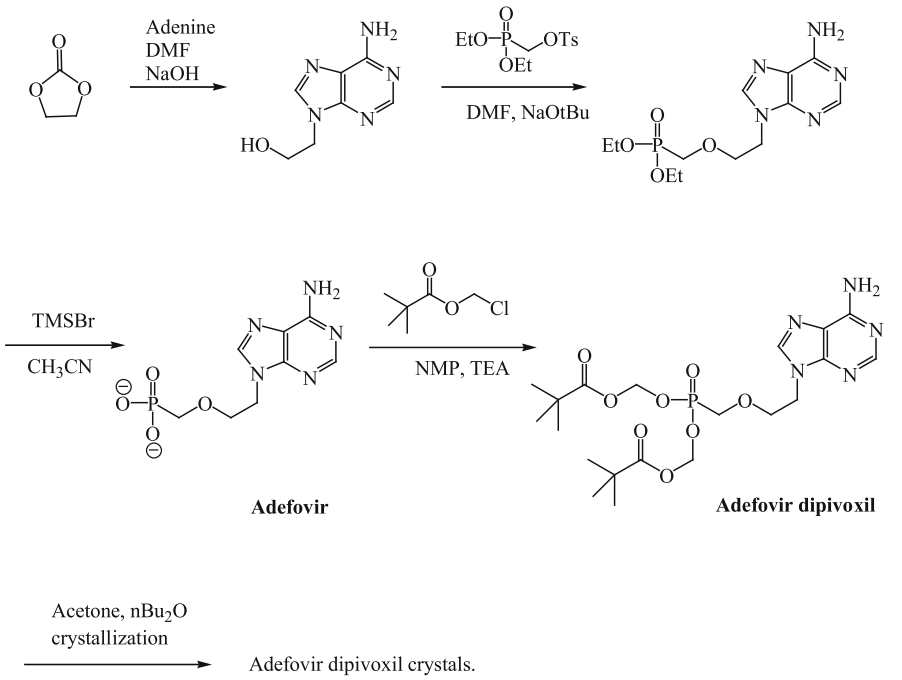
of viral replication. The inhibition constant (K_i) for adefovir diphosphate binding to HBV DNA polymerase is 0.1 μM, while the K_is for binding to human DNA polymerases α and γ are 1.18 μM and 0.97 μM, respectively (Hepsera Package Insert 2002). Therefore, adefovir dipivoxil has a sufficient therapeutic index for clinical utility.

Mimicking a nucleoside monophosphate with a phosphonate as in adefovir has two advantages: it avoids the requisite but slow initial phosphorylation of nucleosides by host kinases, and it prevents dephosphorylation in blood plasma by widely occurring phosphatases, since the phosphonate moiety is stable to hydrolytic cleavage to the nucleoside. In addition, it has been suggested that the diphospho-phosphonate metabolites that are formed in cells are better substrates for viral polymerases than the corresponding nucleoside 5'-triphosphates because the ether oxygen of the phosphonate-bearing side chain coordinates more favorably with the metals present in the active site of polymerases (Sigel, 2004). In summary, acyclic nucleoside phosphonates provide suitable and active substrates for viral DNA polymerases.

Phosphonate-containing drugs such as adefovir exist as dianions at typical physiological pH, making them very polar (pK_{a1} = 2, pK_{a2} = 6.8). Such polar species do not readily undergo passive diffusion across cellular membranes and intestinal mucosa, resulting in poor oral bioavailability (Naesens *et al.*, 1996). To improve the oral absorption of adefovir, the polar phosphonic acid functionality has been esterified with two pivaloyloxymethyl promoieties. The oral bioavailability of adefovir dipivoxil in human subjects is estimated at 59%, based on a cross study comparison (Hepsera Package Insert 2002). A food effect was not observed; the AUC and the oral bioavailability of the drug remained unchanged (59%).

Synthesis

The preparation of adefovir dipivoxil was first described in the literature over 10 years ago (Starrett *et al.*, 1992; Starrett *et al.*, 1994). Optimization of the synthesis and formation of a crystalline final product was subsequently described (Scheme 2) (Arimilli *et al.*, 1999; Yu *et al.*, 1999). The synthesis of diethyl *p*-toluenesulfonyloxymethyl-phosphonate is achieved by heating diethyl phosphite and paraformaldehyde under basic conditions at 87°C, followed by addition of *p*-toluenesulfonyl chloride at 0°C. The preparation of 9-(2-hydroxyethyl)adenine is accomplished through heating adenine, molten ethylene carbonate, and sodium hydroxide in DMF. The newly-formed 9-(2-hydroxyethyl)adenine is further elaborated via an SN_2-type substitution in the presence of diethyl *p*-toluenesulfonyloxymethyl-phosphonate and a sodium alkoxide base in DMF. Conversion of the diethyl phosphonate to the diphosphonic acid is performed under standard bromotrimethylsilane/acetonitrile conditions. The synthesis of adefovir dipivoxil is completed by addition of the chloromethyl pivalate to a solution of the corresponding phosphonic acid in NMP, using triethylamine as base.



Scheme 2.

Mechanism and Site of Bioconversion

As described in the previous section, adefovir dipivoxil is an oral prodrug of adefovir in which the phosphonic acid is masked as the bis-(pivaloyloxymethyl) ester. The bioconversion of adefovir dipivoxil to adefovir is mediated by esterases (Naesens *et al.*, 1996). The bioconversion mechanism involves rapid enzymatic hydrolysis of the bis-ester followed by spontaneous decomposition of the hydroxymethyl intermediate (Scheme 1). The monoester most likely undergoes a similar degradation leading to the rapid formation of adefovir. Adefovir is then transported into different cell lines by various mechanisms such as a saturable protein-mediated process in HeLa cells (Cihlar *et al.*, 1995) or fluid-mediated endocytosis in CCRF CEM T-lymphoblastoid tissue (Olsanska *et al.*, 1997). Adefovir is phosphorylated to adefovir monophosphate by various kinases, one of which in lymphoid cells is identified as adenylase kinase 2 (Robbins *et al.*, 1995). A second phosphorylation of adefovir monophosphate provides adefovir diphosphate, which is incorporated into the elongating HBV DNA through HBV DNA transcriptase. Absence of the necessary 3'-hydroxyl group required for chain elongation during transcription in adefovir results in chain termination of HBV DNA. Adefovir is efficiently phosphorylated in hepatocytes to yield adefovir diphosphate, which has a half-life of 33 hours in human Hep G2 cells (Ray *et al.*, 2004).

Toxicity Issues

Adefovir dipivoxil at 10 mg/day has a safety profile that is similar to that of placebo (Hadziyannis *et al.*, 2003) and is well tolerated by healthy, renally and hepatically impaired patients, and lamivudine-resistant HBV patients coinfected with HIV (Benhamou *et al.*, 2001). A potential concern with adefovir dipivoxil is nephrotoxicity, which was observed at doses equal to or greater than 30 mg daily (Marcellin *et al.*, 2003). This observation is consistent with the high clearance of adefovir, which exceeds the glomerular filtration rate (Cundy *et al.*, 1995). Monitoring of renal function may be required during treatment with adefovir dipivoxil. In addition, increase in serum creatinine (>0.5 mg/dl) has been observed at the 10 mg dose in 20% of pre- and posttransplant patients. However, no dose adjustment is required for hepatically impaired patients as no substantial changes in adefovir pharmacokinetics were observed in that patient population.

The bioconversion of adefovir dipivoxil to adefovir leads to the formation of pivalic acid and formaldehyde. An earlier chapter in this book addresses the issue of pivalic acid toxicity. The major clinical toxicological concern resulting from the pivalate released by prodrugs is related to the impact of pivalate on carnitine homeostasis. Adefovir dipivoxil is the only pivalate-containing prodrug used in chronic treatment. The daily pivalate load from adefovir dipivoxil is only 0.04 mmol/day, which is negligible compared to the total body carnitine pool (120 mmol). The daily formaldehyde load of 0.04 mmol/day is considered insignificant as well.

Formulation Issues

Adefovir dipivoxil is a white crystalline powder with high aqueous solubility at pH 2.0 (19 mg/mL) and lower solubility at pH 7.4 (0.4 mg/mL). The degradation kinetics of adefovir dipivoxil are governed by two distinct but interrelated degradation pathways: hydrolysis of the pivaloyloxymethyl moiety and formaldehyde-mediated dimerization of the adenine ring (Yuan *et al.*, 2000). Hydrolysis of adefovir dipivoxil produces one equivalent each of mono-POM adefovir, pivalic acid, and formaldehyde. Formaldehyde can further react with the N^6-amine of adenine to form the corresponding carbinolamine intermediate. Dehydration of the carbinolamine intermediate leads to the formation of the reactive imine cation (Schiff base) of adefovir dipivoxil, which can react with an additional adefovir dipivoxil or mono-POM adefovir molecule to form the methylene-linked dimer. Both degradation pathways are known to be pH-dependent in solution.

Each tablet of Hepsera for oral administration contains 10 mg of the active ingredient adefovir dipivoxil, in addition to the inactive ingredients croscarmellose sodium, lactose monohydrate, magnesium stearate, pregelatinized starch, and talc.

Discussion

According to the World Health Organization, approximately 2 billion people have been infected with hepatitis B and over 350 million have chronic hepatitis B infection
(http://www.who.int/csr/disease/hepatitis/whocdscsrlyo20022/en/index1.html).
Over 1 million patients have been diagnosed with hepatitis B in the U.S. and Europe (http://hepatitis.about.com/od/hepatitisnews). Only 15% of the infected patient population is receiving treatment for hepatitis B. Lack of treatment in patients with high HBV replication will lead to cirrhosis of the liver within a few years. Patients with liver cirrhosis have a short life-expectancy due to liver failure and/or hepatocellular carcinoma (Beasley, 1988).

Chronic HBV infection commonly develops in patients who were exposed to HBV in childhood. Approval of a hepatitis B vaccine in 1982 has resulted in a decline in chronic HBV cases in recent years.

Currently available treatments for hepatitis B include interferon alfa-2b (Intron® from Schering Corporation), lamivudine (Epivir-HBV®, GlaxoSmithKline), and adefovir dipivoxil (Hepsera®, Gilead Sciences). Interferon alfa, which is administered parenterally, is associated with a number of adverse effects such as depression, fatigue, irritability, and influenza-like symptoms, as well as bone marrow suppression. Lamivudine, a nucleoside also referred to as 3TC, is administered at a daily dose of 100 mg. Lamivudine is well tolerated and reduces the viral load significantly. However, viral resistance develops in approximately two-thirds of the patients after a three-year treatment period (Papatheodoridis and Hadziyannis, 2004).

Adefovir is a potent antiviral agent with activity against HIV, HSV, SIV, and HBV. Despite its broad spectrum of activity, it is not clinically useful as it suffers from poor oral bioavailability. The pharmacokinetics and bioavailability of adefovir have been studied at doses of 1.0 or 3.0 mg/kg in human clinical trials (Cundy *et al.*, 1995). The terminal half-life of adefovir dosed by intravenous infusion is 1.6 ± 0.5 h. Over 98% of the dose is recovered unchanged in the urine within 24 hours. Serum clearance of adefovir is 223 ± 53 mL/h/kg, which is similar to the renal clearance of the drug (205 ± 78 mL/h/kg). Considering the low protein binding (Qaqish *et al.*, 2003), active tubular secretion accounts for approximately 60% of the clearance of adefovir. The steady-state volume of distribution of adefovir is large (418 ± 76 mL/kg) and suggests complete distribution of the compound throughout body water. The oral bioavailability of adefovir at 3.0 mg/kg dose is variable and less than 12%. The subcutaneous bioavailability of adefovir at 3.0 mg/kg is over 100%.

The pre-clinical and human pharmacokinetics data in combination with a low permeability coefficient across Caco-2 cells (Shaw and Cundy, 1993) indicate that the low oral bioavailability observed is due to poor permeation across intestinal epithelium rather than metabolic degradation (Cundy *et al.*, 1994). A number of prodrugs were prepared to improve the bioavailability of adefovir by increasing the lipophilicity of the compound (Naesens *et al.*, 1995; Shaw *et al.*, 1997).

Adefovir dipivoxil demonstrated the most favorable properties and was advanced through clinical trials.

Adefovir dipivoxil is dosed orally at 10 mg per day. It is effective in the treatment of e antigen-positive and e antigen-negative HBV patients and patients that are resistant to lamivudine (Perrillo *et al.*, 2004; Peters *et al.*, 2004) with a median reduction of serum HBV DNA of 4.3 $\log_{10}$ copies/mL. Following oral dosing, the prodrug is very efficiently cleaved and adefovir is released. In animal studies, no intact adefovir dipivoxil or monoester were detected in plasma following oral dosing (Cundy *et al.*, 1994).

Upon initiation of treatment of HBV patients with adefovir dipivoxil, clearance of HBV DNA is observed in a biphasic curve (Rivkin, 2004). Initially, a sharp drop in HBV DNA levels (corresponding to clearance of viral particles from plasma) is observed with a half-life of one day. In the second, slower phase the infected virus-producing cells are eliminated. A single-dose PK study using adefovir dipivoxil 10 mg showed a C_{max} = 18.4 ± 6.26 ng/mL at T_{max}= 1.75 hours (Hepsera Package Insert 2002). The terminal elimination half-life of adefovir in plasma is 7.48 ± 1.65 hours while the *in vitro* intracellular half-life is approximately 17 hours. The efficacy and toxicity of adefovir dipivoxil were studied in two phase III clinical trials. The 48-week studies demonstrated improved liver histology in 53% of HBV e antigen-positive and in 64% of HBV e antigen-negative patients. Adefovir dipivoxil also demonstrated efficacy in patients that were resistant to lamivudine. In addition, levels of serum alanine aminotransferase (ALT), which is a marker for biochemical response to hepatitis B treatment, were normalized (Tong and Tu, 2004). Adefovir dipivoxil is well tolerated and was found to have a safety profile similar to placebo.

A low incidence of resistance is associated with administration of adefovir dipivoxil. Recently, two cases of adefovir resistance have been described in which the patients still responded to subsequent lamivudine therapy (Brunetto and Bonino, 2004). Mutant N236T in domain D of the HBV polymerase causes a reduction in susceptibility of HBV to adefovir both *in vivo* and *in vitro* (Angus *et al.*, 2003). The *in vitro* experiments demonstrated decreased replication capacity associated with these resistant viruses. Chronic dosing of adefovir dipivoxil is necessary; as with lamivudine, acute exacerbation of infection is observed upon termination of treatment with adefovir dipivoxil (Kumar and Agrawal, 2004).

Conclusion

Adefovir dipivoxil (Hepsera®) is an excellent example of a prodrug that can overcome the oral delivery problem associated with poor permeation across the intestinal mucosa. Since its launch in 2002, Hepsera has become an important agent for the treatment of hepatitis B patients with evidence of active viral replication. A safety profile similar to that of placebo has been observed for Hepsera, which allows it to be prescribed as an effective drug for chronic treatment of hepatitis B.

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5.2

Case Study: Amifostine: (Ethyol®)

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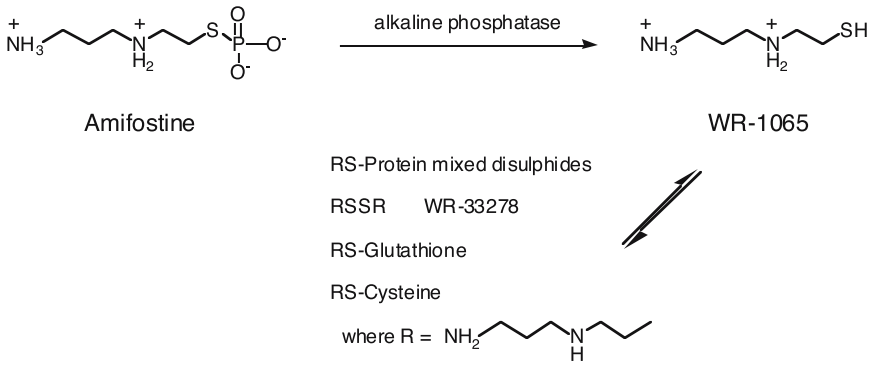
Table of Contents

Name:

Amifostine, 2-[(3-aminopropyl)amino]ethanethiol dihydrogen phosphate (ester), S- (N-(3-aminopropyl)-2-aminoethyl)thiophosphoric acid, Gammaphos, Ethiphos, APAETP, NSC-296961, YM-08310, Ethyol®

Structures and Bioconversion Pathway



Rationale for Amifostine

Amifostine is a prodrug of its free thiol, active metabolite, WR-1065. The thiophosphate promoiety protects the free thiol group of WR-1065 from oxidation to the symmetrical disulfide, WR-33278, while providing a handle for high levels of WR-1065 production at the target tissue. The thiophosphate promoiety of amifostine is cleaved by alkaline phosphatase to produce WR-1065, which accumulates in non-cancerous tissues to a greater extent than in cancerous tissues (Capizzi, 1996).

In the United States amifostine is indicated to reduce cumulative renal toxicity associated with repeated administration of cisplatin in patients with advanced ovarian or non-small cell lung cancer. It is also indicated to reduce the incidence of moderate to severe xerostomia in patients undergoing post-operative radiation treatment for head and neck cancer (Ethyol® Product Insert). A number of clinical trials have been undertaken to evaluate the efficacy of amifostine in other radiation and antineoplastic treatment regimes (Spencer and Goa, 1995; Foster-Nora and Siden, 1997; Gonzalez San Segundo and Calvo Manuel, 2002).

Amifostine originated in the 1950s in an anti-radiation development program at the Walter Reed Army Institute of Research (WRAIR). The WRAIR program evaluated over 4000 compounds for *in vivo* radiation protection, and amifostine and other phosphothioates provided the greatest protection with the lowest incidence of limiting toxicities (Davidson *et al.*, 1980). The use of amifostine as a clinical radioprotector began in the 1980s.

Amifostine is freely soluble in water with a reported aqueous solubility of greater than 200 mg/mL in water, normal saline, or pH 7.4 phosphate buffer. Amifostine is given via IV infusion or, more recently, as a subcutaneous injection (Bonner and Shaw, 2002).

Synthesis

The synthesis of amifostine is covered in detail in U.S. Patent 3,892,824 (Piper and Johnston, 1968). Briefly, a solution of 2-(3-aminopropylamino)ethanol was brominated with hydrobromic acid to yield N-(2-bromoethyl)-1,3-propanediamine dihydrobromide. This product was added to a suspension of trisodium phosphorothioate to obtain the final product, which was isolated in 91% yield by anti-solvent precipitation.

Mechanism and Site of Bioreversion

In vitro and *in vivo* studies have shown greater uptake of amifostine in normal tissues relative to tumor tissues (Yuhas, 1980; Ritter *et al.*, 1982; Tanaka, 1984; Calabro-Jones *et al.*, 1988). Amifostine may be taken up in normal tissues via active transport (Yuhas, 1980), however, most evidence suggests that the differential protection of normal tissue relative to tumor tissue lies in tissue differences in the pre-uptake dephosphorylation of amifostine and absorption of WR-1065 (Calabro-Jones *et al.*, 1988).

Amifostine is converted to WR-1065 predominantly through the action of alkaline phosphate, a cell-surface enzyme present in high concentration in capillaries and arterioles (Calabro-Jones *et al.*, 1988). Several mechanisms have been proposed to explain the preferential uptake of WR-1065 into normal tissues (Yuhas *et al.*, 1982; Calabro-Jones *et al.*, 1985; Shaw *et al.*, 1986, 1988; Calabro-Jones *et al.*, 1988; Rasey *et al.*, 1988). Suggested mechanisms include decreased vascularity of tumor tissues leading to decreased delivery and bioconversion, decreased alkaline phosphatase activity in tumor cells resulting from lower tissue pH (Romanul and Bannister, 1962), and potential pH dependence of WR-1065 cellular uptake.

The cytoprotective effects of WR-1065 may originate from several mechanisms. WR-1065 is capable of scavenging free radicals, depleting oxygen, and covalently binding to active metabolites of antineoplastic agents (Foster-Nora and Siden, 1997). The sulfur-hydrogen bond of WR-1065 can easily donate its hydrogen ion to radiation-induced free radicals and hydrated electrons that can damage DNA, thereby decreasing cell damage (Grdina *et al.*, 1988; McCulloch *et al.*, 1991). WR-1065 can also serve as a nucleophile to sequester charged carbonium ions, thereby eliminating a major alkylation pathway for many antineoplastic agents (McCulloch *et al.*, 1991; Treskes *et al.*, 1992; Treskes and van der Vijgh, 1993; van der Vijgh and Peters, 1994). While the generation of WR-33278, the disulfide oxidation product of WR-1065, has historically been thought to be non-productive, *in vitro* studies have demonstrated that WR-33278 protects

isolated cells from radiation (Shigematsu *et al.*, 1994). In addition to existing *in vivo* as the disulphide WR-33278, WR-1065 can exist as the mixed disulphides of cysteine (Butler *et al.*, 1985; Calabro-Jones *et al.*, 1988) and glutathione (Shaw *et al.*, 1994).

Toxicity Issues

The most common adverse effects associated with amifostine therapy are hypotension, nausea, and vomiting (Turrisi *et al.*, 1986). The effects are dose dependent with no adverse effects reported at doses less than 250 mg/m^2. Shorter infusion times (3–5 min) decrease the incidence and severity of the effects relative to constant rate infusions. Recently, it has been demonstrated that these toxicities could be reduced through subcutaneous administration of amifostine (Koukourakis *et al.*, 2000, Cassatt *et al.*, 2002, 2003).

Formulation Issues

Amifostine is sold for clinical use as Ethyol®, the trihydrate form of amifostine. The original formulation of Ethyol® evaluated at the National Cancer Institute was an amorphous lyophile containing a 1:1 (w/w) ratio of amifostine and mannitol. The amorphous formulation was thermally unstable and required storage temperatures of less than –20°C to maintain potency (Kennedy *et al.*, 1995). The currently marketed Ethyol® formulation is a room-temperature stable, vacuum-dried product of crystalline amifostine trihydrate. The product is reconstituted with 0.9% Sodium Chloride for Injection, USP prior to administration (Ethyol® Product Insert).

Solutions of amifostine are most stable at neutral pH values (Risley *et al.*, 1986). Reconstituted amifostine for injection (5 – 40 mg/mL) is stable for up to 5 h at room temperature and up to 24 h under refrigeration (Ethyol® Product Insert).

Discussion

Amifostine is rapidly cleared from plasma following IV administration with the amifostine concentration decreasing 94% within 6 min following dosing (Shaw *et al.*, 1986). Carbon-radiolabled amifostine does not bind to plasma proteins including human α-1-acid glycoprotein and albumin (Shaw *et al.*, 1997). However, WR-1065 is highly bound to plasma proteins (Shaw *et al.*, 1994). The WR-1065 protein binding, probably through mixed disulphide linkages, is reversible, allowing transport of WR-1065 via plasma proteins (Bonner and Shaw, 2002).

A study conducted by Bonner and Shaw (2002) in 12 healthy volunteers dosed with 200 mg/m^2 amifostine (7.5 minute infusion) found a time to maximum concentration of 6.3 ± 1.7 minutes, a half-life of 15.4 ± 9.6 minutes and a clearance of 1.48 ± 0.46 L/min. In this study, protein-bound WR-1065 following IV administration of amifostine gave a time to maximum concentration of 11.5 ±

7.8 min and a half-life of 79 ± 30 min. These values were in the range of previously reported data (Shaw *et al.*, 1999). The relative AUC to the last time point (240 min) values of amifostine and WR-1065 suggest that amifostine is quantitatively converted to WR-1065 on IV administration. The amount of protein-free WR-1065 was approximately 16% of the total WR-1065 generated. The results suggest that a sustained presence of WR-1065 relative to amifostine is available to the target tissues from circulating, protein-bound WR-1065.

The same study included a crossover that included oral and subcutaneous administration arms. The oral study did not produce plasma levels of amifostine or WR-1065 that were measurable. The subcutaneous studies produced amifostine levels that resulted in an AUC that was 40% of the IV study. The time to maximum amifostine concentration for the subcutaneous study was 23.0 ± 13.6 min, the half-life was 49.4 ± 40.5 min and the clearance was 1.66 ± 1.82 L/min. There were no statistical differences between the IV and subcutaneous studies with respect to amifostine. In the subcutaneous study, the bound WR-1065 time to maximum concentration was 65 ± 36 min and the half-life was 542 ± 655 min. While the average half-life was longer for the subcutaneous study relative to the IV study, the difference in the two routes was not significant. The difference in the time to maximum concentration was found to be significant. The AUC of WR-1065 from the subcutaneous study, surprisingly, was 68% of that obtained from the IV study. Additionally, measurable levels of WR-1065 were still detected at 240 min in the subcutaneous study. In total, the results suggest that the total exposure to WR-1065 from the subcutaneous study would be equivalent to that from IV administration of amifostine.

Conclusion

Amifostine is the current standard for radioprotectant agents. With additional subcutaneous administration studies to support the bioavailability and decreased toxicity data, the use and utility of amifostine should continue to increase.

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5.3

Case Study: Capecitabine: A Prodrug of 5-Fluorouracil

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Table of Contents

List of Abbreviations

Ara-C.....Cytosine arabinoside (cytarabine)
Ara-U .....Uracil arabinoside
CE.....carboxylesterase
DPD .....dihydropyrimidine dehydrogenase
dThdPase.....thymidine phosphorylase
5'-DFCR.....5'-deoxy-5-fluorocytidine
5'-DFUR .....5'-deoxy-5-fluorouridine
F-dUMP .....2'-deoxy-5-fluoro-5'-monophosphate
5-FU.....5-fluorouracil
FUH2 .....5-fluorodihydrouracil
PyNPase.....pyrimidine nucleoside phosphorylase
UdPase.....uridine phosphorylase

Name:

Capecitabine, 5'-deoxy-5-fluoro-N-[(pentyloxy)carbonyl]-cytidine, Xeloda®

Structures and Bioconversion Pathway

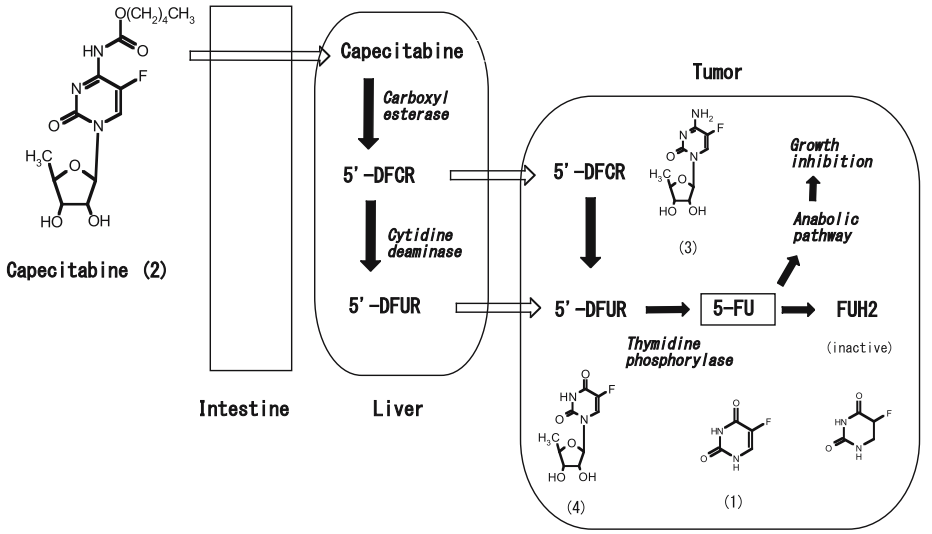

Figure 1. Metabolic pathway for capecitabine.

Rationale for Capecitabine

Capecitabine (**2**), is an off-white crystalline solid with and aqueous solubility of 26 mg/mL. It is sold as Xeloda® and is a prodrug said to achieve selective delivery of 5-fluorouracil (5-FU, **1**) to tumors by sequential activation of the prodrug with endogenous enzymes preferentially located in the human liver and tumor tissues see Fig. 1). Capecitabine was first approved in the US in 1998 for the treatment of metastatic breast cancer that is refractory to standard chemotherapy with paclitaxel and anthracycline-containing regimen; it has now been used for the treatment of metastatic breast and colorectal cancers in more than 70 countries. It was designed to achieve greater selectivity than its active form, 5-FU (**1**)

Design and Discovery of Capecitabine, A Tumor-activated Prodrug of 5-Fluorouracil

5-FU (**1**) is one of the antitumor agents most frequently used for treating solid tumors, such as breast, colorectal, and gastric cancers, in either monotherapy or combination (Chabner and Longo, 1996). Because of its short plasma half-life, 5-FU (**1**) is often administered by continuous infusion, as well as in combination with other cytotoxics or with biochemical modulators, such as leucovorin. 5-FU is

poorly tumor selective and causes high incidences of toxicity in bone marrow, gastrointestinal tract, central nervous system, and skin.

Several oral 5-FU derivatives, such as tegafur (**5**), carmofur (**6**), and furtulon (5'-deoxy-5-fluorouridine; 5'-DFUR: **4**), have been developed in Japan to improve such drawbacks (Fig. 2). Nevertheless, there still needs to be improvement in terms of tumor selectively, efficacy, and safety.

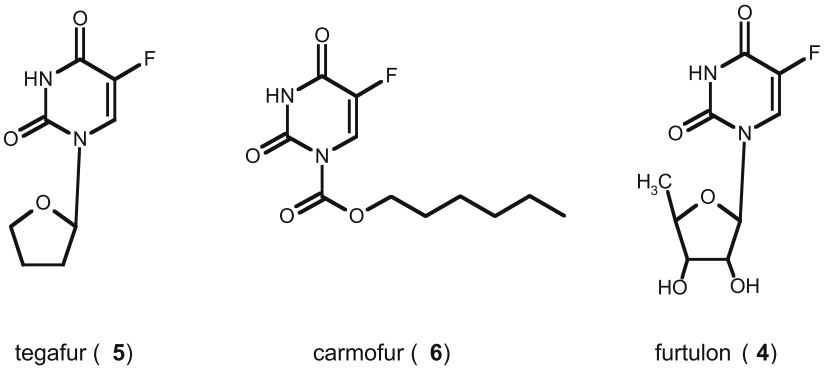

Figure 2. Some 5-fluorouracil (5-FU) prodrugs.

Cook *et al.* (1979) first synthesized 5'-DFUR (**4**), which had higher antitumor activity at broader dose ranges than did 5-FU in many tumor models. Lacking a 5'-hydroxyl group, 5'-DFUR cannot be directly activated by phosphorylation at the 5'-OH. Ishitsuka *et al.* (1980) found that 5'-DFUR acts as a prodrug to generate the active drug 5-FU by the action of pyrimidine nucleoside phosphorylase (PyNPase). In the case of mice, uridine phosphorylase (UdPase) is the enzyme responsible and in humans it is thymidine phosphorylase (dThdPase) (Kono *et al.*, 1983; Miwa et al, 1998). 5-FU is further converted to 2'-deoxy-5-fluoro-uridine

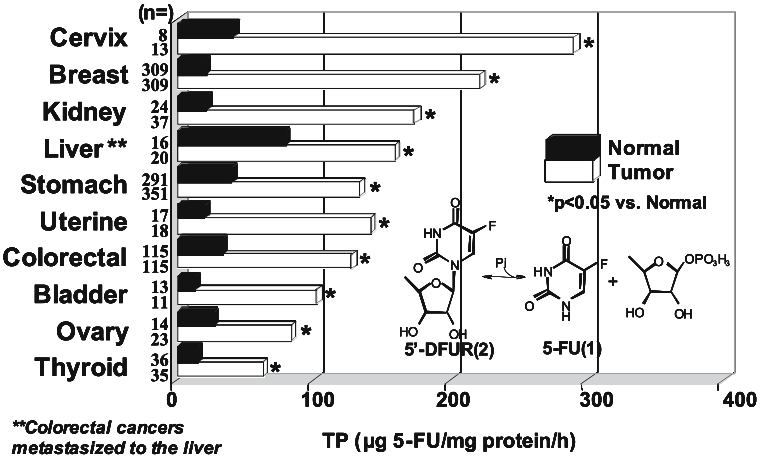

Figure 3. Distribution of TP activity in human tissues.

and finally to F-dUMP, a thymidylate synthase inhibitor, to exert antitumor activity. Thymidine phosphorylase activity is significantly higher in various human tumor tissues than in the adjacent normal tissues (Fig.3). This preferential localization of dThPase explains the high tumor-selective activity of **4**.

Oral 5'-DFUR was approved in Japan in 1987 for the treatment of breast, colorectal, and gastric cancer. However, the efficacy of 5'-DFUR is not strictly tumor selective and caused intestinal toxicity (diarrhea) when given orally at high dose. Since dThdPase also exists in intestinal tissues, 5-FU is generated when a high concentration of **4** passes through the intestinal tract. 5'-DFUR may also cause myelotoxicity, particularly when given at high doses, although the degree is generally mild and the incidence is low.

Taking 5'-DFUR as a lead, Miwa *et al.* (1998) and Shimma *et al.* (2000) developed a new orally available 5-FU prodrug that has a higher tumor-selective activity associated with little myelotoxicity and intestinal toxicity and, therefore, could be safely given for daily oral treatment over long periods. Namely, they designed new N^4-alkoxycarbonyl-5'-deoxycytidine derivatives (N^4-alkoxycarbonyl-5'-DFCR), which can be sequentially converted to 5-FU by enzymes preferentially located in the human liver and tumors after oral administration. This design was made based on the following considerations:

(1) The bone marrow toxicity of 5'-DFUR could be further reduced by 5'-deoxy-5-fluorocytidine (5'-DFCR) since it is known that cytidine deaminase acitivity is high in the liver, kidney, and solid tumors of humans, but low in leukemic myeloblast cells and immature, growing bone marrow progenitor cells compared to that in mature, normal granulocytes (Fig. 4). Thus, 5'-DFCR is not expected to be converted to 5'-DFUR

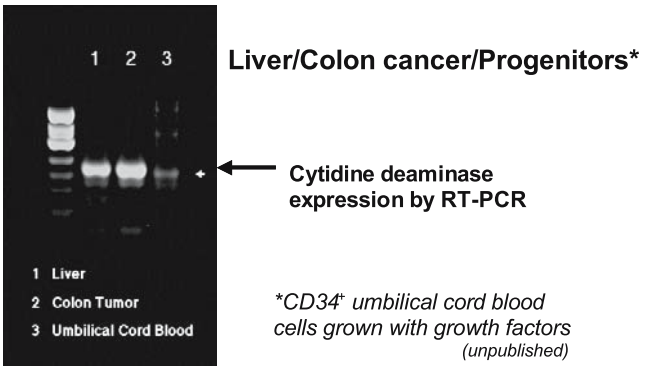

Figure 4. Cytidine deaminase low expression in granulocyte progenitors.

in rapidly growing bone marrow progenitor cells, but will be efficiently converted in solid tumor tissues. This design was supported by the fact that Ara-C (**7**), a potent DNA polymerase inhibitor, is active against leukemia but inactive against solid tumors because of its conversion to the inactive metabolite, Ara-

U, by cytidine deaminase (Chabner and Longo, 1996) (Fig. 5). Capecitabine, a derivative of 5'-DFUR, was later demonstrated to show minimal myelotoxicity in clinical trials.

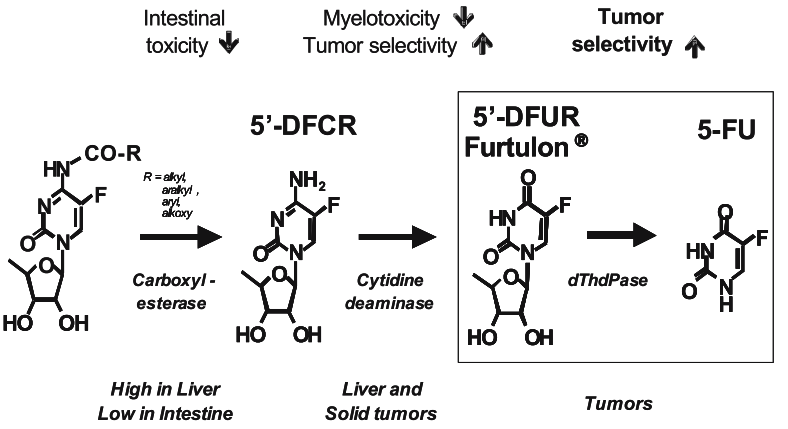

Figure 5. Design of tumor-activated prodrug of 5-FU.

(2) The intestinal toxicity of 5'-DFUR could be avoided by masking the 4-amino group of 5'-deoxy-5-fluorocytitdine (5'-DFCR) with an acyl or alkoxycarbonyl group. This prevents the conversion via 5'-DFCR and 5'-DFUR to 5-FU by the action of cytidine deaminase and dThdPase in the intestinal tract (certain levels of activities of both enzymes were found in human intestinal tract). The prodrugs were expected to then generate 5'-DFCR via an enzyme located in the liver.

Tumor selective Delivery of 5-FU

One of the most critical issues in the prodrug approach is the significant species difference in the substrate specificity of the key enzymes. Shimma *et al.* (2000) synthesized various N^4-alkoxycarbonyl-5'-DFCRs and screened them by the use of crude enzyme extracts from human liver and intestine for their liver-selective hydrolysis. Miwa *et al.* (1998) found that one isozyme of the 60 kDa carboxylesterase family (CE), an enzyme that converts N^4-alkoxycarbonyl-5'-DFCR to 5'-DFCR, was preferentially located in human liver. Very little was found in other normal tissues, including the intestine (Fig. 6). There was a clear correlation between the chain length of the N^4-alkoxy moiety and the rate of hydrolysis: *n*-pentyloxy (capecitabine) and *n*-hexyloxy were the optimal chain length to this liver-specific CE (Fig.7). The pharmacokinetic profiles of capecitabine indicate that it can pass through the intestinal mucosa unchanged and then be hydrolyzed into 5'-DFCR (3) by hepatic carboxylesterase.

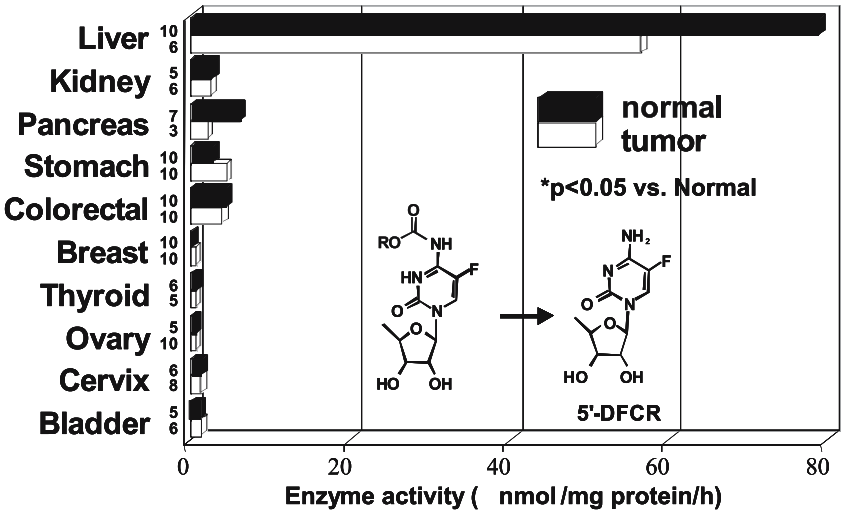

Figure 6. Distribution of carboxylesterase activity in human tissues.

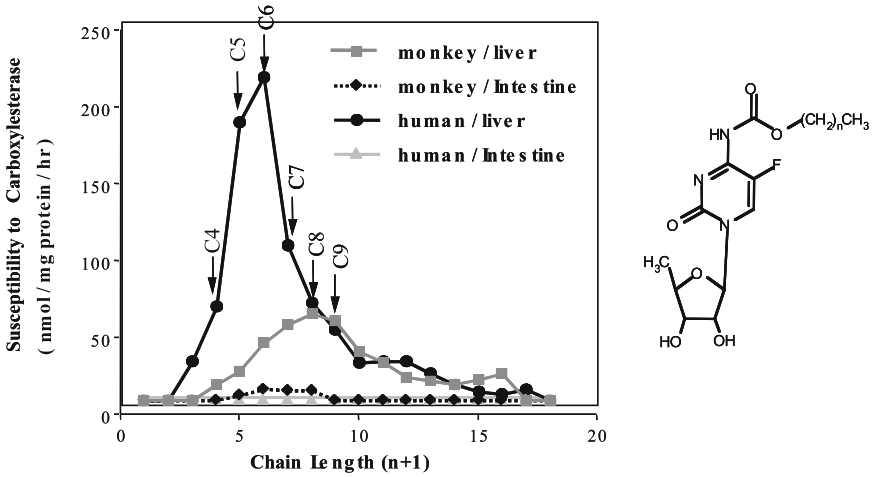

Figure 7. Correlation between the chain length and the susceptibility to carboxylesterase (monkey and human).

In separate experiments, the higher tumor selectivity of orally administered capecitabine *versus* intraperitoneal (i.p.) 5-FU in the HCT116 human colon cancer xenograft model in mice was confirmed by measuring 5-FU levels in tumor, muscle, and plasma after a single administration (Ishikawa *et al.*, 1998a,b,c; Fig. 8). In accordance with these results, capecitabine showed only mild intestinal tract and bone marrow toxicity in monkeys.

The degree of toxicity was less than that of oral 5'-DFUR even though capecitabine gave higher AUC and C_{max} values of 5'-DFUR in plasma (Kawashima and Horii, 1994). Moreover, capecitabine exhibited higher efficacy than 5'-DFUR and 5-FU in various human cancer xenograft models in nude mice, including colon cancer/CXF280, HCT116; gastric cancer/GXF97, MKN-45; and breast

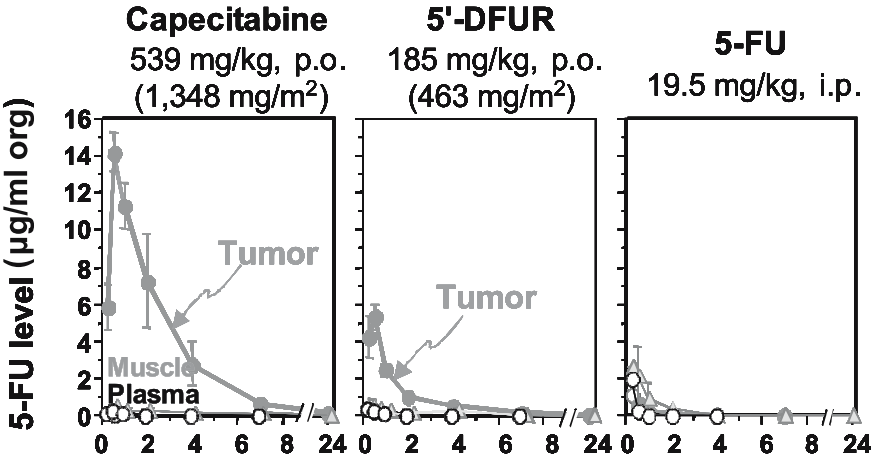

Figure 8. Tumor-selective conversion of capecitabine (p.o.) to 5-FU in human colon cancer xenograft model as compared with 5'-DFUR (furtulon, p.o.) and 5-FU (i.p.) at MTD.

cancer/MX-1, MAXF401 (Ishikawa *et al.*, 1998a,b,c). Capecitabine; N^4-pentyloxy-carbonyl-5'-deoxy-5- fluorocytidine (**2**) was finally identified as a tumor-activated and orally available prodrug of 5-FU.

Capecitabine Bioconversion

Capecitabine passes intact through the intestinal mucosa and selectively delivers 5-FU to tumor tissues by the following enzymatic conversion: first to 5'-deoxycytidine (5'-DFCR: **3**) by carboxylesterase in the liver, then to 5'-deoxy-5-fluorouridine (5'-DFUR: **4**) by cytidine deaminase in the liver and tumors (Kreis *et al.*, 1978), and finally to 5-FU by dThdPase, preferentially localized in tumor tissues (Fig. 1). This sequential metabolic activation was supported by PK studies in humans (Reigner *et al.*, 2001).

Formulation Issues

Capecitabine is formulated for oral use in tablet form. It is sold in both a 150 mg and a 500 mg dosage.

Discussion

One of the most interesting features of capecitabine is that its efficacy can be enhanced by dThdPase upregulation. The antitumor activity of capecitabine in mice depended on the activity of thymidine phosphorylase (dThdPase) in tumor tissues as well as the ratio of dThdPase to dihydropyrimidine dehydrogenase (DPD), a catabolic enzyme of 5-FU (Ishikawa *et al.*, 1998a,b,c). Some inflammatory cytokines, such as TNFα, IFNγα, IFNγ, and IL-1α, upregulated the dThdPase mRNA expression in tumor cells, making the tumor cells more

susceptible to 5'-DFUR (Eda *et al.*, 1993). Moreover, some cytotoxic drugs (Sawada *et al.*, 1998) such as taxans, cyclophosphamide, mitomycin C, and oxaliplatin as well as X-ray irradiation (Sawada *et al.*, 1999) also upregurated the activity of dThdPase in human cancer xenograft models. Interestingly, a similar dThdPase upregulation was not observed in the liver or intestine. The mechanism of this preferential effect on tumor tissues has not yet been fully clarified. If this process were also the case in humans, the efficacy of capecitabine would be optimized by selecting appropriate patients whose tumors highly express dThdPase or by combining it with dThdPase upregulators. O'Shaughnessy *et al.* (2002) reported the result of a Phase III clinical trial in which capecitabine used in combination with taxotere was shown to significantly improve survival compared with taxotere alone in patients with anthracycline-pretreated breast cancer. This is the first cytotoxic combination regimen to significantly improve survival over standard monotherapy.

Another approach for modulation of the efficacy of capecitabine is combination with a DPD inhibitor. Hattori *et al.* (2003) reported the tumor-activated prodrug of DPD inhibitor, RO0094889, for the enhancement of the antitumor activity of capecitabine xeloda, using the same prodrug strategy. The details of this approach are described in Chapter 3, Section 3.10., miscellaneous functional groups.

Conclusion

Capecitabine is the oral tumor-activated prodrug of 5-FU that can be sequentially converted to 5'-DFCR by carboxylesterase in the liver, to 5'-DFUR by cytidine deaminase highly expressed in the liver and tumor tissues, and finally to 5-FU by dThdPase preferentially located in various types of cancer tissues (Fig 1). Capecitabine monotherapy addresses the unmet need for an agent suitable for convenient, home-based treatment of patients with metastatic breast and colorectal cancers. Oral capecitabine enables chronic dosing and mimics continuous infusion of 5-FU without the associated inconvenience, cost and complications. Various clinical studies with capecitabine in combination with other antitumor agents are currently in progress.

Capecitabine studies demonstrate that a prodrug activation approach utilizing enzymes that are specifically expressed in certain tissues or tumors is useful for the targeting of cytotoxic drugs to tumor tissues.

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5.4

Case Study: Cefditoren Pivoxil: An Oral Prodrug of Cefditoren

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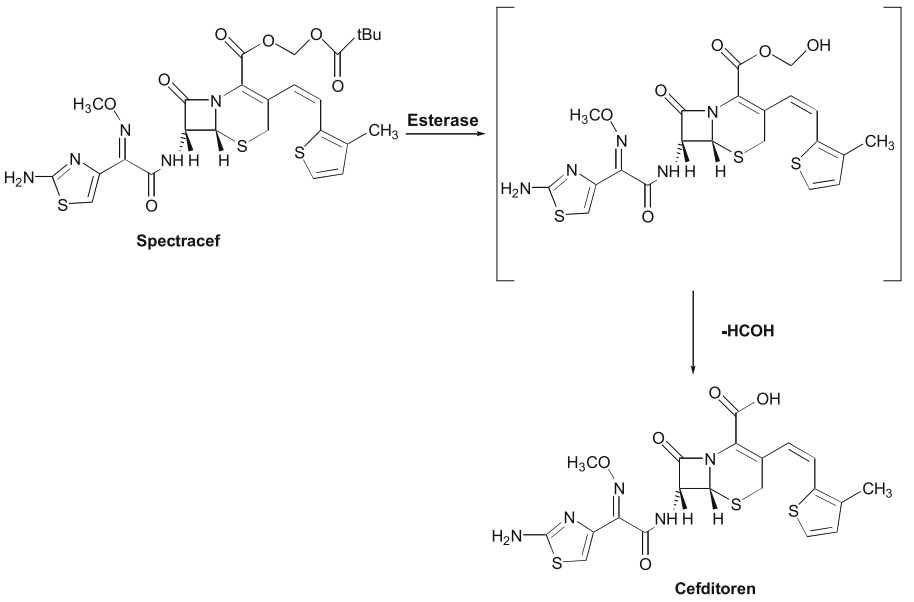
Table of Contents

Name:

Cefditoren pivoxil; (-)(6R, 7R)-2,2-dimethylpropionyloxymethyl 7-[(Z)-2-(2-aminothiazol-4-yl)-2-methoxyiminoacetamido]-3-[(Z)-2-(4-methylthiazol-5-yl)ethenyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate; Spectracef.®

Structures and Bioconversion Pathway



Rationale for Cefditoren Pivoxil

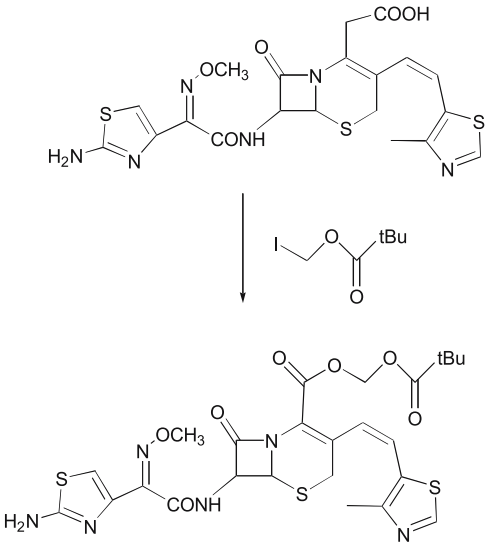
There is a continual need for new broad-spectrum antibiotics and, with this in mind, Sakagami and coworkers developed cefditoren pivoxil (Spectracef®) as an orally available prodrug of cefditoren (1990). Cefditoren has a 2-aminothiazole methoxime ring that imparts gram-negative activity (Balbisi, 2002) and a methylthiazole-substituted vinyl group at C-3 that maintains gram-positive activity (Sakagami *et al.*,1990). However, the hydrophilic carboxyl group of the parent drug makes cefditoren orally inactive due to poor permeation across the intestinal mucosa (Kees and Grobecker, 1995; Stoeckel *et al.*, 1995). Esterfication of the polar carboxyl group increases lipophilicity and allows intestinal absorption to occur (Kees and Grobecker, 1995). That is, cefditoren pivaloyl methyl ester is not biologically active, but after absorption esterases readily hydrolyze the prodrug to the biologically active cefditoren (von Daehne *et al.*,1970; Li *et al.*, 1997; Brass *et al.*, 2003).

The pivaloyloxymethyl functional group in Spectracef® is the same promoiety used to modify hydrophilic carboxyl groups in other penicillins and cephalosporins (von Daehne *et al.*, 1970; English *et al.*, 1990; Blouin and Stoeckel,

1993; Parsons *et al.*, 1997; Brass, 2002; Brass *et al.*, 2003). In fact, the pivaloyloxymethyl ester is one of many acyloxymethyl derivatives used to functionalize a wide range polar drugs and increase their bioavailability (Jansen and Russell, 1965; Stella, 1975; Bodor *et al.*, 1983; Bundgaard, 1985; Stella *et al.*;1999). While the alkyl group provides increased lipophilicity and absorption across biological membranes, the oxymethyl spacer group permits rapid hydrolysis by esterases and release of the active parent drug (von Daehne *et al.*, 1970; Yoshimura *et al.*, 1985; Silverman, 2004).

Synthesis

Sakagami *et al.* (1990, 1991) first reported the synthesis of cefditoren pivoxil (ME1206). Although the synthesis of cefditoren is complex, pivaloyloxymethyl ester synthesis is a relatively simple process, as illustrated below (Sakagami *et al.*, 1990).



Formation of Pivaloyl Oxymethyl Ester in Cefditoren Pivoxil (Sakagami et al., 1990)

Mechanism and Site of Bioreversion

The bioconversion of cefditoren pivoxil to cefditoren requires hydrolysis by intestinal cell (enterocyte) esterases. Hydrolysis yields pivalate and an unstable hydroxymethyl ester, which rapidly dissociates to formaldehyde and the parent acid, cefditoren (Stoeckel *et al.*, 1995; Li *et al.*, 1997). Thus, the byproducts of hydrolysis are pivalate and formaldehyde (Balbisi, 2002). The metabolism and toxicity of formaldehyde and pivalic acid, the released promoieties, are discussed below as well as in other chapters of this book. The parent cefditoren is not

extensively metabolized. Li and co-workers were only able to detect minor amounts of other metabolites stemming from cefditoren pivoxil dosing (Li *et al.*,1997).

Toxicity Issues

A previoius chapter discusses pivalic acid toxicity. As mentioned earlier, pivalic acid has been used to functionalize drugs for enhanced drug delivery in the past (English *et al.*, 1990; Boulin and Stoeckel, 1993; Parsons *et al.*, 1997; Brass *et al.*, 2003). Pivalate production can lead to a decrease in biological carnitine levels, an important component in metabolic processes involving coenzyme A (Brass, 2002). Since much of cartinine is stored in tissue, urinanalysis alone cannot accurately assess the total body content of cartinine (Brass *et al.*, 2003). Brass and coworkers report that short-term administration of cefditoren pivoxil leads to a 10% decrease in the body stores of cartinine, an amount not expected to result in adverse clinical effects; however, precautions need to be observed in renally impaired patients receiving this drug (Anon., 2003; Brass *et al.*, 2003). Caution is also recommended in cases where patients have had adverse reactions to other drugs containing the pivoxil ester promoiety (English *et al.*, 1990; Boulin and Stoeckel, 1993; Parsons *et al.*, 1997; Anon., 2003).

Formaldehyde production is not discussed extensively in the literature of cefditoren pivoxil; however, chromosomal aberrations were apparent in CHO cell incubations with the prodrug (Anon., 2003). This was attributed to formaldehyde production when the pivolate ester is hydrolyzed. However, other assays for mutagenesis and carcinogenesis were negative (Anon., 2003). Specific cases where formaldehyde is generated from prodrug metabolism are discussed elsewhere in this book.

Formulation Issues

Cefditoren pivoxil (Spectracef®), marketed by Purdue Pharma L.P., is a coated tablet containing 200 mg cefditoren as cefditoren pivoxil and inactive ingredients (croscarmellose sodium, D-mannitol, hydroxypropyl cellulose, hypromellose, magnesium state, sodium caseinate and sodium tripolyphosphate). The tablet is coated with carnauba wax, hypromellose, polyethylene glycol, and titanium dioxide (Anon., 2003).

Discussion

Cefditoren pivoxil, (Spectracef®) has been classified as a third-generation cephalosporin because its activity against gram-positive species was similar to that of augmentin and cefuroxime and superior to that of cefaclor and cefixime (Felmingham *et al.*, 1994). Furthermore, its β-lactamase stability was similar to that of cefixime and ceftazidine (Felmingham *et al.*, 1994). Cefditoren pivoxil is particularly active against *Staphylococcus spp*, *Streptococcus pneumoniae* and *Moraxella*

catarrhalis, making it useful for the treatment of respiratory tract infections (Felmingham *et al.*, 1994; Jones *et al.*, 1998; 2001). As such, the FDA approved Spectracef® in 2001. It is indicated for respiratory infections and skin infections (Balbisi, 2002). Cefditoren pivoxil has been used clinically in Japan since the 1990's for both adults and children (Mori *et al.*, 1993; Shimada, 1994; Sugita *et al.*, 1996). In Japan the antibiotic is used primarily to treat respiratory infections (Iwai *et al.*, 1994; Nakamura *et al.*, 1995).

The pharmacokinetics of cefuroxime pivoxil, reported by Li and co-workers, showed that drug bioavailability was increased when the drug was administered with food (1997, see Table 1). Li and coworkers report a 50–70% increase in the C_{max} and AUC when a high-fat meal was consumed with cefditoren pivoxil when compared to dosing with fasting (1997). When cefditoren pivoxil was taken with a low-fat meal, the absolute bioavailability of cefditoren was 16.1% ± 3%; when administered with a high fat meal, the absolute bioavailability increased to 25% (Darkes and Polsker, 2002; Anon, 2003). The bioavailabilities of similar diester prodrug antibiotics, including cefetamet pivoxil, are also reported to increase when administered with food (Finn *et al.*, 1987; Stoeckel *et al.*, 1995). It is possible that the presence of food inhibits pre-absorption esterase hydrolysis of the prodrug (Stoeckel *et al.*, 1995; Ruiz-Balaguer *et al.*, 2002).

Dose (mg)	**100 (fasting)**	**200 (fasting)**	**300 (fasting)**	**200 (after meal)**
Cmax (mg/L)	1.44	2.46	3.62	2.72
Tmax (h)	0.91	1.46	1.52	1.78
AUC (mg/L. h)	4.03	7.93	12.3	10.82
T1/2Ke (h)	1.51	1.22	1.22	1.41
V/F (L)	56.38	46.48	43.85	38.41

Table 1. Pharmacokinetic Parameters of Cefditoren Pivoxil (from Li *et al.*, 1997)

The ability of food to increase the bioavailability of certain prodrugs has been attributed to the presence of food slowing down the rate at which the intact prodrug arrives at its site of absorption in the intestines. There is little published on the mechanism of transport of cefditoren pivoxil across the intestinal mucosa (Saitoh *et al.*, 2002). Some suggest that certain β-lactams have a specialized transport system and that slowing the arrival of prodrug to the site of transport could prevent saturation and increase bioavailability (Tsuji *et al.*, 1987; Bretschneider *et al.* 1999, Ruiz-Balaguer *et al.* 2002; Zmuidinavicius, *et al.* 2003). However, others report that prodrug esters of cephalosporins passively diffuse

across cell membranes (Dantzig *et al.*, 1994; Ruiz-Balaguer *et al.* 2002). The presence of food may simply occupy the active sites of intestinal esterases that could hydrolyze cephalosporin prodrugs prior to absorption and decrease bioavailability (Dantizg *et al.*, 1994; Ruiz-Balaguer *et al.* 2002).

Because cefditoren pivoxil is used for respiratory infections, it is important to know that minimal concentrations of the drug reached respiratory tissue where the infections occur. Figure 1 shows the mean concentrations of drug in plasma, bronchial mucosa, and lining fluid (Darkes and Plosker, 2002) after dosing with cefditoren pivoxil. These concentrations are above the cefditoren MIC_{90} for many strains of bacteria (Darkes and Plosker, 2002)

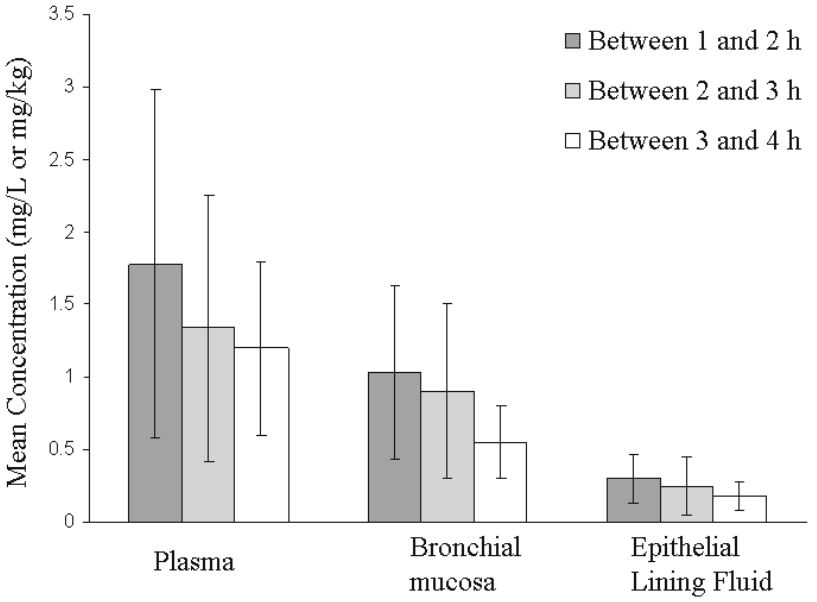


Figure 1. Penetration of cefditoren into respiratory tissue and epithelial lining fluid in patients undergoing fiber-optic bronchoscopy (n = 24). Cefditoren concentrations are expressed as mean values ± standard deviation over three 1-h collecting intervals (adapted from Darkes and Plosker, 2002)

Conclusions

The third-generation cephalosporin prodrug cefditoren pivoxil has reasonable bioavailability, especially when taken with a meal. The pivoxil prodrug is rapidly hydrolyzed to the active cefditoren, which has broad-spectrum antimicrobial properties that are desirable, particularly for the treatment of respiratory infections.

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5.5

Case Study: Cefuroxime Axetil: An Oral Prodrug of Cefuroxime

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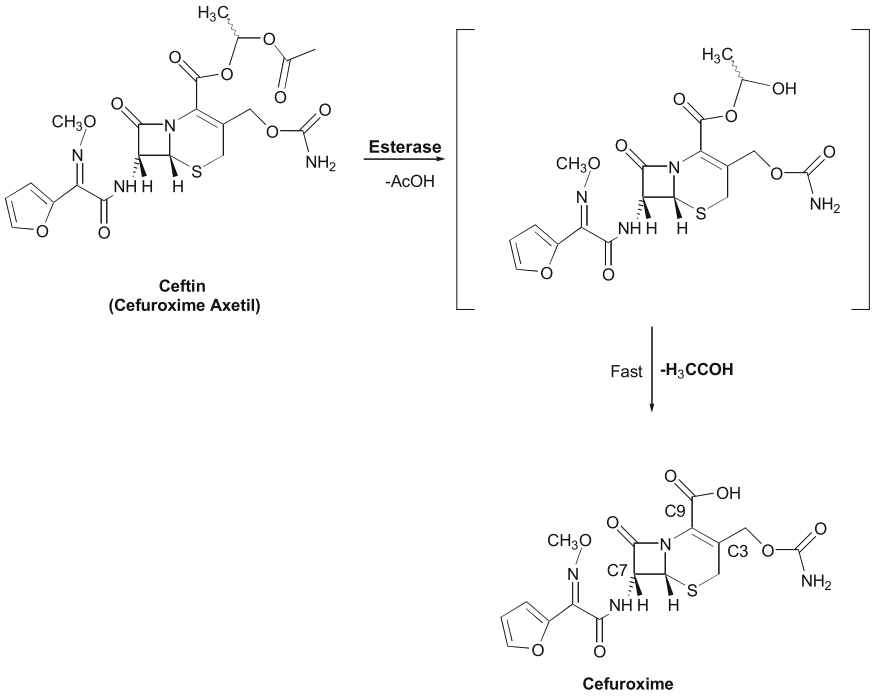
Table of Contents

Name:

Cefuroxime axetil; the 1-(acetyloxy) ethyl ester of cefuroxime; (RS)-1-hydroxyethyl (6R, 7R)-7-[2-(2-furyl)glyoxylamido]-3-(hydroxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]-oct-2-ene-2-carboxylate, 7²-(Z)-(O-methyl-oxime), 1-acetate-3-carbamate; Ceftin®; Zinnat®.

Structures and Bioconversion Pathway



Rationale for Cefuroxime Axetil

Cefuroxime axetil (Ceftin®, Zinnat®) was designed to be an oral form of cefuroxime (Zinacef®), a second-generation cephalosporin antibiotic available by IV and IM injection (O'Callaghan *et al.*, 1976; Brogden *et al.*, 1979; Emmerson, 1988). Cefuroxime is a useful antibiotic because it is a broad-spectrum antibiotic, effective against bacterial strains with β-lactamase activity (Dürckheimer *et al.*, 1985; Kees and Grobecker, 1995). The parenteral cefuroxime, like other cephalosporins, is not orally absorbed because it contains a highly polar carboxyl group that is ionized at intestinal pH, making transport across intestinal mucosa unlikely (Foord, 1976; Tsuji *et al.*, 1986). An attempt to deliver the free acid orally led to only 1% of the drug recovered in the urine (Foord, 1976).

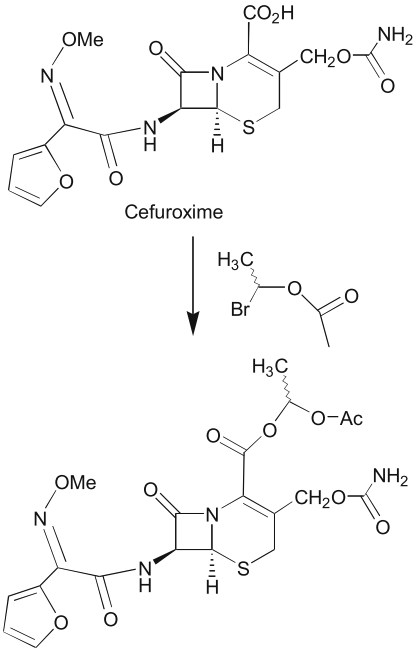
Research to develop an oral form of the parent cefuroxime began because parenterally it showed promise in clinical use (Harding *et al.*, 1984). One

successful approach to developing oral penicillins and cephalosporins has been to mask the carboxylic acid side chains by esterfication (Webber and Wheeler, 1982; Ferres, 1983; Bundgaard, 1985). Esters of these antibiotics have improved lipophilicity and enteral absorption, but they are not biologically active (Kees and Grobecker, 1995). To regain activity, esters must undergo rapid esterase hydrolysis after absorption occurs (Kees and Grobecker, 1995; Sasinowska-Motyl *et al.*, 1995). Since the esters themselves are not typically active, they are considered prodrugs (Kees and Groebecker, 1995). Diester prodrugs (for example, acyloxymethyl esters) of penicillins and cephalosporins have been developed because they are reasonably stable in the GI tract, lipophilic enough to cross the intestinal mucosa, and rapidly hydrolyzed, leading to release of the active parent drug into circulation (Bundgaard, 1985; Finn *et al.*, 1987; Kees and Grobecker, 1995; Silverman, 2004). Ceftin® (cefuroxime axetil) is the acetyloxy ethyl ester of cefuroxime. Ceftin® was the first oral, non-amino side chain cephalosporin antibiotic to be approved for clinical use.

An extensive review of the history of cefuroxime and cefuroxime axetil has been published alone and as part of the literature covering other examples of prodrugs (Moellering and Swartz, 1976; Brogden *et al.*, 1979; Perry and Brogden, 1996; Scott *et al.*, 2001).

Synthesis

The synthesis of cefuroxime axetil builds from 7-amino cephalosporanic acid (Huber *et al.*, 1972; Nomura *et al.*, 1974, Singh *et al.*, 2003). There are many methods patented for attaching the axetil promoiety, for example, reaction of the acid with 1-(acetoxy)ethyl bromide as shown below yields the diester (Hwang and



Ahn, 2002; Felisi *et al.*, 2003; Kansal *et al.*, 2003; Longoni *et al.*, 2003). Cefuroxime axetil is delivered as a diastereomeric mixture (about equal parts (R/S) 1-(acetyloxy)ethyl ester) (Mosher-Gerald *et al.*, 1992). There is no obvious difference in the activity of the diasteriomers since this stereocenter is removed from the cephem core and is hydrolyzed to acetaldehyde when acted upon by esterases (Mosher-Gerald *et al.*, 1992).

Mechanism and Site of Bioreversion

The bioconversion of cefuroxime axetil to cefuroxime requires hydrolysis by intestinal cell (enterocyte) esterases (Foord, 1976; Sasinowska-Motyl *et al.*, 1995). Esterase hydrolysis leads to acetic acid and an unstable hydroxyethyl ester, which rapidly dissociates to acetaldehyde and the parent cefuroxime (Bundgaard, 1985). An *in vitro* study indicates that the half-life of the axetil is 3.5 min in fresh human blood, but *in vivo* hydrolysis is so rapid that the intact axetil cannot be detected in the serum, even if a 1g dosage is administered orally (Harding *et al.*,1984).

Toxicity Issues

Both acetic acid and acetaldehyde can be acted on rapidly by metabolic enzymes and thus are essentially non-toxic byproducts. The parent cefuroxime remains largely unmetabolized and is excreted unmodified in urine (Foord, 1976). As with many antibiotics, disturbances of natural gut flora cause some abdominal distress in patients treated with Ceftin® (Novelli *et al.*, 1995).

Formulation Issues

Ceftin®, marketed by GlaxoSmithKline, is available in two formulations: the film-coated tablet contains 250 or 500 mg of the drug along with inactive ingredients (colloidal silicon dioxide, croscarmellose sodium, FD&C Blue No. 1, hydrogenated vegetable oil, hypromellose, methylparaben, microcrystalline cellulose, propylene glycol, propylparaben, sodium lauryl sulfate, and titanium dioxide) and the oral suspension contains 125 or 250 mg of drug with inactive ingredients (acesulfame potassium, aspartate, povidone K30, stearic acid, sucrose, tutti-frutti flavoring, and xanthan gum), which is to be suspended in 5 mL water (Anon., 2003a).

Discussion

The parent cefuroxime was approved for treatment of respiratory infections caused by *Streptococcus pyogenes* and *pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus* as well as urinary tract infections and uncomplicated gonorrhea (Anon., 2003b). Cefuroxime axetil is used for similar infections as well as skin infections caused by *Stapylococcus aureus* and *Streptococcus pyogenes* (Henry *et al.*, 1995; Langan *et al.*, 1998; Scott *et al.*, 2001; Anon., 2003a). The oral

suspension is approved for pediatric treatment of throat, ear, and skin infections (Carson, 1987; Anon., 2003a).

Pharmacokinetic parameters for oral dosage of the currently marketed film coated tablets are shown in table 1 (Anon., 2003a). Oral suspension pharmacokinetics has also been reported, especially in the pediatric population. Interestingly, the oral suspension is not bioequivalent to the ceftin® tablets.

Dose (cefuroxime equiv.)	Peak Plasma Concentration (mcg/mL)	Time of Peak Plasma Concentration (h)	Mean Elimination Half-Life (h)	AUC (mcg-hr mL)
125 mg tablet	2.1	2.2	1.2	6.7
250 mg tablet	4.1	2.5	1.2	12.9
500 mg tablet	7.0	3.0	1.2	27.4
1,000 mg tablet	13.6	2.5	1.3	50.0
250 mg/5mL suspension	2.23	3	1.40	8.92

Table 1. Showing Pharmacokinetics of Cefuroxime Administered Immediately Following a Meal (Anon, 2003a).

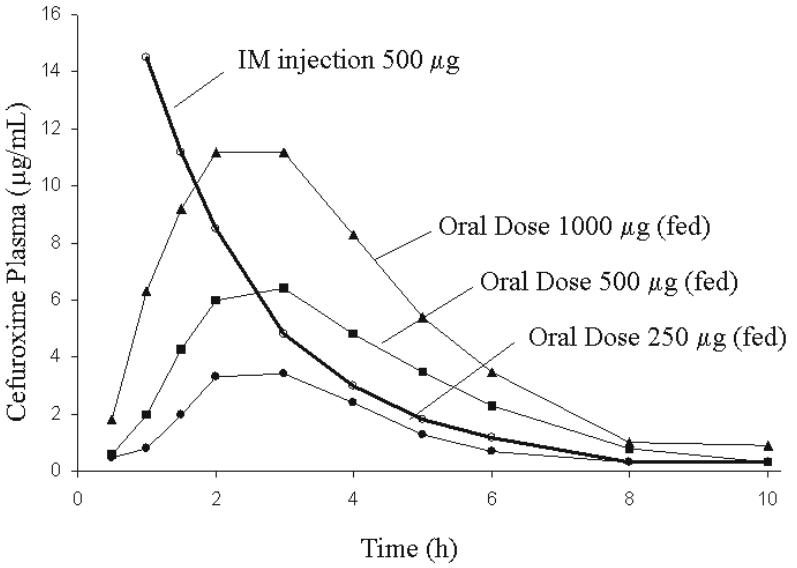


Figure 1. Serum levels of cefuroxime after single 1 g oral dose in 12 male and 11 female volunteers after fasting and after food. NOTE: oral dosage formulation used here may be different from the film coated tablets use today. Plot extracted from Williams and Harding of Glaxo Group Research Limited (Williams and Harding,1984).

By comparing a 1g oral dosage of cefuroxime axetil to a 1g IM dosage of cefuroxime, Williams and Harding determined the absolute bioavailability of the cefuroxime axetil to be 32% when fasting and 43% with food (1984). Finn and workers also compared the IV dosage of parent drug to the oral dosing (1987). Figure 1 shows the plasma concentration vs. time curve comparing a single injection dosage to multiple oral doses with and without food (Finn *et al.*, 1987).

Many oral pharmacokinetic studies were performed with and without food intake to assess the effect of food on the absorption rate and bioavailability. Cefuroxime axetil is not 100% bioavailable. Approximately 25–45% of the total available cefuroxime can be detected in the blood or in the urine in the 24 h after administration of the axetil prodrug (Harding, *et al.*, 1984; Sommers *et al.*, 1984; Finn *et al.*, 1987; Lang *et al.*, 1990; Kees *et al.*, 1991). In contrast to oral administration of many non-prodrug antibiotics (Welling and Tse, 1982), many researchers have consistently found that the cefuroxime axetil is absorbed up to 50% more when administered after food. This is particularly the case after a fatty meal (Harding *et al.*, 1984, Williams and Harding, 1984; Finn *et al.*, 1987; Perry and Brogden, 1996; Ruiz-Balaguer *et al.*, 1997; Scott *et al.*, 2001). Williams and Harding demonstrated a statistically significant increase in urinary recovery and blood serum levels of cefuroxime when cefuroxime axetil was administered after a meal compared to administration with fasting (1984, Harding *et al.*, 1984). Below is a plot of serum level of cefuroxime vs. time from fasting and fed subjects (Harding *et al.*, 1984).

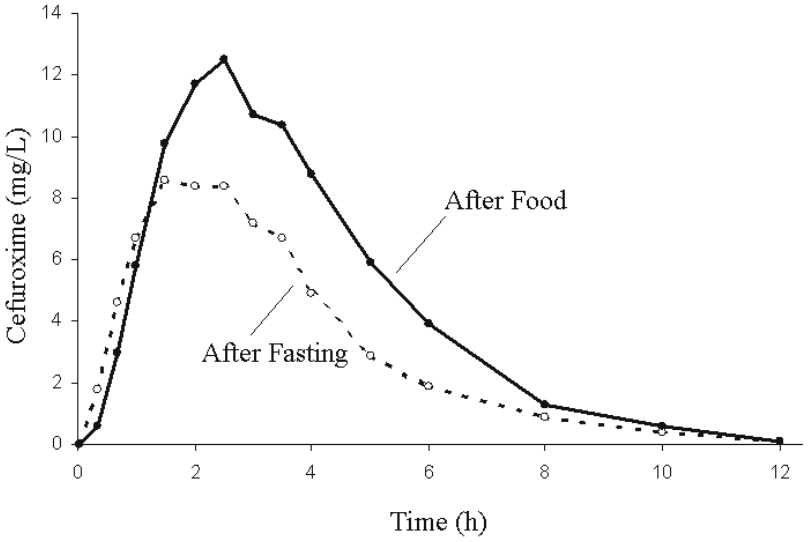


Figure 2. Mean Cefuroxime Concentration. Plot extracted from Finn, Straughn, Meyer and Chubb of Glaxo Inc. (Finn, 1987).

The effect of food is not fully understood (Ruiz-Balaguer *et al.*, 1997; Ruiz-Balaguer *et al.*, 2002). Two factors maybe contribute to the observation. Some suggest that there is a specialized transport system responsible for prodrug uptake

(Dürckheimer, 1985; Kees *et al.*, 1991; Ruiz-Balaguer *et al.*, 2002) that impacts prodrug absorption. The presence of food in the stomach may slow the arrival of the prodrug at the absorption site in the intestinal lumen so that the carriers do not become saturated (Kees *et al.*, 1991; Ruiz-Balaguer *et al.*, 1997). The other possibility is that there are esterases present in the lumen that hydrolyze the prodrug prior to its being absorbed by the mucosa cells (Mosher-Gerold *et al.*, 1992; Dantzig *et al.*, 1994;) and, in the case of fed patients, food may increase bioavailability by occupying luminal esterases as the prodrug makes its way to mucosa cells for absorption (Ruiz-Balaguer *et al.*, 1997; Ruiz-Balaguer *et al.*, 2002). Ruiz-Balaguer and coworkers performed rat-gut perfusion studies in an effort to better understand the mechanism of transport (1997). Their studies do indicate that some type of active-transport system may be operative and that it could become saturated. Their study attempts to add to the understanding of the less than optimal absolute bioavailability of cefuroxime axetil. Ruiz-Balaguer and coworkers also observed hydrolysis of the prodrug in the perfusate, indicating that hydrolysis of the prodrug in the luminal contents is, in part, responsible for the less than 100% bioavailability (1997).

Conclusion

Cefuroxime axetil is an excellent early example of a lipophilic prodrug of a polar drug that has been successfully applied to increase the oral availability of a drug previously available only in its parenteral form.

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5.6

Case Study: Clindamycin 2-Phosphate, A Prodrug of Clindamycin

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Kalamazoo, MI

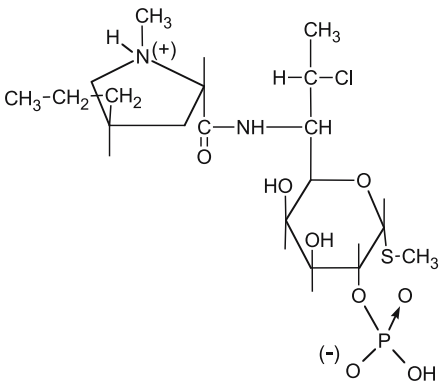
H. A. Karnes
Bridge Organic Chemicals
Vicksburg, MI

Table of Contents

Name:

Clindamycin 2-phosphate; L-threo-α-D-galacto-octopyranoside, methyl 7-chloro-6,7,8-trideoxy-6-[[(1-methyl-4-propyl-2-pyrrolidinyl)carbonyl]amino]-1-thio-,2-di hydrogen phosphate or U-28,508. CAS Registry Number, 24729-96-2.

Structure



Clindamycin 2-Phosphate (I)

$C_{18}H_{34}ClN_2O_8PS$

MW is 504.96

Use

The prodrug clindamycin 2-phosphate is rapidly converted *in vivo* to the parent drug, clindamycin, by phosphatase ester hydrolysis. Clindamycin is an antibacterial agent used orally in treating gram-positive infections, anaerobic infections, and methicillin-resistant staphlococcal and streptococcal infections. Clindamycin 2-phosphate, unlike clindamycin, is highly water soluble and does not produce pain upon injection.

Clindamycin 2-phosphate is marketed in sterile aqueous solutions for parenteral administration as well as in topical (skin) formulations and intravaginal formulations. Slow phosphatase-catalyzed hydrolysis of clindamycin 2-phosphate to clindamycin occurs upon topical application, thereby eliminating the incidence of antibiotic-induced GI side effects. Intravaginal formulations of clindamycin 2-phosphate result in more extensive conversion to clindamycin; however, blood levels are markedly lower than those achieved upon oral administration of clindamycin HCl.

Rationale for the Utility of Clindamycin 2-Phosphate in the Development of IV/IM Parenteral Formulations and Topical Formulations

Clindamycin 2-phosphate was designed as a highly water soluble form of the poorly water soluble parent compound clindamycin with potential for reduced pain upon injection. It was proposed that clindamycin 2-phosphate would be highly water soluble and, therefore, useful in developing IV/IM injectable formulations with reduced injection pain. Later, it was proposed that clindamycin 2-phosphate could be useful in developing topical (skin) as well as intravaginal formulations with reduced systemic absorption and, as a result, reduced GI side effects.

Parenteral Formulations of Clindamycin 2-Phosphate

Rationale for Reduced Pain Upon IV/IM Injection

The proposal of reduced pain upon injection was based on the markedly lower calculated log D value of clindamycin 2-phosphate (log D, –1.87 at pH 7; ACD Labs) compared to that of clindamycin (log D +0.41, pH 7; ACD Labs) and the probable rapid diffusion of clindamycin 2-phosphate away from the injection (IM) site. It was proposed that the highly polar nature of clindamycin 2-phosphate should minimize intercellular diffusion into the surrounding tissues due to the low log D value of the prodrug, and this could reduce the pain upon injection (Morozowich and Lamb, 1969, 1970; Gray *et al.*, 1974). Clindamycin HCl precipitates at the site of injection after IM administration due to the low water solubility of the free base (~0.5 mg/mL, pH 7.4), and a phosphate ester should prevent preciptation (Gray *et al.*, 1974). Highly polar compounds with low log D values are not readily transported across cellular membranes and, thus, clindamycin 2-phosphate had potential for reduced pain upon injection.

Clinical Observations

After synthesizing clindamycin 2-phosphate, a highly soluble, stable aqueous solution formulation was developed with clindamycin 2-phosphate (Riebe and Oesterling, 1972), and the phosphate prodrug was found to be free of necrosis upon intramuscular injection in rats (Gray *et al.*, 1974). The phosphate prodrug was well tolerated by IM and IV administration with less pain and irritation upon injection than was observed with clindamycin HCl as shown in animal and human studies (Gray *et al.*, 1974; PDR, 2005).

Topical Formulations of Clindamycin 2-Phosphate

Rationale for Reduced GI Side Effects with Topical and Intravaginal Formulations

It was proposed that clindamycin 2-phosphate would be slowly hydrolyzed upon topical (skin) application as well as upon intravaginal application due to limited hydrolysis of the prodrug by the phosphatase enzymes on the surface and within the skin (Guin *and Lummis*, 1982; Pena, 1994; Pena *et al.*, 2002; Amr *et al.*, 2001). The subsequent generation of low levels of free clindamycin upon topical application of clindamycin 2-phosphate should reduce the incidence of GI side effects such as diarrhea and pseudomembranous colitis upon either topical or intravaginal application of clindamycin 2-phosphate.

Clinical Observations

In the clinic, topical application (skin) of clindamycin 2-phosphate (Cleocin® T) was found to be effective in treating acne and related skin bacterial infections with very little systemic absorption of clindamycin. There was no change in the colon microflora in humans after topical (skin) application of clindamycin 2-phosphate (Borglund *et al.*, 1984), thus indicating that topical clindamycin 2-phosphate should be free of the GI side effects associated with clindamycin. Topical administration of clindamycin HCl, however, results in appreciable (4–5%) absorption with even higher amounts absorbed in some patients. The risk of GI side effects is considerably higher with topical clindamycin HCl than with topical clindamycin 2-phosphate (Barza *et al.*, 1982).

Topically applied clindamycin 2-phosphate (Cleocin® T Solution) containing the equivalent of 10 mg of clindamycin resulted in recovery of less than 0.020 mg of free clindamycin in the urine in humans (PDR, 2005). The urinary recovery of free clindamycin from patients dosed orally with 150 mg of the parent compound, clindamycin HCl, orally was 15 mg (PDR, 2005). Thus, the % of clindamycin in the urine following a topical dose of clindamycin 2-phosphate (Cleocin® T Solution, 10 mg) is less than 0.13% of the amount of clindamycin excreted in the urine following oral administration of a 150 mg capsule formulation of clindamycin HCl (Cleocin). The exceptionally small amount of clindamycin (<0.020 mg) excreted in the urine following topical administration of clindamycin 2-phosphate (Cleocin T Solution) to the skin suggests that topical application of clindamycin 2-phosphate should be virtually free of GI side effects, and this is borne out in the clinic (Meadowcroft *et al.*, 1998).

In conclusion, topical administration of clindamycin 2-phosphate solution (Cleocin® T Solution) results in zero-order generation of low levels of free clindamycin (Eller *et al.*, 1989) that are effective in treating skin infections. The extent of systemic absorption of free clindamycin is exceptionally low and, as a result, the incidence of GI side effects is virtually zero.

In the case of intravaginal application of clindamycin 2-phosphate, the extent of systemic absorption of clindamycin was 4% with the clindamycin 2-phosphate vaginal cream formulation (Cleocin® T Vaginal Cream) and 30% with the clindamycin 2-phosphate ovule formulation (Cleocin® T Vaginal Ovule). The systemic exposure of clindamycin from the cream and the ovule formulation is 2–20-fold lower than the blood levels achieved with a therapeutic dose (125–250 mg) of clindamycin HCl administered orally (Borin *et al.*, 1999; PDR, 2005).

In conclusion, the incidence of GI side effects with clindamycin 2-phosphate vaginal cream formulations administered topically is very low. The blood levels of clindamycin resulting from the clindamycin 2-phosphate vaginal ovule formulation are about threefold less than the blood levels achieved with a therapeutic dose (125–250 mg) of clindamycin.HCl administered orally (Borin, 1995; PDR, 2005).

Physicochemical Properties

Clindamycin 2-phosphate occurs as a crystalline zwitterionic form (I) with pKa values of 0.9 and 6.5. The pKa values of clindamycin 2-phosphate are 0.96 and 6.06 in water at 21°C (Kipp and Hlavaty, 1991). The ClogP value of clindamycin 2-phosphate is 1.25 (ACD Labs), and the compound is soluble in water at pH 8 (ACD Labs). The solubility in water of clindamycin 2-phosphate is ~80 mg/mL as the hydrate (Resman *et al.*, 1996), and the solubility increases above pH 6.5 due to formation of the mono-anionic species.

Synthesis of Clindamycin 2-Phosphate

Figure 1 shows the scheme for synthesis of clindamycin 2-phosphate starting with conversion of clindamycin to 3,4-O-anisylidene clindamycin followed by phosphorylation with POCl3 to give 3,4-O-anisylidene clindamycin 2-phosphate (Morozowich *et al.*, 1969; Karnes, 1978). The anisylidene protective group is removed with 80% aqueous acetic acid and the product, clindamycin 2-phosphate, is recrystallized from hot ethanol-water. A number of alternate methods for the synthesis of clindamycin 2-phosphate have been reported (Karnes, 1978; Matier *et al.*, 1989; Tobkes *et al.*, 1993; Chen, 2002; Liu *et al.*, 2002).

Assay

Clindamycin 2-phosphate can be determined using reversed-phase chromatography with low wavelength detection along with aqueous acetonitrile or methanol mixtures (Munson and Kubiak, 1985; Ye *et al.*, 2004).

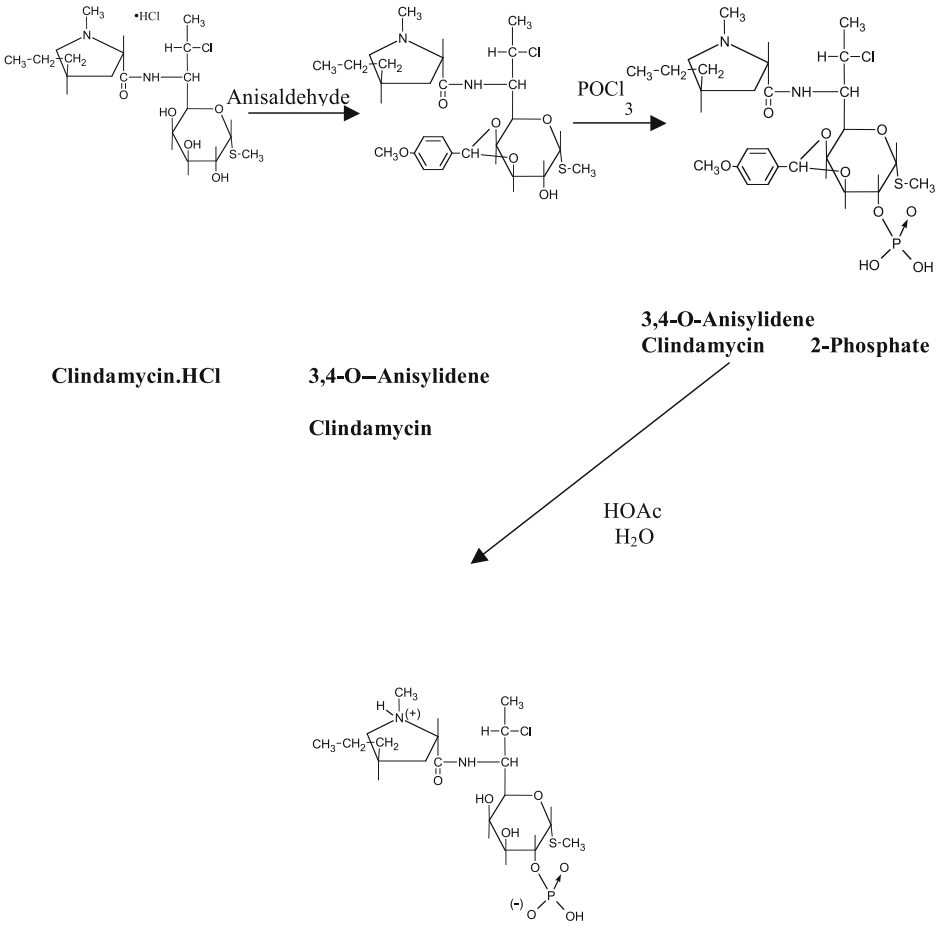


Figure 1. Synthesis of clindamycin 2-phosphate using 3,4-O-anisylidene clinda¬mycin and phosphorylation with POCl3 (Morozowich *et al.*, 1969).

Stability of Clindamycin 2-Phosphate in Water and in Aqueous Dextrose

Aqueous solutions of clindamycin 2-phosphate show a maximum stability at pH 3.5–6.5 (Oesterling and Rowe, 1970; Nahata *et al.*, 1993). The T_{90} for clindamycin 2-phosphate in 5% aqueous dextrose, pH 6, is 55.5–57.8 months at 25ºC (Kipp and Hlavaty, 1991).

Bio-conversion and Blood Levels

Following IV infusion in humans, clindamycin 2-phosphate is rapidly converted to clindamycin by alkaline phosphatase (Figure 2), and the peak concentration of clindamycin is observed shortly after terminating the infusion (Figures 3 and 4).

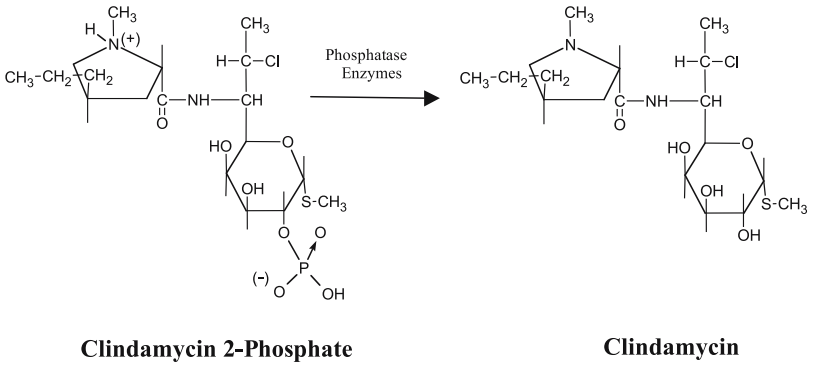


Figure 2. *In vivo* conversion of clindamycin 2-phosphate to clindamycin by alkaline phosphatase.

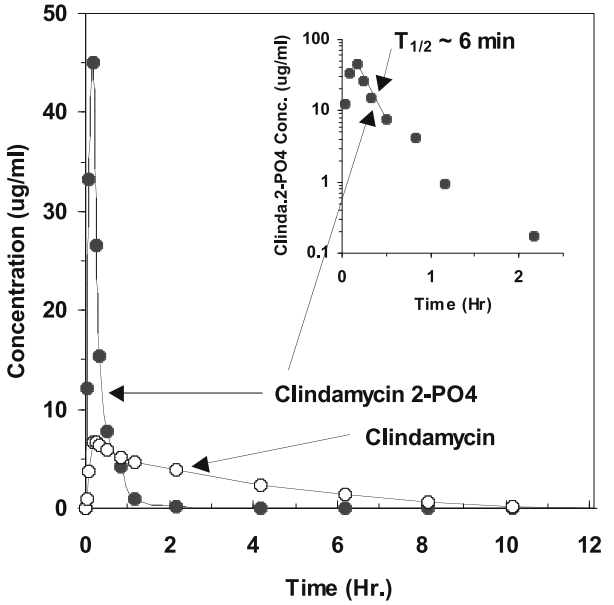


Figure 3. Blood levels in humans (n = 16) of clindamycin 2-phosphate and clindamycin after IV administration of 150 mg of clindamycin 2-phosphate in 50 mL of water using a constant rate infusion over 10 min (PDR, 2005; Smith and Phillips, 1982).

The 2-phosphate ester of clindamycin is rapidly hydrolyzed to clindamycin after IV administration (Cleocin Phosphate® Sterile Solution, ADD-Vantage Vial) in humans with a $T_{1/2}$ of 6 min according to the PDR (PDR 2005; see reference to Smith and Phillips, 1982).

The 3- and the 4-phosphate esters of clindamycin have a lower oral bioavailability in rats (Brodasky and Lewis, 1972). The lower bioavailability of clindamycin 4-phosphate may be due to slow phosphatase hydrolysis of the 4-phosphate ester due to the steric effect at the C4-position. The enzymatic hydrolysis of clindamycin 3-phosphate occurs along with partial isomerization to

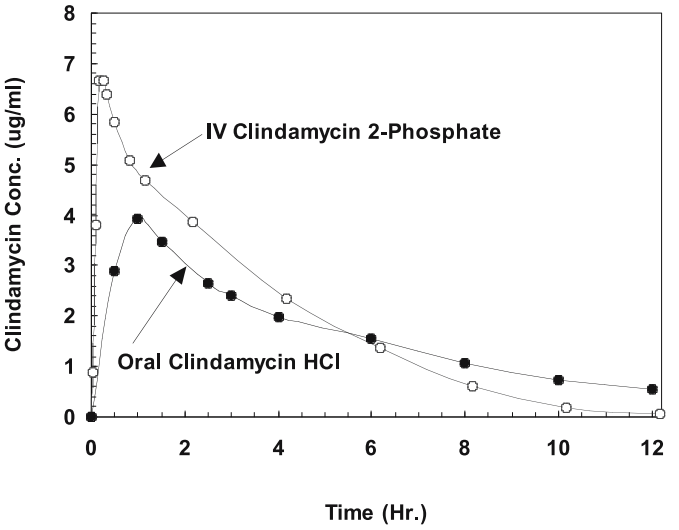


Figure 4. Average blood levels in humans after administration of clindamycin 2-phosphate Ìintravenously (IV) and after oral administration of clindamycin HCl at a dose of 300 mg free base equivalents (PDR, 2005; Smith and Phillips, 1982).

clindamycin 4-phosphate. The 2-phosphate of clindamycin, however, cannot undergo isomerization due to the trans configuration of the C2–C3 diol. However, the C3–C4 diol has a *cis* configuration and, as a result, isomerization can readily occur in the case of clindamycin 3-phosphate.

In conclusion, the synthesis of clindamycin 2-phosphate is achieved by protection of the 3,4-diol moiety in clindamycin, formation of the phosphate ester followed by deprotection, purification and recrystallization of clindamycin 2-phosphate. Although these additional steps increased the bulk drug cost, the phosphate prodrug proved to be essential in developing marketable IV as well as topical delivery formulations of clindamycin. The utility of clindamycin 2-phosphate in overcoming GI side effects with topical formulations is novel.

Safety

Clindamycin 2-phosphate showed an oral LD50 of 1832 (1500–2336) mg/kg and an IV LD50 of 855 (806–908) mg/kg in the rat and these values are somewhat higher than those of clindamycin HCl (Gray *et al.*, 1974).

Marketed Formulations Listed in the Physicians' Desk Reference (PDR)

Benzaclin® Topical Gel (Dermik), 1% Clindamycin Phosphate (free base equivalents), 5% benzoyl peroxide in carbomer, dioctyl sodium sulfosuccinate, and water.

Cleocin Phosphate®, Sterile Solution (Pharmacia & Upjohn), Clindamycin Phosphate, 150 mg/mL (free base equivalents) in water containing 9.45 mg/mL of benzyl alcohol and 0.5 mg/mL EDTA.

Cleocin Phosphate®, Sterile Solution in the ADD-Vantage Vial for IV Use Only (Pharmacia & Upjohn), Clindamycin Phosphate, 150 mg/mL (free base equivalents) in water containing 9.45 mg/mL of benzyl alcohol and 0.5 mg/mL EDTA.

Cleocin Phosphate® Sterile Solution in the Galaxy Plastic Container for IV Use (Pharmacia & Upjohn), Clindamycin Phosphate, Sterile Solution, 150 mg/mL (free base equivalents), in 5% aqueous dextrose.

Cleocin T®, Topical Gel (Pharmacia & Upjohn), 1% Clindamycin Phosphate (free base equivalents) containing allantoin, carbomer 934P, and propylene glycol in water.

Cleocin T®, Topical Lotion (Pharmacia & Upjohn), 1% Clindamycin Phosphate (free base equivalents) containing cetostearyl alcohol, glycerin isostearyl alcohol, sodium lauroyl sarcosinate and water.

Cleocin T®, Topical Solution (Pharmacia & Upjohn), 1% Clindamycin Phosphate (free base equivalents) in 50% isopropyl alcohol, propylene glycol and water.

Cleocin®, Vaginal Cream (Pharmacia & Upjohn), 2% Clindamycin Phosphate (free base equivalents) containing benzyl alcohol, cetostearyl alcohol, mineral oil polysorbate 60, and water.

Cleocin®, Vaginal Ovules (Pharmacia & Upjohn), Clindamycin Phosphate, 100 mg/ovule (free base equivalents) in a triglyceride base.

Clindagel®, Topical Gel (Galderma), 1% Clindamycin Phosphate in carbomer 941, polyethylene glycol 400, and water.

Clindets®, Plegdet Applicator (Stiefel), 1 mL Cleocin T Topical Solution containing 1% Clindamycin Phosphate in 50% isopropyl alcohol, propylene glycol, and water.

Duac™ Topical Gel (Stiefel), 1% Clindamycin Phosphate, benzoyl peroxide, 5% in carbomer 940, in glycerin and water.

Acknowledgments

P. D. Meister, D. J. Lamb, A. A. Sinkula, T. O. Oesterling, K. W. Riebe, E. L. Rowe, E. Novak, F. E. Young, J. R. Munting and S. L. Douglas are acknowledged for their contribution in the synthesis and development of the IV/IM formulations and L. E. Pena and co-workers are acknowledged for developing the topical and vaginal formulations of clindamycin 2-phosphate.

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5.7

Case Study: Enalapril: A Prodrug of Enalaprilat

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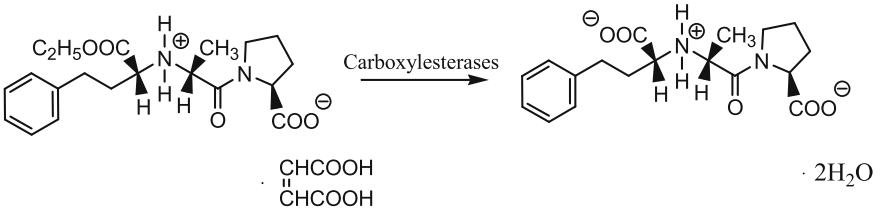
Table of Contents

Name:

Enalapril, (*S*)-[(N-(1-ethoxycarbonyl)-3-phenylpropyl)-L-alanyl)]-L-proline, (*Z*)-2-butenedioate salt (1:1), Vasotec®.

Structure and Bioconversion Pathway



Enalapril Enalaprilat

Rationale for Enalapril

Enalaprilat is a potent and reversible inhibitor of the plasma and tissue angiotensin-converting enzyme (ACE); it is used clinically in the treatment of hypertension (Patchett *et al.*, 1980; Gross *et al.*, 1981). Although enalaprilat has potent and prompt therapeutic activity when administered intravenously, its high polarity and transport characteristics cause it to be poorly absorbed from the gastrointestinal tract. This leads to extremely low bioavailability of enalaprilat when administered orally (Biollaz *et al.*, 1982).

To enhance gastrointestinal absorption of enalaprilat with the intent to increase bioavailability, an ethyl ester promoiety was incorporated into the parent drug. Enalapril as its maleate salt is an orally administered prodrug of enalaprilat. When administered orally, enalapril undergoes *in vivo* ester hydrolysis in the liver to form enalaprilat (Sweet *et al.*, 1981).

Esterification of enalaprilat to enalapril enhances the affinity of enalapril for the intestinal peptide carrier-mediated transport system, thereby increasing the intestinal absorption of enalaprilat and resulting in improved bioavailability of enalaprilat. Enalapril and enalaprilat are dipeptide (Ala-Pro) derivatives, and thus are potential substrates of intestinal carrier-mediated transporters (Friedman and Amidon, 1989, Swaan *et al.*, 1995). Both enalapril and enalaprilat bind to intestinal carrier-mediated transporters; however, it is enalapril that is selectively transported across the intestinal membrane (Swaan *et al.*, 1995).

The absence of an ester moiety on enalaprilat leads to the ionization of both carboxylate moieties at physiological pH. The proline carboxylate moiety is essential for recognition by both the intestinal peptide transporter and the angiotensin-converting enzyme (ACE), therefore the presence of the second ionized carboxylate is thought to have an inhibitory effect on the intestinal transport of enalaprilat (Swaan *et al.*, 1995). Thus, preventing the formation of a

negative charge by conversion of the carboxylate to an ester can reestablish affinity to the intestinal peptide transporter and subsequently increase intestinal absorption.

The prodrugs of ACE inhibitors under investigation were all esterified on the same carboxylate moiety, suggesting that the promoiety plays a structural role in binding the prodrug to the peptide carrier, thereby altering the physicochemical characteristics of the parent drug (Friedman and Amidon, 1989).

Synthesis

Synthesis of enalapril as its maleate salt was first reported by Patchett *et al.* (1980). In a later report Wyvratt *et al.* (1984) presented modifications to the original synthetic methodology. A recent patent (Tien and Liu, 2003) that claims to minimize by-product generation and to enhance yields in the commercial manufacture of enalapril has been issued. However, the currently employed method of commercial manufacture is unknown to this author.

Mechanism and Site of Bioreversion

The bioreversion of enalapril to enalaprilat is performed by the carboxylesterase family of enzymes present in animal and human livers (Ulm, 1983). Subsequent to the gastrointestinal absorption of enalapril, these carboxylesterase enzymes hydrolyze the monoethyl ester in enalapril to produce enalaprilat (Gross *et al.*, 1981; Ulm, 1983). This hydrolysis of enalapril has been reported to occur specifically within the hepatic cells as incubation of enalapril with bile or fresh blood perfusate did not readily yield enalaprilat. In addition, no hydrolysis of enalapril has been reported in human or dog plasma; however, rat plasma has exhibited rapid hydrolysis of enalapril to enalaprilat (Tocco *et al.*, 1982; Pang *et al.*, 1991).

Toxicity Issues

The byproducts of enalapril bioreversion are enalaprilat and ethanol. No further metabolic products have been reported in dogs and humans following the bioreversion of enalapril to enalaprilat (Tocco *et al.*, 1982). Enalapril has been clinically prescribed for hypertension since 1984 and has displayed few adverse effects. Some of the few rare and serious adverse effects from clinical use of enalapril include azotemia, glycosuria and angioedema (Cressman *et al.*, 1982; Slatter *et al.*, 1988).

Formulation

Enalapril is currently sold as tablets for oral administration. An alternate lyophilized formulation of enalapril in a Rapidisc™ (a wafer of enalapril) has been developed. This wafer is designed to disintegrate on the tongue, with subsequent

absorption of enalapril from the gastrointestinal tract. Bioavailability of enalaprilat in this formulation is reported to be equivalent to that of the conventional tablet formulation (Lo *et al.*, 2000). Some patents that claim to minimize enalapril degradation during manufacture or storage have also been issued (Sherman, 1995; Eyjolfsson, 2003; Spireas, 2003; Stofik *et al.*, 2003).

Discussion

Enalapril is a successful orally administered prodrug of the long-acting and potent antihypertensive drug enalaprilat. Enalapril is rapidly absorbed from the gastrointestinal tract and peak serum concentration of enalapril is achieved within 1 h. In human studies, the extent of absorption of enalapril and enalaprilat following oral administration was 60% and 3%, respectively. By comparison with intravenously administered enalaprilat, the bioavailability of enalapril when administered orally was 36–44% (Biollaz *et al.*, 1981; Ulm *et al.*, 1982; Irvin *et al.*, 1984; Till *et al.*, 1984). Swanson *et al.* (1984) have reported that the absorption of enalapril was not influenced by the presence of food in the gastrointestinal tract.

Enalapril rapidly undergoes bioreversion to its active form enalaprilat. As a result, it is found in human serum for a short period of time (4 h), while enalaprilat is detectable 96 h after an orally administered dose of enalapril (Ulm, 1982). Figure 1 is a plot of mean serum concentration of enalapril and enalaprilat in human volunteers versus time (Biollaz *et al.*, 1982). The delay in peak serum concentration of enalaprilat was due to non-instantaneous hepatic hydrolysis of enalapril to enalaprilat.

Subsequent to oral administration of enalapril, approximately 33% of the dose is recovered in feces and 61% in urine (18% as enalapril and 43% as

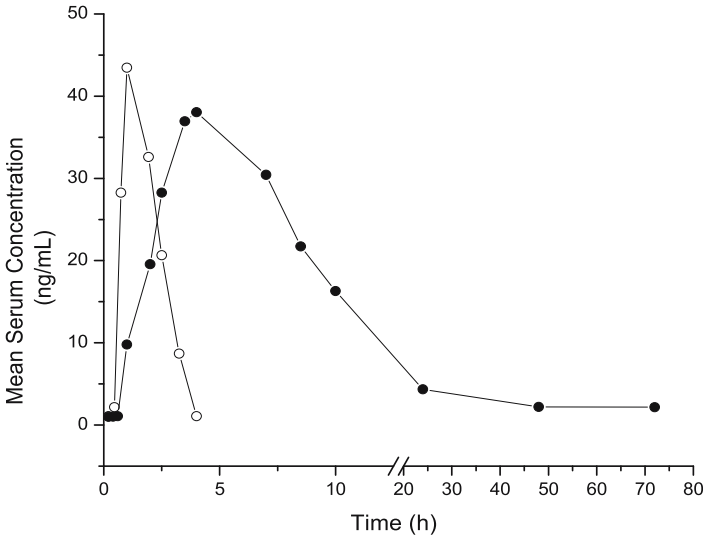


Figure 1. Mean serum concentrations of enalapril maleate (o) and enalaprilat (•) following 10 mg of enalapril maleate given to 12 normal volunteers.

enalaprilat). The drug recovered in feces is a combination of that from biliary excretion and unabsorbed drug (Drummer *et al.*, 1990).

Urinary excretion is the main route of elimination; the mean clearance rates for enalapril and enalaprilat are about 18 and 8–9.5 L/h, respectively (Till *et al.*, 1984). Because enalapril undergoes bioreversion in the liver and the principle route of excretion is the urine, hepatic dysfunction and renal impairment would certainly alter the pharmacokinetics of enalapril. *In vitro* studies using liver tissues from normal and cirrhotic livers have shown slower bioconversion of enalapril in cirrhotic than in normal liver specimens (Larmour *et al.*, 1985; Kelley *et al.*, 1986). These findings suggest that careful monitoring of total dose and dosing interval of enalapril is essential in certain patients that are on hypertension medication.

Conclusion

Enalapril is a successful prodrug that has overcome the low oral bioavailability of enalaprilat.

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5.8

Case Study: Famciclovir: A Prodrug of Penciclovir

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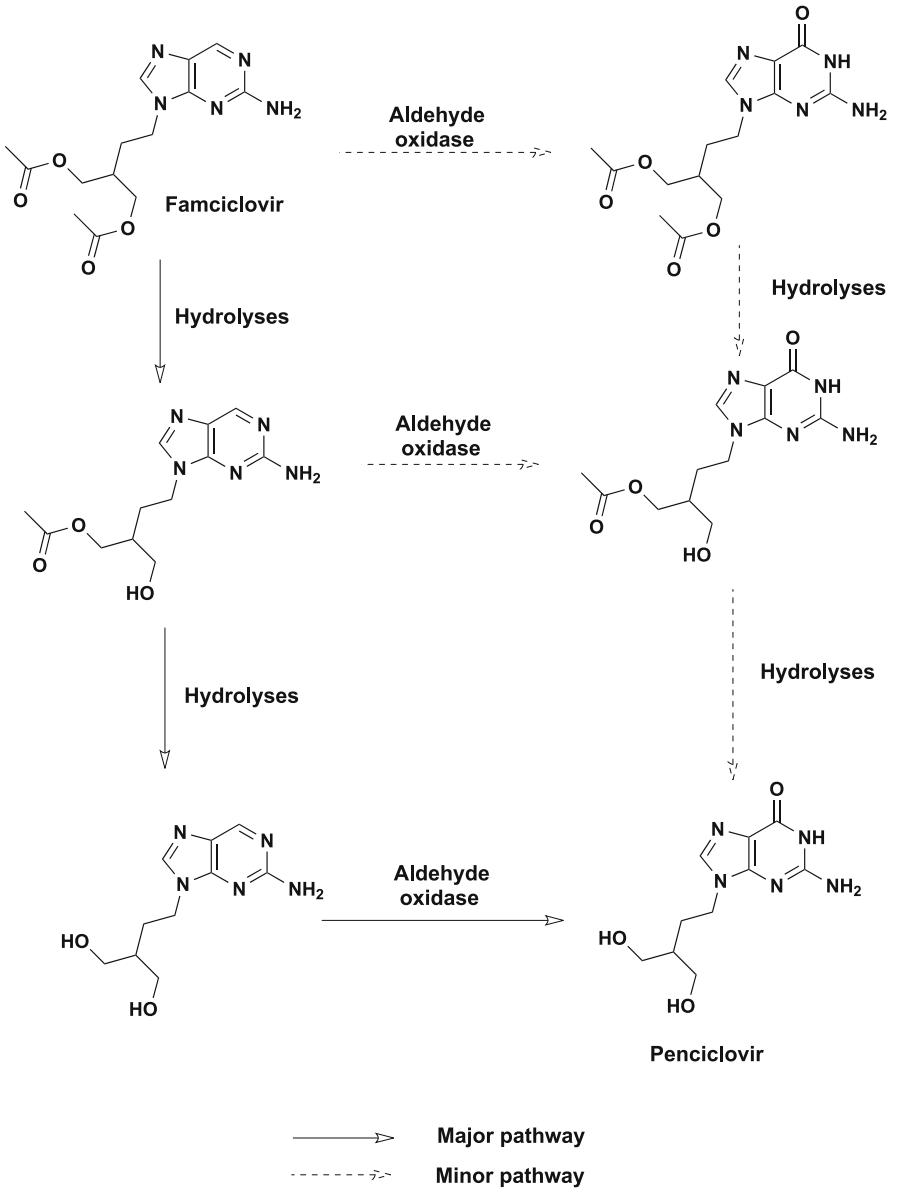
Princeton, NJ 08543

Table of Contents

Name:

Famciclovir, 2-[2-(amino-9H-purin 9-yl)ethyl]-1,3-propanediol diacetate, Famvir®

Structures and Bioconversion Pathway



Rationale for Famciclovir

Famciclovir was designed as an orally bioavailable prodrug of penciclovir, an antiviral agent with activity against herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) and varicella-zoster virus (VZV) (Boyd *et al.*, 1987; 1988). Penciclovir,

similarly to other guanosine analogs (De Miranda and Good, 1992; De Miranda *et al.*, 1981), is poorly absorbed when given orally to rodents and humans (Boyd *et al.*, 1988; Filer *et al.*, 1994). Initially, various mono and dicarboxylic esters of penciclovir were made (Harnden *et al.*, 1987), but none of these compounds resulted in significantly improved plasma concentration, of penciclovir when administered orally (Harnden *et al.*, 1989). The physicochemical properties of penciclovir are, furthermore, influenced by the polar guanine ring, and it was thought that modifications on that ring might lead to improved absorption. The 6-deoxy derivative was made, since it was known from previous work with acyclovir (Krenitsky *et al.*, 1984) that it would be oxidized to form penciclovir. When esters of the 6-deoxy derivative were tested a significant increase in bioavailability was observed (Harnden *et al.*, 1989). After further evaluation of the stability of the 6-deoxy esters (Harnden *et al.*, 1989), the diacetyl derivative, famciclovir, was selected for further development due to its improved stability in human duodenal contents (Jarvest ,1994). For more details on the design of famciclovir the reader is referred to several recent reviews (Jarvest, 1994; Jarvest *et al.*, 1998).

Synthesis

The syntheses of both penciclovir and famciclovir have previously been described by Hernden and coworkers (Harnden and Jarvest, 1985; Harnden *et al.*, 1987; 1989). The method of preparation of the current commercial material is unknown to the authors.

Mechanism and Site of Bioconversion

The mechanism for bioconversion of famciclovir to penciclovir is shown in Scheme 1. Famciclovir has been shown to be relatively stable in human duodenal contents. When incubated with homogenates of the intestinal wall, hydrolysis of one of the acetate ester groups yields predominantly the monoacetate of 6-deoxy-penciclovir (major pathway) and, additionally, a small amount of the oxidation product of the purine ring to the guanine (minor pathway). (Jarvest, 1994; Clarke *et al.*, 1995; Jarvest *et al.*, 1998). Based on these results, it would be expected that majority of famciclovir is absorbed intact and hydrolysis of the first acetate group is carried out by esterases in the intestinal wall after permeation. Removal of the second acetate group and subsequent oxidation of the purine to guanine has been shown to occur predominantly in the liver (Jarvest, 1994; Clarke *et al.*, 1995; Jarvest *et al.*, 1998).

The oxidation of the purine to the guanine is the rate-determining step in the conversion of famciclovir to penciclovir. Initially it was thought that the molybdenum-dependent enzyme xanthine oxidase was responsible for this oxidation (Jarvest, 1994; Clarke *et al.*, 1995). However, when the metabolism of 6-deoxypenciclovir was studied in human liver cytosol in the presence and absence of inhibitors of both xanthine oxidase and the related aldehyde oxidase, it was found that inhibitors of xanthine oxidase did not cause significant inhibition.

Inhibition of the aldehyde oxidase caused extensive inhibition of oxidation to penciclovir. These results strongly indicate that aldehyde oxidase is the main enzyme responsible for the oxidation step in humans (Harrell *et al.*, 1994; Clarke *et al.*, 1995). A schematic representation of the sites of bioconversion for famciclovir to penciclovir following oral administration is shown in Figure 1.

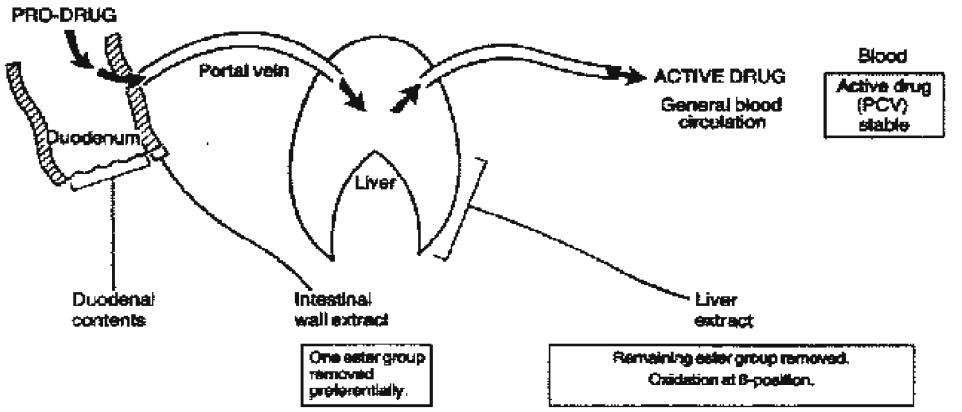


Figure 1. Schematic diagram showing the expected conversion of famciclovir to penciclovir by human tissue after oral administration. (Reproduced by permission from Hodge *et al.*, 1989).

Toxicity Issues

The authors are unaware of any side effects related to the prodrug portion of famciclovir.

Discussion

Initial bioavailability studies for famciclovir were done in rodents (Hodge *et al.*, 1989; Jarvest *et al.*, 1998). In rats the oral bioavailability of penciclovir was only 1–2%. When 6-deoxy penciclovir was dosed, the bioavailability of penciclovir was increased to 9%. However, when the diacetate ester of 6-deoxy penciclovir, famciclovir, was dosed, the oral bioavailability of penciclovir increased to 41% (Hodge *et al.*, 1989; Jarvest *et al.*, 1998). The major metabolite seen in plasma was 6-deoxy penciclovir, indicating that the rate-determining step for the conversion is the oxidation at the 6-position of the purine ring. It should be noted that all of the metabolites shown in Scheme 1, except diacetyl-penciclovir, were observed in some of the rats (Hodge *et al.*, 1989; Jarvest *et al.*, 1998).

When penciclovir was dosed orally to human volunteers, its bioavailability was about 4%, which is similar to what had previously been observed in rats. However, when the prodrug famciclovir was dosed orally, the bioavailability of penciclovir was significantly improved to 75% (Pue and Benet, 1993; Jarvest, 1994; Gill and

Wood, 1996; Jarvest *et al.*, 1998). After oral administration, famciclovir is rapidly absorbed, and the maximum concentration of penciclovir is observed after ~0.75 hours. Famciclovir is rapidly converted to penciclovir, and the only intermediate that has consistently been observed in human plasma is 6-deoxypenciclovir. This metabolite is generally present in plasma for a short time at concentrations up to one-third of the concentrations observed for penciclovir. After oral dosing of famciclovir, the $T_{1/2}$ of penciclovir is 2.27 h; approximately 65% of the dose is excreted in the urine, of which 5% is 6-deoxypenciclovir and the remaining 60% is penciclovir (Pue and Benet, 1993; Jarvest, 1994; Gill and Wood, 1996; Jarvest *et al.*, 1998). For humans, pharmacokinetic parameters for penciclovir are linear after oral dosing of 125–750 mg of famciclovir (Pue and Benet, 1993; Gill and Wood, 1996).

Oral famciclovir exhibits a rapid plasma concentration of penciclovir with high bioavailability. In both rats and humans the main intermediate seen is 6-deoxy penciclovir, indicating that in both species the slow step of the conversion is the oxidation at the 6 position of the guanine ring. In this case, the rat proved to be a good model for the bioconversion of the prodrug in humans even though esterase activity in rodents is significantly higher then in humans.

It is interesting to note that although neither penciclovir nor famciclovir is chiral, the monoacetyl intermediates (namely, monoacetyl-6-deoxypenciclovir and mono-acetyl-penciclovir) are chiral (Hodge and Cheng, 1993; Jarvest, 1994; Jarvest *et al.*, 1998). The removal of the first acetyl group can therefore be enantioselective. When the hydrolysis was studied, the esterase(s) present in the intestinal wall were found to hydrolyze the acetyl group preferentially from the pro-(S)-acetoxymethyl group of famciclovir (Hodge and Cheng, 1993; Vere Hodge

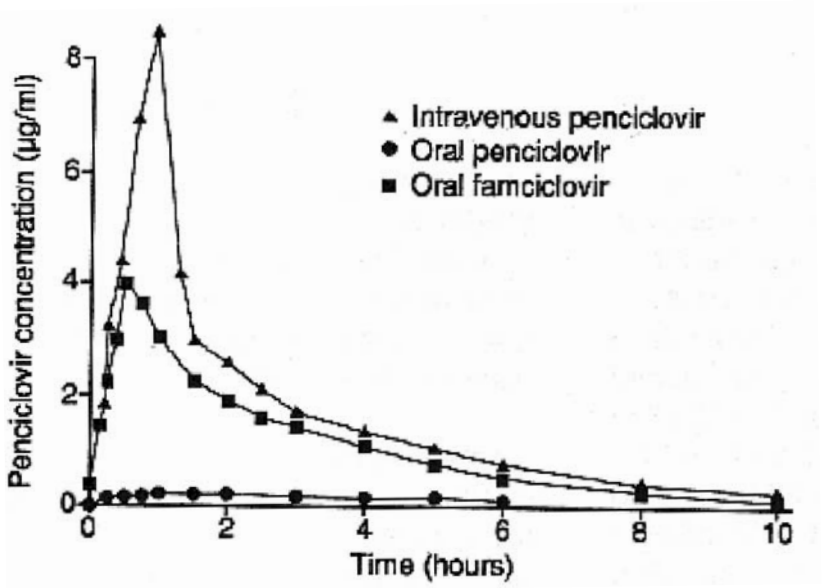


Figure 2. Mean plasma concentration of penciclovir following oral administration of famciclovir or oral or IV administration of penciclovir in healthy volunteers. (Reproduced with permission from Pue and Benet, 1993).

et al., 1993; Jarvest,1994; Jarvest *et al.*, 1998). The specificities of the esterase in forming monoacetyl-6-deoxypenciclovir (R isomer) and monoacetyl-penciclovir (R isomer) are about 77 and 72%, respectively. All of the intermediates independent of the chirality do ultimately form penciclovir that is not chiral (Hodge and Cheng, 1993; Vere Hodge *et al.*, 1993; Jarvest, 1994; Jarvest *et al.*, 1998).

Conclusion

Famciclovir is a good example of the way that increasing the lipophilicity and, thus, changing the physicochemical properties of a compound can lead to a prodrug that is highly orally bioavailable and capable of penetrating the intestinal wall.

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5.9

Case Study: Fosamprenavir: A Prodrug of Amprenavir

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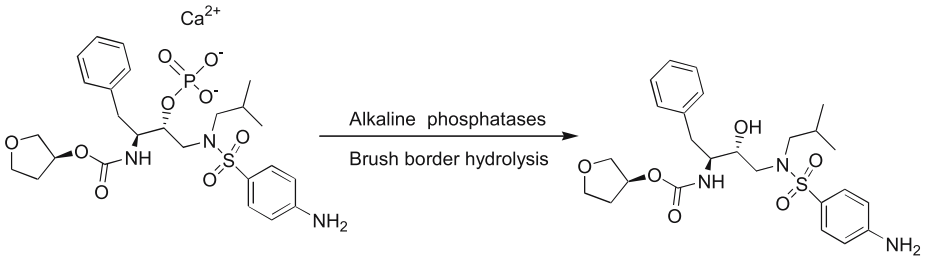
Table of Contents

Name:

Fosamprenavir calcium, *N*-[3-[*N*-(4-aminophenylsulfonyl)-*N*-isobutylamino]-1(*S*)-benzyl-2(*R*)-(phosphonooxyl)propyl]carbamic acid tetrahydrofuran-3(*S*)-yl ester calcium salt, (3*S*)-tetrahydrofuran-3-yl (1*S*, 2*R*)-3-[[(4-aminophenyl)sulfonyl](isobutyl)amino]-1-benzyl-2-(phosphonooxy)propylcarbamate monocalcium salt, Lexiva®

Structures and Bioconversion Pathway



Fosamprenavir (prodrug in the gut)

Amprenavir (drug in the plasma)

Rationale for Fosamprenavir

Fosamprenavir (Lexiva®) is a phosphate ester prodrug of the antiretroviral protease inhibitor amprenavir (Agenerase®). Amprenavir is a white-to-cream-colored solid with a solubility of approximately 0.04 mg/mL in water at 25°C. It was originally marketed as an oral solution at a concentration of 15 mg/mL that included a variety of excipients, such as acesulfame potassium, artificial grape bubblegum flavor, citric acid, TPGS, menthol, natural peppermint flavor, polyethylene glycol 400, propylene glycol, saccharin sodium, sodium chloride, and sodium citrate (GlaxoSmithKline, 2004). Because the recommended amprenavir regimen is 1200 mg twice daily (equal to 80 mL b.i.d.), the large amount of excipients (propylene glycol in particular) in the oral solution posed potential toxicity problems and prohibited its usage in infants and children under the age of 4 years and in certain other patient populations. Amprenavir is also formulated as capsules in strengths of 50 and 150 mg (GlaxoSmithKline, 2002). The content of vitamin E as TPGS in amprenavir capsules (daily dose of 1744 IU) far exceeds the Reference Daily Intake (adults 30 IU, pediatrics 10 IU). Patients are advised not to take additional vitamin E. To reduce the excipient intake and the burden of 8 capsules twice daily, fosamprenavir was designed as a more water-soluble prodrug (0.31 mg/mL in water at 25°C) of amprenavir (Gatell, 2001), which required only two tablets twice a day.

Synthesis

The synthesis of fosamprenavir has been described in detail in various patents from Vertex Pharmaceuticals and GlaxoSmithKline. (Hale *et al.*, 1999; Tung *et al.*, 1999; Armitage *et al.*, 2000; McDade *et al.*, 2000) and has been summarized by Sorbera *et al.* (2001). The method of preparation of the current commercial material is unknown to this author.

Mechanism and Site of Bioconversion

Phosphate prodrugs can be designed to improve the oral absorption of poorly water-soluble compounds. As described in Figure 1 (Fleisher *et al.*, 1996), the highly soluble phosphate prodrug builds up a higher concentrational driving force for absorption in the intestinal lumen than does the parent drug because of its higher solubility. The phosphate prodrug is then converted to its parent drug by alkaline phosphatases on the brush border membrane of the intestinal epithelial cells (Fleisher *et al.*, 1986; Stewart *et al.*, 1986; Heimbach *et al.*, 2003). The released lipophilic parent drug is therefore in the vicinity of the epithelial membrane and can easily partition into the membrane and cross the epithelial cell monolayer into systemic circulation.

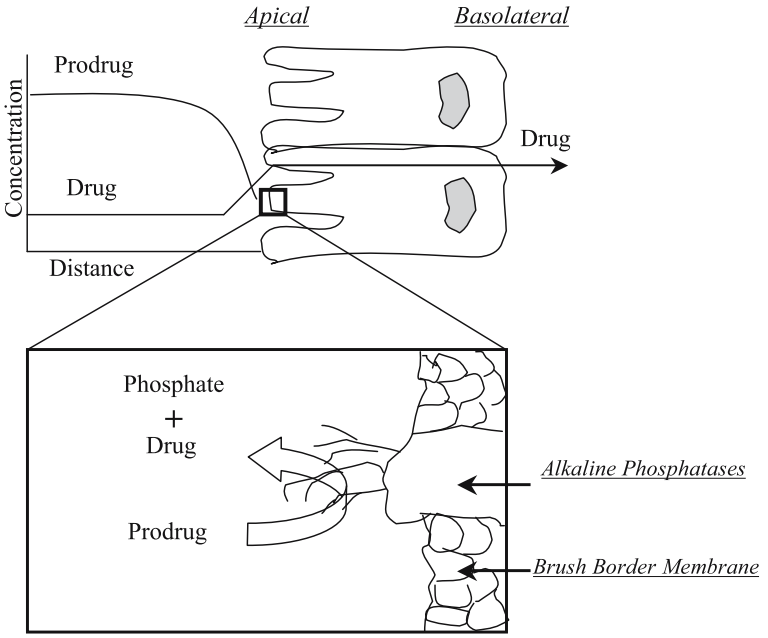


Figure 1. Phosphate prodrug is cleaved by the alkaline phosphatases on the brush border membrane of the intestinal epithelial cells. The hydrophobic parent drug is released close to the apical membrane. The soluble phosphate prodrug provides a great concentrational driving force for the absorption in the intestinal lumen. Reproduced with permission (Fleisher *et al.*, 1996).

Fosamprenavir, as an orally administered prodrug, is hydrolyzed to amprenavir and inorganic phosphate by alkaline phosphatases in the brush border of gut epithelium prior to reaching the systemic circulation (Sorbera *et al.*, 2001). When fosamprenavir was applied to the apical side of Caco-2 monolayers, only amprenavir was detected on the basolateral side (Sorbera *et al.*, 2001). When fosamprenavir was given orally to rats and dogs, no fosamprenavir was detected in the plasma (Sorbera *et al.*, 2001). All the results indicate that the conversion of fosamprenavir to amprenavir is rapid and quantitative during the absorption process.

Toxicity Issues

Fosamprenavir showed drug-related side effects similar to those of amprenavir in clinical trials (GlaxoSmithKline, 2003). These side effects were mostly mild to moderate in severity and did not affect the treatment. The recommended dose of fosamprenavir is well tolerated in clinical studies.

Formulation Issues

Initially, both calcium and sodium salts of fosamprenavir were chosen for clinical trials (Falcoz *et al.*, 2002). The fosamprenavir calcium salt is a crystalline solid whose solubility is pH-dependent, with a maximum solubility of >100 mg/mL at pH 3–4. The fosamprenavir sodium salt is an amorphous solid with a solubility of >100 mg/mL at pH 7.4. Both fosamprenavir salts showed bioequivalency to amprenavir in the phase I clinical trial (Falcoz *et al.*, 2002). Because the sodium salt is extremely hygroscopic and the solubility of calcium salt is adequate at physiological pH, the fosamprenavir calcium salt was selected as the final form of the formulation.

Fosamprenavir calcium salt is currently marketed as 700-mg tablets for oral administration (equivalent to approximately 600 mg of amprenavir) (GlaxoSmithKline, 2003). The tablets contain the inactive ingredients colloidal silicon dioxide, croscarmellose sodium, magnesium stearate, microcrystalline cellulose, and povidone K30. The film coating of the tablet contains hypromellose, iron oxide red, titanium dioxide, and triacetin. This formulation offers a low tablet count and dosing flexibility. It can be given unboosted or boosted with ritonavir, in a once- or twice-daily regimen.

Discussion

Patient compliance is a very important issue during HIV therapy. An orally administered drug with no food influence, a low tablet count, and a once- or twice-daily regimen will significantly enhance the patient compliance and subsequently improve the success rate of HIV drug therapy (Havens, 2003; Park-Wyllie and Phillips, 2003). Amprenavir was launched in 1998 as a long-acting protease inhibitor with a half-life of 7–10 hours (Tung *et al.*, 2002). Amprenavir showed

excellent antiretroviral activity and good tolerability in clinical studies (GlaxoSmithKline, 2002). However, this compound exhibited low aqueous solubility and poor wetting properties that required its formulation as a soft gelatin capsule. Fosamprenavir was initially designed as a highly water-soluble phosphate ester prodrug of amprenavir to reduce the dosing burden from eight capsules b.i.d. to two tablets twice daily (Corbett and Kashuba, 2002).

In an open, randomized, single-dose, crossover phase I clinical trial (Falcoz *et al.*, 2002), very low concentrations of fosamprenavir were detected in the plasma (less than 0.17% of amprenavir concentration), confirming that fosamprenavir was rapidly and completely converted to amprenavir during the absorption process. When given to patients in the fasting state, fosamprenavir tablets were bioequivalent to amprenavir capsules (C_{max} 27% lower) with dose-proportionality (C_{max} 14% lower at higher dose). When given with a low-fat meal, fosamprenavir tablets showed bioequivalence, but amprenavir capsules showed a 23% decrease in bioavailability (C_{max} is also lowered by 46%). When fosamprenavir tablets were given with a high-fat meal, there was no food influence on AUC (C_{max} 12% lower). Overall, the fosamprenavir dosage was well tolerated and was bioequivalent to amprenavir capsules with negligible food effects.

A phase II clinical trial (Wood *et al.*, 2004) comparing amprenavir (1200 mg b.i.d.) and fosamprenavir (1395 mg b.i.d., and 1860 mg b.i.d.) showed equivalent steady-state AUC values, lower maximum plasma amprenavir concentrations ($C_{max,\,ss}$ 30% lower for both doses), and higher plasma amprenavir concentrations ($C_{\tau,\,ss}$ 28% and 46% higher, respectively) at the end of the dosing interval. The median steady-state plasma amprenavir concentration-time profiles for the three dosing regimens at the end of 28 days of treatment are shown in Figure 1. The side effects of fosamprenavir were similar to those of amprenavir. Phase III clinical trials further confirmed that fosamprenavir is comparable to amprenavir in

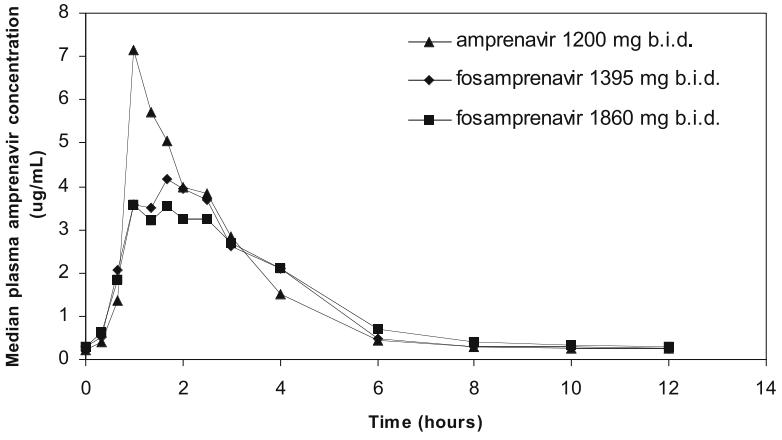


Figure 2. Median steady-state plasma amprenavir concentration-time profiles from amprenavir capsules 1200 mg b.i.d. (▲, n = 53), fosamprenavir 1395 mg b.i.d. (◆, n = 22), and fosamprenavir 1860 mg b.i.d. (■, n = 31) groups at the end of treatment period (day 28). Reproduced with permission (Wood et al., 2004).

efficacy and safety profile, but has a clear dosing advantage (GlaxoSmithKline, 2003).

In addition to its dosing advantage, fosamprenavir provides an effective life-cycle management tool for amprenavir. As mentioned in a previous chapter in this book by Stella, the composition of matter patent for amprenavir will expire in 2013 (Tung, 1996; Tung and Bhisetti, 1996; Tung *et al.*, 1996) while the patent for fosamprenavir will expire later (Tung *et al.*, 1999). The extra years of marketing exclusivity combined with the convenience of the drug dosing should generate a clear economic advantage for the developers of this drug, Vertex Pharmaceutical Inc. and GlaxoSmithKline.

Conclusion

Fosamprenavir is a good prodrug of amprenavir that reduces the tablet burden and is expected to enhance patient compliance during HIV therapy. Fosamprenavir is also a good example of life-cycle management for amprenavir while providing a clear clinical advantage.

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5.10

Case Study: Fosinopril

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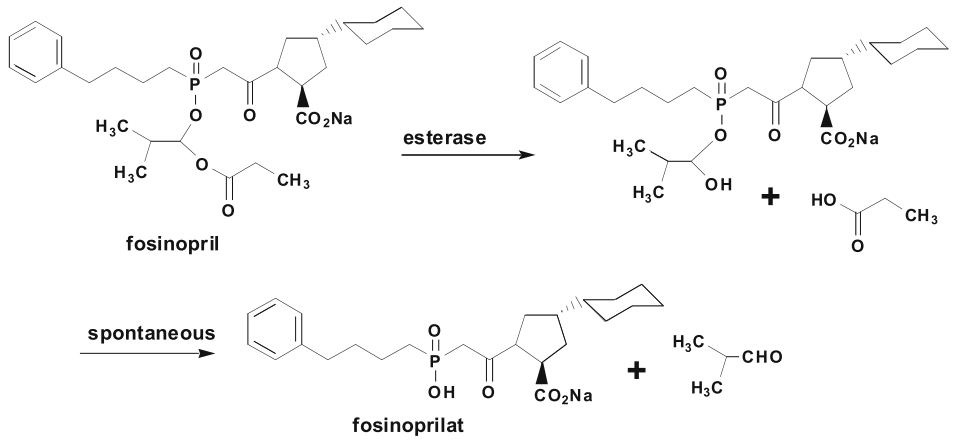
Table of Contents

Name:

Fosinopril, L-Proline, 4-cyclohexyl-1-(((R)-((1S)-2-methyl-1-(1-oxopropoxy) propoxy)(4-phenylbutyl)phosphinyl)acetyl)-, (4S)-; Monopril®

Structures and Bioconversion Pathway



Rationale for Fosinopril

Fosinopril is an acyloxyalkyl ester of fosinoprilat, a phosphinic acid angiotensin-converting enzyme (ACE) inhibitor developed for the treatment of hypertension and congestive heart failure. The hydroxylphosphinylacetyl proline class of inhibitors exhibits high affinity for ACE and a high degree of efficacy for inhibition of the angiotensin I-induced pressor response in animals (Krapcho *et al.*, 1988) when administered intravenously. These inhibitors exhibited a low level of activity upon oral administration, which could be attributed to low oral absorption. The propanoyloxy isobutyl ester of the trans-4-cyclohexyl proline analog exhibited improved oral activity and long duration of action (DeForrest *et al.*, 1989) and was advanced into preclinical and clinical development (Duchin, 1991).

Fosinopril is more extensively absorbed in rats, dogs, and monkeys than is fosinoprilat (Morrison *et al.*, 1990). Some studies concluded that fosinopril is passively absorbed, since absorption was neither concentration-dependent nor inhibited by cephradine, a known inhibitor of peptide transport (Friedman and Amidon, 1989). A later study demonstrated that fosinopril is transported intact by the peptide transporters PEPT1 and PEPT2 in Caco-2 cells by a peptide-coupled, saturable process (Shu *et al.*, 2001).

Synthesis

The synthesis of fosinopril requires 4-trans-cyclohexyl-L-proline, which can be derived from the naturally occurring amino acids trans-4-hydroxy-L-proline or L-pyroglutamic acid (Kleeman *et al.*, 1999). The phenylbutylphosphinylacetic acid moiety is assembled by Arbusov reaction of a derivative of phenylbutyl phosphonous acid with bromoacetic acid. The prodrug moiety is derived in the usual way from an alpha-acyloxy halide. A single diastereomer of the prodrug-containing side chain is used to acylate the proline moiety to provide fosinopril, isolated as the crystalline sodium salt.

Mechanism and Site of Bioreversion

Upon oral administration, fosinopril is absorbed predominantly from the proximal portions of the small intestine, then rapidly and completely hydrolyzed to the active ACE inhibitor diacid fosinoprilat (Duchin, 1991). Studies of radiolabeled fosinopril in dogs demonstrated that >75% of the orally-absorbed dose is hydrolyzed in the gut and <25% in the liver. Blood and lung contributed insignificantly to bioconversion of the prodrug (Morrison *et al.*, 1990).

Toxicity Issues

Propionic acid, one byproduct of the bioconversion of fosinopril, is a normal product of fatty acid and sterol metabolism. It is widely encountered in foodstuffs, occurring either naturally or as an added preservative. Isobutyraldehyde, the other byproduct, is metabolized to isobutyric acid, an intermediate in valine metabolism. The moderate doses of these byproducts arising from fosinopril ingestion appear to have no discernable effect on its toxicity.

Formulation issues

Fosinopril sodium is marketed as 10, 20, and 40 mg tablets. The sodium salt forms micelles in water above a critical concentration of 1.5 mg/mL, leading to concentration-dependent stability behavior and reduced solubility in the presence of metal ions (Wang *et al.*, 1995). Divalent metal ions, especially magnesium, manganese and cobalt, catalyze the degradation of fosinopril in aqueous solution. Formation of a magnesium enolate leads to acylation of the acetyl side chain by the propanoyl prodrug moietyl and loss of phenylbutyl phosphinic acid. This mode of degradation is a consideration in the use of magnesium stearate lubricant in tablet manufacture (Thakur *et al.*, 1993).

Discussion

Captopril (Capoten®), the first orally active inhibitor of ACE, had an immediate and profound impact on the therapy of hypertension and congestive heart failure upon its introduction into the marketplace in 1980 (Ondetti, 1994). The modulation of the renin-angiotensin-aldosterone system by this new drug provided benefits that were not available from the cardiovascular agents available at that time. The success of captopril stimulated a worldwide effort to develop newer ACE inhibitors with properties superior to those of captopril. Research on ACE inhibitors was guided by the groundbreaking hypotheses of Ondetti and Cushman concerning the mechanism by inhibition of ACE and related zinc metalloproteases by captopril and its analogs (Cushman and Ondetti, 1999). The Cushman/Ondetti model described the requirements for a zinc-chelating group appropriately situated in a dipeptide mimetic with a C-terminal carboxylic acid. Captopril employs a mercaptan as the zinc-binding group, but all other ACE inhibitor classes employ an acidic group such as carboxylic acid, hydroxamic acid, phosphonamide, phosphonic or phosphinic acid. Prodrug strategies have been essential in realizing the therapeutic potential of this drug family. Among the ten marketed ACE inhibitors, only captopril (a mercaptan) and lisinopril (an amino diacid) are dosed orally in the active form. There are six carboxylic acid ester prodrugs (enalapril, benazepril, moexipril, quinapril, ramipril and trandolapril), and one phosphinic acid ester prodrug (fosinopril). [Enalaprilat, the active diacid form of enalapril, is marketed for parenteral use (Fischler and Follath, 1999)].

Among marketed prodrugs, the acyloxyalkyl phosphinate ester moiety of fosinopril is unique. Application of the prodrug strategy has resulted in a drug with absorption and bioconversion characteristics that enhance its intended use. Thus, fosinopril compares favorably with all other ACE inhibitor prodrugs with respect to onset of action. The rate, but not the extent, of prodrug hydrolysis is reduced in patients with poor liver function, but this effect does not appear to be a factor in the use of the drug.

Fosinopril has found widespread clinical use in the treatment of hypertension and congestive heart failure. The Sixth Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC-VI, 1997) recommended diuretics or beta-blockers for first-line use in uncomplicated hypertension, with ACE inhibitors recommended as first-line therapy in diabetics, in the presence of heart failure, and following myocardial infarction. Clinical studies reported after publication of JNC-VI supported broader use of ACE inhibitors than was recommended in the report (Hilleman, 2000). Significant reductions in cardiovascular events and complications of diabetes were observed with ACE inhibitors when compared to other therapeutic approaches. In the management of heart failure, fosinopril increased exercise duration and improved symptoms to an extent comparable with those of other ACE inhibitors (Davis *et al.*, 1997). In both hypertension and congestive heart failure, the property that most clearly differentiated fosinopril from other ACE inhibitors was the absence of a requirement to adjust dosage in patents with renal or hepatic impairment (White, 1998). Excretion of fosinoprilat is balanced

approximately equally between hepatic and renal routes, and each route of elimination appears to respond in compensatory fashion if the other route is compromised. As a result, fosinopril can be used in a wider range of patients without dosage adjustment.

Conclusion

Fosinopril represents a highly successful application of prodrug strategy. Clinical and market experience with the drug have demonstrated the therapeutic utility of fosinoprilat, the active ACE inhibitor, in hypertension and congestive heart failure. It is unlikely that the highly favorable pharmacokinetics and metabolism/excretion profile of the phosphinylacetyl proline class of inhibitors could have been realized without reliance on the prodrug approach.

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5.11

Case Study: Fosphenytoin: A Prodrug of Phenytoin

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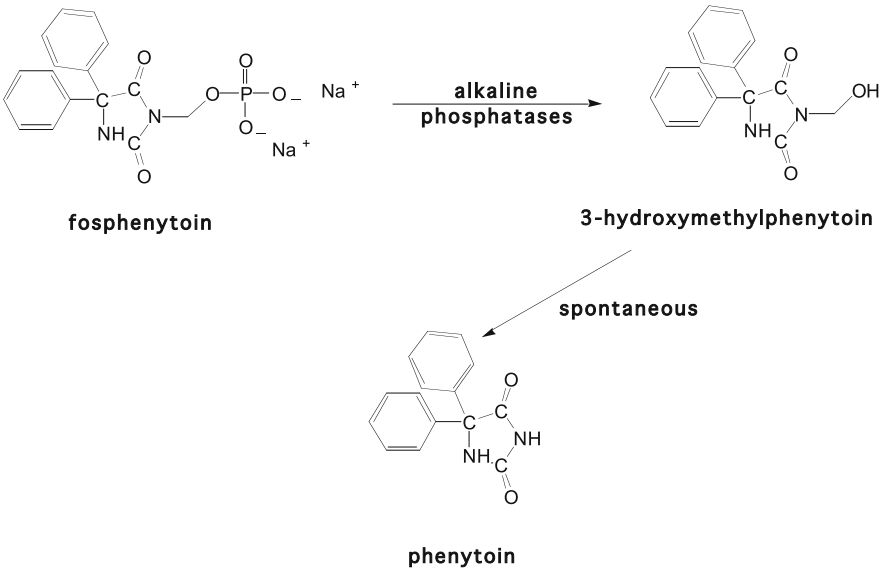
Table of Contents

Name:

Fosphenytoin, 3-phosphoryloxymethyl-5,5-diphenylhydantoin disodium salt, phosphoric acid mono-(2,5-dioxo-4,4-diphenyl-imidazolidin-1-ylmethyl) ester disodium salt, Cerebyx®

Structures and Bioconversion Pathway:



Rationale for Fosphenytoin:

Fosphenytoin was designed to be a water-soluble, parenterally safe form of phenytoin, or 5,5-diphenylhydantoin, a sparingly water-soluble (20–30 μg/mL), weakly acidic drug (pKa 8.3) useful in the treatment of epilepsy. To achieve an aqueous solution suitable for parenteral administration, phenytoin was originally formulated as its sodium salt (sodium phenytoin) in a vehicle consisting of 40%propylene glycol and 10% ethanol with a final pH of 12. This formulation can be very toxic, causing severe irritation and pain at the local site of injection and occasional fatalities if injected too rapidly. Sodium phenytoin cannot be administered by intramuscular (IM) injection. Its water-soluble prodrug, fosphenytoin, is formulated in a purely aqueous solution at a pH of about 8.5; it has shown superior safety compared to sodium phenytoin while also permitting IM dosing. Two patents covering fosphenytoin (Stella and Sloan, 1979, 1981) have expired, but two formulation patents are still valid (Herbranson *et al.*, 1990; Stella 2000). An extensive review of the history of fosphenytoin has been published; it includes other examples of prodrugs of phenytoin (Stella, 1996).

Synthesis

The synthesis of fosphenytoin was initially a challenge. It was first reported by Varia *et al.* (1984a), and some successful modifications have been reviewed (Stella, 1996). The method of preparation of the current commercial material is unknown to this author.

Mechanism and Site of Bioreversion

The bioreversion mechanism of fosphenytoin to phenytoin is due to the action of the phosphatase family of enzymes followed by spontaneous hydrolysis of the N-hydroxymethyl intermediate. The byproducts of bioreversion are inorganic phosphate and formaldehyde. The role of phosphatases (presumably alkaline phosphatases) has been demonstrated both *in vitro* and *in vivo* (Varia *et al.*, 1984b,c,d; Stella, 1996 and references therein). The very high total body clearance values for fosphenytoin after parenteral administration, close to total cardiac output, strongly support extensive and rapid cleavage of fosphenytoin in all accessible tissues including endothelial cell surface phosphatases. Although fosphenytoin is not administered orally, in dogs it has been shown to be an excellent candidate for cleavage by alkaline phosphatases imbedded in the brush-border of intestinal enterocytes (Tenhoor and Stewart, 1995; Heimbach *et al.*, 2003). After cleavage of the phosphate group, dehydroxymethylation has been shown to be rapid (less than 2 s) and quantitative at pH 7.4 and 37°C (Varia *et al.*, 1984b).

Toxicity Issues

Questions are always raised when formaldehyde is a byproduct of prodrug reversion. It is this author's understanding that the FDA raised this issue during fosphenytoin registration. Since fosphenytoin was approved for human use, presumably this issue was addressed to the satisfaction of the agency. The inorganic phosphate byproduct can be a concern in renally impaired patients. A side effect of fosphenytoin, as well as some other phosphate ester prodrugs, is a pruritis/parethesia or tingling sensation noted especially in the extremities. This side effect is transitory (Lippik *et al.*, 1990) and seems to follow the time profile of the prodrug or the reversion process. This effect has been observed with other phosphate prodrugs after parenteral administration (Fechner *et al.*, 2003).

Formulation issues

Fosphenytoin is currently sold as a ready-to-use solution, pH approximately 8.5, which requires refrigeration. This pH was chosen because fosphenytoin degradants at pH values >8.5 are water-soluble (Herbranson *et al.*, 1990). Although fosphenytoin is more stable at pH 7.5–8, the poorly water-soluble phenytoin is a major degradant in this range . A recent study has shown that a

room-temperature formulation of fosphenytoin can be prepared at this lower pH range by adding the cyclodextrin SBE_{7M}-β-CD or Captisol® to help solubilize the formed phenytoin (Narisawa and Stella, 1998). This new formulation is undergoing testing as a room-temperature stable alternative to the current refrigerated formulation.

Discussion

Fosphenytoin has been a successful product since it was launched in 1996–97. The competing sodium phenytoin product continues to be sold despite its less than desirable properties, presumably due to its lower cost.

Fosphenytoin is quantitatively converted to phenytoin after parenteral IV and IM administration. This was first demonstrated in dogs and rats (Varia *et al.*,1984c,d) where the areas under the plasma concentration versus time curve for phenytoin from fosphenytoin versus that of phenytoin from sodium phenytoin were compared. From these studies, after both IV and IM administration, phenytoin absolute availability from fosphenytoin was shown to be 100%. No adverse effects from fosphenytoin that could not be accounted for from the released phenytoin were noted in animals.

Since these early studies in animal species, numerous studies in humans have yielded similar results (Rivenburg *et al.*, 1987; Gerber at al., 1988; Boucher *et al.*, 1988, 1989; Walton *et al.*, 1989; Jamerson *et al.*, 1990; Leppik *et al.*, 1990, Wilder *et al.*, 1993; Jamerson *et al.*, 1994; Ramsay *et al.*, 1997; Aweeka *et al.*, 1999; Pryor *et al.*, 2001). Early human studies with fosphenytoin were designed to answer safety and the quantitative nature of fosphenytoin conversion to phenytoin relative to the use of sodium phenytoin. Gerber *et al.* (1988) estimated clearance of fosphenytoin to be 14 ± 2 l/hr, and its Vd, to be 2.6 ± 0.5 L, 3.4% of body weight, with a conversion half-life to phenytoin of 8.1 ± 1.5 minutes. No non-linearities for fosphenytoin metabolism to phenytoin were noted, but later fosphenytoin was found to displace phenytoin from its plasma protein binding site/s with some clinical implications. Figure 1 is a plot of phenytoin plasma levels versus time from fosphenytoin versus a molar-equivalent sodium phenytoin injection. The slight delay in phenytoin appearance from fosphenytoin is due to the conversion rate being fast but not instantaneous. Because of greater safety, fosphenytoin can be infused at a higher rate than sodium phenytoin, resulting in phenytoin levels comparable to those seen with sodium phenytoin at the slower sodium phenytoin infusion rates (needed for safety). Jamerson *et al.* (1994) noted significantly lower adverse events at the injection site relative to those from the use of sodium phenytoin. This has been confirmed in numerous follow-up studies.

Because of the rapid clearance from its injection site, IM fosphenytoin resulted in complete and rapid release of phenytoin to systemic circulation, something that was not possible with sodium phenytoin (Boucher *et al.*, 1988, 1989; Leppik *et al.*, 1990; Pryor *et al.*, 2001; Ramsay *et al.*, 1997; Wilder *et al.*, 1993). Sodium phenytoin results in phenytoin precipitation at the IM site causing slow and short-term incomplete release of phenytoin. Figure 2 shows a plot of

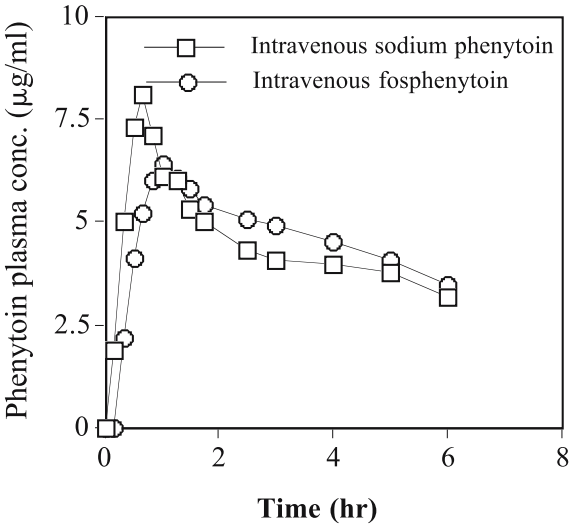


Figure 1. Mean plasma phenytoin levels after IV infusion over 30 minutes of equimolar doses of 250 mg sodium phenytoin (□) and fosphenytoin (○) to 12 subjects. Reproduced with permission (Jamerson *et al.*, 1990).

phenytoin levels from IM fosphenytoin compared to those of IV sodium phenytoin. Relative percent bioavailability was 100%.

Clearance values of fosphenytoin to phenytoin did not appear to be different in cirrhotic or renally impaired patients on hemodialysis (Aweeka *et al.*, 1999) than in healthy subjects.

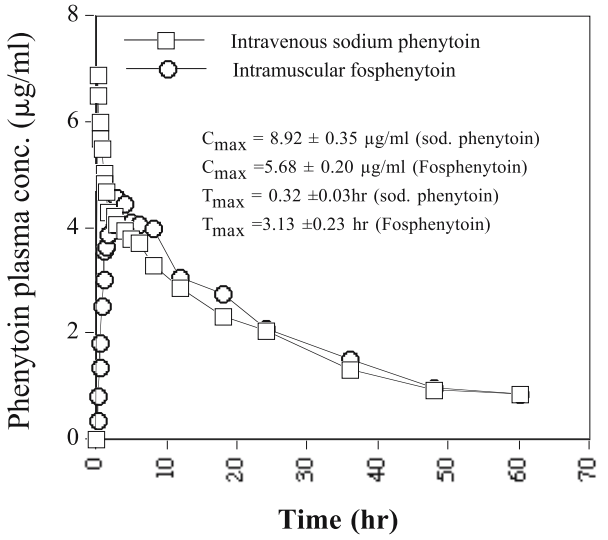


Figure 2. Mean plasma phenytoin levels after equimolar doses of IV sodium phenytoin (□) and IM fosphenytoin (○) to 12 subjects . Reproduced with permission (Browne *et al.*, 1990).

Numerous studies have attested to the greater safety of fosphenytoin relative to sodium phenytoin, but Jamerson *et al.* (1994) were the first to present these observations quantitatively. Lower to no pain on injection and less phlebitis or cording were noted with fosphenytoin, whereas significant adverse events were seen with sodium phenytoin. In this and other studies, fosphenytoin demonstrated clear safety advantages over sodium phenytoin.

Conclusion

Fosphenytoin is an excellent example of a prodrug that can overcome the parenteral delivery problems associated with the sparingly water-soluble drug phenytoin.

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5.12

Case Study: Irinotecan (CPT-11), A Water-soluble Prodrug of SN-38

M. J. Hageman and W. Morozowich
Pfizer Inc., Kalamazoo, MI

Table of Contents

Name:

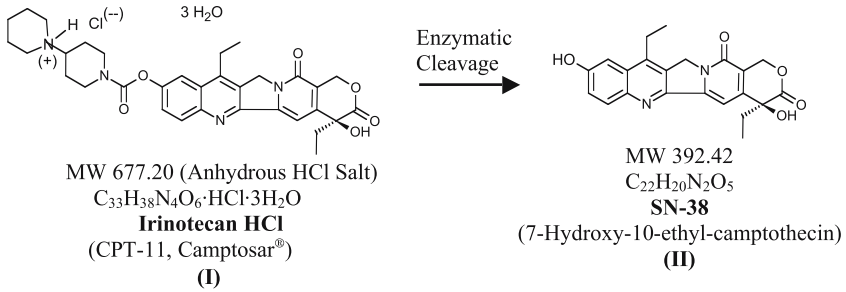
Irinotecan.HCl; CA Index Name: 1,4'-Bipiperidine]-1'-carboxylic acid, (4S)-4,11-diethyl-3,4,12,14-tetrahydro-4-hydroxy-3,14-dioxo-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-9-yl ester, monohydrochloride, trihydrate (9CI).

Other Names:

Camptosar®; CPT 11; Campto;
U-01440E. Chemical Abstracts Registry Number: 136572-09-3

Introduction

Irinotecan (CPT-11) is a potent anticancer agent approved for use in the treatment of advanced colorectal cancer, as either a first-line therapy in combination with 5-fluorouracil or in the treatment of 5-flurouracil refractory cases (Sawada *et al.*, 1995; Saltz, 1997). Irinotecan is a prodrug designed to deliver the poorly soluble parent molecule SN-38, an analog of a highly potent plant alkaloid camptothecin (CPT). Camptothecin and its analogs inhibit the nuclear enzyme topoisomerase I, inhibiting replication and demonstrating antitumor activity as an S-phase-specific drug (Garcia-Carbonero and Supko, 2002). The structure and enzymatic bioconversion of CPT-11 to SN-38 is shown in Scheme 1.



MW 677.20 (Anhydrous HCl Salt)
$C_{33}H_{38}N_4O_6 \cdot HCl \cdot 3H_2O$
Irinotecan HCl
(CPT-11, Camptosar®)
(I)

MW 392.42
$C_{22}H_{20}N_2O_5$
SN-38
(7-Hydroxy-10-ethyl-camptothecin)
(II)

Scheme 1. Structure and bioconversion of the prodrug irinotecan HCl (I) to the parent drug SN-38 (II).

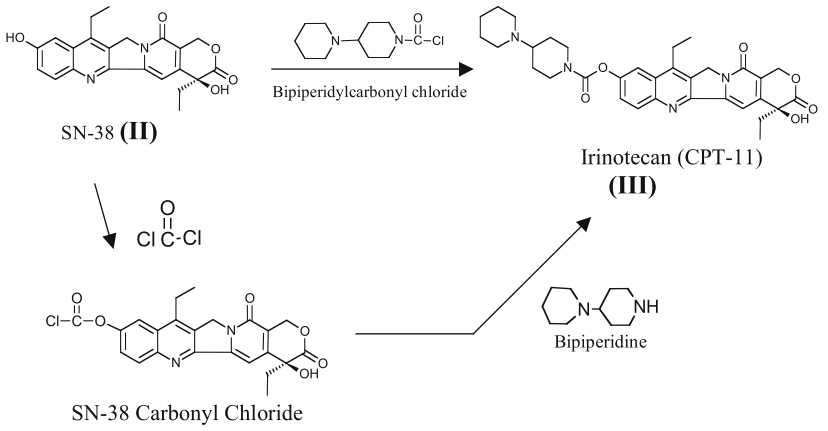
Rationale for CPT-11

Early clinical studies of CPT demonstrated encouraging signs of antitumor activity but were discontinued due to unpredictable toxic effects. The toxic effects were later linked, in part, to the very poor water solubility of CPT (2–3 μg/mL) (Wall *et al.*, 1966) and difficulty with intravenous (IV) administration. In an effort to improve the properties of camptothecin, a large number of analogs and

prodrugs were designed to improve solubility and facilitate IV administration. The bipiperidyl carbamate ester of SN-38 was synthesized as a soluble prodrug (irinotecan), permitting solubilization at low pH due to protonation of the tertiary amino group of the piperidyl ring (Miyasaka *et al.*, 1986; Sawada *et al.*, 1991, 1995). Irinotecan was sufficiently water-soluble to permit a stable aqueous solution formulation that is marketed for IV administration (Camptosar®, Pfizer).

Synthesis of Irinotecan

Irinotecan can be synthesized by direct reaction of SN-38 with bipiperidyl-carbonyl chloride (Scheme II). Alternatively, it can be synthesized by reaction of SN-38 with phosgene to give SN-38 carbonyl chloride followed, in turn, by reaction with piperidylpiperidine to give irinotecan (Scheme 2). Irinotecan is isolated as the crystalline trihydrate hydrochloride salt from both routes (Miyasaka *et al.*, 1986; Sawada *et al.*, 1991, 1995, 1996; Henegar, 2000).

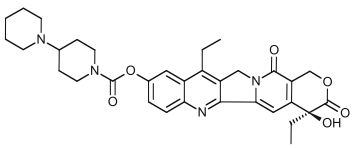


Scheme 2. Synthesis of irinotecan (CPT-11) by the amine carbamoyl chloride and SN-38 carbonyl chloride routes (Miyasaka *et al.*, 1986).

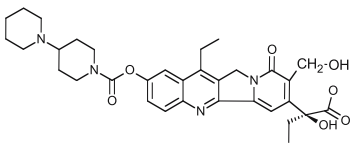
Physicochemical Properties

Irinotecan hydrochloride is a pale yellow to yellow crystalline trihydrate. The Clog P (ACD software) of irinotecan is 3.8 and the estimated pKa value (ACD) is 9.3 for the piperidino group, yielding a calculated log D (ACD) of 1.5 at pH 7. Given the highly conjugated ring system, the pKa of the quinoline functionality would be expected to be around 1, almost 4 units lower than that of quinoline itself (pKa 4.8) (Fassberg and Stella, 1992). The introduction of the basic piperdino group in the promoiety permits solubilities of greater than 20 mg/mL at pH 3–4, providing adequate solubility for an IV formulation.

Irinotecan, like the other camptothecins, contains an α-hydroxy-δ-lactone ring, which undergoes rapid, pH-dependent equilibration with the corresponding open chain carboxylic acid (irinotecan carboxylate) (Scheme 3). Only the lactone form (III) acts to inhibit topoisomerase and tumor growth, making this equilibrium of importance *in vivo* (Sano *et al.*, 2003). Extensive studies have been carried out to characterize this equilibrium for camptothecins (Fassberg and Stella, 1992; Burke and Mi, 1994; Sano *et al.*, 2003). The lactone form of camptothecins is favored at pH < 5, while the carboxylate form is favored at pH > 8. The rates of lactone hydrolysis and formation are similar between pH 6 and 7 for most of the camptothecins, yielding similar amounts of each species at equilibrium (Fassberg and Stella, 1992). Irinotecan lactone (III) is present at about 13% when in equilibrium at pH 7.4, 37oC, in phosphate buffer with a half-life for *in vitro* hydrolysis of about 26 min (Burke and Mi, 1994). The presence of 290 μM human serum albumin results in a slight reduction in the rate of hydrolysis with an increase in % lactone at equilibrium from 13 to 21%, hence an almost twofold reduction in rate of lactonization of the irinotecan carboxylate in the presence of albumin. It is of interest that the parent drug, SN-38, shows a slightly greater reduction in hydrolysis rate, with a greater than fivefold reduction in lactonization rate in the presence of albumin, leading to the % lactone at equilibrium being closer to 40% for the parent SN-38.



Irinotecan
(III)



Irinotecan Carboxylate
(IV)

Scheme 3. Equilibrium of closed and open forms of the lactone ring of irinotecan.

Formulation

Irinotecan is marketed as Camptosar® in 2 and 5 mL vials, as a pale yellow sterile aqueous solution of the hydrocholoride salt intended for single dose IV administration. Each mL contains 20 mg irinotecan·HCl trihydrate, 45 mg of sorbitol, and 0.9 mg of lactic acid formulated at pH 3.0-3.8. Camptosar® IV injectable aqueous solution formulation is marketed as a room temperature-stable formulation. Camptosar® must be diluted before IV administration with, preferably, 5% Dextrose Injection USP or alternatively, 0.9% Sodium Chloride Injection, USP, to a final irinotecan concentration of 0.12–2.8 mg/mL. Diluted Camptosar® solutions are physically and chemically stable for up to 24 h at room temperature under fluorescent lighting (PDR, 2005).

Assay for Irinotecan and SN-38

Irinotecan, SN-38, and the related metabolites of irinotecan are readily separated by reversed-phase HPLC using water-acetonitrile mixtures followed by detection with UV, fluorescence, or mass spectrometry (Ragot *et al.*, 1999; Kahn *et al.*, 2003; Poujol *et al.*, 2003). The lactone and carboxylate forms of irinotecan as well as SN-38 are also easily separated by HPLC (Burke and Mi, 1994). However, most pharmacokinetic studies determine the total amount of irinotecan and SN-38 in the plasma samples by treating the plasma extracts with low pH to force the lactone-acid equilibrium exclusively to the lactone form and then determining the lactone levels by HPLC (Slatter *et al.*, 2000; Poujol *et al.*, 2003). The T1/2 for equilibration of the lactone-carboxylic acid forms under these conditions is less than 1 h (Burke and Mi, 1994).

Bio-conversion and Pharmacokinetics

The *in vitro* cytotoxic activity of the prodrug irinotecan ranges from 100- to almost 3000-fold lower activity than that of the highly cytotoxic parent SN-38, depending on the cell lines used. The desired *in vivo* activity is primarily related to its conversion to SN-38 by carboxylesterase enzymes (Slatter *et al.*, 1997; Haaz *et al.*, 1998). These enzymes are present in high density in the liver (Humerickhouse *et al.*, 2000; Sanghani *et al.*, 2004). It also appears that there may be some potential for conversion in rat plasma due to butyrylcholinesterase (Morton *et al.*, 2000). Significant species differences in efficiency for conversion of irinotecan to SN-38 have been noted (Senter *et al.*, 1996; Danks *et al.*, 1999).

Intravenous administration of irinotecan results in an extremely complex pharmacokinetic profile that is influenced by the lactone to carboxylate conversions and dependent on a host of enzymes involved in metabolic transformation as well as active transport proteins regulating intestinal absorption and hepatobiliary secretion mechanisms (Mathijssen *et al.*, 2001). Upon IV administration, the conversion of the irinotecan lactone to the carboxylate form occurs rapidly *in vivo* with a mean half-life of 9.5 min (Rivory *et al.*, 1994), resulting in a rapid equilibration *in vivo* between the open and closed forms of both the prodrug and the active SN-38. This rapid equilibration results in the lactone to carboxylate AUC ratios of both irinotecan and SN-38 remaining relatively constant with dose and time (Rivory *et al.*, 1994).

The human metabolic scheme for irinotecan has been summarized (Slatter *et al.*, 2000; Mathijssen *et al.*, 2001). In addition to the desired carboxylesterase-mediated conversion of CPT-11 to SN-38, CPT-11 can also undergo cytochrome P-450 isoform 3A (CYP3A4)-mediated oxidation of the distal piperdine group to form a 5-aminopentanoic acid derivative (APC) on the piperdino group of CPT-11. Alternatively, the CYP3A4-induced cleavage of the distal piperidine group of CPT-11 leads to formation of the amino derivative (NPC) of the piperdino group on CPT-11. Unlike APC, NPC can still undergo carboxylesterase-mediated generation of SN-38.

The active molecule, SN-38, is converted to the inactive glucuronide derivative (SN-38G) and is excreted into the intestinal lumen via the bile. The histological damage in the intestines by IV administered CPT-11 is likely related to the liberation of SN-38 through deglucuronidation by intestinal ß-glucuronidase. The resulting blood level curves of irinotecan and SN-38, along with those of related metabolites following IV administration of irinotecan in a clinical study, are shown in Figure 1. The plasma samples are treated with dilute acid to convert the irinotecan and SN-38 to the lactone form before quantitation.

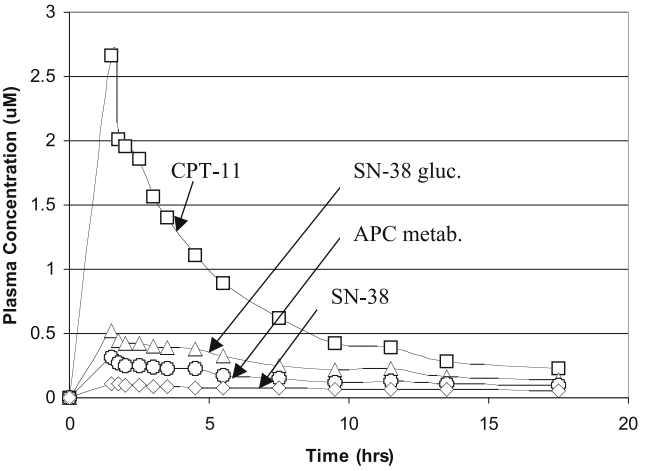


Figure 1. Mean plasma concentrations of intact CPT-11 (□), SN-38 (◇), glucuronidated SN-38 (△), and the 5-aminopentanoic acid (APC) metabolite (○) following a single dose IV infusion of CPT-11 (adapted from Slatter et al., 2000).

The $T_{1/2}$ of irinotecan in humans is 5.8–11.7 h at doses of 124–340 mg/cm^2, respectively, and the $T_{1/2}$ of SN-38 is 10.4–21.0 h, at doses of 124–340 mg/cm^2, respectively, when examined in patients with solid tumors (PDR, 2005). The cumulative urinary and fecal excretion recoveries of irinotecan and metabolites were: SN-38, 8.67%; SN-38G, 3.29%; APC, 10.5% and NPC, 1.5% (Slatter *et al.*, 2000) and the combined SN-38 and SN-38G values are 12%. Upon IV infusion of irinotecan in humans, the lactone form of SN-38 accounts for 60-70% of the total plasma SN-38 levels in humans (Mathijssen *et al.*, 2001; Sano *et al.*, 2003).

Safety

The major dose-limiting toxicities of CPT-11 therapies are diarrhea and leukopenia. Biliary excretion of SN-38 is largely responsible for the diarrhea that is often seen with IV administration of irinotecan in the clinic (Mick *et al.*, 1996). Diarrhea can be reduced by a number of strategies such as co-administering irinotecan (IV) along with oral administration of neomycin to prevent β-

glucuronidase conversion of SN-38 glucuronide to SN-38 or by oral alkalinization and controlled defecation (Takeda *et al.*, 2002; Alimonti *et al.*, 2004).

Summary

Irinotecan, the prodrug form of SN-38, is water soluble at low pH values, and this has allowed marketing of a stable aqueous solution formulation under the trade name of Camptosar®. The conversion of irinotecan to the parent drug SN-38 is incomplete. However, the resulting low levels of SN-38 are effective in treating colorectal cancers. Strategies to reduce the level of SN-38 in the intestine are effective in reducing the occurrence of diarrhea and in allowing administration of larger doses of irinotecan. In effect, irinotecan is a prolonged release prodrug form of SN-38, and this effectively reduces the side effects of the parent drug.

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5.13

Case Study: Latanoprost: Isopropylester of a Prostaglandin F2α Analog

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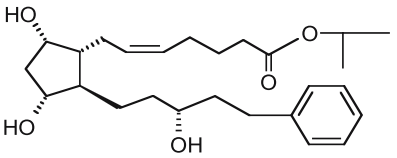
Table of Contents

Name:

Latanoprost, isopropyl-(Z)-7[(1R,2R,3R,5S)3,5-dihydroxy-2-[(3R)-3-hydroxy-5-phenylpentyl]cyclopentyl]-5-heptenoate, Xalatan®. Its molecular formula is $C_{26}H_{40}O_5$ (M.W. 432.58) and its chemical structure is:



Rationale for Latanoprost

Latanoprost is an esterified prodrug and does not render its potent interaction with the prostaglandin $F_{2\alpha}$ receptor until hydrolyzed to its free acid. The phenyl-substituted esters of $PGF_{2\alpha}$ analogs were also found not to be metabolized by 15-hydroxyprostaglandin dehydrogenase, which is found in various ocular tissues, allowing them to penetrate the cornea where they are hydrolyzed to the active free acid form by esterases (Basu *et al.*, 1994). In addition, latanoprost is also thought to be less irritating than the $PGF_{2\alpha}$ isopropyl free ester when applied topically to the eye (Astin *et al.*, 1994). Naturally occurring prostaglandins may cause marked microcirculatory changes in the eye; however, structural analogs with increased affinity and selectivity for prostaglandin F2 receptors, such as latanoprost, have little or no such effect (Stjernschantz *et al.*, 2000).

Synthesis

The original work on the synthesis of the phenyl-substituted PGF2α analogues has been described by Resul *et al.* (1993) and Stjernschantz and Resul (1992). A description of the syntheses of several prostaglandin therapeutic agents has also been described by Collins and Djuric (1993).

Mechanism and Site of Bioreversion

Latanoprost is a prostanoid-selective FP receptor agonist that is believed to reduce the intraocular pressure (IOP) by increasing the outflow of aqueous humor (Bito *et al.*, 1993). Studies in animals and man also support that the main mechanism of action is increased uveoscleral outflow (Toris *et al.*,1993). Latanoprost is absorbed through the cornea where the isopropyl ester prodrug is hydrolyzed to the acid form by esterases to become biologically active. Studies in man indicate that the peak concentration in the aqueous humor is reached about

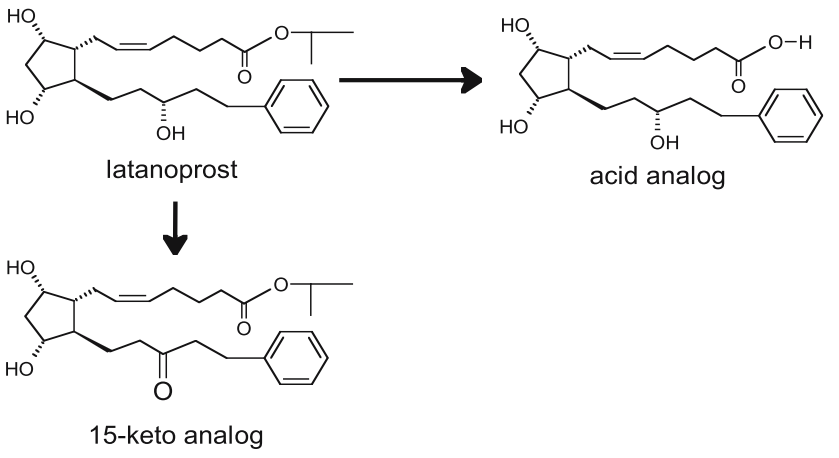
two hours after topical administration. The acid of latanoprost can be measured in aqueous humor during the first four hours, and in plasma only during the first hour after local administration.

Toxicity Issues

The main side effects of PGF2α are conjunctival hyperemia and symptoms of ocular irritation (Giuffre, 1985). The hyperemia may be due in part to PGF2α-mediated activation of nitric oxide synthase in the eye (Astin *et al.*, 1994; Yousufazi *et al.*, 1996). In monkeys, latanoprost has been shown to induce increased pigmentation of the iris (Selen *et al.*, 1997). The results from the preclinical program demonstrated that the increased pigmentation is unlikely to be associated with proliferation of melanocytes. It appears that the mechanism of increased pigmentation is stimulation of melanin production in melanocytes of the iris stroma (Linden *and Alm*, 1999). In ocular toxicity studies, administration of latanoprost at a dose of 6 g/eye/day (4 times the daily human dose) to cynomolgus monkeys has also been shown to induce increased palpebral fissure. This effect is reversible and occurs at doses above the standard clinical dose level. Thus in patients latanoprost may gradually change eye color, increasing the amount of brown pigment in the iris by increasing the number of melanosomes (pigment granules) in melanocytes. The long-term effects on the melanocytes and the consequences of potential injury to the melanocytes and/or deposition of pigment granules to other areas of the eye are currently unknown. The change in iris color occurs slowly and may not be noticeable for several months to years.

Formulation Issues

Latanoprost is a colorless to slightly yellow oil, which is practically insoluble in water. The commercial product, Xalatan™, is supplied as a sterile, isotonic, buffered aqueous solution of 0.005% latanoprost. Benzalkonium chloride, 0.02%, is added as a preservative and also helps in solubilizing the drug. The product



must be refrigerated to avoid potency loss due to absorption of latanoprost to the plastic packaging material (low-density polyethylene). The major degradation products at higher temperatures are its oxidation product, 15-keto latanoprost, and its hydrolysis product, the free acid form. The pH of Xalatan solution is 6.7. Latanoprost is also susceptible to degradation in the presence of light (Morgan *et al.*, 2001)

Discussion

Latanoprost was one of the first of the major prostaglandin drugs with high potency for the treatment of open angle glaucoma. A large number of ring-substituted prostaglandin analogs have been studied (Resul *et al.*, 1997) and optimal activity was seen in those molecules with terminal phenyl ring attached to carbon 17 in $PGF_{2\alpha}$. In latanoprost, the double bond between carbons 13 and 14 has been saturated, which further improved its receptor selectivity. Thus, latanoprost exerts its action through selective stimulation of the FP prostanoid receptor; its reduced hyperemic effect could be due in part to reduced stimulation of the vasodilatory FP prostanoid receptors and concomitant reduction in the release of nitric oxide (Astin *et al.*, 1994). It has a low rate (5–15%) of hyperemia (Alexander *et al.*, 2002). Its major side effect is the hyperpigmentation of the iris. This hyperpigmentation is not due to the proliferation of melanocytes but, rather, a stimulation of the production of melanin (Lindquist *et al.*, 1999). After topical administration to the eye, latanoprost is hydrolysed to the free acid in the cornea. Using autoradiography, it was shown that latanoprost acid was mainly confined to the anterior part (peak concentration $\sim 10^{-7}$ M) of the eye in monkeys, and very little reached the posterior segment (Sjoquist *et al.*, 1999). Similar concentrations of latanoprost acid have been determined in the aqueous humour in man after topical administration of the clinical dose (Sjoquist and Stjernschantz, 2002). The active acid of latanoprost reaching the systemic circulation is primarily metabolized by the liver to the 1,2-dinor and 1,2,3,4-tetranor metabolites via fatty acid oxidation. Following hepatic oxidation, the metabolites are mainly eliminated via the kidneys. The elimination of the acid of latanoprost from human plasma was rapid (t = 17 min) after both intravenous and topical administration. Systemic clearance is approximately 7 mL/min/kg. Approximately 88% and 98% of the administered dose is recovered in the urine after topical and intravenous dosing, respectively (Sjoquist and Stjernschantz, 2002).

In finding the optimal dose of latanoprost a delicate balance was required between its IOP-lowering effect and its potential side effects. Studies by several researchers indicated that 0.005% latanoprost was very close to the top of the dose-response curve (Villumsen and Alm 1992; Alm *et al.*, 1993). No significant increase in IOP lowering effect was seen at doses above 60 μg/mL.

Latanoprost produces additional reduction in IOP when used in combination with other classes of antiglaucoma agents such as beta-blockers (timolol), carbonic anhydrase inhibitors (acetazolamide), and cholinergic agents (pilocarpine) (Hoyng *et al.*, 1997). The IOP lowering effects of latanoprost in combination with

timolol (Timoptic®, Merck) and drozolamide and pilocarpine have been reported in the literature (Calissendorff *et al.*, 2002; Toris *et al.*, 2002). In all cases, however, it is recommended that Xalatan and other drugs be applied to the eye at least 5 min apart to prevent any possible precipitation of latanoprost due to formulation incompatibilities.

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5.14

Case Study: Moexipril Hydrochloride: A Prodrug of Moexiprilat

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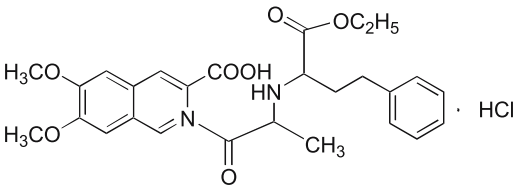
Table of Contents

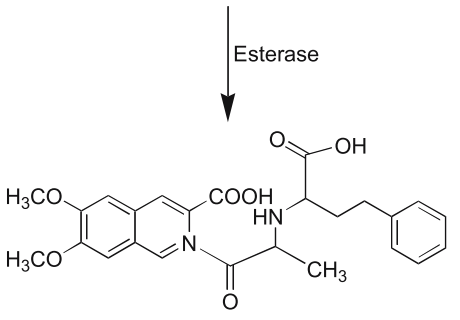
Name:

3–Isoquinolinecarboxylic acid, 2–[(2S)–2–[[(1S)–1–(ethoxycarbonyl)–3–phenylpropyl]amino]–1–oxopropyl]–1,2,3,4–tetrahydro–6,7–dimethoxy–monohydrochloride, (3S)– (9CI); 3–Isoquinolinecarboxylic acid, 2–[2–[[1–(ethoxycarbonyl)–3–phenylpropyl]mamino]–1–oxopropyl]–1,2,3,4–tetrahydro–6,7–dimethoxy-, monohydrochloride, [3S–[2[R*(R*)],3R*]]–; CI 925; Moexipril hydrochloride; RS 10085–197; SPM 925; Univasc

Structures and Bioconversion Pathway



Moexipril hydrochloride



Moexiprilat

Rationale for Moexipril Hydrochloride

Moexiprilat is an orally active, nonsulfhydryl, long-acting dipeptide angiotensin-converting enzyme (ACE) inhibitor used to lower blood pressure (Stimpel *et al.*, 1995; Brogden and Wiseman, 1998; Chrysant and Chrysant, 2003). Moexipril hydrochloride shows improved bioavailability compared to moexiprilat, most likely because esterification reduces the charge of the molecule and the pKa of its amine group ($pKa_{Moexprilat} = 7.7$; $pKa_{Moexipril} = 5.4$). Based on the comparable oral bioavailabilities of various esters of enalapril with different lipophilicities, it appears that lipophilicity is not the only factor that enhances oral bioavailability (Wyvratt and Patchett, 1985).

Synthesis

Moexipril hydrochloride, which was synthesized by (Hoefle and Klutchko, 1982; Klutchko *et al.*, 1986), incorporates structural features of the active ACE inhibitors enalapril and conformationally constrained acyl phenylalanines.

Mechanism and Site of Bioreversion

Moexipril hydrochloride is a substrate for esterase(s), which catalyze its metabolism to moexiprilat. It has been reported that this bioconversion occurs primarily in the liver, however, based on similar ACE inhibitors, the intestine and portal blood are likely to be involved in first pass effects. Moexiprilat binds to ACE present in various tissues and body fluids, thus inhibiting its action (Unger and Gohlke, 1994; Edling *et al.*, 1995). ACE is part of the renin-angiotensin system and catalyzes the conversion of angiotensin I to angiotensin II, a vasoconstrictor. In addition, ACE degrades the potent vasodilator bradykinin. Reduced levels of angiotensin II result in increased renin activity, decreased levels of aldosterone, and, consequently, reduced renal Na^+ reabsorption. Ultimately, the extracellular fluid volume in the body is reduced; this is reflected in lowered blood pressure. Whereas several studies (Unger and Gohlke, 1994; Edling *et al.*, 1995; Friehe and Ney, 1997) indicate that blockage of angiotensin II production is the main mechanism responsible for blood pressure reduction, the effect of inhibition of the degradation of bradykinin on hypertension is not clear.

Toxicity Issues

Long-term studies demonstrated that moexipril hydrochloride is, in general, a safe drug with no significant toxicity (White *et al.*, 1994; Lucas *et al.*,1995; White and Stimpel, 1995). The most common side effects that have been observed with this prodrug include cough, fatigue, headache, and dizziness. Its safety profile is similar to those of other ACE inhibitors. Coadministration with other drugs (*e.g.*, warfarin) did not cause any relevant adverse reactions. Moreover, it has been shown that combination therapy with hydrochlorothiazide (Drayer *et al.*, 1995; White *et al.*, 1997) is more effective in lowering blood pressure than when either drug is used alone. However, concomitant administration of this drug with potassium salts, potassium-sparing diuretics, and non-steroidal antiinflammatory agents is not advised.

Formulation Issues

Moexipril hydrochloride is supplied in tablet form (7.5 or 15 mg). A long terminal half-life ($t_{1/2\beta}$) of about 30 h as well as prolonged ACE inhibition permits once-daily oral administration (Cawello *et al.*, 2002). A controlled-release dosage form (Fig. 1) does not have an advantage over an immediate-release dosage form, possibly because of site-specific absorption of moexipril hydrochloride (Grass and

Morehead, 1989). It has been reported (Gu *et al.*, 1990) that excipients in general have a destabilizing effect on moexipril hydrochloride in the solid state. In addition, moisture as well as basic agents promotes instability. In contrast, basic agents in wet granulations improve stability. Hydrolysis and cyclization yield the main degradation products (Gu and Strickley, 1987, 1988; Strickley *et al.*, 1989; Gu *et al.*, 1990) (moexiprilat, moexipril diketopiperazine, and the diacid analog of moexipril diketopiperazine) both in solid state and aqueous solution. The pH rate profile for moexipril conversion to moexiprilat in aqueous solution shows maximum stability at approximately pH 4.5 and 10, respectively. The lyophilized powder form of moexipril hydrochloride was most stable at pH 8. The U-shaped pH-solubility profiles of moexipril and moexiprilat show minimum aqueous solubility at pH 4.2 and 2.5, respectively.

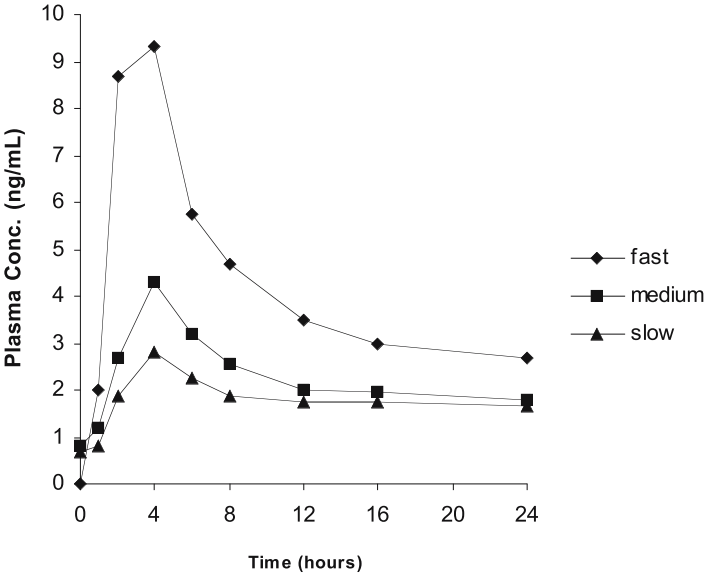


Figure 1. Mean plasma concentrations of moexiprilat in male human volunteers (n = 12) following oral administration of 30 mg of moexipril as a controlled-release dosage form with various release rates. Reproduced with permission from *Pharm Res* 1989; 6:759–765.

Discussion

Moexipril hydrochloride is approved in Europe and in the United States; it shows clinical efficacy similar to those of other commonly used antihypertensive compounds (Drayer *et al.*, 1995; Abernethy *et al.*, 1995; Stimpel *et al.*, 1996) Furthermore, it is relatively cost-effective. Although orally active, moexipril shows a relatively low bioavailability (22%). Moreover, the rate and fraction of drug absorbed decreases even more in the presence of food (Fig. 2) (Grass and Morehead, 1989; Cawello et al, 2002). However, this did not negatively impact the efficacy of the drug.

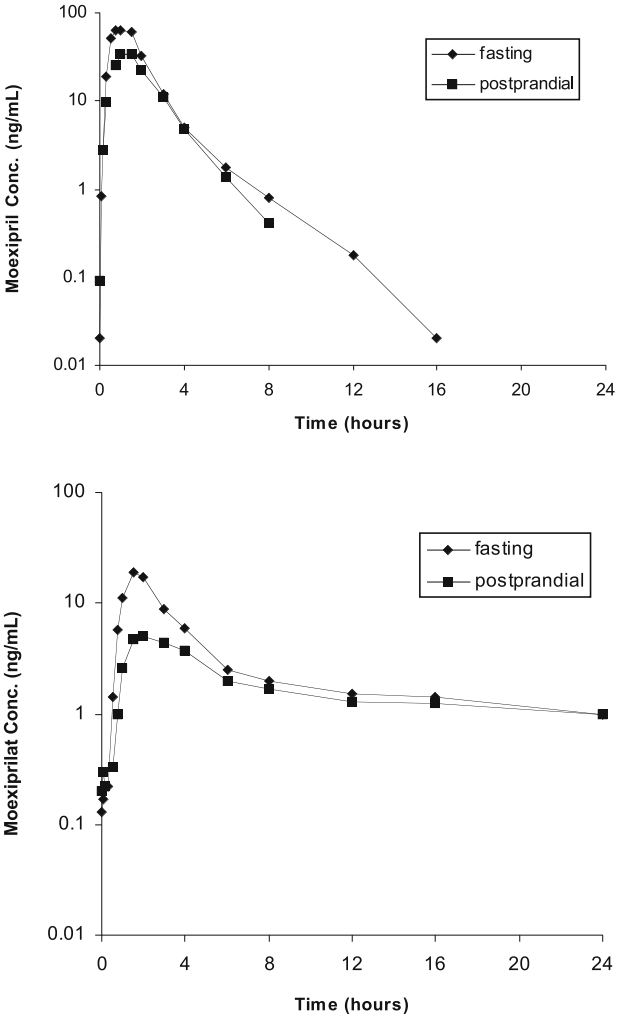


Figure 2. Mean plasma concentrations of moexipril and moexiprilat in fasted and fed male human volunteers (n = 24) over 24 h after single oral administration of 15 mg moexipril, mean log-linear data. Reproduced with permission from *Int J Clin Pharmacol Ther* 2002; 40:9–17.

Stimpel *et al.* (1995) studied many aspects of the pharmacokinetics of moexipril. The maximum plasma concentration (C_{max}) is reached relatively rapidly (within 2 h) after oral administration of a 7.5–30 mg dose. Compared to healthy volunteers, hepatically impaired patients showed increased C_{max} values for moexipril and decreased C_{max} values for moexiprilat after receiving single doses of moexipril. AUC values were increased for both compounds. Renally impaired patients as well as elderly people also showed increased AUC values after moexipril administration. Moexiprilat is highly protein bound (~70%) and has a volume of distribution (Vd) of about 183 L and a clearance of 13.9 L/h. Moexipril hydrochloride has a clearance of 26.5 L/h. Independent of dose, moexiprilat has an elimination half-life ($t_{1/2}$) of about 10 h and a terminal half-life of about 30 h.

After oral and i.v. administration, about 53% and 20%, respectively, of the drug is eliminated in feces, mainly as moexiprilat, and about 13% and 66%, respectively, is eliminated by renal excretion (Stimpel *et al.*, 1995).

Besides its antihypertensive effect, moexilpril provides additional medical benefits as reviewed by Pines and Fisman (2003).

Conclusion

ACE inhibitors are in general interchangeable; however, each ACE inhibitor has unique characteristics that make it more or less suitable for different individuals (White, 1998). Moexipril is a safe and very well tolerated addition to the portfolio of ACE inhibitors used to control hypertension.

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5.15

Case Study: Mycophenolate Mofetil

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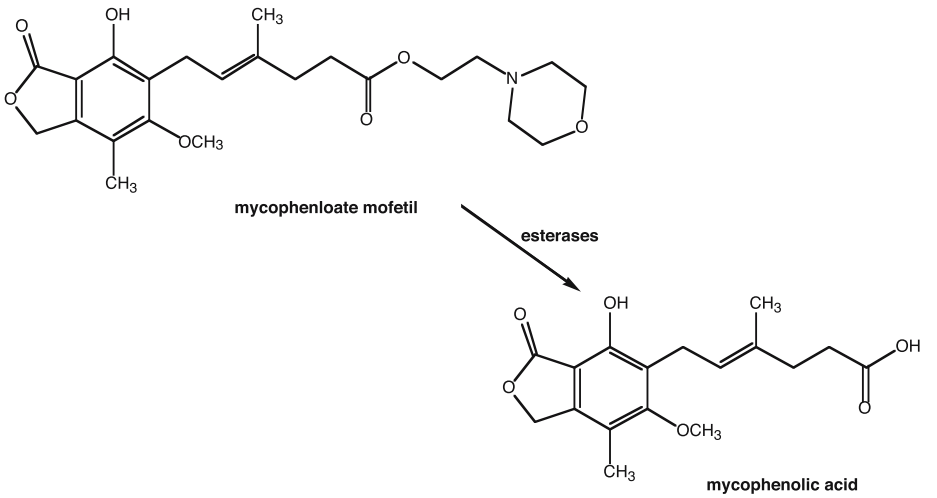
Table of Contents

Name:

Mycophenolate mofetil, 2-morpholinoethyl (E)-6-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-methyl-4-hexeneoate, CellCept®

Structures and Bioconversion Pathway



Rationale for Mycophenolate Mofetil

Mycophenolate mofetil (MMF) is an orally bioavailable prodrug of mycophenolic acid (MPA), a potent immunosuppressive agent. MPA acts by inhibiting inosine monophosphate dehydrogenase (IMPDH), which catalyzes the first step in *de novo* synthesis of guanine (Ransom, 1995). This process is critical to mitogen-stimulating lymphocytes, which gives rise to MPA's therapeutic efficacy.

MPA is poorly bioavailable after oral administration (<40%) (Lee *et al.*, 1990). Further, the bioavailability is quite variable in monkeys, resulting in C_{max} values ranging over tenfold (Lee *et al.*, 1990). MPA's poor partition coefficient and/or its low solubility does not fully explain its low oral bioavailability. Lee *et al.* (1990) compared the intrinsic solubilities, pKas, and partition coefficients over a range of pH values for MPA with those of naproxen, a related compound with excellent bioavailability, and found these properties to be almost identical. Therefore, they concluded that other phenomena (*i.e.*, drug complexation in the GI tract, a narrow absorption window, and/or presystemic metabolism) must be responsible for the low oral bioavailability of MPA.

Although the mechanism is not fully understood, the mofetil ester of MPA, MMF, exhibits much better oral bioavailability with less variability. As a base, MMF is more soluble in the acidic regions of the GI tract and partitions into

lipophilic phases better in more neutral/basic environments (Lee *et al.*, 1990). This situation is the reverse of that of MPA and thus may alleviate any narrow absorption window issues. Regardless, the bioavailability of MPA from MMF is 94% relative to intravenous administration of this prodrug (Roche, 2003). This improved bioavailability leads to more predictable blood levels, with C_{max} values ranging only threefold in monkeys compared to the tenfold range seen with MPA (Lee *et al.*, 1990). Presumably, this translates into safer dosing in humans.

Synthesis

The synthesis of MMF as well as various other MPA derivatives was first described by Nelson *et al.* (1988).

Mechanism and Site of Bioconversion

The mechanism of bioreversion of MMF to MPA is esterase catalyses, with the byproduct being 4-(2-hydroxyethyl)morpholine. Lee *et al.* (1990) concluded that the site of bioconversion after oral dosing is presystemic, probably in the liver. Conversion in human and monkey plasma proceeded with half-lives of 3.8 and 8.5 h, respectively. However, upon oral dosing to monkeys, only MPA and the MPA-glucuronide conjugate were detectable in plasma even minutes after dosing; MMF was not detected. Because plasma esterases could not account for the rapid conversion (from *in vitro* experiments), this suggests a possible alternate site of bioconversion prior to the plasma. Half-lives for chemical conversion of MMF to MPA in aqueous solution at 37°C range from 19 to 118 h across a pH range of 2.0 to 7.4, making chemical degradation in the GI tract unlikely. Thus, Lee *et al.* (1990) concluded that this presystemic site was most likely the liver, with its known high esterase activity. This was supported by the evidence that the half-life of MMF in mouse liver homogenate is less than 5 s. Other presystemic sites, such as the enterocytes, cannot be ruled out.

It should be noted that the major metabolite of MPA seen in the plasma is the inactive MPA glucuronide conjugate, which undergoes enterohepatic recirculation (Fulton and Markham 1996; Hood and Zarembski, 1997). This may account, in part, for the sustained blood levels of MPA.

Toxicity Issues

Conaway *et al.* (1984) reported that 4-(2-hydroxyethyl)morpholine, the promoiety in this case, induced genotoxicity in rats. Since this prodrug was approved by the FDA, genotoxicity is apparently not an issue in humans.

Formulation Issues

MMF (CellCept®) is currently sold as capsules and tablets that may be stored at room temperature. The tablets must be stored in a light-resistant container

(Roche, 2003). MMF is also available as a reconstitutable powder for oral suspension, and the hydrochloride salt of MMF is also available for intravenous administration (Roche, 2003).

Discussion

MMF (CellCept®) clearly offers an improvement in pharmacokinetic properties over MPA. However, in several clinical studies conducted with capsules of MPA for the treatment of psoriasis (Jones *et al.*, 1975; Marinari *et al.*, 1977; Epinette *et al.*, 1987), doses similar to that of MMF were used, resulting in an adverse events profile similar to that of MMF (Roche, 2003).

MPA was first isolated in 1896 (Gosio, 1896), and the structure was elucidated by Birkinshaw *et al.* in 1952; thus, MPA was not a patentable chemical entity. Utilizing the prodrug, which presented some documented bioavailability advantages, offered a means to avoid this barrier by providing protection from competition for the life of the prodrug.

Conclusion

CellCept® is a good example of a prodrug that is able to improve pharmacokinetic properties as well as provide patent protection for a previously disclosed compound.

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Roche Laboratories. CellCept® Complete Product Information. 2003

5.16

Case Study: Olmesartan Medoxomil: A Prodrug of Olmesartan

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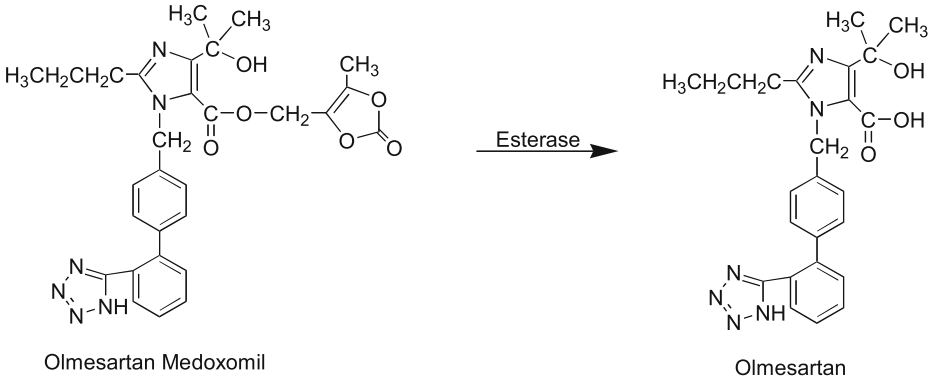
Table of Contents

Name:

1H–Imidazole–5–carboxylic acid, 4–(1–hydroxy–1–methylethyl)–2–propyl–1–[[2'–(1H-tetrazol–5–yl)[1,1'–biphenyl]–4–yl]methyl]–, (5–methyl–2–oxo–1,3–dioxol–4–yl)methyl ester (9CI); 1,3–Dioxole, 1H–imidazole–5–carboxylic acid derivative; Benicar; CS–866; Olmesartan medoxomil

Structures and Bioconversion Pathway



Olmesartan Medoxomil

Olmesartan

Rationale for Olmesartan Medoxomil

Olmesartan is a durable, specific, and competitive nonpeptide angiotensin II receptor antagonist used to treat hypertension. To achieve better oral bioavailability its ester prodrug, olmesartan medoxomil, was developed. Olmesartan medoxomil is suitable for once daily oral administration and is available in tablet form.

Synthesis

Olmesartan medoxomil was synthesized and evaluated for its biological activity among a series of related compounds by Yanagisawa *et al.* (1996). In this study, it represented one of the most potent orally active compounds with long inhibitory activity.

Mechanism and Site of Bioconversion

Both *in vivo* and *in vitro* studies demonstrated rapid bioconversion of olmesartan medoxomil to olmesartan in plasma and biological tissues (*e.g.*, liver, intestine) by esterases (Kobayashi *et al.*, 2000; Laeis *et al.*, 2001; Schwocho and Masonson, 2001; von Bergmann *et al.*, 2001). In plasma, arylesterase catalyzed the bioconversion. After oral administration of olmesartan medoxomil to human

volunteers, only olmesartan was detected in plasma, urine, and fecal samples, indicating that the prodrug is rapidly hydrolyzed to the pharmacologically active drug before it reaches systemic circulation. Potential inactive metabolites generated by the ester hydrolysis include carbon dioxide and diacetyl, the latter being reduced to acetoin and 2,3–butanediol. Olmesartan is stable and does not break down to any further metabolites.

Toxicity Issues

No clinically relevant adverse reactions were observed for olmesartan medoxomil that would distinguish its safety profile from that of the placebo group; only dizziness (2.8%) occurred more frequently in the olmesartan medoxomil group (Puechler et al, 2001; Brunner, 2002; Neutel *et al.*, 2002). In contrast to angiotensin-converting enzyme (ACE) inhibitors, another class of drugs used to reduce blood pressure, angiotensin II receptor blockers (ARBs) do not cause coughing, probably due to their more specific mechanism of blocking angiotensin II. Olmesartan medoxomil is not a substrate, inhibitor or inducer for the cytochrome P450 (CYP) system; thus, no drug interactions are expected with compounds that do interact with this system. Consistent with this, coadministration of olmesartan medoxomil with other drugs (e.g., digoxin, warfarin) did not significantly alter the drug response in patients (Laeis *et al.*, 2001). Minimal drug interactions might be particularly desired for certain groups (*e.g.*, the elderly) that are on multiple drug therapy and in which drug interactions are more likely to occur. In addition, no food-drug interactions were observed.

Formulation Issues

Olmesartan medoxomil is available as film-coated tablets (5, 20, and 40 mg) and, due to its long terminal half-life (~12–18 h), can be administered once daily (Schwocho and Masonson, 2001). A dose larger than 20–40 mg does not provide an improved therapeutic response, neither does dosing twice a day versus dosing once a day (Neutel *et al.*, 2002). In addition, food intake does not have any significant impact on its bioavailability (26%); therefore, the drug can be taken with or without food (Song and White, 2001). Olmesartan has also been evaluated as an intravenous formulation (Schwocho and Masonson, 2001); however, it is not distributed as such.

Discussion

Olmesartan medoxomil was first introduced in 2002, and has been shown to be safe and highly effective in treating high blood pressure. Additionally, it has minimal side effects and drug interactions.

Various clinical studies determined the pharmacokinetic parameters of olmesartan (Laeis *et al.*, 2001; Schwocho and Masonson, 2001, von Bergmann *et al.*, 2001). After oral administration of olmesartan medoxomil, maximum plasma

concentrations of olmesartan were reached in approximately 2 h independent of dose (Fig. 1), and steady-state levels were reached within a few days. Peak plasma concentration (C_{max}) and AUC increased linearly with increasing dose well above the maximum daily recommended oral dose of 40 mg. In addition, it has been shown that C_{max} and AUC increased in the elderly as well as in renal- and hepatic-impaired patients. However, only in severely renal-impaired patients may this necessitate dosage adjustment. Oral doses (up to 80 mg/once daily) of olmesartan medoxomil received for ten days did not result in drug accumulation. Olmesartan medoxomil is highly protein bound; therefore, the volume of distribution determined after oral and intravenous administration was relatively low (34.9 and 25.3 L, respectively). After oral administration of olmesartan medoxomil, approximately 10% of the drug was excreted in urine. This fraction increased to about 40% when olmesartan was given intravenously; the remaining drug was eliminated in faeces via biliary excretion.

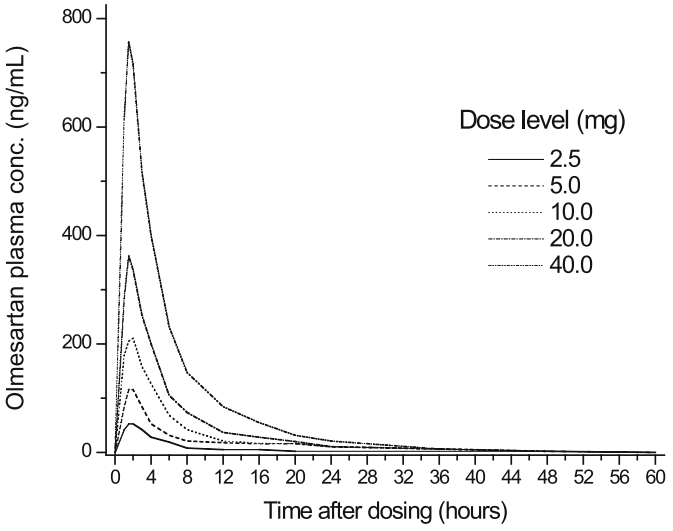


Figure 1. Mean plasma concentrations of olmesartan in male human volunteers (n = 30) after seven daily oral doses of olmesartan medoxomil. Reproduced with permission from *J Hypertens* 2001;19 Suppl 1:S21–32.

As demonstrated in several studies and reviews (Ball *et al.*, 2001; Oparil *et al.*, 2001; Greathouse, 2002; Oparil, 2002; Stumpe and Ludwig, 2002; Chrysant *et al.*, 2003; Gardner and Franks, 2003; Simons, 2003), olmesartan medoxomil compares well with other antihypertensive drugs in terms of cost-effectiveness, safety, and efficacy. Studies in animals and humans have indicated that olmesartan medoxomil may have the potential to treat both atheroscleris in addition to controlling blood pressure (Miyazaki and Takai, 2002; Schiffrin, 2002; Takai *et al.*, 2003) and diabetic nephropathy (Lewis, 2002; Mizuno *et al.*, 2002).

Conclusion

The prodrug olmesartan medoxomil shows improved bioavailability compared to olmesartan, making oral administration possible.

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5.17

Case Study: Omeprazole (Prilosec®)

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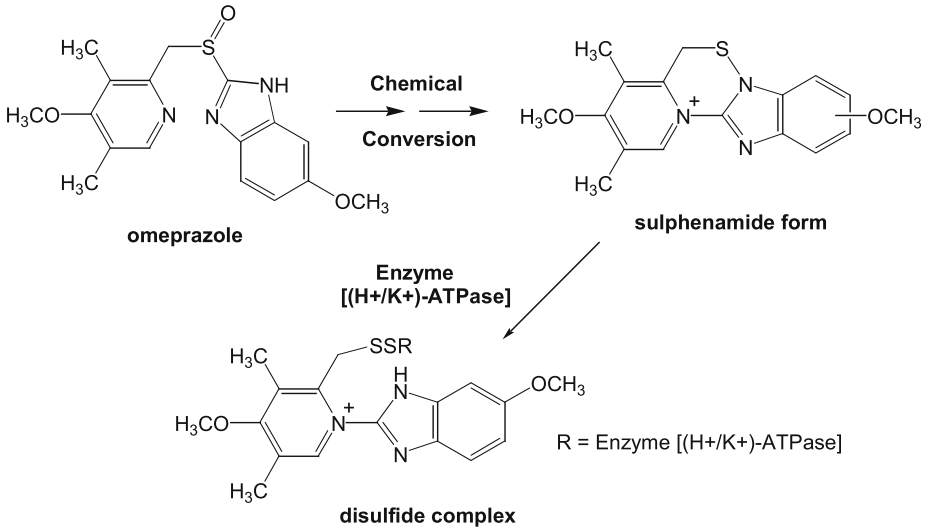
Table of Contents

Name

Omeprazole, 1H-Benzimidazole, 5-methoxy-2-[[(4-methoxy-3,5-dimethyl-2-pyridinyl)methyl]sulfinyl]- (9CI), Prilosec®

Structures and Bioconversion Pathway



Rationale for Omeprazole

Omeprazole, currently marketed as Prilosec and generic omeprazole, has been shown to reduce gastric acid secretion in animals and man. It reduces the secretion of acid by inhibiting the proton pump of the parietal cell (Fellenius *et al.*, 1981). Omeprazole was not designed as a prodrug. It was first synthesized by Junggren *et al.* (1981) along with 29 other derivatives of substituted 2-(2-benzimidazolyl)-pyridines and was found to have more potent gastric acid secretion inhibitory effects in dogs than did previously tested structurally related compounds. However, omeprazole was later found not to be the active inhibitor of the proton pump per se. Instead, it acts as a prodrug and is converted to the active inhibitor in the acidic compartments of the parietal cell, near the enzymes it inhibits (Lindberg *et al.*, 1986).

The pharmacology and therapeutic use of omeprazole is extensively reviewed in the literature (Clissold and Campoli-Richards, 1986; McTavish *et al.*, 1991; Hetzel, 1992; Wilde and McTavish, 1994; Andersson, 1996). Omeprazole is extremely effective in the treatment of duodenal and gastric ulcers, peptic ulceration refractory to treatment with H_2-receptor antagonists, gastro-oesophageal reflux disease, and Zollinger-Ellison syndrome. It is also useful in the eradication of *Helicobacter pylori* infection and is particularly effective in

combination with one or more antibacterial agents (Logan 1996; Langtry and Wilde 1998; Ulmer *et al.*, 2003).

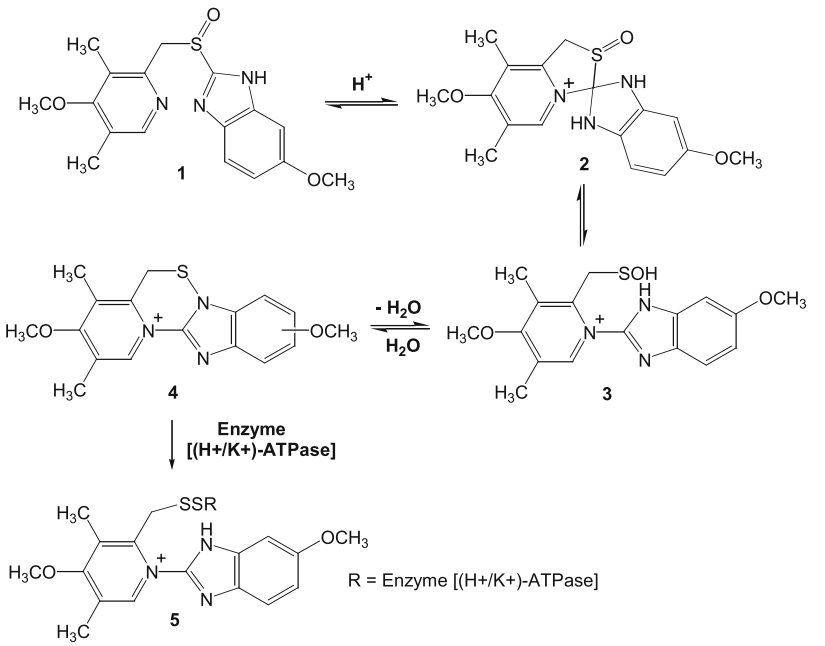
Prilosec® was launched in the United States in 1989 and in 1996 became the world's top-selling pharmaceutical product (AstraZeneca). The original omeprazole patent expired in 2001, and generic omeprazole became available in late 2002. Prilosec® was approved by the FDA for over-the-counter sale in 2003.

Synthesis

Synthetic preparations of omeprazole are found almost exclusively in the patent literature. The synthesis of omeprazole was first reported by Junggren *et al.* (1981). There have been many patents filed over the years claiming improved synthetic procedures (Brandstrom, 1991; Slemon and Macel, 1994; Smahovsky *et al.*, 1998; Baldwin et, al., 1999; Correia *et al.*, 2003).

Mechanism and Site of Bioreversion

Omeprazole is a weak base with a pK_a of 3.97 (Brandstrom *et al.*, 1985). Weak bases are known to accumulate in acidic environments such as the acidic canaliculi of the parietal cells, deep in the gastric mucosa (Wallmark *et al.*, 1984). The parietal cell is the only cell in the body that exhibits such a low pH. The proton pump, hydrogen/potassium adenosine triphosphatase [(H^+/K^+)-ATPase], is located in the apical membrane and tubulovesicles bordering the secretory canaliculi of the parietal cell. Omeprazole in converted to its active sulphenamide form by



Scheme 1.

rearrangement under acidic conditions near the acid-producing enzyme it inhibits. The active sulphenamide form reacts with sulfhydryl groups of [(H^+/K^+)-ATPase], irreversibly binding to form a disulfide complex, which inhibits the proton pump.

The conversion of omeprazole to its active sulphenamide form (Scheme 1) is a reversible chemical process resulting from the dehydration of a sulfenic acid (3) formed from the acid-promoted rearrangement of omeprazole. The process begins with the protonation of omeprazole (1), which rearranges to form a highly reactive sulfenic acid (3). This sulfenic acid is believed to form via a spiro intermediate (2). The sulfenic acid (3) can lose water to give the active sulphenamide form (4). The active sulphenamide form of the drug irreversibly binds to [(H^+/K^+)-ATPase] forming a disulfide complex (5), which blocks the enzyme function. This reaction pathway was purposed by Lindberg *et al.* (1986), who first isolated the sulphenamide (4). This conversion pathway has been supported by extensive studies of the chemical reactions of omeprazole (Senn-Bilfinger *et al.*, 1987; Sturm *et al.*, 1987; Brandstrom *et al.*, 1989).

Toxicity Issues

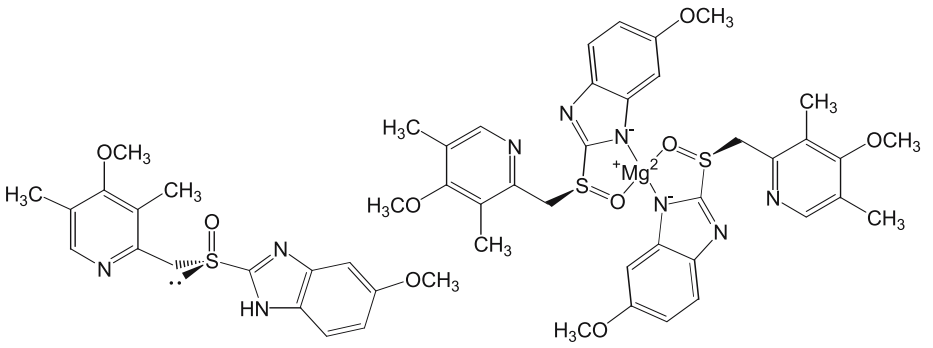
Toxicological studies on omeprazole have been performed in several species of animals with no significant clinical signs of adverse effects (Ekman *et al.*, 1985; Carlsson, 1989; Evans, 1990). Short- and long-term tolerability of omeprazole in human clinical trials has been reviewed (Nelis, 1989; Arnold and Koop, 1989; Solvell, 1990; Joelson *et al.*, 1992). It has now been used by millions of patients and is well tolerated in both long- and short-term use; most adverse events are considered to be minor and infrequent. Omeprazole does show some drug-drug interactions associated with its interference with the cytochrome P450 enzyme system. Omeprazole can potentially inhibit the metabolism of drugs such as diazepam, phenytoin, and the R isomer of warfarin, but not propranolol, theophylline, or the S-isomer of warfarin (Andersson, 1991; Humphries, 1991). These effects are limited and not reproducible in all patients.

Formulation Issues

Omeprazole is supplied in the form of delayed-release capsules for oral administration. It is formulated as enteric-coated granules containing 10, 20, or 40 mg of omeprazole. The drug is known to be acid labile, and the enteric-coating protects the drug from pre-absorption degradation in the acid environment of the stomach (Cederberg *et al.*, 1989; Howden, 1991). Absorption begins after the granules leave the stomach, with peak plasma concentrations 1–3 h after dosing.

The sulfoxide sulfur of omeprazole creates a chiral center, and omeprazole is a racemic mixture of S and R enantiomers. Esomeprazole is the S-enantiomer of omeprazole. It is the first proton pump inhibitor to be developed as a single stereoisomer. Esomeprazole is optically stable in humans (Hassan-Alin *et al.*,

2000). It is apparently metabolized by hepatic enzymes to a lesser extent than is omeprazole and results in greater systemic delivery (Dent, 2003). For a review of the pharmacology of esomeprazole see Spencer and Faulds (2000). Nexium® is a formulation of the magnesium salt of esomeprazole, which was launched in the United States in March 2001.



esomeprazole **esomeprazole magnesium salt**

Discussion

When a prodrug strategy is considered, it usually starts with a well-characterized drug that has some unfavorable drug-like property (*e.g.*, solubility, permeability). A prodrug strategy is then developed in an attempt to overcome this problem. That is, however, not the case with omeprazole. While omeprazole the prodrug has been well characterized, very little is known about the active form of the drug. The active form of the drug was not discovered until five years after omeprazole was found to inhibit gastric acid secretion (Lindberg *et al.*, 1986).

Omeprazole was included here as a case study to exemplify a very effective prodrug strategy that was unknown at the time of its creation. Nonetheless, omeprazole is a very good example of a prodrug that is targeted specifically to its site of action. As a weak base, omeprazole accumulates from plasma to the acidic environment near the proton pumps it inhibits and is converted under acidic conditions to its active form at the site of action. Omeprazole has a 0.5–1 h half-life in plasma and is almost entirely metabolized to inactive compounds in the liver, which are excreted in the urine and feces (Howden, 1991). The gastric acid inhibitory effects remain long after plasma levels are diminished.

Conclusions

Omeprazole is an excellent example of a prodrug that is targeted specifically to its site of action. Although omeprazole was not initially intended to work as a prodrug, it functions very efficiently as one.

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5.18

Case Study: Oseltamivir: An Orally Bioavailable Ester Prodrug of Oseltamivir Carboxylate

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Table of Contents

Name

(3R,4R,5S)-4-acetylamino-5-amino-3 (1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid, ethyl ester, oseltamivir phosphate (1:1), Tamiflu®.

Structures and Bioconversion Pathway

Figure 1. Structures of oseltamivir and oseltamivir carboxylate and bioconversion pathway.

Rationale for Oseltamivir

Oseltamivir is an ethyl ester prodrug requiring ester hydrolysis for conversion to the active form, oseltamivir carboxylate, which is a potent carbocyclic transition-state analog inhibitor of influenza virus neuraminidase with activity against both influenza A and B viruses *in vitro*. The proposed mechanism of action of oseltamivir carboxylate is via inhibition of influenza virus neuraminidase by alteration of virus particle aggregation and release.

The oral absorption of oseltamivir carboxylate has not been studied in humans but its low bioavailability in rat and marmoset (< 5%) suggested a low bioavailability in humans also. In contrast, oseltamivir, the ethyl ester prodrug of oseltamivir carboxylate, exhibited good oral bioavailability as the parent in multiple species (rats, mice, dogs, ferrets, and marmosets) with bioavailability ranging from 11 to 73% (Li *et al.*, 1998). In humans, the prodrug is readily absorbed from the gastrointestinal tract after oral administration of oseltamivir phosphate and is extensively converted predominantly by hepatic esterases to oseltamivir carboxylate. At least 80% of an oral dose reaches the systemic circulation as oseltamivir carboxylate (He *et al.*, 1999).

Synthesis

The original synthesis was first reported by Kim *et al.* (1997). Later, successful modifications were added by Rohloff *et al.* (1998), and the synthesis was reviewed by Abdel-Magid *et al.* (2001). Oseltamivir phosphate, (3R,4R,5S)-4-acetylamino-5-amino-3-(1-ethylpropoxy)1-cyclohexene-1-carboxylic acid, ethyl ester, phosphate

(1:1), is a highly water-soluble and non-hygroscopic prodrug of the active metabolite oseltamivir carboxylate. In the current commercial method, the starting material in the synthesis of oseltamivir phosphate is the epoxide, which itself is synthesized in five steps from either (–)-shikimic acid or (–)-quinic acid, both of which are derived from biological sources (plant or fermentation origin). Shikimic acid originates from star anise or from fermentation (using genetically engineered E. coli), and quinic acid is derived from cinchona bark. There are three chiral centers in the molecule. The chirality of the epoxide starting material has been confirmed, and mechanistic, spectroscopic and X-ray structure analyses have shown that oseltamivir phosphate, as used in the pre-clinical and clinical development program and marketed, is in the 3R,4R,5S configuration (Rohloff *et al.*, 1998).

Mechanism and Sites of Biotransformation

The mechanism of conversion of oseltamivir to oseltamivir carboxylate is the ethyl ester hydrolysis initiated by carboxyesterases, which are ubiquitous enzymes widely distributed throughout the body. The byproduct of the reaction is ethyl alcohol. The ethyl ester prodrug was chosen because, once absorbed, it is readily converted to the parent compound by esterase activity in plasma and tissues (Stella *et al.*, 1985).

Multiple dose preclinical pharmaco/toxicokinetic studies were carried out in rats, mice, rabbits, ferrets, and dogs. Peak concentrations of oseltamivir carboxylate were usually observed between 0.5 and 1 hour after dosing with oseltamivir. Species-dependent variations in the pharmacokinetics of oseltamivir carboxylate were observed. There was a tendency for the maximum concentrations of the parent compound to appear later at higher doses, suggesting that its rate of formation was saturable. Despite a typically wide range in carboxyesterase activity in humans potentially rising from pharmacogenetic differences or disease, a 75 mg oral dose of oseltamivir has been shown to generate a consistent systemic exposure to oseltamivir carboxylate with little inter-subject variability (He *et al.*, 1999).

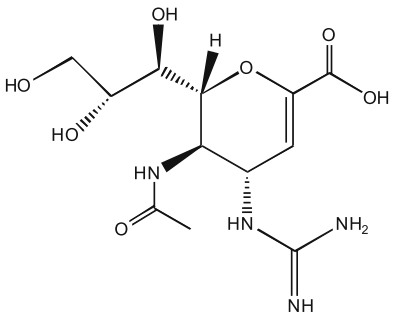
Formulation Issues

Oseltamivir phosphate is a highly water-soluble and non-hygroscopic prodrug of the active metabolite oseltamivir carboxylate. It is currently available in two pharmaceutical forms: hard capsules containing 75 mg oseltamivir, and a powder for oral suspension containing 12 mg/mL oseltamivir. After reconstitution with 52 mL of water, the volume of oral suspension is sufficient for 10 doses, each of 75 mg oseltamivir. When stored at 25°C, the hard capsules and powder for oral suspension have shelf-lives of 48 and 18 months, respectively.

Discussion

Influenza virus infections afflicted humans throughout history (Langmuir *et al.*, 1985, Tampey *et al.*, 2002) and continue to be a serious health concern. Each year, influenza infects an estimated 120 million people in the United States, Europe, Japan and Australia and is a major cause of morbidity and mortality. In the United States, out of an estimated 20 million cases of flu every year, up to 40,000 Americans die and more than 400,000 are hospitalized (Lui and Kendal, 1987; Naem, 2001). In periods of flu pandemics, infection rates have been even higher (Assaad *et al.*, 1984). In 1918–1919, the influenza commonly called "Spanish flu" killed more than 20 million people with some one billion people affected by the disease—half of the total human population at the time. New, emerging influenza viruses such as the H5N1 avian flu are also of major concern (Abbott and Pearson, 2004; Pilcher, 2004). Vaccines provide only partial protection due to their underutilization and the occasional failure to accurately predict annual changes in the antigenic determinants of the surface glycoproteins (Couch, 1993). If the current H5N1 avian influenza outbreak ever assumes the role of a pandemic, vaccines will become available only after a significant period of time and then to only a small percentage of the population. As a result, the use of antivirals could be critical in limiting the initial spread of a new pandemic. Until recently, only limited options existed for the control of influenza virus infections. M2 proton channel blockers (amantadine and rimantadine) have a narrow spectrum of antiviral activity. They are not active against influenza B or the H5N1 influenza virus (Abbott and Pearson, 2004). Also, virulent strains resistant to these agents develop quickly (Belshe *et al.*, 1989). Thus, there was and is an urgent need to identify new antiviral agents that can be used to prevent and treat influenza virus infections. In the last few years, new developments in the prevention and treatment of influenza have been introduced.

The influenza virus neuraminidase (sialidase) has long been considered a potential antiviral target. This enzyme, which is expressed on the surfaces of influenza A and B viruses, hydrolyzes terminal sialic acid residues from glycoproteins, glycolipids, and oligosaccharides. The influenza virus neuraminidase is required for the aggregation and release of newly synthesized viral particles from infected cells and is essential for virus replication (Palese and Compans, 1976). In the 1990s, several sialic acid-based neuraminidase inhibitors were shown to effectively inhibit influenza A and B virus replication (zanamivir, described by von Itzstein *et al.*, 1993 and Hayden *et al.*, 1997; BCX-1812/RWJ-270201, described by Gubareva *et al.*, 1999 and Babu *et al.*, 2000 and, more recently, A-315675 described by Kati *et al.*, 2002. While BCX-1812 and A-315675 were under development, their preclinical and clinical pharmacokinetic data were not widely available, but zanamivir has been well characterized (Hayden *et al.*, 1997; Cass *et al.*, 1999). Zanamivir (Figure 2) seems to have many attractive physicochemical properties. It is a small molecule, soluble, chemically stable, and highly active against influenza A and B viruses. However, the highly polar, zwitterionic nature of zanamivir results in low oral bioavailability; thus, it must be given by inhalation.



Zanamivir

Figure 2. Structure of carbocyclic sialic acid analogue zanamivir.

In an attempt to identify potentially orally bioavailable influenza virus neuraminidase inhibitors, a series of carbocyclic transition-state analog inhibitors of the influenza virus neuraminidases have been designed and synthesized by Kim *et al.* (1997). In these compounds, lipophilic side chains replaced the polar glycerol moiety, and an amino group replaced the guanidino group of the sialic acid-based inhibitors. Oseltamivir carboxylate, the lead candidate from this series, was comparable to zanamivir in terms of its ability to inhibit influenza virus neuraminidase activity (Ki, ~1 nM) and virus replication when tested *in vitro*. Although oseltamivir carboxylate is more lipophilic than zanamivir, its oral bioavailability in rats was still poor. However, a lack of the polar guanidino and glycerol groups present in zanamivir and the carboxylic acid group offered an opportunity to make the molecule orally bioavailable through the prodrug approach. Ester prodrugs have often been used to increase the bioavailability of carboxylic acid-containing compounds (Stella *et al.*, 1985; Beaumont *et al.*, 2003). Once absorbed, these prodrugs are readily hydrolyzed by a variety of esterases ubiquitously present in the blood and tissues of many species (Leinweber *et al.*, 1987) and the parent compound is released. An ideal ester prodrug should not exhibit pharmacological activity but should demonstrate high aqueous solubility and chemical stability across a pH range, good transcellular absorption, resistance to hydrolysis during the absorption phase, and rapid and quantitative breakdown to the active component upon absorption (Beaumont *et al.*, 2003). The ethyl ester prodrug of oseltamivir carboxylate appears to satisfy all of these requirements. After oral administration, oseltamivir is readily absorbed from the gastrointestinal tract and extensively converted to the active metabolite, oseltamivir carboxylate. To illustrate the improvement in oral bioavailability of the prodrug vs. the parent, Figure 3 shows plasma pharmacokinetics of oseltamivir carboxylate and zanamivir following an oral dose of oseltamivir carboxylate, the oseltamivir prodrug or zanamivir.

While both zanamivir and oseltamivir carboxylate show poor oral bioavailability, oseltamivir is readily absorbed from the gastrointestinal tract and extensively converted to the parent compound. The absolute bioavailability of the

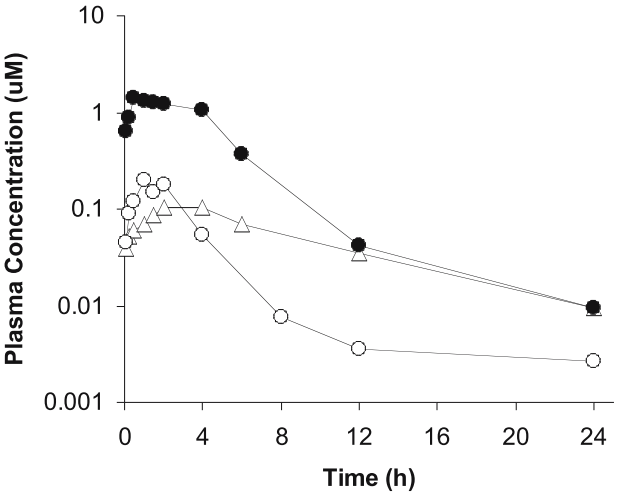


Figure 3. Concentration-time profiles of influenza virus neuraminidase inhibitors in rat plasma. Concentration of oseltamivir carboxylate following oral administration of a 10-mg/kg dose of oseltamivir carboxylate (△), oral administration of a 10-mg-equivalent/kg dose of the prodrug oseltamivir (●) or zanamivir following oral (○) administration of a single 10-mg/kg dose. Each data point represent the mean value from 4 animals except for zanamivir where the mean value from 3 animals was used (Figure 2 is adapted from Li et al., 1998).

parent from orally administered oseltamivir in rats is 43%. The oral administration of oseltamivir also led to higher and more sustained concentrations of the active form of the drug in plasma than did oral administration of oseltamivir carboxylate itself. This was particularly evident in dogs and ferrets, the species with detectable levels of intact prodrug in their plasma (Li *et al.*, 1998).

Interestingly, in the case of the prodrug oseltamivir, intravenous and oral administration of the prodrug oseltamivir to rats gave similar apparent terminal half-lives for the parent (6.2 and 7.0 h, respectively (not shown, Li *et al.*, 1998). These data suggest that elimination of the parent compound after administration of prodrug is rate limited by conversion to oseltamivir carboxylate rather than by absorption. While the stability of the prodrug in plasma would be expected to affect its rate of conversion to oseltamivir carboxylate, the ability of prodrug to aid delivery of the compound to extravascular tissues may also play a role in maintaining the concentrations of oseltamivir carboxylate in plasma. Eisenberg *et al.* (1997) have demonstrated that oseltamivir carboxylate is present in bronchoalveolar lavage fluid following oral administration of the prodrug to rats. This ability of oseltamivir carboxylate to accumulate in lung tissue may also affect its antiviral activity at the primary site of influenza virus infection and replication. Of particular interest, the peak concentration of oseltamivir carboxylate in the bronchoalveolar lavage fluid was similar to that in plasma; however, its rate of decline was slower than that in plasma, indicating that the antiviral activity in lung tissue may be more persistent than is suggested by the plasma concentration-

versus-time profile. The accumulation of prodrug in lung tissue is likely to be even more pronounced in humans which have greater amounts of circulating prodrug (Wood *et al.*, 1997).

In humans, the pharmacokinetic profile of oseltamivir is simple and predictable, and twice daily treatment results in effective antiviral plasma concentrations over the entire administration interval. After oral administration, oseltamivir is readily absorbed from the gastrointestinal tract and extensively converted to oseltamivir carboxylate with an absolute bioavailability of 80%. (He *et al.*, 1999). Oseltamivir carboxylate is detectable in plasma within 30 min and reaches maximal concentrations after 3–4 h. After peak plasma concentrations are attained, the concentration of oseltamivir carboxylate declines with an apparent half-life of 6–10 h. Oseltamivir is eliminated primarily by conversion to and renal excretion of the parent. Renal clearance of both prodrug and the parent exceeds the glomerular filtration rate, indicating that renal tubular secretion contributes to their elimination via the anionic pathway. The pharmacokinetic profile of oseltamivir carboxylate is linear and dose-proportional, with less than twofold accumulation over a dosage range of oseltamivir 50 to 500 mg twice daily. Steady-state plasma concentrations are achieved within 3 days of BID administration and, at a dosage of 75 mg BID, the steady-state plasma trough concentrations of the parent remain above the minimum inhibitory concentration for all influenza strains tested. Exposure to oseltamivir carboxylate at steady state is approximately 25% higher in the elderly than in young individuals, which is likely due to the decreased renal clearance; however, no dosage adjustment is necessary. No clinically significant drug interactions were found.

Two recently developed neuraminidase inhibitors, oseltamivir carboxylate and zanamivir, have been shown to be almost equally effective in inhibiting influenza A and B virus replication (Dreitlein *et al.*, 2001; Brooks *et al.*, 2004). Oseltamivir is an example of a successful prodrug strategy employed to improve oral bioavailability of oseltamivir carboxylate. Zanamivir, in contrast to oseltamivir carboxylate, possesses highly polar diol groups as well as the guanidino group, which is charged at physiological pH, leading to low oral bioavailability of the drug (2% in humans, reported by Cass *et al.*, 1999). As a result of the insufficient oral bioavailability, zanamivir had to be developed as a topical treatment (inhaler) and, as such, is not as widely utilized in clinical practice as oral oseltamivir. Also, aside from the dosing convenience alone, a systemically acting oral agent may have improved efficacy in influenza treatment and prophylaxis. It has been reported, for instance, that the avian H5N1 influenza virus causes systemic infection in the affected species with high virus titers and pathology in multiple organs (Sturm-Ramirez *et al.*, 2004). A locally acting drug may be ineffective if virus can escape from the site of inoculation and replicate elsewhere. When zanamivir, which is highly effective in mouse and ferret models of influenza virus infection (von Itzstein *et al.*, 1993), was assessed as a prophylactic agent in the prevention of infection of chickens with highly pathogenic avian influenza viruses, it produced only a slight delay in the onset of pyrexia and death with one strain of fowl plague virus but not with two other strains (McCauley *et al.*, 1995). At the same time, oral oseltamivir was reported to be highly effective in prevention of

death in mice infected with A/Hong Kong/156/97 (H5N1), mouse-adapted A/Quail/Hong Kong/G1/97 (H9N2), or human A/Hong Kong/1074/99 (H9N2) viruses (Leneva *et al.*, 2000).

Conclusion

The simple and predictable pharmacokinetic profile of oseltamivir and convenient oral regimen resulting in effective antiviral systemic concentrations over the entire dosing interval made it a useful agent that has become by far the most frequently prescribed neuraminidase inhibitor for the treatment and prevention of influenza.

Acknowledgments

The author wishes to thank Dr. Choung Kim and Dr. Adrian Ray for a critical review of the manuscript.

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5.19

Case Study: Parecoxib: A Prodrug of Valdecoxib

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Table of Contents

Name:

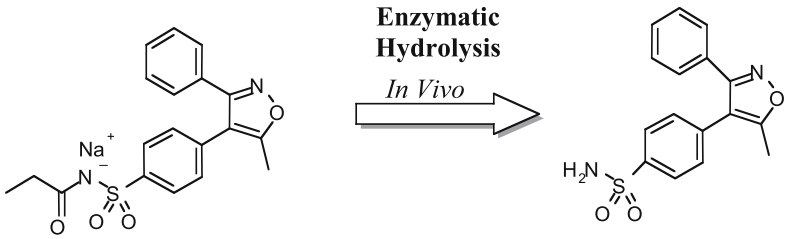
Parecoxib, N-[[4-(5-methyl-3-phenyl-4-isoxazolyl)phenyl]sulfonyl]propanamide, sodium salt, Dynastat® or Rayzon®.

Introduction

Parecoxib, a weakly acidic drug (pK_a 4.9), was developed as a COX-2 inhibitor for the management and treatment of acute pain. It was designed to be a water-soluble (> 50 mg/mL in normal saline), parenterally safe prodrug form of valdecoxib, or 4-(5-methyl-3-phenyl-4-isoxazolyl) benzenesulfonamide, a diaryl-substituted isoxazole. Valdecoxib is a sparingly water soluble (10 μg/mL), weakly acidic drug with a pK_a of 9.8. Valdecoxib is commercially available as Bextra, an oral formulation for the management of acute pain, chronic pain, osteoarthritis (OA), rheumatoid arthritis (RA), and primary dysmenorrhea.

Structure and Bioconversion Pathway

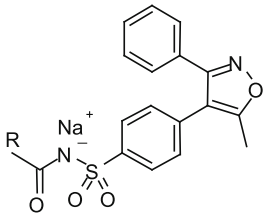
Parecoxib itself shows weak *in vitro* inhibitory activity against both cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). Parecoxib sodium is rapidly (half-life = 15–30 min) and essentially completely converted via enzymatic hydrolysis to the pharmacologically active moiety valdecoxib and sodium propionate. This bioconversion occurs mainly in the liver. Valdecoxib is a potent and selective inhibitor of COX-2.



Parecoxib Sodium **Valdecoxib**

Rationale for Parecoxib

Due to the low solubility of valdecoxib, a parenteral formulation might need to utilize a high concentration of co-solvents and surfactants that will likely result in pain upon injection or hemolysis. Hence, a prodrug approach was utilized. N-acylsufonalmides have been described as potential pro-drug derivatives (Larson *et al.* 1987 and 1988). A series of acylated derivatives of isoxazole sulfonamide (valdecoxib) were synthesized (Talley *et al.*, 2000) and are illustrated below.



Acylated Derivatives of Valdecoxib

1 when R=CH_3

2 when R = CH_2CH_3

3 when R = $CH_2CH_2CH_3$

Compound 1 had good water solubility (44 mg/mL in PBS) and was rapidly converted to valdecoxib in rats. However, IV administration of 1 to canines or cynomolgus monkeys did not result in complete conversion, and a significant amount of unchanged molecule was detected in urine. Both 2 and 3 were rapidly and completely converted to valdecoxib in all three species. The higher aqueous solubility of 2 compared to 3 led to the development of 2 (parecoxib). Parecoxib is the first injectable, selective COX-2 inhibitor available in the world.

Synthesis and Physico-chemical Properties

The acylation of 4-(5-methyl-3-phenylisoxazol-4-yl)-benzenesulfonamide, valdecoxib, with propionic anhydride by means of triethylamine and 4-dimethylaminopyridine in tetrahydrofuran gives *N*-[4-(5-methyl-3-phenylisoxazol-4-yl)phenylsulfonyl]propion-amide, which is then treated with NaOH in ethanol to obtain a sodium salt (Talley *et al.*, 2000; 2002; 2003; Sorbera *et al.*, 2001).

The bulk drug substance is dried at high temperature to obtain a consistent solid state form. Various solvates, hydrates, and anhydrous solid state forms of parecoxib sodium have been observed (Sheikh *et al.*, 2003).

The solubility of parecoxib increases with increasing pH and temperature. The major degradation route of parecoxib is hydrolysis to valdecoxib.

Mechanism and Site of Bioreversion

The mechanism of bioreversion of parecoxib to valdecoxib is predominantly via enzymatic hydrolysis by liver esterases. The by-product of the hydrolysis is sodium propionate.

Toxicity Issues

No significant toxicity has been reported during the clinical evaluation of parecoxib (Hubbard *et al.*, 2000a,b; Karim *et al.*, 2000a,b; 2001a,b). In particular, multiple dose administration of parecoxib is safe and well tolerated with decreased risk of gastroduodenal mucosal injury compared to ketorolac (Harris *et al.*, 2001; Stoltz *et al.*, 2002). The incidence of any adverse effects observed during clinical testing was not different than that noted for placebo (Cheer *et al.*, 2001).

Formulation Issues

Parecoxib sodium is commercially available as a sterile, white to off-white, preservative-free, lyophilized powder in single use vials containing 21.18 mg or 42.36 mg parecoxib sodium (equivalent to 20 or 40 mg parecoxib free acid, respectively). Inactive ingredients include dibasic sodium phosphate heptahydrate, phosphoric acid, and/or sodium hydroxide to adjust pH. Parecoxib was developed as a lyophilized powder to afford maximum stability of the product since solutions of parecoxib degrade predominantly to the highly insoluble valdecoxib (Kararli *et al.*, 2002). The solubility of the degradation product, valdecoxib, is higher in the presence of solutions of parecoxib due to the self-association properties of the parecoxib. The 20 mg strength is reconstituted with 1 mL of 0.9% sodium chloride to produce a 20 mg/mL parecoxib solution; the 40 mg strength is reconstituted with 2 mL of 0.9% sodium chloride to produce an identical 20 mg/mL solution. The reconstituted solution will be clear and colorless, contain 0.011 mEq of sodium per mg of parecoxib, and have a pH of approximately 8.0. The lyophilized powder can be safely reconstituted with the following diluents (Crane *et al.*, 2003): bacteriostatic 0.9% sodium chloride injection USP, 5% dextrose injection USP, and 5% dextrose/0.45% sodium chloride injection USP. The use of lactated Ringer's injection USP or 5% dextrose in lactated Ringer's injection USP is not recommended for reconstitution because they will cause drug precipitation. The use of water for injection is not recommended since the resulting solution will be hypotonic. After reconstitution, the resulting solution, when stored at room temperature, should be used within a few hours to prevent microbial contamination as the solution does not contain any preservatives; however, the solution is chemically stable for 48 hours (Crane *et al.*, 2003). Reconstituted solution should not be refrigerated. Both the freeze-dried powder and reconstituted solution should be protected from light.

Alternate dosage forms and routes of administration for parecoxib sodium have been investigated, including buccal, transdermal (Lu *et al.*, 2002), topical, and oral (Karim *et al.*, 2003). A patent describing a stable liquid parenteral parecoxib formulation has been issued (Gokarn *et al.*, 2004).

Discussion of Pharmacokinetics and Clinical Studies

Parecoxib is a member of the pharmacological class of cyclooxygenase-2 (COX-2)-specific inhibitors (Lipsky *et al.*, 1998; Jain, 2000). The COX-2 specific inhibitors currently marketed or being developed (celecoxib, valdecoxib, etoricoxib, lumiracoxib, and rofecoxib) are all available only as oral formulations. Parecoxib sodium (the water-soluble prodrug of valdecoxib) allows parenteral delivery of this COX-2 inhibitor by either the intravenous or intramuscular route. Parecoxib sodium was developed to provide a safe, effective analgesic alternative to conventional NSAIDs (e.g., ketorolac tromethamine), which suffer from increased risk of upper gastrointestinal tract ulceration, inhibition of platelet function, and reduction of renal function (Anonymous, 1993a,b; Choo *et al.*, 1993;

Gillis *et al.*, 1997; Noveck *et al.*, 2001). Additionally, it may be used in combination with opioids. It offers increased flexibility in perioperative pain control by reducing requirements for opioids needed for effective pain control and providing improved safety to patients through reduction of adverse side effects associated with opioids such as nausea, vomiting, constipation, paralytic ileus, respiratory depression, somnolence, and dizziness (Bowdle, 1998; McArdle, 1999; McQuay, 1999; Power *et al.*, 1999; Rawal *et al.*, 1999; Tong *et al.*, 1999).

Talley *et al.* (2000) completed numerous studies that identified and confirmed the selection of parecoxib as the appropriate prodrug for valdecoxib. Their work included the following pre-clinical research: studies in the rodent, dog, and monkey demonstrated that parecoxib was rapidly and completely converted to parecoxib (Figure 1 illustrates the pharmacokinetic profile of the conversion of parecoxib sodium to valdecoxib in the male rat); *in vitro* metabolic studies with human liver microsomes demonstrated that parecoxib was completely converted to valdecoxib; chronic anti-inflammatory activity was achieved in the rat adjuvant arthritis model (ED_{50} = 0.08 mg/kg); the acute anti-inflammatory activity of parecoxib was demonstrated in the carrageenan air pouch assay (98% inhibition at 0.3 mg/kg); and strong analgesic activity for parecoxib was noted in the carrageenan-induced paw edema model in rats.

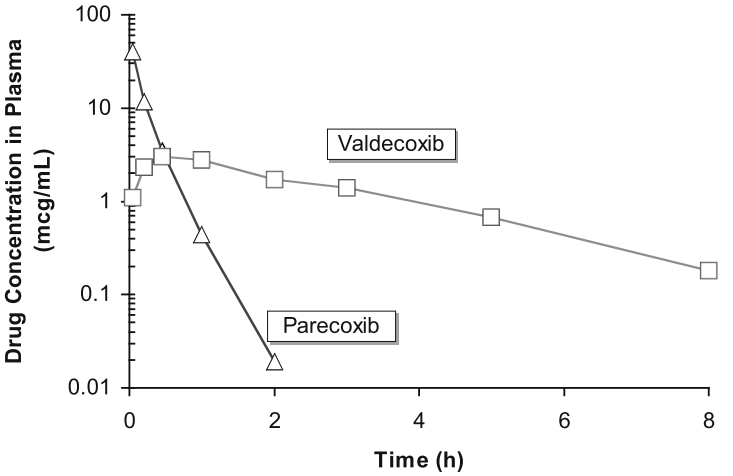


Figure 1. Pharmacokinetic profile of the conversion of parecoxib sodium to valdecoxib in male rats after IV administration of a parecoxib solution at a dose of 12.5 mg parecoxib/kg. Reproduced with permission. (Talley *et al.*, 2000).

Numerous clinical studies on parecoxib have been performed in humans to evaluate the pharmacokinetics (Daniels *et al.*, 2000; Hubbard *et al.*, 2000a; Karim *et al.*, 2000a,b, 2001a,b; Mehlish *et al.*, 2000; Anonymous 2001). Figure 2 describes the metabolic pathway for the fate of parecoxib after administration to humans. The pharmacologically inactive parecoxib is rapidly converted by hepatic enzymes to the active moiety, valdecoxib. The valdecoxib undergoes

conversion involving both cytochrome P-450-mediated and noncytochrome P-450-mediated metabolic pathways to a hydroxylated metabolite (active, but at lower concentrations). Figure 3 illustrates the plasma concentrations with time of parecoxib, valdecoxib, and the metabolite after a single 20 mg IM injection. Single dose disposition kinetics of parecoxib at a 20 mg dose in healthy patients noted that a peak concentration (C_{max}) of valdecoxib is achieved in approximately 30 min for IV administration and approximately 1 h for IM administration.

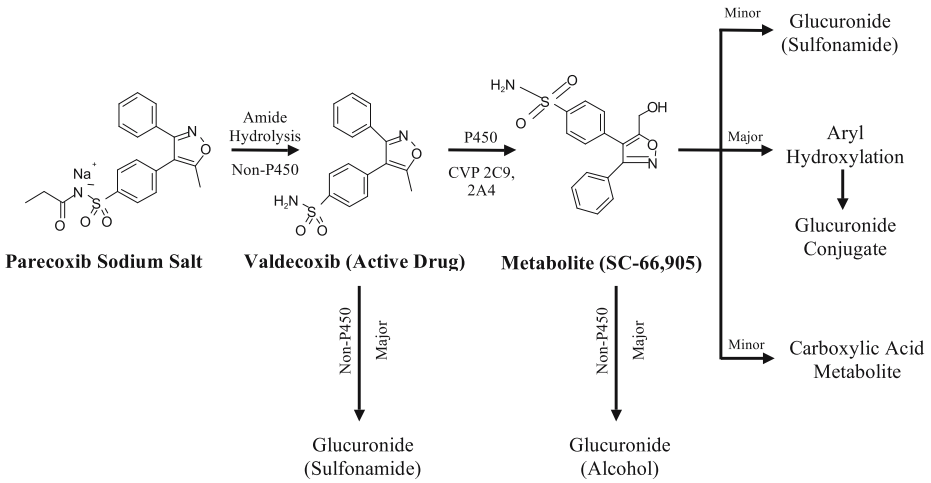


Figure 2. Metabolic pathway for parecoxib after injection in humans. Reproduced with permission. (Karim *et al.*, 2001a).

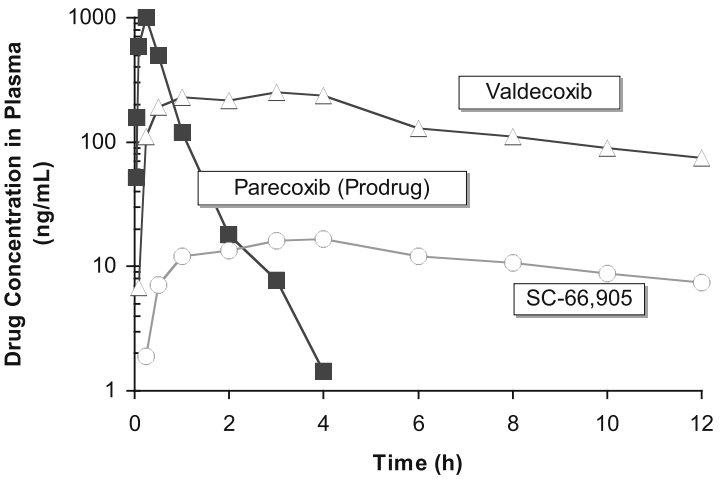


Figure 3. Mean semi-logarithmic plasma concentrations of unchanged parecoxib, hydrolyzed active drug valdecoxib, and the active hydroxylated metabolite M1 (SC-66905) following a single 20 mg dose of parecoxib solution (as the sodium salt) administered IM to 8 healthy adult male subjects. Reproduced with permission. (Karim *et al.*, 2001a).

Exposure to valdecoxib was similar with respect to area under the plasma level curves (AUC) and C_{max} following IV and IM administration. Exposure to parecoxib was similar after IM and IV administration with respect to AUC. The C_{max} of parecoxib after IM administration was lower than after IV dosing. The plasma half-life of parecoxib is approximately 22 min. The plasma clearance (CL_p) for valdecoxib is about 6 L/h. The elimination half-life of valdecoxib is about 8 h. Dose proportionally has been demonstrated when administered in the range of 1–40 mg parecoxib. Doses up to 40 mg and concentrations up to 20 mg/mL have been well tolerated.

Clinical efficacy has been studied in various clinical models of pain (Daniels *et al.*, 2000; 2001; Wender *et al.*, 2001; Barton *et al.*, 2002; Joshi *et al.*, 2002; Rasmussen *et al.*, 2002; Rowbotham, 2002; Barden *et al.*, 2003; Desjardins *et al.*, 2003; Hubbard *et al.*, 2003; Malen *et al.*, 2003; Mehlisch *et al.*, 2003; Ott *et al.*, 2003). Specifically, parecoxib, after single dose treatment, was shown to effectively relieve the following types of pain: oral, gynecologic, and orthopedic surgical pain. After multiple dose treatment, parecoxib was shown to effectively relieve pain due to bunionectomy, gynecologic, orthopedic, and mixed general surgery, and after coronary artery bypass graft surgery. Finally, parecoxib was shown to reduce opioid consumption when used in combination with opioids to treat post-surgical pain. Parecoxib was well tolerated in various clinical studies, with side effects similar to those noted for placebo.

Conclusions

Parecoxib is a superb example of the development of a water-soluble prodrug that was designed to circumvent problems associated with the parenteral delivery of the poorly water-soluble active moiety valdecoxib.

Acknowledgment

The authors would like to thank Yatin Gokarn and Ingrid Crane for their work on parecoxib sodium formulation development and characterization.

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5.20

Case Study: Tenofovir Disoproxil Fumarate: An Oral Prodrug of Tenofovir

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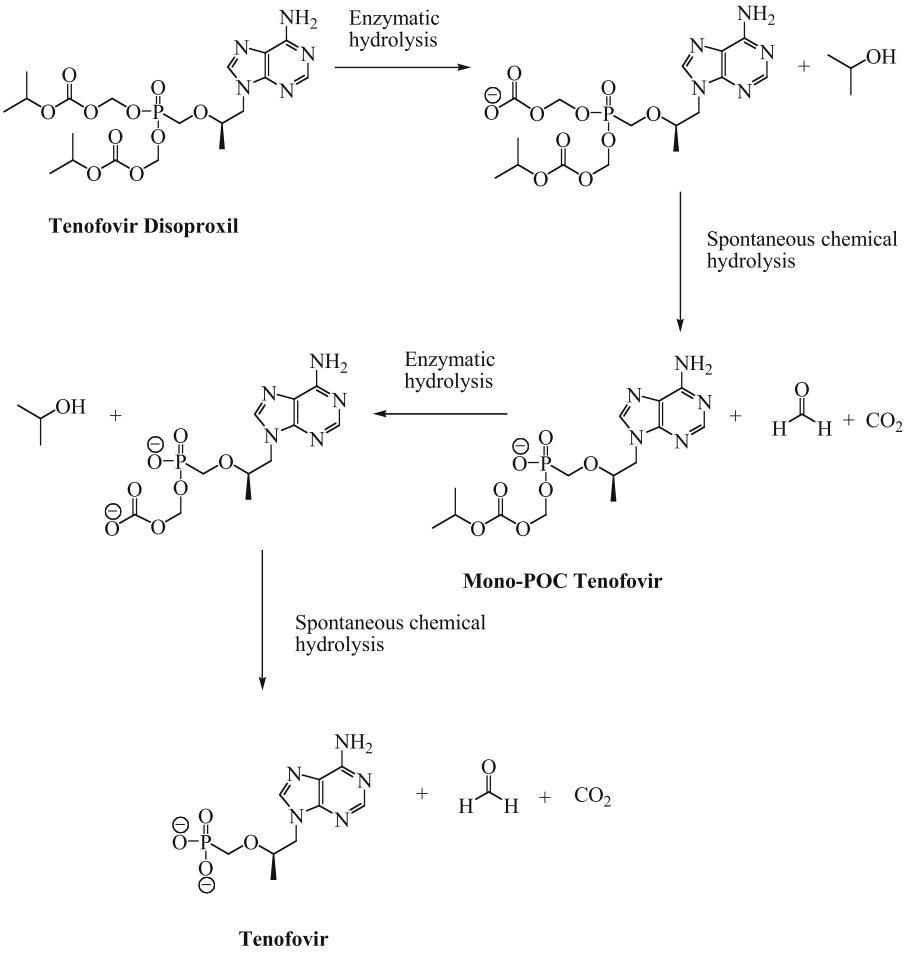
Table of Contents

Name:

Tenofovir disoproxil fumarate, tenofovir DF, 9-[(R)-2-[[Bis[[(isopropoxy-carbonyl)oxy] methoxy]phosphinyl]methoxy]propyl]adenine fumarate (1:1), (R)-5-[[2-(6-amino-9H-purin-9-yl)-1-methylethoxy]methyl]-2,4,6,8,-tetraoxa-5-phosphanonanedioic acid, bis(1 methylethyl)ester, 5-oxide, (E)-2-butenedioate (1:1), Viread®

Structures and Bioconversion Pathway



Scheme 1. Structure of tenofovir disoproxil, tenofovir, and the bioconversion pathway.

Rationale for Tenofovir Disoproxil Fumarate

Tenofovir (PMPA) is a novel nucleotide analog that belongs to the class of acyclic nucleoside phosphonates. The potential antiviral effect of tenofovir was

demonstrated by Holˇ and DeClercq (Balzarini *et al.*, 1993). In this class of compounds, the ribose-phosphate group is replaced with the isopolar phosphonopropyl ether functionality. Tenofovir is recognized by host kinases and is phosphorylated *in situ* to the virologically active tenofovir diphosphate. Tenofovir diphosphate inhibits the human immunodeficiency virus (HIV-1 and HIV-2) DNA polymerase (reverse transcriptase) in addition to other viral DNA polymerases (Naesens *et al.*, 1997). This inhibition results in DNA chain termination and impairment of viral replication. The IC_{50} values of tenofovir inhibition of HIV-1 reverse transcriptase is between 0.04 and 8.5 μM (Viread package insert).

Mimicking a nucleoside monophosphate with a phosphonate as in tenofovir has two advantages: it avoids the requisite but slow initial phosphorylation of nucleosides by host kinases, and it prevents dephosphorylation in blood plasma by widely occurring phosphatases, since the phosphonate moiety is stable to hydrolytic cleavage to the nucleoside. In addition, it has been suggested that the diphospho-phosphonate metabolites that are formed in cells are better substrates for viral polymerases than the corresponding nucleoside 5'-triphosphates because the ether oxygen of the phosphonate-bearing side chain coordinates more favorably with the metals present in the active site of polymerases (Sigel, 2004). In summary, acyclic nucleoside phosphonates provide suitable and active substrates for viral DNA polymerases.

Preclinical properties of tenofovir attracted attention because the compound had an orthogonal resistance profile to the nucleosides used for the treatment of HIV at the time. In addition, parenteral administration of tenofovir to human immunodeficiency virus type-1 (HIV-1)-infected individuals was well tolerated and effective since it resulted in a significant dose-related anti-viral activity (Deeks *et al.*, 1998). Tenofovir was initially dosed by parenteral administration due to low oral bioavailability.

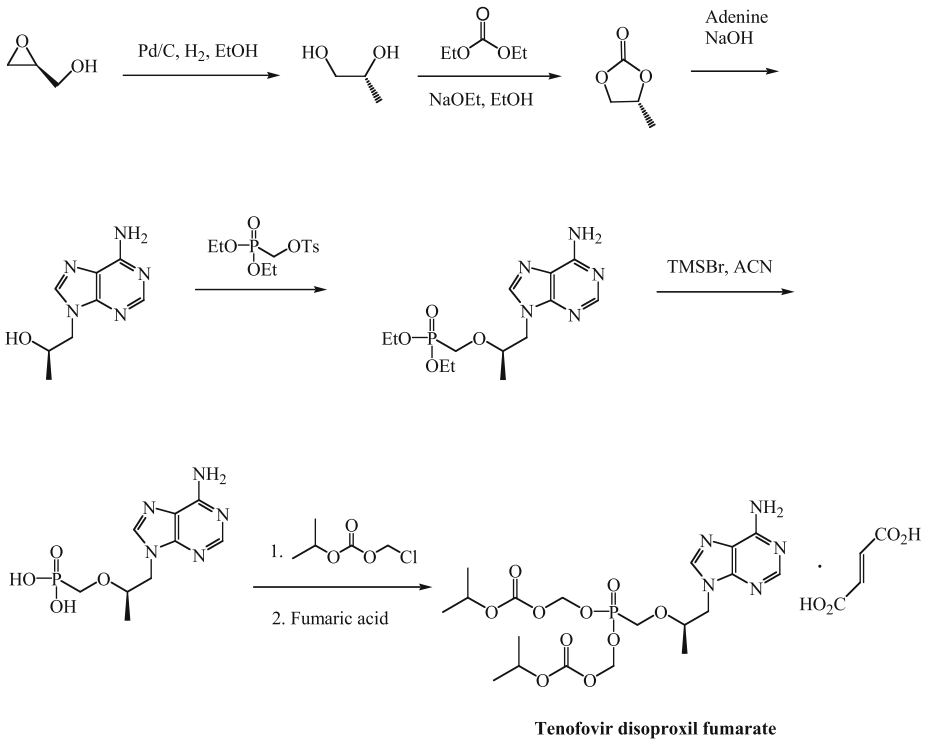
Phosphonate-containing drugs such as tenofovir exist as a dianion at most physiological pH values, making them very polar (pK_{a1} = 2, pK_{a2} = 6.8). Such polar species do not readily undergo passive diffusion across cellular membranes and intestinal mucosa, resulting in low bioavailability after oral administration (Cundy *et al.*, 1998; Naesens *et al.*, 1998).

A series of novel prodrugs of tenofovir were designed to overcome the pharmacokinetic limitations of tenofovir (Arimilli *et al.*, 1997; Shaw *et al.*, 1997). These prodrugs were engineered to mask the polar phosphonic acid functionality using a novel oxycarbonyloxymethyl linker to permit passive diffusion in the intestinal tract. The resulting alkyl methyl carbonate prodrugs of tenofovir were synthesized and evaluated both *in vitro* and *in vivo*. While the bis-[(pivaloyloxy)methyl] prodrug of tenofovir was also evaluated and demonstrated high oral bioavailability in fasted beagle dogs, this compound was not selected for clinical development (Shaw *et al.*, 1997). The amount of pivalic acid that would be released from the breakdown of the bis-[(pivaloyloxy)methyl] tenofovir was a concern considering the necessary high dose of the prodrug. Instead, the bis-isopropyl-carbonate ester (tenofovir disoproxil) was selected for clinical evaluation based on its chemical stability, aqueous solubility, intestinal homogenate stability, and bioavailability following oral dosing in dogs.

The bioconversion of tenofovir disoproxil to tenofovir leads to the formation of isopropyl alcohol, carbon dioxide, and formaldehyde. In contrast to pivaloyloxymethyl-containing prodrugs, tenofovir disoproxil when chronically administered has no impact on carnitine homeostasis.

Synthesis

Preparation of tenofovir disoproxil was described by Arimilli *et al.* initially in 1997 followed by enclosure of further details in a patent in 2000. Preparation of tenofovir (PMPA) starts with catalytic reduction of (S)-glycidol to (R)-1,2-propanediol, which is subsequently protected using diethyl carbonate to provide (R)-1,2-propylene carbonate. Reaction of this carbonate with adenine under basic conditions gives (R)-9-[2-(hydroxy)propyl]adenine, which is further alkylated with diethyl-*p*-toluenesulfonyl-oxymethylphosphonate using a base such as lithium *t*-butoxide to provide (R)-9-[2-(diethylphosphonomethoxy)propyl]adenine. Diethyl-*p*-toluenesulfonyl-oxymethylphosphonate is prepared by reacting diethyl phosphite and paraformaldehyde followed by formation of the tosylate using *p*-toluenesulfonylchloride. Synthesis of tenofovir is completed by deprotection of the diethylphosphonate using trimethylsilylbromide in acetonitrile. Tenofovir is further purified by recrystallization from water to provide tenofovir monohydrate.



Scheme 2. Synthetic scheme to tenofovir disoproxil fumarate.

The chloromethyl isopropyl carbonate necessary for the synthesis of tenofovir disoproxil is prepared by slow addition of pyridine to a cooled solution of *i*-propanol and chloromethyl chloroformate in diethyl ether under an inert atmosphere. Preparation of the prodrug is achieved by addition of chloromethyl isopropyl carbonate to tenofovir in DMF. The fumarate salt of the prodrug is prepared by addition of fumaric acid in *i*-propanol to the prodrug to give tenofovir disoproxil product.

Mechanism and Site of Bioconversion

As described in the previous section, tenofovir disoproxil is an oral prodrug of tenofovir in which the phosphonic acid is masked as the bis-isopropyl-carbonate ester. Tenofovir disoproxil has a long half-life ($t_{1/2}$ > 7 hours) at both pH 2.0 and 7.4 (Yuan *et al.*, 2001). The bioconversion of tenofovir disoproxil to tenofovir is mediated by nonspecific carboxylesterases (Shaw *et al.*, 1997). The bioconversion mechanism involves rapid enzymatic hydrolysis of bis-ester followed by spontaneous decomposition of carbonic acid monomethyl phosphonate ester (Scheme 1). The mono-POC tenofovir undergoes a similar degradation leading to the rapid formation of tenofovir. The conversion process is rapid, as demonstrated in preclinical and clinical studies. In the intracellular environment, tenofovir is phosphorylated to tenofovir diphosphate, which inhibits viral DNA reverse transcriptase by competing with the natural substrate deoxyadenosine 5'-triphosphate. Once incorporated into viral DNA, chain elongation is halted as the necessary 3'-hydroxyl group present in nucleotides is absent in tenofovir.

Toxicity Issues

The clinical safety of tenofovir disoproxil fumarate has been examined in various clinical studies in HIV-infected subjects. Tenofovir disoproxil fumarate is generally well tolerated. In a pooled analysis, the severity and incidence of adverse events were similar for those receiving either a 300-mg daily dose of tenofovir disoproxil fumarate or placebo. The bioconversion of tenofovir disoproxil to tenofovir leads to the formation of formaldehyde in addition to carbon dioxide and isopropyl alcohol. The daily formaldehyde load of 0.945 mmol/day is considered insignificant.

Formulation Issues

Tenofovir disoproxil fumarate is a white crystalline powder with a high aqueous solubility of 13.4 mg/mL in water. Hydrolysis of tenofovir disoproxil to mono-POC tenofovir produces formaldehyde as well as other byproducts as discussed earlier (Yuan *et al.*, 2001). Formaldehyde can further react with the N^6-amine moiety of adenine to form the corresponding carbinolamine intermediate (Yuan *et al.*, 2000). Dehydration of the carbinolamine intermediate leads to the formation of the reactive imine cation (Schiff base) of tenofovir disoproxil, which

can react with an additional tenofovir disoproxil or mono-POC tenofovir molecule to form the methylene-linked dimer. Both degradation pathways are known to be pH-dependent in solution.

The degradation kinetics of tenofovir disoproxil is therefore governed by two distinct but interrelated degradation pathways: hydrolysis of the isopropyloxycarbonylmethyl moiety and formaldehyde-mediated dimerization of the adenine ring (Yuan *et al.*, 2001). A crystalline fumarate salt of tenofovir disoproxil was developed to reduce the rate and extent of dimerization in solid state. Viread oral tablets contain 300 mg of tenofovir disoproxil fumarate in addition to croscarmellose sodium, lactose monohydrate, magnesium stearate, pregelatinized starch, and microcrystalline cellulose as the inactive ingredients.

Discussion

According to estimates from the UNAIDS/WHO AIDS Epidemic Update (December 2004), 37.2 million adults and 2.2 million children were living with HIV at the end of 2004. This is more than 50% higher than the figures projected by WHO in 1991 on the basis of the data then available. During 2004, some 4.9 million people became infected with HIV, which causes AIDS. The current approach to treating HIV-1 infection is a regimen of highly active antiretroviral therapy (HAART), with the goal of sustained and comprehensive suppression of viral replication. The HAART regimen requires agents with activity against HIV-1 and HIV-2, excellent safety profile, durable response, and, more importantly, a simplified dosing schedule.

The clinical efficacy and safety of tenofovir in HIV-infected subjects were initially demonstrated using an intravenous formulation (Deeks *et al.*, 1998). A randomized, double-blind, placebo-controlled, dose-escalation clinical trial of intravenous tenofovir monotherapy was conducted in 20 HIV-infected adults with plasma HIV RNA of >10,000 copies/mL. The dose levels of 1 and 3 mg/kg of body weight/day were evaluated. All subjects tolerated the dosing without significant adverse events. Following 7 consecutive days of tenofovir administration at 3mg/kg/day, the median change in plasma HIV-1 RNA from baseline was $-1.1 \log_{10}$.

Preclinical studies aimed at examination of the pharmacokinetics and metabolism of tenofovir were performed in beagle dogs using tenofovir dosed by intravenous, intraperitoneal, and oral routes (Cundy *et al.*, 1998; Naesens *et al.*, 1998). Tenofovir was excreted mainly in urine by filtration and tubular secretion. Tenofovir demonstrated low oral bioavailability in animal models. Therefore, a prodrug of tenofovir, tenofovir disoproxil, characterized by enhanced pharmacokinetic profile was selected for clinical development (Arimilli *et al.*, 1997; Shaw *et al.*, 1997).

The pharmacokinetics, safety, and activity of oral tenofovir disoproxil fumarate in HIV-infected subjects were evaluated in a randomized, double-blind, placebo-controlled, dose-escalating study at doses of 75, 150, 300, and 600 mg given once daily (Barditch-Crovo *et al.*, 2001). After oral administration, tenofovir disoproxil was readily absorbed from the gastrointestinal tract and extensively

converted to tenofovir. The absorption pathway was apparently not saturated at doses used in the study. As a result, the plasma exposure to tenofovir for patients receiving tenofovir disoproxil fumarate was dose-proportional. The time required to reach maximum drug concentration (T_{max}) in fed cohorts ranged from 0.5 to 1 h and was independent of the dose. The plasma concentration-versus-time curves were dose-proportional and showed no change with repeated dosing. The oral bioavailability of tenofovir from tenofovir disoproxil fumarate 300 mg dose was estimated to be 39% in the fed state. Oral bioavailability of tenofovir disoproxil is increased following a high fat meal, but a light meal did not affect the pharmacokinetics of the drug. All other pharmacokinetic parameters were not affected by repeated administration of tenofovir disoproxil fumarate at any dose level. The median decrease in log10 HIV-1 RNA after 28 days of dosing in the 300 mg dose group was –1.22 compared to the placebo arm (Barditch-Crovo *et al.*, 2001). The largest decrease in HIV-1 RNA levels was observed with the 300-mg dose.

In human clinical trials, the change in HIV viral load was threefold greater after oral administration of tenofovir disoproxil fumarate than after an equivalent molar exposure of intravenously administered tenofovir (Cundy, 1999). The enhanced anti-HIV activity observed in patients with the oral prodrug relative to the intravenously administered parent drug may be attributable to an increase in the intracellular concentration of tenofovir, which is likely the result of better intracellular distribution of the oral prodrug. Interestingly, in preclinical studies in dogs, the intracellular levels of tenofovir in peripheral blood mononuclear cells (PBMCs) were fivefold greater after oral administration of tenofovir disoproxil fumarate than following an equivalent subcutaneous exposure of tenofovir (Cundy, 1999).

The safety and antiviral activity of tenofovir disoproxil fumarate were further established in multiple large, well designed, placebo-controlled clinical trials at 300 mg per day dose (Schooley *et al.*, 2002; Squires *et al.*, 2002). Tenofovir disoproxil fumarate at a 300 mg daily dose was shown to be effective in antiretroviral-experienced and treatment naïve patients.

The emergence of viral resistance to tenofovir disoproxil fumarate therapy has been low. The K65R mutation occurred in isolates from 3% of patients treated with tenofovir disoproxil fumarate (Margot *et al.*, 2002). No clinically significant clinical drug interaction has been reported when tenofovir disoproxil fumarate and lamivudine, efavirenz, indinavir, or lopinavir/ritonavir were coadministrated in two crossover studies in healthy volunteers (Flaherty *et al.*, 2001; Kearney *et al.*, 2001).

Conclusions

Tenofovir disoproxil fumarate (Viread®) is an excellent example of a prodrug that can overcome the pharmacokinetic limitations associated with poor permeation of tenofovir across intestinal mucosa. Viread has been approved worldwide in combination with other antiretroviral agents for the treatment of HIV-1 infection in adults. Viread has become a pivotal product for treatment of

HIV since it was launched in 2001. The antiviral activity, excellent safety profile, superior resistance profile, and convenient once-a-day oral regiment has made Viread one of the most frequently prescribed antiretroviral agents in the United States and Europe.

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5.21

Case Study: Travoprost: A Potent PGF2α Analog

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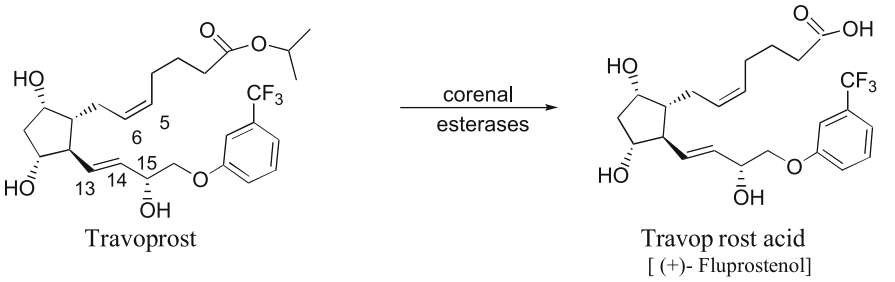
Name:

Travoprost; isopropyl (Z)-7-[(1R,2R,3R,5S)-3,5-dihydroxy-2-[(1E,3R)-3-hydroxy-4[(α,α,α - trifluoro-m-tolyl)oxy]-1-butenyl]cyclopentyl]-5-heptanoate, Travatan®

Structures and Bioconversion Pathway



Travoprost

Travop rost acid
[(+)- Fluprostenol]

Rationale for Travoprost

Travoprost, an analog of prostaglandin $PGF_{2\alpha}$, was designed to be a potent agent for lowering intraocular pressure (IOP) as well as a selective FP prostaglandin receptor agonist in the treatment of glaucoma.

Natural prostaglandins (PGs) were initially used in the treatment of glaucoma. However, they presented two significant challenges: (i) poor corneal penetration; and (ii) severe ocular side effects (Giuffré, 1985). These two factors made natural prostaglandins ineffective drug candidates. Corneal penetration was a major issue, but a terminal carboxylic acid made drug delivery difficult, and it was known that this carboxylic acid was important to the biological activity of the drug (Bito, 1984). To increase corneal epithelium penetration and reduce the required dose but still maintain the biologically active acid once absorbed, an ester prodrug moiety was introduced (Bito and Baroody, 1987). The addition of an isopropyl ester moiety to $PGF_{2\alpha}$, increased the drug penetration more then 200 times faster then $PGF_{2\alpha}$, but did not eliminate undesirable side effects (Camber and Edman, 1987). The rationale for the intense side effects is thought to be drug binding at multiple PG receptors. This is supported by the fact that natural PGs are known to act upon multiple PG receptors and, specifically, ocular hypotensive PGs act on PG receptors characterized as DP, EP, and FP (Lindén and Alm, 1999). In glaucoma treatment it is desirable to target the FP receptor as binding to a variety of PG receptors leads to unwanted side effects, especially eye redness and discomfort. These side effects are a problem not only as a cosmetic factor to the patient but also with respect to patient compliance, which is especially important because this type of drug is rapidly cleared and requires frequent dosing to insure efficacy (Feldman, 2003).

Travoprost is unique in that it is the only ocular hypotensive prostaglandin analog on the market that is a full agonist of the prostaglandin receptor (Whitson, 2002). It shows a greater affinity for the FP receptor than for other prostanoid receptors, thereby reducing the ocular side effects as well as increasing the potency (Sharif *et al.*, 1999). Travoprost is the isopropyl ester prodrug of (+)-fluprostenol, a known potent FP prostaglandin receptor agonist. The active pharmaceutical ingredient in travoprost, (+)-fluprostenol, referred to as travoprost acid when formed from travoprost, has a twofold higher potency for the FP prostaglandin receptor than racemic fluprostenol and a ninefold higher potency than $PGF_{2\alpha}$ acid (Sharif *et al.*, 2002).

Synthesis

The synthesis of Travoprost is modeled after that of $PGF_{2\alpha}$. Owing to its elaborate structural makeup of five chiral centers and two stereochemically defined double bonds, the synthetic pathway is a 22-step process with the formation of the ester prodrug moiety mid-synthesis (Boulton *et al.*, 2002). The rights to production and utilization of this process are held by Chirotech Technology Limited, UK (Jackson and Lennon, 2000; Anonymous, 2002).

Mechanism and Site of Bioreversion

Travoprost lowers IOP by increasing the uveoscleral outflow of aqueous humor by means of selective binding to critical FP receptors. IOP in the eye is maintained by the circulation of aqueous humor. A buildup of IOP results when there is a decreased outflow of aqueous humor (Brubaker, 1991). There are two aqueous humor drainage routes: the trabecular route and the uveoscleral route (Lindén and Alm, 1999). PGs are known to increase the uveoscleral outflow, thereby lowering ocular pressure. The eye is a highly localized site for FP receptors, and when these receptors are activated, an increase in aqueous humor outflow results (Lindén and Alm, 1999, and references therein). It is thought that travoprost lowers IOP by binding to the FP receptors, causing an increase in aqueous humor outflow; however, the precise mechanism of action is not fully understood.

Travoprost is hydrolyzed by corneal esterases to give the biologically active free acid, travoprost acid. $PGF_{2\alpha}$ analog ester prodrugs, like travoprost, are rapidly hydrolyzed during passage through the cornea by butyryl-cholinesterase and carboylesterase esters (Camber and Edman, 1987; Cheng-Bennett *et al.*, 1994). Travoprost metabolizes rapidly and does not accumulate significantly (Alcon, 2004). There are several sites susceptible to metabolic enzymes, including the C13-C14 double bond, the C15 hydroxyl group, and the carboxylic acid side chain. All metabolites were shown to be non-toxic (Hellberg *et al.*, 2001). Travoprost was also determined to be safe for renally and hepatically impaired patients (Anonymous, 2004).

Toxicity Issues

Travoprost eye drops show no systemic toxicity (Anonymous, 2004).

Formulation issues

The formulation issues for Travoprost were drug stability and storage. Travoprost is formulated with Polyoxyl hydrogenated castor oil 40 as the excipient and solubilizing agent. The formulation is made isotonic with a trometamol/borate/mannitol buffer system. Benzaldonium chloride, 0.015% , is used as a preservative. Boric acid and disodium EDTA are added as anti-bacterial and anti-fungal agents. The current marketed formulation of travoprost maintains stability for 52 weeks in an unopened container and after opening has a shelf-life of 28 days with no special storage conditions (Anonymous, 2004).

Travoprost provided a unique storage container challenge. The standard low-density polyethylene (LDPE) bottles resulted in adsorption of the active ingredient. Classical polypropylene (PP) containers alleviated the absorption problem but were not compatible with eye drop delivery due to a squeezabilty factor. A new syndiotactive polypropylene (sPP) was developed that increased container flexibility while providing lower moisture transfer than LDPE containers (Anonymous, 2004). This container is patented by Alcon as the DROP-TAINER® (Schneider *et al.*, 2000).

Discussion

Glaucoma is the second leading cause of vision loss worldwide, with an estimated 67 million people affected (Quigley, 1996). The need for an effective glaucoma treatment that minimizes side effects is great. Clinical trials have

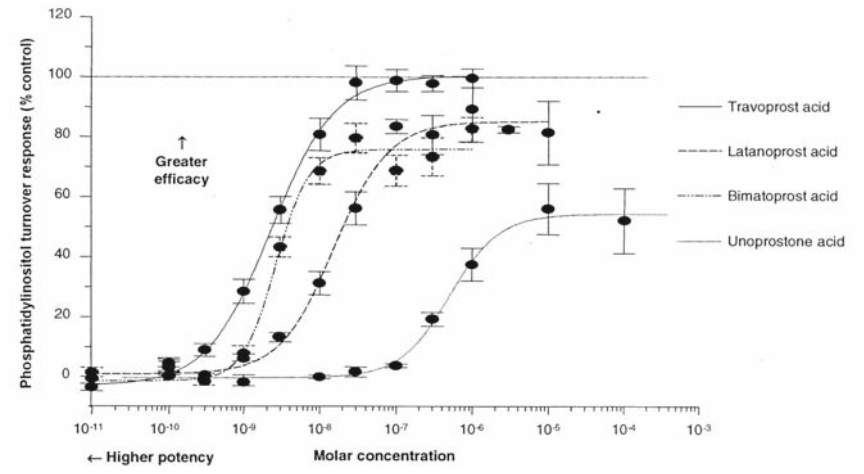


Figure 1. Efficacy and potency of prostaglandin analogs acting on prostanoid FP receptors of Swiss 3T3 cells (reproduced with permission from Whitson, 2002)

proven the effectiveness of travoprost as a first-line drug therapy comparable to other currently marketed $PGF_{2\alpha}$ ocular hypotensive agents: latanoprost, bimatoprost, and unoprostone (Hoyng and Kitazawa, 2002; Waugh and Jarvis, 2002; Whitson, 2002; Anonymous, 2003). The high potency of travoprost can be attributed to its highly selective binding affinity to the FP prostaglandin receptor. With a binding affinity of K_i = 52 nM in bovine corpus lumen membranes, travoprost free acid binding affinity is 1.8 and 2.5 times greater than that of latanoprost acid and $PGF_{2\alpha}$, respectively (Hellberg *et al.*, 2001).

Travoprost reduces IOP more efficiently as a result of selective FP receptor binding. Hellberg *et al.* (2002) completed a large SAR study of FP and DP prostaglandin receptor agonists. They concluded that the saturation level and stereochemistry of the C5-C6 and C13-C14 carbons in travoprost were important to the FP receptor binding affinity of the drug (Hellberg *et al.*, 2002). Figure 2 shows the enhanced selective binding to the FP receptor compared to the $PGF_{2\alpha}$ biologically active acid.

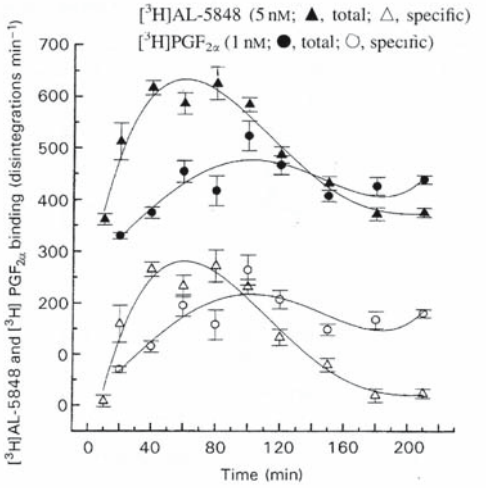


Figure 2. Time-course of carboxylic acid of Travoprost {[^{3}H]AL-5848} and $PGF_{2\alpha}$ binding to washed total particulate bovine corpus luteum homogenates at 37°C. Vertical lines show s.e.m. (reproduced with permission from Sharif *et al.*, 1999)

Travoprost's highly selective FP binding also reduces unwanted side effects compared to $PGF_{2\alpha}$. It has been seen that side effects such as ocular swelling and discharge are less with the selective FP agonist then with previous non-selective $PGF_{2\alpha}$ isopropyl esters, proving that these local side effects result from drug binding to multiple prostaglandin receptors (Hellberg *et al.*, 2001). These side effects were found to be dose-dependent and consisted predominantly of hyperemia and eyelash changes, but also included ocular irritation, foreign body

sensation, photophobia, and changes in iris pigmentation in a small minority of patients (Alcon, 2004).

Various clinical trials also support the effectiveness of travoprost as an adjunctive drug, especially with timolol (Orengo-Nania *et al.*, 2001; Waugh and Jarvis, 2002; Whitson, 2002). It has also been shown in Phase II clinical trials that travoprost has an increased IOP-lowering effect of up to 2 mm Hg for African America patients compared to non-African Americans (Waugh and Jarvis, 2002; Whitson, 2002).

There are assorted general reviews about prostaglandin analogs in the treatment of glaucoma (Linden and Alm, 1999; Schachtschabel *et al.*, 2000; Alexander *et al.*, 2002; Hoyng and Kitazawa, 2002; Hylton and Robin, 2003) as well as specific reviews covering travoprost (Sorbera and Castaner, 2000; Eisenberg *et al.*, 2002; Waugh and Jarvis, 2002; Whitson, 2002; Al-Jazzaf *et al.*, 2003). Numerous patents are also held in the areas development/dosing (Jackson and Lennon, 2000; Stjernschantz and Resul, 2000; Hellberg and Nixon, 2002; Robertson, 2002) and formulation/stability (Schneider *et al.*, 2000; Weiner *et al.*, 2000; Kararli *et al.*, 2002) for PG derivatives, including travoprost.

Conclusion

Travoprost is a good example of an improved prostaglandin analog product whose designed FP receptor selectivity helps to make the drug more potent while decreasing side effects.

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5.22

Case Study: Valacyclovir: A Prodrug of Acyclovir

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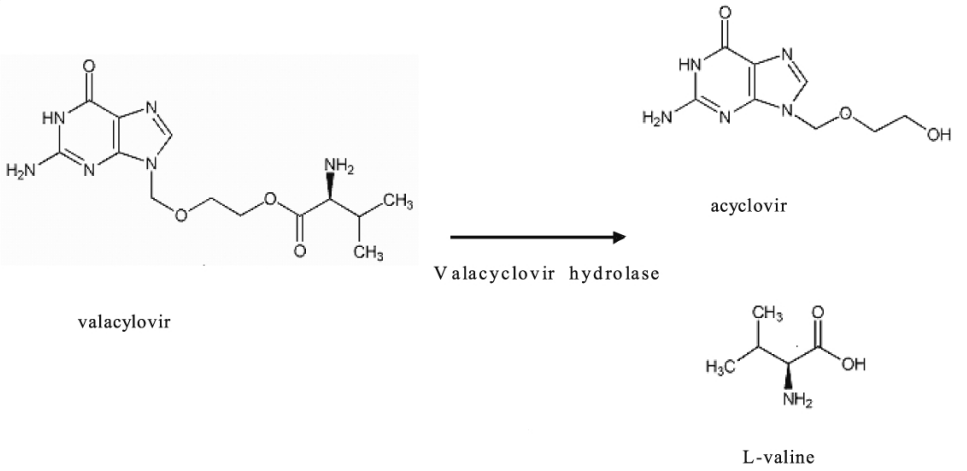
Table of Contents

Name:

Valacyclovir hydrochloride; L-valine, 2-[(2-amino-1,6-dihydro-6-oxo-9 H-purin-9-yl)methoxy]ethyl ester monohydrochloride; Valtrex®

Structures and Bioconversion Pathway



Rationale for Valacyclovir

Valacyclovir is an L-valyl ester prodrug of acyclovir that is used for the treatment of herpes, varicella zoster, and cytomegaloviruses. Valacyclovir was developed to increase the oral absorption and plasma levels of acyclovir. Increased plasma concentrations of acyclovir are important in maintaining antiviral activity, especially in immunocompromised patients and in the treatment of less sensitive viruses such as VZV and CMV (Beauchamp *et al.*, 1992). Suboptimal exposures can lead to more resistant viral strains. To achieve high enough exposures, acyclovir must be dosed intravenously or in multiple high doses (de Miranda and Burnette, 1994). In the design of valacyclovir, the following criteria were met: it was as safe as acyclovir, efficiently converted, and gave exposures after oral administration that were comparable to plasma levels of intravenously dosed acyclovir. Several reviews describe the development, pharmacokinetics, and efficacy of valacyclovir (Crooks and Murray 1994; Beutner, 1995; Perry and Faulds, 1996).

Initially, two prodrugs of acyclovir with modifications on the purine ring were synthesized. The development of these prodrugs was discontinued due to incomplete conversion and to chronic toxicity that was worse than that of acyclovir (Beauchamp *et al.*, 1992; de Miranda and Burnette, 1994) The toxicity was suspected to be due to phosphorylation of the prodrug (Beutner, 1995). Valacyclovir is not phosphorylated.

Eighteen amino acid esters of acyclovir were synthesized (Beauchamp *et al.*, 1992). The bioavailabilities were measured following oral dosing to rats. Of the 18 prodrugs, ten exhibited greater recovery of acyclovir in the urine than did acyclovir itself when dosed orally. Of the ten, the L-valyl ester, valacyclovir, performed the best with 63% excreted as acyclovir in the urine.

Synthesis

The synthesis of valacyclovir was described by Beauchamp *et al.* (1992). The preparation method of the current commercial compound is unknown to the authors.

Transport

The high absorption of valacyclovir is due to carrier-mediated transport. The absorption of acyclovir and valacyclovir was studied in cynomologus monkey intestinal brush border membrane vesicles, where the influx of valacyclovir into the vesicles was 6- to 10-fold higher than the influx of acyclovir (Perry and Faulds, 1996) Additional studies in Caco-2 cells showed that transport of valacylovir was 7x higher than acyclovir transport. In rats, L-amino acid ester analogs of acyclovir show better absorption than D- or D-L analogs, indicating that the transport is stereoselective (Beauchamp *et al.*, 1992)

Many studies in Caco-2 and transfected cell lines have shown that valacylovir is transported by rat and human PEPT1, even though the prodrug does not have a peptide bond (Balimane *et al.*, 1998; de Vrueh *et al.*, 1998; Ganapathy *et al.*, 1998; Han *et al.*, 1998; Sinko and Balimane, 1998; Guo *et al.*, 1999; Sawada *et al.*, 1999). Recent work has, however, shown that several additional transporters may be involved in valacyclovir transport in humans and that PEPT1 may not be the predominant transporter of this prodrug in humans. Most recently it has been suggested that valacyclovir transport by hPT1 may be the most important transporter involved in the oral absorption of this prodrug (Landowski *et al.*, 2003)

Mechanism and Site of Bioconversion

Valacyclovir is converted to acyclovir by enzymatic hydrolysis in the intestine and the liver. A valacyclovir hydrolase hydrolyzes valacyclovir to produce acyclovir and L-valine. The rat and human enzymes have been purified and characterized (Burnette *et al.*, 1995; Kim *et al.*, 2003). The enzyme is also found in rat intestine and kidney, although it is predominately expressed in the liver (Smiley *et al.*, 1996). All of the natural amino acid esters of acyclovir were substrates for the rat valacylovir hydrolase. Recent work indicates that, in humans, the hydrolysis is predominantly due to a biphenyl hydrolase-like protein that hydrolyzes valacyclovir as well as valganciclovir, another amino acid ester prodrug of a nucleoside analog (Kim *et al.*, 2003).

Valacyclovir is rapidly absorbed and almost completely converted to acyclovir in all species. The plasma profiles of acyclovir and valacyclovir following oral administration of valacyclovir to humans is shown in Figure 1 (Soul-Lawton *et al.*, 1995). Valacyclovir is stable in aqueous buffers in the gut lumen, but it is rapidly hydrolyzed in rat liver and intestinal homogenates (Beauchamp *et al.*, 1992; Burnette and de Miranda, 1994; Smiley *et al.*, 1996). After iv dosing in cynomologus monkeys, there is less extensive conversion to acyclovir than when valacyclovir is dosed orally.

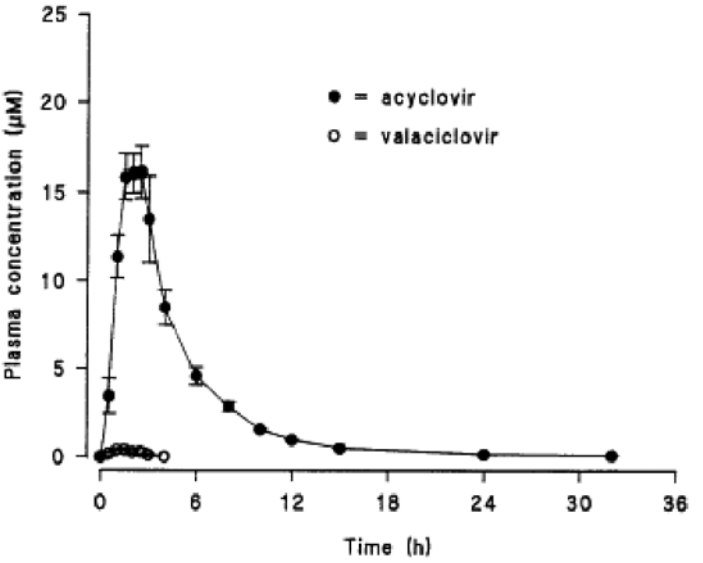


Figure 1. Mean plasma acyclovir and valacyclovir profiles after administration of 1000 mg of [^{14}C]valacyclovir to four healthy volunteers (Soul-Lawton *et al.*, 1995).

Discussion

Valacyclovir is rapidly absorbed and converted to acyclovir following oral dosing. Unlike the purine-modified analogs of acyclovir, valacyclovir is not phosphorylated prior to conversion because it lacks a free hydroxyl group (Beutner, 1995). It is well tolerated, with a safety profile similar to that of acyclovir.

The absolute bioavailability of acyclovir when 100 mg or oral valacyclovir prodrug is dosed to healthy human subjects is 54%, compared to only 15–20% (600–200 mg doses) after acyclovir was dosed orally (de Miranda and Blum, 1983; Soul-Lawton *et al.*, 1995). Larger variability was also observed after dosing of acyclovir than following valacyclovir dosing. The bioavailability of acyclovir from orally dosed valacylovir is similar in rats and cynomologus monkeys (de Miranda and Good, 1992; Burnette and de Miranda, 1994). Over 99% of valacyclovir that is not absorbed is converted to acyclovir.

When valacyclovir is administered orally, there is a slightly less than dose proportional increase in acyclovir exposure. T_{max} also increases as the dose increases. The slightly reduced absorption of valacyclovir with increasing dose is probably not due to saturable conversion to acyclovir because of low urinary recovery of valacyclovir and because the valacyclovir/acyclovir ratio remains the same with increasing dose. The reduced absorption may be due to saturation of absorption sites along the gastrointestinal tract (Weller *et al.*, 1993; Perry and Faulds, 1996).

Although valacyclovir is more soluble than acyclovir (174 mg/mL versus 1.3 mg/mL) (Beauchamp *et al.*, 1992), solubility is unlikely to limit absorption of either compound. This is supported by the fact that many of the other amino acid ester analogs that have been studied also exhibit improved solubility over acyclovir. However, these analogs have very diverse bioavailabilities, most likely due to differences in their carrier-mediated transport (Beauchamp *et al.*, 1992).

Conclusion

Valacyclovir is a good example of how a poorly absorbed drug can be modified to take advantage of carrier-mediated transport.

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5.23

Case Study: Valganciclovir: A Prodrug of Ganciclovir

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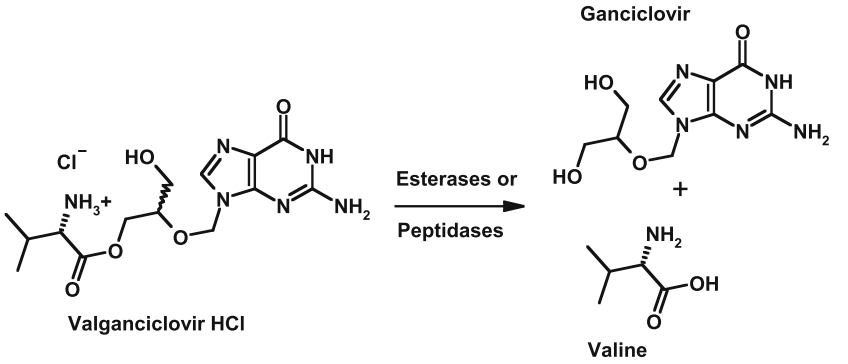
Table of Contents

Name:

Valganciclovir; L-Valine, 2-[(2-amino-1,6-dihydro-6-oxo-9H-purin-9-yl)methoxy]-3-hydroxypropyl ester, monohydrochloride; Valcyte™

Structures and Bioconversion Pathway



Rationale for Valganciclovir

Valganciclovir (Valcyte™) is the commercially successful example of a number of prodrugs investigated to improve the oral bioavailability of ganciclovir (Cytovene®), a potent antiviral agent for the treatment of cytomegalovirus (CMV) infections (Markham and Faulds, 1994). Ganciclovir is a fairly polar compound (logP = -1.65) limiting its uptake by passive routes and has moderate solubility in water at pH 7 (6 mg/mL at 37°C). Ganciclovir is supplied in two formulations: a) a intravenous formulation (IV) in the form of a lyophilized powder of the sodium salt to be reconstituted with water and b) a oral tablet formulation (Physician's Desk Reference 2004). The IV formulation is quite basic and has to be administered slowly in dilute form. The oral formulation is adversely impacted by the physical properties of the compound, resulting in an oral bioavailability of approximately 7%. Therefore, the oral formulation is used mainly for maintenance therapy, while for induction therapy, IV formulations are being used to assure adequate exposure levels.

The primary objective of the prodrug project was the increase in the oral bioavailability of ganciclovir. As with most prodrug approaches, the prodrug modifier of the active drug molecule has to be non-toxic and needs to be cleaved rapidly and completely from the active drug after absorption. Ester prodrugs appeared to be most appealing first due to the presence of two, symmetrically disposed, primary hydroxyl groups and a number of alkyl bis-esters have been prepared (Martin *et al.*, 1987). However, the increase in bioavailability in rats achieved with these modifications was at best about threefold (to 15 to 20%) and the compounds were not advanced further. A double prodrug approach was

pursued by Hoechst with HOE 602 (Rociclovir), a compound with isopropoxy ether modifications of the primary hydroxyl groups and a 6-deoxy base modification (Winkler *et al.*, 1990). This prodrug showed impressive properties in animal models but has not been extensively studied in man.

Amino acid esters of lipophilic amino acids have been employed very successfully as prodrug modifiers, for instance for antibiotics and nucleosides, and these prodrugs are typically substrates for small peptide transporters benefiting therefore from an active uptake mechanism (Rubio-Aliaga and Daniels, 2002). The initial efforts with ganciclovir bis-amino-acid esters were rather disappointing as these compounds showed only slightly better oral bioavailabilities compared with simple ganciclovir bis-alkyl esters. An astute observation in a pharmacokinetics experiment led to the investigation of mono-amino acid esters of ganciclovir. These efforts culminated in valganciclovir, which demonstrated significantly enhanced bioavailability in animals as well as in humans and has now been approved for the treatment of CMV retinitis in AIDS patients (Martin *et al.*, 2002) and for the prevention of CMV disease in high-risk kidney, kidney-pancreas, Liver (EU), and heart transplant patients (Paya *et al.*, 2004).

Synthesis

The synthesis of valganciclovir follows the route for ganciclovir with the major modification being the differential protection of the dihydroxy-isopropoxy sidechain to allow for the selective introduction of the mono-valine ester prodrug moiety towards the end of the synthesis (Sorbera *et al.*, 2000). The scheme exemplifying this approach is shown in Figure 1 and details an approach to the enantio-selective synthesis of one of the diastereomers of valganciclovir (Ho, 1995).

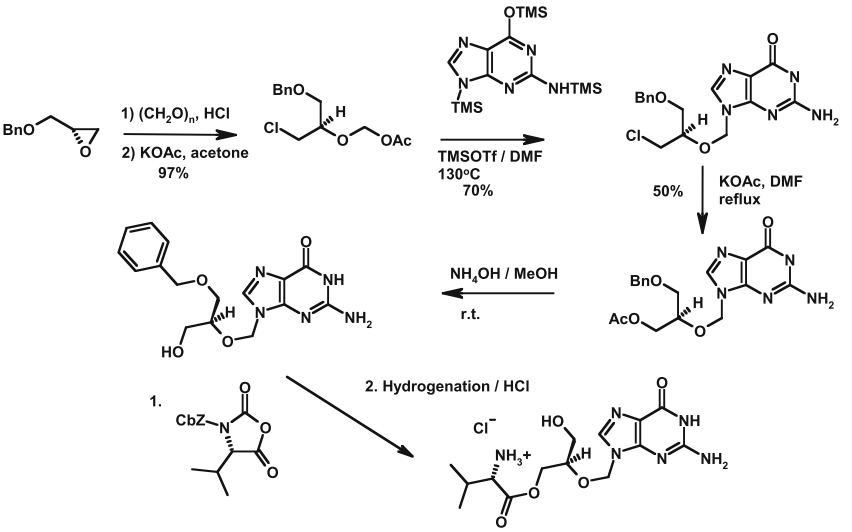


Figure 1. Stereospecific synthesis of one (R,S') Diastereomer of Valganciclovir

Mechanism and Site of Bioreversion

Valganciclovir is a substrate of the peptide transporter PEPT1 present in intestinal epithelial cells as evidenced by experiments in Caco-2 cell layers using Gly-Phe as a diagnostic inhibitor of PEPT1 function (Table 1) (Wan 1995). Sugawara *et al.* (2000) have shown that valganciclovir is in addition also a substrate for the PEPT2 transporter, a transporter mainly present in the kidney. Concomitant to the active uptake mechanism, considerable cleavage occurs in the Caco2 cell layer as shown in Table 1. This rapid metabolic conversion occurs preferentially with the mono-valine ester, valganciclovir, while the bis-ester is both a poor substrate for the transporter as well as for the cleavage steps. This strongly suggests that the two steps, uptake transporter and enzymatic prodrug cleavage are tightly linked.

Compound(s) Applied to Apical Side	Flux (nmol/min/cm^2)	Compound Mixture Detected at Basolateral Side
Ganciclovir	1.2	100% Ganciclovir
Ganciclovir plus Gly-Phe	1.0	100% Ganciclovir
Valganciclovir	10.3	80% Ganciclovir, 20% Valganciclovir
Valganciclovir plus Gly-Phe	1.6	40% Ganciclovir, 60% Valganciclovir
Ganciclovir bis-valine ester	2.8	45% Ganciclovir, 25% Valganciclovir, 30% Ganciclovir bis valine ester
Ganciclovir bis-valine ester plus Gly-Phe	0.01	(Amounts on basolateral side below detection limits)

Table 1. Compound Flux and Activation Experiments in Caco2 Cell Layers

Further evidence of this very efficient cleavage process stems from the clinical pharmacokinetic experiments showing only very low levels of valganciclovir with the overwhelming majority present as ganciclovir in the systemic circulation.

More recently, it has been demonstrated that cleavage of the prodrug moiety is carried out by a biphenyl hydrolase like protein with high efficiency (Kim *et al.*, 2003). This enzyme is present in Caco2 cells and most likely also present in normal epithelial intestinal cells in humans. Thus the ideal prodrug conditions are fulfilled: a) active uptake transport by the PEPT1 transporter and b) efficient cleavage by an enzyme present in the epithelial cell layer of the intestine.

Toxicity Issues

Ganciclovir underwent the required battery of toxicology testing in support of the clinical trials. In these tests, the prodrug did not display any additional (or unexpected) toxicity and all observations could readily be explained by the ganciclovir exposure levels. Ganciclovir is known to cause neutropenia and anemia in animals as well as in humans and early animal studies suggest a potential to cause cancer (Physician's Desk Reference 2004a).

Formulation Issues

Valcyte is sold in the convenient form of tablets containing 450 mg of valganciclovir in the form of the hydrochloride salt. The hydrochloride salt is highly soluble, particularly in moderately acidic aqueous media (70 mg/mL at pH 7 and 24ºC and 580 mg/mL at pH 3 and 24ºC, respectively). Clinical studies have demonstrated a beneficial effect from taking the dose with food. In volunteers fed a high fat meal of 600 calories along with the administration of Valcyte showed higher AUC (+30%) and Cmax (+14%) without a change in the elimination parameters. Therefore it is recommended to take Valcyte along with food at a typical dose of 900 mg either twice daily (bid) (active CMV disease) or once daily (qd) (maintenance therapy) (Brown *et al.*, 1999).

Discussion

Valcyte was first introduced in 2001 and has advanced to being the primary treatment option in prophylactic, induction and maintenance treatments of CMV infections in transplant and AIDS patients. It allows for a convenient alternative to the IV therapy with ganciclovir (Cytovene) as it affords similar exposure levels by the oral route compared with the IV route, which is less convenient and more costly to administer.

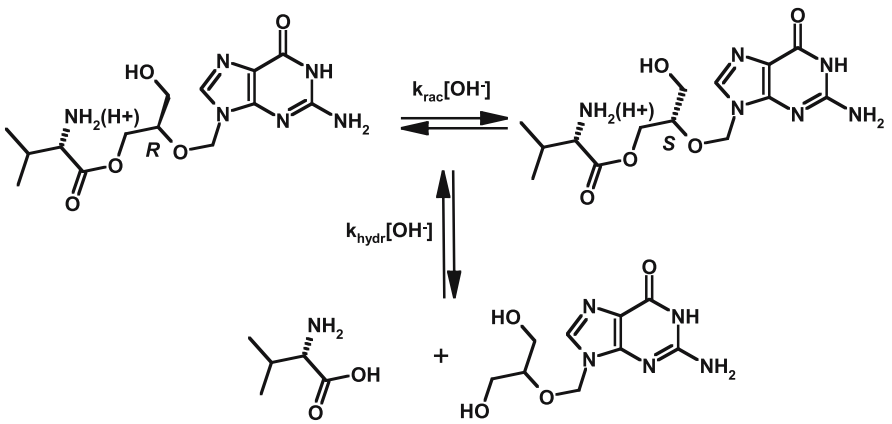


Figure 2. Diastereomer Interconversion and Hydrolysis Reaction Scheme.

The attachment of a single natural (L) amino acid to the symmetric diol of ganciclovir leads to a diastereomeric mixture of mono esters. While diastereomeric and enantiomeric mixtures are in many cases a concern for regulatory or safety reasons, biophysical experiments in the case of valganciclovir showed that there is a facile intramolecular transesterification occurring leading to a rapid equilibration between the two diastereomers (Stefanidis and Brandl, 2005) (Figure 2).

The rapid interconversion made it impractical to develop a single diastereomer for clinical use. This equilibration is about ten times faster than the chemical hydrolysis rate to the parent (Table 2).

Any remaining safety or regulatory concerns were eliminated through preliminary experiments carried out in Caco2 cells and in the cynomolgus monkeys (*in vivo*) with single diastereomers, carefully prepared by total synthesis. These experiments failed to demonstrate significant transporter kinetics and

pH	'Racemization'		Hydrolysis	
	K_{obs} (hr^{-1})	$t_{1/2}$ (hr)	K_{obs} (hr^{-1})	$t_{1/2}$ (hr)
3.81	0.0013	533	0.00013	5300
7.05	0.702	1	0.060	11
11.45	144	0.005	3.82	0.2

Table 2. First Order Rate Constants for Hydrolysis and Diastereomer Interconversion ('Racemization') of Valganciclovir in Aqueous Buffers at 37°C

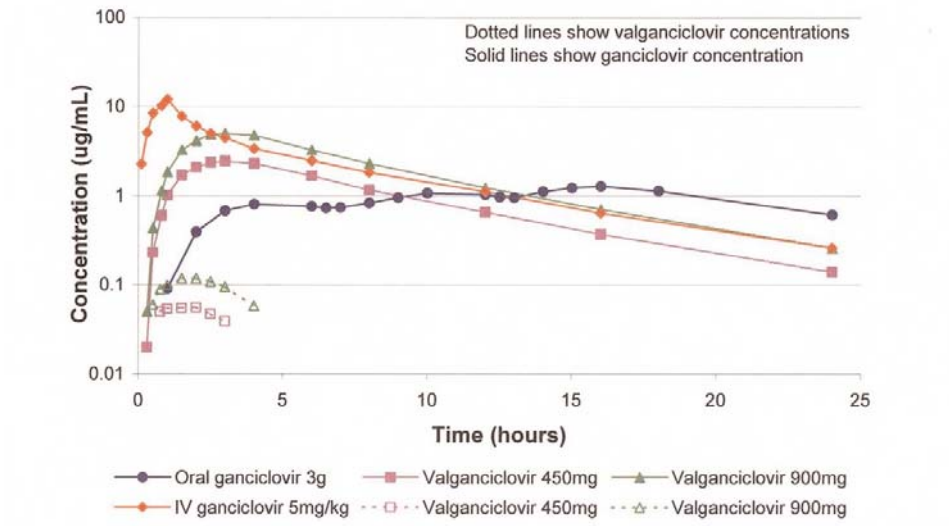


Figure 3. Mean Plasma Concentrations of Ganciclovir and Valganciclovir in Liver Transplant Recipients

pharmacokinetics differences between the two diastereomers. Therefore it was justified to develop a diastereomeric mixture in this case.

In preclinical tests, valganciclovir provided good oral bioavailabilities in mice (100%), rat (56%), dog (100%) and cynomolgus monkey (50%) as measured by exposure to the parent, ganciclovir.

The clinical experience parallels the animal data very closely. The prodrug provides an oral bioavailability as measured as the parent, ganciclovir, of about 60%. The absorption is not limited by either solubility or polarity as it is in the case of ganciclovir. Therefore, Valcyte can provide the same systemic exposure as measured by the area under the curve (AUC) as can be achieved by the ganciclovir IV formulation (Cytovene®–IV) (Figure 3), making it an ideal drug for both the induction and maintenance parts of CMV therapy. Dosing is limited by the side effects of the parent drug as commonly manifested by granulocytopenia, anemia and thrombocytopenia.

The major elimination route of ganciclovir, and thus valganciclovir, from the systemic circulation is by the renal route. Accordingly special dose adjustments need to be made for patients with renal impairment (Czock *et al.*, 2002).

Conclusion

Valcyte is a nearly perfect example of a prodrug for a medicine limited by absorption. It is taken up by active transport and efficiently converted to the parent resulting in high systemic exposures of ganciclovir in animals as well as in man. Very low levels of prodrug are detectable in the systemic circulation of all species tested and exposure to the prodrug is further reduced by the faster elimination half life of the prodrug over that of the parent. This allows for the convenient treatment of all CMV infections, from prophylactic treatment in the transplant patient population through maintenance therapy and the treatment of acute infections in both AIDS and transplant patients (Razonable and Paya, 2004).

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5.24

Case Study: Vantin: A Prodrug of Cefpodoxime

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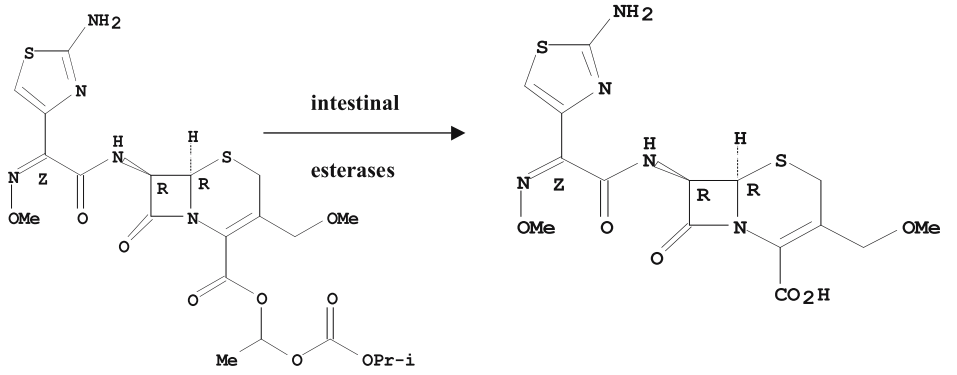
Table of Contents

Name:

Cefpodoxime proxetil, (RS)-1-(isopropoxycarbonyloxy)-ethyl-(+)-6R,7R)-7-[2-(2-amino-4-thiazolyl-2-{(Z)-methoxy-imino}acetamido]-3-methoxymethyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate, Vantin®.

Structures and Bioconversion Pathway



Rationale for Cefpodoxime Proxetil

Cefpodoxime proxetil is an ester prodrug of the extended spectrum antibiotic cefpodoxime, developed by Sankyo Co., Ltd, Japan. It was designed to overcome the poor oral bioavailability of the parent drug. Cefpodoxime (pKa ~2.20 ± 0.10) exists predominantly in the ionic form at intestinal pH and thus exhibits poor permeability (Fujimoto *et al.*, 1987). The free carboxylic acid moiety in cefpodoxime offers opportunities for the preparation of prodrugs. Esterification of this carboxylic acid functional group with an isopropyloxycarbonyloxyethyl group removes the ionizable group and improves lipohilicity, enabling the compound to be absorbed by passive diffusion after oral administration (Fujimoto *et al.*, 1987). Approximately 50% of the drug is reported to be absorbed systemically as cefpodoxime (Tremblay *et al.*, 1990).

Synthesis

The synthesis of cefpodoxime proxetil was first reported by Nakao *et al.* (1982). An improved method for its preparation was recently reported by Rodriguez *et al.* (2003).

Mechanism and Site of Bioreversion

The bioreversion of cefpodoxime proxetil to cefpodoxime occurs by the enzymatic hydrolysis of the ester bond. Studies by Crauste-Manciet *et al.* (1997a)

have suggested that the cytosolic cholinesterase present in the intestinal lumen is mainly responsible for the cleavage of the ester bond. The absolute bioavailability of a 100 mg dose tablet in humans is about 50%, and the majority of the administered dose of cefpodoxime proxetil was recovered as free cefpodoxime in urine (Tremblay *et al.*, 1990). These observations support the idea that bioreversion of the prodrug occurs in the intestinal mucosa during absorption.

Toxicity Issues

Toxicity arising from the promoeity of the drug has not been reported. Incidences of toxicity with cefpodoxime were similar to those of other commonly used beta-lactam antibiotics (Borin, 1991).

Formulation Issues

Cefpodoxime proxetil is poorly water-soluble at neutral pH (0.4 mg/mL at pH 7) (Hamaura *et al.*, 1995a,b). It is currently marketed in two forms, tablets and an oral suspension under the brand name Vantin®. Being a poorly water-soluble drug, it poses some challenges to drug formulators. The major objectives for an oral formulation for cefpodoxime proxetil are to increase its solubility and decrease the enzymatic attack by extracellular esterases present in the intestinal lumen. A recent study by Nicolaos *et al.* (2003) has shown that an o/w submicron emulsion formulation of cefpodoxime proxetil provides greater bioavailability of cefpodoxime by solubilizing the prodrug and protecting it from enzymatic attack.

Discussion

Cefpodoxime proxetil has proven to be a successful product since its launch in 1992. It is currently sold in 57 countries worldwide for the treatment of a variety of disorders such as pneumonia, bronchitis, pharyngitis, tonsillitis, pyelonephritis, cystitis, gonococcal urethritis, otitis media, and sinusitis. It is effective against a wide spectrum of Gram-positive, Gram-negative, and anaerobic bacterial infections. Cefpodoxime acts by binding to the penicillin-binding proteins and thus interfering with the cell wall synthesis. It was shown to display greater resistance to many beta-lactamases than other cephalosporins (Todd, 1994).

Given orally, cefpodoxime proxetil undergoes bioreversion to cefpodoxime in the intestinal mucosa. *In vitro* permeability studies of cefpodoxime proxetil using rabbit mucosa have demonstrated that complete bioreversion of the prodrug to cefpodoxime occurs within 90 min of incubation, suggesting the release of metabolizing cytosolic esterase from the intestinal tissue into the lumen (Crauste-Manciet *et al.*, 1997b). This extracellular enzymatic bioreversion of cefpodoxime proxetil in the lumen could explain the partial biovavailability (~50%) of the orally administered prodrug. The absorption half-life of cefpodoxime proxetil ranges from 0.5–1.0 h with a lag time of 0.3–0.4 h (Borin *et al.*, 1991). The volume of distribution of the drug is estimated to be 32 L with an elimination half-life of

2.5–3.0 h (Tremblay *et al.*, 1990, Borin *et al.*, 1991). It is eliminated from the plasma mainly by renal clearance (Borin, 1991). The renal clearance values of cefpodoxime were estimated to be 10 L/h after intravenous administration of the sodium salt (Tremblay *et al.*, 1990). Total body clearance values (CL/F) following the oral administration of cefpodoxime proxetil ranged from 14.3 to 20.3 L/h in adults with normal renal function (Frampton *et al.*, 1992). Plasma protein binding of cefpodoxime ranged from 18–23% (Borin *et al.* 1991). The majority (80%) of the drug was shown to be recovered in urine as unchanged cefpodoxime, indicating no significant metabolism of the drug in the body after its absorption (Tremblay *et al.*, 1990).

Comparative pharmacokinetic studies of orally administered cefpodoxime proxetil and intravenously administered cefpodoxime sodium did not show any significant difference in renal clearance and elimination half-life values of cefpodoxime.

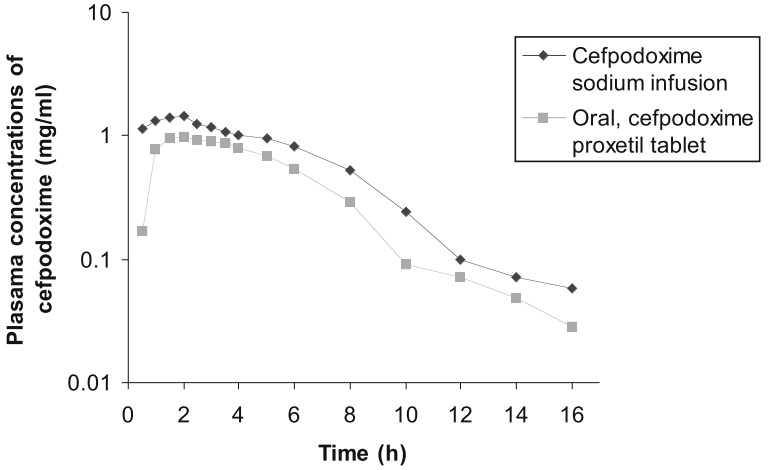


Figure 1. Mean of the plasma concentrations of cefpodoxime in mg/L after administration of a single dose equivalent to 100 mg cefpodoxime iv, cefpodoxime sodium salt by infusion in 2 h at a constant rate. Reproduced with permission (Tremblay *et al.*, 1990).

Linear pharmacokinetics were observed in the dose range of 100–400 mg, above which non-linearity was displayed (Tremblay *et al.*, 1990). The extensive distribution of cefpodoxime in tissues and bodily fluids (V_d = 32 L) results in drug concentrations that exceed the MIC_{90} values for a broad spectrum of pathogens (Frampton *et al.*, 1990). Food has been shown to influence the extent of absorption, but the rate of absorption was unaffected (Hughes *et al.*, 1990). The long elimination half-life (2.5–3.0 h) of cefpodoxime enables the drug to be given only twice daily, resulting in greater patient compliance (Wise, 1990).

	IV cefpodoxime sodium salt	Oral cefpodoxime proxetil	ANOVA Effect of route of administration
C_{max} (mg/L)	2.97 ± 0.13	0.96 ± 0.97	b
T_{max}	2.00	2.25 ± 0.19	ND
AUC (mg.h/L)	10.3 ± 0.41	4.75 ± 0.28	b
U (mg)	80.5 ± 4.7	42.8 ± 3.8	b
Cl_r (L/h)	8.06 ± 0.44	9.46 ± 0.81	NS
Cl (L/h)	9.88 ± 0.39	–	
Cl_{er}	2.09 ± 0.48	–	
$T_{1/2}$	2.296 ± 0.080	2.421 ± 0.080	NS
V (L)	32.3 ± 1.6	–	

Table 1. Mean ± SEM, and ANOVA of the pharmacokinetic parameters of cefpodoxime after administration of a dose equivalent to 100 mg of cefpodoxime. Reproduced with permission (Tremblay et al., 1990).
ANOVA, Summary of analysis of variance
NS, $P > 0.05$; b, $P < 0.001$
ND, not done.

Conclusion

Cefpodoxime proxetil is a good example of the application of the prodrug approach to improve the absorption of a promising broad-spectrum antibiotic, cefpodoxime.

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5.25

Case Study: Ximelagatran: A Double Prodrug of Melagatran

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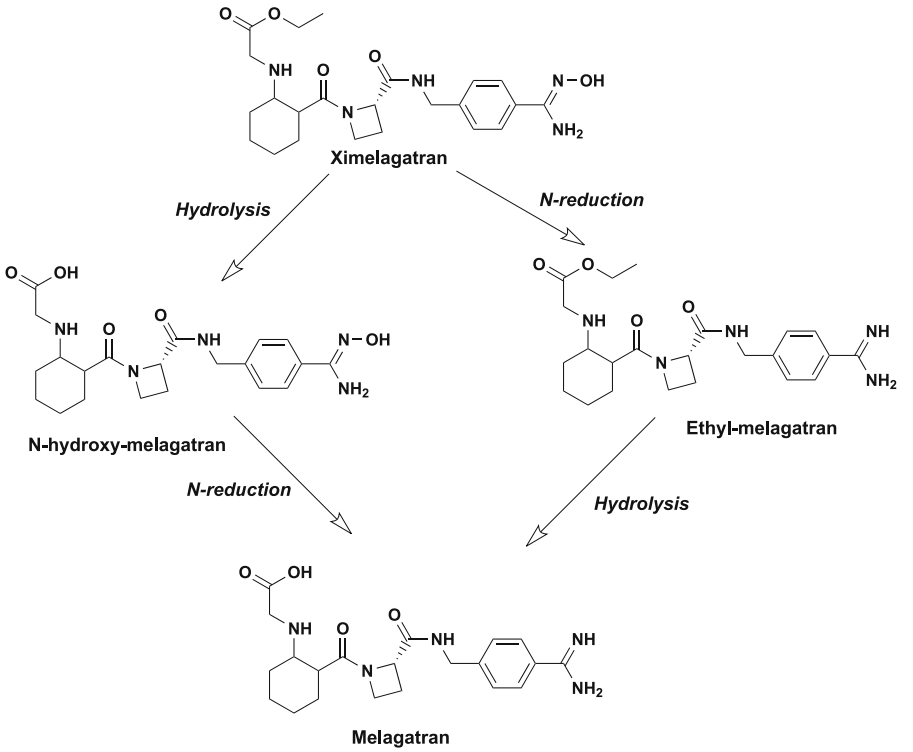
Table of Contents

Name:

Glycine, N-[(1R)-1-cyclohexyl-2-[(2S)-2-[[[[4-amino(hydroxyimino) methyl] phenyl] methyl] amino] carbonyl]-1-azetidinyl]-2-oxo-ethyl]-,ethyl ester, Exanta®

Structures and Bioconversion Pathway



Rationale for Ximelagatran

Ximelagatran was designed to increase the bioavailability of melagatran, a potent thrombin inhibitor, by eliminating charges and thus increasing the lipophilicity of the molecule. Melagatran, like most other thrombin inhibitors, contains a strongly basic benzamidine group with a pK_a of 11.5 that is protonated at the pH of the intestinal tract and hinders intestinal absorption. Furthermore, melagatran also contains an acidic carboxylic group with a pK_a of 2.0, which is ionized at physiological pH. Melagatran also contains a secondary amine with a pKa of 7.0. The effect of the charges on the oral absorption of melagatran was observed when the compound was initially dosed orally and a low and variable bioavailability of 3–7% was observed (Gustafsson *et al.*, 2001).

The prodrug ximelagatran was developed to try to improve the oral absorption of melagatran. The double prodrug ximelagatran is composed of an ethyl ester group in place of the carboxylic acid and an N-hydroxyamidine group

in place of the amidine. By introduction of the oxygen atom into the amidine functional group, the basicity is lowered to a pK_a of 5.2. The ethyl ester of the carboxylic acid not only eliminates its charge but also, in conjugation with the hydroxyamidine, reduces the pK_a of the secondary amine to 4.5. These two protecting groups significantly change the apparent physicochemical properties of the molecule, such that greater then 90% of the ximelagatran molecules is neutral at pH above 6.2. When ximelagatran was dosed orally, the bioavailability of melagatran was observed to be in the range of 18–24% (Gustafsson *et al.*, 2001).

Synthesis

The synthesis of melagatran and ximelagatran has previously been described in two patents by AstraZeneca (Antonsson *et al.*, 1994; 1997). The method of preparation of the current commercial material is unknown to the author.

Mechanism and Site of Bioconversion

The bioconversion of ximelagatran to melagatran involves the reduction of the hydroxyl benzamidine functional group and the hydrolysis of the ester to the carboxylic acid functional group. As previously mentioned, the reduction on one side or the hydrolytic pathway on the other side lead to the formation of one of two possible intermediates, N-hydroxy-melagatran and ethyl-melagatran, that subsequently undergoes a second transformation to form melagatran (See Scheme 1)

The ester of ximelagatran or ethylmelagatran is cleaved by unspecific carboxylic esterases to form hydroxy-melagatran or melagatran, respectively (Clement and Lopian 2003).The hydroxy-benzamidine of ximelagatran and hydroxy-melagatran is reduced to ethyl-melagatran or melagatran, respectively, by reductases in the kidneys, liver, brain, lungs, and/or gastrointestinal tract

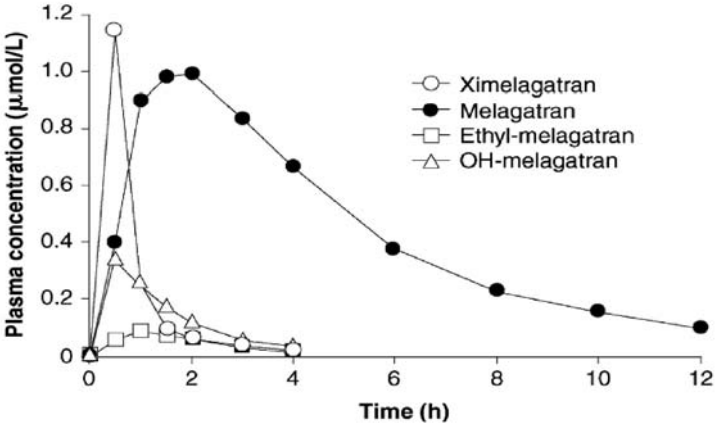


Figure 1. Mean plasma concentration of ximelagatran, melagatran, ethyl-melagatran, and hydroxy-melagatran versus time (hours) after administration of a single 98 mg oral dose of ximelagatran to 5 human subjects. Reproduced by permission (Eriksson *et al.*, 2003b).

(Bredberg *et al.*, 2003; Clement and Lopian, 2003) with the highest enzymatic activity found in liver mitochondria (Bredberg *et al.*, 2003).

In rats the bioconversion of ximelagatran is so rapid that it cannot be detected after oral or IV administration. Both melagatran and hydroxy-melagatran could be detected in rat plasma samples, which is consistent with high esterase activity in blood and tissues of rats. Furthermore, the concentration of hydroxy-melagatran decreases rapidly in the rat and has declined below LOQ 30 min after dosing, indicating relatively rapid reduction to melagatran once the ester hydrolysis has taken place (Eriksson *et al.*, 2003a). In dogs and humans both intermediates could be detected in plasma, with the concentration of the two intermediates dependent on whether the compound was dosed orally or IV. In general, the trend was that the plasma concentration of the hydroxy-melagatran intermediate appeared to be higher than that of the ethyl-melagatran intermediate, indicating that the ester hydrolysis is more rapid than the reduction of the hydroxy-benzamidine.

Toxicity Issues

No known toxicity issues related to the conversion of ximelagatran to the parent molecule have been reported to the knowledge of the author. In general, it is recognized that nitric oxide can be formed after incubation of hydroxy benzamidines, at least *in vitro* (Mansuy *et al.*, 1995). However, the amount of nitric oxide is low to negligible *in vivo* (Mansuy *et al.*, 1995; Clement, 2002). For phase II metabolism, an O-glucuronide or an O-sulfate conjugate of the hydroxy benzamidine is also possible, but is most often observed for unsubstituted hydroxy benzamidines. Neither conjugate has been reported to be mutagenic in the Ames test (Mansuy *et al.*, 1995; Clement *et al.*, 2001; Clement, 2002). It should be pointed out that, depending on which prodrug group is converted initially, two different intermediates that may have very different distribution and toxicity profiles can be formed.

Formulation issues

Melagatran (parent molecule) is currently marketed in Europe as a S.C. formulation. Ximelagatran is currently marketed in Europe as a filmed coated tablet taken orally twice a day.

Discussion

Low and variable GI absorption (approximately 3–7% (Gustafsson *et al.*, 2001; Eriksson *et al.*, 2003a)) is a problem for most direct thrombin inhibitors, and melagatran is no exception. The suboptimal bioavailability of melagatran is caused by the presence of a carboxylic acid, a secondary amine, and an amidine residue that render melagatran charged at physiological pH conditions. The

prodrug ximelagatran was developed to try to improve the bioavailability of melagatran by masking the charges and thus making melagatran more lipophilic.

Ximelagatran is derived from melagatran by ethylation of the carboxylic acid group and hydroxylation of the amidine group. By adding these protecting groups, the highly hydrophilic and charged melagatran molecule is converted into a much more lipophilic (170 times more lipophilic) molecule that is uncharged at physiological pH. This change in physicochemical properties of the compound results in an approximately 80-fold increase in penetration of ximelagatran compared to melagatran across human colon epithelial cell (Caco-2 cell) monolayers (Gustafsson *et al.*, 2001; Gustafsson, 2003).

The improved absorption of ximelagatran was, furthermore, demonstrated by an increase in bioavailability to 18–24% of that of melagatran when ximelagatran was dosed orally; this is three to five times greater than the bioavailability of melagatran itself after oral administration. Ximelagatran also has improved pharmacokinetic properties relating to lower variability in bioavailability and lack of food effects. This is in contrast to the large variabilities in bioavailability as well as in food effects that were observed when melagatran was dosed orally by itself.

Following absorption, ximelagatran is rapidly converted to melagatran with maximum plasma concentration of melagatran observed 1.5–2 hours postdosing (Eriksson *et al.*, 2003a; Sarich *et al.*, 2004). After oral administration of ximelagatran, the volume of distribution of melagatran is large and its plasma half-life increased to 2.5–3.5 hours compared to 1.5–2 hours when melagatran itself was dosed parenterally. Renal excretion by glomerular filtration of unchanged melagatran is reported to be the main route of elimination, which is consistent with the hydrophilic nature of melagatran (Eriksson *et al.*, 2003a; Gustafsson and Elg, 2003).

In humans, the relative bioavailability of melagatran is 43% after IV administration of ximelagatran. Assuming that the metabolism of ximelagatran is the same after oral and IV administration, this would indicate that 40–70% of the oral dose of ximelagatran is absorbed. As the bioavailability of melagatran after oral dosing of ximelagatran to humans is only 18–24%, this is presumably due to either incomplete oral absorption of ximelagatran or first-pass metabolism of ximelagatran with sequential biliary excretion of the formed metabolites.

Conclusion

Ximelagatran is a good example of how elimination of charges can be used to increase oral bioavailability and reduce variability. Furthermore, the compound is an excellent example of the way in which a double prodrug strategy can be used to overcome development hurdles for new and challenging drug targets, where lead molecules are not compatible with oral administration due to their charge and/or hydrophilic nature.

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