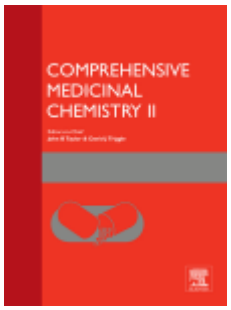

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Personal Essays

Case Histories

8.01 Introduction

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8.01.1 Introduction

The role of a medicinal chemist in drug discovery is the design, synthesis, and registration of the best compound for treating a particular disease condition. The job is not finished when the most potent compound for a given target (receptor, ion channel, enzyme) is identified. The best compound of the series for bioavailability to the target must also be found, the active compound with least metabolism or most predictable pharmacokinetics, and certainly the compound with fewest other effects or greatest selectivity for the desired effect must be identified. The convergence of these properties is the best compound for the disease until a more relevant mechanism or target is found that provides a more effective compound.

My perspectives on drug discovery have developed over nearly 50 years that I have worked in the field, but many of the views were clear early in this time before combinatorial chemistry, high-throughput screening, and cloned and expressed receptor subtypes. The case histories I describe here focus on whether to discontinue or redirect projects; only the time required to complete these projects is shortened by new technologies, but the strategic lessons are still valid today.

8.01.2 Case 1

My first project in industry was to inhibit the body's synthesis of cholesterol because my employer sold the resin Questran which absorbed bile salts as an effective way of lowering serum cholesterol, but compliance was poor because of constipation and the unpleasant task of 'eating sand' several times a day. Hydroxymethylglutaryl (HMG) CoA reductase was known to be the rate-limiting step of cholesterol synthesis so inhibition of this enzyme was the obvious target and assays were quickly established. A series of aryl pentadienoic acids was optimized within a year; they were effective by oral dosing to cholesterol-fed rabbits which were sacrificed and fatty deposits in the aorta measured for drug effect. But what was the disease and how would we measure patient benefit? This was 1962 before ultrasound was used to measure a decrease in fatty deposits in femoral arteries as an indicator of coronary artery disease; the link between serum cholesterol levels and coronary artery disease had not yet been made. The project was put on hold until the company developed a clinical strategy for disease intervention, but that was not a high priority and the clinicians waited for someone else to show the way. A few years later, compactin was identified by Sankyo and mevinolin by Merck as research clinicians quantitated fatty blockages with ultrasound; the statins were born. Two lessons emerged from this shelved project: (1) having clinical strategies and measurements of disease progression is a fundamental part of the drug discovery process and must be available for a seamless and timely development; (2) stepping back from the cutting edge of an emerging field concedes failure because you are no longer first and the competition may be better.

8.01.3 Case 2

Also in the early 1960s, I joined a company effort to explore beta-adrenergic agents because of clinical use of epinephrine for cardiac rescue and isoproterenol for bronchodilation in asthma. Pharmacologic identification of the first beta-blocker, sotalol, concurrent with ICI's pronethalol which soon yielded to the more potent propranolol, hit the same wall as inhibiting cholesterol synthesis – what is the disease condition and clinical measurement? The marketing director clearly asked, "What kind of patient needs their betas blocked?" In 1964, the British physician Brian Pritchard

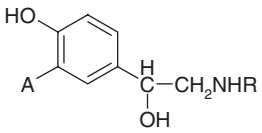

Figure 1 A = HO, R = *i*Pr, isoproterenol; A = $MeSO_2NH$, R = *i*Pr soterenol; A = $HOCH_2$, R = *t*Bu, albuterol.

reported significant lowering of blood pressure in hypertensives by propranolol, opposite to what was predicted because of its predominant effect on cardiac function rather than peripheral vasculature. By following rather than leading with clinical trials, sotalol was registered first in Europe but its sales were dwarfed by propranolol. In later years, probing clinical research established a significant role for sotalol as an antiarrhythmic, a use not anticipated by early animal pharmacology.

More lessons emerged from the adrenergic studies. Our major thesis for structurally modifying the catecholamines was to replace phenolic –OH groups with the methanesulfonamide group in hopes of achieving some organ or functional selectivity. With sotalol (4-$MeSO_2NH$–) we achieved good beta-blocking potency, some $beta_1$/$beta_2$-selectivity, and good oral bioavailability. The more interesting experiment was replacing one phenolic –OH at a time with $MeSO_2NH$ in the catecholamines to retain beta agonist potency (**Figure 1**). The 4-OH, 3-$NHSO_2Me$ analog was equipotent to isoproterenol with much longer $t_{1/2}$ and more $beta_2$-selective, whereas the 3-OH, 4-$NHSO_2Me$ was over 10 000 times less active. The more acidic proton of $MeSO_2NH$ had to be *meta* to the phenethanolamine for beta-adrenergic potency, and the group is more ionized at blood pH than the catechol, whereas the ionized group *para* to the side chain does not function as an agonist. Furthermore, the *meta* $MeSO_2NH$ group is not a good substrate for catechol-*o*-methyltransferase (COMT) methylation, which explains why these bioisosteres were not rapidly metabolized and have a longer $t_{1/2}$. Three compounds from this series (different amine substituents) were clinically evaluated and found to be very safe, effective, and selective agonists.[1,2] Further studies showed the 3-$MeSO_2NH$ group to be a useful bioisostere in phenylalanines, but just as it was not a good substrate for COMT it did not permit decarboxylation with L-DOPA decarboxylase. This meant the methanesulfonamide bioisostere of L-DOPA would not provide an alternative Parkinson's drug, nor did this methanesulfonamide bioisostere provide an effective long-lasting alternative to estradiol, and its usefulness remained primarily in the catecholamines.

A major lesson came belatedly from the clinical trials and toxicological studies of the beta agonists. Soterenol, the bioisostere of isoproterenol, had almost completed phase III clinical trials as a bronchodilator for asthmatics, and mesuprine was in phase III for peripheral vascular disease and premature labor as a uterine relaxant when preliminary results from chronic toxicity and carcinogenicity studies became available. At necropsy 20 of 50 rats in the high dose group had benign tumors, mesovarial leiomyomas, which appeared like a third ovary. No tumors were found after 18 months, only after 24 months. With a heightened concern for tumors and carcinogenicity at the Food and Drug Administration (FDA) in the 1960s, all clinical trials were stopped and all patients who had received drug were to be followed for their lifetime. The FDA even assumed the $MeSO_2NH$ group was responsible for the leiomyomas, although the rats were dosed with 10 000 times the amount of compound that would have killed them with epinephrine. The concern at that time was not benefit/risk ratio or separation between active and toxic dose, but an absolute concern for tumors. All development projects with sulfonamido beta-adrenergic agonists were stopped and no products were marketed.

About 7 years later, Glaxo developed a similar compound salbutamol/albuterol, which differed from isoproterenol and soterenol only by having the *meta* OH or $MeSO_2NH$ isostere replaced by CH_2OH and *t*-butylamine in place of *i*-propylamine. The amine substituent was a trivial change because all three series of catecholamines had *t*-butyl > *i*-Pr > Me in potency with no appreciable difference in pharmacologic or kinetic/disposition properties. The pK_a of the catechol or 'mixed catechol' group was $MeSO_2NH > OH > CH_2OH$, but all somewhat acidic due to the *para* OH, and only the OH was readily metabolized by COMT to an inactive analog. At that time, the Mead Johnson (Bristol-Myers) management agreed to repeat the carcinogenicity studies to see if the leiomyomas were truly drug related, even though it cost nearly 3 years and > $2 million at that time. The new study included four arms with a small group necropsied at 18 months: (1) were the results reproducible? (2) were they reproducible with a different strain of rats? (3) were they reproducible if drug was administered orally by gavage or in feed? (4) were they reproducible if a similar dose of albuterol was used instead of soterenol? The answer to all questions was yes with nearly identical results. Albuterol had been licensed to Schering Plough for the US market, and the carcinogenicity study was conducted by Schering Plough, but no mesovarial leiomyomas were found. We called the head of R&D at Schering Plough who asked their pathologist to reexamine the preserved rat ovaries from their study. Although surprised, they found a similar incidence of leiomyomas when they knew what to look for. At that time, our tissue pharmacologists measured the effect of soterenol on isolated mesovarial tissue and found the density of beta-adrenergic receptors to be much greater than the density in

rat uterus, the classical tissue for measuring beta-adrenergic potency. We were convinced that the unusual benign tumors were an extension of the compounds' pharmacology at very high doses and lifetime dosing rather than a specific chemical toxicity. Subsequent to these findings, Glaxo repeated the carcinogenicity studies with albuterol and found a similar incidence of tumors at their laboratories, but their convincing finding came from a parallel treatment arm with enough propranolol added to block the adrenergic effects of albuterol – no tumors!

I believe this was one of the first examples clearly showing that animal tumors were the result of high dose pharmacology rather than chemical toxicity. The FDA's conclusion was to accept these findings as establishing the dose-limiting toxicity, and the likely dosage or even overdose consumed by people did not indicate significant risk, so albuterol was approved and Bristol-Myers was allowed to resume clinical trials with soterenol and mesuprine. After patients were followed for over 11 years, no major adverse effects or toxicities were reported. Although the sulfonamide adrenergic drugs were first-of-type to be developed by many years, they would be second to the marketplace, and Bristol-Myers management did not see a marketing advantage at that time, so they were never commercialized. By contrast, Glaxo marketed both oral and inhaled formulations of albuterol (Ventolin) as it quickly became the leading drug for asthma and obstructive lung diseases. It has remained champion of its class without significant competition for over 25 years.

Several perspectives or morals are to be learned from this example – primarily, do not accept an unusual effect or toxicity in a nonhuman species as a basis for killing a clinical project – first, understand it, especially if it is an extension of the desired pharmacology at very high doses. Design the toxicology protocols so you can understand what is responsible for unexpected effects. Another perspective I learned as a medicinal chemist from this work is that you cannot deviate very far from the structure of neurotransmitters or hormones if you want to develop receptor agonists. whereas there can be considerable variation for receptor antagonists. This holds up well for adrenergics, serotonin, acetylcholine, glutamate, glycine, and estrogen. The $MeSO_2NH$ mimic for OH was most effective as part of a catechol group for agonists. Even the $–OCH_2–$ linker for propranolol compared to pronethalol retains a similar conformation for the side chain and improves affinity for antagonists but not agonists. Simple replacement of functional groups with bioisosteric groups that do not change stereochemistry, pK_a, hydrophilicity, or bioavailability are about all that is permitted for a true transmitter or hormone mimetic.

8.01.4 Case 3

A major new perspective was learned in the 1970s and 1980s when therapeutic area teams of chemists, biologists, and clinicians were assigned to intervene in major disease conditions. Our understanding of mechanism, relevant animal models, and preferred points for disease intervention was very limited at that time. We made structural assumptions for prototypical compounds with known activity, synthesized a few grams from multistep pathways, tested this compound in several in vitro assays for potency and selectivity, and assumed their mechanism of action would translate to clinical activity. Research groups from other companies were following the same process and would occasionally announce exciting new structural leads at scientific meetings. We often made the mistake that we were competing within structural classes or mechanism (usually unknown) rather than disease class. Bristol-Myers took an early lead at that time by licensing lead compounds from other companies or academic groups and probing disease targets with clinical trials; the mechanism or target was learned only after clinical efficacy was established. Trazodone (Desyrel) blazed the trail for other serotonergics in depression, and was replaced by the nonsedating nefazodone from internal discovery. Treatment of cancer became an achievable goal and a significant business with cyclophosphamide, bleomycin, mitomycin, cisplatin, and Taxol – structurally diverse, different mechanisms but clinically effective. Each of these first-of-type products spawned improved drugs from in-house research, but the structural lead came from licensing and was still novel.

8.01.5 Case 4

The final perspective I would like to pass to younger medicinal chemists is the need to optimize structure for treating the disease, not just the molecular target. The typical drug discovery process today is to screen more than 100 000 compounds from small molecule libraries against a cell-based or cell-free target in high-throughput manner. The iterative process between synthesis and screening is so fast now because many analogs are prepared at the same time by parallel synthesis in tubes or 96-well plates, then screened against molecular targets in high-throughput mode. There is a tendency in many organizations to assign enough chemists to optimize the 'hit' structure for potency, then explore the most active compounds by in vivo assays. It is my opinion that the optimal compound in a series for clinical development can be identified more quickly if most compounds synthesized are evaluated for aqueous solubility and partition coefficient, cytotoxicity, induction of P450 liver enzymes, and a conscious animal model of the disease being investigated.

Seldom does the structure–activity relationship (SAR) for the primary molecular target parallel the SAR for oral bioavailability (indicated by in vivo potency and water solubility) or for safety (indicated by cytotoxicity and P450 enzyme induction). Convergence of SAR for in vivo potency, safety, bioavailability, and drug disposition ($t_{1/2}$) is essential to select an optimal compound for clinical development. Obviously this perspective pertains to systemic diseases but not to invading microbes (bacteria, viruses, fungi, etc.). With high-throughput synthesis capabilities, the assays are rate-limiting rather than the preparation of compounds, so the convergence of different SAR can be achieved by getting multiple data points on each compound as it is synthesized.

The disparity of SAR has never been greater in my experience than for the project with which I am currently involved, inhibiting brain levels of $A\beta_{42}$ for Alzheimer's disease. We use a demanding human-cell-based assay and look for nanomolar potency in the primary screen, then we use mixed brain culture to confirm activity and potency for decreasing $A\beta_{42}$. Early in our efforts to optimize structure, we learned that removing a methyl group from a heteroaromatic ring decreased potency slightly but increased brain levels of drug and induced P450 enzyme activity. Then when we varied the substituent pattern on an aromatic ring, we found the most potent series lacked aqueous solubility and oral bioavailability. Even slight structural changes that improved potency worsened several other properties which made the newer analogs less desirable. We recognized the need for convergence of SAR for all of these properties before we pursued multiple dose pharmacology or other receptors/ion channels/enzymes in search of specificity. To achieve optimal clinical efficacy for this series, we wanted a compound to have some water solubility, good oral bioavailability and brain levels (brain/plasma ratio >1), no cytotoxicity or P450 induction, significant plasma $t_{1/2}$, and area under the curve (AUC), in order to safely dose patients orally and daily in clinical trials for several months. The simultaneous study of potency, bioavailability, and preliminary pharmacokinetics shortened the time to select an optimal clinical candidate. This perspective is certainly aided by the high-throughput screening of many compounds against these drug properties.

In concluding my perspectives on the foremost considerations for medicinal chemists, probably top of the list is patentability of the lead compounds. In this day of court decisions on infringement, issued patents on drug targets or mechanism of action and diagnostic tools enable the patent holder to use them and possibly license them but not prevent infringement if used by others for drug development. This makes the composition of matter patents, plus synthetic process and formulation patents, king of intellectual property and sole protector of a product in the market place. For this reason, the medicinal chemist needs to synthesize compounds beyond the optimal clinical candidate which might be anticipated as second generation products, then secure broad patent coverage to prevent 'me-too' developments. Do not try to patent too broadly and include inactive compounds because that will weaken the patent for unanticipated active analogs. Good patent strategy drives much of the synthetic effort for a first-of-type series.

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Biography

William T Comer in March 2000, was co-founder and Chairman of Neurogenetics, Inc. (re-named TorreyPines Therapeutics in 2005), serving as Interim CEO until April 2002 and Chairman until 2005; he remains a Director of the

Board. From April 1991 until November 1999 he was President and CEO of SIBIA Neurosciences, Inc. which became publicly traded in May 1996 and was acquired by Merck in November 1999. From 1961 to 1991 he was employed by Mead Johnson where he was responsible for discovery and worldwide clinical development of pharmacodynamic agents and was President of Pharmaceutical Research and Licensing until the merger in 1989 to become Bristol-Myers Squibb when he was Senior Vice President of Strategic Management.

Dr Comer has served as Board Director for Trega Biosciences (formerly Houghten Pharmaceuticals) 1993–96, Epimmune (formerly Cytel Corporation) 1994–2005, Board Chairman of Prescient Neuropharma 2000–02, and Tetragenex Pharmaceuticals (formerly Innapharma) February 2001–present. He also serves the Boards of UCSD Foundation 2000–06, UCSD Cancer Center 1992–present, UCSD Department of Chemistry and Biochemistry 1992–present, Dean's Advisory Board for UCSD Skaggs School of Pharmacy, La Jolla Institute of Molecular Medicine 2000–present, and has served the California Governor's Council on Biotechnology, California Breast Cancer Research Council, BIOCOM Board, and several national and divisional offices of the American Chemical Society. He received a BA in Chemistry from Carleton College in 1957 (Alumni Achievement Award 1997) and a PhD in Organic Chemistry and Pharmacology from the University of Iowa in 1961.

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8.02 Reflections on Medicinal Chemistry Since the 1950s

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8.02.1 The Years of Learning

When I started to study chemistry at the Vrije Universiteit in Amsterdam in 1956, it was just one hundred years since the history of modern synthetic medicines had started. Perkin had synthesized the first synthetic dye (1856), and in Germany pharmacology became more and more an experimental discipline. Series of chemically related compounds became available, and were subjected to a pharmacological screening. When in 1868 Crum Brown and Fraser published their famous paper "On the connection between chemical constitution and physiological action," some scientists became euphoric and predicted that soon new medicines would be designed and, moreover, for each disease a specific medicine would become available.

The optimism during the last part of the nineteenth century was not based on evidence. At that time the chemical structure of compounds was poorly understood and also the knowledge of physiological and pathological mechanisms was very limited. Developing new medicines remained for almost a complete century a matter of trial and much error.

When I arrived as a fresh student, the subdiscipline of medicinal chemistry was hardly recognized in the Netherlands. In the USA, the Division of Medicinal Chemistry (American Chemical Society) got its name in 1948; earlier it was known as the Division of Pharmaceutical Chemistry (1909–20), and from 1920 as the Division of Medicinal Products. At the Dutch universities there were no courses in medicinal chemistry, but at the Vrije Universiteit, my alma mater, the organic chemist Wijbe T. Nauta was a lecturer, and at the same time research director of the Dutch pharmaceutical company Brocades (now acquired by Yamanouchi-Fujisawa).

Nauta has been in the Netherlands – and Europe – instrumental in the process that eventually led to the birth of the new discipline of medicinal chemistry. Nauta, however, preferred the use of the word 'pharmacochemistry.' He had two reasons: first, a medicinal chemist (pharmacochemist) does not make medicines, but active ingredients of medicines, pharmaca. Second, Nauta liked to include the fields of crop protectants in this discipline. In the Netherlands and several other countries the term pharmacochemistry is in use as more or less the equivalent of medicinal chemistry.

The manner in which Nauta transferred from a 'classical' organic chemist into a medicinal chemist may be an example of what happened in the years after World War II when medicinal chemistry emerged as a new chemical subdiscipline. Immediately after World War II, the countries that had suffered got free access to patents owned by German companies. The Dutch pharmaceutical wholesaler Brocades asked Nauta to explore the possibilities of starting a research program on the basis of selected patents. This happened, and met with good success, and subsequently Nauta initiated the search for new active molecules. As an organic chemist he had worked on tetraphenylethanes and studied the influence of substitution patterns in the aromatic groups on radical formation from these derivatives. When Parke-Davis introduced the diphenylmethane derivative diphenhydramine as an antihistamine (H_1 blocker), Nauta investigated the influence of substitutions in the benzhydryl group on the histamine-blocking properties. He identified compounds with a strongly enhanced effect, and 'gaps' in the Parke-Davis' patents as well. Subsequently, alkyl-substituted diphenhydraminines were introduced (e.g., orphenadrine for Parkinsonism, based rather on antimuscarinic properties, however).

Figure 1 Professor E.J. (Eef) Ariëns at a Camerino–Noordwijkerhout symposium in Camerino.

Nauta brought his success at Brocades to his university group in the early 1950s. He was absolutely convinced that for a successful research program seeking new active agents, pharmacological studies should be included: not separated groups for chemistry and pharmacology, but a department to which both the disciplines contribute.

In the late 1950s, I joined Nauta's group as an undergraduate and later became a PhD student. I had bought at a booksale a book called *Chemie in Dienst der Mensheid*, which may be translated as "Chemistry at the service of mankind." I was extremely impressed by the stories about Ehrlich, Domagk, the penicillins and other antibiotics, DDT, and on many other scientists and compounds. At that time several of my fellow students considered the choice of this kind of organic chemistry quite negative, the easy way. Applied science was apparently not a popular choice.

I was very lucky to join Nauta's group because of the vision of its leader. But there was more. At the same time the Dutch pharmacologist Eef (E.J.) Ariëns (**Figure 1**) published the book *Molecular Pharmacology*. The way Ariëns treated pharmacology has influenced my way of thinking very much. The ligand–receptor interactions were described in simple mathematics, the pharmacological testing was carried out on isolated organs in a very reproducible manner, and, most importantly, structure–activity relationships (SARs) got much (at best it is qualitative) attention. It is the combination of synthetic chemistry, pharmacology at the molecular level, and the study of the relationship(s) between structure and biological activity, which defines the current discipline of medicinal chemistry.

During the period I worked on my PhD thesis, the pioneering work of Corwin Hansch changed the face of medicinal chemistry in a fundamental way. The SAR studies were transformed into quantitative SAR (QSAR) studies. Many scientists became as euphoric as those who predicted about 75 years earlier that new medicines would be designed. The enthusiasm reached a remarkable level: the IUPAC Committee on Medicinal Chemistry deemed it necessary to initiate a study on the question whether the Hansch approach would allow one to predict the activity of a given compound to such a level that there would not be any innovative aspect in proposing the given compound(s) as a potential medicinal agent. If such were the case, patents for new biological compounds might become complicated – or even impossible – such was the fear. The fear was not justified, concluded the IUPAC study.

Indeed, the Hansch approach has influenced the field of SARs enormously, together with other methods of study. Too often, however, it is forgotten, even nowadays, that the QSAR approaches have a very important limitation. Comparing activities of compounds only makes sense when one has made sure that the biological activities of all compounds do stem from one and the same interaction between the compounds and the site of interaction in the biological system. This limitation makes, e.g., a QSAR study of chemically unrelated compounds using LD_{50} values as the biological parameter an absolute nonsense. But often the limitation is neglected, though it is clear that in many cases the condition is not fulfilled at all. In studies applying mutants of given receptors it has, e.g., been shown that the influence of the mutation may differ even to an absolute extent from one compound to another compound of a chemically closely related series of derivatives. In such cases, a QSAR approach does not make any sense any more! The results have a statistical meaning only, just as in the case of QSARs of LD_{50} values. QSAR studies, with series of compounds without the guarantee that when all have a similar way of interfering with the target, may be considered as an example of what Ariëns has called for another case 'sophisticated nonsense' (see later discussion).

Medicinal chemistry does require the scientist's understanding of both chemical and pharmacological principles. In this sense the pharmacologist Ariëns was a medicinal chemist. This eminent scientist defended on the same day two PhD theses: one in chemistry and the other in medical sciences. One of the many contributions of Ariëns to medicinal chemistry concerns the role of chirality in drug action. The different levels of activity of enantiomers had long been

known, but the important practical consequences had been neglected. In the 1920s, the well-known pharmacologist Cushny considered it of no practical interest that in a given compound the activity resided in one isomer: it is not needed, he claimed, to use the pure enantiomer, you just double the dose when using the mixture. Ariëns explained in a very effective way that in case one isomer does not contribute to a given biological profile, it should be removed. He used the term isomeric ballast for racemic mixtures of both medicines and agricultural preparations. Scientists who used racemic mixtures in pharmacokinetic studies, not knowing whether they were measuring concentrations of one of the other isomer or of mixtures, were condemned as practitioners of 'sophisticated nonsense.'

8.02.2 Pharmacochemistry at the Vrije Universiteit Amsterdam

It was in this atmosphere of a continuous meeting of pharmacologists and (originally) synthetic chemists that I learned the metier of medicinal chemistry and I saw that a new discipline had been born. This new discipline became a star. Its products have contributed to a large extent to the increase of quality of life, even to solving some medical problems. Medicinal chemistry became a mature science, both independent and interdependent, as Alfred Burger once said.

It is currently relatively easy to identify compounds that interfere with a given target; the problem is of course that it is necessary to ensure that the target is meaningful and can be used as the point of attack for a certain disease. In other words, when a target is available the modern approaches in medicinal chemistry allow the identification of a potential medicinal agent. In a way it is permissible to say that the critical step in the process aiming at new medicine is no longer the identification of an active compound or a series of active compounds for a given target. The new issue is much more the question of whether an active compound can be converted into a medicine which is better than the already available therapies. Has medicinal chemistry then no further role to play? Can it not contribute in other ways than by the routinely making compounds which have an attractive level of activity at a certain target? No, I would say, medicinal chemistry will continue to play a major role in drug research and development; the way this role will be played is however changing all the time, especially because of the increased knowledge in the field of life sciences.

Both research and development in medicinal chemistry should become more and more transdisciplinary. As in the years of Ariëns, biological phenomena still require special attention. Let us focus for a while on receptors as targets for medicines; receptors – especially G protein-coupled receptors (GPCRs) – are by far the largest class of drug targets in any case. In my own research program I have always used the histamine receptors as examples for receptors – now GPCRs – in general, and I would like to describe how within a period of about 25 years the field changed dramatically, especially as a consequence of more precise information from the pharmacology of these histamine receptors. I would also like to stress that in this essay I use the histamine field as an example.

Nauta started to work on antihistamines in the 1950s in the traditional way: making compounds and testing compounds. He detected qualitative regularities in the relationship between the substitution pattern and the histamine-blocking properties of extended series of especially alkyl-substituted diphenhydramines. At that time nothing was known about the structure of 'receptor.' In the classic book *Molecular Pharmacology* (Ariëns and associates), a receptor was compared with a beautiful lady to whom you might write letters; you received occasionally an answer, but nobody could claim ever to have seen this remarkable lady. On another occasion – a meeting of the New York Academy of Sciences in 1967 – Ariëns, who had concluded that it was unlikely that agonists and antagonists of a given receptor would bind in the same way to the receptor, said: "when I am talking about receptors, I am talking about something I know nothing about."[1] Indeed, at that time the lock-and-key theory was the accepted concept, without any information about the nature of the lock, never mind the mechanism of the lock.

It was Nauta who proposed, possibly being the first to do so, that a receptor might be a protein in a helix shape. He and his associates published a model[2] in which histamine and the antagonist diphenhydramine were shown to bind in a reversible way to selected amino acid units of a hypothetical protein (**Figure 2**). This proposal unfortunately did not get much attention and it was not followed up by Nauta and his team either.

The Nauta's group – of which I had become a member – continued the search for 'better' antihistamines, including nonsedating ones. Several interesting compounds were identified, such as a quaternary compound (pirfonium) with high activity; poor oral activity made the compound unfit for development as a medicine, but due to its quaternary ammonium function it has been used as a research tool.

In fact, it was not difficult to identify highly potent antihistamines, but at the same time it was not easy to add an extra feature making the compound interesting for development as a therapeutic agent. In the diphenhydramine series, we arrived at pA_2 values up to around 11.0, but no compound except for the early ones went into clinical use.

When the Hansch approach was introduced, the group in Amsterdam started to follow it. It was Roelof Rekker who came up with the so-called 'fragmental constant methodology' to calculate log *P* values. His method was different from

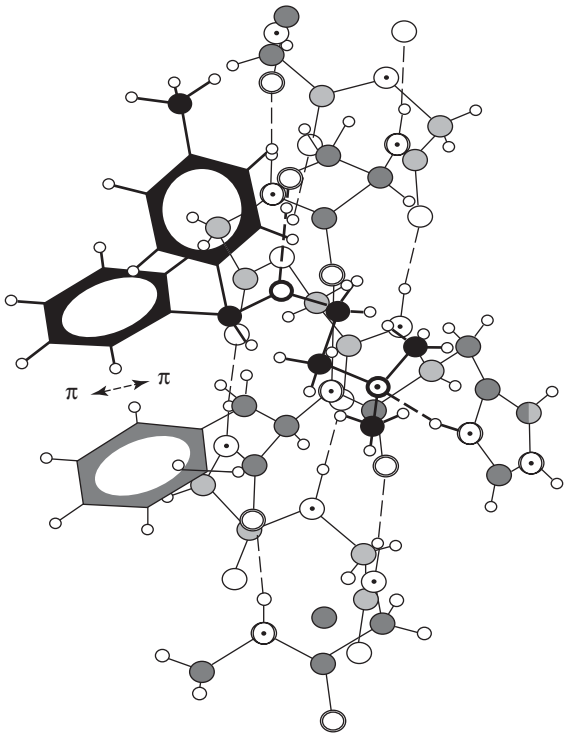

Figure 2 The receptor scheme of Nauta, showing the proposed histamine receptor to which diphenhydramine is bound.

the Hansch system, as Rekker was using a value (established from large series of compounds) for each, including a hydrogen atom, fragment of a molecule,[3] whereas Hansch (and Leo) 'neglected' a value for hydrogen atoms. Rekker and Hansch never settled the straightforward competition between them; however, Rekker became the most-cited scientist of the Faculty of Chemistry of the Vrije Universiteit Amsterdam. The QSAR studies of Rekker, especially using antihistamines as examples, continued into the 1980s, but in the histamine field they did not lead to a discovery of any better compound.

When the research team of Sir James Black at Smith, Kline & French in the UK identified a new class of antihistamines (the H_2 antagonists) during the 1970s, the Amsterdam group failed to pick up the new theme. Ariëns, who was a consultant of the Brocades company (already known then as Gist-brocades, for which company I worked at that time, and of which Nauta was still the director of research), asked that attention should be given to the new ideas, but Nauta was apparently not persuaded by Arien's pharmacological expertise and did not take up this suggestion. How unfortunate this attitude was; the group lost contacts in the (small) world of histaminologists, which focused more and more on what became known as the H_2 receptors than on H_1, the classic field.

8.02.3 Being a Professor in Amsterdam

Toward the end of the 1970s, Nauta had to retire (because of his age, in accordance with the strict Dutch laws) and I was appointed as his successor (**Figure 3**). I decided to revive the (anti) histamine research of the group. As I did not feel any need to develop a new medicine I selected to work on H_2 agonists rather than antagonists. It is my sincere conviction that academics should use their freedom for selecting research fields; they should never imitate what is done by their colleagues working in the pharmaceutical industry; they would be in a poor position anyhow when doing so. At that time it had been proposed that for activating the H_2 receptor, a proton transfer via the tautomeric species of the imidazole nucleus of the histamine molecule was essential. We could show by the relatively high H_2 agonist activity of properly subsisted thiazole analogs of histamine that this could not be true. In the meantime we identified a highly selective H_2 (versus H_1) agonist amthamine, a substituted thiazole analog of histamine, a compound that became a much-used research tool.

What had happened with the H_1 antagonist in the 1950s was seen in the H_2 blockers in the 1980s. The first compounds were moderately active (cimetidine), but soon extremely potent H_2 antagonists were identified. Again,

Figure 3 The author pays tribute to his professor W. Th. (Wijbe) Nauta during a lecture at his alma mater, the Vrije Universiteit Amsterdam.

it was shown that when an interesting target is available, medicinal chemistry will come up with ligands. Not long thereafter it seemed that the histamine book could be closed for the second time; medicinal chemistry of the field was finished.

However, one question related to the clinical profile of the classical antihistamines (H_1 antagonists) had so far not been solved. All compounds caused severe sedation, but the mechanism by which it was caused was unknown. Many investigators thought that the effect was not related to an interaction with the histaminergic system but was rather caused by blockade of the muscarinic receptor (at that time only one type). It was generally accepted that histamine had no function as a neurotransmitter.

But this opinion changed when Schwartz showed by elegant ex vivo studies that there was a clear relationship between the level of occupation of histamine receptors in the central nervous system (CNS) and the level of sedation caused by H_1 antagonists. The results of Schwartz led to two important conclusions: histamine is likely a neurotransmitter and H_1 antagonists cause sedation by blocking H_1 receptors in the brain. Both conclusions proved to be true soon thereafter.[4]

Subsequently, H_1 blockers attracted new attention of the pharmacochemical industry. The first nonsedation or second-generation H_1 blocker, terfenadine, was found by chance; it had been developed as a Ca-entry-blocking agent. The nonsedating properties of this moderately active H_1 antagonist were the result of a poor capacity to enter the brain; this approach had been tried earlier, but without success. But now the principle has been proved to be productive; new nonsedating compounds reached the market and became blockbusters; after terfenadine, for example, cetirizine and loratadine were introduced.

All new-generation derivatives caused only minimal blockade of H_1 receptors in the CNS, and textbooks stated that this was 'because of a high hydrophilicity.' However, the nonsedating compounds showed log *P* values which according to a rule of thumb in respect of lipophilic character would allow them to pass the blood–brain barrier readily. We tried to explain the finding by applying the $\Delta\log P$ theory: a high $\Delta\log P$ (log *P* octanol–water minus log *P* cyclehexane–water) would mean a high hydrogen-binding capacity and therefore a strong binding to plasma proteins and consequently a poor

CNS penetrating capacity. We could explain the findings only by using, besides the Δlog *P*, additional properties of the compounds. It seemed almost impossible to design non-CNS-penetrating compounds; it was largely a matter of chance. Later on the real mechanism became clear, when it was shown that all second-generation compounds are substrates of the P-gP transporter. Indeed, the new compounds had been the result of especially good luck.

8.02.4 The 'New' Histamine Receptors

In the early 1980s Schwarz showed that histamine, being a neurotransmitter, could regulate its own synthesis and release from specific neurons by an interaction with the newly defined H_3 receptor. This presynaptic receptor was subsequently shown to be both a histamine autoreceptor and a heteroreceptor present on other neurons, such as cholinergic and dopaminergic neurons. The receptor is especially, but not exclusively, located in the CNS. Shortly after the H_3 receptor had been postulated, histaminergic neurons were observed by applying immunocytochemical techniques. Selective highly potent ligands, both agonists and antagonists, were identified and potential uses of such ligands were predicted. The pharmaceutical industry showed minimal interest, however. It was the time of 'cloning the genes,' but efforts of several teams – including ours in Amsterdam – to clone the H_3 receptor gene failed. We have, when presenting our findings on new H_3 ligands, often been asked: "Are you sure there is indeed such a thing as an H_3 receptor?" We were sure, especially as the level of selectivity of agonists (H_3 versus H_1 or H_2) was very high.

This situation lasted until the late 1990s, when the gene was finally found, and indeed, found in a database. Now it became clear why high selectivity was often seen: the homology with both H_1 and H_2 is extremely low, much lower than for example that observed for the subtypes of the dopamine or muscarine receptors. Now, the industry became interested and several companies, including big pharma started research programs, especially for antagonists. Large series of patent applications appeared, but as of now (2006) no H_3 ligand has reached the marketplace as a medicine.[5]

But, again, not all aspects of the histamine-related physiology or pathology had been addressed by the medicinal chemist. Histamine has strong bronchoconstrictory properties but the efficacy of histamine H_1 blockers against in allergy-related bronchoconstriction is rather limited. The reason of this lack of efficacy is not only due to the fact that other factors besides histamine are involved in the process (e.g., acetylcholine), but is also as a consequence of the absence of an anti-inflammatory property in H_1 antagonists; in asthma inflammation plays a major role and histamine has proinflammatory properties. Just a few years ago, around the turn of the century, a newly detected histamine receptor, H_4, was found to be present especially on leukocytes. For histamine antagonist ligands for use in asthma it is most likely that compounds which block both H_1 and H_4 receptor properties will be needed; information on such compounds has so far not been published. As the homology between H_3 and H_4 receptors is significant, it is not easy to identify selective H_4 ligands.

8.02.5 Histamine, Histamine Receptors, and Ligands as Research Tools

The story of the histamine receptors so far identified is an intriguing one. The role of histamine in allergic reactions was discovered around 1930. The compounds developed in the 1940s and 1950s as antihistamines were not very active against the asthmatic condition, owing to the lack of efficacy and a high incidence of side effects, especially drowsiness. The latter problem has been solved; the first might find a solution in the coming years. The story can be used for explaining the need of interaction between chemical and biological disciplines when the aim is to find new medicines and also when 'better' medicines are desired. Recent developments in 'receptorology' may lead to compounds with an improved profile for use in medicinal preparations.

Since the introduction of the lock-and-key hypothesis for interactions between a ligand and an enzyme (Emil Fischer) or receptor (Ehrlich, Clark), agonists of a receptor have been described as a key able to open a lock (e.g., receptor), whereas an antagonist fits the lock, but cannot open it, thereby inhibiting the real key from reaching the lock. Ariëns used the following metaphor. An agonist is a piano player, who sits on a piano stool and plays the piano; an antagonist is only able to sit on the stool, but he blocks this stool for the real piano player. We may add that there are more people who do not play the piano than there are piano players; it is indeed easier to identify antagonists of a receptor than to find new agonists; in an agonist two properties have to be present: affinity and intrinsic activity; for an antagonist the former suffices.

The lock-and-key paradigm has however been seriously challenged during recent years. Our group has contributed to these new developments, again using the histamine receptors and their ligands as examples. I am referring to such principles as spontaneously active receptors, inverse agonism versus classical antagonism. In the early 1980s the term inverse agonism had appeared in the literature; it was used to explain the opposite effects of ligands that stimulated

the so-called diazepam receptor (ion channel linked) and ligands that inactivated this system, the two classes were called agonists and inverse agonists. At the same time compounds that blocked both the agonist and the inverse agonists became available. In the 1990s, a comparable principle was shown to be operative in GPCRs. Several GPCRs show a certain level of spontaneous activity (i.e., production of the second messenger, e.g., cyclic AMP in the absence of an agonist); an agonist increases this level. Several compounds, especially many of those known as antagonists, have the opposite effect and they behave as inverse agonists.

We were able to show that most of the known H_2 antagonists are in fact inverse agonists; most of them, but not all, e.g., burimamide, are antagonists of both histamine and the inverse agonists. For both effects, blocking an agonist or an antagonist, burimamide behaved as it should be, the same level of antagonism (i.e., affinity). Later on comparable situations were observed for H_1 (all established H_1 antagonists are in fact inverse agonists) and H_3 receptors; H_4 receptors also showed a spontaneous activity.

Spontaneous activity of receptors is especially observed in artificial systems, i.e., receptors expressed in isolated cells. It reaches sometimes high levels when certain mutants are used. This spontaneous activity has been shown to be the cause of certain diseases: due to a mutation, a receptor is always 'on'; inverse agonists can switch off such systems. In pharmacological experiments inverse agonism has been shown to be operative in vivo for, e.g., H_3 receptors, using thioperamide as the inverse agonist.

The crux of the new model, the difference from the old principle, is of course that many ligands that were considered to be silent at their receptor (they blocked the lock only) are in fact active as an inverse agonist, causing the opposite effect of an agonist. Is such a mechanism important? It might very well be. Just as agonists can downregulate receptor densities, the inverse agonists have been shown to upregulate the system. Such an upregulation may have serious consequences, as it has for the downregulation, and may even be dangerous when therapy with such a compound is stopped abruptly. Indeed, true antagonists may have an advantage, especially for long-lasting treatments.

The developments around agonism versus inverse agonism constitute a perfect example of the benefit of transdisciplinary research for both parties. The new principle would however have been difficult to prove without the availability of several ligands. At the same time the principle opens new vistas for drug development. And, again, the relatively easily accessible histamine receptors have been very useful for understanding the matter. It is sometimes too easily forgotten that for mapping out biological systems the availability of biologically active compounds has been essential: atropine and adrenaline were needed to differentiate the autonomous neuron system into the parasympathetic and the sympathetic components; it has not been the reverse.

8.02.6 Looking Back

During a period of about 40 years I have been very lucky in having excellent teachers, and also in the fact that new theories and models, and especially equipment, have become available. I refer to molecular pharmacology in the beginning and later on the molecular biology. I saw the arrival of QSAR, though I later became quite skeptical about the meaning of the approach; molecular modeling using individual compounds would seem to deliver more useful information.

I have, from the beginning, also been skeptical about combinatorial synthetic methods. For me, the technological achievements were impressive, but for a true medicinal chemist it seemed to be rather a step backward, not making the required compounds, but the compounds which were possible to be synthesized; back to screening large numbers of compounds. That was also done in the nineteenth and the first part of the twentieth century.

When medicinal chemistry is considered to be a science that can produce new and better medicines, the medicinal chemist should admit that there is a need to live close to the pharmacologists. The new ideas come especially from physiology and pharmacology. Medicinal chemistry when applying its skills properly will be able to come up with new attractive compounds to be used in therapy, as soon as targets have been defined.

One new development emerging from modern pharmacology causes concern for me. Through the enormous increase of the output of gene-profiling technologies more and more mutations in important genes encoding proteins can be identified as the real cause of a certain condition; such a condition, however, in the given patient may be seen only in a relatively small group of 'comparable' patients. Individualized medicine, 'tailor-made treatment,' is what then becomes possible. Such individualized medicines are something like the opposite of blockbuster drugs. The consequence is obviously that because the costs for development of an individualized medicine will be as high as for a drug which could become a blockbuster, such tailor-made treatment will become extraordinarily expensive. It is an odd situation: here we have something like an orphan disease for which nevertheless a medicine will be developed. Who can pay for this? This question seems seldom to be asked.

It is indeed not the need for a drug that determines whether a drug will be developed; it is the need for a drug to be used by patients who can pay for it. In a way, it is just because AIDS emerged in the Western world at a relatively early

period that anti-HIV agents have been developed. If such had not been the case, if the outbreak of AIDS had been limited to Africa, it is likely that no active medicine would have become available. Who can bear such costs as around €15 000 per year for an anti-HIV treatment, the costs for 1-year treatment with a fusion protein inhibitor developed in the early years of the current century? It is a very disturbing situation that enormous amounts of money are used to reduce the health problems of only a small part of the world's population, knowing that with the same money life-threatening conditions for much larger groups could be cured. As I said earlier in this paragraph, for emerging countries it is not the science, but the political and economical will that determines whether the major health problems in such countries will be improved or not.

Would it be correct to blame the pharmaceutical industry in particular for this situation? It is certain that nobody can justify a situation where medicines that are available are not made available, just because of cost. But who has to bear the costs? Let us compare this situation with another major problem of the emerging countries: famines and the enormous stocks of food in the Western countries. Who has to make the food available? The producers, the farmers? Or would it be an international organization like the Food and Agriculture Organization or the World Health Organization? And could such a distribution system be organized for medicines as well? It is my true conviction that too often the pharmaceutical industry is blamed for the medicines issue, while for food the responsibility is seen to be society-wide.

8.02.7 My Histamine

I have often considered histamine to be my amine. It has brought much to me. The results of histamine research in physiology, pharmacology, and medicinal chemistry have throughout the years contributed much to the progress in these fields. The effective drugs from this research are very useful in allergic diseases (H_1) and in treating gastric ulcers (H_2, though the proton-pump inhibitors took over the role of H_2 blocking agents). General principles, developed by searching the histamine field, are, however, probably even a more important result of about hundred years of histamine-related investigations than the new medicines developed in this field. I am pleased that I was able to contribute during the last decades a little to a continuation of a program that started a century ago.

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Biography

Henk Timmerman (b. 1937) obtained his PhD in 1967 at the Vrije Universiteit Amsterdam under the guidance of Wijbe T Nauta. He worked until 1979 for the Dutch pharmaceutical company Gist-brocades, until at the end of that year he succeeded his teacher as professor of pharmacochemistry at the Vrije Universiteit Amsterdam.

His research concentrated on histamine receptors and their ligands, studying receptor mechanisms, and SARs: in his research group the interplay between the disciplines contributing to medicinal chemistry received much attention. Several of the new ligands the group developed became research tools, such as the H_2 agonist amthamine and the H_3 antagonist clobenpropit.

Henk Timmerman is (co-)author of about 500 published papers or chapters; he supervised 35 PhD students. He has been on many national and international bodies, committees, etc., having been, e.g., president of the Royal Netherlands Chemical Society (KNCV) and the European Federation of Medicinal Chemistry (EFMC). He has been honored by, e.g., the honorary membership of the KNCV and honorary degrees of the Gadjah Mada University in Yogyakarta (Indonesia) and the Medical University of Lodz (Poland). In 2002 he retired from his chair in Amsterdam.

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8.03 Medicinal Chemistry as a Scientific Discipline in Industry and Academia: Personal Reflections

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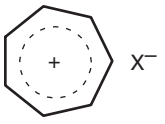
8.03.1 Introduction

There was probably a strong genetic determinant for me to become a chemist. My father was a chemist working for a firm of public analysts (Dr Bernard Dyer & Partners) in the City of London and one of my maternal uncles (A. Cluer) was an oil chemist. I have a cousin (Dr Brian D Ross) who is a medically qualified biochemist and Director of Magnetic Resonance Spectroscopy at Huntingdon Medical Research Institutes, Pasadena, CA, and my daughter is a pharmacist. I was born in East London and studied Chemistry at Queen Mary College, London University, receiving a PhD in organic chemistry with Professor Michael JS Dewar for research on tropylium chemistry. I started my PhD research in 1955 and the tropylium cation (**1**) had been synthesized for the first time by von E Doering and Knox the year before.[1] I found this research to be very challenging and extraordinarily exciting. For example, I discovered that I could oxidize cyclooctatetraene (C_8H_8) directly to the tropylium cation ($C_7H_7^+$).[2]



1 Tropylium cation

I was amazed to see this apparently simple loss of CH! Part of the challenge for me at the time was that I was working as an organic chemist synthesizing molecules that had inorganic properties, since tropylium existed as an ionic salt. This apparent contradiction had a profound influence in making me very aware of the relationship between chemical structure and chemical properties. There was, of course, a theoretical basis for this phenomenon, which had been analyzed and predicted by Hückel[3] in 1938 and so I was also introduced to the concept of using molecular orbitals and computational

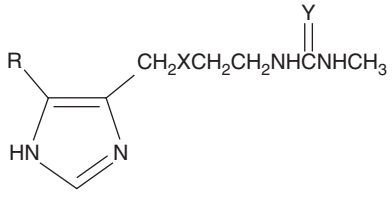
chemistry to make explanations and predictions. This was in any case the forte of Michael Dewar, who was using theoretical chemistry to predict and explain the course of reactions in organic chemistry.[4] Calculations had to be rather simple in those days because we did not have access to electronic computers. I mention this because I believe that it removed a barrier for me as an organic chemist so that, later on, I was not afraid to collaborate with computational chemists. I believe that education is a mixture of imparting information and techniques but also of removing barriers to further learning. Furthermore, I subsequently realized that working with Michael Dewar had strongly imprinted me with an interest in chemical properties. Also there was no spoon-feeding; we were left to get on with our work with only very occasional discussion and so one learnt to develop one's own resourcefulness to overcome the many problems which PhD research posed. I do not know whether these observations are causally related to the subsequent manner in which I conducted my own research or whether it is simply a convenient post hoc rationalization.

8.03.2 SmithKline & French Laboratories

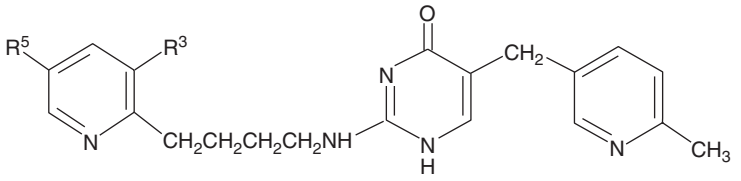
8.03.2.1 Early Years

I joined SK&F in 1958 because it happened to be near where I was living at the time in London and its local organization, although small, appeared to me to be flexible and have potential for expansion; I was also impressed by the people I would report to, namely David Jack (later to become Research Director at Allen & Hanburys and then Glaxo Research Laboratories) and Geoffrey Spickett (who eventually became Research Director at Laboratorios Almirall in Barcelona, Spain). The research, however, turned out to be unexciting. It was based upon testing compounds in animal models using very-low-throughput screening. We sought a novel antidepressant, or an analgesic, or anticonvulsant, or anti-inflammatory drug. The chemical leads were either thrown up by the screens or they were reported compounds and we synthesized analogs. For example, we made partial structures of reserpine[5] and tetrabenazine.[6] We made some interesting indene isosteres of indoles[7] and some 4-piperidones.[8] These were patented and taken as far as clinical studies as potential antidepressants, but none was successful enough for full clinical trials, let alone to reach the market. I found this to be very disappointing because of the complete lack of any biological mechanistic understanding.

There was certainly no quantitative way of relating chemical properties to the biological properties. I began to wonder whether this pharmaceutical research could ever be as exciting again as I had found the research during my PhD years. Meanwhile SK&F moved out of London to Welwyn Garden City and then underwent an internal reorganization that brought in several scientists from ICI Pharmaceuticals. In particular James Black came in like a breath of fresh air to head Pharmacology and I instantly knew that here was somebody with whom I wanted to collaborate. Black initiated several lines of research but eventually all work was concentrated (because of limited resources) on to the search for compounds to block a new type of histamine receptor, the putative H_2 receptor. This was a novel approach to controlling the secretion of gastric acid: the therapeutic aim was to treat peptic ulcer disease. This was a real challenge for the medicinal chemists because there was no chemical lead other than the chemistry of the natural transmitter, histamine, and I was given the responsibility of directing the chemistry. Much has already been published[9–11] about the work that gave rise to the prototype drug burimamide[12] (**2**) that was used to characterize histamine H_2 receptors for the first time, and to validate the pharmacology and provide proof of principle in human volunteer studies. The further development of the failed drug metiamide[13] (**3**) and the first of the modern 'blockbuster' drugs, cimetidine[14,15] (**4**), has also been well publicised. So I will, instead, discuss aspects of my philosophy as a medicinal chemist at this time.



2 Burimamide R=H, X=CH_2, Y=S
3 Metiamide R=CH_3, X=S, Y=S
4 Cimetidine R=CH_3, X=S, Y=N–C≡N



8.03.2.2 Structure–Activity Analysis

Having been trained as an organic chemist I had to learn medicinal chemistry 'on the job.' I realized that there was a major problem in communicating with other disciplines that arises from the language, and concepts, which we have been taught and then take for granted. The words we use with other scientists appear to be the same but our own appreciation of them depends upon the context in which they were presented to us. The nuances and shades of meaning can differ, sometimes markedly. In my discussions with Black, I soon learnt that I could not take the words for granted or at face value, but had to get behind them to understand what he meant, that is, to appreciate what lay underneath the words and what were the ideas that he was expressing. The discussions were very stimulating and from them I learnt about the chemical issues that were of importance in pharmacology. Of course we also discussed the results from testing our compounds and what were the implications to us as chemists for projecting to the next compounds to synthesize. To the chemist, the first appreciation of this is based on pattern recognition. However, sometimes the results appeared to defy rational explanation and then Black would throw down his final challenge: "my rats can see a big difference in these structures, why can't you chemists see it?"

For me, the essence of medicinal chemistry is structure–activity analysis. We have to combine two disciplines continuously: we have to select what to make and identify how to synthesize it. These two aims are not always achievable in an ideal form and sometimes we have to compromise or reconcile contradictions. This is why it is preferable to retain the two disciplines within each medicinal chemist. Furthermore we have to understand the language and limitations that express 'activity.' This will not be news to most medicinal chemists but in my experience the major problem for chemists is to 'read' the chemistry contained in the structures of their compounds. The problem lies in the way chemistry is taught at university. As organic chemists we mainly learn about synthesis and reactions, that is, making and breaking covalent bonds. In biology, most of the productive drug–receptor interactions are noncovalent. Of course enzymes eventually react covalently but, even so, the initial interaction is noncovalent.

In relating chemical structure to biological activity, I realized that we should use properties as a bridge; that is, to relate chemical properties to the biological properties. This may appear obvious but it poses the major issue of how to appreciate the chemical properties. Most chemists think that their problem is to understand biology but it is my contention that their real problem is to understand the chemistry. This analysis leads naturally into the dual exercise of determining the chemical properties of drug molecules and of trying to discern which properties are most critical for biological activity. This becomes part of the iterative process whereby one continuously analyzes for relationships between chemical properties and biological activity, then predicts the next compounds to be made and then determines how to synthesize them. It places a different emphasis in comparison with the approach that selects compounds on the basis of synthetic availability. This does not mean that one should ignore synthetic accessibility but it does mean that the research should be property driven, not synthetically driven.

Molecular interactions between molecules are determined fundamentally by molecular size, shape, and charge distribution. Inspecting molecules from this standpoint leads to an awareness that they rarely have a unique description and that there may be several different forms or species in equilibrium.

8.03.2.3 Dynamic Structure–Activity Analysis

We know that writing a chemical structure on paper is misleading since the molecule may not be planar and the formula does not adequately represent a three-dimensional stereochemistry; also various conformers may exist in equilibrium. Furthermore, many drug molecules have ionizable protons and these too can give rise to prototropic equilibria. An interesting question arises from these considerations: if one changes drug structure to alter the equilibria, can one relate the consequences to changes in biological activity? If the answer is yes, then perhaps this may provide some insight into the chemical mechanism of drug action and give a method for drug design. These thoughts led me to formulate[16] a concept of DSAA and to apply it to histamine. That histamine may interact with its receptor as a monocation was also used as an argument to relate this property to partial agonism at histamine receptors; this led to the search for a noncationic compound.[10,11] The result gave a pure antagonist which led on to burimamide (**2**), the first H_2 receptor histamine antagonist.[12] Although histamine, being an imidazole derivative, is tautomeric, I was surprised to

find that nobody had published on its tautomeric properties, and so we measured the tautomeric ratio for histamine.[17] Later on the same approach of DSAA was used to develop metiamide (**3**) from burimamide.[13]

One of the problems I had experienced early on at SK&F was the difficulty of having chemical properties measured. The analytical laboratory had a background of chemical analysis to service chemical and pharmaceutical production and the resources were arranged specifically to conduct these tasks. There was simply no conception of what might be needed to investigate the open-ended questions posed by a medicinal chemistry research laboratory. So I became involved in an unedifying internal struggle to set up a physical-organic chemistry laboratory that would establish evidence for structures and purity, but that also had some spare capacity to make measurements and answer some of the questions posed by medicinal chemistry. The key was to have it report to Research Management instead of Production. Eventually we were able to do this and to put in charge an excellent organic chemist, Dr PMG Bavin, who built up a department that in the 1960s could study conformation and pK_as by nuclear magnetic resonance, could determine hydrogen-bonding by infrared spectroscopy, and could measure solvent/water partition and pK_a values.

8.03.2.4 University Collaborations

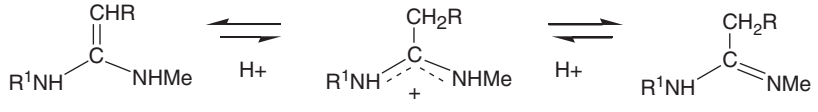
As part of our studies of histamine's properties I started collaborations with university scientists. We needed more information about properties but I did not wish to divert our resources from synthesis into these studies. At that time the UK government had instituted research grants which combined university and industrial collaboration in PhD degrees (Cooperative Awards in Science and Engineering: CASE) and I started a project with Dr Graham Richards at Oxford University for molecular orbital computations on histamine,[18,19] for both its conformational and tautomeric preferences. I also started working with Dr Keith Prout,[20,21] a crystallographer at Oxford University. The advantage of such collaborations was not only the data that they produced, but also the perspective these colleagues gave about properties; this was gained through discussion with them as chemists who viewed chemistry in a different manner, especially when it came to nonbonded interactions between atoms in molecules.

Another consequence of considering properties is that it allows one to formulate questions where the answers can be found in the literature, especially when this concerns quantitative data. This became very fruitful when seeking pK_a data and applying the Hammett equation to predict the pK_a values of novel compounds. This approach led us to identify that cyanoguanidine could be a bioisostere for thiourea and led to the synthesis of cimetidine[15] (**4**).

Meanwhile we were also seeking properties that might provide a quantitative correlation between structure and H_2 receptor antagonist activity by using multiparameter correlation analysis. Since methyl on the thioureido group (the 'polar group') increased the potency to give burimamide or metiamide, and since thioureas were much more potent than ureas (by about 20-fold) we wondered whether desolvation of the drug molecule was playing a critical role prior to its binding to the receptor. Support for this idea was obtained from an excellent correlation between activity of the whole molecule and octanol/water partition of the polar group for a series where only the structure of the polar group was changed. There were, however, certain outliers, which are discussed below.

8.03.2.5 Diamino-Nitroethene as a Bioisostere

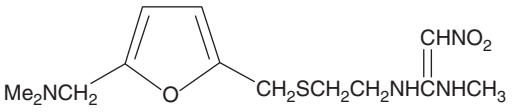
About this time we also became interested in replacing the N of cyanoguanidine by a CH on the grounds that it might be less readily hydrated and have a higher octanol/water partition ratio and therefore may be more potent. However this leads to another problem because the resulting diamino-cyanoethene tautomerizes to a cyanoacetamidine (**Figure 1**). One of my younger colleagues, HD Prain, drew my attention to a publication describing a synthesis of diamino-nitroethene (**5**). Encouraged to make it, he synthesized the corresponding analog of cimetidine but it was only similarly active, i.e., it was not more potent and, surprisingly, the group had a much lower octanol/water partition than did the corresponding cyanoguanidine. Based upon this latter finding, the compound should have been much less active. These considerations of DSAA and pK_a analysis led us to identify the diamino-nitroethene (**5a**, R = NO_2) bioisosteric group,[16] which we published in a patent.[22] It was subsequently taken up by Glaxo Laboratories to produce the second marketed H_2 receptor



5a **5b** **5c**

Figure 1 R = CN, the diamino-cyanoethene (**5a**) in equilibrium with the cyanoacetamidine, **5c**, via the cyanoacetamidinium conjugate acid, **5b**. R = NO_2, the diamino-nitroethene (**5a**) in equilibrium with the nitroacetamidine, **5c**, via the nitroacetamidinium conjugate acid, **5b**.

antagonist 'blockbuster' drug, ranitidine[23] (**6**), since the Glaxo researchers found that replacing cyanoguanidine by diamino-nitroethene in their furan series unexpectedly increased potency by 10-fold.



6 Ranitidine

Outliers from a linear correlation can be extremely valuable because they can indicate that another chemical property is affecting the biological activity. In the above case, a cyclic analog was much less potent, possibly due to tautomerism into another structural form. On the other hand, the diamino-nitroethene was much more potent than predicted (see **Figure 1** in [16]). This led us to investigate quantitatively the polarity of the polar group and we collaborated with Professor Ted Grant of Queen Elizabeth College, London University, who measured the dipole moments[24] in water, but this did not provide a correlation. Now, dipoles are also directional, and so we used molecular orbital calculations to predict the dipole vector; this latter gave an excellent correlation which depended on an optimum orientation of the dipole together with the octanol/water partition value.[25] Intuitively this sounds very sensible, i.e., that an interaction of a very polar molecule should require an appropriate orientation, but it is rather unusual to be able to dissect it out and demonstrate a relationship. It would be very interesting to do this for other series of H_2 antagonists where the ring structure–activity relationship follows a different pattern from the imidazoles, as occurs for the furan series with ranitidine.[26]

8.03.2.6 Medicinal Chemistry Summer School

My interest in medicinal chemistry as a scientific discipline also led me to become involved with the Royal Society of Chemistry (RSC) summer school. My colleague, Dr AM Roe, was Chairman of the RSC Education Committee which advised the RSC on the various courses mounted for postgraduate chemists on specialist topics. These were usually residential and each lasted for approximately 1 week. One such course had started as a week's summer school in medicinal chemistry but it had not attracted enough chemists from the pharmaceutical industry, possibly because it appeared to be overly dependent on techniques of structure determination and chemical analysis. Anthony Roe invited me to think up a suitable syllabus.

Our aim was to provide a rapid and concentrated conversion course for recently hired postdoctoral research chemists in the pharmaceutical industry; in the main these were organic chemists who needed to know what was required to become a practicing medicinal chemist. My past experience had shown me that such courses usually dealt with diseases and their test models but I decided to avoid this approach. It seemed to me that the basic discipline for medicinal chemists was to understand structure–activity analysis and the interface with the other disciplines involved in drug discovery. Thus the core lectures would be on physicochemical properties (octanol/water partition, pK_a and hydrogen-bonding, conformational analysis), multiparameter correlation analysis and computation, bioassay, receptors and enzymes, drug disposition (DMPK, drug metabolism and pharmacokinetics) and the drug development process. We would also include several case histories of drug discovery (something which I had previously encountered in a Society for Drug Research symposium). Lecturers were mainly industrial, and the number of participants was limited to around 100 to foster a more intimate and informal atmosphere.

The first summer school of this type was mounted in 1981 and the result was a resounding success. It has since been repeated every other year, and is always oversubscribed, with a healthy participation of delegates from continental Europe. It has received further accolade by providing the model for the annual course put on in the USA since 1987 at Drew University, Madison, NJ. It has also been publicised within the International Union of Pure and Applied Chemistry (IUPAC).[27]

8.03.2.7 Research Post-Cimetidine

Our aims at SK&F after cimetidine had been to make improved compounds for inhibiting gastric acid secretion. I was appointed Director of Histamine Research and the aims were broadened to explore other potential therapeutic applications of histamine H_2 receptor ligands, as in inflammation, the central nervous system, and immunology.

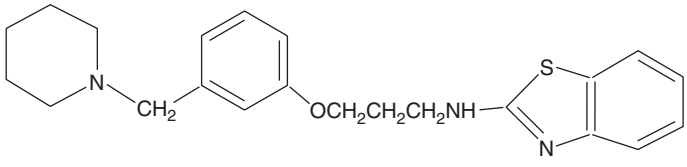
The vasculature contains both H_1 and H_2 receptors and it is important to block both simultaneously if the aim is to suppress the histamine-induced inflammation (vasodilatation and edema) in the skin. To this end we set out to combine H_1 and H_2 antagonist properties in the same ligand; by using our discovery of pyridine analogs of imidazole and cyclized

versions of acylguanidines which gave 2-amino-4-pyrimidones (isocytosines), we were able to use appropriate substituents in the pyridine and isocytosine rings to provide a compound, icotidine (**7**), which was equipotent as an antagonist at both H_1 and H_2 receptors.[28] Unfortunately it ran into problems during safety studies in animals and was discontinued.

An unexpected finding with icotidine, however, was that it had no propensity to enter the brain. This gave us an opportunity to use molecular modification to engineer out the H_2 antagonist property and increase the H_1 receptor potency. Again, this depended on the appropriate use of substituents; temelastine (**8**) went into development as an H_1 antihistamine that did not penetrate into the brain and did not cause the usual drowsiness associated with conventional antihistamines.[29] Although the compound was very effective in rats and guinea-pigs it was, unfortunately, too short-lived for human studies and was abandoned.[30] Temelastine has a very unusual structure for an antihistamine since it is not a basic tertiary amine and the main part of its structure is very similar to those of some H_2 receptor antagonists. Although the H_1 and H_2 receptors have only 40% amino acid sequence homology[31] for the transmembrane domains, it is fascinating to find potent antagonists that are so similar in chemical structure.

			pA_2 H_1	pA_2 H_2
7	Icotidine	R^3=OCH_3 R^5=H	7.8	7.5
8	Temelastine	R^3=CH_3 R^5=Br	9.55	c. 5.9

Almost all of the potent H_2 receptor antagonists are very polar molecules which do not penetrate into the brain. Since there are H_2 receptors in the brain, we decided to seek an H_2 receptor antagonist that would penetrate. This led us to study a series of compounds by radiolabeling them and determining brain penetration The compounds were carefully selected and it became apparent that having a high octanol/water partition value (log *P*) was no guarantee of brain penetration. Even H_2 antagonist compounds with log $P > 4$ did not penetrate. This was surprising and ran counter to the wisdom which Corwin Hansch had propounded.[32,33] My colleagues Drs RC Mitchell and RC Young were able to show that hydrogen-bonding was critical in reducing brain penetration and they obtained a very interesting inverse correlation between the difference between octanol/water and cyclohexane/water partitions and brain penetration – the so-called Δ log *P* hypothesis. Using this hypothesis we were able to design zolantidine (**9**), where we had reduced the donor hydrogen-bonding capability (small Δ log *P*) so that the compound became a very effective brain-penetrating H_2 receptor antagonist.[34] Sadly, this interesting compound could not be pursued into human studies because of the management decision to abandon this project.



9 Zolantidine

The results from immunological studies of possible involvement of H_2 receptors were rather difficult to pin down. We now know that there are both H_2 and H_4 receptor components involved in the immunological actions of histamine, for example in interleukin-16 release from human T lymphocytes,[35] and it is probable that the effects of the H_4 receptor dominate.

About this time it was my feeling that management pressure was too intense and so I followed up an offer that I had received from Sir James Lighthill, the Provost of University College London (UCL), to take a Chair of Medicinal Chemistry in the Chemistry Department. There was not an adequate financial backing for this position and subsequent negotiations led to the establishment of the SK&F Chair of Medicinal Chemistry. I took this up in 1986. This turned out to be a remarkable year for me personally, since I had been awarded the DSc degree from London University for my studies on the medicinal chemistry of histamine, and had also been admitted as a Fellow of the Royal Society (the UK National Academy of Science).

8.03.3 University College London

8.03.3.1 University College London Medicinal Chemistry

It is not that usual to go from industry to academia and I joined UCL at a difficult time since there was considerable financial pressure on the universities in general. The pressures on the Chemistry Department had led to its contraction from 35 academic (faculty) staff to 21. The Department no longer funded research and the majority of grant applications to the UK government-funded Science Councils were refused for lack of funds, even though the Council may have deemed them worthy of support. If technical staff left the Department they were not replaced. The infrastructure of the building was also slowly deteriorating. This was the result of Prime Minister Margaret Thatcher's policy and the consequential decline in the support for physical sciences in British universities. Yet the UCL Chemistry Department increased the number of students and the number of publications, although at a cost to the academic staff: they had to spend more time teaching and as a result they tended to do 'safe' research. Furthermore their pay was continuously eroded as it did not keep up with increases in the cost of living.

The Department was unusual in that it provided an undergraduate education in medicinal chemistry, which had been initiated by Professor James Black (when Head of the UCL Pharmacology Department), together with Professor Charles Vernon, a biological chemist in the Chemistry Department. It sacrificed much of the usual inorganic chemistry teaching from the BSc Chemistry degree and replaced it by units (or half-units) in physiology, pharmacology, and biochemistry during the 3-year period. In the final year, the students attended a half-unit on the principles of drug design. I injected a healthy amount of the physical-organic basis for structure–activity analysis into this course and also invited industrial speakers to give case histories of drug discovery (leading to about 30–35 lectures). The students also did a 3-month practical research project for which they wrote a thesis and sat an oral examination.

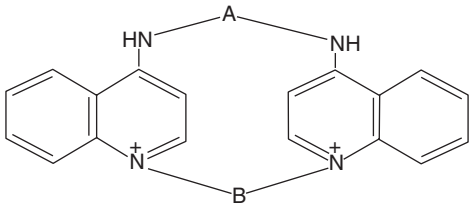
My appointment to a Chair in Medicinal Chemistry was meant to give a bigger research presence to the subject. Yet I arrived with no research funding and no equipment or glassware. I also knew that the Science Research Council was not interested in funding medicinal chemistry, first because one did not develop novel organic synthesis methodology and second, because the Council took the view that this is what the pharmaceutical industry did. I also faced another problem. It is usual that an academic is appointed to a Chair after having produced a good volume of interesting research and having generated an accelerating research momentum, which will be expanded on taking up the professorship. In my case I had to leave all my research behind to SK&F and start again from scratch. I decided not to choose projects that might be in competition with work that was conducted in the pharmaceutical industry. My approach was to collaborate with biologists to help them solve a biological problem by providing appropriate compounds; my hope was that this might lead to new areas for drug discovery by producing useful chemical substances as tools which could ultimately give prototype drugs. In essence this was ligand design for new areas of biology. Critically important in an academic setting was whether the project could be funded.

The work at UCL encompassed a wide range of biological applications, from G protein-coupled receptors (for histamine and serotonin), enzyme inhibitors for cholecystokinin-inactivating peptidase and human immunodeficiency virus (HIV)-aspartyl peptidase, potassium ion channels, through to phosphatidyl inositol transfer protein, transport P, and persistent sunscreens. This involved collaborations with various biologists but two, in particular, stood out for their excellent science and manner of working with chemists.

8.03.3.2 Potassium Ion Channels

At UCL, Professor DH Jenkinson in the Pharmacology Department had strong interests in calcium-activated potassium ion channels. We were fortunate to obtain a 5-year grant from the Wellcome Trust to fund both medicinal chemistry and pharmacology (electrophysiology). In chemistry this provided the seed that we were able to grow by accommodating project students and academic visitors over many years.

The small-conductance Ca^{2+}-activated K^+ channel (SK_{Ca}) is found in many cell types and was originally defined electrophysiologically using a natural peptide toxin apamin. To find a simpler molecule, the drug dequalinium was taken as a μmol L^{-1} lead. Since dequalinium is a 4-aminoquinoline, the influence of the amino group was investigated in a small series of substituted analogs and an excellent correlation was obtained between blocking potency and the σ_R substituent constant.[36] This was extended to a much larger series in which activity was correlated with the energy of the Lowest Unoccupied Molecular Orbital (LUMO).[37] The effects of conformational restriction in the linking chain were also investigated[38] and then the dequalinium analogs were cyclized to give tetra-aza-cyclophanes that were particularly interesting. Thus, UCL 1530 (**10**) provided the first evidence[39] for pharmacological differentiation between the SK_{Ca} channels in liver and neuronal cells, while UCL 1684 (**11**) was the first[40] nonpeptidic nanomolar inhibitor ($IC_{50} = 3$ nmol L^{-1}), and this was followed[41] by UCL 1848 (**12**).



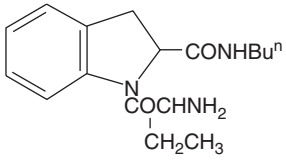
10	UCL 1530	A= $-(CH_2)_{10}$	A= $-(CH_2)_{10}$
11	UCL 1684	A= $-CH_2-C_6H_4-CH_2-$	A= $-(CH_2)_{10}$
12	UCL 1848	A= $-(CH_2)_{10}$	A= $-(CH_2)_{10}$

8.03.3.3 Cholecystokinin

My other highly productive collaboration was with Professor Jean-Charles Schwartz, Director of an Institut National de la Santé et de la Recherche Médicale (INSERM) Unit in Paris, France. I had previously had a strong collaboration with him on histamine research when I was at SK&F. We were very lucky to share a large grant from the Upjohn Company (Kalamazoo, MI, US) for a proposal to discover an inhibitor of the peptidase that inactivates the octapeptide neurotransmitter, cholecystokinin-8 (CCK-8). It was hypothesized that such a compound would prolong the natural lifetime of CCK-8 and promote a feeling of satiety, thereby reducing food intake in a natural way.

The enzyme had not been fully purified but its activity was isolated from rat brain in Schwartz's laboratory and characterized as a serine proteinase. Compounds were to be synthesized at UCL and tested at INSERM. Our approach was to avoid incorporating a serine-reactive group (for a transition state or irreversible inhibitor) but to seek a reversible inhibitor since this should be more likely to be selective and nontoxic. To do this we used molecular probes to seek noncovalent molecular interactions with the enzyme active site; the aim was to achieve closely matched stereospecific interactions between the enzyme and the putative inhibitor. The strategy was first to characterize the binding opportunities of the enzyme subsites using a series of systematically varied dipeptides and tripeptides by screening commercially available compounds supplemented by some which we synthesized.[42] Peptides were selected from those with alkyl or aryl side chains to determine the accessible volume for binding and to probe the potential for hydrophobic interactions. Dipeptides were also derivatized at the NH_2 or CO_2H termini.

From the above work there emerged a submicromolar dipeptide amide (P_1P_2NHR) as a lead. The side chains of the amino acids (in $P_1P_2NH_2$) were then optimized with respect to activity by synthesizing and testing analogs as primary amides in which the two amino acids were systematically varied to afford Abu.Pro.NHR (R = H), then R was optimized. Fusion of a benzene ring to Pro gave an indoline derivative, butabindide (**13**), a prototype drug which is a selective competitive reversible nanomolar inhibitor ($K_i = 7\,\text{nmol}\,L^{-1}$) that does not contain a serine-reactive group.[43] This compound was shown to be active in potentiating the action of CCK-8 and to reduce food intake (as a result of the satiating effect of CCK-8) in starved mice. Analogs of butabindide then yielded potent subnanomolar inhibitors.



13 Butabindide

As a result of the above work, the identity of the proteinase that inactivates CCK-8 was shown to be tripeptidylpeptidase II (TPP II), a known enzyme of previously unknown function. Thus, searching for the noncovalent interactions around the enzyme active site and exploiting hydrophobic effects led to a potent, reversible

competitive and selective peptidase inhibitor, the first known inhibitor of TPP II. This strategy has the potential to provide a general approach to the design of peptidase inhibitors provided that the enzyme possesses an accessible lipophilic pocket, even though the structure of the enzyme may be unknown.

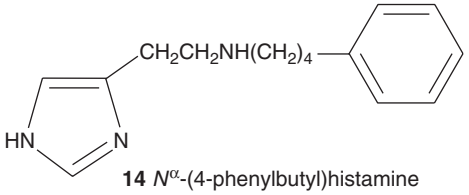
8.03.3.4 Histamine H_3 Receptors

I also collaborated with Professor Schwartz and his colleagues on designing ligands for the histamine H_3 receptor. For many years all the known appropriately active ligands were imidazole derivatives. For several reasons a nonimidazole was preferred but all attempts to replace imidazole by other heterocycles led only to inactive or weakly active compounds. We therefore went back to first principles and applied thoughts that had been proposed by EJ Ariens[44,45] in the 1960s.

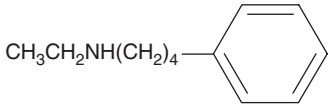
It is possible to convert an agonist into an antagonist by introducing additional groups into the molecule which can locate binding sites in the vicinity of the receptor. Whether the resulting molecule will be a partial agonist or a pure antagonist probably depends on whether the agonist moieties continue to engage the receptor in the critical manner required to elicit a receptor response. If they do not, then the molecule will be an antagonist and one may question whether the agonist moieties actually make any useful contribution to the affinity. If the additional groups are correctly positioned and interact appropriately with the receptor, the resultant molecule should achieve a considerable increase in affinity.

For histamine, the thought arose that it might be possible to convert histamine into an antagonist by the addition of appropriate groups, and then to remove the imidazole ring to yield a nonimidazole antagonist molecule. It therefore seemed to be worthwhile applying this analysis to the interaction of histamine at the H_3 receptor. The difficulty of the approach resides in finding out what may be appropriate groups to incorporate into the histamine molecule and in which positions they should be introduced to achieve a sufficient increase in affinity.

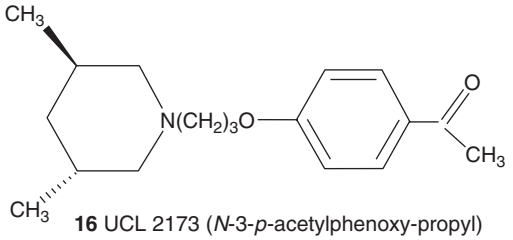
Of various attempts made, the one that appeared to hold promise was the finding that N^{α}-(4-phenylbutyl)histamine (**14**) was a pure antagonist of histamine at the H_3 receptor with a $K_i = 0.63\,\mu mol\,L^{-1}$. Removal of the imidazole ring from this structure led to the synthesis and testing of *N*-ethyl-*N*-(4-phenylbutyl)amine (**15**) which, remarkably, was found to have a $K_i = 1.3\,\mu mol\,L^{-1}$ as an H_3 receptor histamine antagonist. The removal of the imidazole ring had led merely to a twofold drop in affinity and had successfully produced the necessary lead to generate a nonimidazole H_3 receptor histamine antagonist. Inserting an O or S atom in the chain at the position α to the phenyl ring simplified the synthesis for a structure–activity exploration.[46] Investigating the effect of substituents in the phenyl ring, and altering the chain length and the type of amino group led to the very potent antagonist,[47,48] UCL 2173, *N*-(3-*p*-acetylphenoxy-propyl)-*trans*-3,5-dimethyl-piperidine (**16**), $K_i = 1.8\,nmol\,L^{-1}$, $ED_{50} = 0.12\,mg\,kg^{-1}$, which, in vivo, is considerably more potent than the reference drug, thioperamide. These discoveries were made before the availability of the human recombinant receptor. However, following the cloning[49] of the human H_3 receptor cDNA in 1999, many pharmaceutical companies set up high-throughput screens to seek other nonimidazole H_3 receptor antagonists and several such compounds have since entered the drug development process.[48]



14 N^{α}-(4-phenylbutyl)histamine



15 *N*-ethyl-N-(4-phenylbutyl)amine



16 UCL 2173 (*N*-3-*p*-acetylphenoxy-propyl) -*trans*-3, 5-dimethyl-piperidine)

8.03.4 Conclusion

This has been a personal account focusing on the approaches I have taken in attempting to discover new potential drugs as medicines. The success rate has been extraordinarily low, but at the very least, one has tried to construct molecules having specific biological properties that may serve as tools to help unravel physiological mechanisms. The key to success has been to collaborate with outstanding biologists and outstanding chemists.

Along the way, one has also aimed at helping to develop the scientific discipline of medicinal chemistry and to inspire others also to enjoy research. I cannot help wondering, though, on how things would have turned out if James Black had not come to SK&F and if I had not been involved in the discovery of cimetidine. Would I have found other opportunities for new drug design, or would I have retreated from medicinal chemistry and gone back to researching problems in organic chemistry?

Research has been very stimulating and it has sometimes been very exciting; it has been taxing and occasionally it has generated strong emotions. Of one thing I am sure: I have been one of the fortunate few who has been paid to work on a hobby. The real bonus has been to be involved in a discovery (cimetidine) that helped millions of people fight their disease.

Would it have been like that now, or could it be like that in the future? Nowadays there seems to be so much pressure on chemists in the pharmaceutical industry. They appear to be ruled by technologies such as high-throughput screening for lead generation, parallel synthesis to develop their structure–activity relationship database, and ready-made computer programs to assist structure–activity analysis. Will they take time to stand back and think as scientists, or will they be regarded as technicians carrying out instructions? Medicinal chemists will have to be very careful in the future not to let the technologies dominate them.

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Biography

C Robin Ganellin studied chemistry at Queen Mary College, London University (BSc and PhD) and in 1960 was a Research Associate with Prof A C Cope, at the Massachusetts Institute of Technology (MIT). Then he joined SmithKline & French Laboratories (SK&F) in the UK, and from 1966 collaborated with Sir James Black, to lead the chemical research for the discovery of the H_2-receptor histamine antagonists. He is coinventor of the drug cimetidine (Tagamet) which revolutionised the treatment of peptic ulcer disease. He became Director of Histamine Research and, subsequently, Vice-President for Research, at SK&F Welwyn, UK. In 1986 he was elected as a Fellow of the Royal Society and appointed to the SK&F Chair of Medicinal Chemistry at University College London, where he is now Emeritus Professor. He is an author of some 250 scientific publications and named coinventor on over 160 US patents. He has received various awards for medicinal chemistry. He is a past Chairman of the UK Society for Drug Research (1985–87). He was a member of the IUPHAR Committee for Receptor Nomenclature and Drug Classification (1990–98), was President of the Medicinal Chemistry Section of IUPAC (1999–2001), and is currently Chairman of the IUPAC Subcommittee on Medicinal Chemistry and Drug Development.

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8.04 Some Aspects of Medicinal Chemistry at the Schering-Plough Research Institute

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8.04.1 Introduction

Since the discovery of penicillins during the Second World War, pharmaceutical companies around the world have made spectacular contributions toward curing many diseases. In the area of infectious diseases, the introduction of newer antibiotics has saved many lives, although challenges lie ahead because of the emergence of bacterial resistance to these antibiotics. Similarly, the use of protease inhibitors has helped millions of AIDS patients around the globe, and in this area also there is ongoing research to discover better drugs. A few CCR5 antagonists are in clinical trials, and they are expected to inhibit viral entry into the cells. Hepatitis C infection is being treated with interferon and ribavarin. Interferon was the first biotechnology-derived product to be introduced in human medicine. The work in biotechnology started in earnest in the 1980s, with some in the industry not completely convinced of its importance. Today, biotechnology-based companies are making major contributions to medicine, in areas as diverse as growth factors, arthritis, diabetes, and vaccines.

The introduction of enzyme inhibitors such as angiotensin-converting enzyme (ACE) inhibitors and statins has revolutionized the management of cardiovascular diseases, and the general concept of drug discovery using enzyme inhibitions has been used for drugs in other disease conditions.

Receptor antagonists, for example H_1 antagonists, have been in use in the clinic for a long period of time, and, more recently, using the knowledge of G protein-coupled receptors, several new drugs have reached the clinic, and many more exciting drugs in this category are in development.

Besides infectious and cardiovascular diseases, cancer is one of the major causes of death in the world. Cytotoxic agents, including paclitaxel and temzolomide, have been in use for a number of years, and the longevity of cancer patients has greatly improved. Recently, understanding how kinases work intracellularly, several pharmaceutical companies have discovered novel anticancer agents. Imatinib is already in the clinic, and others are in various stages of development.

No attempt has been made here to present a catalog of drugs synthesized; instead, an attempt has been made to very briefly capture the trend in drug discovery.

Several new technologies have been introduced in the drug discovery process since the start of the 1990s. Amongst these, combinatorial synthesis, genomics, proteomics, and high-throughput assays must be highlighted. Many articles and reviews have been written on these subjects. It is our expectation that, with time, one would learn how to use these technologies to the fullest extent, which is already happening in the pharmaceutical industry, and, as a result, many new drugs involving these new technologies are expected to reach clinical trials soon. There has been a lot of unreal expectation that the use of these technologies would shorten the time required to discover drugs, and, therefore, there has been disappointment in some corners.

With the advent of biotechnology, the cloning and purification of receptors and enzymes has almost become a routine in the industry. Biologist are able today to establish an in vitro assay in a short period of time, and, with the help of high-throughput assays, an active lead is generally – but not always – found in the collection of compounds in the corporate libraries. It is not an uncommon experience in the industry to find a lead structure from their past collections, because the compounds in the file were analogs of biologically active compounds. It has therefore become very important to have good-quality compounds in the library, and, here, chemists have a challenge and responsibility to cleverly use combinatorial chemistry and synthetic organic chemistry to achieve this goal.

In recent years, the number of structures of proteins solved by x-ray analysis has vastly increased. In some instances this information has greatly helped in the drug discovery process. In an ideal world, the x-ray crystal structure of a protein of interest bound to a ligand would be available prior to chemists starting their work. This situation, however, is rarely the case, and certainly nonexistent in receptor-based drug design.

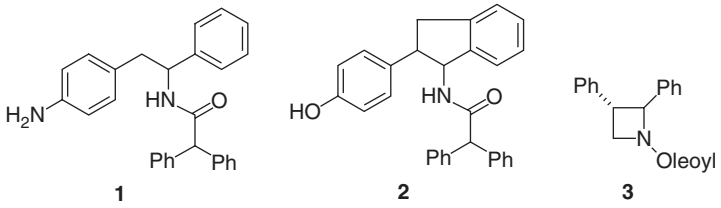
This perspective article is based on my Hershberg award lecture (American Chemical Society, 2003) and also on several reviews and articles written by my colleagues. I have attempted to capture different journeys taken toward the discovery of ezetimibe (Zetia), posaconazole (Noxafil), and lonafarnib (Sarasar). A common theme among these drugs is that they are all used in curing diseases where previously there was an unmet need for a pharmaceutical. The work on ezetimibe began with finding an acyl coenzyme A-cholesterol *O*-acyltransferase (ACAT) inhibitor, and ended with the discovery of a compound with potent activity in inhibiting the absorption of cholesterol. The mechanism of its action, which is different to ACAT inhibition, was discovered after the drug was approved by the US Food and Drugs Administration (FDA) for its use in the clinic. Vytorin, a combination of ezetimibe and simvastatin, jointly developed by Schering-Plough and Merck, has also been approved by FDA. Posaconazole is a novel azole antifungal that has demonstrated broad-spectrum activity in the clinic, particularly against *Aspergillus*, infection which is difficult to cure with existing drugs. *Aspergillus* infection is common amongst AIDS and cancer patients. It is hoped that posaconazole will soon be approved by the FDA for use in humans. In the case of lonafarnib, the discovery of an anticancer agent was based on the inhibition of farnesyl protein transferase (FPT), an important enzyme involved in the posttranslational modification of Ras that is required for the protein to attach to the cell membrane. Mutation of Ras has been found in a significant number of human tumors. During our work, the x-ray crystal structure of FPT bound to an initial lead compound was available, which allowed us to rationally design and synthesize lonafarnib. Lonafarnib is presently undergoing clinical trial against several cancer targets.

8.04.2 The Discovery of Ezetimibe

Extensive clinical trials and epidemiological studies have unequivocally established the importance of lowering the low-density lipoprotein (LDL) level in the treatment and prevention of coronary heart disease. Lowering of the LDL level has been achieved in humans using 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, an

enzyme responsible for the biosynthesis of cholesterol in the liver. Several extremely potent HMG-CoA reductase inhibitors (statins), including lovastatin, simvastatin, and atorvastatin, have been extensively used in the clinic with much success. Bile acid sequestrate inhibitors, including resins such as colestipol and cholesteryl amine, have also been used, with limited success. There are two sources of the cholesterol in our body: approximately 70% is from biosynthesis in the liver, and the remaining 30% comes from the food that we consume. Intensive effort led to the discovery of ezetimibe[1] at the Schering-Plough Research Institute (SPRI) as a potent inhibitor of cholesterol absorption. It has been approved by the FDA as a monotherapy for lowering the serum cholesterol level. In addition, Vytorin, a combination of ezetimibe and simvastatin, has also been approved recently as a potent agent for lowering serum cholesterol. Vytorin was developed jointly by the Schering-Plough Corporation and the Merck Corporation.

Before our work began in this area it was known[2] that ACAT was involved in cholesterol trafficking in hamsters; however, its relevance in nonrodents was unclear. In the hamster model,[3] when the animals were fed with on a high-cholesterol diet they showed a significant increase in cholesterol ester in their liver without much change in their serum cholesterol level. Among the initial compounds synthesized, **1** and **2** showed in vitro ACAT inhibition, and also showed in vivo activity in the hamster model by lowering the cholesterol ester level in the liver without changes in the serum cholesterol level. As the enzyme inhibitory activity of **2** was considerably superior to **1**, it was decided to prepare further conformationally constrained azetidine analogs, represented by structure **3**.



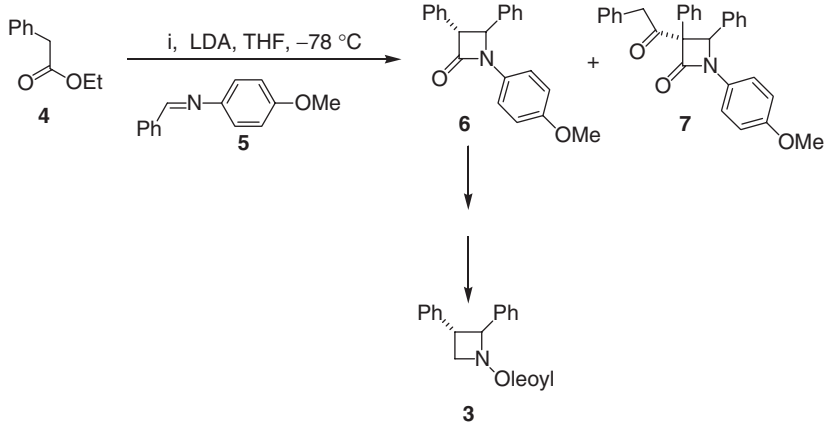
8.04.2.1 The Synthesis and Biological Activities of Initial Leads

The ester enolate derived from **4** was condensed with the Schiff base **5**, to yield a mixture of **6** and **7** (Scheme 1). Compound **6** was treated with ceric ammonium nitrate, and the reaction product was reduced and then acylated, to yield **3**.[4,5]

Several azetidinones were synthesized, and their activities determined. Compounds represented by structure **7**, when administered orally in the hamster model, showed modest serum cholesterol lowering activity, even though the ACAT activities of these analogs were no better than, for example, **1** and **2**. The activities of **1**, **2**, **3**, **6**, and **7** are presented in Table 1.

8.04.2.2 The Discovery of SCH 48461

After synthesizing several analogs of **7**, it became clear that there was no correlation between ACAT activity and the ability of these analogs to lower the serum cholesterol level when they were administered orally in the hamster. At this



Scheme 1 Reprinted with permission from Clader, J. W. *J. Med. Chem.* **2004**, *47*, 2. Copyright (2004) American Chemical Society.

Table 1 Activities of azetidinones **1**–**3**, **6**, and **7**

Parameter	*Compound*				
	1	*2*	*3*	*6*	*7*
ACAT IC_{50}	900 nM	40 nM	4% inhibition at 25 μM	64% inhibition at 25 μM	4% inhibition at 25 μM
Hamster (100 mg kg^{-1}) serum cholesterol level	NE	NE	NE	NE	− 10%
Cholesterol ester	− 80%	− 88%	NE	NE	− 26%

NE, not effective.
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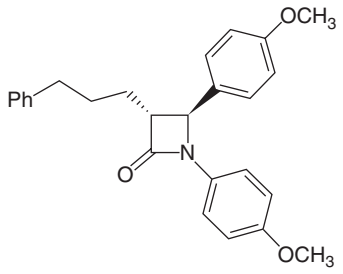


SCH 48461 **8**

ACAT: IC_{50}: 26 μM

Cholestrol-fed animal models, ED_{50}:

Hamster:	2.2 mg kg^{-1}
Rat:	2.0 mg kg^{-1}
Monkey:	0.2 mg kg^{-1}
Dog:	0.1 mg kg^{-1}

Figure 1 SCH 48461: structure, ACAT inhibition and ED_{50} values. (Reprinted with permission from Clader, J. W. *J. Med. Chem*. **2004**, *47*, 5. Copyright (2004) American Chemical Society.)

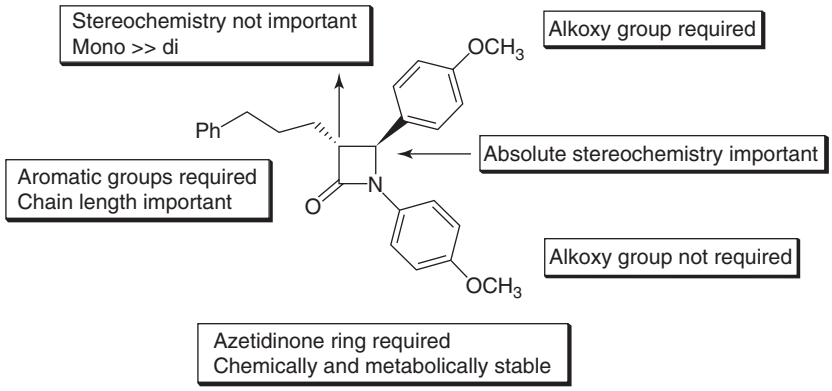


Figure 2 Structure–activity relationship of SCH 48461. (Reprinted with permission from Clader, J. W. *J. Med. Chem*. **2004**, *47*, 7. Copyright (2004) American Chemical Society.)

point it was decided that the structure–activity relationships in azetidinones needed to be established following in vivo results, which was challenging for both chemists and biologists. Extensive work in this area led to the discovery of SCH 48461 (**8**) as the most potent inhibitor of cholesterol absorption.[6] The activity of SCH 48461 is summarized in Figure 1, and a summary of the structure–activity relationship in this series is presented in Figure 2.

8.04.2.3 The In Vivo Activity of SCH 48461

The effect of SCH 48461 in cholesterol-fed rhesus monkeys is summarized, along with control animals, in Figure 3. The total serum cholesterol level in the control animals steadily increased over a period of 3 weeks compared with the monkeys dosed with SCH 48461 at 1 mg kg^{-1} over the course of the same period. The serum cholesterol levels did not show any significant change in the SCH 48461 group, and remained at the baseline. At the end of 3 weeks the control

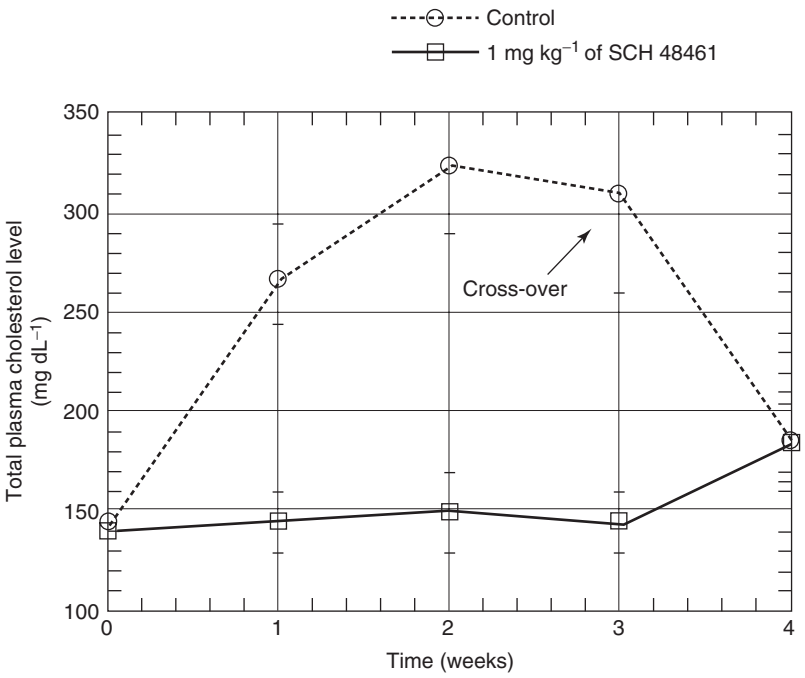
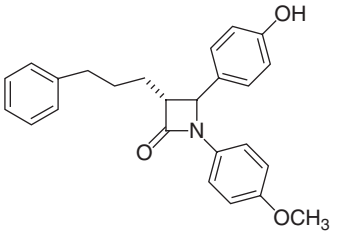


Figure 3 Effect of SCH 48461 in cholesterol-fed rhesus monkeys versus controls. (Reprinted with permission from Clader, J. W. *J. Med. Chem*. **2004**, *47*, 5. Copyright (2004) American Chemical Society.)

animals were administered SCH 48461 at 1 mg kg^{-1}, and it was observed that their cholesterol levels returned back to the baseline in a short period of time. The withdrawal of SCH 48461 from the second group of monkeys resulted in the rise of their serum cholesterol levels, again in a very short period of time. These results unequivocally established that SCH 48461 is a potent inhibitor of cholesterol absorption in various species of animals, and, based on its lack of ACAT inhibitory activity, it was obvious that SCH 48461 inhibited cholesterol absorption by an unknown mechanism. We shall return to this point later on in this chapter.

Although the above results were very promising, SCH 48461 however underwent extensive metabolism in vivo, thus it became important to identify the structures of the major metabolites, and then incorporate the information toward the design and synthesis of future analogs. Biologists at the SPRI established,[7] in a cleverly designed experiment using an intestinally cannulated bile duct diverted rat model, that one of the major active metabolites of SCH 48461 was the glucoronide of compound **9**.



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8.04.2.4 The Design of Ezetimibe

Based on the activity of **9**, the phenols **10**, **11**, **12**, **13**, **14**, and **15** were synthesized, and their in vivo activities determined. The results are summarized in Figure 4.

Based on the structure–activity relationship as described in Figure 2 and also on the biological activities of the possible metabolites as described in Figure 4, several new analogs were synthesized. Ezetimibe was found to be the most potent among all the analogs synthesized in inhibiting the absorption of cholesterol. The structure of ezetimibe[8] and a summary of its design is presented in Figure 5.

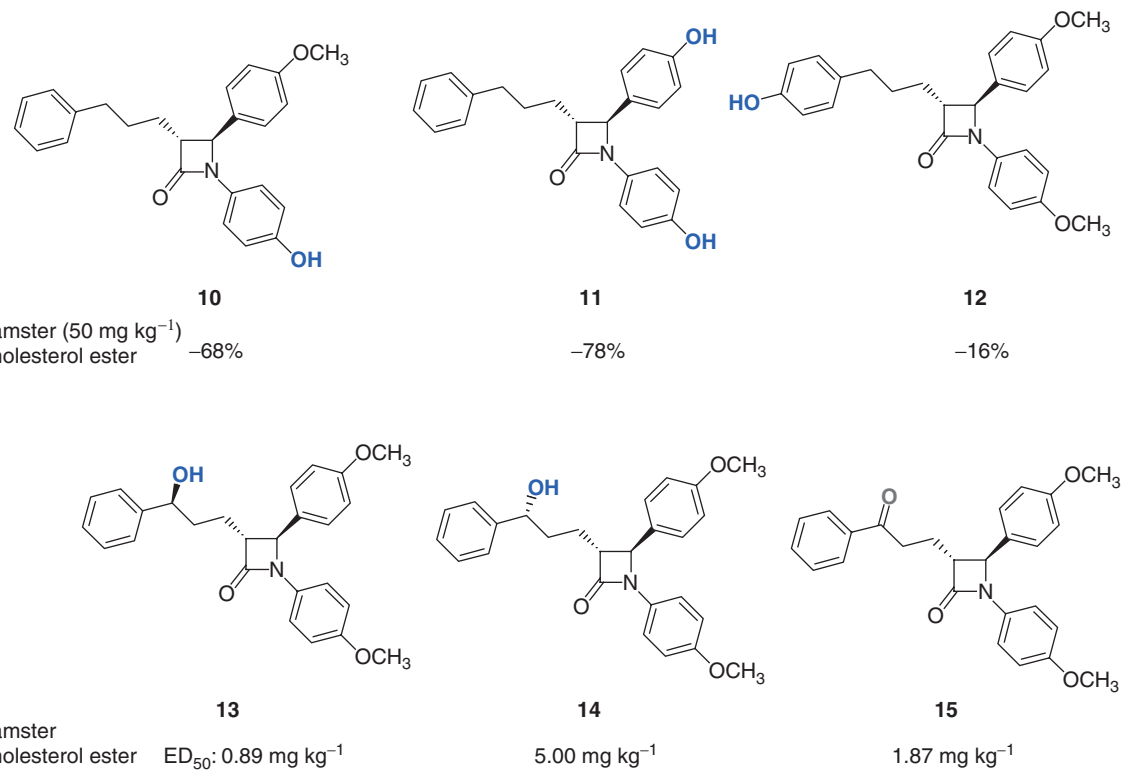


Figure 4 In vivo activities (in the hamster) of phenols **10**–**15**. (Reprinted with permission from Clader, J. W. *J. Med. Chem.* **2004**, *47*, 7. Copyright (2004) American Chemical Society.)

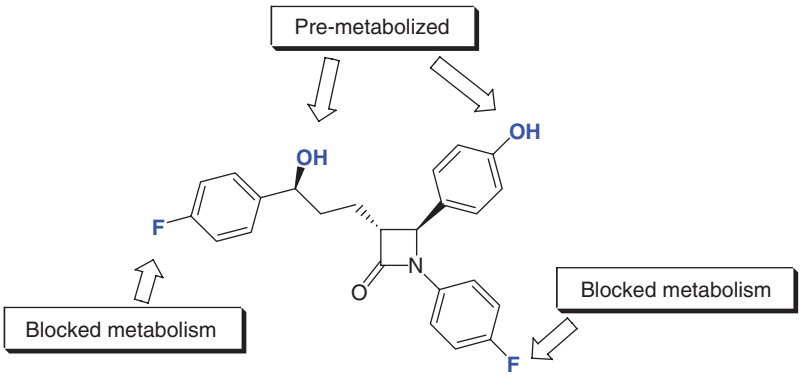


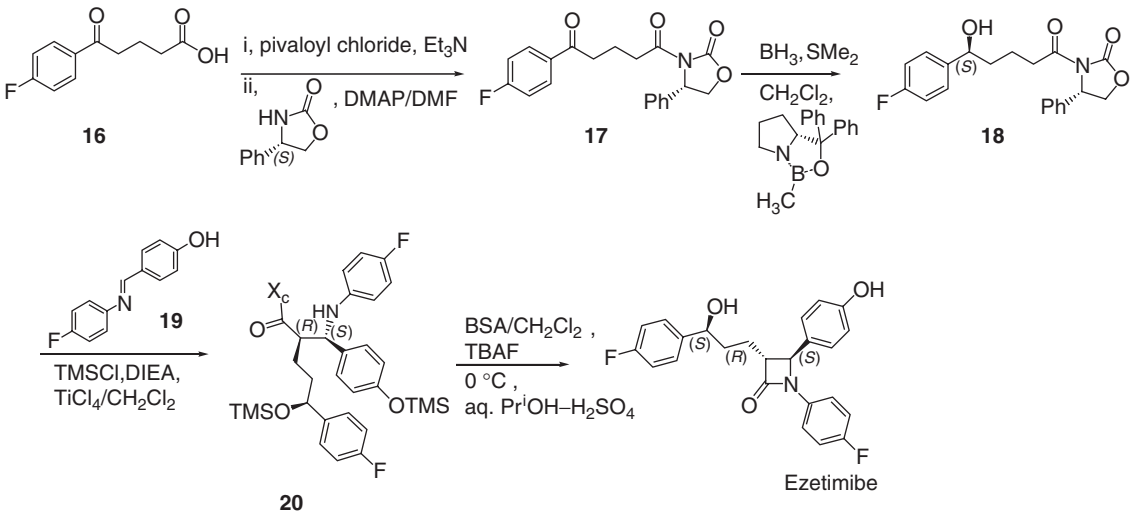
Figure 5 Structure–activity relationship of ezetimibe. (Reprinted with permission from Clader, J. W. *J. Med. Chem.* **2004**, *47*, 7. Copyright (2004) American Chemical Society.)

8.04.2.5 The Synthesis of Ezetimibe[9]

The keto acid **16** was converted to **17**, and then reduced to **18** using Corey's oxazaborolidine catalyst (Scheme 2). The titanium enolate of the silylated derivative of **18** was condensed with the silylated phenolic Schiff base **19**, to yield **20**. The basic nitrogen atom of **20** was silylated in situ, and then treated with tetrabutyl ammonium fluoride, to form the azetidinone ring. Final deprotection of the silyl groups with acid yielded ezetimibe.

8.04.2.6 The Biological Activity of Ezetimibe

The cholesterol absorption inhibitory property of ezetimibe was compared with SCH 48461, and the results are summarized in Table 2. In every species studied, ezetimibe showed dramatic improvement in activity when compared with SCH 48461, and, in monkeys, ezetimibe showed the greatest activity.



Scheme 2 Reprinted with permission from Clader, J. W. *J. Med. Chem*. **2004**, *47*, 8. Copyright (2004) American Chemical Society.

Table 2 Cholesterol absorption inhibition of ezetimibe and SCH 48461[a]

Species	*ED_{50} (mg kg^{-1})*	
	SCH 48461	*Ezetimibe*
Hamster	2.2	0.04
Rat	2.0	0.03
Monkey	0.2	0.0005
Dog	0.1	0.007

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[a] Blood levels significantly lower for ezetimibe.

In parallel, we studied the synergistic effect of ezetimibe, along with a statin for lowering cholesterol levels. Thus, ezetimibe (0.007 mg kg^{-1}) and lovastatin (5 mg kg^{-1}) were administered orally to two different sets of chow-fed dogs for 14 days. Neither ezetimibe nor lovastatin showed significant activity; however, the combination showed a dramatic reduction in serum cholesterol levels (**Figure 6**). Based on all these results, ezetimibe has progressed to the clinic as a monotherapy agent and also in combination with simvastatin. The combination drug, named Vytorin, was jointly developed by Schering-Plough and Merck.

8.04.2.7 The Results of Clinical Trials of Ezetimibe

Based on all these observations, ezetimibe was advanced alone and also in combination with simvastatin and atorvastatin in the clinic, and the results are presented in **Figure 7**. In the clinic,[10–13] ezetimibe (10 mg) when administered alone reduced serum cholesterol levels by 18.5%, on average, and in combination with simvastatin (10 mg), serum cholesterol levels were reduced by 51.9%.

Based on safety studies and clinical response, ezetimibe has been approved for human use as a monotherapy, and Vytorin (ezetimibe plus simvastatin) has also been approved by the FDA for reducing serum cholesterol levels.

8.04.2.7.1 The mechanism of action of ezetimibe

Scientists at the SPRI have recently discovered that ezetimibe blocks the activity of the cholesterol transporter NPC1L1 that is expressed at the apical surface of enterocytes.[14] It is believed to be the transporter for dietary cholesterol absorption. As a further proof, it was demonstrated that in NPC1L1 knockout animals, ezetimibe was ineffective in preventing the absorption of cholesterol.

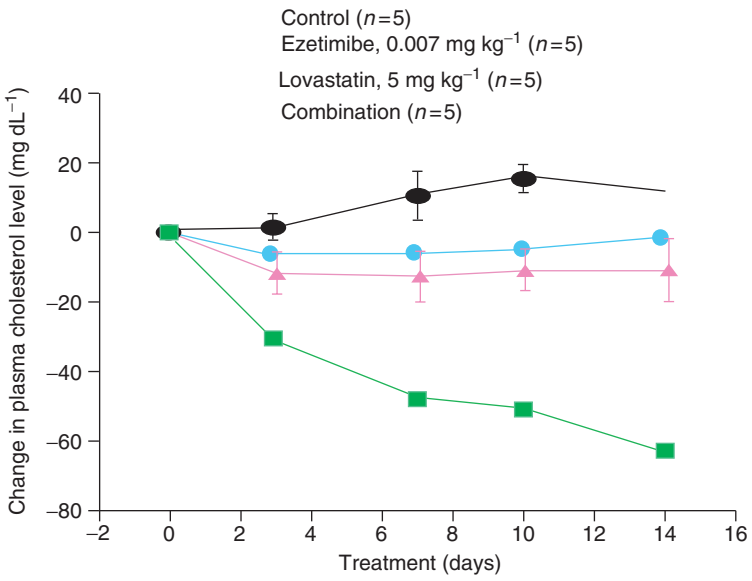


Figure 6 Effects of ezetimibe and lovastatin on cholesterol levels. (Reprinted with permission from Clader, J. W. *J. Med. Chem*. **2004**, *47*, 7. Copyright (2004) American Chemical Society.)

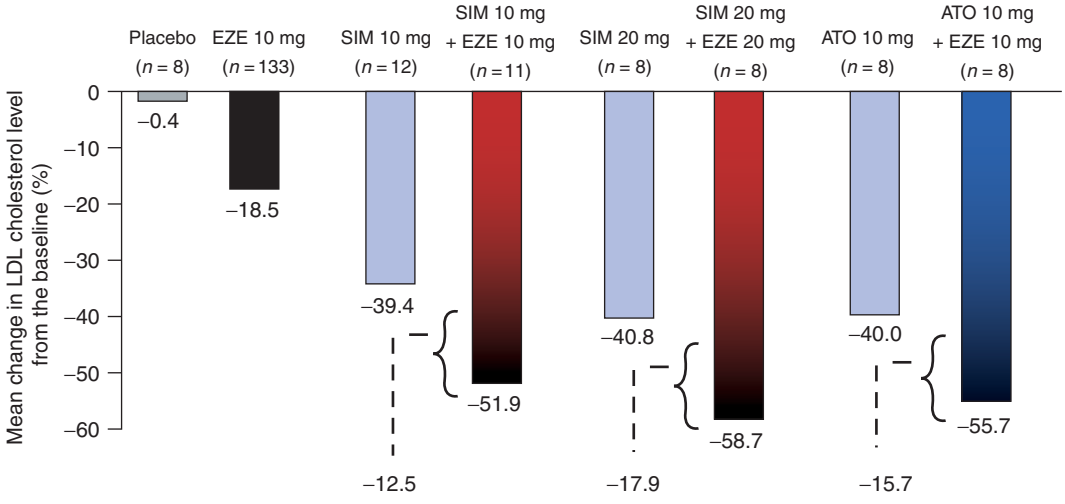
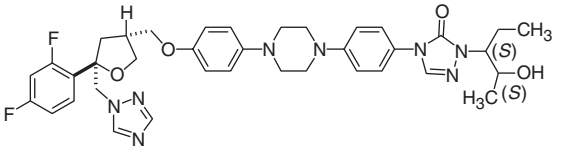


Figure 7 Results from a clinical trial of ezetimibe (EZE) alone and in combination with simvastatin (SIM) and atorvastatin (ATO) on cholesterol levels. (Reprinted with permission from Clader, J. W. *J. Med. Chem*. **2004**, *47*, 8. Copyright (2004) American Chemical Society.)

8.04.3 The Discovery of Posaconazole

Posaconazole[15] (**21**) is a novel triazole that has broad-spectrum antifungal activity against *Aspergillus* spp., *Cryptococcus* spp., *Histoplasma* spp., and a variety of other pathogens. In the clinic, posaconazole has been found to be well tolerated, with common side effects being gastrointestinal in origin. Life-threatening opportunistic fungal infections occur in AIDS patients, and also in patients undergoing chemotherapy for cancer, or those who have undergone organ transplants. The older antifungals do not work well with these patients, and the use of amphotericin B, a broad-spectrum antifungal, is limited in its use by its inherent toxicity. Posaconazole has been demonstrated in extensive clinical trials to be a potent orally active antifungal agent that works very well in the above-mentioned patient population.



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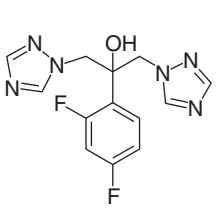
Based on the above observations, Schering-Plough has submitted a New Drug Application to the FDA, and is waiting for its approval to use posaconazole in the clinic.

It has been established for a long time that 'azoles'[16] (i.e., fluconazole[17] (**22**), ketoconazole (**23**), itraconazole (**24**), and saperconazole (**25**)) work as antifungals by inhibiting the biosynthesis of ergosterol using fungal cytochrome P450 enzyme lanosterol 14α-demethylase. Ergosterol is an essential component of the fungal cell membrane, and therefore inhibiting its synthesis will prevent the fungus growing. It is imperative for the above process to be selective over mammalian cytochrome P450, to avoid toxic side effects.



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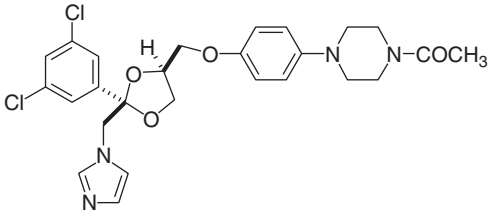
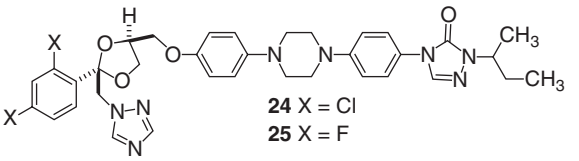


23

Fluconazole (**22**) is orally active, and is very widely used against *Candida* and *Cryptococcus* infections; however, it lacks activity against the important pathogen *Aspergillus*. Ketoconazole (**23**) was the first example of an orally active antifungal used in the clinic, but exhibits hepatotoxicity and also interferes with testosterone biosynthesis.

Itraconazole[18] (**24**) is an orally active broad-spectrum antifungal that shows activity against *Aspergillus*.

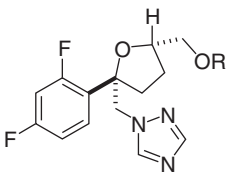


24 X = Cl
25 X = F

8.04.3.1 The Synthesis of Initial Leads

Our aim at the SPRI was to discover an orally active antifungal, comparable to fluconazole (**22**) in its activity against *Candida* and also having activity against fluconazole-resistant strains of *Candida glabrata* and *Candida krusei*. In addition, our compound should have activity against *Aspergillus*, and should be superior to itraconazole (**24**), be safe, and not be an inducer of human cytochrome P450 enzymes.

As has been pointed out already, it will be important to have an azole moiety in our new antifungal; however, it should not have the 1:3-dioxolane ring system present in the structures of **23**, **24**, and **25**, because it is expected that such a ring system might induce instability of the drug under the acidic conditions in the stomach. Thus, we wished to explore whether other oxygen-containing heterocycles represented by **26**, **27**, **28**, **29**, and **30** will possess activities of interest. We have synthesized[19] all these novel structures with appropriate R groups in the racemic form, and determined their activities, and the results are as follows: **26**, **27**, and **28** were more active than **23** and **24**, and, among these compounds, **27** was the most active. In this chapter we focus on the synthesis of this class of compounds that led to the discovery of posaconazole (**21**).



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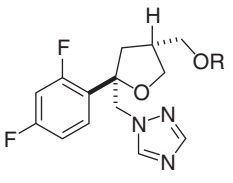

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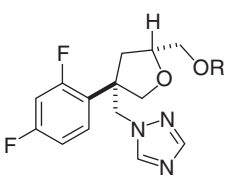

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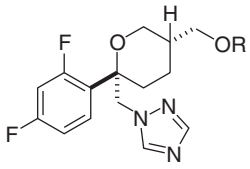

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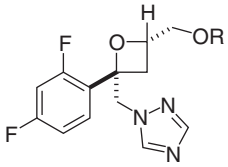
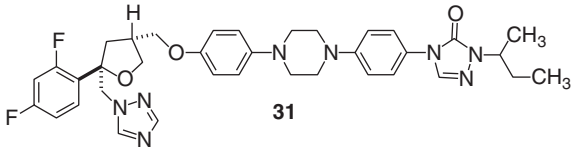


30

In our initial studies the importance of the aromatic spacer group –OR, and the presence of an azole moiety for its broad-spectrum activity, potency, and oral bioavailability, became apparent. Among the initial compounds synthesized, SCH 45012 (**31**)[20] was more active than itraconazole (**24**) and saperconazole (**25**) against systemic *Candida* and pulmonary *Aspergillus* infection models.

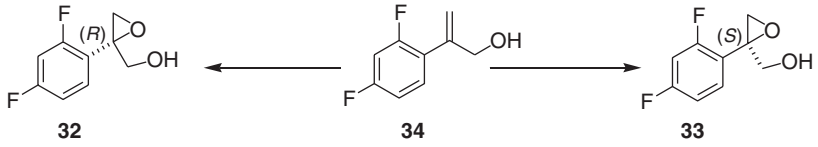


31

8.04.3.2 The Synthesis and Antifungal Activities of SCH 45012 and Its Isomers

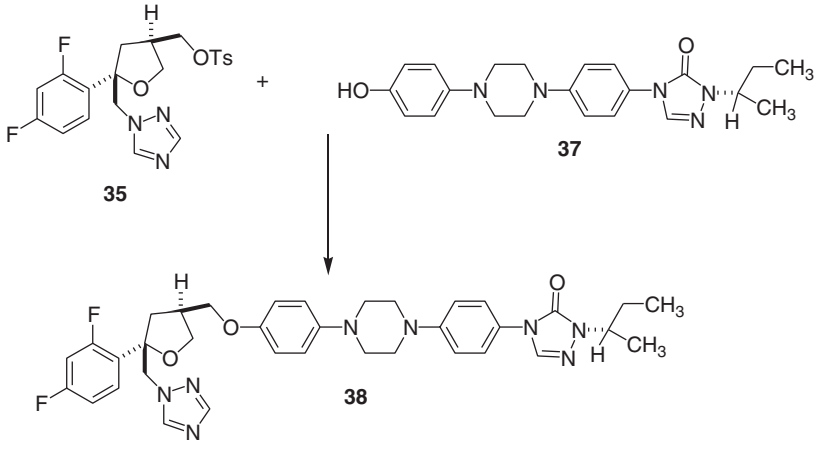
SCH 45102 has three chiral centers, two of which have an established *cis* stereochemical relationship, and therefore it presents as a mixture of four optical isomers. Using Sharpless oxidation the chiral epoxides **32** and **33** were prepared from the allylic alcohol **34** (Scheme 3). Enantiomerically pure **32** and **33** were converted to the tosylates **35** and **36**, respectively, as described in our earlier publication. The reaction of **35** with **37** gave SCH 49999 (**38**) (Scheme 4), whereas the reaction of **36** with **37** yielded SCH 50002 (**40**) (Scheme 5). Alternatively, when **35** was treated with **41** it yielded SCH 50000 (**42**) (Scheme 6), and **36** on treatment with **41** yielded SCH 50001 (**43**) (Scheme 7). The phenol **37** was prepared by reacting **44** with the enantiomerically pure tosylate **45**, followed by *O*-demethylation (Scheme 8). Similarly, **41** was prepared by reacting **44** with the enantiomerically pure tosylate **46**, followed by *O*-demethylation (Scheme 8).

With all the four isomers of SCH 45012 in hand, we determined their antifungal activities. Interestingly, SCH 49999 and SCH 50000 possessing 5-(*S*) stereochemistry were inactive, whereas SCH 50001 and SCH 50002, possessing 5-(*R*) stereochemistry, were highly active as antifungals, and were both equally active. These results emphasize the importance of synthesizing possible stereoisomers to determine their biological activity in drug discovery, because there



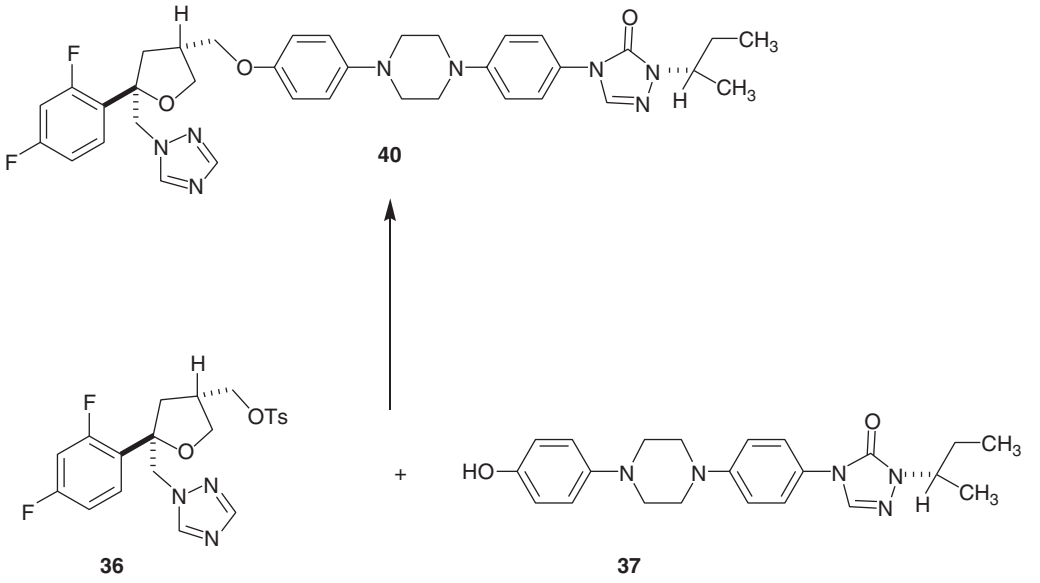
32 34 33

Scheme 3

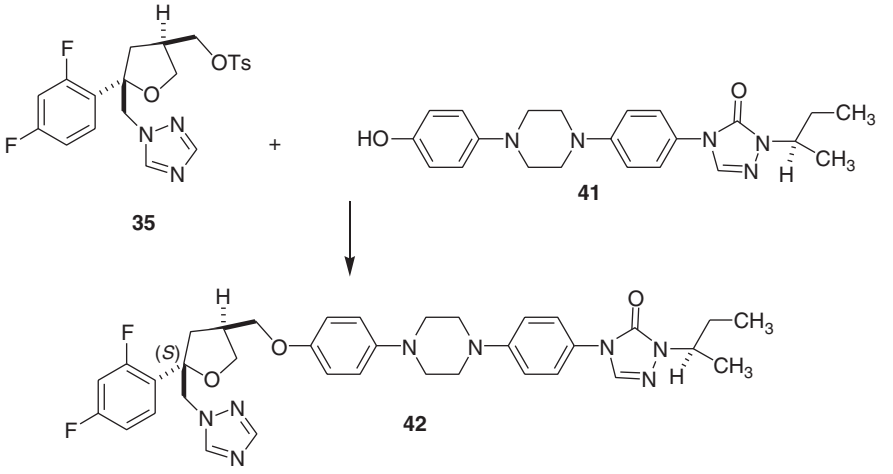


35 37 38

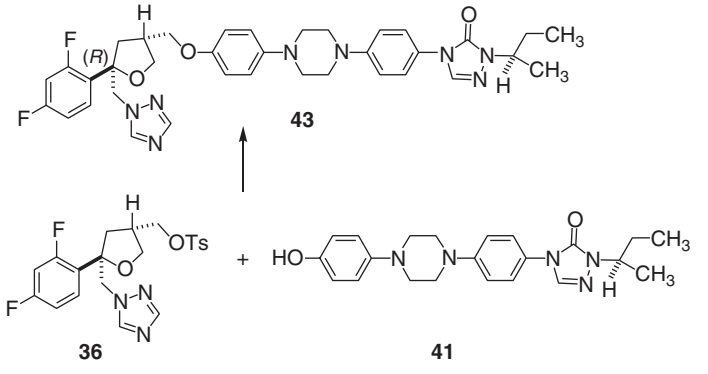
Scheme 4



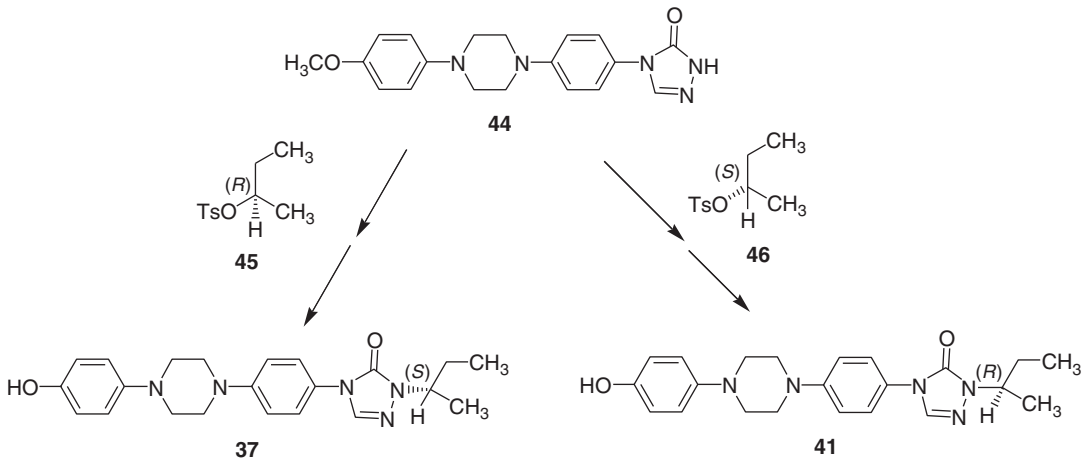
Scheme 5



Scheme 6

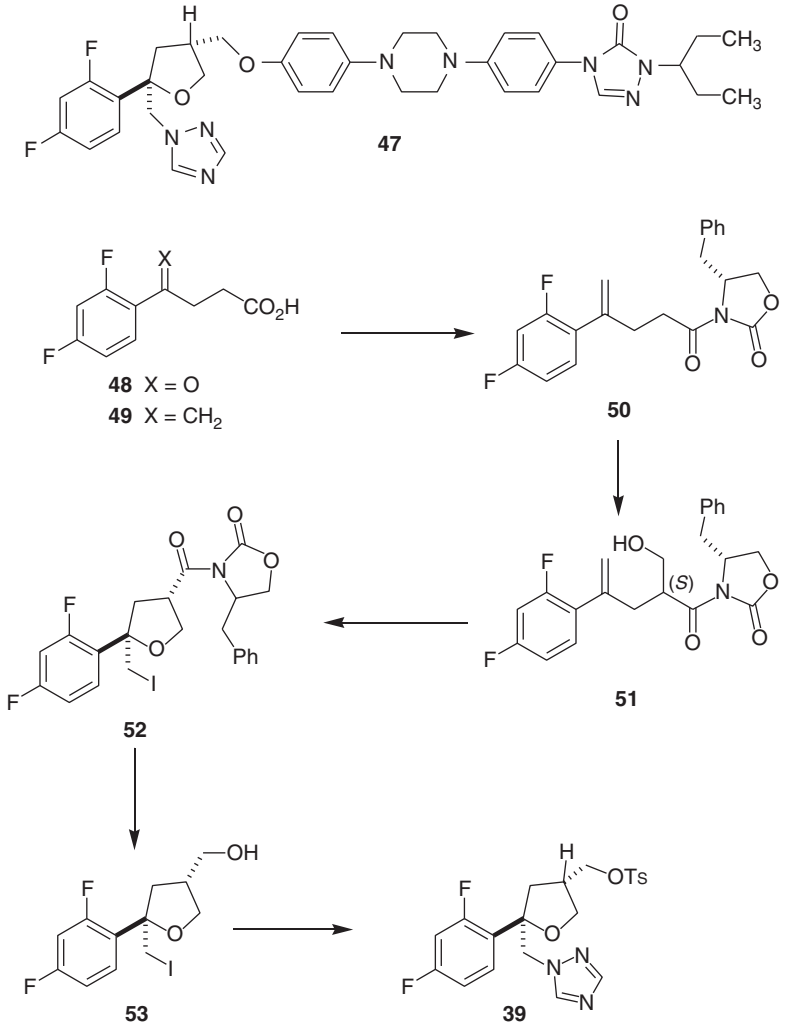


Scheme 7



Scheme 8

is no other way at the outset to know which stereoisomer will be of importance. SCH 50001 and SCH 50002 being equiactive also suggests that the stereochemistry of the side chain is not important. With this information in hand, we synthesized SCH 51048 (**47**),[21] following similar schemes as used for the synthesis of SCH 50001 and SCH 50002, thus eliminating one asymmetric center, which of course makes the synthesis much simpler.

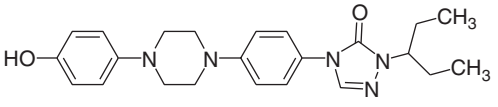


Scheme 9

SCH 51048 was much more potent than either SCH 50001 or SCH 50002, and also had a much broader spectrum of activity as an antifungal. Thus, eliminating one asymmetric center in SCH 51048 resulted in a compound that was more active and easier to synthesize.

8.04.3.3 The Improved Synthesis of SCH 51048[22]

The Friedel–Crafts reaction of 2,4-difluorobenzene with succinic anhydride yielded the ketoacid **48**, which was converted in Wittig reaction to the olefin **49** (Scheme 9). Stereoselective hydroxymethylation of **50** obtained from **49** yielded **51**. Iodocyclization of **51** yielded **52**, which on reduction with lithium borohydride yielded the alcohol **53**. Displacement of the iodo compound with sodium triazole followed by tosylation yielded **39**. The synthesis of SCH 51048 was completed by reacting **39** with the phenol **54**.



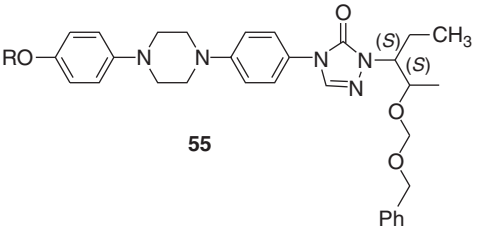
54

8.04.3.4 The Design of Posaconazole

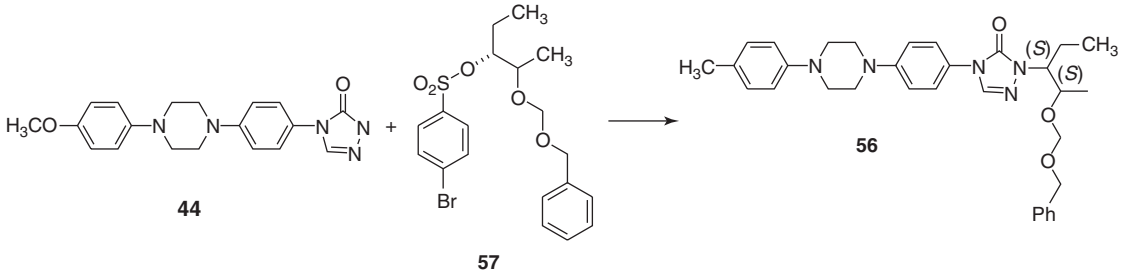
During in vivo studies it was found that SCH 51048 produced an active metabolite, and it was concluded from mass spectrometry studies that the metabolic hydroxylation occurred at one of the carbons in the side chain of SCH 51048. As hydroxylation of the tertiary center will produce an unstable compound, the metabolic hydroxylation must have occurred either at the primary (two isomers) or secondary carbon (four isomers) center. We synthesized all the possible isomers, and determined their activities. Based on their spectrum of activity, potency, and pharmacokinetic properties, posaconazole (**21**) was selected as a clinical candidate.

8.04.3.5 The Synthesis of Posaconazole[15,22]

The synthesis of posaconazole was achieved by reacting **36** with the phenol **55** followed by deprotection of the alcohol protecting group. Compound **55** was prepared by *O*-demethylation of **56**, which in turn was prepared by reacting **44** with **57** (Scheme 10). Compound **57** was derived from (*S*)-lactic acid.



55



44 **57** **56**

Scheme 10

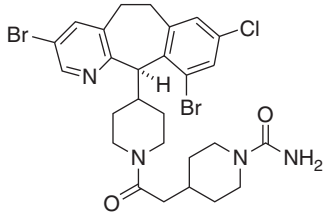
8.04.3.6 The Antifungal Activity of Posaconazole

Posaconazole was tested against 285 strains of yeasts and filamentous fungi comprising 37 different species of fungi, including fluconazole-sensitive and -resistant strains of *Candida*, and also against *Aspergillus*. The mean minimum inhibitory concentration (MIC) against *Candida* was $0.018\,\mu g\,mL^{-1}$, and that against *Asperigillus* was $0.048\,\mu g\,mL^{-1}$. These MIC values are superior when compared with other clinically used antifungals. It has already been pointed out in this chapter that posaconazole was found in the clinic to be safe and efficacious as an antifungal – it is active against *Candida* and, particularly, serious infections caused by *Aspergillus*.

8.04.4 The Discovery of Lonafarnib

8.04.4.1 Overview

In recent years, considerable progress has been made in cancer chemotherapy, with the discovery of paclitaxel, hercepetin, imatinib, etc., yet there is a growing need for new anticancer agents with fewer side effects. Among several possible approaches, we[23,24] and others[25–27] decided to attempt to discover an anticancer agent that would work by inhibiting the enzyme FPT. In this chapter we discuss the path we took to the discovery of lonafarnib (**58**), a potent inhibitor of FPT, and discuss its anticancer activity.



58

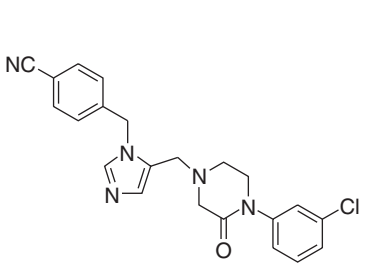
It was well documented[28] at the outset of our work that approximately 30% of all human tumors contained a mutated Ras protein. Three mammalian Ras genes (i.e., H, K, and N), with mutations at positions 12, 13, and 61, have been identified in these tumors. Each of these genes encodes closely related proteins known as Ras P-21 proteins, containing 189 amino acids.

Ras proteins undergo a series of post-translational modifications before attaching to the cell membrane. In the 'off' state the modified Ras protein is bound to guanosine diphosphate (GDP); however, during cell activation, Ras-GDP is exchanged to the guanosine triphosphate form (Ras-GTP). In the normal cellular process, Ras-GTP (the 'on' state) is hydrolyzed by a GTPase (GTP-activating protein) to Ras-GDP, and cellular function returns to the 'off' state. However, in cancer cells the above hydrolysis step is much slower, and cellular proliferation continues in an uncontrolled manner.

At the C-terminus of the Ras protein there is a CAAX sequence (where C is cysteine, A is an aliphatic amino acid, and X is a variable such as methionine). However, when X is leucine the protein becomes geranyl geranylated, which is a more common event in a normal cellular process, and it uses a related enzyme called geranyl geranyl protein transferase (GGPT). It is therefore important that a desirable FPT inhibitor should be selective over GGPT to avoid potential toxicity problems.

The post-translation modification[29,30] of the RAS protein involves modification of the CAAX box using the following steps: (1) farnesylation of the cysteine residue using FPT, (2) cleavage of the CA bond using Ras-converting enzyme, and (c) methylation of the newly generated carboxylic acid group using carboxymethyl transferase. Thus, the transformed Ras protein; attaches to the cell membrane. It should be noted that selective inhibition of any of the above three steps will provide an anticancer agent.

Several different approaches have been taken in different laboratories to discover potent inhibitors of FPT, and as a result four compounds have entered clinical trials: L-778123 (Merck)[25] (**59**), BMS-214662 (Bristol Meyers-Squibb)[26] (**60**), R-115777 (Janssen)[27] (**61**), and lonafarnib (Schering-Plough)[23,24] (**58**). In this chapter we focus on the discovery of lonafarnib.



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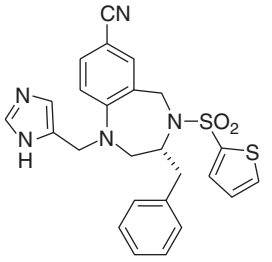

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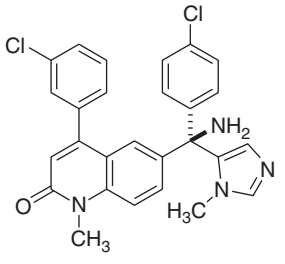


61

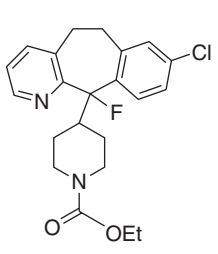
8.04.4.2 Screening of the SPRI Library of Compounds to Discover Initial Leads

The discovery of lonafarnib began with the screening of SPRI compound libraries. Several tricycles, including compounds **62**, **63**, and **64**, were found to possess weak to moderate activity against FPT, and these compounds had selectivity over GGPT; however, they showed poor cellular activity (Figure 8).

8.04.4.3 Structure–Activity Relationships and the Discovery of SCH 44342

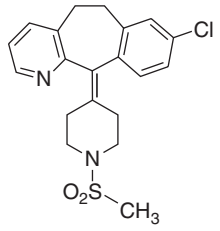
Following the above leads, several amides were prepared. In Table 3, the activities of a few of these amides are summarized to highlight structure–activity relationships. SCH 44342[31,32] was one of the most active analogs synthesized.

It should be noted in Table 3 that the aliphatic amide **65** was less active than the aromatic amides **66** and **68**, and within the aromatic amides the introduction of a CH_2 spacer group significantly improved their activities. The reason for the improvement of activity from **66** to **67**, and from **68** to **69** (SCH 44342), became clear when an x-ray analysis of



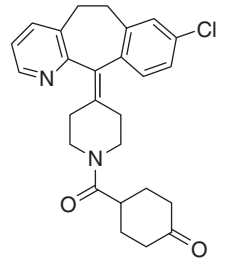
62

H-Ras FPT IC_{50} = 3.7 μM
GGPT IC_{50} > 49 μM
COS cell IC_{50} = 15.4 μM



63

H-Ras FPT IC_{50} = 1.9 μM
GGPT IC_{50} > 49 μM
COS cell IC_{50} = 13 μM

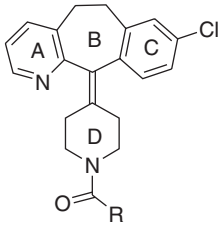


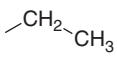
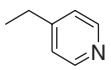
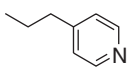
64

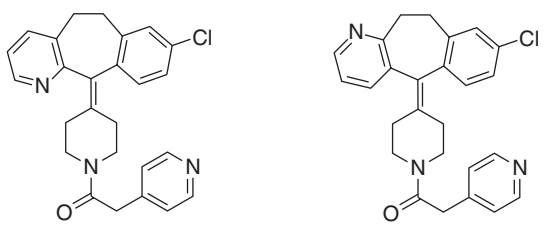
H-Ras FPT IC_{50} = 1.7 μM
GGPT IC_{50} > 49 μM
COS cell IC_{50} = 12 μM

Figure 8 Activities of initial lead compounds. (Reprinted with permission from Ganguly, A. K.; Doll, R. J.; Girijavallabhan, V. M. *Curr. Med. Chem.* **2001**, *8*, 1421 © Bentham Science Publishers Ltd.)

Table 3 Activities of amides



Compound	*R*	*FPT IC_{50} (μM)*
65		8.4
66		5.3
67		0.8
68		4.7
69 (SCH 44342)		0.25
70		15.8



SCH 44342
FPT IC_{50} = 0.25 μM

SCH 55387
FPT IC_{50} = 2.5 μM

SCH 55686
FPT IC_{50} = 48.4 μM

SCH 55778
FPT IC_{50} = 25.8 μM

Figure 9 A change in position of the pyridine nitrogen or from ring cleavage results in inactivity.

an inhibitor bound to FPT was available, and we shall return to this point later. When the spacer group was increased in length, the resulting compounds were inactive. We also found that when the position of the pyridine nitrogen atom was changed, or when the rings B and D were cleaved, the resulting compounds were essentially inactive (Figure 9).

FPT is an obligatory dimer composed of α and β subunits. The crystal structure of unliganded FPT shows that the α unit is composed of seven pairs of anti-parallel α helices packed to form a crescent shape. The six pairs of helices in the β subunit are arranged as a double-walled barrrel. The active site cavity is situated near the center of the protein, and lined with residues of both the subunits. The catalytic zinc is liganded by three side chains arising from the β subunit, and a water molecule occupies the fourth site.

8.04.4.4 X-ray Crystallography Results and the Design of Future Analogs

An x-ray crystallographic study[33] of SCH 44342 bound to FPT (Figure 10) demonstrated an extended interaction between SCH 44342 and FPT. SCH 44342 binds in the center of the active site cavity, and the tricyclic ring A-B-C is situated deep in the cavity, and fits well in the lipophilic pocket and near the farnesyl pyrophosphate residue. Ring E is closest to the molecular surface. In three dimensions, the tricyclic ring is at a right angle to the rest of the molecule. The pyridine nitrogen atom is hydrogen bonded to a water molecule, which in turn is hydrogen bonded to the Ser99β residue. Aromatic rings A and C stack against the aromatic amino acid residues of the α and β subunits. One of the most important interactions involves the hydrogen bonding of the amide carbonyl with a water molecule, which is hydrogen bonded in turn to the Phe360β and Tyr361β residues of the protein backbone. It was also clear from x-ray studies that a few more interactions could be gained in SCH 44342 if there was a substituent at the 3-position of the pyridine ring. Thus, a number of analogs with different substituents at the 3-position (Br, Cl, F, and CH_3) were synthesized, and their activities are summarized in Table 4. Although **74** was highly active, as the methyl group is expected to undergo metabolic oxidation we decided to substitute bromine at the 3-position in ring A for the synthesis of future analogs.

At this stage we also investigated[34] the importance of having an sp^3 center at C-11, and our results are summarized in Figure 11. It was intriguing that the C-11 enantiomers **75** and **76** had equal activities, as did the enantiomers **77** and **78**. These compounds were premetabolized to the pyridine *N*-oxides, and, as expected, they had better pharmacokinetic properties compared to the pyridine analogs. X-ray analysis of the desbromo enantiomers **79** and **80** bound to FPT revealed that the two enantiomers bind in a similar way, and when the bound structures were superimposed they overlapped almost perfectly. The (*S*)-(−) enantiomer binds in the same location as the (*R*)-(+)

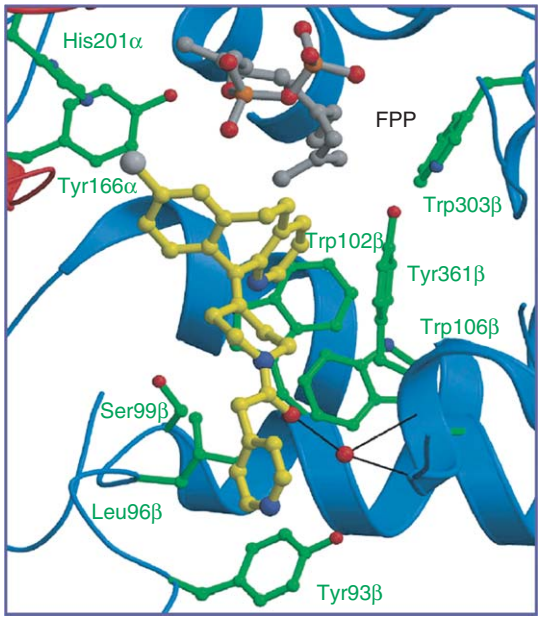
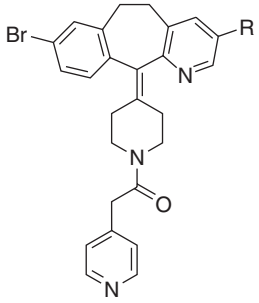
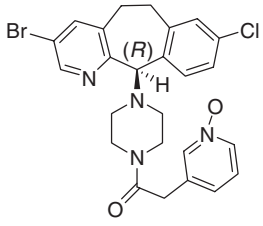


Figure 10 X-ray crystal structure of SCH 44342 bound to FPT ($IC_{50} = 250$ nM). (Reprinted with permission from Strickland, C. L.; Weber, P. C.; Windsor, W. T.; Wu, Z.; Le, H. V.; Albanese, M. M.; Alvarez, C. S.; Cesarz, D.; del Rosario, J.; Deskus, J. *et al. J. Med. Chem.* **1999**, *42*, 2125. Copyright (1999) American Chemical Society.)

Table 4 Activities of analogs of SCH 44342 with different substituents



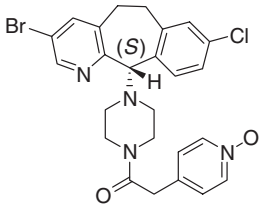
Compound	*R*	*IC_{50} (μM)*
SCH 44342 (**69**)	H	0.25
71	F	0.65
72	Cl	0.07
73	Br	0.06
74	CH_3	0.04



75

(*R*)–(+)

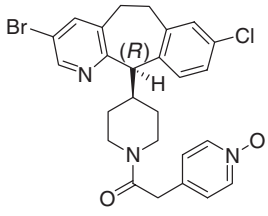
FTP IC_{50} = 32 nM
COS cell IC_{50} = 500 nM



76

(*S*)–(–)

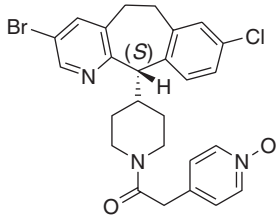
FTP IC_{50}= 38 nM
COS cell IC_{50} = 125 nM



77

(*R*)–(+)

FTP IC_{50}= 34 nM
COS cell IC_{50} = 560 nM



78

(*S*)–(–)

FTP IC_{50}= 28 nM
COS cell IC_{50} = 2000 nM

Mouse pharmacokinetics (25 mg kg^{-1})

AUC (p.o.) = 32 μg h ml^{-1}	AUC (p.o.) = 35 μg h ml^{-1}	AUC (p.o.) = 9.2 μg h ml^{-1}	AUC (p.o.) = 4.8 μg h ml^{-1}
$t_{1/2}$ (i.v.) =1.2 h	$t_{1/2}$ (i.v.) =1.9 h	$t_{1/2}$ (i.v.) =1.8 h	$t_{1/2}$ (i.v.) =1.0 h
Bioavailability = 100%	Bioavailability = 100%	Bioavailability = 54%	Bioavailability = 48%

Figure 11 Properties of analogs of SCH 44342 with an sp^3 center at C-11 (AUC, area under the plasma concentration–time curve).

enantiomer, except that the tricyclic ring is rotated 180° relative to the active site. The increased potency of the 3-bromo analogs was due to the additional interactions with the aromatic amino acids, as mentioned above. A summary of the interactions revealed in the x-ray analysis of **79** and **80** are depicted in Figure 12.

8.04.4.5 Lonafarnib and Its Biological Activity

From these studies it was clear that there is enough flexibility at C-11, and that it could be either a C–C bond or a C–N bond. Several amide analogs were synthesized, and, as indicated in the x-ray studies, this portion of the molecule is exposed to the surface, and, as a result, most of them were active. Among these amides, **81** and its enantiomer were highly

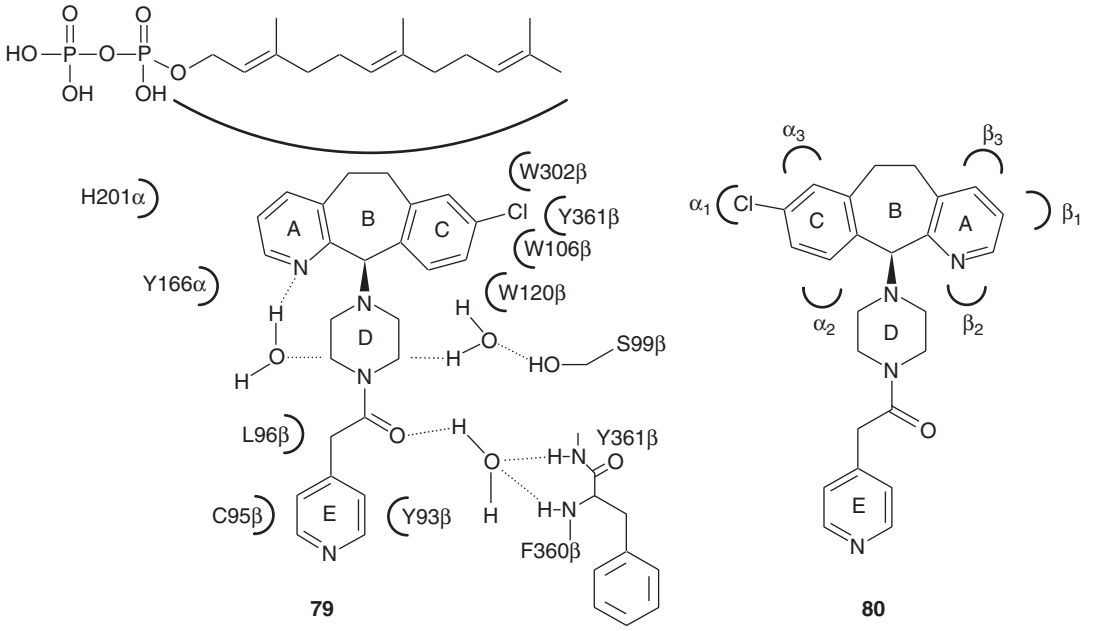
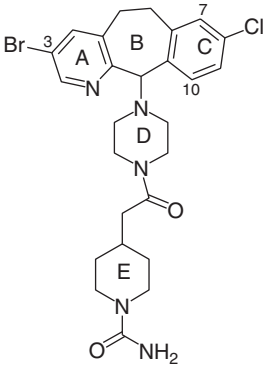


Figure 12 X-ray analysis of the des bromo enantiomers (**79**) and (**80**). (Reprinted with permission from Strickland, C. L.; Weber, P. C.; Windsor, W. T.; Wu, Z.; Le, H. V.; Albanese, M. M.; Alvarez, C. S.; Cesarz, D.; del Rosario, J.; Deskus, J. *et al. J. Med. Chem.* **1999**, *42*, 2129. Copyright (1999) American Chemical Society.)

active. X-ray studies with these enantiomers bound to FPT indicated, besides the interactions noted above, that further substitutions of these compounds at either the 7- or 10-position should be beneficial in improving their in vitro potency.



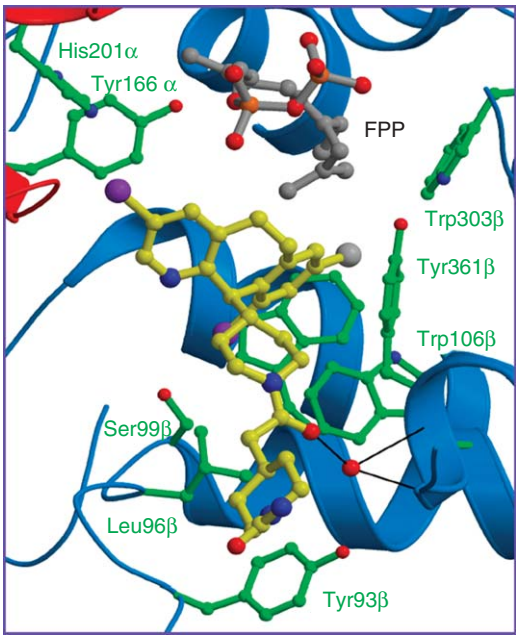
We synthesized both of these classes of compounds with different amide side chains, and C-11 substituents. Among all of the compounds synthesized, lonafarnib (SCH 66336) was one of the most potent analogs, possessing desirable pharmacokinetic properties. It also showed potent cell-based activity and antitumor activity. Unlike the enantiomers **79** and **80**, which were equally active in vitro, in the case of lonafarnib the (*R*)-(+) enantiomer was active and the (*S*)-(−) enantiomer was inactive.

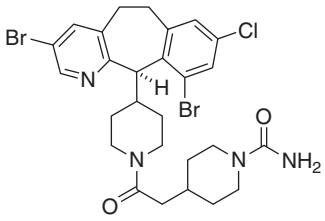
The x-ray crystal structure[33] of lonafarnib bound to FPT is presented in Figure 13. As the (*S*)-(−) enantiomer was inactive, it could not be co-crystallized with FPT for x-ray studies.

The profile of the biological activity of lonafarnib is summarized in Table 5.

8.04.4.6 Synthesis of Lonafarnib

An overview of the synthesis of lonafarnib[23,24] is given in Scheme 11.





58

Figure 13 X-ray crystal structure of lonafarnib bound to FPT ($IC_{50} = 1.9$ nM). (Reprinted with permission from Strickland, C. L.; Weber, P. C.; Windsor, W. T.; Wu, Z.; Le, H. V.; Albanese, M. M.; Alvarez, C. S.; Cesarz, D.; del Rosario, J.; Deskus, J. *et al. J. Med. Chem.* **1999**, *42*, 2130. Copyright (1999) American Chemical Society.)

Table 5 Biological activity of lonafarnib

Inhibition
H-ras FPT $IC_{50} = 1.9$ nM
K-ras FPT $IC_{50} = 5.2$ nM
N-ras FPT $IC_{50} = 2.8$ nM
GGPT $IC_{50} > > 50\,000$ nM
COS cell $IC_{50} = 10$ nM
Soft agar
NIH-H-ras $IC_{50} = 75$ nM
NIH-K-ras $IC_{50} = 500$ nM
Mouse pharmacokinetics (25 mg kg^{-1})
AUC = 24.1 mg h μM^{-1}
C_{max} (p.o.) = 8.84 μM
$t_{1/2}$ (i.v.) = 1.4 h
Bioavailability = 76%
Monkey pharmacokinetics (10 mg kg^{-1})
AUC = 14.7 mg h μM^{-1}
C_{max} (p.o.) = 1.9 μM
$t_{1/2}$ (i.v.) = 3 h
Bioavailability = 50%

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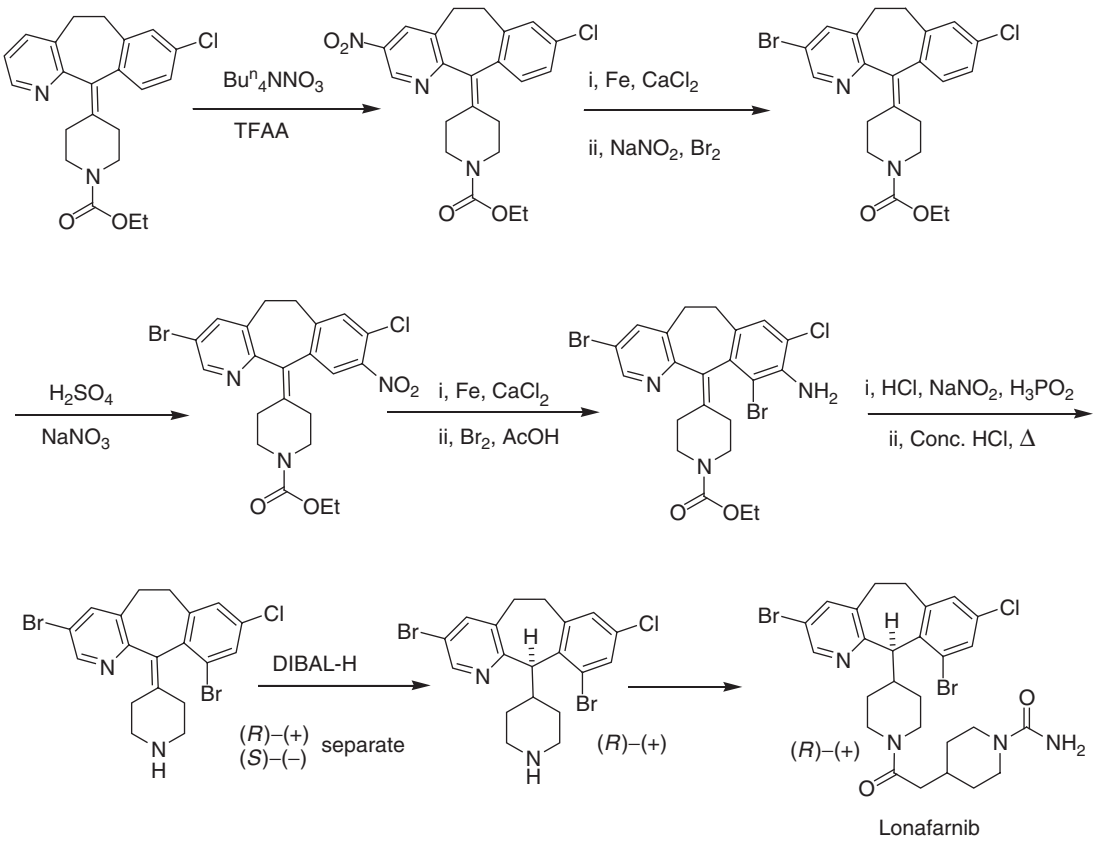

Scheme 11 Reprinted with permission from Ganguly, A. K.; Doll, R. J.; Girijavallabhan, V. M. *Curr. Med. Chem.* **2001**, *8*, 1427 © Bentham Science Publishers Ltd.

8.04.5 Summary

Based on all the above observations lonafarnib was recommended for clinical studies, and presently it is in third phase of the trial in cancer patients.

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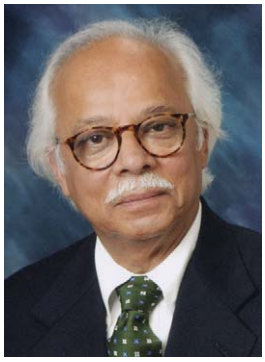
Biography



A K Ganguly was born in India and educated partly in India and in England. He received his PhD degree from Imperial College, London under the supervision of Sir Derek Barton.

Prof Ganguly immigrated to the United States in 1967 and worked with Sir Derek at the Research Institute of Medicine and Chemistry, Cambridge, Massachusetts before joining the Schering-Plough Research Institute, Kenilworth, NJ in 1968 as a Senior Scientist. At Schering-Plough Research Institute he progressed to the position of Senior Vice President of Chemical Research in which capacity he directed all aspects of Chemical Research at the

institute. In September 1999 he joined Stevens Institute of Technology, Hoboken, NJ as a Distinguished Research Professor of Chemistry where he teaches Medicinal Chemistry and directs research programs for graduate students.

Prof Ganguly has made many significant contributions in drug discovery. He is associated with the discovery of Ezetimibe (Zetia), a cholesterol absorption inhibitor; Noxafil (Posaconazole), a potent antifungal and Lonafarnib (Sarasar), a highly selective farnesyl protein transferase inhibitor for the treatment of cancer. Prof Ganguly is also recognized for his many contribution toward synthesis of biologically active molecules and determining structures of complex oligosaccharide antibiotics such as Ziracin. Prof Ganguly has published 176 papers and is a coinventor of 80 patents. He has been a plenary lecturer at many international meetings and received several awards. In 2003, Prof Ganguly received the prestigious Hershberg award from the American Chemical Society for making important contributions in Medicinal Chemistry. In 2004, Prof Ganguly received Doctor of Engineering (Honoris Causa) from Stevens Institute of Technology and was also the recipient of the 'Life time achievement award in Chemical Sciences' from the Indian Chemical Society. Prof Ganguly remains as a consultant at Schering-Plough Research Institute.

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8.05 Viread

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8.05.1 Introduction

According to estimates from the United Nations acquired immunodeficiency syndrome (UNAIDS)/World Health Organization (WHO) AIDS Epidemic Update (December 2004), 37.2 million adults and 2.2 million children were living with human immunodeficiency virus (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, most importantly, convenient dosing schedule.

The clinical efficacy and safety of tenofovir in HIV-infected subjects were initially demonstrated using an intravenous formulation.[1] 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 $\geq 10\,000$ copies mL^{-1}. Dose levels of 1 and 3 $mg\,kg^{-1}$ of body weight day^{-1} were evaluated. All subjects tolerated these doses without significant adverse events. Following 7 consecutive days of tenofovir administration at 3 $mg\,kg^{-1}\,day^{-1}$, the median change in plasma HIV-1 RNA from baseline was -1.1 $\log_{10}$.

Tenofovir (9-[2-(phosphonomethoxy)propyl]adenine, PMPA) is a novel nucleotide analog that belongs to the class of acyclic nucleoside phosphonates (**Figure 1**). The potent antiviral effect of tenofovir was demonstrated by Balzarini *et al*.[2] 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 HIV-1 and HIV-2 DNA polymerase (reverse transcriptase) in addition to other viral DNA polymerases.[3] This inhibition results in DNA chain termination and impairment of viral replication. The IC_{50} value of tenofovir inhibition of HIV-1 reverse transcriptase is between 0.04 and 8.5 $\mu mol\,L^{-1}$.[4]

Mimicking a nucleoside monophosphate with a phosphonate, as in tenofovir, has two advantages: (1) it avoids the requisite but slow initial phosphorylation of nucleosides by host kinases; and (2) 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.[5] In summary, acyclic nucleoside phosphonates act as facile false substrates for viral DNA polymerases 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.

The preclinical properties of tenofovir attracted attention since the compound had a resistance profile that was complementary to the nucleosides used for the treatment of HIV at the time. In addition, parenteral administration of tenofovir to HIV-1-infected individuals was well tolerated and effective, resulting in significant dose-related antiviral activity.[1]

Tenofovir was initially dosed by parenteral administration. 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

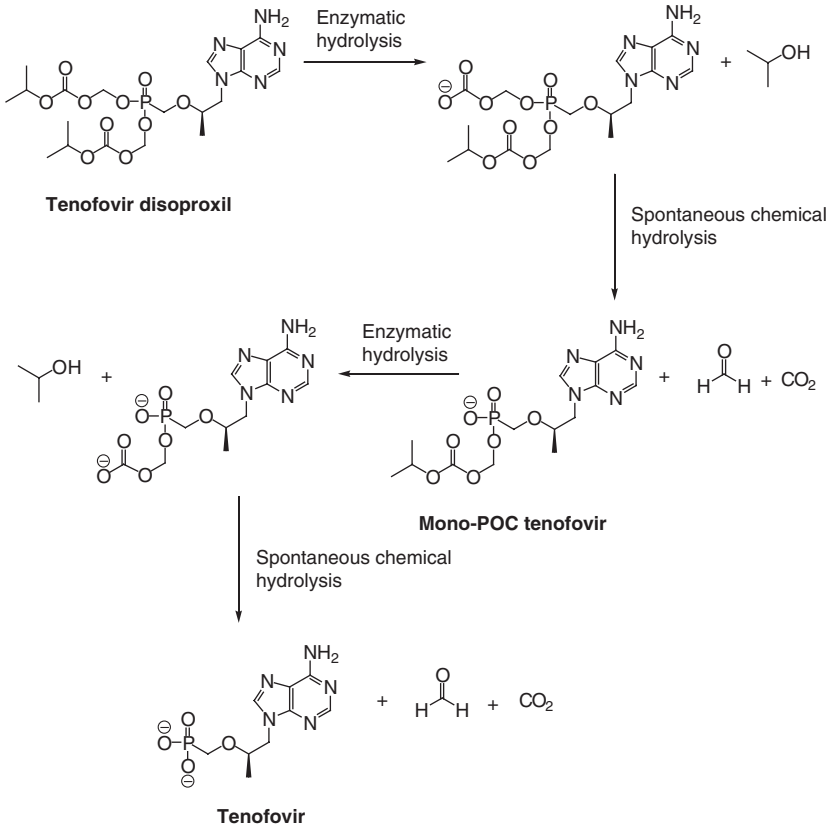

Figure 1 Structure of tenofovir disoproxil, tenofovir, and the bioconversion pathway.

readily undergo passive diffusion across cellular membranes and intestinal mucosa, resulting in low bioavailability after oral administration.[6,7]

A series of novel prodrugs of tenofovir was designed to overcome the pharmacokinetic limitations of tenofovir.[8,9] These prodrugs were engineered to mask the polar phosphonic acid functionality using a novel oxycarbonyloxymethyl linker to allow for 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.[8] The amount of pivalic acid that would be released from the breakdown of the bis-[(pivaloyloxy)methyl] tenofovir was a concern, considering the necessarily 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.

8.05.2 Pharmacokinetics of Tenofovir Disoproxil Fumarate

Preclinical studies examining the pharmacokinetics and metabolism of tenofovir were performed in beagle dogs using tenofovir dosed by intravenous, intraperitoneal, and oral routes.[6,7] The drug was mainly excreted in urine by filtration and tubular secretion. The oral bioavailability was low. Therefore a prodrug of tenofovir, tenofovir disoproxil, characterized by an enhanced oral pharmacokinetic profile, was selected for clinical development.[8,9]

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.[10] 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. The oral bioavailability 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 $\log_{10}$ HIV-1 RNA after 28 days of dosing in the 300-mg dose group was –1.22 compared to the placebo arm.[10] The largest decreases in HIV-1 RNA levels were 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.[11] This enhanced effect 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 were fivefold greater after oral administration of tenofovir disoproxil fumarate than following an equivalent subcutaneous exposure of tenofovir.[11]

The safety and antiviral activity of tenofovir disoproxil fumarate were further established in multiple large, well-designed, placebo-controlled clinical trials.[12,13] Tenofovir disoproxil fumarate 300 mg daily was shown to be effective in antiretroviral-experienced and treatment-naive 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.[14] No clinically significant 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.[15,16]

8.05.3 Synthesis and Formulation

The preparation of tenofovir disoproxil was initially described by Arimilli *et al.* in 1997[9] followed by disclosure of further details in a patent in 2000.[17] The synthesis 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 the reaction of diethyl phosphite with paraformaldehyde, followed by formation of the tosylate using *p*-toluenesulfonylchloride. The synthesis of tenofovir is completed by deprotection of the diethylphosphonate using trimethylsilylbromide in acetonitrile. Tenofovir is further purified by recrystallization from water to provide the product as the monohydrate.

The chloromethyl isopropyl carbonate necessary for the synthesis of tenofovir disoproxil is prepared by the 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 *N,N*-dimethylformamide (DMF). The fumarate salt is prepared by addition of fumaric acid in *i*-propanol to the prodrug to give the product (**Figure 2**).

Tenofovir disoproxil fumarate is a white crystalline powder with high aqueous solubility of 13.4 mg mL^{-1} in water. The hydrolysis of tenofovir disoproxil to mono-POC tenofovir produces formaldehyde as well as other by-products, as discussed previously.[18] Formaldehyde can further react with N^6 of the adenine moiety to form the corresponding carbinolamine intermediate.[19] Dehydration of the carbinolamine intermediate leads to the formation of a 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 pseudo-dimer. Both of these processes are known to be pH-dependent in solution.

The degradation kinetics of tenofovir disoproxil is therefore governed by two distinct but interrelated degradation pathways: (1) hydrolysis of the isopropyloxycarbonylmethyl moiety; and (2) formaldehyde-mediated dimerization.[18] A crystalline fumarate salt of tenofovir disoproxil was developed to reduce the rate and extent of dimerization in the 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.

8.05.4 Mechanism and Site of Bioconversion

Tenofovir disoproxil has a long half-life ($t_{1/2} > 7$ h) at both pH 2.0 and 7.4.[18] The bioconversion of tenofovir disoproxil to tenofovir is mediated by nonspecific carboxylesterases.[8] The mechanism involves rapid enzymatic hydrolysis followed

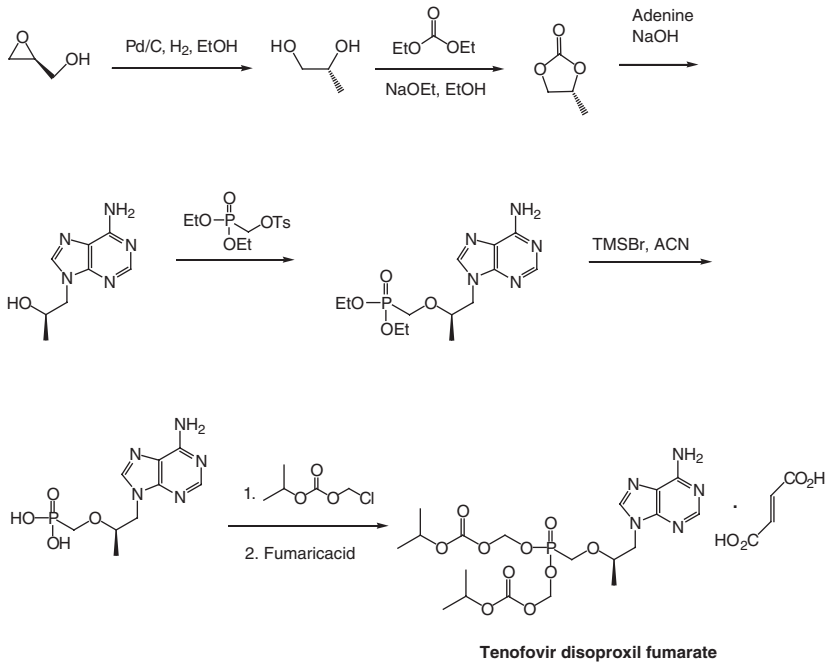

Figure 2 Synthetic scheme to tenofovir disoproxil fumarate. TMSBr, bromotrimethylsiline; ACN, acetonitrile.

by spontaneous decomposition of carbonic acid monomethyl phosphonate ester (Figure 1). The resulting monoester tenofovir undergoes a similar degradation leading to the 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.

8.05.5 Toxicity Issues

The clinical safety of tenofovir disoproxil fumarate has been examined in various clinical studies in HIV-infected subjects. The drug is generally well tolerated. In a pooled analysis, the severity and incidence of adverse events were similar for those receiving 300-mg daily dose of tenofovir disoproxil fumarate and 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^{-1} is considered insignificant.

8.05.6 Conclusion

Tenofovir disoproxil fumarate (Viread) is an excellent example of a prodrug that can overcome the pharmacokinetic limitations associated with poor permeability of the parent drug across intestinal mucosa. Viread has been approved worldwide in combination with other antiretroviral agents for the treatment of HIV-1 infection in adults and has become a pivotal product for the treatment of HIV since it was launched in 2001. The antiviral activity, excellent safety profile, superior resistance profile, and convenient once-daily oral regimen have made Viread one of the most frequently prescribed antiretroviral agents in the western world.

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8.06 Hepsera

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8.06.1 Introduction

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.[1] Over 1 million patients have been diagnosed with hepatitis B in the US and Europe.[2] Only 15% of the infected patient population is receiving treatment for hepatitis B. Lack of treatment in patients with high hepatitis B virus (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.[3]

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 is administered parenterally and 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 patients after a 3-year treatment period.[4]

Adefovir is a potent antiviral agent with activity against human immunodeficiency virus (HIV), herpes simplex virus (HSV), simian immunodeficiency virus (SIV), and HBV (**Figure 1**). 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^{-1} in human clinical trials.[5] 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 h. Serum clearance of adefovir is 223 ± 53 mL h^{-1} kg^{-1}, which is similar to the renal clearance of the drug (205 ± 78 mL h^{-1} kg^{-1}). Considering the low protein binding,[6] 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^{-1}) and suggests complete distribution of the compound throughout body water. The oral bioavailability of adefovir at 3.0 mg kg^{-1} dose is variable and less than 12%. The subcutaneous bioavailability of adefovir at 3.0 mg kg^{-1} is over 100%.

The preclinical and human pharmacokinetics data in combination with the low permeability coefficient across Caco-2 cells[7] indicate that the low oral bioavailability is due to poor permeation across intestinal epithelium, rather than metabolic degradation.[8] A number of prodrugs were prepared to improve the bioavailability of adefovir by increasing the lipophilicity of the compound.[9,10] Adefovir dipivoxil demonstrated the most favorable properties and was progressed through clinical trials.

Adefovir dipivoxil is dosed orally at 10 mg day^{-1}. It is effective in the treatment of the e antigen-positive and e antigen-negative HBV patients, and in patients who are resistant to lamivudine,[11,12] with a median reduction of serum HBV DNA of 4.3 $\log_{10}$ copies mL^{-1}. Following oral dosing, the prodrug is very efficiently cleaved and adefovir is released. No intact adefovir dipivoxil or monoester was detected in plasma following oral dosing in animal studies.[13]

Upon initiation of treatment of HBV patients with adefovir dipivoxil, clearance of HBV DNA is observed in a biphasic curve.[14] Initially, a sharp drop in HBV DNA levels (corresponding to clearance of viral particles from plasma) is observed with a half-life of 1 day. In the second, slower phase the infected virus-producing cells are eliminated. A single-dose pharmacokinetic study using adefovir dipivoxil 10 mg showed a $C_{max} = 18.4 \pm 6.26$ ng mL^{-1} at $T_{max} = 1.75$ h (C_{max}, the maximum observed plasma concentration; T_{max}, time to reach the maximum observed plasma concentration).[15] The

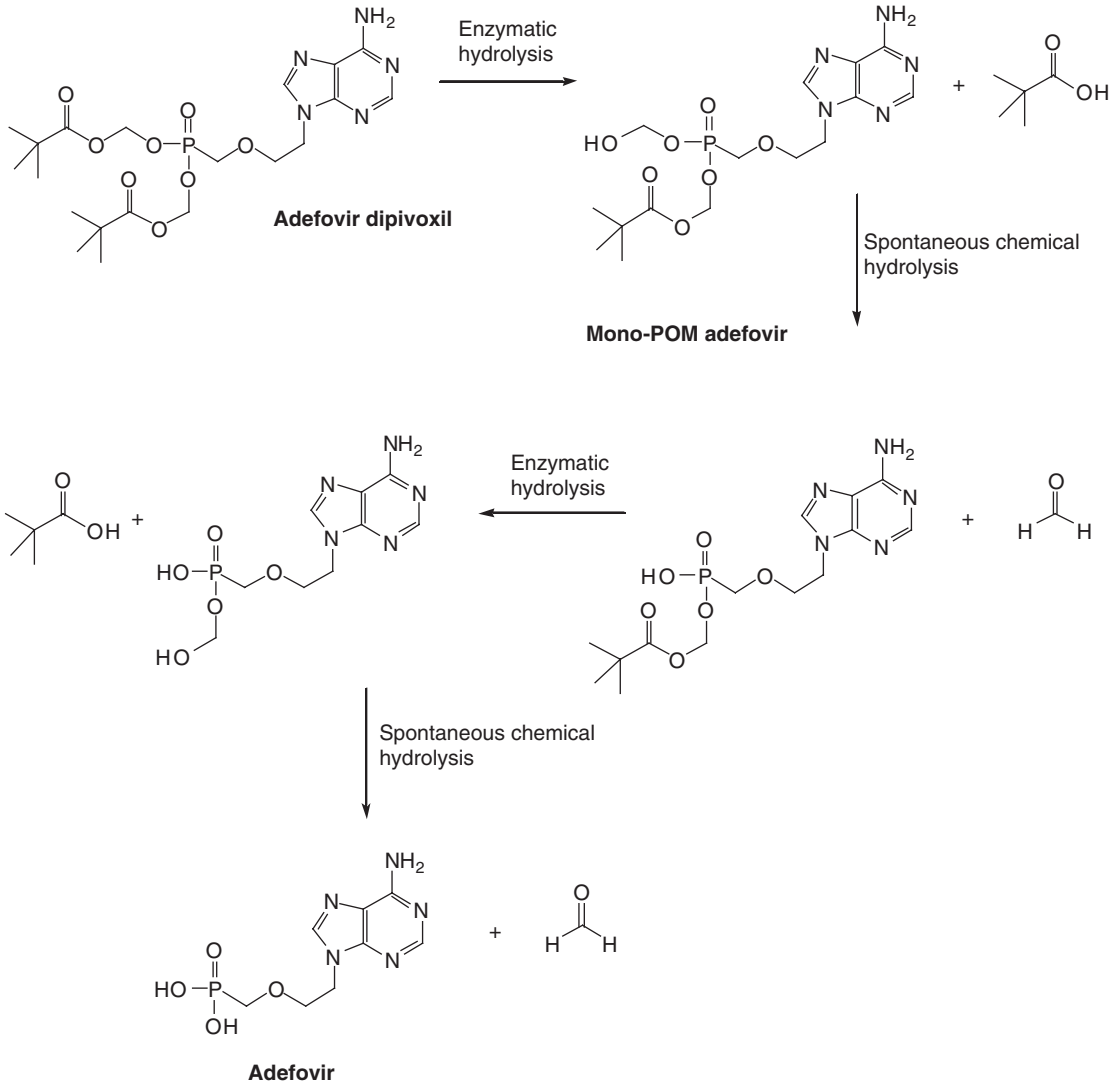

Figure 1 Structures of adefovir dipivoxil, adefovir, and the bioconversion pathway.

terminal elimination half-life of adefovir in plasma is 7.48 ± 1.65 h while the in vitro intracellular half-life is approximately 17 h. 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 64% of HBV e antigen-negative patients. Adefovir dipivoxil also demonstrated efficacy in patients who were resistant to lamivudine. In addition, levels of serum alanine aminotransferase, which is a marker for biochemical response to hepatitis B treatment, were normalized.[16] 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.[17] Mutant N236T in domain D of the HBV polymerase causes a reduction in susceptibility of HBV to adefovir both in vivo and in vitro.[18] 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.[19]

8.06.2 Synthesis and Formulation

The preparation of adefovir dipivoxil was first described in the literature over 10 years ago.[20,21] Optimization of the synthesis and formation of crystalline final product was subsequently described (**Figure 2**).[22,23] The synthesis of

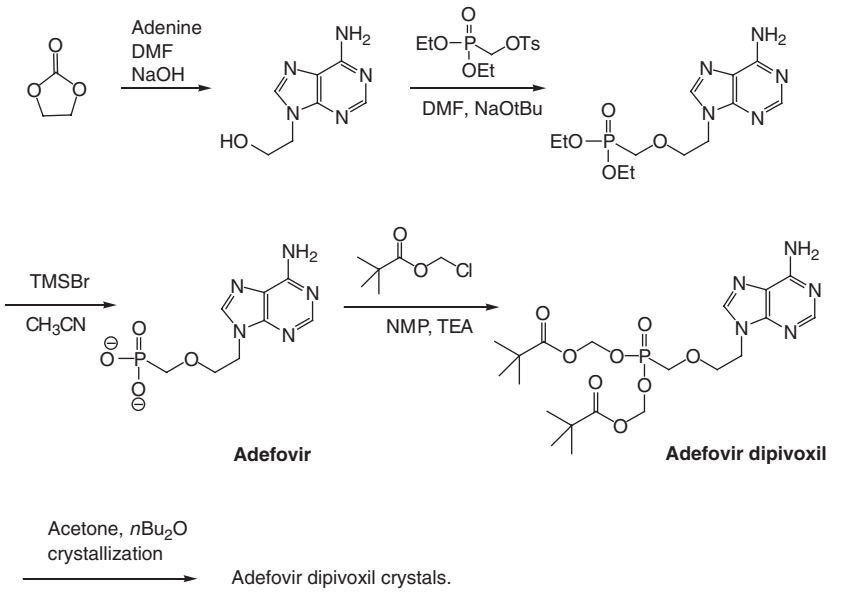

Figure 2 Synthetic scheme of crystalline adefovir dipivoxil.

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 *N*,*N*-dimethylformamide (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 *N*-methylpyrrolidone (NMP), using triethylamine as base.

Adefovir dipivoxil is a white crystalline powder with high aqueous solubility at pH 2.0 ($19\,mg\,mL^{-1}$) and lower solubility at pH 7.4 ($0.4\,mg\,mL^{-1}$). The degradation kinetics of adefovir dipivoxil is governed by two distinct, but interrelated degradation pathways: (1) hydrolysis of the pivaloyloxymethyl moiety; and (2) formaldehyde-mediated dimerization of the adenine ring.[24] 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.

8.06.3 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.[25] The bioconversion mechanism involves rapid enzymatic hydrolysis of the *bis*-ester followed by spontaneous decomposition of the hydroxymethyl intermediate. 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[26] or by fluid-mediated endocytosis in human, caucasian, peripheral blood, leukemia, acute lymphoblastic (CCRF CEM) T-lymphoblastoid tissue.[27] Adefovir is phosphorylated to adefovir monophosphate by various kinases, one of which present in lymphoid cells is identified as adenylase kinase 2.[28] 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 yielding adefovir diphosphate, which has a half-life of 33 h in human Hep G2 cells.[29]

8.06.4 Toxicity Issues

Adefovir dipivoxil 10 mg day^{-1} has a safety profile that is similar to that of placebo[30] and is well tolerated by healthy, renally, and hepatically impaired patients, as well as lamivudine-resistant HBV patients coinfected with HIV.[31] A potential concern with adefovir dipivoxil is nephrotoxicity, which was observed at doses $\geq$30 mg daily.[32] This observation is consistent with the high clearance of adefovir which exceeds the glomerular filtration rate.[5] Monitoring of renal function may be required during treatment with adefovir dipivoxil. In addition, increase in serum creatinine (>0.5 mg dL^{-1}) 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. 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^{-1}, which is negligible compared to the total body carnitine pool (120 mmol). The daily formaldehyde load of 0.04 mmol day^{-1} is considered insignificant as well.

8.06.5 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 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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8.07 Ezetimibe

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8.07.1 Introduction

Atherosclerotic coronary artery disease remains a major cause of death and morbidity worldwide and a significant drain on healthcare resources, especially in developed countries. Cardiovascular disease claimed over 900 000 lives in the USA in 2002. The World Health Organization (WHO) estimates that worldwide 17 million people die every year from cardiovascular disease, especially heart attack and stroke. The direct and indirect costs of cardiovascular disease are estimated to reach $393 billion in 2005.[1]

Of the many risk factors for cardiovascular disease, dyslipidemia is among the best understood and one that has clearly lent itself to both lifestyle and pharmacological intervention. Several large clinical studies conducted over the last few decades have indicated that overall mortality rates from cardiovascular disease can be significantly reduced with aggressive pharmacological intervention and risk factor management. Dietary counseling, exercise, and the use of drug therapy to reduce low-density lipoprotein (LDL) levels can significantly reduce the risk of developing coronary artery disease. While statins have dominated the market for pharmacotherapy, recent changes in the target LDL levels recommended by the National Cholesterol Education Program as well as a desire to limit the required dose of statins have highlighted the need for even more effective therapies.[2–4]

Ezetimibe is the first of a new class of drugs that treat hypercholesterolemia by inhibition of absorption of cholesterol from the intestines. While some intestinal cholesterol comes from the diet, the majority of the cholesterol that is absorbed from the intestines comes from the liver and has been excreted into the intestines in bile. Ezetimibe alone reduces LDL-C 15–18%, and adding ezetimibe to a starting dose of a statin produces a reduction in cholesterol levels equivalent to that seen with an eightfold higher statin dose.[5–8] Ezetimibe has been the subject of a number of reports in the biological and medical literature.[9–12]

This case history traces the development of ezetimibe from the discovery of the first azetidinone cholesterol absorption inhibitors (CAIs) through the most recent information on the mechanism of action of this class of drugs. Beyond its significance as a new treatment for hypercholesterolemia, ezetimibe is of considerable interest because of the unusual path that led to its discovery. Although it began as a traditional medicinal chemistry effort, early in the program it became clear that ezetimibe and related azetidinone CAIs were not inhibiting cholesterol absorption via any known mechanism. Because of the absence of clear understanding of the molecular target, no in vitro assay existed to assess activity. As a result, every compound in this class had to be evaluated exclusively using in vivo models, most commonly in the cholesterol-fed hamster. Despite this limitation, these compounds displayed structure–activity relationships (SARs) that were well behaved and consistent with interaction with a structurally well-defined molecular target, although the nature of this target was unknown. Thus, the path that led to ezetimibe was arguably less

technologically driven and more biology-based than typical drug discovery programs. The process relied heavily on hypothesis and experimentation to understand both the nature of the molecular target and how the activity of these compounds could be optimized. The history that is presented attempts to capture not only the facts and chronology of the discovery but also the thoughts, hypotheses, and milestones that led to an evolving understanding of the nature of the unknown biological target and how the activity of compounds could be optimized when a more targeted approach was not possible.[13]

8.07.2 Discovery of the Prototype Azetidinone Cholesterol Absorption Inhibitor

The discovery program that led to ezetimibe began as a traditional drug discovery program to discover novel acyl-coenzyme A cholesterol acyltransferase (ACAT) inhibitors.[14] Although ACAT was known to be involved in a variety of cholesterol trafficking events including cholesterol absorption in rodents, the relevance of ACAT in nonrodent species was still unclear at the time this program began. Nonetheless, a variety of structural classes were known to be potent ACAT inhibitors in vitro and to be active in rodent animal models that reflect a potential for lowering cholesterol levels. Among these models was the cholesterol-fed hamster.[15] A high-cholesterol diet dramatically increases liver cholesteryl ester (CE) levels in these animals, making them especially sensitive to ACAT inhibition. By contrast, serum cholesterol (SC) levels are not dramatically changed by cholesterol feeding in these animals, and most ACAT inhibitors have minimal effect on serum cholesterol levels. **Figure 1** shows the in vitro and in vivo profiles of **1** and **2**,[16,17] which are typical early compounds from this effort, as well as a reference ACAT inhibitor **3** (CI-976).[18]

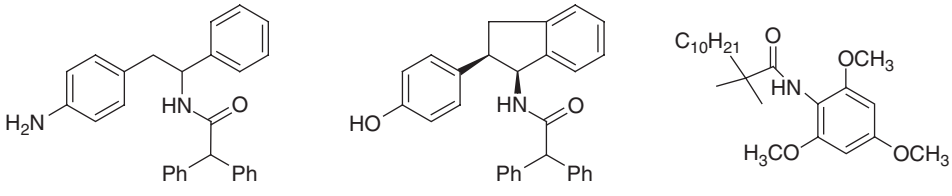
As would be expected with a well-defined molecular target, ACAT inhibitors displayed clearly defined SARs in these models that followed logically from structural changes. For instance, proper conformational constraint of **1** led to indane **2**, which showed both a significant increase in in vitro potency as well as a commensurate improvement in potency in the cholesterol-fed hamster.[16,17]

In addition to **2**, a number of alternate conformationally constrained analogs were prepared to probe their impact on in vitro and in vivo potency. Among these was azetidinone **4** proposed by Burnett *et al.*[19] These compounds were prepared by ester-enolate condensation to give azetidinone **5**, which was then deprotected by CAN oxidation, reduced, and acylated with a variety of acids (**Figure 2**).

In practice, the ester-enolate condensation gave a very modest yield of the desired azetidinone **5** accompanied by a small amount of a by-product **6** apparently derived from deprotonation of **5** followed by Claisen condensation with the ethyl phenylacetate starting material. Both the desired product **4** as well as the intermediate **5** and by-product **6** were evaluated for in vitro activity against ACAT as well as for in vivo activity in the cholesterol-fed hamster (**Figure 3**).[19]

None of the compounds was a potent ACAT inhibitor, although compound **6** did show some modest ACAT activity with an IC_{50} of about 7 μM. Despite this relatively weak activity, **6** showed moderate in vivo activity in the cholesterol-fed hamster assay. This included effects on both CE as well as a modest but reproducible effect on SC. Initial follow-up of this lead structure by Burnett and co-workers (**Figure 4**) demonstrated that several analogs of **6** displayed a similar profile of weak ACAT activity accompanied by a modest but reproducible effect on CE and SC in the hamster.[20]

Even at this early stage, elements of clear structure–activity trends were apparent, such as the loss in activity with aliphatic derivative **9**. Borrowing elements of known ACAT inhibitors such the 2,4,6-trimethoxy moiety of **3**, the ACAT activity of these compounds could be improved to give compounds such as **11**, but this had little or no impact on the



1
ACAT: IC_{50} = 900 nM
Hamster: at 50 mg kg^{-1} PO
SC: NE
CE: –80%

2
ACAT: IC_{50} = 40 nM
Hamster: at 50 mg kg^{-1} PO
SC: –7%
CE: –88%

3 (CI-976)
ACAT: IC_{50} = 4200 nM
Hamster: at 50 mg kg^{-1} PO
SC: –10%
CE: –67%

Figure 1 Prototype ACAT inhibitors.

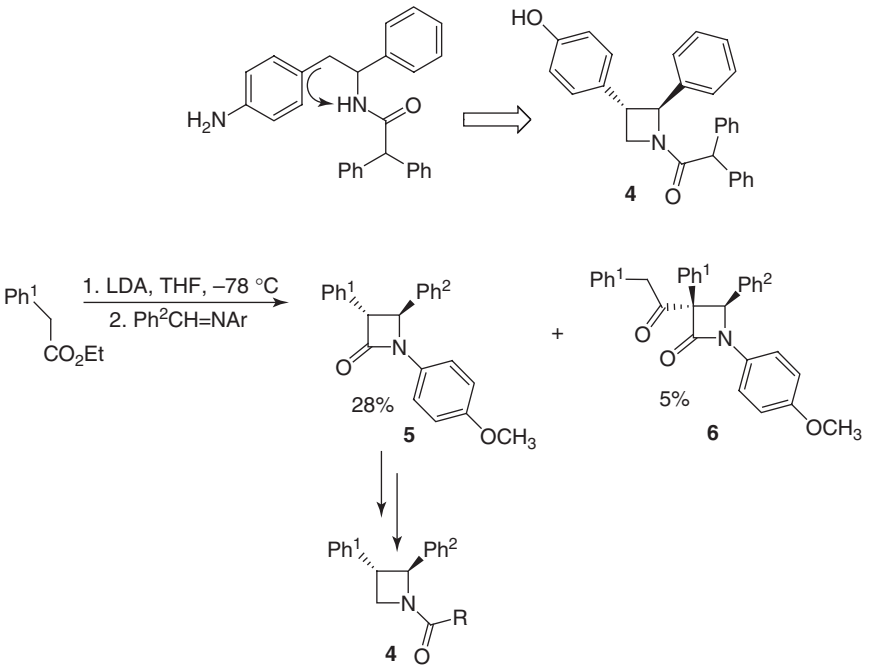
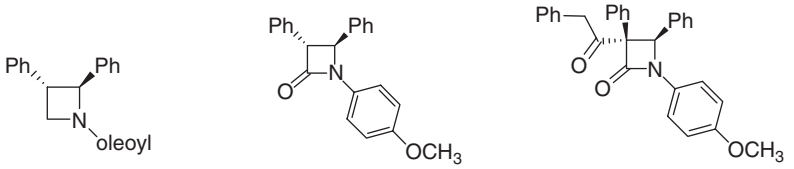


Figure 2 Design and synthesis of early azetidinones.



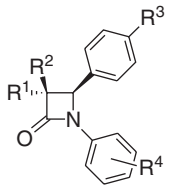
4	**5**	**6**
ACAT (25 μM): 4% Inh.	ACAT (25 μM): 64% Inh.	ACAT(25 μM): 83% Inh.
		IC_{50}: 7.5 μM
Hamster (100 mg kg^{-1})	Hamster (100 mg kg^{-1})	Hamster (100 mg kg^{-1})
SC: NE	SC: NE	SC: –10%
CE: NE	CE: NE	CE: –26%

Figure 3 In vitro and in vivo activity of first azetidinones.

in vivo activity of compounds. Based on this observation, Burnett *et al.* opted to disregard the ACAT activity of subsequent analogs and focus on optimizing the in vivo activity of compounds, guided solely by activity in the cholesterol-fed hamster. The absence of an in vitro assay clearly made this an extraordinarily challenging medicinal chemistry effort. Nonetheless, this work culminated in the discovery of azetidinone **13** and its resolved form **14**, the prototype azetidinone cholesterol absorption inhibitors and the starting point for all subsequent work. In an interesting portent of things to come, the difference between **13** and less active analogs such as **12** is the addition of a single well-placed methoxy group at the C4 phenyl. Figure 5 shows the in vitro and in vivo profile of **14**.[21]

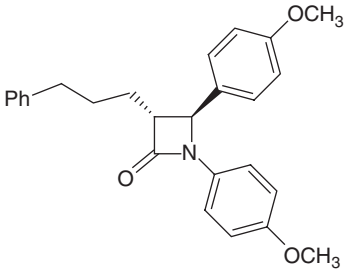
In addition to blocking accumulation of hepatic CEs in the hamster, **14** reduces SC levels in the cholesterol-fed rat, dog, and monkey. Of particular significance is the activity in the cholesterol-fed rhesus monkey. Figure 6 shows the effects of **14** on rhesus monkeys fed a high-cholesterol diet for 4 weeks.[21] Control animals show a profound hypercholesterolemia after 3 weeks which is completely blocked by 1 mg^{-1} kg dose of **14** administered in diet. When the control animals are then treated with 1 mg^{-1} kg of **14**, their cholesterol levels return to nearly baseline within 1 week, while withdrawal of drug causes a gradual rise in cholesterol levels over the same time period.

While ACAT inhibitors are known to inhibit cholesterol absorption in rodents, such potent antihypercholesterolemic activity in nonrodents was unprecedented with ACAT inhibitors. In addition, Salisbury *et al.* demonstrated that ACAT



Compound	R^1	R^2	R^3	R^4	ACAT IC_{50}	Dose (mg kg^{-1})	% Change SC	% Change CE
6	$PhCH_2CO$	Phenyl	H	4-CH_3O	7.5 μM	50	−10	−26
7	Phenyl	Ethyl	H	4-CH_3O	>10 μM	50	−15	−39
8	$Ph(CH_2)_3$	Ethyl	H	4-CH_3O	>10 μM	50	−12	−29
9	*n*-$C_{10}H_{21}$	H	H	4-CH_3O	>10 μM	50	NE	NE
10	$Ph(CH_2)_3$	Phenyl	H	4-CH_3O	6 μM	50	−11	−16
11	Ethyl	$Ph(CH_2)_3$	H	2,4,6-CH_3O	1.7 μM	50	NE	−31
12	$Ph(CH_2)_3$	H	H	4-CH_3O	>10 μM	50	NE	−15
13	$Ph(CH_2)_3$	H	CH_3O	4-CH_3O	18 μM	10	−21	−60
14	(R)-$Ph(CH_2)_3$	H	(S)-CH_3O	4-CH_3O	26 μM	10	−43	−93

Figure 4 Early azelidinone structure–activity relationships.



14

ACAT: IC_{50} = 26 μM
Cholesterol-fed animal models:

ED_{50}:	Hamster:	2.2 mg kg^{-1}[a]
	Rat:	2.0 mg kg^{-1}
	Monkey:	0.2 mg kg^{-1}
	Dog:	0.1 mg kg^{-1}

[a] Endpoint in hamster is hepatic cholesteryl esters, others are total plasma cholesterol.

Figure 5 Profile of **14** in cholesterol–fed animal models.

inhibitors and **14** have different effects on intracellular cholesteryl levels.[21] In these experiments, the ACAT inhibitor **3** blocked the accumulation of ^{14}C-cholesteryl esters but had no effect on free cholesterol in the intestinal wall of cholesterol-fed hamsters. By contrast, **14** inhibited the accumulation of both esterified and unesterified cholesterol. The reduction in cholesteryl ester levels was due entirely to reduction in free cholesterol substrate, while ACAT activity remained essentially unchanged. Thus, both the in vitro data and the in vivo pharmacology suggested that the azetidinone cholesterol absorption inhibitors act via a unique mechanism that is upstream from ACAT.

8.07.3 Defining the Nature of the Target

8.07.3.1 Structure–Activity Relationship on the Azetidinone Nucleus

Starting from the initial discovery of **14** by Burnett *et al.*, follow-up studies focused on establishing the SAR profile around the azetidinone nucleus.[20] While important in any medicinal chemistry effort, this was an especially critical early step in this program since it established that the observed in vivo activity followed predictable SARs despite the potential complications associated with use of an in vivo rather than an in vitro model. Among other things, these early studies confirmed the importance of the C4 *p*-methoxyphenyl moiety of **14** (Figure 7).

Activity decreases as the 4-methoxy group is moved to the 3- and 2-positions or is replaced by either a 3,4-dimethoxy or methylenedioxy group (**13–18**). On the other hand, adding a 2-hydroxyl group in addition to the

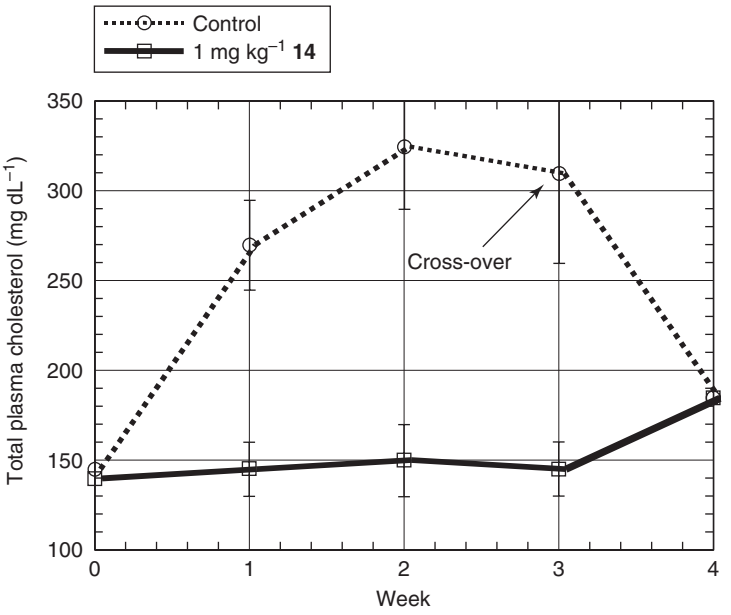
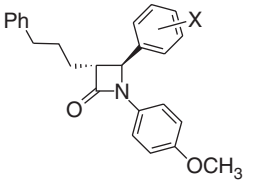


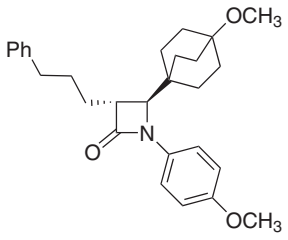
Figure 6 Effect of **14** in the cholesterol-fed rhesus monkey.



Compound	X	Dose (mg kg^{-1})	% Change CE	% Change SC
13	4-CH_3O	50	–78	–29
15	3-CH_3O	50	–24	–17
16	2-CH_3O	50	0	0
17	3,4-CH_3O	50	–33	–11
18	3,4-OCH_2O	50	–19	0
19	2-OH, 4-CH_3O	10	–95	–30
20	4-OH	50	–48	–16
21	3,4-OH	50	–39	0
22	CH_2OH	50	–46	–21
23	CH_2OCH_3	50	–56	–27
24	NMe_2	50	–60	–29
25	NH_2	50	–0	0
26	F	50	–12	0

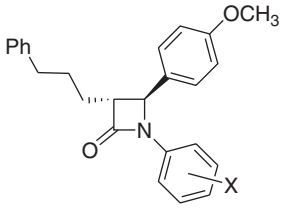
Figure 7 SAR on the C4 phenyl.

4-methoxy group (**19**) increases activity.[22] Some activity is retained when the 4-methoxy group is replaced by similar hydrogen bonding moieties (**20–25**), but is essentially abolished when this group is replaced by nonpolar groups such as fluorine (**26**). These observations are consistent with subsequent work by Van Heek *et al.* described below that suggests that phenol **20** is the bioactive form of **14**. More drastic changes to the C4 phenyl itself, i.e., **27**, cause a marked reduction in activity even in the presence of a methoxy substituent (Figure 8).



27
CE: NE at 20 mg kg^{-1}

Figure 8 Compound **27**.



Compound	X	% Change CE	% Change SC
28	3-CH_3O	–91	–38
29	2-CH_3O	–87	–49
30	3,4-OCH_2O	–52	–19
31	4-OH	–90	–35
32	H	–95	–40
33	F	–95	–38
34	CH_3	–90	–38

Figure 9 SAR on the N1 phenyl group.

Unlike the C4 position, the N-aromatic ring, while required for activity, is tolerant of a variety of substituents or no substituent while retaining good activity in the 7-day cholesterol-fed hamster model (**28–34**) (Figure 9).

An aromatic moiety directly attached to the azetidinone nitrogen is also required for good activity (Figure 10). Simple alkyls, aralkyls, N-H, and N-acyl derivatives **35–39** are devoid of activity, although the N-cyclopropyl derivative **40** retains some activity.

The remaining aromatic ring tethered to the azetidinone 3-position via a 3-carbon chain is also an important part of the pharmacophore, in that activity can be modulated by changing the tether length (Figure 11) (**41–44**) or nearly abolished by removing the aromatic moiety (**45**). Activity is also reduced by disubstitution at C-3 (**46–47**), which may be related to the conformational requirements of the tethering chain. Unlike the chiral center at C4, which shows a clear preference for the 4-*S* configuration, both the 3-(*R*) and 3-(*S*) forms often show comparable activity with no consistent preference.

One remaining issue concerned the importance of the azetidinone itself. Unlike drugs such as antibiotics or elastase inhibitors which are also built on an azetidinone nucleus, there is no evidence that a reactive azetidinone is required for cholesterol absorption inhibition. Compound **14** is completely unchanged after 7 days' incubation at 37 °C with 0.1 N benzylamine, while a variety of beta-lactam antibiotics are completely consumed by these conditions. The azetidinone nucleus of **14** is also stable in vivo, although interestingly its enantiomer is rapidly hydrolyzed in vivo to the amino acid **48**.[13] Neither enantiomer of **48** has appreciable CAI activity, a fact that confuses the interpretation of the apparent preference for the 4-*S* configuration. Although the azetidinone carbonyl is required for good activity, activation of the azetidinone by N-acylation with a variety of groups (e.g., **37**) essentially abolishes the cholesterol absorption inhibitory activity. On the other hand, some activity is retained by analogous gamma-lactams and related compounds **50–51** as well as sultams **52–53**, all of which suggests that the primary role of the azetidinone nucleus is to provide a suitable scaffold (Figure 12).[23]

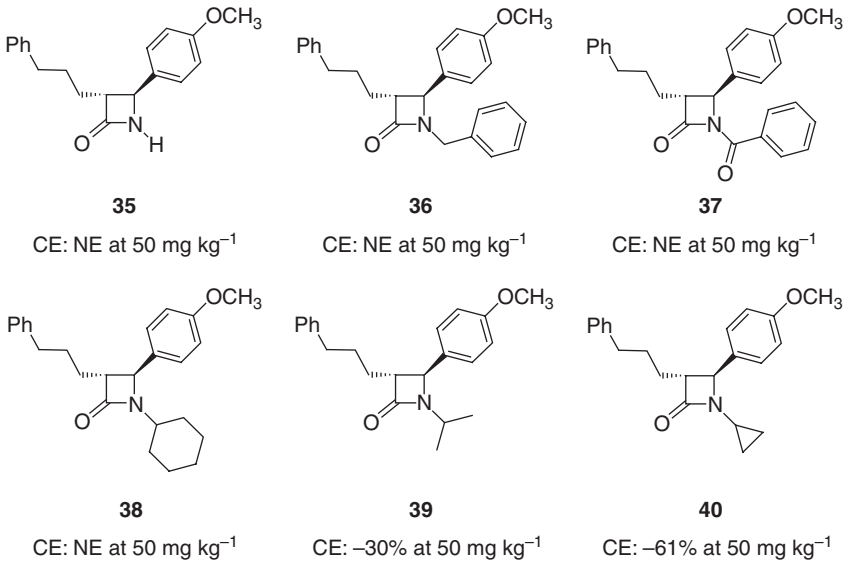
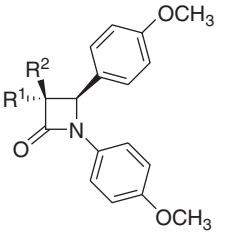


Figure 10 Modifications to the N1 phenyl group.



Compound	R^1	R^2	% Change CE	% Change SC
41	$Ph(CH_2)_2$	H	0	0
42	H	$Ph(CH_2)_2$	–19	0
43	$Ph(CH_2)_4$	H	–23	–8
44	H	$Ph(CH_2)_4$	–72	–28
45	$C_6H_{11}(CH_2)_3$	H	–28	–12
46	$Ph(CH_2)_3$	CH_3	–21	0
47	CH_3	$Ph(CH_2)_3$	–56	0

Figure 11 SAR at C3.

In the absence of an in vitro assay, it is impossible to unequivocally determine which of these effects on CAI activity reflect changes in intrinsic affinity for the unknown target of these compounds and which reflect purely pharmacokinetic influences. The striking difference in the metabolic stability of SCH 48461 versus its enantiomer is a clear example of the dangers of overinterpreting these data. Nonetheless, these initial studies of azetidinone CAIs demonstrated that activity in this series follows clearly defined SARs consistent with a well-defined molecular target. None of the compounds showed significant ACAT activity.

8.07.3.2 Rigid Analogs

Some follow-up studies focused on defining the active conformation of azetidinone CAIs, with the dual purpose of improving the potency of compounds as well as providing additional tools to understand the nature of the molecular target. That the conformation of the sidechain tether was important for activity was first suggested by the difference in activity of the *E*- and *Z*-propenyl derivatives **54** and **55** (Figure 13).[13] Additionally, the fact that potent compounds

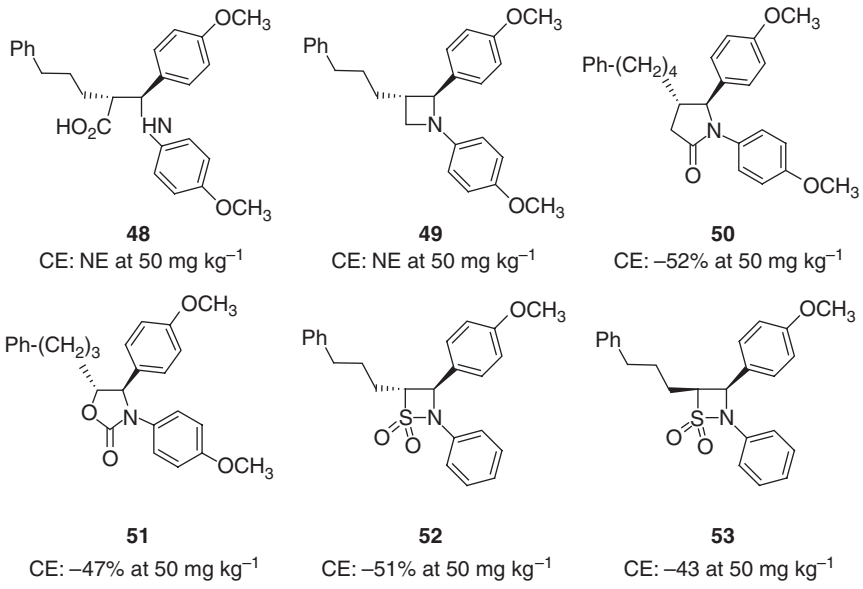


Figure 12 Nonazetidinone compounds.

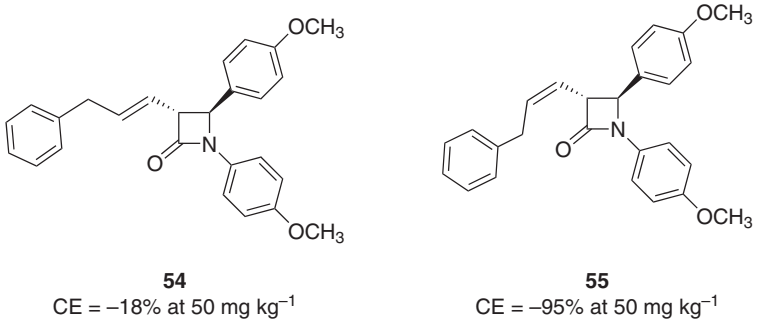


Figure 13 Activity of compounds **54** and **55**.

exist with either the 3-(*R*) or 3-(*S*) stereochemistry led Dugar *et al.* to propose that in the binding conformation, the tethered aromatic ring is located in a position in space that is equally accessible from both the 3-(*R*) and 3-(*S*) stereochemistries (Figure 14).[24] According to this model, the reduced potency of C3-disubstituted compounds results from steric hindrance that prevents the side chain from adopting the required conformation. Based on this model, they proposed a series of azaspirononanone derivatives which could exist in either a *syn* or *anti* form (Figure 14), both of which were consistent with the conformational model.

In practice, the *anti* derivative **56** (Figure 15) is slightly more active than **14** in the cholesterol-fed hamster model, while *syn* isomer **57** is much less active. Other aspects of the SARs in the spirocyclic series follow similar trends in the acyclic series, including the absolute stereochemistry and substitution pattern on the phenyl at the position equivalent to C4 of nonspirocyclic azetidinones. Unlike the acyclic compounds, enantiomers **60** and **61** in the spirocyclic series show reasonable metabolic stability, indicating that the stereochemical preference likely reflects differences in intrinsic activity. This provides some of the most compelling evidence that the azetidinone CAIs have a well-defined molecular target.

8.07.4 The Discovery of Ezetimibe

While the data discussed so far provided encouragement that the activity of this class of compounds could be optimized, the absence of an in vitro assay made it difficult to separate potential effects on intrinsic potency from effects on pharmacokinetics. This issue was confounded by the fact that **14** is extensively metabolized in vivo, making

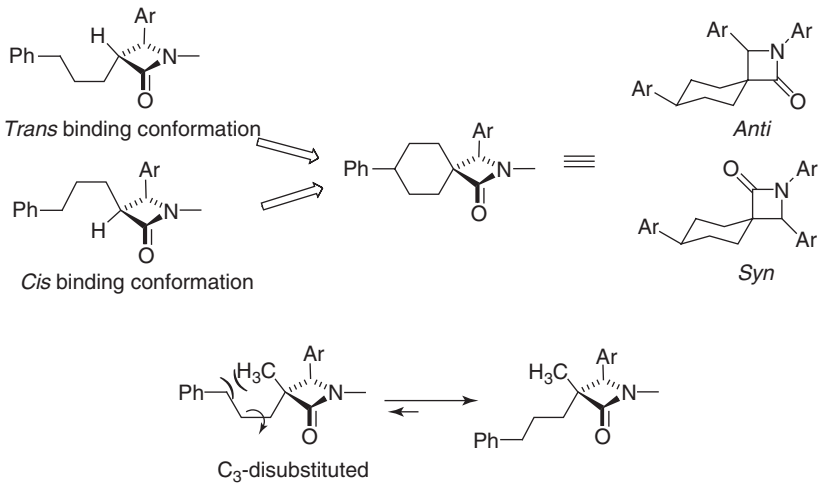


Figure 14 Conformational model leading to design of spirocyclic compounds.

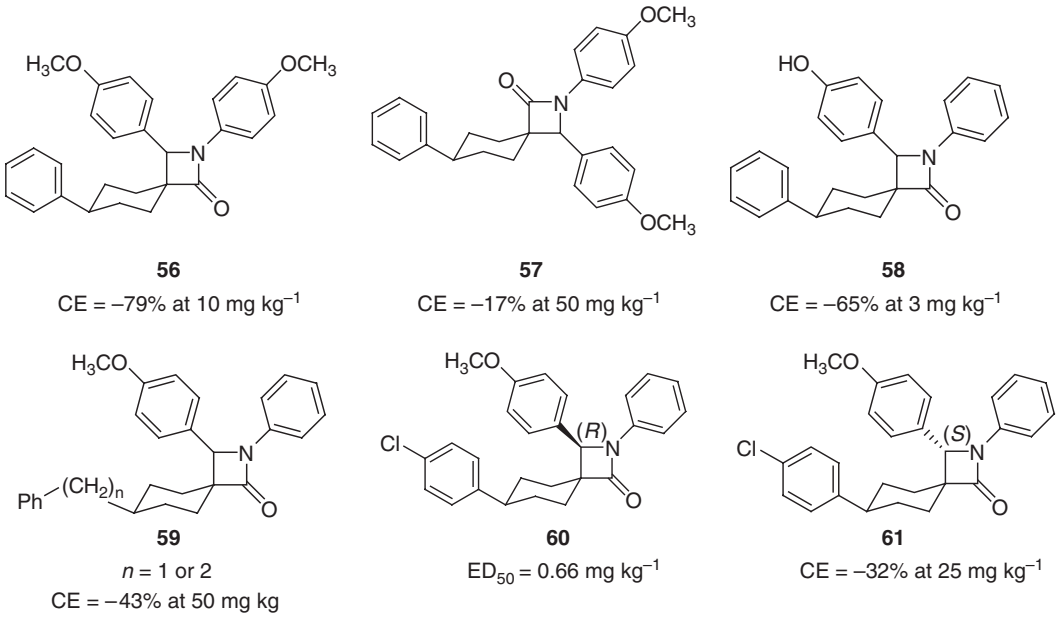


Figure 15 Spirocyclic derivatives.

the identity of the true active species unclear.[13] To address this latter issue, Van Heek and colleagues devised a unique experimental protocol designed to determine if there were active metabolites and how they contributed to the overall in vivo profile of **14**.[25] The experiment was divided into two parts, both of which used an intestinally cannulated, bile duct-diverted rat model (Figure 16).

In the first part of the experiment, animals were dosed via intraduodenal cannula with ^{3}H-**14**. Bile from these animals was collected via the bile duct cannula to give so-called 'metabolite' bile, which based on previous experiments was known to contain the majority of the metabolites of **14**. Concurrently, control animals were dosed with vehicle and their bile was also collected. ^{3}H-**14** was added directly to this bile to match the specific activity of the metabolite bile to give so-called 'parent' bile. In the second part of the experiment, both the metabolite bile and the parent bile were again dosed intraduodenally into a second group of diverted animals along with ^{14}C-cholesterol, and the counts of both ^{14}C and ^{3}H in various tissues were measured. In this way, both the pharmacological effects as well as the disposition of drug could be measured in a single experiment by following counts of ^{14}C and ^{3}H, respectively. Furthermore, because of

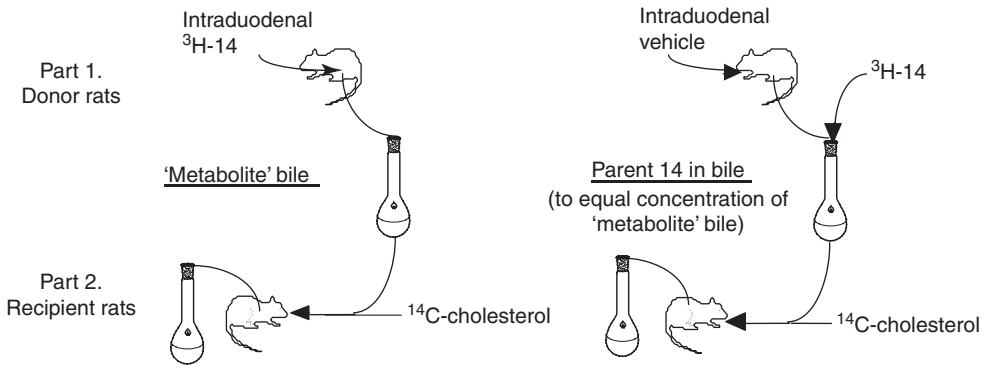


Figure 16 Intestinally cannulated bile duct-diverted rat protocol. (Reprinted with permission from Clader, J. W. *J. Med. Chem.* **2004**, *47*, 1–9. Copyright 2004 American Chemical Society.)

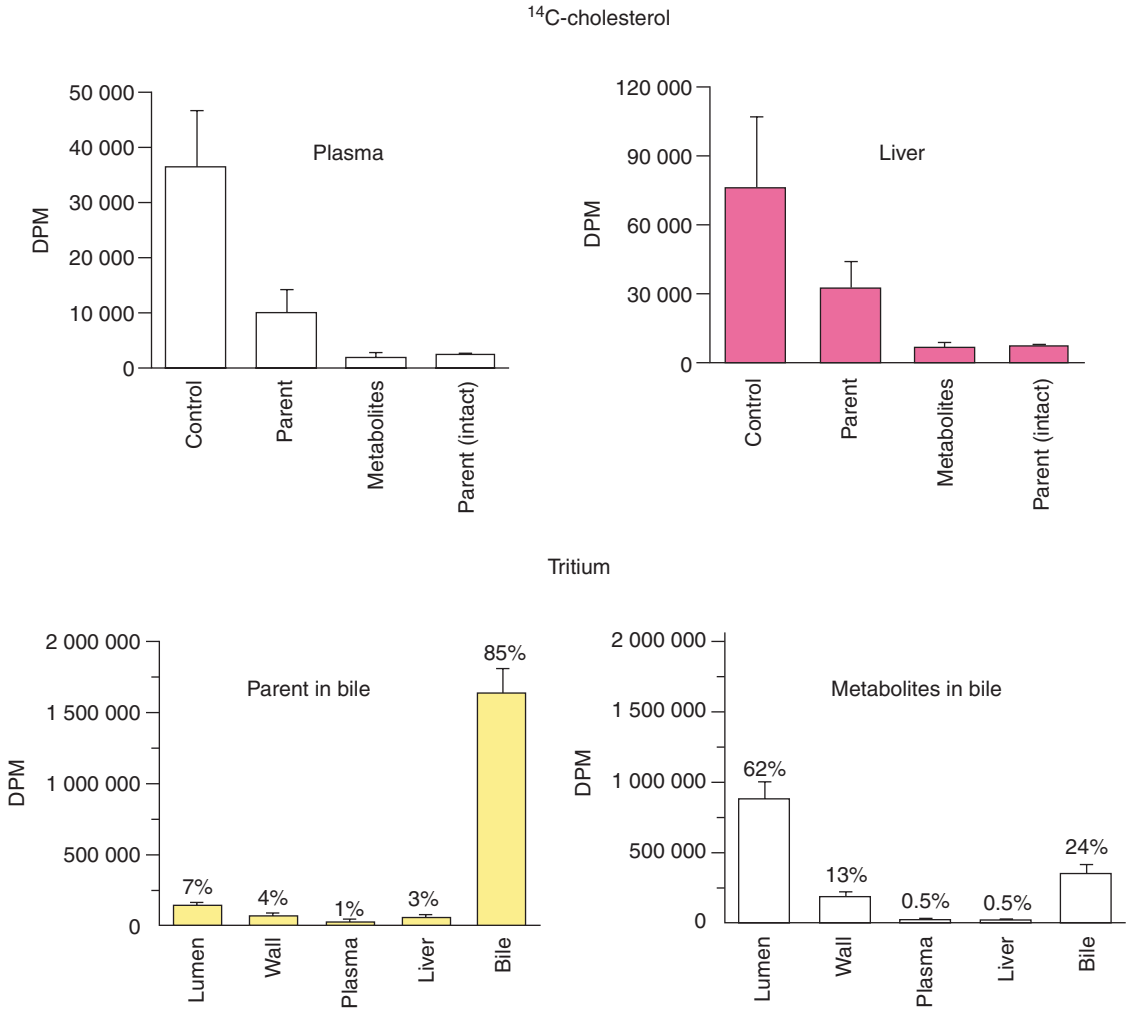


Figure 17 Distribution of ^{14}C-cholesterol and ^{3}H-**14** metabolites in bile duct-diverted rats. DPM, disintegrations per minute.

the bile duct diversion, the pharmacological effects reflected primarily the activity of the species being dosed and not the effects of any subsequently produced metabolites. In a third control arm of part 2, parent bile was dosed into intact, undiverted animals, where formation of active metabolites could still contribute to activity. Figure 17 shows the results of these experiments.

While the parent bile reduced the appearance of ^{14}C cholesterol in both the plasma and liver, the metabolite bile was clearly more effective and reduced ^{14}C levels comparably to the parent compound **14** in intact animals. These data strongly suggested that formation of one or more active metabolites plays a significant role in the in vivo activity of **14**. Consistent with this, when animals were dosed with parent bile the majority of the tritium counts were recovered in the bile of recipient animals. However, when ^{3}H metabolite bile was dosed, the majority of the counts remained in the intestinal wall and lumen. These data suggest that not only are there active metabolites but that these metabolites localize at the putative site of action more efficiently than **14** itself.

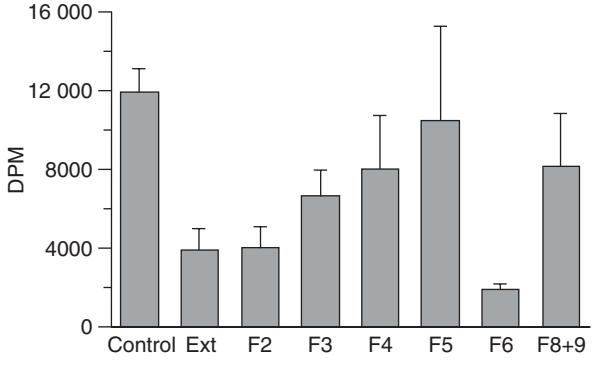
Clearly the next important question was the identity of these active metabolites. To address this, metabolite bile was fractionated by high-performance liquid chromatographs (HPLC) and each fraction was evaluated according to the paradigm described in part 2 above. The results of this experiment are shown in Figure 18. Based on the appearance of ^{14}C-cholesterol in plasma, these data showed that the bulk of the activity resided in fraction 6, and subsequent analysis showed that this fraction was composed primarily of the glucuronide of compound **20**, a phenolic metabolite of **14**.

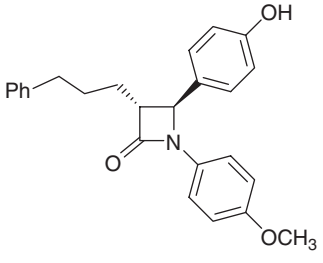
To complete the experiment, the activity of the metabolite bile, crude fraction **6**, and authentic **20** were compared in bile duct-diverted rats (Figure 19). These data show that the activity of **20** is identical to that of fraction 6 and both are substantially more active than the crude mixture of metabolites.

In total, these data strongly suggested that much of the in vivo activity of **14** was due to the formation of **20**. Furthermore, they suggested that metabolism of **14–20** helped to localize the compound in the intestines at the putative site of action of the compound.

The experiments by Van Heek *et al.* provided compelling evidence for the presence of at least one active metabolite of **14**. Nonetheless, there were also other less prominent metabolites whose formation could either contribute to the activity of **14** or could diminish it. To understand this, Rosenblum *et al.* prepared authentic samples of a number of known or putative metabolites which were evaluated for activity in the cholesterol-fed hamster (Figure 20).

Among the various putative metabolites of **14** were a variety of phenols produced by dealkylation of either or both of the methoxy groups or via aromatic hydroxylation. Both phenol **62** and bisphenol **63** showed substantial activity in the cholesterol-fed hamster, although neither was more active than **14** or **20**, suggesting that metabolism on the N-aryl moiety was not required for activity. On the other hand, phenol **64** was less active than **14**, suggesting that this route of metabolism might be detrimental to activity. In addition to the phenols, another route of metabolism involved hydroxylation of the 3-phenylpropyl side chain to produce alcohols and ketones. (*S*)-alcohol **65** was substantially more active than **14**, the (*R*)-alcohol **66** was less active, and the corresponding ketone **67** had intermediate activity. This





20

Figure 18 Efficacy of total bile extract (Ext) and fractionated bile.

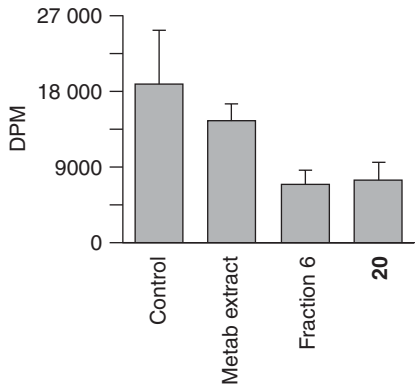
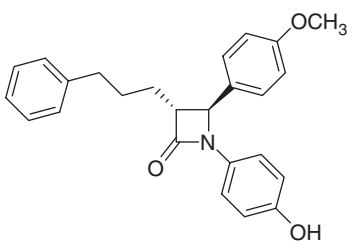
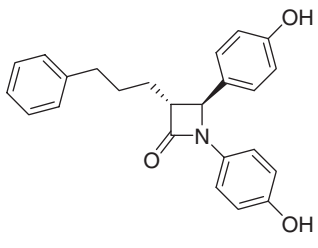


Figure 19 Efficacy of fraction 6 versus **20** in bile duct-diverted rats.



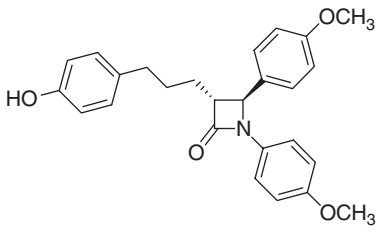
62

CE: –68% at 50 mg kg^{-1}



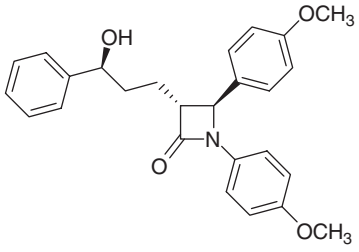
63

CE: –78% at 50 mg kg^{-1}



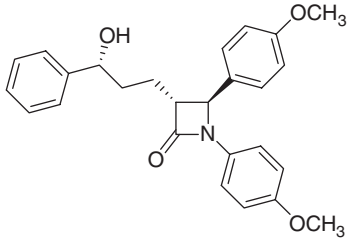
64

CE: –16% at 50 mg kg^{-1}



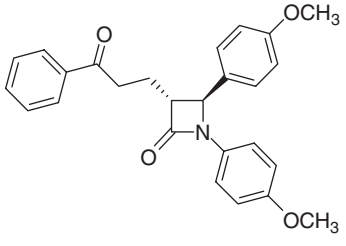
65

CE: ED_{50} = 0.9 mg kg^{-1}



66

CE: ED_{50} = 5.0 mg kg^{-1}



67

CE: ED_{50} = 1.9 mg kg^{-1}

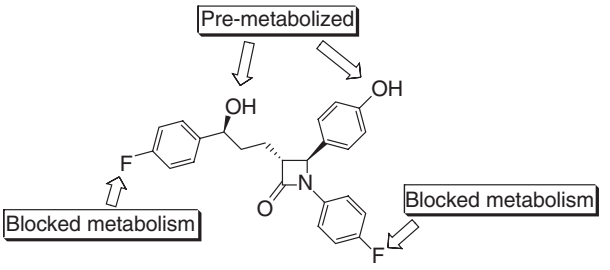
Figure 20 Activity of possible metabolites of **14** in cholesterol-fed hamster.

suggested that metabolism to the (*S*)-alcohol, if not required for activity, might improve the activity of the compound. Other combinations of these routes of metabolism were also investigated with similar results.

Based on the combined observations of experiments in bile duct-diverted rats and the activity of various putative metabolites, a strategy emerged for the design of a second-generation compound, namely:

1. Premetabolize profitable sites of metabolism on the C4 aryl and the phenylpropyl sidechain to improve activity, minimize plasma levels, and localize the compound in the intestines.
2. Block unprofitable sites of metabolism to maximize activity and limit further oxidative metabolism.

This strategy was in fact applied to a number of chemical series related to **14**,[26–29] but most successfully by Rosenblum *et al.* to give azetidinone **68**, now known as ezetimibe.[30]



68 (Ezetimibe)

ED_{50}:	**14**	Ezetimibe
Hamster:	2.2 mg kg^{-1}	0.04 mg kg^{-1}
Rat:	2.0 mg kg^{-1}	0.03 mg kg^{-1}
Monkey:	0.2 mg kg^{-1}	0.0005 mg kg^{-1}
Dog:	0.1 mg kg^{-1}	0.007 mg kg^{-1}
Blood levels:		<< 14

Figure 21 Comparison of structure and in vivo profile of **14** and ezetimibe. (Reprinted with permission from Clader, J. W. *J. Med. Chem.* **2004**, *47*, 1–9. Copyright 2004 American Chemical Society.)

Figure 21 compares the activity of ezetimibe to **14** in a number of cholesterol-fed animal models.[25] In every case, but most dramatically in the monkey, ezetimibe is substantially more active than **14** and shows substantially lower plasma levels.

8.07.5 Synthesis

A number of syntheses of ezetimibe and related compounds have been reported, several of which were utilized in the course of these investigations.[31–38] Many of these are based on an Evans-type oxazolidinone condensation to establish the correct stereochemistry on the azetidinone ring. Figure 22 shows a representative synthesis of ezetimibe. In addition to the use of the oxazolidinone, this synthesis also features a Corey oxazaborolidine reduction to set the (*S*) stereochemistry of the side chain hydroxyl group.

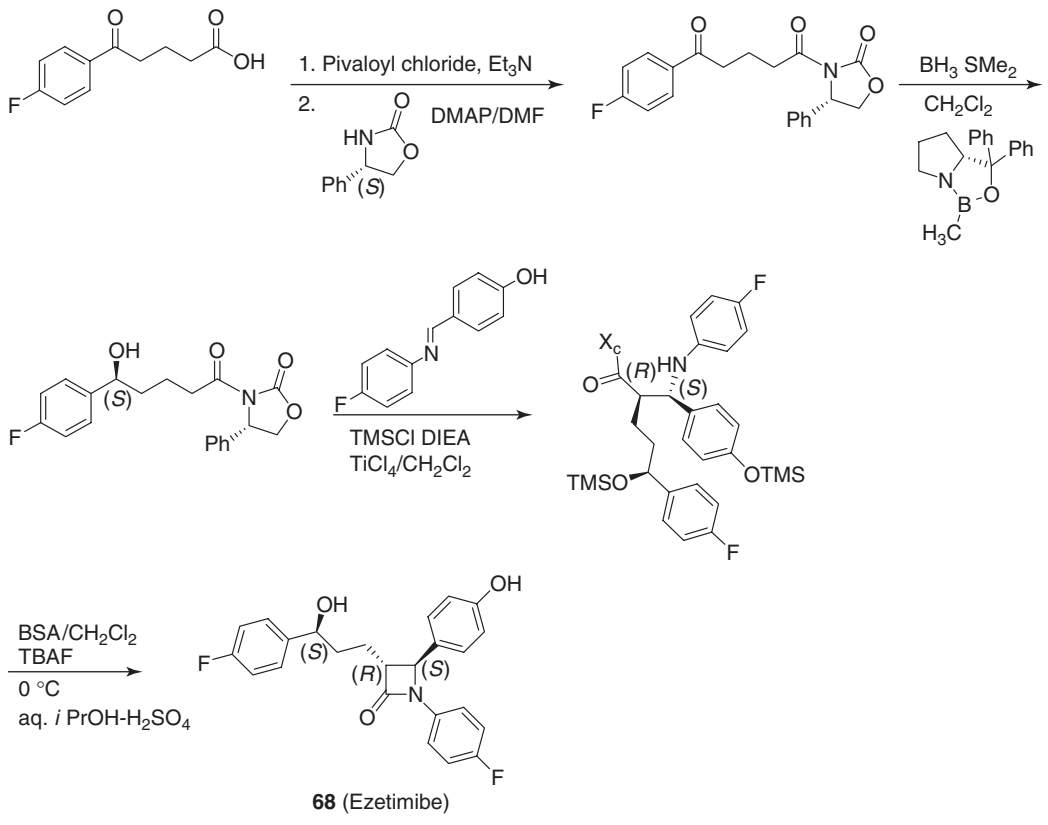
8.07.6 Ezetimibe and Statins

All of the in vivo data described thus far involve animals fed diets that are substantially higher in fat and cholesterol than the animals' normal chow diet. Despite the substantial activity of ezetimibe and other azetidinone CAIs in these models, none of the compounds tested significantly reduced plasma cholesterol levels in animals fed a normal chow diet. While this was initially a concern, this observation ultimately led to one of the most important aspects of the profile of ezetimibe. In considering the possible reasons for the lack of substantial effect on serum cholesterol in the absence of a high cholesterol diet, Davis reasoned that a CAI might stimulate hepatic hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase activity. This could compensate for a reduced cholesterol load due to inhibition of intestinal cholesterol absorption. If this were the case, then coadministration of a CAI and an HMG-CoA reductase inhibitor should produce an enhanced reduction in serum cholesterol at doses that were less effective or ineffective as monotherapy. To test this hypothesis, Davis *et al.* administered ezetimibe (0.007 mg kg^{-1}) or lovastatin (5 mg kg^{-1}) to chow-fed dogs over 14 days.[39] While neither compound had a substantial effect on serum cholesterol alone, the combination produced a profound reduction in serum cholesterol (Figure 23).

Similar experiments demonstrated a comparable effect in other species and with other statins. These data suggested that ezetimibe would be effective at reducing cholesterol levels in humans and would be particularly effective in combination HMG-CoA reductase inhibitors.

8.07.7 Clinical Results

Human clinical trials with ezetimibe supported the expectations of animal studies with ezetimibe both as monotherapy and in combination with statins.[40–43] Table 1 shows the results of phase III human trials with ezetimibe as



68 (Ezetimibe)

Figure 22 Representative synthesis of ezetimibe.

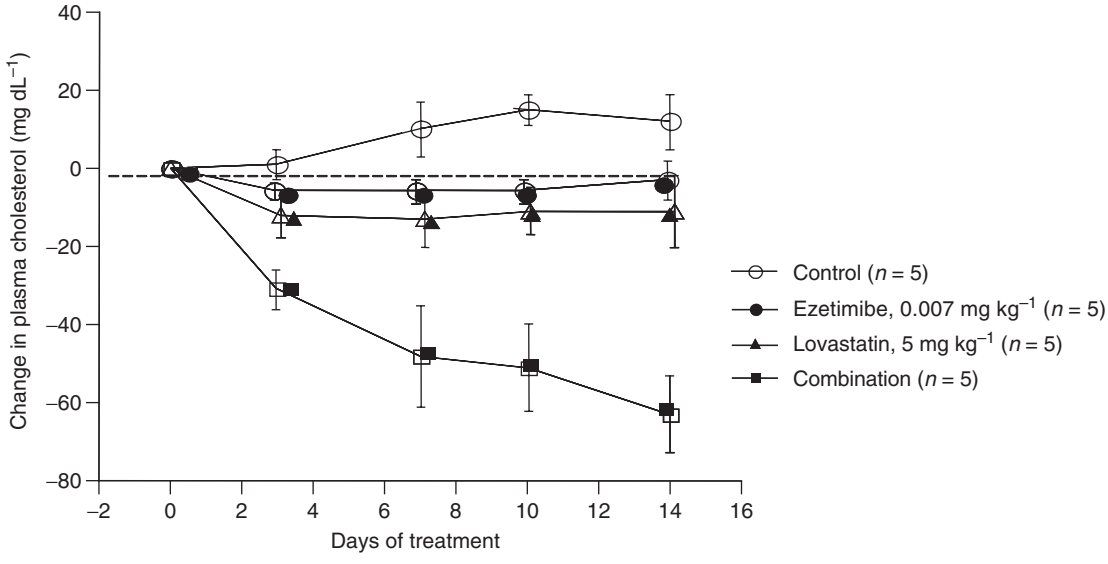


Figure 23 Effect of ezetimibe and lovastain in chow-fed dogs.

monotherapy. Ezetimibe produced a significant reduction in total cholesterol, LDL cholesterol, and triglycerides as well as a small but significant increase in HDL cholesterol.

Figure 24 compares the effect of simvastatin or atorvastatin either alone or when coadministered with ezetimibe on LDL cholesterol. In each case, ezetimibe produced an additional 15–18% reduction in LDL cholesterol above that

Table 1 Ezetimibe phase III monotherapy efficacy results

Treatment	*Mean % change from baseline at endpoint*			
	LDL cholesterol	*Total cholesterol*	*HDL cholesterol*	*Triglycerides*
Placebo ($n = 226$)	+ 0.4	+ 0.8	− 1.6	+ 5.7
Ezetimibe 10 mg ($n = 666$)	− 16.9*	− 12.5*	+ 1.3*	− 5.7*

*Significantly different from placebo ($p < 0.01$).

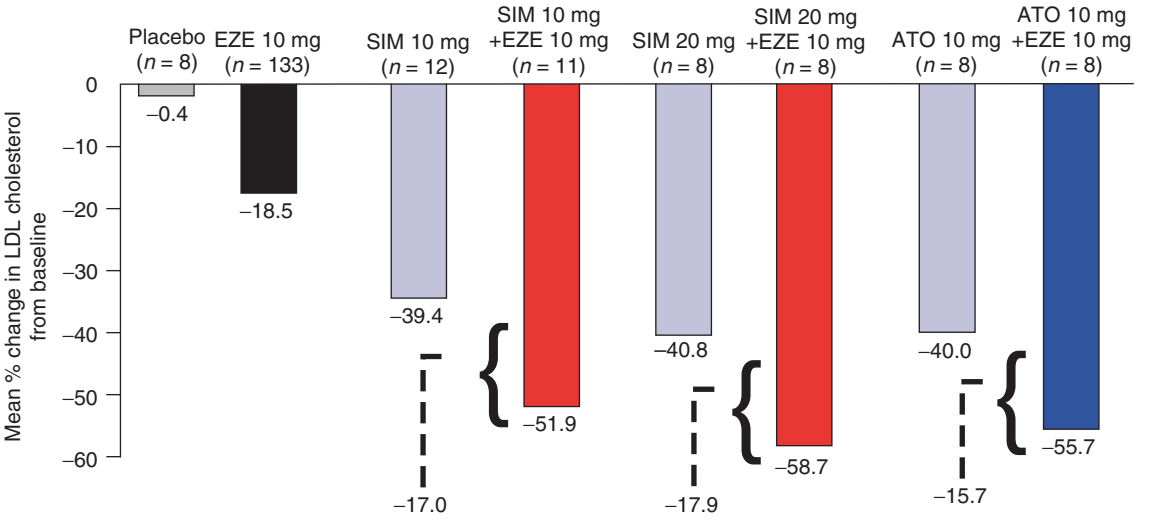


Figure 24 Ezetimibe (EZE) coadministered with simvastatin (SIM) or atorvastatin (ATO).

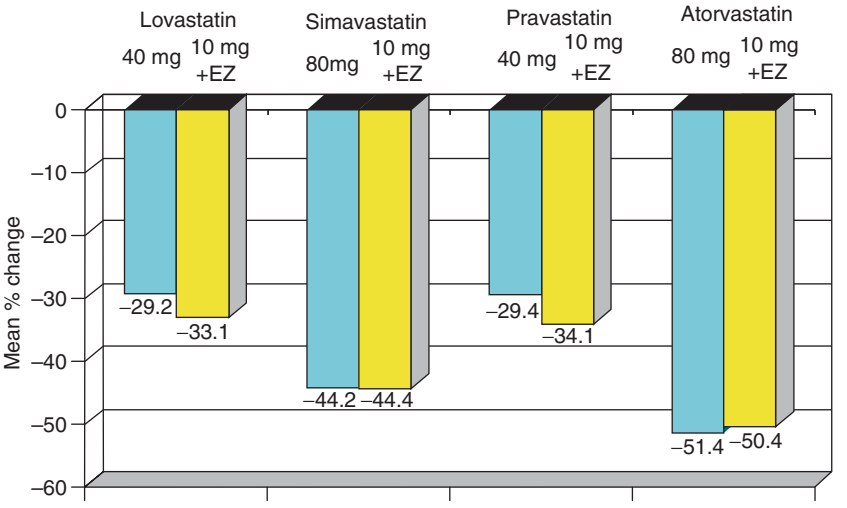


Figure 25 Effect of high and low dose coadministered with ezetimibe (EZ).

achieved by the statin alone. Finally, **Figure 25** compares the effect of a high dose of statin alone with a low dose of statin coadministered with ezetimibe. In each case, coadministration of ezetimibe and low-dose statin produced an equivalent reduction in LDL cholesterol as the high dose of statin alone. Combined, these data demonstrate that ezetimibe alone or coadministration with statins provides favorable effects on the major lipid parameters in patients with hypercholesterolemia.

8.07.8 Mechanism of Action Studies

Clearly one of the important goals in this area has been identification and characterization of the molecular target of these compounds and the establishment of an in vitro assay. Considerable progress has been made in this area since the discovery of ezetimibe, with the primary focus being on Niemann–Pick C1 Like 1 Protein (NPC1L1). Altman, Davis *et al.* have shown that NPC1L1 is critical for the uptake of cholesterol by intestinal enterocytes and is a key modulator of whole-body cholesterol homeostasis. NPC1L1-null mice were completely insensitive to ezetimibe, suggesting that this or an associated protein may be the molecular target of this class of compounds.[44,45] Recent data showing that several ezetimibe analogs bind to NPC1L1 provide compelling evidence that this protein is in fact the molecular target of ezetimibe.[46] The identification of this protein is an important scientific achievement not just for its role in promoting the development of this class of drugs but also for furthering our understanding of the mechanisms of cholesterol absorption and cholesterol homeostasis.

8.07.9 Conclusion

The past 30 years of drug discovery have seen a procession of breakthrough technologies designed to improve the efficiency and overall success rate of drug discovery programs. Ironically, the fact that drug discovery remains a difficult and risky endeavor despite the use of these technologies has caused some to question the value of these technologies compared to more traditional biologically driven approaches towards drug discovery, with ezetimibe often cited as a case in point. The technological advances of the past decades have unquestionably had a profound and positive impact on the discovery process, and new technologies such as genomics, which played a pivotal role in elucidating the mechanism of action of ezetimibe, will likely continue to shape how we discovery drugs in the future. The fact that ezetimibe was discovered without the benefit of many of these technologies is less a testament to the relative value of biological versus technological approaches than it is a reaffirmation of the continued importance of science and scientists in drug discovery.

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Biography

John W Clader received his PhD in organic chemistry in 1980 from Indiana University. After two years at the University of Notre Dame he began his career in medicinal chemistry at Hoffmann-La Roche in Nutley, New Jersey. He moved to the Schering-Plough Research Institute in 1985, where he is currently Distinguished Research Fellow in Medicinal Chemistry. In addition to ezetimibe, his research interests at Schering-Plough have included potential treatments for Alzheimer's disease and HIV infection as well as the application of chemoinformatics and other computer methods to facilitate drug discovery.

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8.08 Tamoxifen

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8.08.1 Background and Introduction

In 1896 George Beatson[1] demonstrated that removal of the ovaries from premenopausal women could cause the regression of breast cancer. By the turn of the century it was established[2] that about one-third of all premenopausal women with advanced breast cancer could benefit from oophorectomy and from that time, a principal strategy for the treatment and prevention of breast cancer has been either to block or to restrict the action of estradiol in its target tissue, the breast. However, the successful clinical development of the antiestrogenic drug tamoxifen did not initially focus on the therapy for breast cancer but evolved to this application by drawing upon expertise in several unrelated disciplines. Most of the early interest in antiestrogens was focused on reproductive endocrinology but it was clear from the beginning of clinical studies that the effects of the drugs on cholesterol biosynthesis would play a pivotal role in assessing safety considerations for long-term therapy. Ultimately the discovery of the estrogen receptor[3,4] in the 1960s and the application of this basic knowledge to understand hormone-dependent breast cancer growth[5] focused interest on the development of tamoxifen as a targeted agent to block estrogen action in the tumor directly.

8.08.2 Nonsteroidal Antiestrogens

Tamoxifen was not the first antiestrogen but the value of the drug slowly increased as the fashions in research changed. During the late 1950s and throughout the 1960s there was a focus on the development of new contraceptives in the wake of the success of oral contraceptives. These medicines did not treat a disease but altered lifestyle so there was huge potential for widespread use. But the application of antiestrogens as contraceptives failed and the compounds were drugs looking for a disease to treat! This perspective changed following the start of the 'War on Cancer' declared on 23 December 1971. There were now incentives to conduct translational cancer research and introduce new treatments. Regrettably, the process was slow for the introduction of targeted treatments as all hopes were initially pinned on combination cytotoxic chemotherapy to cure cancer.

In 1958, Lerner and coworkers at the William S. Merrell Company reported the biological properties of the first nonsteroidal antiestrogen, MER25 (**Figure 1**).[6] The discovery of nonsteroidal antiestrogens was an example of serendipity and is best described in Lerner's own words[7]:

> As 1954 was drawing to its end a triphenylethanol compound was synthesized, not for the purpose of investigation for estrogen antagonism but for testing by the cardiovascular research section at Merrell since it had been

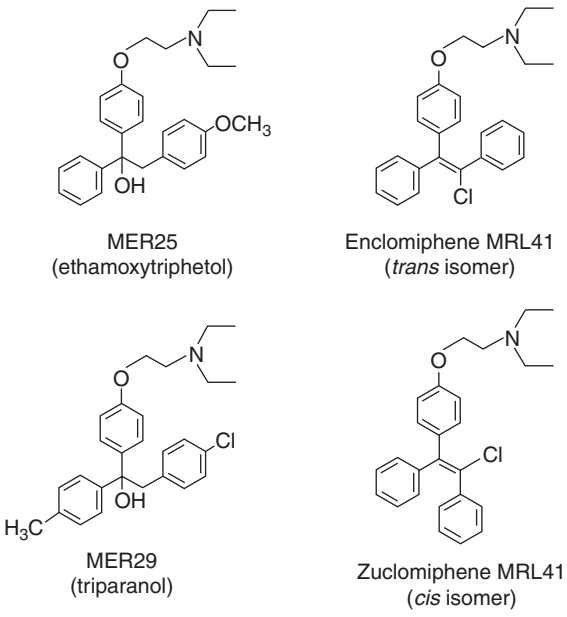


Figure 1 The formulae of the first nonsteroidal antiestrogen, MER25, and the structurally related compound MER29 used to lower circulating cholesterol. MER29 was withdrawn from the market because of the increased incidence of cataracts. The next antiestrogen, MRL41, was a mixture of the *cis* and *trans* isomers of a substituted triphenylethylene later named clomiphene.

reported that a related compound had some effect on blood flow. A request for a sample of that compound for study as a possible estrogen antagonist was answered by the cardiovascular system pharmacologist with his entire supply since it was essentially inactive in his studies. This compound, 1-(*p*-1 diethylamino-ethoxyphenyl)-2(*p*-methoxyphenyl)-1-phenylethanol was tested in immature mice at the arbitrary 3-day screening dose of 5 mg. It was administered subcutaneously twice daily for three days alone or in combination with 0.03 µg of estradiol benzoate, and the uterine weight and intraluminal fluid served as the end points to be measured on the day after the last treatment. The results of this study were highly questioned since neither the uteri of the mice administered the compound alone nor the uteri of the animals receiving the compound plus estrogen were significantly heavier than those of controls treated with olive oil vehicle alone. It was thought that this was a 'bad study.' The compound, however, was retested and the results were identical to those of the first study. The increase in uterine weight and intraluminal fluid by estradiol treatment was completely prevented by simultaneous administration of the compound that was eventually to be called MER25 or ethamoxytriphetol.

The compound was found to be an antiestrogen in all species tested and was found to have no other hormonal or antihormonal properties. However, the discovery MER25 was considered to be of importance at the time because the compound was a postcoital contraceptive in laboratory animals.[8–10] Obviously, one application could have been as a 'morning-after pill' but after clinical evaluation in numerous situations the results were disappointing. MER25 underwent initial evaluation for the induction of ovulation[11] and the treatment of chronic cystic mastitis, breast and endometrial carcinoma[12,13] but the low potency and severe side effects on the central nervous system prohibited further clinical development.[7]

It is relevant to point out that the antiestrogen MER25 is a structural derivative of the cholesterol-lowering drug triparanol (MER29) (**Figure 1**). In the late 1950s there was initial enthusiasm about the potential benefits of triparanol as a hypocholesterolemic drug.[14] However, the finding that triparanol caused an accumulation of desmosterol (an intermediate in cholesterol biosynthesis)[15–18] and the linking of this biochemical effect to cataract formation,[19–21] caused withdrawal of the drug in 1962 (**Figure 2**). Nevertheless, triparanol was first evaluated as a potential therapy for breast cancer[22] but again the results were disappointing.

A successor compound to MER25, MRL41 or clomiphene (**Figure 1**), was a more potent antiestrogen but drug development for long-term use was to be retarded because of toxicological concerns. Clomiphene is an effective antifertility agent in laboratory animals[23] but paradoxically induces ovulation in subfertile women.[24–26] Again, the prospect of developing a 'morning-after pill' for women was not realized. Although clomiphene showed some activity in

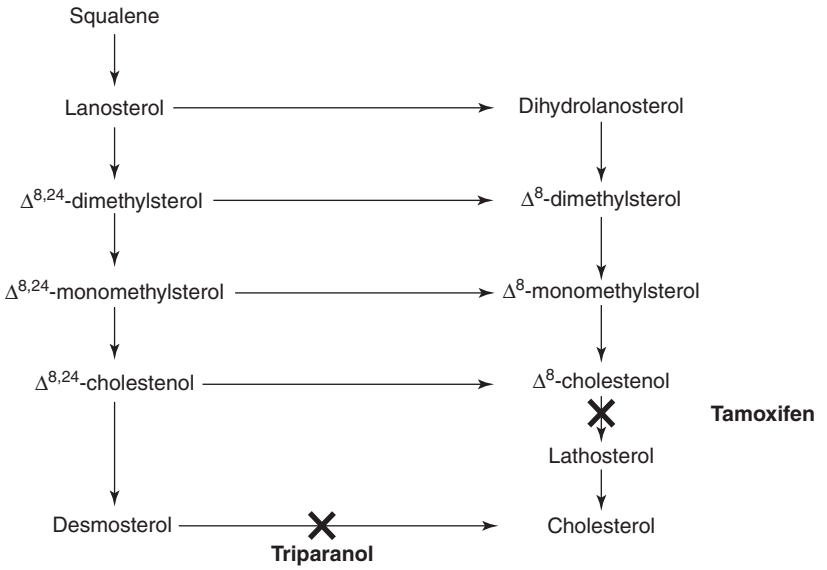
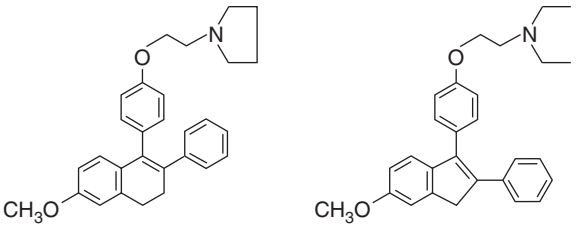


Figure 2 The mechanism of action of triparanol is to inhibit cholesterol biosynthesis but also to increase demosterol levels which is implicated in cataract formation. In contrast, tamoxifen has an alternate mechanism of action to lower cholesterol levels.



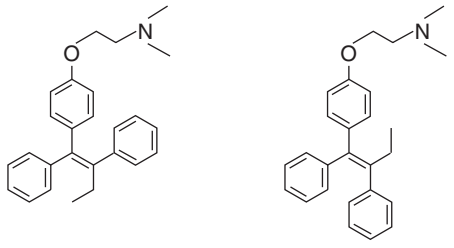
U-11,100A (nafoxidine) U-11,555A

Figure 3 The formulae of compounds discovered by the Upjohn Company as antifertility agents. Nafoxidine subsequently was tested as a therapy for breast cancer but development was not pursued because of toxic side effects experienced by the majority of patients. Two observations merit comment. The fixed ring structure confirmed the idea that a '*trans*-like' structure was necessary for potent antifertility and, later, antiestrogenic activity. The compound U-11,555A became a template for numerous agents developed throughout the 1990s and into the twenty first Century.

the treatment of advanced breast cancer,[27,28] the drug was only developed for short-term use for the induction of ovulation[29] because desmosterol was noted in patient sera during prolonged treatment.[30]

Clomiphene is marketed as an impure mixture of geometric isomers (**Figure 1**) which have opposing biological activities: one isomer is an estrogen and one isomer is an antiestrogen.[31] Unfortunately the isomers were initially (1967) given the incorrect designation but this was corrected by 1976.[32] Although breast cancer clinical trials were still being reported with the impure mixture of isomers in 1974,[28] the antiestrogenic isomer eventually entered into clinical trial for breast cancer treatment but the studies were dropped by the National Institutes of Health (NIH) due to the interest in tamoxifen (**Figure 2**).[33]

In contrast, the compound nafoxidine (U-11,100A)[34,35] and U-11,555A[36] are dihydronaphthalene and indene derivatives respectively and therefore cannot isomerize (**Figure 3**). The drugs are potent antiestrogens with antifertility properties in laboratory animals.[37–39] Subsequent studies by Terenius[40] demonstrated tight and somewhat irreversible binding of nafoxidine to the estrogen receptor derived from mouse target tissues. Nafoxidine exhibits antitumor properties in laboratory models of kidney cancer in the hamster[41] and the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model[42] but following extensive testing as a treatment for breast and renal cancer, the drug was not developed further because of unacceptable side effects experienced by all patients.[43,44]



ICI46,474 (tamoxifen) ICI47,699 (*cis* isomer)

Figure 4 The formula of the mixed estrogen/antiestrogen ICI46,474 and its estrogenic isomer ICI47,699 discovered in antifertility screens at ICI Pharmaceuticals Division. The *trans* isomer designated ICI46,474 was eventually developed as the breast cancer drug tamoxifen (Nolvadex).

8.08.3 The Discovery of ICI46,474

Although the discovery of ICI46,474 (tamoxifen) (**Figure 4**), the antiestrogenic, pure *trans* isomer of a substituted triphenylethylene, was made by Harper, Richardson, and Walpole[45–47] as part of the Fertility Control Program at ICI Pharmaceuticals (now AstraZeneca), Cheshire, UK, the study of cancer therapies was Walpole's long-term interest.[48]

In the late 1940s Walpole was a member of staff at ICI's Dyestuffs Division Biological Laboratory in Wilmslow, Cheshire. This establishment was the fledgling predecessor of the Pharmaceuticals Division Research Laboratories built in 1956–57 at Alderley Park near Macclesfield, Cheshire. Walpole was asked to establish animal models for the bioassay of potential alkylating agents[49–54] to evaluate compounds as bladder carcinogens[55] and to assess the potential health hazards for workers in the dyestuffs industry.[56] Walpole made the important discovery that tris-ethyleneimino-*S*-triazine (M9500) was an active anticancer agent in the Walker rat carcinoma 256[50] and conducted extensive structure–activity relationship studies with many mono and bifunctional compounds. Although tris-ethyleneimino-*S*-triazine is now only of historical interest, the related compound, hexamethylmelamine (M10,567) was also found to be active by Walpole.[52] Hexamethylmelamine is active in a broad spectrum of tumors including ovarian carcinoma resistant to other alkylating agents. However, Walpole's interest in alkylating agents and carcinogenesis provides only part of the background which led to his suggestion that ICI46,474 should be tested in breast cancer. Walpole was also interested in estrogens and was aware in 1963 that antiestrogens could be used for the treatment of breast cancer. The story of ICI's involvement with the hormonal treatment of breast cancer goes back to the early 1940s following the laboratory discovery by others that chlorotriphenylethylene is an orally active estrogen.[57] ICI supplied the triphenylethylenes initially used by Haddow, Watkinson, and Paterson in their landmark study of the antitumor effects of synthetic estrogens in advanced breast and prostate cancer.[58] These studies paved the way for the standard use of high-dose estrogen therapy to treat both breast and prostate cancer for the next two decades. In 1949, Walpole and Paterson[59] studied the antitumor actions of nonsteroidal estrogen therapy on breast cancer in the hope of finding the reason why some patients responded and others did not. These early studies by Walpole were unsuccessful but the subcellular mechanism of hormonal-dependent growth was eventually discovered by Jensen with his pioneering work on the estrogen receptor.[3,60]

8.08.4 Studies Published by Scientists at ICI Pharmaceuticals Division

The focus of the fertility control research program at ICI Pharmaceuticals Division was to investigate the physiology of reproduction in laboratory animals, use interesting antihormonal agents to block the reproductive process and to propose possible clinical evaluations of novel compounds.[45,46,61–77]

The work of others, testing compounds from competing pharmaceutical companies, established the activity and potential value of antiestrogens for the induction of ovulation in subfertile women.[12,13,24,26,78–80] Cancer therapy was a remote possibility as the treatment strategy at the time for advanced (metastatic) breast cancer was either endocrine ablation or additive high-dose hormonal therapy.[81] Although additive hormonal therapy was cheap, only one in three patients responded and the average response duration was 1 year. Nevertheless, a few unsuccessful attempts to test antiestrogens had occurred (**Table 1**).

Walpole's team of endocrinologists concentrated exclusively on the study of ovulation so that it could be prevented. 'No egg no pregnancy,' to quote Gregory Pincus, the Director of the Worcester Foundation, when describing how oral contraceptives worked. Additionally, the team at ICI Pharmaceuticals Division studied implantation so that the process

Table 1 Preliminary clinical trials of antiestrogens for the treatment of metastatic breast cancer

Compound	*Reference*	*Daily dose (mg)*	*Total patients (% response)*	*Toxicities*
Triparanol	Kraft (1962)[22]	250–1000	8(11)	None[a]
MER25	Kistner and Smith (1960)[12]	500–4500	4(25)	Acute psychotic episode[b]
Clomiphene	Herbst *et al.* (1964)[27]	100–300	56(34)	None reported or mild[c]
	Hecker *et al.* (1974)[28]			
Nafoxidine	Legha *et al.* (1976)[44]	180–240	198(31)	Bilateral cataracts, ichthyosis Cutaneous photophobia[d]
Tamoxifen	Cole *et al.* (1971)[87]	20–40	114(31)	Transient thrombocytopenia[e]

[a] Withdrawn from the market by the William S. Merril Co. in cooperation with the Food and Drug Administration in April 1962.
[b] Does not include patients treated by Dr Roy Hertz when therapy was stopped due to hallucinations.[7]
[c] Visual symptoms.[30]
[d] Affecting 80–100% of patients.[44]
[e] "The particular advantage of this drug is the low incidence of side effects"[87]; "The side effects were usually trivial."[88]

could be prevented to avoid pregnancy. Walpole, Harper, and subsequently Labhsetwar all published extensively throughout the 1960s on the physiology of reproduction and the use of inhibitory compounds, discovered through a screen in vivo, to dissect the mechanisms of the reproductive process in animals.

The compound ICI46,474 was discovered in the screening process for antifertility agents in the rat. Many of the laboratory studies with ICI46,474 focused on the application of an antiestrogen to confirm that the implantation of the blastocyst in the rat requires an estrogen surge on day 4 following mating.[67,68] In other words, an understanding of reproductive mechanisms was the primary goal of the research group. ICI46,474 was subsequently shown to be an antiestrogen in the rat but an estrogen in the mouse (**Figure 4**). The *cis* geometric isomer ICI47,699 (**Figure 4**) was an estrogen in the rat and the mouse.[45] Only at the end of Walpole's career at ICI Pharmaceuticals Division did interest turn to the interaction of compounds with the estrogen receptor. The geometric isomers of tamoxifen and clomiphene all inhibited the binding of tritiated estradiol to rat and mouse estrogen receptor derived from uterus and pituitary gland.[69] Unfortunately, no firm conclusion could be drawn to explain estrogen/antiestrogen action in different species. This species difference in the pharmacology of antiestrogens also raised the question of whether ICI46,474 would be an estrogen or an antiestrogen in humans. Estrogens were already used in the treatment of breast cancer but an antiestrogenic compound would be of value clinically because there may be fewer side effects. Nevertheless, based on the earlier experience at Merrill in the USA, only compounds that did not cause an increase in desmosterol could be used for long-term treatment in humans.

The reproductive endocrinology team advanced clinical testing in several areas primarily endocrinology and gynecology. Indeed in 1972, Walpole reviewed all of the progress in advancing ICI46,474 to become a clinically useful drug. These data were either just published at the time or subsequently published a few years later.

There was, at the time, an ever-expanding literature on the use of clomiphene for the induction of ovulation in subfertile women.[82] ICI46,474 was successfully tested as an agent for the induction of ovulation[83,84] and was approved for use in clinical practice in 1975 in the UK and several other countries around the world. Additionally, ICI46,474 was noted to block the uptake of ^{3}H-estradiol in the human uterus in vivo[85] and had some benefit in diminishing bleeding for patients with menometrorrhagia.[86] As noted earlier, Walpole was interested in cancer therapy and had connections at the Christie Hospital in Manchester.[59] A small preliminary study using ICI46,474 to treat late breast cancer showed some benefit in 10 of 46 patients[87] and in a preliminary dose response study[88] found that 12 out of 33 (33%) of patients receiving 10 mg twice daily and 14 out of 35 (40%) of patients receiving 20 mg twice daily had definite responses.

At that time, in 1973, there was little or no enthusiasm at ICI Pharmaceuticals Division to pursue a major program of drug development for the treatment of breast cancer. Walpole, in contrast, was optimistic about exploiting ICI46,474 as a breast cancer drug and agreed not to take early retirement if the antiestrogen was supported for clinical approval. This was achieved in the UK through the Committee for Safety of Medicines in 1973 and in December 1977 by the Food and Drug Administration (FDA) in the United States.

The reasons for the reluctance to pursue global development of ICI46,474 were the perceived vulnerability of the product in the absence of a patent in the US market and the assessment, which was correct at the beginning of the 1970s, that there was virtually no market. Only one in three patients with advanced breast cancer would respond to endocrine therapy for about a year. This market would amount to no more than £50 000 of sales per annum and the competitor products of high-dose estrogen or androgen cost only pennies per dose whereas the new antiestrogen ICI46,474 would, by necessity, have to cost 10–20 times more per dose. The only advantage for ICI,46,474 was a reduction in adverse side effects (**Table 1**).

The scientists at ICI Pharmaceuticals Division did not conduct any systematic study of the mechanism of action or antitumor properties of ICI46,474. These studies were conducted outside ICI Pharmaceuticals Division with academic collaborators (the process was advanced through the good offices of Walpole who remained as a consultant for ICI after his retirement in the early 1970s). Laboratory programs were established at the Worcester Foundation for Experimental Biology in Shrewsbury, MA (1972–74) in collaboration with ICI Americas (Stuart Pharmaceuticals) and subsequently at Leeds University, UK (1974–79) with a formal Leeds University/ICI joint research scheme. Tamoxifen was to be reinvented as a pioneering targeted antihormonal treatment for breast cancer by using appropriate laboratory models to create a scientific basis for pursuing rational clinical trials.

8.08.5 Patenting Problems

Adequate patent protection is required to develop an innovation in a timely manner. In 1962, ICI Pharmaceuticals Division (now AstraZeneca and formerly Zeneca) filed a broad patent in the UK.[228]

> The application stated: The alkene derivatives of the invention are useful for the modification of the endocrine status in man and animals and they may be used for the control of hormone-dependent tumours or for the management of the sexual cycle and aberrations thereof. They also have useful hypocholesterolemic activity.

This was published in 1965 as UK Patent GB 1013907 which described the innovation that different geometric isomers of substituted triphenylethylenes had either estrogenic or antiestrogenic properties. The original patent was enhanced with UK Patent GB 1064629 published 1967 which is a process for the manufacture of *cis* and *trans* isomers.

In 1973, Nolvadex, the ICI brand of tamoxifen (as its citrate salt), was approved by the Committee on the Safety of Medicines in the UK for the treatment of breast cancer. Although tamoxifen was approved for the treatment of advanced breast cancer in postmenopausal women on 30 December 1977 in the US (ICI Pharmaceuticals Division received the Queen's Award for Technological Achievement in the UK on 6 July 1978), the patent situation was unclear. ICI Pharmaceuticals Division was repeatedly denied patent protection in the USA (with an exclusion of claims for a cancer treatment) until the 1980s because of the perceived primacy of the earlier Merrill patents[229] and because no advance (that is, a safer, more specific drug) was recognized by the US Patent Office. In other words, the clinical development of tamoxifen advanced steadily for more than a decade in the USA without the assurance of exclusivity. This situation also illustrates how unlikely the usefulness of tamoxifen was considered to be by the pharmaceutical industry in general. In theory, tamoxifen could have been marketed by other companies as a generic drug. Remarkably, when tamoxifen was hailed as the adjuvant endocrine treatment of choice for breast cancer by the National Cancer Institute in 1984, the patent application, initially denied in 1984, was awarded through the court of appeals in 1985. This was granted with precedence to the patent dating back to 1965! So, at a time when worldwide patent protection was being lost, the patent protecting tamoxifen started a 17-year life in the USA. The unique and unusual legal situation did not go uncontested by generic companies but AstraZeneca rightly retained patent protection for their pioneering product, most notably, from the Smalkin Decision in Baltimore, 1996.[230] Nevertheless, one generic company (Barr Pharmaceuticals), in a separate out-of-court agreement, did distribute tamoxifen supplied by ICI Pharmaceuticals Division throughout the 1990s. The tablets were priced slightly lower than Nolvadex. Worldwide there are now dozens of generic brands of tamoxifen supplied to healthcare systems outside the USA. Most notable is the Hungarian brand of tamoxifen marketed under the name of Zitozonium. Worldwide sales probably exceeded $10 billion for AstraZeneca and its earlier founder companies which provided the resources for all subsequent drug discovery and development in cancer. Early successes were the antiandrogen Casodex and the sustained release luteinizing hormone-releasing hormone superagonist Zoladex. The actual figures of total worldwide sales for generic tamoxifen are hard to estimate.

8.08.6 A Conversation between the Laboratory and the Clinic

In 1973, Nolvadex was approved by the UK Committee on the Safety of Medicines for the treatment of breast cancer. Nolvadex subsequently became available in more than 110 countries as first-line endocrine therapy for the treatment of breast cancer. The early remarkable clinical success of tamoxifen encouraged a closer examination of its pharmacology with a view to further development and wider applications.

The metabolism of tamoxifen in animals and humans was first described by Fromson and coworkers at ICI Pharmaceuticals Division.[89,90] The major metabolic route to be described was hydroxylation to form 4-hydroxytamoxifen, which was subsequently shown to have high binding affinity for the estrogen receptor[91] and be a potent antiestrogen in its own right, with antitumor properties in the DMBA model.[92] Indeed, it was an advantage for tamoxifen to be metabolically activated to 4-hydroxytamoxifen but this was not a prerequisite for antiestrogen action.[93,94] The metabolite was subsequently shown to localize in target tissues after the administration of radioactive tamoxifen to rats.[95] Remarkably 4-hydroxytamoxifen is still being evaluated as a therapeutic agent but now as a rub-on preparation for local therapy of the breast.[96]

Originally, 4-hydroxytamoxifen was believed to be the major metabolite of tamoxifen in patients but Adam at ICI Pharmaceuticals Division demonstrated that *N*-desmethyltamoxifen was the principal metabolite found in patients.[97] There was usually a blood-level ratio of 2 : 1 for *N*-desmethyltamoxifen to tamoxifen in patients maintained on tamoxifen therapy, since *N*-desmethyltamoxifen had twice the plasma half-life of tamoxifen (14 days vs. 7 days). Recent studies demonstrate that a new metabolite 4-hydroxy-*N*-desmethyltamoxifen could play a role in breast cancer therapy of select patients.[98–100]

The ubiquitous use of tamoxifen has resulted in the publication of numerous methods to estimate tamoxifen and its metabolites in serum.[101–107] The metabolites that have been identified in patients are shown in **Figure 5**. The minor

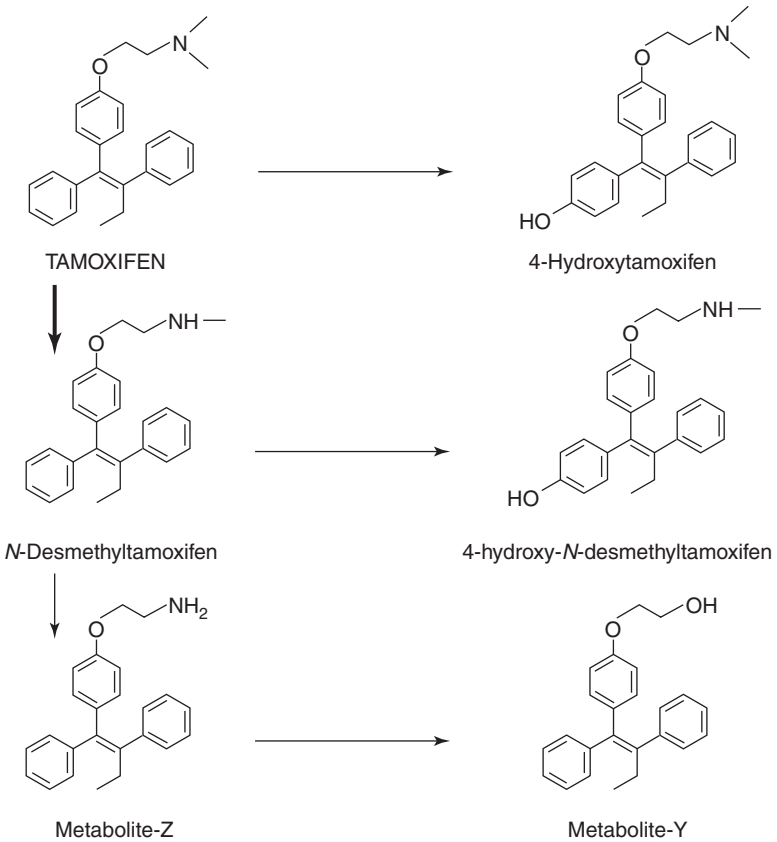


Figure 5 The metabolism of tamoxifen is humans. The principal metabolic route is via *N*-desmethylation but this occurs only because *N*-desmethyltamoxifen accumulates and is more slowly excreted than tamoxifen. In contrast, the 4-hydroxymetabolites are rapidly excreted via phase II metabolism and low levels are observed in patient sera. Nevertheless, the 4-hydroxymetabolites are potent binders to the estrogen receptor, approximately 50–100 more potent than tamoxifen.

metabolites – metabolite-Y, metabolite-Z, and 4-hydroxy-*N*-desmethyltamoxifen – all contribute to the antitumor actions of tamoxifen because they are all antiestrogens that inhibit the binding of estradiol to the estrogen receptor.[108]

The next significant advance in the understanding of tamoxifen action in breast cancer came with the availability of hormone-dependent human breast cancer to study antitumor mechanisms in the laboratory.[109–111] In 1975, Lippman at the National Cancer Institute was the first to describe the ability of tamoxifen to inhibit the growth of macrophage chemotactic factor 7 (MCF7) estrogen receptor-positive breast cancer cells in culture.[112,113] He was also the first to demonstrate that the addition of estrogen could reverse the action of tamoxifen. Nearly a decade later, Osborne and Sutherland independently described the blockade by tamoxifen of breast cancer cells at the G1 phase of the cell cycle.[114,115]

Throughout the 1970s and 1980s, the development of tamoxifen became a conversation between the laboratory and the clinic. The initial success of adjuvant monotherapy with L-phenylalanine mustard or combination chemotherapy in the mid-1970s to delay the recurrence of node-positive breast cancer[116,117] helped encourage the investigation of other, perhaps less toxic, therapies. Laboratory studies using the DMBA-induced rat mammary carcinoma model[118] were first used to explore whether tamoxifen would be effective and whether the drug produced a tumoristatic or tumoricidal effect in vivo.

Previous in vitro studies by Lippman had indicated that tamoxifen could be a tumoricidal drug[112]; but the results from the in vivo DMBA studies (first reported at a breast cancer symposium at King's College, Cambridge, UK in September 1977)[119] demonstrated that a 1-month course of tamoxifen therapy in rats given 1 month after the carcinogenic insult only delayed the appearance of mammary tumors; continuous therapy for 6 months, on the other hand, resulted in 90% of the animals remaining tumor free.[92,119–121] Indeed, tumors appeared whenever tamoxifen therapy was stopped. Thus, tamoxifen was shown to have a tumoristatic component to its mode of action and the laboratory results indicated that long-term (up to 5 years) or indefinite therapy might be the best clinical strategy for adjuvant tamoxifen treatment.

Subsequent laboratory studies using *N*-nitrosomethylurea-induced rat mammary tumors[122] or human breast cancer cell lines inoculated into athymic mice[123,124] have all supported that initial observation. However, attention now focused on the clinical evaluation of the laboratory concept.

Several trials of tamoxifen monotherapy as an adjuvant to mastectomy were initiated in the mid to late 1970s. The majority of clinical trials organizations selected a conservative course of 1 year of adjuvant tamoxifen therapy. This decision was based on a number of reasonable concerns. Patients with advanced disease usually respond to tamoxifen for 1 year, and it was expected that estrogen receptor-negative disease would be encouraged to grow prematurely during adjuvant therapy. If this growth occurred, then the physician would have already used a valuable palliative drug and would have only combination chemotherapy to slow the relentless growth of recurrent disease. A related argument involved the changing strategy for the application of adjuvant combination chemotherapy. Recurrent treatment cycles (2 years) were found to be of no long-term benefit for patients. In contrast, it seemed that aggressive, short-term courses of treatment (6 months) with the most active cytotoxic drugs had the best chance to kill tumor cells before the premature development of drug resistance. The same intuitive sense that 'longer might not be better' contributed to a reluctance on the part of the researchers to start with long-term tamoxifen therapy.

Finally there were sincere concerns about the side effects of adjuvant therapy and the ethical issues of treating patients who might never have recurrent disease. Although this argument primarily focused on chemotherapy and node-negative patients, it is fair to say that few women in the 1970s had received extended therapy with tamoxifen; therefore, long-term side effects were largely unknown. Most tamoxifen-treated patients had only received about 2 years of treatment for advanced disease before drug resistance developed. Potential side effects such as thrombosis and osteoporosis were only of secondary importance. The evaluation of tamoxifen as a chemopreventive in the 1990s would, however, change that perspective.

In 1977, the first evaluation of long-term tamoxifen therapy was started in node-positive patients treated with combination chemotherapy plus tamoxifen. This pilot study was initiated to determine whether patients could tolerate 5 years of adjuvant tamoxifen therapy and whether metabolic tolerance would occur during long-term treatment.[125,126] No unusual side effects of tamoxifen therapy were noted and blood levels of tamoxifen and its metabolites *N*-desmethyltamoxifen and metabolite-Y remained stable throughout the 5 years of treatment. Although this study was not a randomized trial, those patients who received long-term tamoxifen therapy continued to make excellent progress and many patients were to take the drug for more than 14 years. Tamoxifen did not produce metabolic tolerance during 10 years of administration. Serum levels of tamoxifen and its metabolites were maintained.[127]

These data and the DMBA rat mammary carcinoma data were used to support randomized Eastern Cooperative Oncology Group (ECOG) trials EST 4181 and 5181. An analysis of EST 4181, which compared short-term tamoxifen with long-term tamoxifen (both with combination chemotherapy), has demonstrated an increase in disease-free survival with long-term tamoxifen therapy. In fact, the 5-year tamoxifen arm went through a second randomization, either to stop the tamoxifen or to continue the antiestrogen indefinitely.[128,129]

The 2-year adjuvant tamoxifen study conducted by the Nolvadex Adjuvant Trials Organization or NATO (an acronym chosen to encourage American investigators to read *Lancet* papers because they might believe the work had been done by the North Atlantic Treaty Organization!) was the first to demonstrate a survival advantage for patients receiving tamoxifen alone.[130,131]

Similarly, a Scottish trial that evaluated 5 years of tamoxifen versus no treatment demonstrated a survival advantage for patients who take tamoxifen.[132] The Scottish trial is particularly interesting because it addresses the question of whether to administer tamoxifen early as an adjuvant or to save the drug until the disease recurs. This comparison was possible because most patients in the control arm received tamoxifen at recurrence. Early concerns that long-term adjuvant tamoxifen would result in premature drug resistance were unjustified since patients on the adjuvant tamoxifen arm had a survival advantage.

Building on the success of their earlier trials that demonstrated the efficacy of tamoxifen in receptor-positive postmenopausal patients,[133] the National Surgical Adjuvant Breast and Bowel Project (NSABP) conducted a 2-year registration study of combination chemotherapy (L-PAM, 5-FU) plus tamoxifen, with an additional year of tamoxifen alone. Overall, these investigators concluded that 3 years of tamoxifen confers a significant advantage over 2 years of tamoxifen.[134]

Extensive testing as an adjuvant therapy and proven efficacy to enhance survival[130,132,133,135,136] led to FDA approval for the use of tamoxifen as an adjuvant therapy with chemotherapy (1985), as an adjuvant therapy alone (1986) in node-positive, postmenopausal patients, and in pre- and postmenopausal patients with estrogen receptor-positive, node-negative disease (1990). Tamoxifen was approved for the treatment of male breast cancer in 1993.

Until recently, the accepted strategy for the adjuvant treatment of estrogen receptor-positive breast cancer was to employ at least 5 years of therapy. This duration became the standard used in clinical trials in the US because a comparison of 5 with 10 years of adjuvant tamoxifen showed an increase in side effects and a decreased efficacy.[137]

8.08.7 Twenty-First Century View of Tamoxifen as a Treatment for Breast Cancer

The impact of the clinical introduction of tamoxifen on healthcare can be assessed through the work of the Early Breast Cancer Trialists Collaborative Group (EBCTCG) that meets in Oxford, UK every 5 years. The international group has met since 1984 to evaluate the impact of therapies on the treatment of breast cancer. The method is to integrate the positive and negative findings of all the world's randomized prospective clinical trials to reach a consensus on the merits of a treatment approach. Reports on the value of long-term adjuvant tamoxifen therapy in the treatment of node-positive and node-negative estrogen receptor-positive breast cancer can be documented through the EBCTCG publications.[138–140] Overall, the reports document the enhanced survival and disease-free survival conferred by tamoxifen. The recent report by the EBCTG[141] analyzes the results from 145 000 women in 194 trials of chemotherapy or hormonal therapy begun before 1995. The treatment of estrogen receptor-positive breast cancer with about 5 years of adjuvant tamoxifen reduces the annual breast cancer death rate by 31% (± 3 SE) irrespective of the use of chemotherapy, age, progesterone receptor status, or other tumor characteristics. Tamoxifen given for 5 years is significantly ($p < 0.01$) more effective than for 1–2 years. Most importantly, the annual mortality rates during the first few years and the subsequent 5–14 years are similar. This results in the cumulative reduction in mortality being twice as large at 15 years than at 5 years. Overall, the EBCTG conclude that for women under 70 years of age with estrogen receptor-positive breast cancer, their death rate could be halved over the next 15 years if treated with 6 months of an anthracycline-based chemotherapy followed by 5 years of adjuvant tamoxifen.

These important analyses suggest that the introduction of widespread long-term tamoxifen treatment to postmenopausal patients (two-thirds of the total incidence of breast cancer) during the 1980s would contribute substantially to the decreases in mortality by 25–30% observed by others in the USA and UK.[142]

8.08.8 Concerns about Tamoxifen

During the 1990s several important aspects of the pharmacology of tamoxifen emerged that had an impact on the clinical use of the drug. Each aspect was to create severe problems for developing the medicine from a treatment to a chemopreventive. Nevertheless, the rigorous evaluation of tamoxifen proved to be invaluable to understand the actions of the drug fully so that a solid clinical data base could be used to introduce improved medicines.

There were three principal areas of concern. First, an increased incidence in endometrial cancer was reported[143,144] in tamoxifen-treated patients and one study found higher-grade disease and patients with a poorer prognosis associated with tamoxifen treatment compared to patients with de novo disease.[145] These clinical observations were supported by laboratory evidence.[124,146] Second, emerging laboratory studies demonstrated that tamoxifen was a liver carcinogenic in the rat[147,148] and associated concerns that tamoxifen could produce second primary tumors in the liver, stomach, colon, and rectum of women.[149] Third, laboratory evidence[150–153] demonstrated that tamoxifen-stimulated breast tumors can develop indicated that the antitumor action of long-term adjuvant therapy could eventually fail. Long-term tamoxifen might ultimately encourage tumor growth in patients. However, each of these concerns was placed in perspective using the available clinical data base of 8 000 000 women–years of experience accumulated from the worldwide use of tamoxifen in patients for nearly a quarter of a century.

Much controversy surrounded the associations between tamoxifen use and the detection of endometrial cancer. However, it was possible to provide a reasonable picture of the actual incidence of endometrial cancer and provide a balanced view of the concerns. Reviews[154–156] of the literature in the mid-1990s only identified about 400 cases of endometrial cancer associated with tamoxifen use worldwide. Millions of women had taken tamoxifen over many years. The increase in the incidence of endometrial cancer was found predominately in postmenopausal women and there was not a strong association between the duration of tamoxifen use and the risks of developing endometrial carcinoma. Based on the known long genesis of cancer in humans, it was inappropriate to suggest that the detection of endometrial cancer was caused by short courses of tamoxifen. It is also important to point out that DNA adducts were found to be absent from uterine samples of patients taking tamoxifen.[157] Detection bias, through the investigation of symptoms, may have been responsible for the disease found in many patients receiving tamoxifen. It is important to note that epidemiology studies at the time did not show a statistically relevant increase in the incidence of endometrial cancer after a short (2 years) course of tamoxifen.[158,159]

The finding by Magriples *et al.*[145] that tamoxifen use was associated with poor prognosis of disease was not confirmed by any other study.[144,149,160] Overall, the stage and grade of endometrial cancer associated with tamoxifen use was proportionally the same as Surveillance Epidemiology and End Results (SEER) data.[154] Therefore, it is fair to say that the overall consensus was that the benefits of tamoxifen in the treatment of breast cancer far outweighed the risks associated with a twofold elevation in early stage low-grade endometrial carcinoma.[139,161–164] However, as a precaution, patients were prospectively screened to determine whether they had pre-existing endometrial carcinoma before starting a course of adjuvant tamoxifen therapy. Additionally, as a general rule, it was advised that patients who presented with spotting and bleeding during treatment must be investigated with a thorough gynecological examination. There was, however, no justification for an extensive screening program to detect endometrial cancer in asymptomatic women taking tamoxifen.[165]

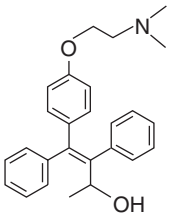
The move to evaluate tamoxifen as a chemopreventive in women at risk for breast cancer resulted in a mandatory re-evaluation of tamoxifen's toxicology. The studies required to market tamoxifen as an anticancer drug in the 1970s were modest but now lifetime rat studies were necessary to treat well women.

The findings in the 1990s that tamoxifen was a liver carcinogen in the rat had a profound impact on drug sales and clinical development. Several investigators reported that tamoxifen is both an initiator and a promoter of rat liver carcinogenesis.[147,166–170] Tamoxifen, at high doses, causes DNA adducts in rat liver.[148,167,171] However, only low adduct formation is noted in mouse liver DNA,[171] a species that does not produce tumors in response to high daily doses of tamoxifen.[172] It was also reassuring to note that there is no increase in DNA adduct formation in the livers of patients receiving tamoxifen.[173] As a result, it was been argued that the rat studies were not relevant to human usage.[164,174]

An examination of the data from the rat carcinogenesis studies demonstrated that the animals receive tamoxifen (5–50 mg kg^{-1} daily) from puberty for more than 50% of their life.[147] In contrast, the therapeutic dose of tamoxifen as an anticancer agent in rats was 250 µg kg^{-1}[1175] which is comparable to the therapeutic dose in a 70 kg patient of 285 µg kg^{-1} or 20 mg of tamoxifen administered twice daily. The duration of adjuvant therapy for postmenopausal patients is usually 5 years. This would be equivalent to 8% of a woman's life. Thus, the animal experiment at the lowest dose to produce tumors, 5 mg kg^{-1}, was equivalent to a teenage girl (i.e., 14 years of age) receiving 20 times the daily dose of tamoxifen until she is 40 years old. This would be 40 tablets a day!

The reason that such large doses have to be administered to the rat to produce drug levels comparable to the human is that the drug is cleared from the rat ten times faster than in humans. Thus, artificially high levels of drug must be given, far outside the therapeutic range, that ultimately cause damage in the rat liver. In recent years, concerns about carcinogenesis with tamoxifen have led to a report of increases in colorectal cancer and stomach cancer.[149] These results were not supported by either individual reports from clinical trials[144] or from the Oxford overview analysis.[140]

The finding of liver carcinogenesis in the rat would be cause for concern with any new drug that was about to go into clinical trial. However, tamoxifen had been used extensively for 20 years before the investigation of rat liver



Alpha-hydroxytamoxifen

Figure 6 The metabolite of tamoxifen believed to be responsible for rat liver carcinogenesis.

carcinogenesis. There had not been a significant increase in hepatocellular carcinoma since the two initial cases reported in 1989.[143] Similarly, epidemiology studies[176] had not shown a rise in hepatocellular carcinoma in breast cancer patients since tamoxifen was approved for use in the USA in 1978. In contrast, oral contraceptives cause a tenfold increase in the risk for the development of hepatocellular carcinoma,[177] but this risk is considered to be acceptable to regulatory authorities because of the rarity of the disease. Clearly there was a problem in translating laboratory studies of the toxicology of tamoxifen to clinic experience with the drug. Unfortunately, this was not relevant to the media and those scientists promoting other products to replace tamoxifen.

During the early years of the 1990s there was intense interest in discovering the initiating event for tamoxifen-induced rat liver carcinogenesis and determining the relevance for humans. Han and Lehr[148] first noted an accumulation of DNA adducts in the liver of Sprague–Dawley rats on repeated injections of 20 mg kg^{-1} (cf. human dosage of 0–3 mg kg^{-1}). This observation was adequately confirmed by numerous investigators and the focus of investigation turned to the identification of the actual DNA adduct. Several candidates were proposed: an epoxide,[178–180] 4-hydroxytamoxifen,[181,182] Metabolite E,[183] or alpha-hydroxytamoxifen.[184–186] Osborne *et al.*[187] prepared alpha-acetoxytamoxifen which is able to react with DNA to a greater extent (1 in 50 bases) than alpha-hydroxytamoxifen (1 in 10^5 DNA bases). The products of the reaction were identical to those isolated from DNA of rat hepatocytes or the livers of rats treated with tamoxifen. The adduct of tamoxifen and DNA was identified at the nucleoside deoxyguanosine in which the alpha position of tamoxifen is linked covalently to the exocyclic amino of deoxyguanosine.

These important observations provided a framework to study the metabolic activation of tamoxifen in human systems and to identify any DNA adducts in human tissues. The metabolic activation of tamoxifen and its metabolite alpha-hydroxytamoxifen (**Figure 6**) were compared using primary cultures of rat, mouse, and human hepatocytes.[180] Although DNA adducts were readily identified in rat and mouse hepatocytes (90 and 15 adducts per 10^8 nucleotides respectively), DNA adducts were not detected in tamoxifen-treated human hepatocytes. Additionally, human hepatocytes also appeared to produce 50-fold lower levels of alpha-hydroxytamoxifen from tamoxifen compared to rat hepatocytes. Further studies showed that if cells were treated with alpha-hydroxytamoxifen human hepatocytes had 300-fold lower levels of adducts compared to rat hepatocytes.

Studies in humans have confirmed that the human is not as susceptible as the rat to DNA adduct formation with tamoxifen. The pattern of DNA adducts found in the rat liver was not found in humans treated with tamoxifen,[173] DNA adducts were not found in lymphocytes,[188] and there is a lack of genotoxicity of tamoxifen in human endometrium.[157] In the latter studies, DNA adducts could be produced in endometrial samples with alpha-hydroxytamoxifen but not with tamoxifen. The authors proved that tissue was capable of metabolizing tamoxifen to alpha-hydroxytamoxifen but apparently it is incapable of producing adducts. Endometria from patients taking tamoxifen for up to 9 years were analyzed for DNA adducts. No evidence for any DNA adducts induced by tamoxifen was found in any of the patients examined. The authors concluded that the genotoxic events observed with tamoxifen in the rat may not apply to the human endometrium.[157] This conclusion supports the previous suggestion that tamoxifen, or indeed any new antiestrogen which has partial agonist actions, will cause the activation and detection of pre-existing disease.[174]

The results from the EBCTCG (2005) provide a current evaluation of the benefits and side effects experienced with adjuvant tamoxifen in clinical trials during the past two decades.[141] A comparison of all patients receiving adjuvant tamoxifen versus those not receiving tamoxifen reveals that there is no significant excess of deaths from any particular cause. The average non-breast cancer death rate was calculated to be 0.8% per year for women receiving tamoxifen or not. There is a small excess of deaths in women receiving tamoxifen from thromboembolism and uterine cancer (but not liver cancer) but these data are nonsignificant. Presuming there is a real excess of death for both side effects combined for the 60 000 women–years of tamoxifen exposure in the trials, this would represent an absolute risk of death of about 0.2% per decade. The EBCTG suggest this risk is small in comparison with the 10-year benefit in reducing breast cancer mortality by 5.3% (node-negative) and 12.2% (node-positive).

The trend in clinical practice of using longer and longer treatment regimens with tamoxifen stimulated the investigation of the development of drug resistance to tamoxifen. Drug resistance to tamoxifen therapy can take many forms.[189,190] Obviously, if tumors are estrogen receptor-negative there is only a small probability of a response to antiestrogen therapy. In the case of metastatic breast cancer about 10% of estrogen receptor-negative and progesterone receptor-negative patients respond to any form of endocrine modulation.[191] Similarly the overview analysis[140] of clinical trials shows that postmenopausal, node-positive patients with receptor-poor disease do not benefit from adjuvant tamoxifen.

In the laboratory, tamoxifen was found to inhibit estrogen-stimulated growth of MCF7 breast tumors implanted into athymic mice.[123] Nevertheless, continuous therapy with tamoxifen results in the emergence of tamoxifen-stimulated breast tumors that will grow in response to either estrogen or tamoxifen.[150–152,192] Since there were clinical reports of tamoxifen-stimulated tumors that have a withdrawal response to tamoxifen,[193,194] new second-line agents (or first-line agents) were becoming necessary to control tumors that grow after extended tamoxifen treatment. New nonestrogenic agents were introduced to improve response rates and reduce side effects. However, this goal was only to become successful in the twenty-first century (*see* Section 8.08.12). Tamoxifen was first destined to be tested as the first chemoproventive to reduce the incidence of breast cancer in high-risk women.

8.08.9 Selective Estrogen Receptor Modulation

In the 1960s and 1970s, antiestrogenicity was correlated with antitumor activity. However, the finding that nonsteroidal antiestrogens expressed increased estrogenic properties, i.e., vaginal cornification and increased uterine weight in the mouse, raised questions about the reasons for the species specificity. One obvious possibility was species-specific metabolism, i.e., the mouse converts antiestrogens to estrogens via novel metabolic pathways. However, no species-specific metabolic routes to known estrogens were identified but knowledge of the mouse model created a new dimension for study that ultimately led to the recognition of the target site-specific actions of antiestrogens. This concept was subsequently referred to as selective estrogen receptor modulation (SERM) to describe the target site-specific effects of raloxifene (*see* 8.09 Raloxifene), an antiestrogen originally targeted for an application in breast cancer but now used, paradoxically, as a preventive for osteoporosis. Now the whole class of drugs is known as SERMs.

The estrogen receptor-positive breast cancer cell line MCF7[110] can be heterotransplanted to immune-deficient athymic mice but the cells will only grow into tumors with estrogen support.[123] Paradoxically, tamoxifen, an estrogen in the mouse, does not support tumor growth but stimulates mouse uterine growth with the same spectrum of tamoxifen metabolites present in both the uterus and the human tumor.[195] To explain the selective actions of tamoxifen in different targets of the same host, it was suggested that the estrogen receptor complex could be interpreted as a stimulatory or inhibitory signal at different sites. The concept was consolidated with experimental evidence from two further models. First, tamoxifen and raloxifene maintain bone density in the ovariectomized rat but both compounds inhibit estradiol-stimulated uterine weight and prevent carcinogen-induced mammary tumorigenesis.[122,196] Second, the finding that tamoxifen would partially stimulate the growth of a human endometrial carcinoma transplanted into athymic mice allowed the investigation of two human tumors bitransplanted in the same mouse to determine whether tamoxifen could inhibit estrogen-stimulated growth of two tumors in the same host equally. Tamoxifen demonstrated target site specificity: breast tumor growth was controlled but endometrial tumors continued to grow.[197] Again, the spectrum of tamoxifen metabolites was consistent in all target tissues despite the contrasting biological responses, so it was concluded that the estrogen receptor complexes must be interpreted differently in different target tissues.

The laboratory principle of selective estrogen receptor modulation translated to the clinic with the findings that tamoxifen maintained bone density[198] and lowered circulating cholesterol.[199] These were extremely important findings because there were justifiable concerns that tamoxifen, an 'antiestrogen,' might prevent breast cancer but increase risks for osteoporosis and coronary heart disease. The beneficial effects of SERM action on bones and circulating cholesterol were important to advance clinical studies testing the worth of tamoxifen as a chemopreventive in high-risk women. Additionally, the recognition that tamoxifen increases the risk of endometrial cancer was an advantage for screening volunteers for trials. Nevertheless, the reports of carcinogenicity associated with tamoxifen naturally created major problems for recruitment to chemoprevention trials.

8.08.10 Tamoxifen and Breast Cancer Prevention

Thirty years ago, tamoxifen was shown to prevent the induction and promotion of carcinogen-induced mammary cancer in rats.[92,200] Similarly, tamoxifen was also shown to prevent the development of mammary cancer induced by ionizing radiation in rats. These laboratory observations, coupled with the emerging preliminary clinical observation that

adjuvant tamoxifen could prevent contralateral breast cancer in women,[201] provided a rationale for Powles to start a toxicology study at the Royal Marsden Hospital, London, UK to test whether tamoxifen would be acceptable to prevent breast cancer in high-risk women. This vanguard study opened for recruitment in 1986[202] and was to provide important toxicological and compliance data for subsequent trialists.

This toxicology and compliance study was supplemented by parallel investigations of tamoxifen as a chemopreventive in animal models of tumorigenesis[122] and the safety studies of tamoxifen to establish the effects on bone and circulating lipids (*see* Section 8.08.9).

In the decade following the Powles initiative, several studies were started to answer the question: "Does tamoxifen have worth in the prevention of breast cancer in select high-risk women?" Eventually four studies were available to evaluate the veracity of the question - the Royal Marsden study, the NSABP/NCI study, the Italian study, and the International Breast Intervention Study (IBIS). The results have been adequately summarized by Cuzick and coworkers[203] but the NSABP Study will be presented in detail because it was the only prospective study to achieve its recruitment goal.

The NSABP P-1 study opened in the USA and Canada in May of 1992 with an accrual goal of 16 000 high-risk women to be screened and recruited at 100 North American sites. It closed after accruing 13 338 in 1997 due to the high-risk status of the participants. Those eligible for entry included any woman over the age of 60 or women between the ages of 35 and 59 whose 5-year risk of developing breast cancer, as predicted by the Gail model,[204] was equal to that of a 60-year-old woman. Additionally, any woman over age 35 with a diagnosis of lobular carcinoma in situ (LCIS) treated by biopsy alone was eligible for entry to the study. In the absence of LCIS, the risk factors necessary to enter the study varied with age, such that a 35-year-old woman must have a relative risk (RR) of 5.07, whereas the required RR for a 45-year-old woman was 1.79. Routine endometrial biopsies to evaluate the incidence of endometrial carcinoma in both arms of the study were also performed.

The breast cancer risk of women enrolled in the study was extremely high with no age group having an RR of less than 4, including the over-60s group. Recruitment was also balanced with about one-third younger than 50 years, one-third between 50 and 60 years old, and one-third older than 60 years. Secondary end points of the study included the effect of tamoxifen on the incidence of fractures and cardiovascular deaths. Most importantly, the study planned to provide the first information about the role of genetic markers in the etiology of breast cancer. Unfortunately the question of whether tamoxifen has a role to play in the treatment of women who are found to carry somatic mutations in the *BRCA-1* and *BRCA-2* gene could not adequately be answered[205] because of the low incidence of women with mutations in the P-1 study overall.

The first results of the NSABP study were reported in September 1998, after a mean follow-up of 47.7 months.[206] There were a total of 368 invasive and noninvasive breast cancers in the participants; 124 in the tamoxifen group and 224 in the placebo group. A 49% reduction in the risk of invasive breast cancer was seen in the tamoxifen group and a 50% reduction in the risk of noninvasive breast cancer was observed. A subset analysis of women at risk due to a diagnosis of LCIS demonstrated a 56% reduction in this group. The most dramatic reduction was seen in women at risk due to atypical hyperplasia where risk was reduced by 86%.

The benefits of tamoxifen were observed in all age groups with a relative risk of breast cancer ranging from 0.45 in women aged 60 and older to 0.49 for those in the 50–59-year age group and 0.56 for women aged 49 and younger. A benefit for tamoxifen was also observed for women with all levels of breast cancer risk within the study, indicating that the benefits of tamoxifen are not confined to a particular lower risk or higher risk subset. Benefits were observed in women at risk on the basis of family history and those whose risk was due to other factors.

As expected, the effect of tamoxifen occurred on the incidence of estrogen receptor-positive tumors which were reduced by 69% per year. The rate of estrogen receptor-negative tumors in the tamoxifen group (1.46 per 1000 women) did not significantly differ from the placebo group (1.20 per 1000 women). Tamoxifen reduced the rate of invasive cancers of all sizes but the greatest difference between the groups was the incidence of tumors 2.0 cm or less. Tamoxifen also reduced the incidence of both node-positive and node-negative breast cancer. The beneficial effects of tamoxifen were observed for each year of follow-up in the study. After year 1 the risk was reduced by 33% and in year 5 by 69%.

Tamoxifen also reduced the incidence of osteoporotic fractures of the hip, spine, and radius by 19%. However, the difference approached, but did not reach, statistical significance. This reduction was greatest in women aged 50 and older at study entry. No difference in the risk of myocardial infarction, angina, coronary artery bypass grafting, or angioplasty was noted between the groups.

This study confirmed the association between tamoxifen and endometrial carcinoma. The relative risk of endometrial cancer in the tamoxifen group was 2.5. The increased risk was seen in women aged 50 and older whose relative risk was 4.01. All endometrial cancers in the tamoxifen group were grade 1 and none of the women on the

tamoxifen died of endometrial cancer. There was one endometrial cancer death in the placebo group. Although there is no doubt that tamoxifen increases the risk of endometrial cancer, it is important to recognize that this increase translates to an incidence of 2.3 women per 1000 per year who develop endometrial carcinoma.

More women in the tamoxifen group developed deep vein thrombosis (DVT) than in the placebo group. Again, this excess risk was confined to women aged 50 and older. The relative risk of DVT in the older age group was 1.71 (95% CI 0.85 to 3.58). An increase in pulmonary emboli was also seen in the older women taking tamoxifen, with a relative risk of approximately 3. Three deaths from pulmonary emboli occurred in the tamoxifen arm, but all were in women with significant comorbidities. An increased incidence of stroke (RR 1.75) was also seen in the tamoxifen group, but this did not reach statistical significance.

An assessment of the incidence of cataract formation was made using patient self-report. A small increase in cataracts was noted in the tamoxifen group: a rate of 24.8 women per 1000 compared to 21.7 in the placebo group. There was also an increased risk of cataract surgery in the women on tamoxifen. These differences were marginally statistically significant and observed in the older patients in the study. These findings emphasize the need to assess the patient's overall health status before making a decision to use tamoxifen for breast cancer risk reduction. These observations are also particularly interesting based on the early controversy in the 1960s (*see* Section 8.08.2) about the safety of this drug group.

An assessment of quality of life showed no difference in depression scores between groups. Hot flushes were noted in 81% of the women on tamoxifen compared to 69% of the placebo group and the tamoxifen-associated hot flushes appeared to be of greater severity than those in the placebo group. Moderately bothersome or severe vaginal discharge was reported by 29% of the women in tamoxifen group and 13% in the placebo group. No differences in occurrence of irregular menses, nausea, fluid retention, skin changes, or weight gain or loss were reported.[207,208]

8.08.11 Current Chemoprevention

Based on a thorough review of all the available data, the FDA approved tamoxifen for the reduction of breast cancer incidence in high-risk pre- and postmenopausal women in 1998. However, the report that tamoxifen caused a small but significant increase in uterine sarcoma[209] resulted in an industry request for a black box inclusion for tamoxifen from the FDA. Additionally, the IBIS-1 study noted an unacceptable increase in deaths from tamoxifen treated patients who inadvertently had surgery during the study acceptability of tamoxifen as a chemopreventive.[210] This led to the development of IBIS-2 using an aromatase inhibitor to prevent breast cancer. Aromatase inhibitors have fewer side effects than tamoxifen and it is known that during adjuvant treatment, they reduce the incidence of contralateral breast cancer even more than tamoxifen.[211–213]

Another approach is the evaluation of the SERM raloxifene as a preventive for breast cancer in high-risk postmenopausal women. The Study of Tamoxifen and Raloxifene (STAR) has reduced its recruitment goal of 19 000 volunteers and the results will be available by July 2006.

The promise of the chemoprevention for breast cancer is becoming a reality. However, there are many challenges. Tamoxifen, the pioneering medicine, is considered by many to be too controversial to be widely used as a chemopreventive. However, there are no alternatives for the premenopausal woman at high risk for breast cancer and the good news is that this risk group has the best risk–benefit ratio.[214] For postmenopausal women, where the side effects are well defined, the future depends on the results of current clinical trials with raloxifene or aromatase inhibitors. Unfortunately, there are no comparisons of a SERM with an aromatase inhibitor so the choice of a chemopreventive strategy will need to be made on a patient-by-patient basis. In other words, the options are the use of raloxifene or an aromatase inhibitor with bone monitoring and a bisphosphonate to avoid osteoporosis.

8.08.12 Tamoxifen's Legacy: A Menu of Medicines

Tamoxifen became the most investigated anticancer agent over the 40 years of its development. The success of the drug as an adjuvant therapy has been quantified: 400 000 women with breast cancer are alive because of long-term tamoxifen treatment. Most importantly, the development of tamoxifen demonstrated that there was an advantage for patients by targeting the estrogen receptor specifically. This in turn encouraged the pharmaceutical industry to invest in research to discover both safer and more effective drugs. This is best illustrated by comparing treatment options for advanced breast cancer in 1970, i.e., before tamoxifen (**Figure 7**) with the therapeutic options for all stages of breast cancer in 2005 (**Figure 8**).

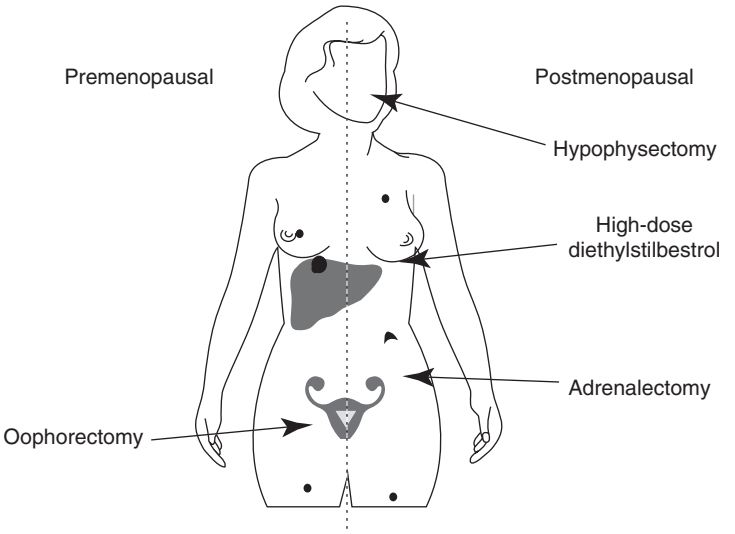


Figure 7 The endocrine options in the early 1970s for the patient with metastatic breast cancer. Surgery to remove endocrine organs (ablative surgery) which secreted estrogenic hormones or their precursors. In the case of postmenopausal patients, additive high-dose estrogens, androgens, or progestins were standard therapy.

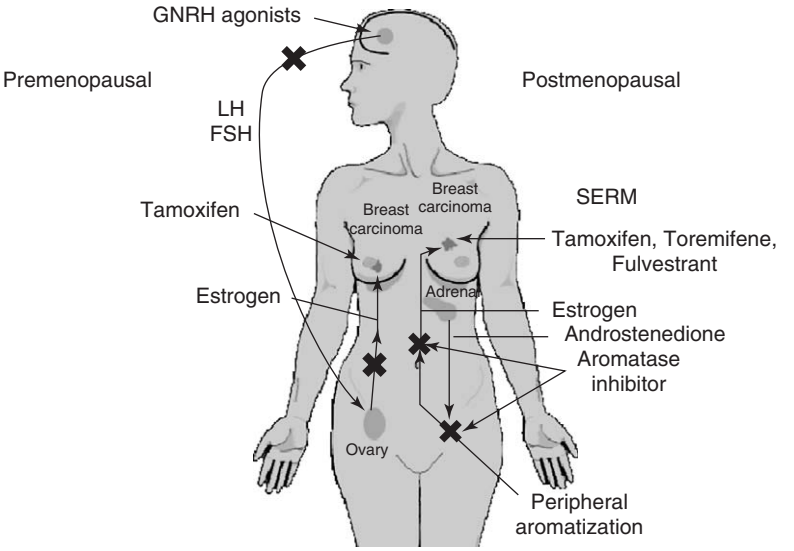


Figure 8 The current menu of medicines used to block the exposure and action of estrogens in breast tumors. The ultimate target in all cases is to prevent the formation of the estrogen–estrogen receptor complex within tumor cells. FSH, follicle-stimulating hormone; GNRH, gonadotropin-releazing hormone; LH, luteinizing hormone; SERM, selective estrogen receptor modulation.

The clinical success of tamoxifen encouraged an important re-evaluation of inhibitors for the aromatase enzyme system. In other words, block the synthesis of estrogens from androgen precursors. Early nonspecific inhibitors such as aminoglutethimide had many side effects, especially since there was a necessity to coadminister the drug with a glucocorticoid. Aminoglutethimide faded out of fashion during the 1970s as tamoxifen became the endocrine treatment of choice for breast cancer. However, the development of the suicide inhibitor formestane[215] based on Brodie's original laboratory work[216,217] created an opportunity for the development of a whole class of new drugs. The process took about 20 years, i.e., throughout the 1980s and 1990s.[218] Today aromatase inhibitors are used to treat postmenopausal women with estrogen receptor-positive disease instead of tamoxifen[211,219] or after tamoxifen.[212,213] In general, there are fewer side effects such as endometrial cancer or blood clots and there are significant improvements in disease control.

Examination of the pharmacology of the drug in great detail taught many lessons. The finding that tamoxifen was a carcinogen in rat liver advanced the development of toremifene that did not induce rat liver tumors. Despite the fact that toremifene was not a complete carcinogen, the drug also increased endometrial cancer to the same extent as tamoxifen.[220] No major increase in human liver cancer has been reported with tamoxifen. Additionally, the development of aromatase inhibitors[221] for the treatment of breast cancer obviated the need for another SERM for treatment. More importantly, the studies with tamoxifen in rats exposed a weakness in the toxicity testing methods. Had tamoxifen been tested for carcinogenicity 30 years ago, there would have been no tamoxifen, lives would have been lost, and the goal of targeting tumors would have been retarded for perhaps a decade. Tamoxifen became a success story which proved that targeting could save lives.

Interestingly enough, there are those in the research and clinical community that consider that tamoxifen will continue to have a role in treating select patients and will certainly continue to be a useful medicine in underdeveloped countries that cannot afford more expensive medicines such as aromatase inhibitors. It is also fair to say that the pure antiestrogen fulvestrant would not have been discovered at ICI Pharmaceuticals Division in the early 1980s[222,223] if research on tamoxifen had not been continued. Tamoxifen is a pioneering medicine not a perfect medicine so it was only rational that work on other approaches to breast cancer treatment, targeted to the estrogen receptor, should have started in the hope of exploiting tamoxifen's expanding clinical market in the 1980s. The serendipitous discovery of the pure antiestrogens is an example of drug discovery by talented scientists in industry who discovered a new class of drugs, the estrogen receptor downregulators which not only are useful in the clinic but provided a new insight into regulatory processes in cancer cells.[224]

Tamoxifen is the first SERM and without the developing pharmaceutical database during the 1980s, raloxifene, originally a failed breast cancer drug called keoxifene (*see* 8.09 Raloxifene), would not have been reinvented as a treatment and preventive for osteoporosis with breast and endometrial safety.[225,226]

The discovery of SERM action with tamoxifen[227] has opened the door to discovering selective activity for all members of the steroid hormone receptor superfamily. A huge effort is under way to discover agonist and antagonists drugs for the androgen receptor, progesterone receptor, glucocorticoid receptor, thyroid hormone receptor, and the peroxisome proliferator-activated receptor (PPAR).

Overall, the impact of tamoxifen on healthcare, drug discovery, and cancer cell biology has proved to be extremely beneficial but the process took more than 40 years. This story illustrates the real difficulties that industry scientists, often working in a restrictive environment governed by intellectual property rights, have in exploiting the benefits of novel molecules. The development of tamoxifen is unique but its inception depended on individuals, in at the right place at the right time, and a change in the fashions of medical research first from contraception to cancer research and then from cancer research to women's health and preventive care.

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8.09 Raloxifene

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8.09.1 Background and Introduction

The female sex hormone estrogen plays an essential role in reproduction and is important for the overall maintenance of physiologic homeostasis in a woman's body.[1,2] During menopause, which occurs in women at an average age of 51, the amount of estrogen produced by the ovaries decreases and this estrogen deficiency causes menstrual periods to become less frequent and then stop.[3–5] The loss of estrogen is responsible for many of the uncomfortable symptoms associated with menopause, including hot flashes, mood swings or depression, sleep disorders, vaginal dryness, and urinary dysfunction.[6] Osteoporosis or bone loss is another consequence of reduced estrogen levels after menopause.[7–11] In women, bone density increases until ages 30–35,[12] but slowly declines after menopause.[13] Postmenopausal women are also at increased risk for coronary heart disease (CHD)[14,15] and Alzheimer's disease,[16–18] as a result of estrogen deficiency. The realization that the symptoms reported by postmenopausal women were capable of amelioration by exogenous estrogens led to the development of estrogen replacement therapy (ERT) more than 30 years ago.[19–22] ERT is defined as therapy to replace estrogen no longer made by a woman's body because she is postmenopausal or her ovaries have been damaged or removed. The most commonly used form of estrogen is known as conjugated estrogens, such as Premarin.[23] Conjugated estrogens are physiologically inactive but are converted within the body to active compounds. The physiologically active form of estrogen, 17-β-estradiol (E_2), is not well absorbed when taken by mouth; however, it is well absorbed through the skin and is the form of estrogen used in the newer estrogen patches. Estrogen patches are preferable to conjugated estrogens because they deliver the natural form of estrogen directly into the bloodstream in a slow, sustained manner. Application of a single patch maintains a relatively constant serum level of E_2 for approximately 3.5 days; therefore the patches must be changed twice a week.[24,25]

ERT gained widespread popularity in the US in the 1960s and early 1970s[26–28] and, by 1974, approximately 28 million prescriptions were filled for noncontraceptive use of estrogens. Previous studies have shown that ERT slows the rate of postmenopausal bone loss[22,29] and reduces the incidence of osteoporotic fractures,[22] and until recently, it was generally believed that ERT might be beneficial for reducing the risk of heart disease.[30,31] The idea that ERT might have cardioprotective effects was based on observational studies[32–37] which suggested that women who take estrogen have a lower risk of CHD compared to women who do not take estrogen. Previous studies[38–40] have shown that estrogen therapy reduces plasma levels of the 'bad cholesterol' low-density lipoproteins (LDL) and increases the levels of the 'good cholesterol' high-density lipoproteins (HDL), changes that are associated with a reduced risk of cardiovascular disease. Estrogen also prevents oxidation of LDL cholesterol, which is thought to protect against the development of atherosclerosis.

Long-term use of ERT, however, does increase the risk of endometrial cancer. The first definitive study demonstrating a causal relationship between endometrial cancer and ERT was published in 1975. Ziel and Finkle[41] reported that women taking estrogen were between four and 13 times more likely to develop cancer of the uterus than women who were not taking estrogen. The increased incidence of endometrial cancer attributed to estrogen led initially to a dramatic decline in the number of prescriptions of this category of drugs; however, the finding that endometrial cancer could be entirely eliminated if estrogen therapy was combined with progestins (medroxyprogesterone acetate),[42–45] given either sequentially or continuously during a monthly cycle, paved the way for the development of combined hormone replacement therapy (HRT). With its ability to treat the symptoms of menopause effectively while at the same time reducing the risk of osteoporosis and endometrial cancer with possible cardioprotective effects, HRT was considered the 'perfect drug' during the 1990s[46] and was routinely prescribed by physicians to postmenopausal women.

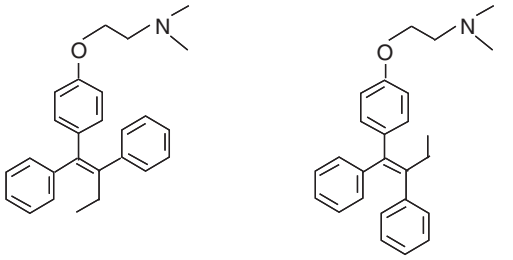
However, there was a plethora of epidemiologic studies[47–51] which suggested a link between HRT (combined estrogen–progestin therapy) and an increased risk of breast cancer. Metaanalyses of the published studies indicated a direct correlation between a longer duration of use of HRT and a higher risk of breast cancer in postmenopausal women.[52] Apart from these studies, there was also an interest in determining if estrogen plus progestin therapy altered the risk for CHD events in postmenopausal women with established coronary disease, and between January 1993 and September 1994, 2763 women were enrolled in the Heart and Estrogen/progestin Replacement Study (HERS). After a follow-up of 4.1 years, Hulley and colleagues[53] reported that estrogen plus progestin did not reduce the overall rate of CHD events in postmenopausal women with established coronary disease; however, estrogen plus progestin did increase the rate of thromboembolic events and gallbladder disease. A subsequent open-label observational follow-up for 2.7 years (HERS II), carried out between 1993 and 2000, indicated no overall effect of estrogen plus progestin therapy on cardiovascular disease event rates[54]; however, there was a slight decrease in the incidence of both deep-vein thrombosis and pulmonary embolism in this group.[55]

Due to the conflicting reports regarding HRT, the Women's Health Initiative (WHI), a randomized controlled primary prevention trial, was established in 1992. The objective of the WHI study was to assess the major risks and health benefits of estrogen plus progestin therapy (planned duration of 8.5 years) in postmenopausal women. Between 1993 and 1998, the WHI enrolled a total of 161 809 postmenopausal women aged 50–79 years into a set of clinical trials at 40 clinical centers in the US.[56] On May 31, 2002, after a mean of 5.2 years of follow-up, one component of the WHI study, the trial of combined estrogen and progestin in women with an intact uterus, was stopped prematurely at the recommendation of the data and safety-monitoring board because the women receiving the active drug had an increased risk of invasive breast cancer (hazard ratio (HR), 1.26; 95% confidence interval (CI), 1.00–1.59) and CHD, and an overall measure of health effects suggested that the treatment was causing more harm than good.[57–60] In March 2004, a second component of the WHI study, which examined estrogen-only therapy in women who had undergone hysterectomy and therefore did not require a progestin, was also stopped early due to an increase in the risk for strokes.[61]

The Million Women Study, a cohort study of 1 084 110 British women aged 50–64 years, was established to investigate the relation between various patterns of use of HRT and breast cancer incidence and mortality.[62,63] This is the largest study of incidence published to date and women were recruited between 1996 and 2001, and followed up using National Health Service central registers. Half of the women had used HRT, and during an average follow-up of 2.6 and 4.1 years, 9364 incident invasive breast cancers and 637 breast cancer deaths were registered. Current users of HRT at recruitment were more likely than never users to develop breast cancer (adjusted relative risk (RR) 1.66; 95% CI 1.58–1.75) and die from it (1.22). Past users of HRT were, however, not at an increased risk of incident or fatal disease. Incidence was significantly increased for current users of preparations containing estrogen only (RR, 1.30), estrogen–progestogen (RR, 2.0), and tibolone (RR, 1.45), but the magnitude of the associated risk was substantially greater for estrogen–progestogen than for other types of HRT. The Million Women Study also examined the effect of estrogen-only HRT or combined estrogen–progestogen on the risk of endometrial cancer in postmenopausal women without a history of hysterectomy. After a follow-up of 3.4 years, it was found[63] that women using estrogen-only HRT had an increased risk of endometrial cancer compared with women who had never used HRT, and that progestogens counteracted the adverse effect of estrogens on the endometrium. Based on these overall findings, it has now been recommended that HRT be used only for management of menopausal symptoms and for the shortest duration possible.[65]

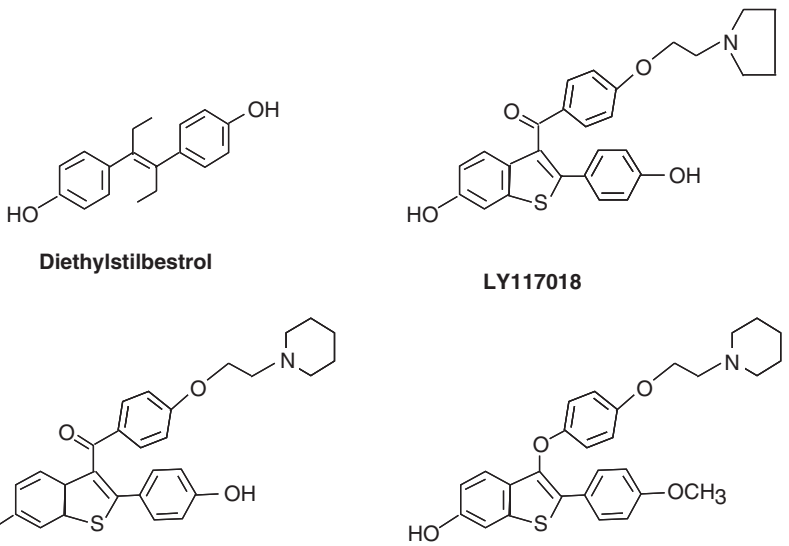
Due to the above-mentioned side-effects associated with HRT, there have been renewed efforts by the academic community and the pharmaceutical industry to develop compounds that mimic the effects of estrogen in some organs (bone and heart) but are neutral, or function to oppose estrogen action in the breast.[66,67] This in turn has ultimately led to the recognition of site-specific estrogen agonist/antagonist therapy and the development of selective estrogen

receptor modulators (SERMs). SERMs are a diverse group of nonsteroidal compounds that potentially offer postmenopausal women many of the advantages of ERT while avoiding undesired effects on reproductive and other tissues. Unlike estrogens, which are uniformly agonists, and antiestrogens, which are uniformly antagonists, SERMs display an unusual tissue-selective pharmacology: they are estrogen agonists in tissues such as bone, liver, and the cardiovascular system, but estrogen antagonists in the breast, uterus, and brain.[66,67] Clinically available SERMs fall into two chemical classes: triphenylethylenes and benzothiophenes. Tamoxifen (**Figure 1**) is the most well-known of the first-generation triphenylethylene SERMs. It has been successfully tested for the prevention of breast cancer in high-risk women[68] and is currently approved for the endocrine treatment of all stages of estrogen receptor-positive breast cancer.[69–71] Other triphenylethylene SERMs in clinical use include clomiphene (used to induce ovulation)[72–74] and toremifene (used to treat postmenopausal women with metastatic breast cancer).[75,76] Raloxifene (**Figure 2**)[77] is a benzothiophene second-generation SERM that is chemically distinct from tamoxifen (*see* 8.08 Tamoxifen). Because of its estrogen agonist activity in bone, raloxifene was evaluated as an agent for the treatment and prevention of osteoporosis in postmenopausal women in the Multiple Outcomes of Raloxifene (MORE) trial.[78–81] It is the first SERM to be approved by the US Food and Drug Administration (FDA: 1997) for the treatment and prevention of postmenopausal osteoporosis and is currently being tested against tamoxifen in the Study of Tamoxifen and Raloxifene (STAR) trial[82] for the prevention of breast cancer in high-risk postmenopausal women. Raloxifene has been evaluated in the Continuing Outcomes Relevant to Evista (CORE) trial,[83] which is a follow-up study to the MORE trial.



ICI 46,474 (tamoxifen) **ICI 47,699 (*cis* isomer)**

Figure 1 Tamoxifen and its estrogenic *cis* isomer.



Diethylstilbestrol

LY117018

LY156758 (keoxifene, raloxifene)

Arzoxifene

Figure 2 Diethylstilbestrol and compounds discovered by Eli Lilly and Company.

Raloxifene also lowers cholesterol and its potential cardioprotective effects are being examined in the Raloxifene Use for the Heart (RUTH) trial.[84,85] The primary endpoint of the RUTH trial is the incidence of myocardial infarction, but the incidence of breast cancer and osteoporotic fractures will also be examined as secondary outcomes. In contrast to its estrogen agonist effect in the bone, raloxifene acts as an estrogen antagonist in breast tissue through competitive binding to the estrogen receptor[77,86] and it inhibits estrogen-induced breast cancer cell proliferation[87–89] and prevents the growth of chemically induced rat mammary tumors.[90–92] Raloxifene also partly inhibits the growth of tamoxifen-stimulated human endometrial cancers in athymic animals.[93] It should be noted that the original direction for raloxifene's clinical development was breast cancer therapy[94]; however, Eli Lilly and Company chose to abandon this approach toward the end of the 1980s because clinical trials showed no activity in tamoxifen-resistant breast cancer patients.[95] The discovery that raloxifene might prevent bone loss and prevent breast cancer,[91,96] however, laid the foundation for subsequent confirmation of bone data in animals.[97–99] These discoveries eventually led to the completion of clinical trials that demonstrated maintenance of bone density in postmenopausal women at risk for osteoporosis.[79] In other words, raloxifene (then named keoxifene) was actually reinvented in the early 1990s as a safer treatment for osteoporosis with breast and endometrial safety. The drug group is now positioned to fulfill some of the promise of ERT, but unfortunately not all of the promise.

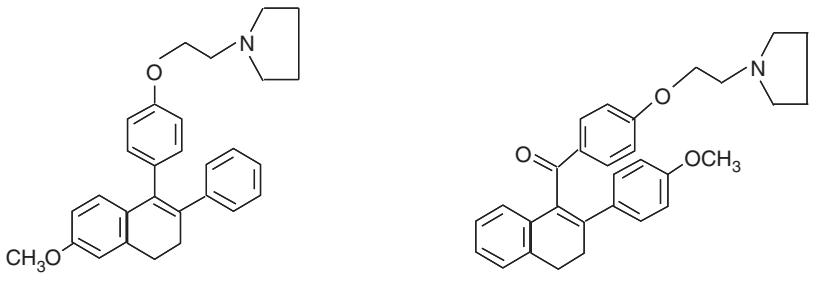
This review will trace the evolution of the development of raloxifene from its failure as a breast cancer drug (then named keoxifene) to its current success as a bone maintenance therapy to prevent osteoporosis in postmenopausal women and its potential as a drug for prevention of breast cancer (i.e., chemopreventive).

8.09.2 Evolution of Antiestrogens to Raloxifene

The scientific knowledge that Eli Lilly and Company utilized to develop raloxifene in the 1990s was based on: (1) earlier studies performed in the 1960s to develop novel contraceptives[100,101]; and (2) knowledge of the structure–function relationships of antiestrogens in the 1970s.[102] While many of the compounds failed as contraceptives, they ultimately evolved into antiestrogens and subsequently SERMs with multiple applications in medicine.[67,103] The path leading to SERM development began with the synthesis of ethamoxytriphetol (MER-25) (*see* 8.08 Tamoxifen), which, interestingly enough, was an antiestrogen with no estrogen-like actions in all tissues and species.[100]

The finding that MER-25 possessed antifertility action in laboratory animals[104] stimulated a search for more potent agents for clinical applications.[105,106] Clomiphene (*see* 8.08 Tamoxifen); originally known as chloramiphene or MRL-41,[107] nafoxidine (U-11,-100A),[108] nitromifene (CI6128 or CN-55, 945–27),[109] and tamoxifen (ICI 46,474) (**Figure 1**)[110,111] were all the result of that search, but clinical application as postcoital contraceptives was found to be inappropriate because these drugs induced ovulation in subfertile women.[112] As a result, clomiphene[113] and initially tamoxifen[114,115] were approved as profertility drugs for the induction of ovulation. A pivotal observation in the 1960s was the description of opposing biological activities for the *E* and *Z* isomers of substituted triphenylethylenes. Tamoxifen (ICI 46,474; **Figure 1**) is the *Z* isomer of *p*-dimethylaminoethoxy-1,2-diphenylbut-1-ene and is an antiestrogen in the rat, whereas ICI 47,699 (**Figure 1**), the *E* isomer, is an estrogen.[110,116] Similarly, clomiphene is a mixture of geometric isomers with opposing biological properties. Unfortunately, the isomers were originally given the wrong designations as isomers A and B[117] to identify the *E* and *Z* isomers, respectively. These were subsequently renamed enclomiphene and zuclomiphene for the antiestrogenic *E* isomers and estrogenic *Z* isomer, respectively.[118,119] The appropriate classification of the compounds was confirmed with the finding that nafoxidine, tamoxifen, and enclomiphene have identical crystal structures and stereochemical features.[120]

During the late 1960s, the pharmaceutical industry began to lose interest in contraceptive research. However, structure–activity relationship studies were still being rigorously pursued at the Central Drug Research Institute in Lucknow, India. Simple acyclic 1,2,3-triarylpropenones were shown to possess antifertility activity, but the *Z* isomers are more potent than the *E* isomers.[121–124] This observation led to the discovery by scientists at Eli Lilly and Company of trioxifene (LY 133,314; [3,4-dihydro-2-(4-methoxyphenyl)-1-naphthalenyl][4-]2-(L-pyrrolidinyl) ethoxy phenylmethanone; **Figure 3**).[125] Trioxifene is a compound related to nafoxidine but diverges from the general triphenylethlene structure by the introduction of a ketone bridge that links the phenyl ring with the *p*-alkylaminoethoxy side chain to the rest of the carrier molecule. Nafoxidine,[108] a first-generation antiestrogen, contains the dihydronaphthalene core and was a clinical candidate for the treatment of breast cancer[126,127] but suffered from extensive side-effects, including phototoxicity. In the presence of light, nafoxidine undergoes a photocyclo addition followed by subsequent air oxidation to a phenanthrene-containing compound. Trioxifene prevents the possibility of photocyclization by hingeing the carbonyl atom placed between the amine-containing side chain and the dihydronaphthalene core. In terms of its pharmacologic profile, trioxifene is very similar to tamoxifen.[128–130] It is a



Nafoxidine **Trioxifene**

Figure 3 Nafoxidine and trioxifene.

potent antiestrogen with slightly less estrogenic activity than tamoxifen.[129] Phase II clinical trials using trioxifene have shown activity in the treatment of breast cancer[131,132]; however, due to the lack of increased efficacy when compared to tamoxifen, this compound was abandoned for development as a treatment of breast cancer in the early 1980s.

The finding that 4-hydroxytamoxifen is a potent antiestrogen in the rat[133] with a binding affinity for the estrogen receptor equivalent to that of estradiol[134,135] stimulated a search for compounds with potential use as new research tools and anticancer agents. The hope was to find an antitumor agent that had negligible estrogen activity but potent antagonist activity with high affinity for the estrogen receptor. To this end, the antiestrogen LY 117018 (**Figure 2**),[136–139] a raloxifene analog, was investigated by Eli Lilly and Company. This compound was shown to have a high binding affinity for the estrogen receptor and low intrinsic estrogenic activity in breast cancer cells.[139] It is capable of inhibiting the uterotropic actions of E_2 in immature rats[137,140] and blocks the growth of 7,12-dimethylbenzanthracene (DMBA)-induced rat mammary carcinomas at low doses but surprisingly enhances tumor growth at high doses.[141] LY 117018, however, had a short duration of action as an antiestrogen,[142] hence, it was quickly realized that a more active compound keoxifene (LY 156758; **Figure 2**)[86] should be pursued for clinical testing as an anticancer agent. The compounds LY 117018 and LY 156758 are both rather similar to the diphenolic estrogen diethylstilbestrol (DES; **Figure 2**). Clinical trials with keoxifene originally showed either no activity or modest activity as a breast cancer therapy,[95] hence, its development as an antitumor agent was abandoned in the late 1980s.

The recognition of SERM activity (*see* 8.08 Tamoxifen) and the possibility of developing multifunctional medicines eventually resulted in the successful reinvention of keoxifene as raloxifene to treat and prevent osteoporosis. This was a direct result of the finding that tamoxifen and keoxifene can maintain bone density[96,97] but reduce mammary cancer incidence in rats.[91] The concept of using SERMs to treat or prevent multiple diseases in women was clearly outlined in 1990.[143]

> We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high risk group to prevent breast cancer.

In response to this proposed medical research strategy, keoxifene was reinvented as raloxifene: the pharmacologically active molecule remained the same but the code name changed from LY 156758 to LY 139481.

8.09.3 Complex Patenting

The claims for exclusivity in product development are, in the case of raloxifene, extremely complex and reflect the changing priorities for Eli Lilly and Company at the beginning of the 1980s and again at the beginning of the 1990s. By that time, scientists outside the company had provided a 'roadmap' for the development of a new class of drugs targeted to specific organs such as bone or circulating cholesterol[143] (see above). There are now more than 40 essentially 17-year-use patents pertaining to raloxifene. These are best summarized in US patent number US 6,906,086 B2, June 14, 2005, that extends or adjusts the original claims of US 5,393,763, published Febuary 28, 1995, for an

additional 98 days. The original raloxifene use patent, as a method for inhibiting bone loss (inventors Larry J. Black and George J. Cullinan) was filed on January 12, 1994 with a publication date of February 28, 1995. The actual use patent for raloxifene would therefore expire on July 28, 2012 + 98 days, because of a prior application date of July 28, 1992.

There is a long evolution of the Eli Lilly patents before the multiple-use patents for raloxifene were filed on January 12, 1994 and awarded as US 5,393,763 on February 28, 1995. The first intended development for the 2-phenyl-3-aroylbenzothiophene compounds that are the active component of raloxifene was as antifertility agents by David Jones and Tulio Suarez (US patent no. 4,133,814, issued on January 9, 1979), with certain of the compounds subsequently being found to be antitumor agents in the DMBA model.[94] An indication for the treatment of breast and prostatic cancer was secured by Jones, with US patent no. 4,418,068 issued November 29, 1983. Keoxifene (now known as raloxifene) was briefly tested unsuccessfully as an antitumor agent before this line of investigation was abandoned in the mid to late 1980s.

The reinvention of raloxifene is summarized as follows: "This invention provides new method for the treatment of bone loss comprising administering to a human in need of treatment an effective amount of a compound of Formula I." This is the generalization of the 2-phenyl-3-aroylbenzothiophene structure. The US patent no. 5,393,763 was a continuation of the application ser. no. 07/920,933 filed on July 28, 1992, which was abandoned. The chain of events that led to filing a patent application on July 28, 1992 is particularly interesting as the translational research published by the academic community had made the claim obvious for this class of drugs and for keoxifene in particular. In fairness, some of the relevant references were listed as 'other publications'[96] in US patent no. 5,393,763. The Love publication[144] on the bone-sparing effects of tamoxifen was a direct result of ongoing research at the University of Wisconsin Comprehensive Cancer Center that showed tamoxifen and keoxifene maintained bone density in ovariectomized rats,[96] prevented rat mammary carcinogenesis,[91] and partially blocked the growth of tamoxifen-stimulated endometrial cancer growth in athymic mice.[93] One can only speculate about the role played by the Wisconsin group on March 26, 1992 in the *New England Journal of Medicine* which demonstrated, in a prospective randomized trial, that tamoxifen could maintain and potentially increase lumbar spine bone density in postmenopausal women. Eli Lilly submitted a patent application 920,933 for their molecules on July 28, 1992! Indeed, all laboratory and clinical studies with tamoxifen,[145–148] except one,[149] showed that it maintained bone density. In contrast, the Feldman study was used in the patent application to suggest uncertainty as to the actions of raloxifene on bone, despite a prior publication[96] which demonstrated similar actions to tamoxifen in a comparison study. A specific patent application for inhibiting the loss of bone using 6-hydroxy-2-(4-hydroxyphenyl)-benzo[b]thien-3-yl-4[2-piperidin-1-ethoxyphenol] methanone hydrochloride was filed on October 26, 1994 (application number 329,396) and granted on October 10, 1995 as patent no. 5,457,117. A broad patent for a method of inhibiting bone loss and lowering serum cholesterol using low-dosage amounts of particular 2-phenyl-3-aroylbenzothiophenes was also filed by Michael Draper, assigning rights to Eli Lilly and Company on March 2, 1994 and issued on December 26, 1995 at patent no. 5,478,847.

Although it is not relevant to consider all the subsequent patents awarded to Eli Lilly and Company, it is perhaps pertinent to observe that these are often patents for formulations of numerous oral preparations. These patents are illustrated by patent numbers 5,811,120 (September 22, 1998), 5,972,383 (October 26, 1999), US 6,458,811 B1 (October 1, 2002), US 6,797,719 B2 (September 28, 2004), and US 6,894,064 B2 (May 17, 2005). Each of these is a new invention for raloxifene-like analogs "optionally containing estrogen or progestin for alleviating the symptoms of osteoporosis, lowering lipid levels, and inhibiting endometriosis, uterine fibroids, and breast cancer."

8.09.4 Raloxifene Structural Characteristics

Recent progress in our understanding of the molecular biology of estrogen receptor action has provided a great deal of evidence which promises to increase our understanding of the mechanism through which SERMs elicit their tissue-specific effects. This in turn has enhanced interest in raloxifene and increased the interest in developing new tissue-specific SERMs. The identification of numerous coactivators and corepressors[150,151] which modulate receptor function and the realization that there are two subtypes of estrogen receptor (ERα and ERβ)[152] attest to the potential complexity through which SERMs produce diverse tissue-specific responses. To date, more than 20 coregulator proteins have been discovered that bind to estrogen receptors and modulate their function, each acting as either a positive (coactivator) or a negative (corepressor) transcriptional regulator. Depending on the unique receptor conformation induced by ligand binding, varying combinations of coregulator proteins can potentially interact with the estrogen receptor complex to modulate its function in a variety of ways.[150,151]

What separates SERMs like raloxifene and tamoxifen from classical estrogen agonists (i.e., E_2) is their ability to function in a similar fashion to the classical estrogens on some tissues while not acting as agonists, or perhaps even behaving as antagonists in other tissues. During the late 1990s there was an increased interest in the molecular

mechanism of action of raloxifene by the scientific community. This in turn enhanced interest in the development of raloxifene as a clinically useful agent. There was also interest in determining the mechanism of drug resistance to tamoxifen. Development of tamoxifen resistance can be characterized by an increase in the partial agonist properties of the antiestrogen in the breast, resulting in loss of growth inhibition and even inappropriate tumor stimulation. Initially, it was thought that a mutation in the estrogen receptor might be involved in changing the pharmacology of tamoxifen from an antiestrogen to an estrogen; however, no such mutations were ever found in clinical samples. Interestingly, the chance finding of a D351Y (Asp-351 to Tyr-351) estrogen receptor mutation in a tamoxifen-stimulated breast tumor in 1994,[153,154] that enhanced the agonist activity of 4-hydroxytamoxifen and altered the pharmacology of raloxifene from an antiestrogen to a partial agonist,[155,156] provided an invaluable starting point to decipher how SERMs modulate estrogen receptor function.

Evidence from co-crystal structures of estrogen receptor ligand-binding domains complexed with an estrogen or a SERM (i.e., tamoxifen or raloxifene) provided important information as to how an estrogen or antiestrogen alters the shape of the estrogen receptor complex.[157,158] A key feature of the estrogen agonist-ERα structure is the ability of the ligand to be enveloped in a hydrophobic pocket that is closed by helix 12 in the ligand-binding domain of ERα. This change in structure (i.e., repositioning of helix 12) facilitates coactivator binding to the AF-2 region of the receptor and is considered an important mechanism for full estrogen action at ERα.[157] Both raloxifene and 4-hydroxytamoxifen fit into the hydrophobic pocket of the ligand-binding domain; however, the bulky alkylaminoethoxyphenyl side chain prevents the reorientation of helix 12 that must seal the ligand into the receptor before coactivators can bind and produce a transcription complex. The high-affinity antiestrogens both interact through phenolic hydroxyls with Glu-353 and Arg-394 to locate the ligands correctly in the binding domain[157]; however, the side chain, which is critical for antiestrogenic activity, interacts with an aspartic acid residue (Asp-351; D351), which lies at the base of ERα helix 3.[157,158] An examination of the surface structure of the raloxifene–estrogen receptor complex has shown that Asp-351 forms a strong hydrogen bond (2.7 Å, 180°) with the tertiary amine of the piperidine ring of the antiestrogenic side chain of raloxifene.[157] This interaction forces the piperidine ring into an awkward high-energy gauche position so that the bulky side chain of raloxifene can shield and neutralize the Asp-351 (D351) on the receptor surface.[159] It is suggested that the shielding or neutralization of D351 by the side chain of raloxifene is responsible for the difference in the intrinsic activity of the raloxifene and tamoxifen–ERα complex. Replacing the aspartate with glycine results in a tamoxifen D351G–ERα complex that has lost estrogen-like activity while retaining antiestrogenic properties.[160,161] The D351G mutation decreases the affinity of raloxifene for ERα, thereby illustrating the important role of the interaction of the piperidine side chain and D351. Similarly, the raloxifene–ERα complex can be modulated through both D351 and the antiestrogenic side chain. A D351E mutation that extends the interactive distance from 2.7 Å in raloxifene D351 to 3.5–5 Å in E351 increases the estrogen-like action of the raloxifene–ERα complex.[159]

8.09.5 Raloxifene and Bone

Beall and coworkers[162] published the first report of the actions of a nonsteroidal antiestrogen clomiphene on bone maintenance in ovariectomized rats. Unfortunately, these studies were flawed, as clomiphene is a mixture of the antiestrogenic *E* isomer and the estrogenic *Z* isomer. Clearly, it was possible that one isomer produced a dominant estrogenic effect in bone but the other isomer produced a dominant antiestrogenic effect in uterus and breast. The uncertainty was clarified with the finding that tamoxifen, the antiestrogenic pure isomer of a triphenylethylene, and raloxifene, a chemically stable antiestrogen, were both estrogen-like in bone but antiestrogenic in uterus and breast.[96]

The 1995 patent and the December 10, 1997 FDA approval of raloxifene for the treatment and prevention of osteoporosis were based, in part, on earlier studies performed in 1987 by Jordan and coworkers[96] which showed that raloxifene preserved bone density in ovariectomized rats (**Figure 4a**) and prevented rat mammary carcinogenesis (**Figure 4b**).[91] The discovery that raloxifene and related compounds might prevent osteoporosis[96] laid the foundation for subsequent confirmation of bone data in animals.[97–99,163] These discoveries also led to clinical trials that demonstrated maintenance of bone density in postmenopausal women at risk for osteoporosis.[78] These data were remarkably similar to those observed with tamoxifen.[144] However, the actual proof that raloxifene could be useful to treat osteoporosis was obtained in the MORE trial (**Figure 5**), which was a multicenter, randomized, blinded, placebo-controlled osteoporosis treatment trial. A total of 7705 women aged 31–80 years in 25 countries who had been postmenopausal for at least 2 years and who met World Health Organization criteria for having osteoporosis were included in the study. The study began in 1994 and had up to 36 months of follow-up for primary efficacy measurements and nonserious adverse events and up to 40 months of follow-up for serious adverse events. Participants were randomized to raloxifene (60 or 120 mg day^{-1}) or placebo. Results from this study[79,164] showed that raloxifene at

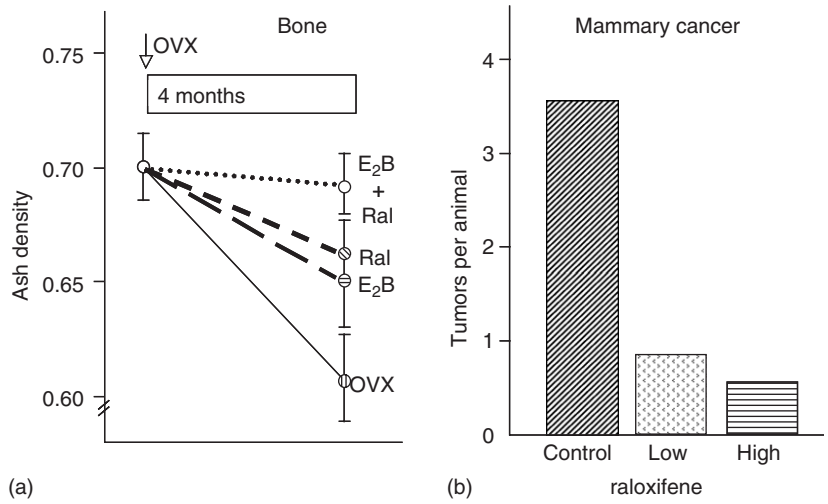


Figure 4 A comparison of the effects of raloxifene (Ra1) on (a) femur ash density in ovariectomized (OVX) rats[96] and (b) the incidence of rat mammary tumors[91] after the administration of *N*-nitrosomethylurea (NMU). For bone density studies, ovariectomized rats (8 per group) were treated daily with raloxifene (100 μg), estradiol-3-benzoate (25 μg), or with a combination of raloxifene and estradiol-3-benzoate for 4 months. One group of 8 ovariectomized rats received only the vehicle (0.2 ml peanut oil). For tumor incidence studies, virgin female Sprague-Dawley rats were injected with 5 mg NMU, and 2 weeks after the injection rats were randomized into three groups of 25 animals and were given injections daily for 10 weeks with 100 (low) and 500 μg (high) raloxifene. The control group received peanut oil alone.

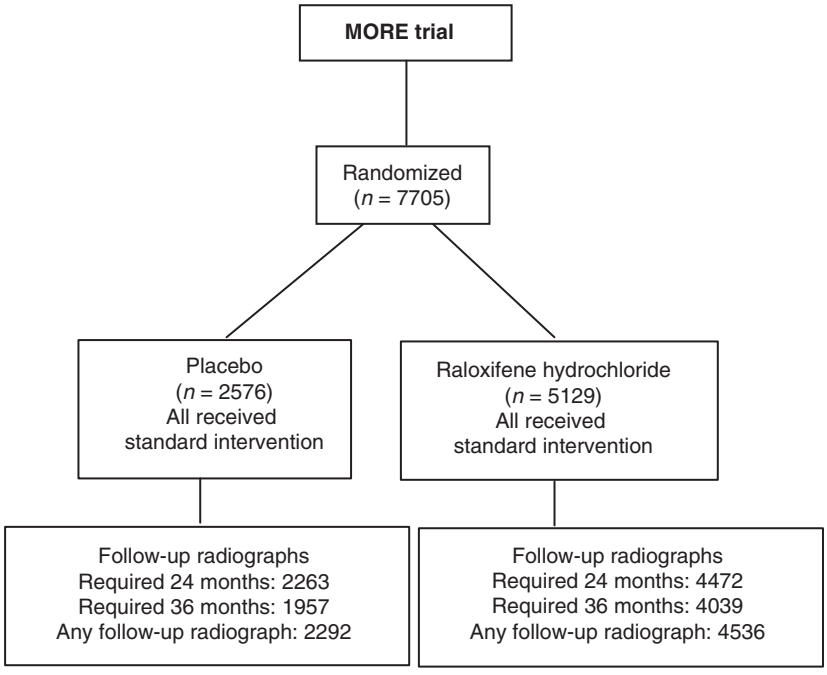


Figure 5 Study design of the Multiple Outcomes of Raloxifene Evaluation (MORE) trial. The MORE trial is a multicenter, randomized, blinded, placebo-controlled trial which was undertaken primarily to examine the effect of raloxifene on the skeleton. A total of 7705 postmenopausal women with osteoporosis and ranging in age from 31 to 80 years participated in this study. The women were divided into two study groups and were then randomized to receive either placebo or 60 or 120 mg day^{-1} of raloxifene. Study group 1 included those whose femoral neck or lumbar spine bone density *t* score was below -2.5. Study group 2 included women who had low bone density and one or more moderate or severe vertebral fractures or two or more mild vertebral fractures. Incident vertebral fracture was determined radiographically at baseline and at scheduled 24- and 36-month visits. Bone mineral density was determined annually by dual-energy x-ray absorptiometry.

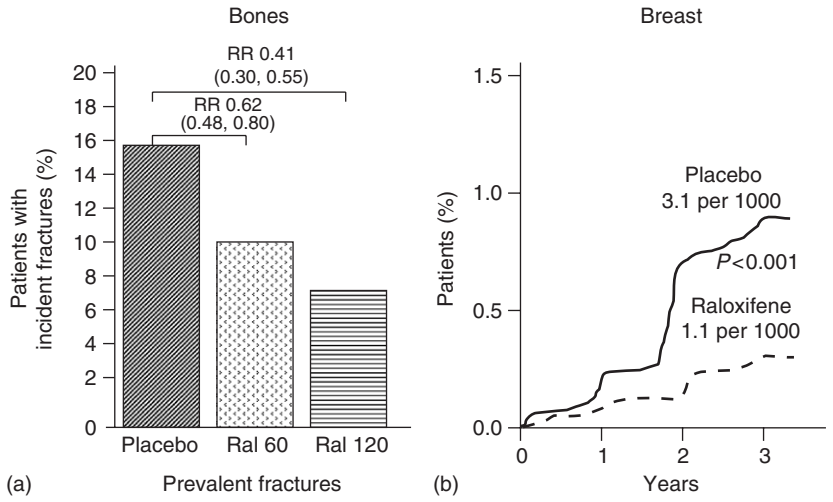


Figure 6 The effect of raloxifene on (a) bone mineral density and vertebral fracture in postmenopausal women with osteoporoisis[79] and (b) breast cancer incidence[168] in the MORE trial. At 36 months of the evaluable radiographs in 6828 women, risk of vertebral fracture was reduced in both study groups receiving raloxifene (60 mg day^{-1} group: RR, 0.62; 95% CI, 0.5–0.8; 120 mg day^{-1} group: RR, 0.5; 95% CI, 0.4–0.7). The cumulative incidence of breast cancer among subjects in the placebo group and those in the combined raloxifene group are represented as a percentage of all patients randomized to either group. Statistical significance of the difference between the groups was tested by a log-rank test ($P < 0.001$).

60 mg day^{-1} reduced the risk of vertebral fractures by 30% (RR, 0.7; 95% CI 0.5–0.8) and by 50% (RR, 0.5; 95% CI 0.4–0.7) at 120 mg day^{-1}, compared to placebo (**Figure 6a**). The risk of nonvertebral fracture for raloxifene versus placebo, however, did not differ significantly.[79] Compared with placebo, raloxifene also increased bone mineral density in the femoral neck by 2.1% (60 mg) and 2.4% (120 mg) and in the spine by 2.6% (60 mg) and 2.7% (120 mg; $P < 0.001$) for all comparisons.[79]

As a follow-up to the MORE trial, the CORE trial (**Figure 7**) was developed. The CORE study was designed to evaluate the long-term efficacy of 4 additional years of raloxifene therapy in reducing the incidence of invasive breast cancer in postmenopausal women with osteoporosis who previously were treated with raloxifene in the MORE trial. Siris and coworkers[83] reported that, after 7 years of the MORE trial, compared with MORE baseline, raloxifene treatment significantly increased lumbar spine (4.3% from baseline, 2.2% from placebo) and femoral neck bone mineral density (1.9% from baseline, 3.0% from placebo). Bone mineral densities were also significantly increased from MORE baseline at all time-points at both sites with raloxifene.

8.09.6 Raloxifene and Breast Cancer Prevention

The rationale for the use of SERMs, including raloxifene, as breast cancer preventives is based on a strategic hypothesis formulated when SERM action was first recognized in the late 1980s.[96,143] The evidence to support the use of raloxifene in this paradigm stems from observations made in the laboratory[91,96] and the clinic[165] along with close monitoring of ongoing osteoporosis placebo-controlled trials. Previous studies have shown that raloxifene inhibits the growth of dimethylbenzanthracene-induced rat mammary carcinoma[94] but it prevents mammary cancer by reducing the incidence of *N*-nitrosomethylurea-induced tumors[91,92] if given after the carcinogen but before the appearance of palpable tumors. However, as would be anticipated with a drug that has a short biological half-life, raloxifene is not superior to tamoxifen at equivalent doses.[91] Studies have shown that raloxifene, when administered orally, is rapidly absorbed from the gastrointestinal tract and undergoes extensive phase II metabolism in the liver.[166] Hence, its absolute availability is around 2%.[166] Because of its low availability, a higher dose of raloxifene must be administrated in vivo to obtain an efficacy equivalent to that with tamoxifen,[91,167] which has a low binding affinity for the estrogen receptor but accumulates and is subsequently converted to the active metabolite 4-hydroxytamoxifen.[134] For this reason, doses above 60 mg raloxifene daily have been tested in clinical trials to prevent osteoporosis. Interestingly, raloxifene, at a high dose of up to 300 mg daily, produced only modest antitumor activity for the treatment of advanced disease.[165]

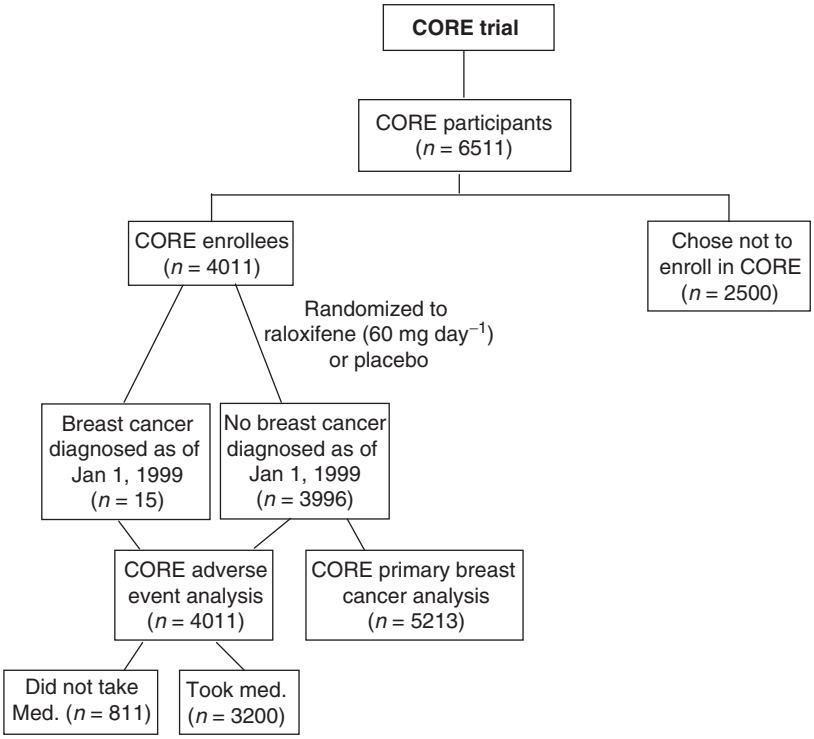


Figure 7 Study design for the Continuing Outcomes Relevant to Evista (CORE) trial. CORE is a multicenter, double-blind, placebo-controlled clinical trial. It is a follow-up to the MORE trial and its purpose is to evaluate the long-term efficacy of raloxifene in reducing the incidence of invasive breast cancer in postmenopausal women with osteoporosis who were previously treated with raloxifene for up to 4 years in the MORE trial. All MORE investigation sites were invited to participate in the CORE trial. From those investigators choosing to participate, all women in the MORE trial ($n = 7705$ participants) were invited to participate in the CORE trial after their completion or discontinuation from the MORE trial, and 4011 of those women chose to participate. Of the 2500 MORE trial participants who chose not to enroll in the CORE trial, 1217 women were still participating in the MORE trial as of January 1, 1999. The remaining 1283 women had completed their participation in the MORE trial before January 1, 1999, and thus did not contribute data for any of the CORE trial analyses. Fifteen CORE enrollees were diagnosed with breast cancer before January 1, 1999, and were excluded from the CORE breast cancer and sensitivity analyses but were included in the safety analyses. Raloxifene (60 mg day^{-1}) was selected as the only active treatment dose for CORE because the 60 and 120 mg day^{-1} doses of raloxifene had shown similar risk reduction efficacies in the MORE trial. Women who had been randomized to receive placebo in the MORE trial received placebo in the CORE trial. The CORE trial was designed to continue for a maximum of 4 years, hence, the planned total treatment period will be approximately 8 years from the time of randomization in the MORE trial.

Based on the hypothesis that SERMs could reduce the incidence of breast cancer as a beneficial side effect of the prevention of osteoporosis[143] and as a safety requirement for FDA approval, the MORE trial was analyzed for changes in breast cancer incidence. As explained above, the MORE trial was an osteoporosis treatment trial conducted in postmenopausal women comparing the efficacy of raloxifene (60 or 120 mg daily) versus placebo, with breast cancer risk reduction as a predefined secondary endpoint. A 3-year follow-up in the MORE trial[168] revealed that raloxifene decreased the risk of of invasive breast cancer by 76% and the risk of estrogen receptor-positive breast cancer by 90%, with no significant effect on estrogen receptor-negative invasive breast cancer. A 4-year follow-up of the MORE trial showed that raloxifene reduced the incidence of all breast cancers by 62% (**Figure 6b**), invasive breast cancer by 72%, and invasive estrogen receptor-positive cancer by 84% in postmenopausal women, with no significant effect on estrogen receptor-negative invasive breast cancer.[169]

Due to the positive results from the MORE trial, the National Surgical Adjuvant Breast and Bowel Project (NSABP) initiated the STAR trial[82] in June 1999. The STAR trial (**Figure 8**) is a phase III, randomized, double-blind trial that will compare the effect of raloxifene (60 mg orally) with that of tamoxifen (20 mg orally) in reducing the incidence of invasive breast cancer in postmenopausal women at high risk for the disease over a 5-year period. Trial participants will

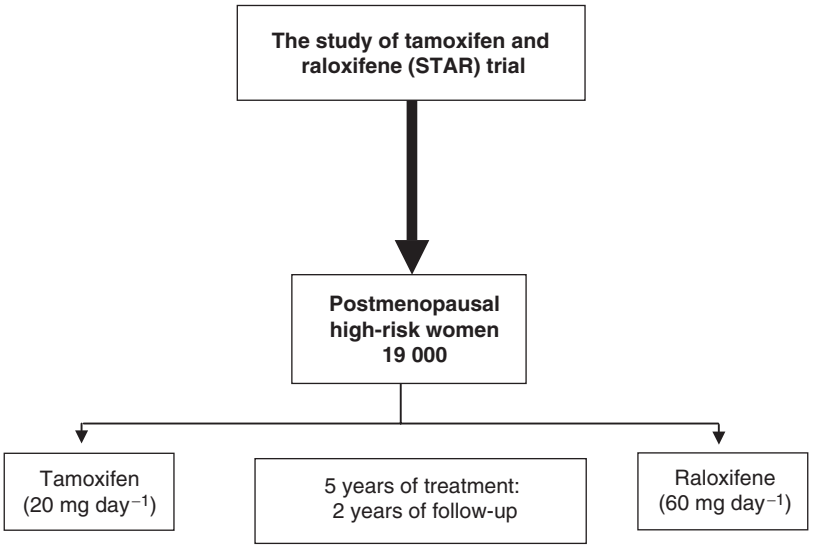


Figure 8 The design of the STAR trial. The STAR trial is a phase III, double-blinded trial that will assign eligible postmenopausal women to either daily tamoxifen (20 mg orally) or raloxifene (60 mg orally) therapy for 5 years. It is the first head-to-head trial comparing the effect of raloxifene with that of tamoxifen in reducing the incidence of invasive breast cancer in postmenopausal women at risk for the disease. Approximately 19 000 postmenopausal women 35 years of age or older having at least a 1.66% estimated Gail risk of developing breast cancer or a history of lobular carcinoma in situ (LCIS) are being enrolled. The trial is double-blinded, and study participants will be randomized to receive either 20 mg day^{-1} tamoxifen or 60 mg day^{-1} raloxifene for 5 years. The STAR trial's primary aim is to determine whether long-term therapy is effective in preventing the occurrence of invasive breast cancer in high-risk postmenopausal women. The comparison is to be made to the established drug, tamoxifen. The secondary aim is to establish the relative safety profiles of raloxifene and tamoxifen.

also complete a minimum of 2 additional years of follow-up after therapy is stopped. The primary aim of the STAR trial is to determine whether long-term raloxifene therapy is effective in preventing the occurrence of invasive breast cancer in postmenopausal women who are identified as being at high risk for the disease. It is the first head-to-head trial comparing tamoxifen with raloxifene. The secondary aim is to establish the net effect of raloxifene therapy, by comparison of cardiovascular data, fracture data, and general toxicities with tamoxifen. The results from the STAR trial are anticipated in 2006.

The significant reduction in risk of invasive breast cancer observed in the MORE trial led directly to the design of the CORE study (Figure 7). Results from the CORE trial[170] revealed that the incidences of invasive breast cancer and estrogen receptor-positive invasive breast cancer were reduced by 59% (HR, 0.41; 95% CI, 0.24–0.71) and 66% (HR, 0.34; 95% CI, 0.18–0.66), respectively, in the raloxifene group compared with the placebo group. Raloxifene, however, did not significantly ($P = 0.86$) alter the incidence of estrogen receptor-negative invasive breast cancer. Over the 8 years of both trials, the incidences of invasive breast cancer and estrogen receptor-positive invasive breast cancer were reduced by 66% (HR, 0.34; 95% CI, 0.22–0.50) and 76% (HR, 0.24; 95% CI, 0.15–0.40), respectively, in the raloxifene group compared with the placebo group.[170]

8.09.7 Raloxifene and Lipids

Estrogen increases HDL cholesterol levels and decreases LDL cholesterol levels in humans[39,171] as well in animal models of atherosclerosis, partly because of estrogen receptor-mediated upregulation of the hepatic LDL receptor.[172] In ovariectomized rats, raloxifene treatment has been shown to reduce serum total cholesterol concentrations,[97,173] and this reduction correlates with the extent of raloxifene binding to the estrogen receptor.[97,173] These results are not surprising for a 'nonsteroidal antiestrogen,' as the original observations for clomiphene analogs and tamoxifen show (*see* 8.08 Tamoxifen). Raloxifene may also have cardioprotective effects because of its antioxidant properties. This is important since oxidative modifications of LDL have been implicated in atherogenesisis.[174] Raloxifene also appears to have a favorable effect on lipid parameters in postmenopausal women. In the published European trial,[78] treatment with raloxifene in a dosage of 30, 60, or 150 mg day^{-1} resulted in significant decreases in the serum concentrations of total and LDL cholesterol over a 24-month period ($P < 0.05$ versus placebo). These decreases were evident during the

first 3 months of therapy and were maintained thereafter. Notably, none of the treatment groups showed any changes in serum concentrations of HDL cholesterol and triglycerides. The effect of raloxifene on serum lipid levels was also assessed in 390 healthy postmenopausal women.[175] In this study, raloxifene (60 and 120 mg day^{-1}) was compared with HRT (0.625 mg day^{-1} of conjugated estrogen and 2.5 mg day^{-1} of medroxyprogesterone given continuously) and placebo. Assessments were made at baseline, 3 months, and 6 months. Over the 6-month study period, both dosages of raloxifene lowered serum LDL cholesterol levels by about 12% compared with placebo ($P < 0.001$). This finding was similar to the 14% reduction that occurred with continuous HRT.[78] The effect of raloxifene on cardiovascular events was also examined in osteoporotic postmenopausal women from the MORE trial. In the study design, patients were randomly assigned to receive raloxifene 60 mg day^{-1} ($n = 2557$), or 120 mg day^{-1} ($n = 2572$), or placebo ($n = 2576$) for 4 years. Barrett-Connor and coworkers[176] reported that raloxifene therapy for 4 years did not significantly affect the risk of cardiovascular events in the overall cohort but did significantly reduce the risk of cardiovascular events in the subset of women with increased cardiovascular risk. In addition, there was no evidence that raloxifene caused an early increase in risk of cardiovascular events.

To address the question of whether raloxifene reduces the risk of CHD, a total of 10 101 women (at increased risk of CHD) have been recruited to receive placebo or raloxifene in the RUTH trial, with cardiovascular disease as a primary endpoint.[177] The RUTH trial (**Figure 9**) is designed to determine whether raloxifene (60 mg day^{-1}), compared with placebo, reduces the risk of coronary events and invasive breast cancer in postmenopausal women at risk for a major coronary event.

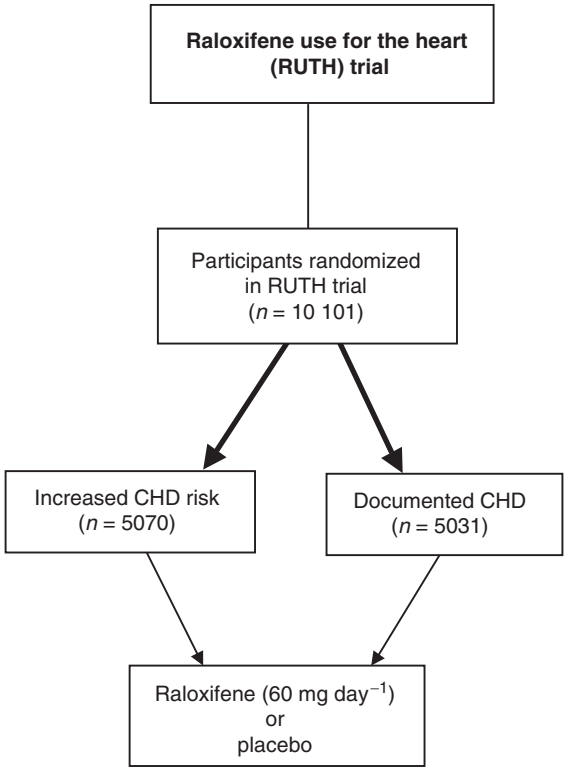


Figure 9 Study design of the Raloxifene for Use in The Heart (RUTH) trial. The RUTH trial is a double-blind, placebo-controlled, randomized clinical trial designed to evaluate whether 60 mg day^{-1} of oral raloxifene compared with placebo reduces the risk of coronary events. Between June 1998 and August 2000, 11 767 women signed an informed consent agreement to participate in RUTH at 187 sites in 26 countries. After excluding 1411 women who did not meet inclusion criteria and 255 women who met more than one exclusion criteria, a total of 10 101 women were randomized to raloxifene 60 mg day^{-1} or placebo. Of these, 5070 were at increased risk for CHD events and 5031 had documented CHD. Women were eligible for randomization if they were aged 55 years or older, at least 1 year postmenopausal, and had documented CHD, peripheral artery disease, or multiple risk factors for CHD. Breast cancer incidence will be determined by mammograms performed 2, 4, and 6 years after the qualifying mammogram. The study is planned to end after the pre-specified number of participants experience their first acute coronary event. The total duration of treatment is projected to range from 5 to 7.25 years.

8.09.8 Other Selective Estrogen Receptor Modulators

Current interest in new SERM molecules has built on the experience of the prototypes with the goal of enhancing bioavailability and selectivity and decreasing side effects (i.e., breast cancer, uterine cancer, and blood clots). All compounds under study have predominantly antiestrogenic effects in the rodent uterus with virtually no estrogen agonist properties. In order to improve upon the raloxifene pharmacophore, some groups have reported on the effect of modifying the benzothiophene nucleus. Particularly noteworthy were two discoveries made by the chemists at Eli Lilly which improved upon raloxifene.[77] One change involved the introduction of a methyl ether on either the 5-OH or the 4′OH position, which resulted in compounds with increased potency in a cholesterol reduction assay in ovariectomized rats.[178,179] The other change involved the replacement of the carbonyl 'hinge' with other atoms or groups, including N, CH_2, S, and O. The change to the oxygen atom resulted in a compound with very little or no uterine effects in preclinical rodent models as well as increased potency in preventing bone loss in the ovariectomized rat model.[180] These combined changes resulted in the development of arzoxifene.

8.09.9 Arzoxifene

Arzoxifene (LY 353,381.HCl; **Figure 2**) is a new benzothiophene analog that is structurally related to raloxifene.[180,181] Its structure differs from that of raloxifene by the replacement of a carbonyl group with oxygen. It was designed to improve the bioavailability of raloxifene and provide sustained antiestrogenic blockade in the treatment of breast cancer without any of the agonist effects seen with tamoxifen. It is classified as a second-generation SERM, based on its differential estrogenic/antiestrogenic effects in vivo on estrogen target tissues.[181] It is metabolized by demethylations and both the parent compound and the metabolite bind to the estrogen receptor with high affinity and inhibit estrogen-dependent growth of MCF-7 breast cancer cells.[182–184] Arzoxifene protects against bone loss and reduces serum cholesterol levels in ovariectomized rats with a potency that is 30–100 times greater than that of raloxifene and it has minimal uterine effects.[181,185] It is highly effective at preventing *N*-methyl-*N*-nitrosourea-induced mammary cancer in rats and is significantly more potent than raloxifene in this regard.[186] Interestingly, arzoxifene has also been shown to be only partially cross-resistant with tamoxifen in models of drug-resistant breast and endometrial cancer[187,188]; however, recent evidence indicates that it is superior to raloxifene as a chemopreventive in rat mammary carcinogenesis.[182,189,190] In a small phase I study in 32 pre- and postmenopausal women with locally advanced or metastatic breast cancer who had previously received endocrine therapy, arzoxifene (10, 20, 50, and 100 mg) did not produce any significant responses, suggesting cross-resistance between arzoxifene and tamoxifen.[191] In a phase II study in 119 pre- and postmenopausal women with advanced or metastatic breast cancer, two doses of arzoxifene (20 versus 50 mg day^{-1}) were compared in patients who had either tamoxifen-sensitive or tamoxifen-resistant disease and 20 mg arzoxifene was found to be as effective as 50 mg in the treatment of metastatic breast cancer.[191] A phase III trial was subsequently initiated comparing arzoxifene (20 mg day^{-1}) with tamoxifen (20 mg day^{-1}) in postmenopausal women with advanced disease; however, at the interim review, the trial was terminated and development of arzoxifene discontinued for this indication.

8.09.10 Bazedoxifene

Bazedoxifene (TSE-424; **Figure 10**) is a novel SERM developed by Wyeth Pharmaceuticals that is currently in phase III clinical trials for the prevention and treatment of postmenopausal osteoporosis. It is an indole-based estrogen receptor ligand that has been stringently selected to ensure an improved profile over its predecessor raloxifene. It was developed using preclinical selection parameters, which included favorable effects on the skeleton and lipid metabolism, demonstrable mammary and uterine safety, and neutral effects on hot flashes.[192] Bazedoxifene treatment maintains bone mineral density, preserves normal bone histology, increases bone compressive strength, and reduces total cholesterol levels in animal models.[192–194] It lacks uterotropic activity[194] and it blocks raloxifene-induced increases in uterine weight[192] and inhibits E_2-induced proliferation in MCF-7 breast cancer cells.[192] Based on the favorable preclinical evaluation, it is suggested that bazedoxifene has the potential to improve the SERM profile beyond that achieved by raloxifene.

8.09.11 Lasofoxifene

Lasofoxifene (**Figure 10**) is a novel nonsteroidal SERM that is in clinical trials for the prevention and treatment of osteoporosis in postmenopausal women.[195] It is a diaryltetrahydronaphthalene derivative referred to as CP336156. The structure of CP336156 is reminiscent of nafoxidine (**Figure 3**) if it were to be demethylated in vivo. There are two diastereometric salts. CP336156 is the L enantiomer that has 20 times the binding affinity of the D enantiomer. Studies

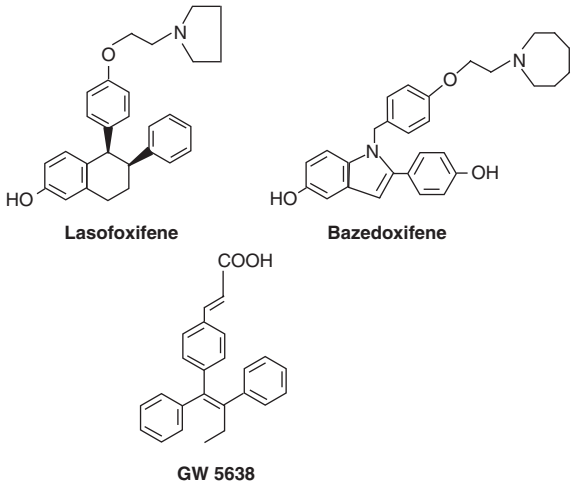


Figure 10 Bazedoxifene, lasofoxifene, and GW 5638.

demonstrated that the L enantiomer had twice the bioavailability of the D enantiomer.[195] It has a high binding affinity for estrogen receptor and preclinical studies indicate that it prevents lumbar vertebral bone loss in the ovariectomized rat model, with greatly enhanced potency relative to raloxifene.[195–197] Lasofoxifene also lowers serum cholesterol levels without induction of uterine hypertrophy in rat models,[195,196] and it inhibits breast tumor formation in mice injected with human MCF-7 breast cancer cells and blocks *N*-nitrosomethylurea-induced mammary carcinomas in rats.[198] In a phase III clinical trial conducted by Pfizer[199] involving 410 postmenopausal women randomly assigned to CP336156 (0.25 or 1 mg day^{-1}), raloxifene (60 mg day^{-1}), or a placebo, CP336156 increased bone mineral density at the lumbar spine by about 2% after 2 years of treatment, compared to no increase with raloxifene and a 2% decrease in the placebo group. Changes in bone turnover markers were also greater with CP336156 and the drug reduced LDL cholesterol levels by a mean of 20% versus 12% with raloxifene. Overall, the drug was well tolerated and there were no reports of increased endometrial hyperplasia or vaginal bleeding.

8.09.12 GW 5638

GW 5638 (**Figure 10**) is a tamoxifen analog (*see* 8.08 Tamoxifen) that was discovered by Willson and colleagues in 1994[200] at Glaxo Wellcome in North Carolina. It functions as a full estrogen receptor agonist in bone and the cardiovascular system in ovariectomized rats, but, unlike tamoxifen, it is a more potent antagonist in breast cancer cells and has no uterotrophic behavior.[201] GW 5638 does not have the usual tertiary amino antiestrogenic side chain but a shorter allylcarboxylic group on a triphenylethylene carrier molecule.[200,201] GW 5638 can induce a unique conformational change in ERα that is recognized by synthetic peptides selected by phage display.[202,203] These peptides recognize GW5638–ERα complexes but not tamoxifen–ERα or other ligand-bound estrogen receptor complexes,[200,201] indicating that conformational changes elicited by GW 5638 and tamoxifen are different. Recent crystallograpy studies[204] of ERα ligand binding domain (LBD) bound to GW 5638 have revealed a new LBD conformation in which AF2 H12 is repositioned by direct contacts between the carboxyl side chain of GW 5638 and the N-terminus of H12. In addition to preventing coactivator recruitment by occlusion of the AF2 cleft, GW 5638 also destabilizes ERα, although less so than the more potent estrogen receptor antagonist ICI 164,380/182,780 (fulvestrant),[204] which suggests that it is a more potent growth inhibitor than tamoxifen and raloxifene. This effect is associated with a rotation of H12, induced by the tethering of Leu-536 and Tyr-537 to the carboxyl moiety on GW 5638/7604, which leads to an increase in the surface hydrophobicity of the ERα LBD and a decrease in ERα stability. The fact that tamoxifen-resistant breast cancers are not cross-resistant to GW 5638[205] suggests that this SERM has significant potential as a therapeutic agent.

8.09.13 Conclusion

During the last 15 years there has been a revolution in understanding the multifaceted aspects of the estrogen receptor as a changeable target. Subtle three-dimensional changes in ligand structures lead to conformational changes between

ligand–receptor complexes that yield distinct physiological responses. However, despite our rapid advance in understanding these complex phenomena, it is still unclear as to exactly how these multisignalling pathways of estrogen ultimately determine certain biological endpoints. Many of the basic building blocks have been identified and they must now be assembled. What is interesting about the SERM story is that the first- and second-generation compounds were available long before they were recognized to have SERM activity.[66] The clinical development of nonsteroidal antiestrogens over the past 40 years has resulted in the first agents (clomiphene and tamoxifen) for the induction of ovulation in subfertile women, the first antiestrogen (tamoxifen) specifically for the treatment of estrogen receptor-positive breast cancer, the first chemopreventive (tamoxifen) to reduce the incidence of breast cancer in high-risk pre- and postmenopausal women, and the first SERM (raloxifene) for the treatment and prevention of osteoporosis. The potential effect of raloxifene to reduce the incidence of invasive breast cancer is currently being evaluated in three prevention trials, the CORE, RUTH, and STAR trials. Each raloxifene trial has enrolled a unique cohort and, when all trials are complete, they will provide important information about the occurrence of invasive breast cancer in diverse populations encompassing over 33 000 postmenopausal women with widely varying breast cancer risks. If the results of these raloxifene trials demonstrate a significant reduction in the incidence of invasive breast cancer, as was observed in the MORE trial, while confirming a better risk–benefit profile than tamoxifen, raloxifene will become an important therapy for the reduction of breast cancer risk among postmenopausal women.

Development of drugs of the SERM class is being advanced by numerous companies; however, the sheer cost of mounting the clinical trials necessary to prove efficacy as a breast cancer preventive, and preventive for osteoporosis and/or CHD is staggering. Nevertheless, new and improved SERMs are available for further development (arzoxifene, Eli Lilly and Company; bazedoxifene, Wyeth Laboratories; lasofoxifene, Pfizer). What is remarkable is the reinvention of the molecules for different indications over the past 50 years (*see* 8.08 Tamoxifen). The basic structures originally discovered as potential antifertility agents in the early 1960s are today continuing to be mined by medicinal chemists as advances in basic knowledge of molecular mechanisms occur.

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8.10 Duloxetine

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I Yalçin, Eli Lilly and Co., Indianapolis, IN, USA

8.10.1 Lower Urinary Tract Function and Dysfunction

The function of the lower urinary tract is to store and periodically release urine.[1] The lower urinary tract is composed primarily of smooth muscle that forms a reservoir (the urinary bladder) and an outlet (the urethra), which has a 'valve' – the urethral rhabdosphincter – that is composed of striated muscle. Regulation and coordination of urine storage and release (i.e., micturition) is accomplished by a series of spinal and spinobulbospinal reflexes, respectively (**Figure 1**). These reflexes can be thought of as a 'hardwired' system, but they can be modulated by various regions of the central nervous system (CNS) allowing voluntary control for closure of the rhabdosphincter and determining when the spinobulbospinal micturition reflex pathway is disinhibited or 'turned on.'

There are two urine storage reflexes, one tonic sympathetic reflex pathway originating from efferent preganglionic neurons in the upper lumbar spinal cord that relaxes the smooth muscle of the bladder and contracts the smooth muscle of the urethra via the hypogastric nerve and a second reflex pathway originating from sacral motor neurons in Onuf's nucleus that contracts the rhabdosphincter via the pudendal nerve. The latter has both a tonic component and a phasic component that rapidly and strongly contracts the rhabdosphincter to resist rapid increases in bladder pressure that accompany valsalva-related increases in abdominal pressure such as coughing, laughing, and sneezing.

The spinobulbospinal micturition reflex is triggered by sacral primary afferent neurons that respond to stretch of the bladder to activate ascending second-order neurons that subsequently activate the pontine micturition center where descending pathways activate sacral parasympathetic preganglionic neurons to produce a bladder contraction via the pelvic nerve. Coordinated with activation of the micturition reflex, the storage reflexes are simultaneously inhibited. This coordination produces a bladder contraction and opening of the urethra to allow for efficient expulsion of urine.

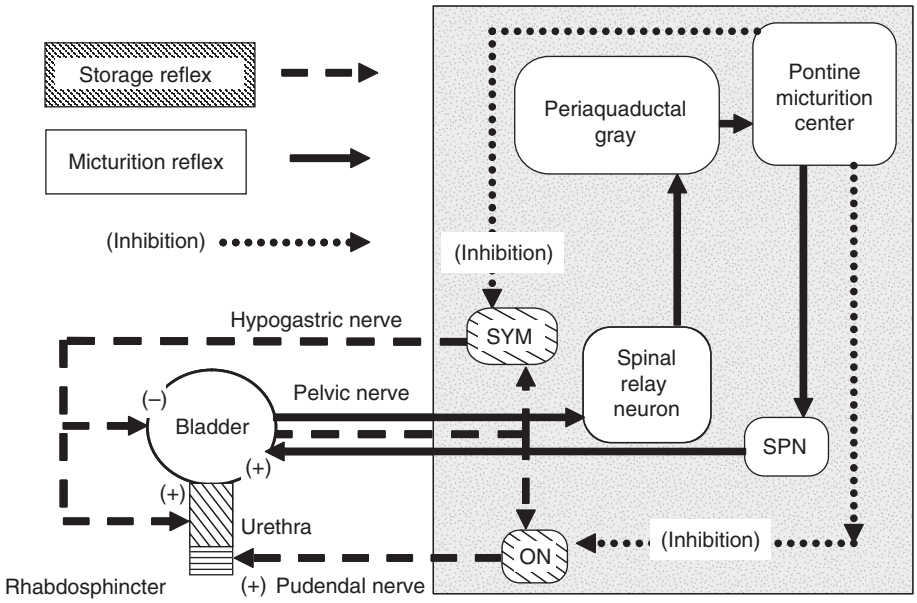

Figure 1 Urine storage and micturition reflexes. SYM, sympathetic preganglionic neurons; ON, Onuf's nucleus rhabdosphincter motor neurons; SPN, sacral parasympathetic nucleus preganglionic neurons.

Inability to store urine is termed urinary incontinence. There are three primary forms of urinary incontinence.

1. Stress urinary incontinence (SUI) is urine leakage resulting from abdominal pressure exceeding urethral resistance during physical 'stress' (i.e., coughing, laughing, or sneezing) and is primarily seen in women.
2. Urge urinary incontinence (UUI) is urine leakage resulting from involuntary activation of the micturition reflex, which in certain circumstances is due to emergence of a pathological spinal reflex (i.e., a 'short circuit' reflex not routed through the brain stem and considerably less influenced by higher levels of the CNS) that is initiated by bladder 'nociceptive' (C fiber) primary afferent (i.e., sensory) fibers.
3. Often, involuntary bladder contractions can occur without leakage of urine but produce symptoms of urinary frequency, urgency, and nocturia. This condition is often referred to as overactive bladder (OAB).

8.10.2 Unraveling the Role of Serotonin (5-hydroxytryptamine, 5HT) and Norepinephrine in Control of Lower Urinary Tract Function

de Groat conducted a series of iontophoretic studies examining the effects of 5HT and norepinephrine on sympathetic[2] and sacral parasympathetic[2,3] preganglionic neurons and showed that these monoamines increased sympathetic neuron activity but inhibited parasympathetic neuron activity. These studies were the first to suggest that 5HT and norepinephrine systems are associated with urine storage and micturition.

Others, including Morrison[4] and McMahon,[5] became interested in serotonergic control of the bladder and also showed that electrical stimulation of the medullary raphe, an area that contains 5HT neurons that project to the sacral spinal cord, could inhibit bladder activity. Later studies by Fukuda[6] also showed a serotonergic link between supraspinal centers and activation of rhabdosphincter pathways. These electrical stimulation studies suggest an endogenous 5HT system might exist to control bladder and sphincter activity. This suggestion was supported by studies showing that bladder distension activates medullary raphe serotonergic neurons.[7,8]

In support of de Groat's iontophoretic studies and the electrical stimulation studies, we found that a 5HT receptor agonist, 5-methoxy-dimethyltryptamine (5-MeODMT), inhibited bladder activity and enhanced sympathetic and somatic outflow to the urethra in cats.[9] Importantly, the doses that inhibited bladder activity were lower than those that excited the sympathetic and somatic outflow to the urethra, which was ascribed to differential affinity of 5-MeODMT for $5HT_1$ and $5HT_2$ receptors. (The plethora of 5HT receptor subtypes was just beginning to be realized when these studies were initiated and selective agonists and antagonists were scarce.)

However, the picture became complicated when studies by Lecci in Maggi's group[10] showed that 8-OH-DPAT, a $5HT_{1A}$ receptor agonist, enhanced bladder activity in rats. Studies in my laboratory with 8-OH-DPAT confirmed Lecci's results in rats but showed completely opposite effect in cats, i.e., an inhibition of bladder activity, in support of my previous studies in cats with 5-MeODMT.[11]

As subtypes of 5HT receptors became discovered, it seemed that understanding the anatomical localization of the receptors and their association with lower urinary tract centers would provide insight into their role for spinal control of lower urinary tract reflexes. Those studies[12] showed that both the $5HT_{1A}$ and $5HT_{1B}$ receptors were very dense in the dorsal horn of the sacral spinal cord (an area where bladder primary afferent terminals make connections with second-order interneurons) while $5HT_2$ receptors were preferentially localized to the ventral horn (where rhabdosphincter motor neurons were localized).

Similar to the complicated findings of in vivo effects of 5HT, the role of norepinephrine in the control of lower urinary tract function was complicated, as subtypes of the alpha adrenoceptors were found to have differential effects. We found that alpha-2 adrenoceptor agonists inhibited sympathetic reflexes to the lower urinary tract[13] suggesting that enhancing the effects of norepinephrine may compromise urine storage. Furthermore, Downie's group at Dalhousie University showed that alpha-2 adrenoceptor agonists also suppress somatic (i.e., voluntary) reflexes to the striated urethral sphincter,[14] which would decrease rhabdosphincter force and possibly worsen incontinence. On the other hand,[15] the Dalhousie group also concluded that alpha-1 adrenoceptor activation could enhance outflow to the rhabdosphincter. Eventually my group[16] and Wyllie and Ramage[17] showed that alpha-1 adrenoceptor activation could enhance sympathetic outflow to the lower urinary tract. Thus, norepinephrine has opposite effects on the sympathetic and somatic storage reflexes depending on which adrenergic receptor subtype to which it binds.

Concurrent with stimulation studies of serotonergic raphe neurons, studies of the effects of locus coeruleus stimulation, the largest group of noradrenergic neurons in the brain, on bladder function were being conducted by various groups in Japan.[18–21] These studies indicated that norepinephrine facilitated parasympathetic efferent outflow to the bladder and enhanced contractility. This indication was supported by recent pharmacological studies by Yoshiyama and de Groat[22] that showed an alpha-1 adrenergic receptor agonist stimulates efferent parasympathetic activity. However, that study also concluded that it also inhibited bladder afferent activity. Similar to serotonergic raphe neurons, noradrenergic locus coeruleus neurons are also activated by bladder distension.[23] Thus, like the story for serotonergic control of the lower urinary tract, the story for noradrenergic control was compelling for an association but was ambiguous regarding the overall effects of the two transmitter systems because of the divergent actions of the various receptor subtypes.

In addition to physiological and pharmacological studies, various groups where applying histofluorescence and immunohistochemical techniques for anatomical localization of 5HT and norepinephrine-containing nerve terminals in the spinal cord and showed that both the sympathetic and parasympathetic preganglionic neurons that innervate the lower urinary tract were densely innervated by these monoamines.[24,25] Importantly, separate laboratories also showed that the motor neurons in Onuf's nucleus, which innervate the urethral rhabdosphincter, contained the highest densities of 5HT and norepinephrine terminals among all motor neuron groups in the spinal cords of dog, monkey, and baboon.

Thus, there was no doubt that both 5HT and norepinephrine transmitter systems were intimately associated, physiologically, pharmacologically, and anatomically, with lower urinary tract control, but their prevailing role in regulating bladder and sphincter function was unclear and the effects of enhancing their influence on lower urinary tract function could not be predicted.

8.10.3 Discovery of Duloxetine's Preclinical Effects on the Lower Urinary Tract

Duloxetine (**Figure 2**) was discovered by Robertson, Krushinski, and Wong, as part of a chemistry effort by Eli Lilly and Co. aimed at finding combined serotonin norepinephrine reuptake inhibitors (SNRIs) as treatments for depression.[26]

In the first series of experiments testing duloxetine's effects on lower urinary tract function,[27] I chose to use the cat as the experimental species because most of the preceding experiments with 5HT and norepinephrine had been conducted in cat and thus provided benchmarks upon which to interpret the effects of duloxetine. I also chose to use a model of bladder irritation, i.e., infusion of dilute acetic acid into the bladder, to induce 'overactive bladder' because the importance of nociceptive (i.e., C fiber primary afferent neurons) stimuli in the etiology of overactive bladder was just beginning to emerge. As luck would have it, both of these choices were critical because subsequent studies showed that duloxetine has very little effects on normal (i.e., saline infused – unirritated) bladder activity, presumably because 5HT and/or norepinephrine have greater effects on 'irritative,' C fiber-mediated bladder activity than normal bladder primary afferent fibers. Subsequent studies also showed that duloxetine has very little effect in the rat, which I believe is due to behavioral differences in the fight-or-flight response between rats (which urinate as a defense mechanisms) and cats and higher species (which suppress micturition in fight-or-flight situations).

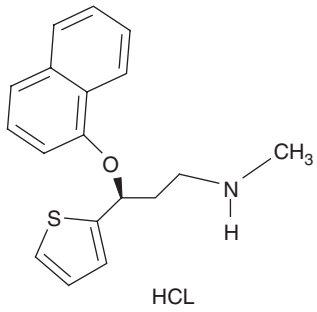

Figure 2 Structural formula of duloxetine.

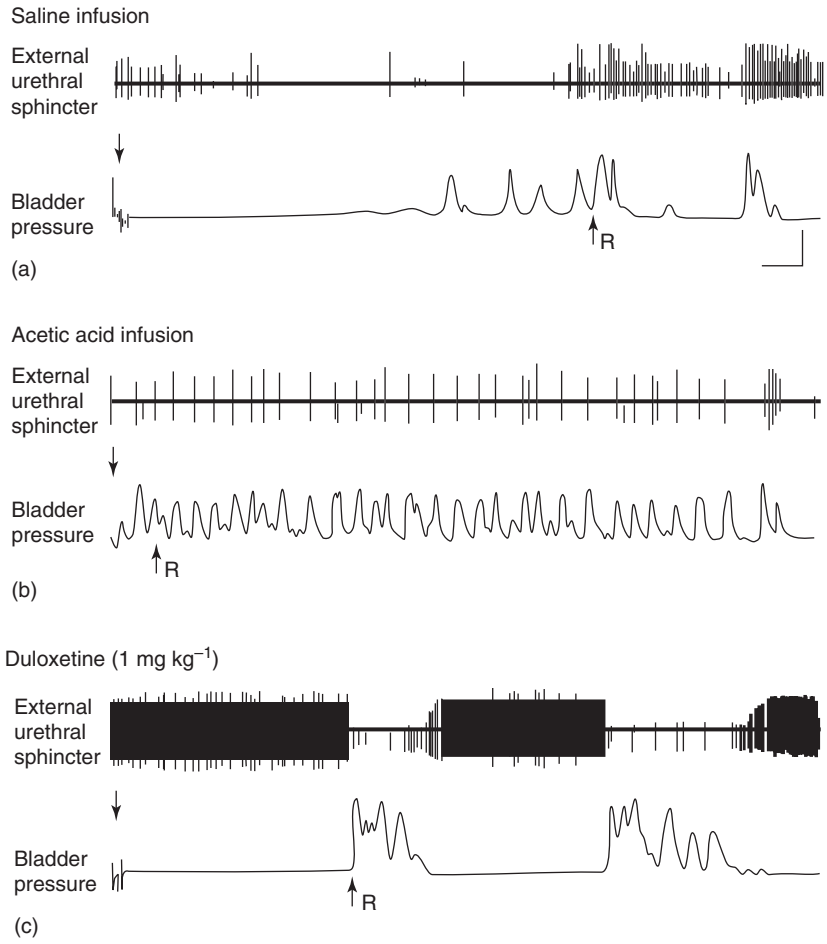

Figure 3 Electromyographic (EMG) activity recorded from the rhabdosphincter (upper traces in panels a–c) and bladder pressure (lower traces in panels a–c) during infusion of saline into the bladder (a), during infusion of dilute acetic acid into the bladder to induce bladder irritation and bladder overactivity (b), and during continued infusion of acetic acid into the bladder following administration of duloxetine (c). (Adapted from Thor, K. B.; Katofiasc, M. A. *Pharmacol. Exp. Ther.* **1995**, *274*, 1014–1024.)

These studies showed that duloxetine inhibited bladder activity (i.e., increased the volume of bladder distension required before triggering the micturition reflex – increased 'bladder capacity') and increased rhabdosphincter electromyogenic (EMG) activity during bladder filling (i.e., during the storage phase) (Figure 3). The effects on bladder indicated that duloxetine might have utility for treatment of urge incontinence – i.e., if the bladder could hold more before triggering bladder contractions then urinary frequency, urgency, nocturia, and incontinence might be reduced. The effects on the rhabdosphincter indicated that duloxetine might have utility for treatment of stress

incontinence – i.e., if the rhabdosphincter were contracting more forcibly it would be more likely to resist the increase in abdominal pressure during a cough, laugh, or sneeze.

Importantly, duloxetine's facilitatory effects on the sphincter 'disappeared' during a micturition contraction. In other words, during a micturition contraction, the sphincter activity was absent. During micturition, it is important for the bladder to contract and the sphincter to relax in synergy to allow efficient voiding. Thus, the fact that inhibition of the sphincter remained during a bladder contraction (i.e., synergy was maintained) indicated that urinary retention should not be a problem. (This has been confirmed in all the clinical trials to date.)

Similar effects were seen with another SNRI, venlafaxine.[28] Remarkably, however, we could not administer a combination of a selective serotonin reuptake inhibitor (SSRI) and a norepinephrine reuptake inhibitor to the same animal and obtain a similar effect to duloxetine and venlafaxine, despite our best efforts. In other words, combining two selective individual reuptake inhibitors was ineffective but having the combination of reuptake inhibition in a single molecule was effective. This remains an enigma to date.

Further studies indicated that the enhancement of sphincter activity was due to increased stimulation of $5HT_2$ serotonergic and alpha-1 adrenergic receptors resulting from increased levels of 5HT and norepinephrine associated with reuptake blockade.[27–29]

Because of the role of the sympathetic nervous system and norepinephrine in mediating contraction of the urethral smooth muscle, we examined the effects of selective norepinephrine reuptake inhibitors,[30] as well as duloxetine, for their ability to augment sympathetic-nerve-induced urethral contractions via the norepinephrine reuptake inhibition properties. We found that increasing synaptic levels of norepinephrine with a reuptake blocker produced no consistent facilitation of urethral contractions due to counteracting effects resulting from enhanced norepinephrine stimulation of relaxatory beta adrenergic receptor stimulation.

8.10.4 Challenges in Bringing Forward the First Therapeutic Treatment for Incontinence

In the early 1990s, therapy for stress urinary incontinence relied on pelvic floor exercises and surgery. Bringing the first drug forward to treat any indication provided a number of challenges, such as extent of medical need and clinical trial design. Unique to duloxetine's trials in incontinence were (1) the fact that urologic thought leaders' prevailing opinion at that time was that stress incontinence was 'an anatomical defect' that would be only amenable to surgery and not pharmacological therapy, and (2) doubts about whether a CNS approach to a urological problem was tenable.

8.10.4.1 Incontinence Markets

Without any historical pharmaceutical sales data for an indication, the market potential is difficult to predict because most financial models are based on sales of competitors' products. Since there were no well-marketed products for stress urinary incontinence, it was difficult to develop a financial model. In 1992, even sales of urge incontinence products were remarkably small; for example, the top UUI medicine was Ditropan, which only had 92 million days of therapy prescribed in the USA, and there were virtually no drug sales in the USA for SUI. This absence of therapy highlighted the need for new therapy with a mechanism of action that was different from previous therapy and was emphasized in 1992 by the Agency for Health Care Policy and Research (AHCPR) which released its first guideline on urinary incontinence and reported:

- 13 million Americans are incontinent; 11 million are women
- 1 in 4 women ages 30–59 have experienced an episode of urinary incontinence
- 50% or more of the elderly persons living at home or in long-term care facilities are incontinent
- $16.4 billion is spent every year on incontinence-related care: $11.2 billion for community-based programs and at home, and $5.2 billion in long-term care facilities
- $1.1 billion is spent every year on disposable products for adults.

Valuable resources for establishing characteristics of unexplored markets with great medical need are found in patient advocacy groups. One of the most prominent patient advocacy groups for incontinence was the Simon Foundation, led by Cheryl Gartley. These patient advocacy groups can provide valuable direction for understanding the patient perspective in regards to why some seek treatment versus those who do not, concerns about surgical treatments, reasons for failure with pelvic floor exercises, and motivation in regards to treating their SUI. For example,

in 1992, only about 20% of all incontinence patients sought treatment, only about half of those received medical therapy, and 90% of those stopped taking their medication within 3 months because of intolerable side effects and low efficacy. While it seemed that just about every publication on surgical procedures for stress incontinence reported 80–90% cure rates, it was not until follow-up reports were published did we become aware that these rates seen immediately after surgery, at the most prestigious academic institutions, in the hands of the best practitioners, were not reflective of long-term results of the overall surgical population and were accompanied by concerning complications.

8.10.4.2 Clinical Trial Considerations

Being the first to initiate regulatory submission quality clinical trials in a therapeutic indication also carries substantial challenges:

1. There are no physicians with regulatory submission clinical trial experience specific to your indication.
2. There are no publications indicating
   - which efficacy measures are best
   - inclusion/exclusion criteria
   - recruitment rates
   - trial duration or design
   - anticipated placebo response rates or intrinsic variability to allow power calculations
   - quality of life instruments.

Fortunately, the pharmaceutical industry and the Food and Drug Administration (FDA) worked together to develop guidelines for regulatory submissions in incontinence and laid the groundwork for the first meeting to establish 'FDA Guidance for Industry for the Development of Incontinence Drugs' in 1998.

One of the take-home messages from that public meeting was that incontinence clinical trials needed both an objective and a subjective measure of patient improvement. For example, a patient might not consider a statistically significant reduction in number of incontinence episodes from four per day to two per day clinically significant. Similarly, one must question a drug that makes the patient less bothered by incontinence if there is no statistically significant reduction in the number of incontinence episodes. Thus, both an objective measure and subjective measure were needed.

Since the American Urological Association was over 100 years old and incontinence constituted a significant portion of a urologist's practice, it was surprising that opinions about the best objective measures of the condition were so disparate. As it turned out, incontinence episode frequency recorded in the micturition diary (which one might question as being objective since it is filled out by the patient) was the most reliable and used in all clinical trials for duloxetine.

Since a psychometric instrument to assess patient perception of their incontinence was needed, Buesching of Lilly, in collaboration with Patrick of University of Washington, developed the Incontinence Quality of Life Questionnaire (IQOL).[31] This questionnaire contained domains for avoidance and limiting behaviors, psychosocial impacts, and social embarrassment and was validated for internal consistency, reproducibility, correlation with other measures, and responsivity.[32]

8.10.5 Clinical Trial Results

8.10.5.1 The First Trial – SAAA

The initial clinical trial[33] examined stress, mixed, and urge incontinent patients because of duloxetine's affects on both the rhabdosphincter and bladder capacity. This preliminary proof-of-concept trial for duloxetine's use in treating incontinence was at a dose of 20 mg q.d., which is substantially lower than that used in later trials (i.e., 40–60 mg b.i.d.). In hindsight, based on the low dose of duloxetine, the small number of SUI patients (22 on duloxetine, 11 on placebo), and the extreme variability typically found in the stress-pad test (one of our efficacy measures), it is remarkable that this trial showed a statistically significant reduction in stress-pad test weights with duloxetine treatment (**Figure 4a**) and a nonsignificant reduction in incontinence episodes (**Figure 4b**). Additionally, the trial showed that a significantly greater proportion of SUI patients on duloxetine showed at least a 30% reduction in incontinence episodes compared to placebo. Nearly 50% of the patients showed 70% improvement in incontinence episodes and stress-pad test, but this stratification was not statistically significant (**Figure 5**). No effects were seen in the mixed or urge incontinent

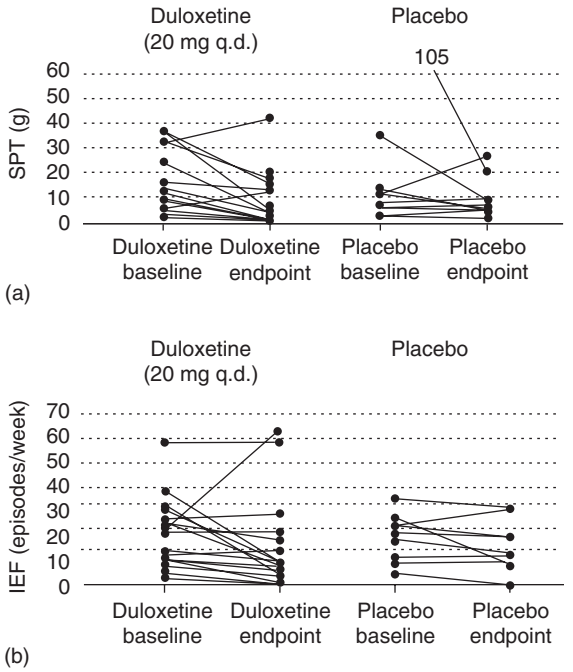

Figure 4 Line graph 'baseline to endpoint' for (a) stress-pad test (SPT) and (b) incontinence episode frequency (IEF). Each line connects an individual SUI patient's baseline and endpoint values. Duloxetine significantly decreased amount of SPT leakage ($p = 0.02$ versus placebo, ANCOVA on ranked changes with baseline values as covariate) and frequency of incontinence, but it was not statistically significant ($p = 0.34$ versus placebo, ANCOVA on ranked changes with baseline values as covariate). (Adapted from Mulcahy, J. J.; Kirkemo, A.; Rudy, D. C.; Blaivas, J. G.; Wahle, G. R.; Sirls, L. T.; Laddu, A. R.; Faries, D.; Debrota, D.; Thor, K. B. *Neurourol. Urodyn.* **1996**, *15*, 92–395.)

patients. These results led to a focus on SUI in subsequent trials. Remarkably, the placebo-treated group actually had a higher proportion of adverse events than the duloxetine-treated group, and the subsequent US trial dosed at 20, 30, and 40 mg q.d.

8.10.5.2 The Japanese Trial

This trial[34] was the first to show efficacy in neurogenic bladder (and is the only trial published for any overactive bladder condition to date). This important trial also supported the early positive results for duloxetine in SUI. This study showed a reduction from 1.7 to 0.3 incontinence episodes per day in neurogenic bladder patients at 20 mg (but no effect at 10 mg) and a reduction from 3 to 1 incontinence episodes/day at both 10 and 20 mg doses in SUI patients. Although the trial was a single-blinded study and contained no placebo group (which was traditional in Japan at that time to ensure all patients were treated with something), these results added to the early suggestions of duloxetine's efficacy.

8.10.5.3 The Second US Trial – SAAB

The next trial measured incontinence episode frequency, IQOL, stress-pad test weights, and 24-h pad weights with about 35 patients in each dose group and restricted itself to stress and mixed UI patients.[35] Unexpectedly, only the 20 mg group showed statistically significant improvement in all measures, while those at dose 40 mg showed significance in only stress-pad test and IQOL, and the 30 mg dose group only showed significance in IQOL (**Figures 6** and **8**). In hindsight, this absence of a dose response is not surprising since the dose increments of 20, 30, and 40 mg duloxetine are proportional to increments of, for example, 1, 1.5, and 2 aspirins. One might not expect to see a dose-dependent reduction in headaches in groups of 35 patients across those doses of aspirin. When all duloxetine arms were pooled, significance was retained for all measures except 24-h pad weights, which still showed twice as much reduction as placebo. As in the first trial, the overall incidence of adverse events on placebo was actually worse than any of the duloxetine groups. However, nausea (now recognized as the most prominent side effect of duloxetine) did show a higher (though not significant) increase with duloxetine compared to placebo. In a dose-related pattern similar to the efficacy described above, the highest rate of

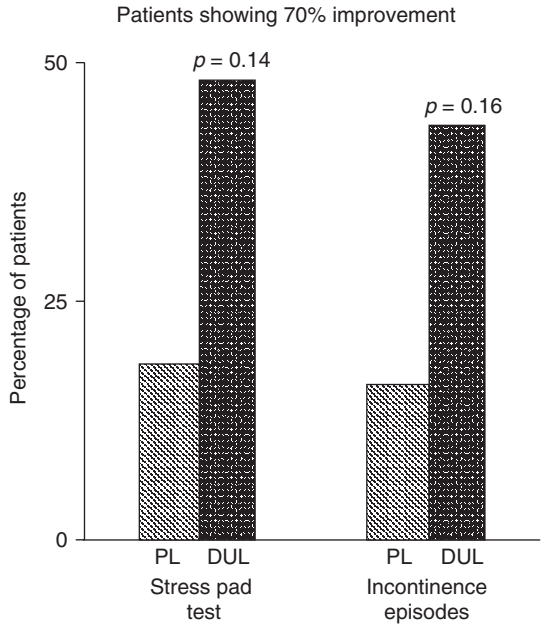

Figure 5 Responder analysis of 20 mg q.d. duloxetine (DUL) versus placebo (PL) as defined by a 70% improvement from baseline in SUI patients. (Adapted from Mulcahy, J. J.; Kirkemo, A.; Rudy, D. C.; Blaivas, J. G.; Wahle, G. R.; Sirls, L. T.; Laddu, A. R.; Faries, D.; Debrota, D.; Thor, K. B. *Neurourol. Urodyn.* **1996**, *15*, 92–395.)

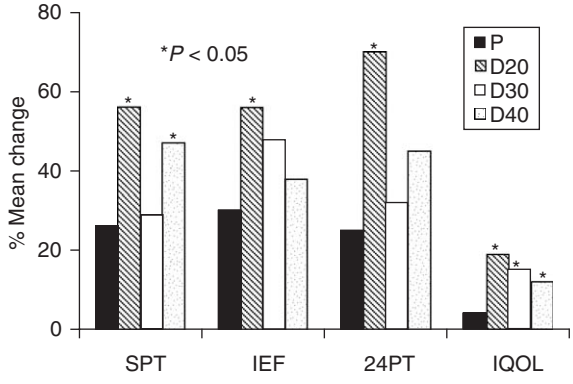

Figure 6 Effects of placebo (P) and duloxetine (D) at 20, 30, 40 mg q.d. on stress-pad test (SPT), incontinence episode frequency (IEF), 24-h pad weight (24PT), and incontinence quality of life (IQOL) in SUI patients. (Adapted from Zinner, N.; Sarshik, S.; Yalçin, I.; Faries, D.; DeBrota, D.; Riedl, P.; Thor, K.B. *Efficacy and Safety of Duloxetine in Stress Urinary Incontinent Patients: Double-Blind, Placebo-Controlled Multiple Dose Study.* International Continence Society, 28th Annual Meeting, Jerusalem, Israel, Sept. 1998.)

nausea was seen in the 20 mg group (17%), followed by the 40 mg group (14%), with the 30 mg group trailing (10%). Again, this rank order for nausea mimicked the rank order for efficacy across these doses.

8.10.5.4 Incontinence Severity Index in SAAB

The fact that all efficacy measures showed improvements with duloxetine at all doses (but lacked statistical significance for all points) suggested that patients were really improving with duloxetine but that the small number of patients coupled with the noise in each of the individual parameters were obscuring the positive signal. Therefore, a factor analysis approach, the incontinence severity index (ISI), was developed by Ilker Yalçin.[36,37]

The underlying supposition in the creation of the ISI was that incontinence is a multifaceted condition that cannot be represented with a single measure. For example, if two women leak three times a day, one at a volume of 5 g and the other at 10 g, episode frequency itself will not reflect the condition or improvement in the condition appropriately.

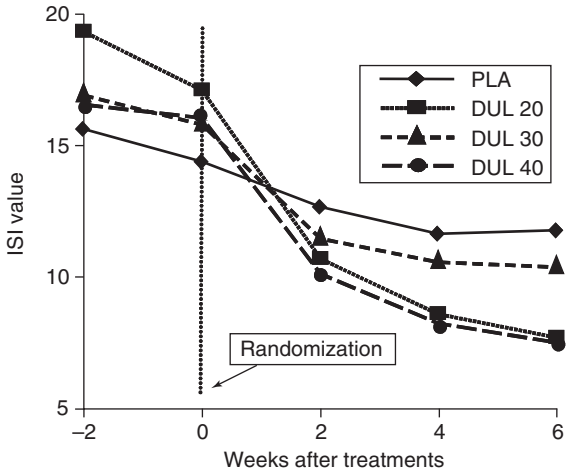

Figure 7 Effects of duloxetine (20, 30, 40 mg q.d.) and placebo (PLA) on Incontinence Severity Index after a 2 week placebo lead-in in SUI patients. (Adapted from Yalçin, I.; DeBrota, D.; Amemiya, Y.; Thor, K. B. *Neurourol. Urodyn.* **1997**, *16*, 80; Yalçin, I.; DeBrota, D.; Thor, K. B. Incontinence Severity Index (ISI) in Measuring Efficacy of Duloxetine in Stress and Mixed Incontinent Patients. International Continence Society, 28th Annual Meeting, Jerusalem, Israel, Sept. 1998.)

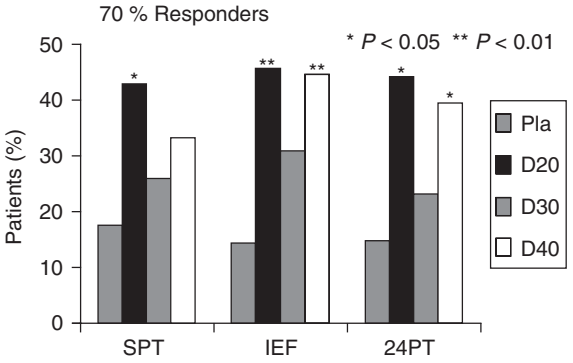

Figure 8 Responder analysis of 20, 30, and 40 mg q.d. duloxetine (D) versus placebo (Pla) as defined by a 70% improvement from baseline in SUI patients. (Adapted from Zinner, N.; Sarshik, S.; Yalçin, I.; Faries, D.; DeBrota, D.; Riedl, P.; Thor, K.B. *Efficacy and Safety of Duloxetine in Stress Urinary Incontinent Patients: Double-Blind, Placebo-Controlled Multiple Dose Study*. International Continence Society, 28th Annual Meeting, Jerusalem, Israel, Sept. 1998.)

Even if both women leak at the same frequency and the same volume, one may be bothered more than the other because, for example, she frequently plays tennis. Factor analysis assumes that number of incontinence episodes (frequency of leaks), stress-pad test and 24-h pad test (volume of leaks), and IQOL (effect of incontinence on quality of life) measure the condition 'incontinence.' The analysis produces weights for each of these variables to create an index score in such a way that the resulting index has the highest reliability.

The ISI revealed immediate, marked improvement (i.e., obvious inflection point at randomization to drug treatment) in stress patients when switched from 2-week placebo lead-in to duloxetine (all doses) at visit 3 (Figure 7). In contrast, patients who were maintained on placebo showed only a gradual, minor improvement throughout the 8-week period (i.e., a straight line with no inflection point at randomization to placebo). Similarly, the ISI showed immediate, marked improvement in mixed patients who were switched from placebo to duloxetine (30 mg and 40 mg doses) at visit 3. Mixed patients who were maintained on placebo or received the lowest dose of duloxetine showed only a gradual, minor improvement.

In comparing baseline to endpoint ISI measures, duloxetine-treated stress patients showed significant (20 and 40 mg) or nearly significant (30 mg) improvement using the ISI. This rapid onset of effects in all treatment groups contrasted with the gradual slope in placebo patients again gave the team confidence that we were seeing real drug effects. Although a valuable tool in certain cases, the ISI may not be an appropriate measure of efficacy measure in very

large clinical trials due to the difficulty performing pad tests on large numbers of patients and the inherent variability in pad tests. The overall greater proportion of side effects in the placebo group, and absence of any serious side effects, allowed us to again increase the dosage of duloxetine in subsequent trials to 40 mg b.i.d. (typically) and 60 mg b.i.d. in the trial in women awaiting surgery.[38]

8.10.5.5 Subsequent Trials in Stress Incontinence

The results from the next trial and all subsequent trials have been or will be published as full-length papers in peer-reviewed journals.[39–42] Briefly, subsequent trials in North America, South America, Europe, Africa, and Australia have continued to show dose-dependent improvements in patients with SUI. The top dose studied and being prescribed in those countries where it has launched is 40 mg b.i.d. Surprisingly (to me at least), duloxetine is just as effective in severe SUI patients as it is in mild SUI patients, while the placebo response drops markedly in severe patients. In general the reduction in mean incontinence episodes ranges from 50% to 60% with half the women showing reductions in incontinence episodes between 50% and 100%. Importantly, when combined with physiotherapy to strengthen pelvic floor muscles, a mean reduction to 75% can be achieved.[43] This is fairly remarkable considering that many opinion leaders felt that SUI was due to an anatomical defect that could not be helped through pharmacology and that surgery was required. Interestingly, one trial has shown that 25% of women scheduled for SUI surgery have opted to cancel surgery at the end of the clinical trial.[38]

Table 1 Timing of critical events that contributed to registration of duloxetine

Critical event	*Contribution*	*Date*
1. June Allyson TV commercials for Depends	Made it acceptable to discuss incontinence	1985
2. Author joins Eli Lilly & Co.	Studied 5HT and norepinephrine reuptake inhibitor effects on lower urinary tract function.	May 1990
3. Duloxetine US patent 5,023,269 granted	Protected duloxetine composition of matter	11 September 1990
4. American Health Care Policy and Research (AHCPR) first guideline on urinary incontinence published	US Government-validated review of prevalence, medical need, and short-comings of curent therapies. Highlighted issues	1992
5. Method of use for treating incontinence US patent 5,744,474 granted	Extended patent protection for use of duloxetine to treat incontinence to 2015	20 April 1995
6. Publication of first manuscript for duloxetine effects on LUT	Disclosed proposed mechanisms of action for duloxetine on lower urinary tract	July 1996
7. FDA Guidance for Industry for the Development of Incontinence Drugs	Provided basic guidance for regulatory concerns regarding incontinence medicines in general	1998
8. Method of use for treating interstitial cystitis US patent 6,150,396	Extended patent protection for use of duloxetine to treat interstitial cystitis to 2017	21 November 2000
9. New Drug Application to FDA for duloxetine for SUI	Required for regulatory approval	October 2002
10. Division of Reproductive and Urologic Drug Products issues an Approvable Letter	First step in approval process but not binding on FDA	September 2004
11. US launch for depression		August 2004
12. European Medicines Agency (EMEA) approval for SUI	Allowed marketing authorization	12 August 2004
13. Approval for diabetic neuropathic pain		September 2004
14. Withdrawal from FDA		January 2005

In the subsequent trials, the most commonly reported adverse events were nausea, although it is reportedly mild to moderate and resolved within 1 week to 1 month in most patients. Less prevalent adverse events include headache, insomnia, constipation, dry mouth, dizziness, and fatigue, which tend to be nonprogressive and mild to moderate in almost all patients. Importantly, none of the SUI clinical trials have indicated that duloxetine impedes voluntary micturition or increases residual urine volume, indicating that bladder sphincter synergy is maintained.

8.10.5.6 Urge Urinary Incontinence and Overactive Bladder

Interestingly, the impetus for studying duloxetine was for discovering a treatment of urinary urge incontinence and overactive bladder. Insertion of EMG electrodes into the rhabdosphincter was a compulsion instilled in me during my dissertation studies to obtain as much data as practical from every experiment conducted. Even after seeing pronounced effects on the rhabdosphincter, I was still more impressed with the effects on the bladder and anticipated clinical benefits for urge incontinence and overactive bladder to supersede clinical benefit for SUI. Fortunately we did have the preclinical data regarding enhancement of sphincter activity or we might not have included SUI patients in the initial trials and we might not have been as aggressive in post hoc analyses of the initial low-dose clinical trial results that supported additional trials at higher doses.

Interestingly, there are some indications for benefit by duloxetine in urge incontinence and overactive bladder. The first is the Japanese study in neurogenic bladder patients which showed a reduction in the median number of daily incontinence episodes from 1.7 to 0.3.[34] The second is that there was a decrease in urinary frequency seen in SUI patients.[39] Finally, at the European Association of Urology 2005 Annual Meeting in Istanbul, Turkey, Bump of Eli Lilly and Co. indicated that "some proof of concept and confirmatory studies with duloxetine have been performed in populations with mixed and urge urinary incontinence, but the results are not yet in the public domain" in response to a question from the audience about duloxetine's effects in urge incontinence and overactive bladder. It would be very interesting if a single agent provided benefit in both stress and urge urinary incontinence. However, this global attribute of reduced urinary leakage fits with the overarching concept of 5HT and norepinephrine being involved in attention, arousal, and fight-or-flight situations where urine leakage and concerns about micturition would be a detriment.[44]

8.10.6 Regulatory Approval

In August 2004, duloxetine was given marketing authorization for SUI in Europe (**Table 1**). It will be marketed under the trade names Yentreve and Aricept. The FDA issued an approvable letter for Yentreve in September 2003. However, Eli Lilly and Co. withdrew its application from the FDA in January 2005 but did not stop clinical trials. Duloxetine is already approved for neuropathic pain and depression in the USA and for incontinence (as well as depression and neuropathic pain) in Europe and Mexico.

8.10.7 Conclusions

Duloxetine as a medicine for SUI appears to be effective and well tolerated. The clinical data available to the public to date indicate that the most predominant side effect is mild nausea that resolves in a couple of weeks in most patients.

As in most clinical trials aimed at registration of the first drug for a new indication, the benefits of the pioneering trials extend beyond understanding of the drug's safety and efficacy. The duloxetine SUI clinical trials are increasing public awareness of the condition, focusing debate on diagnostic criteria,[45] standardizing trial efficacy measurements, generating quality-of-life psychometric instruments to determine patient's perceived benefits, standardizing the terminology, providing insights into the relationship between incontinence episode frequencies and patient perceptions of condition severity,[46] generating insight into the placebo response,[47] expanding our understanding of the prevalence of the condition, and providing substantial baseline data for more academic studies of the condition of urinary incontinence.

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Biography

Karl B Thor, PhD, formed Urogenix, Inc., as a subsidiary of Astellas Pharmaceuticals, Inc., in March of 2007 and holds the position of vice-president of research. In addition, Dr Thor holds Adjunct Research Associate Professor positions in the Department of Surgery/Division of Urology and the Department of Obstetrics and Gynecology at Duke University Medical Center in Durham, North Carolina. He is Co-director of the Laboratory of Neurourology at the Veterans Affairs Medical Center, Surgical Research Services, also in Durham. The Laboratory of Neurourology receives grant support from the NIH, Veterans Administration, and Christopher Reeves Foundation for studies of neural control of the lower urinary tract under pathological conditions including spinal cord injury.

Dr Thor received his PhD in Pharmacology from the University of Pittsburgh School of Medicine where he trained under William (Chet) de Groat, PhD and was supported by a PhARMA predoctoral fellowship. He held a National Research Service Award postdoctoral fellowship from the NIH at Uniformed Services University of the Health Sciences in Bethesda, Maryland, and was a Senior Staff Fellow in the Laboratory of Neurophysiology at the NIH. He joined Eli Lilly in the Neuroscience Division in 1990, where he discovered duloxetine (Yentreve) as a treatment for stress urinary incontinence. In 1998, he formed PPD GenuPro as a subsidiary of PPD Inc., where he discovered the clinical potential of dapoxetine as a therapy for premature ejaculation. These two drugs are the first agents to be submitted to regulatory agencies for their respective indications. In 2002, he founded Dynogen Pharmaceuticals Inc., a neuroscience-based drug discovery and development company targeting genitourinary and gastrointestinal diseases, where two clinical program for overactive bladder and two clinical programs for irritable bowel syndrome were initiated.

Dr Thor has published numerous articles and book chapters on CNS control of lower urinary tract function and holds several patents for methods of treating urinary bladder and sexual dysfunction. He is a member of the Urodynamics Society, the International Continence Society, and the Society for Neuroscience. He served as a member of the FDA-PhARMA Industry Guidelines for Urinary Incontinence Trials in 1998. He has served as a peer reviewer for the NIH on the Urology Special Emphasis Study Section, the National Institutes of Neurological Disorders and Stroke Neurology B Study Section, and the Small Business Initiative Review Study Section. He has served on the World Health Organization's International Consultation on Incontinence since its inception.

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8.11 Carvedilol

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8.11.1 Historical Overview

Carvedilol (Coreg) is commonly referred to as a third-generation beta adrenoceptor blocker (beta blocker) with vasodilatory and antioxidant properties. These varied activities are attributed to different parts of the carvedilol molecule (Figure 1). Carvedilol was originally discovered in the early 1980s as a novel beta blocker for the primary therapeutic indications of angina and hypertension, which were traditional uses of drugs of this class at the time. However, the true novelty behind carvedilol is the fact that it represents the first beta blocker to be approved for the treatment of chronic congestive heart failure, which is a serious progressive disease that typically results in death. Until the time that carvedilol was approved by the Food and Drug Administration (FDA), beta blockers were 'contraindicated' in patients with heart failure because of the well-known cardiac depressant effects of this class of drug, as well as the prevailing view that such drugs would worsen heart failure and potentially increase mortality. The paradoxically beneficial effects of carvedilol in patients with heart failure changed not only our thinking about this serious disease, but resulted in a new treatment paradigm for patients with heart failure, as well as a new standard of care. Most importantly, carvedilol removed the death sentence from many patients with this invariably progressive and fatal disease.

More than 20 years ago, research commenced in the laboratories of SmithKline Beecham Pharmaceuticals (now GlaxoSmithKline) with the intent to explore the potential of carvedilol's unique pharmacological profile, consisting of beta blockade, alpha blockade, vasodilatation, and antioxidant activity, as a potential therapy for chronic heart failure.

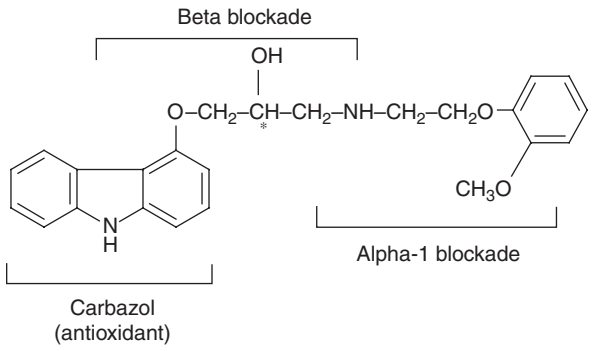

Figure 1 Chemical structure of carvedilol. The active moieties responsible for the antiadrenergic and antioxidant actions are noted. Asterisk denotes the point of asymmetry.

Over the next decade, compelling data were generated in a variety of in vitro and in vivo experimental systems that highlighted novel and unusually effective cardioprotective properties of the drug that resulted in part from the beneficial hemodynamic effects emanating from beta blockade and alpha blockade, and in part from the unique antioxidant, antiapoptotic, and antiproliferative properties of the molecule. Based on these extensive studies, the highest levels of corporate and R&D management at SmithKline Beecham took the brave, risky, controversial, and highly innovative decision to support long-term clinical trials of carvedilol in patients with congestive heart failure, for whom such drugs remained contraindicated. The importance of this decision cannot be overstated given the dogma prevalent at the time that such a drug might actually harm patients, as well as the resulting concerns related to liability and the view that the commercial value to the corporation after taking such unprecedented risks was low.

During the lengthy and costly development program for carvedilol in heart failure, clinical trials were often delayed, and the priority of the program changed regularly, as is commonly the case in the risky environment of drug development. The fact that these trials did, in the end, continue through to completion can be attributed to the vision, persistence, and courage of a few basic research and clinical scientists, including these authors among others, and the trust in them provided by corporate and R&D management, including Jan Leschly (former chief executive officer), Jean-Pierre Garnier (chief operating officer), and George Poste (former chairman of R&D). Certainly there were more doubters than supporters at the time, making the support received from SmithKline Beecham executives all the more important.

The highlight of the entire 15–20 year discovery and development effort on carvedilol was the day that an independent Data Safety and Monitoring Board (DSMB) terminated the studies of carvedilol in heart failure prematurely because of an 'unexpected beneficial effect' of the drug in heart failure patients compared to those patients receiving placebo, making it, in the judgment of the DSMB, unethical to maintain patients in the placebo part of the study. In other words, the DSMB felt that the clinical trial needed to be stopped so that the patients receiving placebo could be immediately treated with this new life-saving medicine. The reduction in mortality observed with carvedilol in patients with this fatal disease was an unprecedented 65%. In hindsight, the decision taken by SmithKline Beecham to develop carvedilol in heart failure, against the recommendations of many experts in the field at the time, represents the pharmaceutical industry at its best. This extraordinary risk taken by SmithKline Beecham, its management, and its scientists has resulted in a dramatic improvement in human health, and in this case, created a new standard of care for seriously ill patients with heart failure to extend their lives, decrease their need for hospitalization, and decrease the burden of this devastating disease on healthcare systems. The ultimate beneficiary of this extraordinary gamble taken by SmithKline Beecham is indeed the patient.

8.11.2 The Pharmacology of Carvedilol: Historical Perspectives

8.11.2.1 Adrenergic Receptors

SmithKline Beecham originally acquired rights to carvedilol in the US from Boehringer Mannhein (now part of Roche) with the obligation to develop the drug for angina and hypertension. The original pharmacological profile of carvedilol was that of a vasodilating beta blocker.[1,2] A long-standing core team of scientists at SmithKline Beecham, consisting of the authors, as well as Hieble, Nichols and Ohlstein, devoted between one and two decades of their respective lives to understanding every aspect of this drug, and to determining exactly how it might work in heart failure. Early reports suggested that the vasodilatory effects of carvedilol resulted from calcium channel blockade.[2] Through a detailed series of experiments, it was established unequivocally that the primary vasodilatory properties of carvedilol resulted from alpha-1 adrenergic receptor blockade, and not through inhibition of calcium channels.[3] This finding was initially viewed as a significant disappointment, since another combined alpha and beta blocker was known (i.e., labetalol), making carvedilol appear to be less novel. However, through other investigations, the core team subsequently discovered many additional important activities of carvedilol, and these activities made carvedilol unique from any other drug in the world.

The extreme potency of carvedilol as an alpha-1 blocker was thought to be important in the pharmacodynamic response of the drug in patients with heart failure, and would result in a beneficial reduction in afterload, making it easier for the failing heart to eject its contents, thereby improving general circulatory status. The role of alpha-1 receptor blockade in the pharmacological profile of carvedilol has proven to be critical in the ability of patients with heart failure to tolerate the beta blocking actions of the drug. Thus, the reduction in afterload produced by alpha-1 blockade helped the heart to compensate for the known cardiac depressant effects of the beta blocking actions.[4] It is also likely that alpha-1 receptor blockade contributes to the favorable metabolic profile of the drug.[5] In retrospect, establishing that the alpha-1 blocking action of carvedilol was responsible for the vasodilatory properties of the drug, and not calcium channel blockade, was a blessing in disguise inasmuch calcium channel blockers have subsequently been shown to increase risk of cardiac morbidity in certain populations of patients with heart disease.[6,7]

8.11.2.2 The Multiple Actions of Carvedilol: Antioxidant Properties

However, following detailed medical and commercial analyses, the company decided not to aggressively pursue the indications of angina and hypertension for a number of reasons: (1) there was little medical need for another drug to treat these disorders, (2) there were, at the time, no known properties of carvedilol to differentiate the drug from others in the class, (3) generic beta blockers were already available and in wide use, and (4) due to the high promotional expenditure that would be required to penetrate these well-satisfied markets, the commercial return would be low. So in the mid- to late-1980s, the carvedilol program at SmithKline Beecham hit a roadblock, and sat on the verge of termination; all for good and logical medical and commercial reasons.

However, the team of scientists listed above began to refocus the program toward the general area of 'cardiac protection,' which was also a well-known property of beta blockers. But our thinking at the time was that the highly unique pharmacological properties of carvedilol might distinguish it from other drugs in the class and provide a greater degree of cardioprotection than had been seen previously with any other drug. Thus, the reduction in afterload mentioned previously might improve cardiac work efficiency, especially in oxygen-deprived conditions such as myocardial infarction, and this, combined with the cardioprotective effects afforded by beta blockade might provide a far greater degree of cardioprotection than commonly observed with other beta blockers. An extensive series of studies were conducted in our laboratories over many years using a variety of experimental models to explore the cardioprotective effects of carvedilol, all of which confirmed our hypothesis.[8–14] Depending on the model, we observed reductions in infarct size secondary to ischemia (a measure of cardioprotection) of as much as 80–100%, which represented a degree of cardioprotection that had not been seen previously with any drug. This extraordinary degree of efficacy clearly had to result from something other than beta blockade, and our relatively naive thinking at the time was that this enhanced cardioprotection observed with carvedilol resulted from the additional alpha blocking actions of the drug (which it certainly did in part). But we could never obtain similar degrees of cardioprotection by combining a beta blocker with an alpha blocker, indicating that something else must be occurring as well. The data were quite puzzling, but we were content to believe at the time that the 'surplus efficacy' of carvedilol compared to other beta blockers resulted from simple hemodynamic effects that could be attributed to alpha blockade.

A different potential explanation for the extraordinary cardioprotective effects of carvedilol in models of cardiac ischemia and infarction surfaced in the literature. At that time, Weglicki and colleagues suggested that the antioxidant properties of some antihypertensive drugs, including some beta blockers at extremely high doses, could provide cardioprotection by inhibiting the generation of toxic oxygen free radicals.[15] Although the antioxidant actions of the beta blockers studied were far too weak to be therapeutically relevant, this work triggered a series of experiments in our laboratory by Yue, a biochemist with interests in 'redox' (oxidation–reduction) reactions and drugs that inhibit oxygen free radical formation. He investigated carvedilol in experimental systems of oxygen radical formation, primarily in cell membranes from the brain, and demonstrated that carvedilol was an extremely potent antioxidant. Most importantly, these antioxidant actions of carvedilol were of sufficient potency that they would occur at blood levels that were well within the projected clinical dosing range.[16–23] Of course we not inclined to believe his results initially, and so we insisted that he repeat his studies, which he did, and he returned to tell us that he had obtained the same results. Still doubting that carvedilol possessed antioxidant activity, we finally insisted that he demonstrate these antioxidant effects not in brain cell membranes, but rather in cardiac cell membranes, which were far more relevant to our interests in cardioprotection and heart failure. His findings were that carvedilol was even more potent as an antioxidant in heart cell membranes (and subsequently in intact heart cells) than in brain membranes, and ultimately we needed to come to the realization that carvedilol was an extremely potent antioxidant.

Additional studies with many derivatives of carvedilol indicated clearly that the carbazol moiety (**Figure 1**) was responsible for this novel antioxidant property, and was different from the parts of the molecule that were responsible for beta and alpha blocking effects; it was clear that carvedilol was a complex multiple action drug. Furthermore, some of carvedilol's metabolites that were produced in vivo (including humans) turned out to be even more potent antioxidants than carvedilol, making them among the most potent antioxidants ever discovered.

A unique component of the investigation of the antioxidant properties of carvedilol came from Mason, one of our external collaborators. His studies deployed low-angle x-ray diffraction methodology that determined the spatial interactions of antioxidants within engineered lipid bilayers or intact cell (cardiac myocyte) membranes. These studies suggested that carvedilol, because of its highly lipid-soluble carbazol function, could occupy a unique position within the plasma membrane bilayer so that a conduit for electron transfer from the inner membrane toward the outside enabled protection of critical unsaturated fatty acids within the membrane, and thereby preventing lipid peroxidation with exquisitely high potency. Molecular modeling of various beta blocking agents by this technique revealed that only carvedilol had the capacity to occupy this unique spatial position in the plasma membrane.[24]

The identification and confirmation of the antioxidant properties of carvedilol had a significant impact on the subsequent development prospects for the drug. We now had a potentially important distinguishing feature of carvedilol that might translate into an important clinical benefit. Equipped with compelling data on the cardioprotective efficacy of carvedilol in diverse models of cardiac ischemia/infarction (which were of known translational significance to humans), and a novel molecular explanation residing in antioxidant properties of the drug, we were able to launch a plausible argument that the drug should be developed for the prevention and treatment of myocardial ischemia and acute myocardial infarction. Although the scientific case was compelling, little enthusiasm could have been garnered for this proposal because this 'phenomenon' of cardioprotection is not recognized by regulatory agencies as a drug indication, and clinical trials to demonstrate efficacy in humans would require prohibitively expensive and impractical morbidity and mortality endpoints. So the decision to pursue a cardioprotection indication was a 'no go,' which was the correct decision scientifically, medically, and commercially. We were disappointed, but the decision was a right one.

8.11.3 Enter Heart Failure

8.11.3.1 Beta Blockers in Heart Failure: Perspectives

The decision not to develop carvedilol for cardioprotection caused us to rethink what we could do with this very novel drug; a drug unlike any other we had ever studied. We discussed the absurd possibility of studying carvedilol, a beta blocker, in congestive heart failure with a number of prominent cardiologists, and most were not interested in this prospect, and some were, quite frankly, astonished by such a radical proposal to use a contraindicated drug in such seriously ill patients. But we did team up with four cardiologists who felt that our proposal, and more importantly our data, could perhaps justify such a seemingly inappropriate study. The four cardiologists were Lukas and Shusterman (both from SmithKline Beecham) and Bristow and Packer, both external consultants. Bristow had advocated the use of beta blockers in heart failure for some time, and had been personally involved in small exploratory trials with beta blockers in heart failure with Swedberg and Waagstein, who themselves had experimented with beta blockers in the treatment of heart failure in the mid-1970s, also in small exploratory studies. The original logic behind the potential utility of beta blockers in the treatment of heart failure emanated from observations regarding the prevalence of tachycardia (high heart rate) in patients with severe heart failure, along with a decrease in heart rate variability (an independent risk factor in heart failure). High heart rate was considered to play an important role in decompensation of cardiac output, and it followed, therefore, that 'toning down' heart rate with beta blockers was logical.

Indeed, Waagstein,[25] Swedberg,[26] and Hirschberger[27,28] obtained results in small, uncontrolled studies that suggested improved cardiac function in patients with heart failure. Although these studies were useful in drawing attention in the cardiology community to the potential utility of beta blockers in heart failure, progress was slow and interest in the field had waned. Several problems could not be easily overcome, such as the fact that academic medical centers (even with extensive government funding) could never afford to do the extensive clinical trials that would be required to establish a benefit of beta blockers in heart failure, as well as the lingering doubt about the safety of this class of drugs in seriously ill patients for which they were officially contraindicated by all regulatory agencies in the world. And finally, the most important issue of all; which beta blocker would be studied if one could indeed address all of the other issues? After a prolonged debate lasting several years, the availability of carvedilol and the convincing data that supported the use of this drug in heart failure, as well as the resources, finances and commitment (not to mention bravery) of SmithKline Beecham to invest in such studies changed everything.

8.11.3.2 Carvedilol and Heart Failure: Banging our Heads against the Textbooks

At the time, there was no solid proof that beta blockers would have beneficial effects on morbidity and mortality in heart failure, and there was an overwhelming fear that these drugs might hurt, or even kill, patients with heart failure. Leading textbooks in pharmacology,[29,30] medicine,[31,32] cardiology, and heart failure[32–36] all warned of the potential of beta blockers to exacerbate the symptoms of heart failure and to negatively impact cardiac output (due to cardiac depressant effects) and cardiac conduction disturbances.

To compound the situation, in the compilation of beta blockers by Cruickshank and Prichard,[29] while the emerging reports on the potential benefits of beta blockers in heart failure were acknowledged, there was also emphasis on the widely held belief (which is probably true in the short term) that sympathetic tone, mediated by beta receptor stimulation in the heart, is important in maintaining cardiac function in heart failure, and there were reports of cardiogenic failure and severe hypotension precipitated by some beta blockers in these patients.

Likewise, the 'bible' of pharmacology, *Goodman and Gilman*: *The Pharmacological Basis of Therapeutics*,[30] the leading teaching textbook of pharmacology for the biomedical sciences, highlighted special precautions for beta blockers, including the fact that "heart failure that may develop suddenly or slowly usually in severely compromised heart" with the use of these drugs. Likewise, *Harrison's Principals of Internal Medicine*[31] lists as adverse effects of beta blockers "the precipitation of heart failure in patients whom cardiac compensation depends upon enhanced sympathetic drive," and in the *Textbook of Medicine*,[32] the suggestion is made that "beta adrenoceptor blockers should not be used in patients with Asthma, COPD [chronic obstructive pulmonary disease] or congestive heart failure."

The skepticism around the utility of beta blockers in the treatment of heart failure was further reinforced by the early termination of a clinical trial with xamoterol, a beta receptor 'partial agonist,' which is somewhat like a beta blocker, in severe heart failure due to an increase in mortality in patients taking the drug.[33,34] The bias at that time against the use of carvedilol was very strong and at least one vocal opponent of the study suggested that the proposed studies were 'extreme in nature.' So strong was the bias against the use of beta blockers in heart failure that even after the utility of carvedilol had been clearly demonstrated in heart failure through large controlled phase III clinical trials, some textbooks continued to warn of their use in this disease, or recommend their use with extreme caution, and warned of the potential to worsen the status of patients with heart failure.[35,36]

It was against this perceived background of caution, fear, and hesitation, and the anticipation of tough regulatory hurdles (which would require the demonstration of reductions in morbidity and mortality) as well as serious commercial doubts about the return on what would be an extraordinary financial investment, that a decision needed to be made on whether to proceed with the development of carvedilol in heart failure. The stakeholders, who consisted of corporate and R&D management, as well as the core team of Discovery scientists and a few internal and external cardiologists who supported this program, faced a very tough decision indeed.

8.11.4 Science, Medicine, and Leadership Win Out: The Pendulum Swings in Carvedilol's Favor

Through the persistence and advocacy of the basic research team in Discovery and a small group of external heart failure leaders, the decision was made to undertake this extraordinary risk of resources (people and money, not to mention the potential liability) and initiate the clinical development of carvedilol in heart failure. In the end, science won out over historical bias.

The data that swung the pendulum were extensive. Significant new science on the role of the activated adrenergic nervous system in heart failure, including the growing body of evidence that suggested that enhanced adrenergic tone in heart failure could have negative (as opposed to the generally accepted positive) consequences by increasing preload and afterload, and thereby exacerbate work load in the failing heart. Furthermore, there were emerging data to suggest that adrenergic stimulation of the heart might be responsible for hypertrophy and phenotypic remodeling of the heart due to direct signaling of both alpha-1 and beta adrenergic receptors.[37–39] In addition, norepinephrine in the heart acting on both the alpha-1 and beta adrenergic receptors located on cardiac myocytes and fibroblasts, was shown to induce expression of genes that transcribe and translate into growth factors[40,41] that have been thought to cause cardiac remodeling,[42] which have negative effects in heart failure. This emerging 'neurohormonal hypothesis' involving a paradoxically negative effect of the sympathetic nervous system in heart failure began to develop as a critical factor in the relentless progression of this disease, and supported our belief that inhibiting the sympathetic nervous system with carvedilol, which would block all of the negative activities of the sympathetic nervous system due to its dual beta and alpha blocking actions, might indeed provide benefit to these patients as opposed to the generally accepted notion that such an intervention would cause harm.

In addition, the other properties of carvedilol, namely its antioxidant actions, could also provide added benefit inasmuch as oxygen radicals were known to be potent activators of signaling pathways that have a short- and long-term negative impact on cardiac cell growth and survival. In particular, the emergence of apoptosis as a primary mechanism of cardiac cell death[43] and remodeling in heart failure mediated in part by intracellular redox imbalance potentially expanded as we had successfully demonstrated in animal studies the potential benefits that this drug might have in heart failure beyond its adrenergic pharmacology. Thus, carvedilol could inhibit apoptotic cell death through modulation of expression of the Fas receptor, which is a cell surface receptor that activates a cell-death-signaling pathway.[44–47] Additionally, carvedilol was also shown to possess antiproliferative actions on vascular smooth muscle cells and anti-inflammatory actions through its ability to inhibit oxygen radical formation.[48] Preservation by carvedilol of vascular wall and kidney function,[49,50] also likely through this mechanism, further supported the use of this drug in heart failure where multiple organs are observed to have compromised functions.

Through an intense campaign of lectures, seminars, consulting meetings, and symposia to the national and international community of cardiologists on this subject, the authors garnered sufficient interest in carvedilol, as well as the data and logic for its use in treating heart failure, to support the development of this drug in a disease indication where it was currently contraindicated. If the studies we proposed worked, the paradigm shift in the therapy of heart failure would be dramatic. We did not want to think of the consequences if the studies failed.

8.11.5 Clinical Trials of Carvedilol in Heart Failure: The Bottom Line

The decision having been taken to launch clinical trials with carvedilol in heart failure was a relief, as well as a concern; what if the drug did not work, or worse, what if it hurt people with heart failure as the textbooks at the time, and conventional wisdom, indicated it would? Because of these concerns, clinical trials progressed slowly at first through phase I and phase II. Notably, in the phase II clinical trials, some of the hoped- for benefits seemed to be occurring. In a small, but well-controlled phase II clinical trial in heart failure, there seemed to be a reduction in the number of deaths and cardiovascular events in the patients taking carvedilol (in addition to standard care for this disease, which included angiotensin-converting enzyme (ACE) inhibitors, digoxin, and diuretics) compared to patients who were given placebo (in addition to the same standard of care). This sign was exciting, but the study was far too small to draw conclusions on the overall benefit of the drug. For this to happen, a large and well-controlled, not to mention very expensive, phase III study would be required.

The phase III program was initiated for carvedilol in heart failure. This study was, at the time, the largest phase III development program ever undertaken in heart failure, and of course, it was initiated with a drug that was still contraindicated for these patients. Because of this, an independent DSMB was set up to monitor the trial, and they were empowered to stop the trial at any time if carvedilol appeared to be having a negative effect on these seriously ill patients; likewise, the DSMB could stop the trial early if there were 'extraordinary' beneficial effects of the drug, which most of us felt was an extremely unlikely event. We will never forget the day when we were informed that the DSMB had prematurely stopped the phase III clinical trial with carvedilol in heart failure, before its scheduled completion date. Our initial reaction to the DSMB's termination of the carvedilol heart failure study was that the drug must have failed, and even worse, that the drug must have caused harm, as many of our critics had warned it would. However, this turned out not to be the message from the DSMB at all. On the contrary, the results of the clinical trials with carvedilol in heart failure were so strikingly beneficial that the DSMB stopped the study early because, in their assessment, the benefits of the drug were so strong that it was unethical to continue to give patients placebo (plus the standard of care for heart failure described above), and that all patients in the study should be given carvedilol immediately (in addition to the standard of care). The reason behind the DSMB's decision became apparent shortly afterward. In the group of patients receiving carvedilol, the reduction in death was 65% compared to patients receiving placebo. This was an astounding outcome given that heart failure is a progressive disease where mortality rates are very high. The critics were wrong, and so was the dogma; and the patients were the true beneficiaries. For the first time, the treatment of heart failure would be radically changed, and carvedilol would become a new standard of care for these seriously ill patients. The feelings of gratification, and vindication, were indescribable. It is not often that a drug can change the course of therapy and totally reverse conventional wisdom. But carvedilol did just that.

Shortly after the completion of the carvedilol trials in heart failure, SmithKline Beecham, so convinced that carvedilol was different from other beta blockers, as our experimental data had indicated, invested in yet another risky study in which carvedilol would be compared, 'head to head,' to metoprolol in a clinical trial called COMET. Again, the drug performed in these clinical trials just as it had in our preclinical studies, and produced a greater reduction in 'all cause mortality' as well as 'cardiovascular mortality' than did metoprolol, although metoprolol was also effective in its own right.[51] So our belief that carvedilol was different from other beta blockers was also borne out, and this was confirmed later when another beta blocker that was studied in heart failure, namely bucindolol, failed to provide any benefit at all to patients with heart failure. Clearly the effects we had observed with carvedilol in experimental systems translated to benefit to heart failure patients in the clinic, but could not be extended to the entire class of beta blockers.

8.11.6 The Food and Drug Administration: The Last Major Obstacle

The story of the development of carvedilol in heart failure did not immediately have a happy ending based on the clinical results described above. The drug still needed approval by the FDA before it could be used widely in patients with congestive heart failure. Based on the striking results from clinical trials, and the known deadly outcome of this

disease, our anticipation was that the FDA would readily (and rapidly) approve carvedilol for use in heart failure. However, we were in for an extremely disappointing outcome in our initial dealings with the FDA. Because of the uniqueness of the type of study we did, specifically taking a contraindicated drug and showing enormous benefit in seriously ill patients with heart failure, the FDA, as they typically do when faced with a new and unusual situation, called for an advisory panel to convene in order to review the data in a public forum. We had mistakenly believed that the overwhelmingly positive results from the clinical trials would carry the day; we were wrong. The FDA Advisory Committee initially focused on a minor statistical nuance, and virtually ignored the enormous benefit that this drug provided to the patients in the study, namely the 65% reduction in death. Even one former FDA Advisory Committee member pleaded in public to convince the new statistician on the panel to consider the breadth and depth of the overwhelmingly positive data, and balance this against a minor statistical issue (spending 'alpha' to be specific). A seemingly misguided Advisory Committee member stated publicly that "if this were an AIDS drug, I would not be concerned [about the minor statistical nuance] and would recommend approval," but went on to state that he would not do this for a heart failure drug (in spite of the fact that by far more Americans die each year from heart failure than from AIDS, which was apparently unknown to him). To add insult to injury, a senior FDA official declared, also publicly, that the carvedilol results "were too good to be true," and so he also chose not believe the outcome of what was the largest and most sophisticated heart failure clinical trial completed up to that point, and which had been conducted to the pre-agreed specifications and standards of the FDA. So, to our astonishment, the FDA Advisory Committee recommended that the drug not be approved for use in heart failure, and subsequently the FDA rejected the drug. In short, the FDA and its Advisory Committee wanted even more data after having become lost in statistical minutia.

Fortunately, additional clinical trials were already under way in Australia and New Zealand, and the results from those trials, when they became available, supported the previous studies that had been reported to the FDA Advisory Committee a year earlier. Now there was no way to deny that carvedilol was highly effective in preventing death, morbidity, and hospitalization in patients with heart failure, and even in the most severe forms of heart failure. The FDA finally approved the drug in 1997 (2 years after the original data from the phase III clinical trials were available) and an important new therapy became available to patients. It is important to note that the delay in making this life-saving medicine available to patients with heart failure caused by the initial FDA rejection resulted in more American deaths than had occurred during the entire Vietnam War. Nonetheless, we are in agreement that the day carvedilol was approved by the FDA for the treatment of heart failure was the highlight of our scientific careers.

8.11.7 Summary and Perspective

The course of drug discovery and development is fundamentally rationale. Molecular targets are identified for pharmaceutical exploitation based on their role in biological pathways that bear on disease processes. Most major pharmaceutical companies rely on a number of important factors in making a decision to invest in an innovative new therapy, including medical need, competition, development time, costs, probability of success, and ability to recoup the substantial investment they make based on marketing forecasts. In the case of carvedilol, most of these factors, with the exception of the medical need, weighed against the development of the drug. But the successful development of carvedilol for heart failure[51–53] reveals additional and critical dimensions of the process that cannot be easily gauged or measured. Carvedilol was launched to the market as a pioneer drug for treatment of heart failure after many significant hurdles and 'roadblocks,' which at that time seemed insurmountable. The public health implications of carvedilol as a life-saving medicine are dramatic as analyses have shown that the number of patients needed to treat with carvedilol in order to prevent one death compares most favorably with other life-saving medicines introduced for cardiovascular diseases (**Table 1**).[54] Considering the prevalence of heart failure in the general population, the number of lives saved through the use of carvedilol is significant, and has had a positive impact on the overall health of the nation.

The dedication persistence and leadership demonstrated by the Discovery team in 'forcing' this drug to the market, and the confidence in them demonstrated by their management, is what turned this 'pipe dream' into a reality. The case history of carvedilol also illustrates the importance of strong biology and translational research aimed to explore fundamental disease mechanisms, and how innovative new drugs can change the practice of medicine. And of course, the 'cross-talk' between Discovery, Development, key opinion leaders, and commercial experts is key to bringing any new therapy to patients.

While carvedilol has proven itself in the cardiology community, and has become a significant financial success, the full potential of the drug may still not yet be realized. The multiple organ protection that carvedilol has demonstrated in preclinical studies suggests the potential for carvedilol to provide 'best in class' therapy for hypertension (for which it also is approved), angina and organ damage/failure, including the renal, vascular, and central nervous systems.

Table 1 Number of patients needed to treat with various drugs based on results from recent trials

Trial [drug]	*Number needed to treat**
HOPE [ramipril]	221
4S [simvastatin]	159
SAVE [captopril]	86
CAPRICORN [carvedilol]	43
MERIT-HF [metoprolol succinate]	26
COPERNICUS [carvedilol]	14

CAPRICORN, Carvedilol Post-Infarct Survival Control in Left Ventricular Dysfunction; COPERNICUS, Carvedilol Prospective Randomized Cumulative Survival; 4S, Scandinavian Simvastatin Survival Study; HOPE, Heart Outcomes Prevention Evaluation Trial; MERIT-HF, Metoprolol Controlled-Release Randomized Intervention Trial; in Heart Failure; SAVE, Survival and Ventricular Enlargement Trial.

*Number of patients needed to treat for 1 year to save one life.

But in the end, the most significant gratification one can feel, other than the birth of a child, is playing a role in the discovery and development of a drug that changes medicine and brings enormous benefit to patients. The similarities between the birth of a child and the birth of a new drug will not be lost on those few who have had the privilege to be involved in both.

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Biographies

Robert R Ruffolo is currently President of Research & Development for Wyeth Pharmaceuticals, and corporate Senior Vice President of Wyeth. He joined Wyeth in 2000 as Executive Vice President, responsible for Pharmaceutical Research and Development. Prior to joining Wyeth, Dr Ruffolo spent 17 years at SmithKline Beecham Pharmaceuticals where he was Senior Vice President and Director of Biological Sciences, Worldwide. Before joining SmithKline Beecham, Dr Ruffolo spent 6 years at Lilly Research Laboratories.

During his career in the pharmaceutical industry, Dr Ruffolo played a significant role in the discovery and/or development of a number of marketed products, including carvedilol (Coreg/Kredex) for the treatment of congestive heart failure and hypertension, dobutamine (Dobutrex) for the acute management of congestive heart failure, ropinerole (Requip) for Parkinson's disease, and eprosartan (Teveten) for hypertension.

Dr Ruffolo received his BS degree in pharmacy *summa cum laude with distinction* in 1973, and his PhD degree in pharmacology in 1976, both from The Ohio State University. Thereafter, he spent 2 years as a postdoctoral fellow at the

National Institutes of Health. Dr Ruffolo has authored nearly 500 full-length publications and 200 abstracts, and has edited 17 books. He is the Editor-in-Chief of *Current Opinions in Pharmacology*, and was the Editor-in-Chief and Founder of both *Pharmacology Reviews and Communications* and *Pharmacology Communications*. Dr Ruffolo has served on the editorial boards of 28 international scientific journals.

Dr Ruffolo has received a number of prestigious awards, including Chief Scientific Officer of the Year (2004), George B. Kolle Award for Scientific Excellence, Lorenzini Gold Medal for Biomedical Research (1999), John Jacob Abel Award in Pharmacology (1988), Prix Galien Special Commendation for Excellence and Innovation in Research (1996), and the Distinguished Alumni Award from The Ohio State University (1989). In 1997, Dr Ruffolo was honored by SmithKline Beecham Pharmaceuticals for his pioneering research on carvedilol (Coreg/Kredex), which radically changed the treatment of congestive heart failure. In 2004, the American Chemical Society recognized Dr Ruffolo for leadership in creating a research environment that promoted innovation, which lead to the Heroes of Chemistry Award. In 2005, *R&D Directions* recognized Dr Ruffolo twice for the transformation of Wyeth's drug development pipeline, first with the Top Ten Pipelines Award, in which they designated Wyeth as *The Pipeline to Watch*, and second for being *Industry Best* with respect to the number of drugs in their list of top '100 Great Investigational Drugs.' Recently, the American Society for Information Science & Technology designated Dr Ruffolo as a *Highly Cited Scientist* for being among the top 100 most cited pharmacologists in the world over the past two decades.

Giora Z Feuerstein joined Wyeth in 2005 as the Senior Director, Translational Medicine. Before joining Wyeth, Giora Feuerstein has maintained Directorship position in discovery of cardiovascular, stroke, and metabolic disease programs for 16 years in other Pharmaceutical Houses. At SmithKline Beecham (1980–98), Giora Feuerstein served as the Director of the Department of Cardiovascular Pharmacology where he lead the Carvedilol (COREG) program which became the first beta blocker launched for treatment of chronic heart failure. In addition, Giora Feuerstein was associated with the discovery and development of eprosartan, enrasartan, lotrafiban, and several other compounds for diverse cardiovascular indications including stroke and anti-arrhythmic drugs.

In 1998, Giora Feuerstein joined DuPont Pharmaceuticals as the head of the Cardiovascular Disease Department leading thrombosis, cardiovascular, and metabolic syndrome programs. Razaxaban, a lead FXa inhibitor was advanced to phase II development prior to acquisition of the DPC by BMS. In 2003 Giora Feuerstein joined Merck Co, Inc, West Point as the Executive Director, cardiovascular diseases where he established a new department leading efforts in hypertension (renin inhibitors), metabolic syndrome and cardiac arrhythmias. In addition, Giora Feuerstein was appointed as member of strategic forums in cardiovascular drug development and chaired licensing and business development committees.

Prior to joining the pharmaceutical industry, Giora Feuerstein held academic position in the USUHS, Bethesda, MD (1981–88) where he was the Director of the Neurobiology Research Laboratories heading research in central and peripheral regulation of the cardiovascular system with focus on adrenergic and peptidergic systems. In addition, Giora Feuerstein developed research lines in stroke, gene expression, and pharmacological strategies. Giora Feuerstein held the rank of Professor (Research).

Giora Feuerstein received his MSc degree in Pharmacology from the Hebrew University, Jerusalem, Israel in 1970 and his MD degree from the Hadassah Medical School, Jerusalem, Israel. Following lectureship position in the Department of Pharmacology, Hadassah Medical School (1976–79) Giora Feuerstein obtained the Fulbright scholarship

for further training at the National Institutes of Health (NIH), Bethesda, MD (1979–81) in the Laboratory of Clinical Sciences (Chief, I Kopin) focusing on sympathetic nervous system control of the cardiovascular system.

Giora Feuerstein holds adjunct position in academic organization (Med College of Georgia, Augusta, GA) and Jefferson Medical College, Philadelphia, PA). He also serves on editorial boards of the *Journal of Pharmacology and Experimental Therapeutics; Biochemical Pharmacology; Journal of Cerebral Blood Flow and Metabolism; Circulation Research; and Stroke*. Giora Feuerstein is the recipient of several national and international awards including Award of Excellent in Cardiovascular Research, AHA, 1987; Prix Galien Award for Drug Discover (endothelin antagonist), 1994; Conrad R Lam Award for Cardiovascular Research, Henry Ford Foundation, 2001.

Giora Feuerstein has authored and co-authored over 650 publications of which over 400 are peer review journals. He is also the coinventor on 12 patents and has edited 8 books.

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8.12 Modafinil, A Unique Wake-Promoting Drug: A Serendipitous Discovery in Search of a Mechanism of Action

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The search for additional indications for modafinil was directed toward diseases associated with wake deficit and somnolence but also to those in which symptoms could be related to cognition deficits, with modafinil showing human efficacy in attention deficit hyperactivity disorder (ADHD). Preclinical studies also showed a beneficial effect of modafinil in models of depression.

Since its discovery, modafinil has proven to be an efficient and safe therapeutic agent. Many biochemical and pharmacological studies, focused on the mechanism of action of modafinil, have failed to provide any conclusive evidence for a discrete target for this novel drug.

8.12.1 Introduction

The story of modafinil (Provigil), that began in the early 1970s, is the discovery of the unexpected activity of an NCE in classical animal models that transitioned to an effective agent that helped define and grow the field of sleep medicine. Additionally, extensive preclinical work on modafinil led to: (1) the identification of additional therapeutic indications that were not fully appreciated from the initial in vivo profile for modafinil; and (2) a continuing search, albeit unsuccessful, to define its mechanism(s) of action.

At the time that modafinil was discovered, the process of drug discovery was neither target-directed nor technology-(genome, high-throughput screening (HTS), or combinatorial chemistry) driven. The search for new drugs was thus mainly based on proven functional and empirical methods. Many of the receptors now known to be involved in sleep–wake regulation, e.g., hypocretin/orexin,[1] had not been identified, while the discrete functional role(s) of better-characterized central nervous system (CNS) neurotransmitter systems, e.g., cholinergic, noradrenergic, dopaminergic, serotonergic, histaminergic, GABAergic, in sleep function were still emerging.[2–4]

8.12.2 The First Step: The Discovery of Adrafinil

In the early 1970s, the Research Department of Laboratoire Louis Lafon, a small family-owned pharmaceutical company located in Maisons-Alfort, a suburb of Paris, had focused its research activities in three areas: (1) cardiovascular; (2) antispasmodics; and (3) nonsteroidal anti-inflammatory drugs (NSAIDs)/analgesics.

In this last field, an empirical test battery was implemented, using the best-characterized in vivo pharmacological methods generally in use at that time (some of which are still used). These included the carrageenan-induced paw edema and mechanical inflammatory pain (Randall and Selitto model) in rats, hot plate test, and the abdominal writhing test in mice.

Initial screening efforts involved the investigation of many NCEs in the abdominal writhing test: five compounds were simultaneously included in the same study. A trained observer, blinded to the randomized compound treatments, watched the mice for 5 min following p.o. dosing of NCEs. Typically, following administration of an agent that elicited

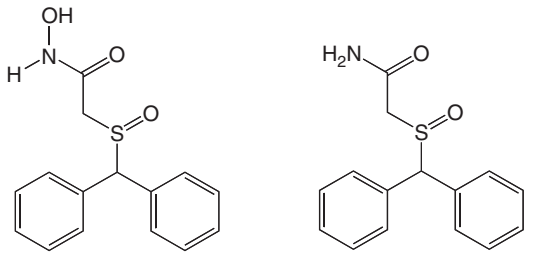

Figure 1 Structures of adrafinil and modafinil.

writhing (given i.p.), the mice were quiet and showed restricted motor activity, probably to avoid experiencing additional pain. However, during one of these experiments, the observer noticed that some mice, usually one in each group of six, did not display this behavior but instead exhibited marked locomotion, exploring their surroundings in a similar manner to control vehicle-treated mice. When the experiment was unblinded, the mice displaying this locomotor activity were all found to have received the same compound, CRL40028 ((benzhydryl sulfinyl) acetohydroxamic acid), later named adrafinil (Figure 1).

As a result of this unexpected finding, Louis Lafon, the founder of the company, immediately switched the objectives of the NSAID/analgesic research team to CNS therapeutic agents, more precisely to the discovery of nonamphetamine-like psychostimulant or antidepressant drugs.

Using a battery of classical behavioral tests in mice, the stimulant potential of adrafinil was confirmed, based on a dose-dependent increase in locomotor activity, antagonism of barbital-induced narcosis, and a decrease in the duration in the forced swim test.[5] Interestingly, adrafinil did not display any of the other effects normally observed with amphetamine and nonamphetamine (methylphenidate-like) stimulants: it failed to induce changes in core temperature; did not produce stereotyped or climbing behavior; and did not increase lethality in aggregated mice. Adrafinil was also devoid of other effects usually seen with classical antidepressants. It thus had no interaction with reserpine-, oxotremorine-, or apomorphine-induced hypothermia (although it slightly potentiated yohimbine-induced toxicity); lacked peripheral sympathetic effects (lack of mydriasis, salivation, piloerection, or antagonism of reserpine-induced ptosis); and lacked peripheral anticholinergic effects (lack of mydriasis or antagonism of oxotremorine-induced salivation or lacrimation).

These results led to the conclusion that, as compared to amphetaminic, anticholinergic, or antidepressant drugs, adrafinil had a unique behavioral profile in mice. This was defined by a specific stimulant activity associated with nonclassical antidepressant-like effects that did not appear to be related to a β-adrenergic mechanism, together with behavioral effects not linked to dopaminergic stimulation.

8.12.3 From Adrafinil to Modafinil

In considering adrafinil as a lead compound in search of therapeutic utility, the Chemistry Department in Laboratoire L. Lafon began to synthesize compounds chemically related to adrafinil. More than 100 compounds were then characterized using the primary behavioral screening used to determine the effects of adrafinil. In April 1976, 2 years after the first assays with adrafinil, CRL40476 was identified. This compound displayed the same pharmacological profile as adrafinil, but was more potent and longer-lasting than adrafinil. This compound, (diphenyl-methyl)-sulfinyl-2-acetamide, was named modafinil (presumably by analogy to 'modified adrafinil' or 'modulated adrafinil'?).

Because of the similar effects of adrafinil and modafinil and the available preclinical experimental data obtained with adrafinil (particularly in toxicological studies), the development of modafinil moved forward, mainly focused on putative therapeutic applications, a search for the mechanism of action, and a differentiation from amphetamine- and nonamphetamine-like stimulants. The initial publication of the unique behavioral profile of modafinil[6] aroused considerable interest from several research groups, resulting in many subsequent animal studies that confirmed, in a variety of species – mice,[7] rats,[8,9] cats,[10] narcoleptic dogs,[11] monkeys,[12,13] and even fruitfly[14] – the stimulant and awakening effects of the compound. Simultaneously, every effort was made to generate additional data to advance knowledge related to modafinil to the same level as that which was known regarding adrafinil, in order to provide a choice between the two compounds for further development. At the same time, studies on the metabolism of adrafinil indicated that this compound was primarily inactivated by conjugation but was, to a slight extent, metabolized to an active metabolite, modafinil.

8.12.4 From Animal Pharmacology to a Human Therapeutic

Following the discovery of the stimulant effects of adrafinil in rodents, it was essential to select a viable therapeutic indication consistent with the effects observed. At that time, Lafon had a research and development agreement with a large pharmaceutical company that wanted to focus clinical studies and indications in 'psychogeriatrics.' That led to a new drug application being filed only in France with an approval for 'vigilance and mood disorders in the elderly.' At the same time Louis Lafon had the opportunity to meet Professor Michel Jouvet, the world-renowned sleep expert from the University of Lyons, France, who was mainly interested in the stimulant properties of the compound. He anticipated that these would be linked to awakening effects. To demonstrate that idea, he initially used a cat model of sleep–wake and in preliminary studies confirmed his hypothesis, that modafinil could produce a quiet wakefulness in cats, an effect that was subsequently confirmed in monkeys.[15] From these results, Jouvet suggested that modafinil be studied in a disease in which patients have difficulty in staying awake and suddenly fall into paradoxical (rapid eye movement or REM) sleep, e.g., narcolepsy or Gélinau's disease.

In spite of the low prevalence of this disease (1 in 1000 of the population), studies were first undertaken in healthy volunteers where the wake-promoting properties of modafinil were confirmed.[16] Additional clinical studies demonstrated the beneficial effects of the compound in hypersomniac narcoleptic patients[17–22] and confirmed the limited abuse liability of the compound as compared to amphetamine and its congeners.[23]

8.12.5 In Search of a Mechanism(s) of Action

As soon as the original profile of adrafinil was described, a number of research teams, among them some with existing collaborations with Laboratoire L. Lafon, used different approaches in attempts to elucidate the mechanism(s) of action of adrafinil and modafinil, in order to differentiate them from amphetamine and methylphenidate. Using classical pharmacological probes known to interact preferentially (but probably not specifically) with neurotransmitter systems and receptors, it was found that centrally but not peripherally acting α_1-adrenoceptor antagonists reduced the stimulant and wake-promoting effects of both adrafinil and modafinil: locomotor activity in mice,[5,6,24] nocturnal activity in monkeys,[12,13] electroencephalogram sleep–wake in cats,[10] and convulsions in quaking mice.[25,26] Moreover, while the motor stimulant and wake-promoting effects of amphetamine and methylphenidate were blocked by the dopamine receptor antagonists, haloperidol or sulpiride, and by α-methyl tyrosine blockade of catecholamine synthesis, these agents did not affect modafinil activity.[27] Moreover, while modafinil was unable to modify the firing pattern of central dopaminergic and noradrenergic neurons in the rat, amphetamine consistently inhibited their activity, leading to the conclusion that, in contrast to amphetamine, the waking effect of modafinil was not mediated by catecholaminergic systems.[28] Similarly, using in vivo voltammetry in mouse caudate nucleus, modafinil was ineffective in modulating presynaptic nigrostriatal function, in contrast to dexamphetamine and methylphenidate.[29] A *c-fos* immunocytochemistry study in cat produced an additional demonstration that the neuronal targets through which modafinil induced wakefulness were different from those of amphetamine and methylphenidate.[30] Additional studies in the rat supported these results and concluded that the brain neuronal targets for modafinil included nuclei of the hypothalamus and amygdala.[31] The wake-promoting effects of modafinil were associated with activation of the tuberomammillary nucleus and orexin neurons, two regions implicated in the promotion of normal wakefulness.[32]

While amphetamine altered glucose utilization in a wide variety of brain regions, modafinil had a relatively restricted pattern of changes (hippocampus, centrolateral nucleus of the thalamus, central nucleus of the amygdala), suggesting that modafinil was acting on a specific subset of brain pathways that regulated sleep and wakefulness, whereas amphetamine activated a greater number of cerebral structures where modafinil had no effect (basal ganglia, nuclei of the thalamus, frontal cortex, nucleus accumbens, ventral tegmental area, and pontine reticular formation), involved in the regulation of sleep and wakefulness.[33]

Despite these findings, controversy exists regarding an indirect involvement of central dopaminergic systems in the wake-promoting effects of modafinil. In a variety of studies, modafinil has shown minimal interactions with a wide variety of receptors, including adrenergic, histaminergic, dopaminergic, P1, and orexin-1 and 2. Modafinil did however bind with low affinity ($Ki \sim 3–4\ \mu mol\ L^{-1}$) to dopamine transporter-binding sites[34] (DAT).

In addition, in vitro [^{3}H]-modafinil (specific activity $20\ Ci\ mmol^{-1}$) was, in our hands, unable to bind specifically to many brain tissues from various animal species (mouse: total brain, cortex, hippocampus, thalamus, hypothalamus, pons medulla; rat: total brain; rabbit: cortex, hippocampus; dog: total brain, cortex) and autoradiography studies in the rat did not display any affinity of the labeled compound for any brain area (unpublished results).

The involvement of dopamine in the wake-promoting effects of modafinil and other CNS stimulants was supported by studies in control and narcoleptic canines.[35] Like amphetamine, modafinil increased caudate extracellular dopamine

at least in narcoleptic dogs, via a mechanism independent of the hypocretin receptor.[36] Additionally, in DAT knockout mice, modafinil-like methamphetamine and the selective DAT blocker, GBR 12909, lacked wake-promoting effects.[36]

Increases in dopamine release in the rat nucleus accumbens were observed following modafinil administration but this was secondary to a reduction in GABAergic transmission that led to a reduction of $GABA_A$ receptor signaling in dopamine terminals.[37] Modafinil dose-dependently reduced γ-amino-butyric acid (GABA) outflow from the cortex of awake guinea-pig,[38] and from the striatum, pallidum, and substantia nigra,[39] and, more importantly, from the medial preoptic area and posterior hypothalamus of the awake rat.[40] The latter are hypothalamic fields where functional inhibition of GABA release by modafinil may be relevant for its vigilance-promoting effects. Modafinil also increased glutamate release in the ventrolateral and ventromedial thalamic areas, hippocampus,[41] medial preoptic area, and posterior hypothalamus, where its effects were dependent on decreased GABA efflux.[42] The decrease in GABA outflow and the concomitant increase in glutamate release appear to be indirect effects on GABAergic and glutamatergic neurons.[43] Additionally, the decrease in cortical GABA outflow appeared to be regulated by a balance between noradrenergic and serotonergic neurotransmission.[44,45]

Modafinil has complex interactions in the central nucleus of amygdala[46] and increases histamine release in the anterior hypothalamus, suggesting that modafinil may promote waking via the activation of histaminergic systems, despite the lack of direct interactions of modafinil with any of the four subtypes of histamine receptor family.[47] Modafinil potentiated the effects of norepinephrine on sleep-promoting neurons in the ventrolateral preoptic (VLPO) nucleus, as did nisoxetine, leading to the suggestion[48] that the wake-promoting effects of modafinil may involve inhibition of the norepinephrine transporter (NET) in VLPO neurons, even though the compound had no effect on NET at concentrations up to 100 $\mu mol\ L^{-1}$. In both orexin-null and wild-type mice, modafinil produced similar patterns of neuronal activation, as indicated by Fos immunohistochemistry. Interestingly, modafinil was more effective in increasing wake time in orexin-null than in the wild-type mice, suggesting that orexin was not mandatory for the wake-promoting effects of modafinil, but might be involved in some aspect of the alerting actions of the compound.[49]

Many of these studies, while providing intriguing evidence for a potential mechanism of action of modafinil, have, in toto, provided little in the way of testable hypotheses that are unequivocal in their focus on the mechanism of action of modafinil. One intriguing question is whether the effects of modafinil are, per se, wake-promoting, or rather are due to a possible inhibition of sleep-inducing systems.

8.12.6 In Search and Discovery of Potential New Therapeutic Indications

The search for additional indications for modafinil naturally focused on diseases associated with wake deficits and somnolence. The effects of the drug in an animal model of sleep-disordered breathing suggested that modafinil might be effective in reducing sleepiness associated with sleep apnea,[50] and this was subsequently demonstrated in the clinic.[51–53] Other disorders where somnolence or sedation was concomitant with the disease, e.g., Parkinson's disease,[54–56] myotonic dystrophy,[57–60] fibromyalgia,[61] amyotrophic lateral sclerosis,[62] multiple sclerosis,[63] cerebral lymphoma,[64] or resulting from the side-effects of other medications such as antidepressants,[65] antipsychotics,[66] dopaminergic D_2 agonists,[67,68] opioids,[69] or valproic acid,[70] have also proven to be amenable to treatment with modafinil.

Likewise modafinil has been applied with equal success to treating the fatigue coexisting with other serious diseases, including multiple sclerosis,[71,72] pain,[73] and acquired immunodeficiency syndrome (AIDS).[74]

Because wake and vigilance are essential requirements for attention, learning, and cognition, research on these topics has also been undertaken in animals. Modafinil was found to induce a faster learning rate in a serial spatial discrimination task, demonstrating an improvement of learning processes following acute[75,76] and chronic administration in mice[77] and facilitating performance on a delayed nonmatching to position swim task in rats.[78] In healthy human volunteers without sleep deprivation, modafinil had subtle stimulating effects on maintenance and manipulation processes in relatively difficult and monotonous working memory tasks, especially in lower-performing subjects.[79] In addition, in healthy volunteers, modafinil produced a selective improvement of neuropsychological task performance, attributable to an enhanced ability to inhibit prepotent responses, leading to a reduction of impulsive responding, that appears to be beneficial in the treatment of ADHD.[80] Based on this result, it was then obvious to try modafinil in ADHD, without any animal prerequisites but by analogy with the established uses of stimulants, even though its mechanism of action was unknown but unquestionably was dissimilar from amphetamine and methylphenidate. Modafinil was found to be effective in ADHD in children[81,82] and in adults[83,84] and has been approved as Sparlon.

Beyond this application, research focused on diseases in which symptoms could be related to cognition deficits. In the five-choice serial reaction time task of attentional function in rats, modafinil had attention-enhancing effects that may be relevant to the treatment of cognitive deficits in schizophrenia.[85] Likewise, in patients with schizophrenia, modafinil

produced a significant improvement in attentional set shifting (despite no effect of modafinil on this task being seen in healthy volunteers), that led to the assumption that the compound may have potential as an important therapy for cognitive impairment.[86] The results of a preliminary open study suggested that modafinil may be an effective and well-tolerated adjunct treatment that improves global functioning and clinical condition in patients with schizophrenia or schizoaffective disorder.[87] Other applications resulted from case observations such as spastic cerebral palsy.[88]

Based on the activity of modafinil in the forced swim test in animals, considered as predictive of some antidepressant activity in humans, several preliminary clinical studies demonstrated that modafinil was able to enhance the effects of antidepressant drugs, especially in patients with residual tiredness or fatigue.[89] It was recently confirmed that modafinil was potentially effective as adjunctive therapy in depressed patients, particularly in those with problematic fatigue and sleepiness.[90,91] Adjunct therapy of modafinil at initiation of treatment with a selective serotonin reuptake inhibitor (SSRI) improved the degree and onset of therapeutic effects in patients with major depressive disorder and fatigue.[92] These beneficial effects may result from an enhancement by modafinil of the increase of extracellular serotonin levels induced by antidepressant drugs, such as fluoxetine and imipramine, in awake rat[93] and a differential enhancement of serotonin efflux in distinct brain regions of the awake rat by modafinil, that could be possibly relevant for wakefulness and depression.[94] Modafinil regulated cortical serotonergic transmission, suggesting that the drug could preferentially act by amplifying the electroneurosecretory coupling via mechanisms that do not involve the reuptake processes.[95] Such puzzling results are not fully elucidated yet, as modafinil does not affect serotonergic transmission from cortical synaptosomes. Also, the serotonin-releasing effects of modafinil are different from those of either DL-fenfluramine or fluoxetine.[96]

Despite its stimulant activity, modafinil did not produce reinforcing or rewarding effects and did not modify the effects of cocaine in rats.[97] Evaluation for cocaine-like discriminative stimulus effects in rats and for reinforcing effects in rhesus monkeys maintained on intravenous cocaine self-administration demonstrated that the reinforcing and discriminative stimulus effects of modafinil required very high doses.[98] The low abuse potential was confirmed via an extensive data set in healthy human volunteers[23,99] and in volunteers with a recent history of cocaine abuse where cocaine and methylphenidate, but not modafinil, produced cocaine-like discriminative stimulus, subject-rated, and cardiovascular effects.[100]

Based on the low potential of addiction and dependence, a preliminary study provided evidence that modafinil improved clinical outcome when combined with psychosocial treatment for cocaine dependence.[101] An anecdotal story in a woman outpatient with social phobia and comorbid amphetamine dependence reported that her craving for amphetamines diminished and her anxiety and depression improved without the same 'high' with modafinil that she experienced with amphetamines.[102]

8.12.7 Conclusions

In the 30 or so years since the initial discovery of its stimulant effect in mice, despite an impressive amount of preclinical, pharmacoclinical, and clinical studies, modafinil has yet to reveal all the secrets of either its mechanism of action or its potential for new therapeutic applications. The fascinating unique profile of this drug (awarded the French Science and Defense Prize in 1994 and the Galien Prize in 1997) is still in search of a mechanism[103] that could help to explain its multiple applications, to find links between these mechanisms and other putative indications, and to develop second-generation agents. With the current state of knowledge and taking into account its therapeutic and chemical class, its yet-to-be-determined mechanism of action, and its multiple indications, modafinil must therefore be regarded as fitting the requirements of portmanteau,[104] using one drug to treat multiple symptoms.

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Biographies

Francis A Rambert was born in Algiers (Algeria) in 1942 and began medical studies at the University of Algiers and then in Paris, but was forced to give up before completion in 1964 and consequently joined Laboratoire Louis Lafon in Maisons-Alfort. He successively led the Antispasmodic Group, the Analgesics/Anti-Inflammatory Group in the Pharmacology Department and in 1974 was appointed Head of the Neuropsychopharmacology Department, following training in behavioral central nervous system pharmacology in the group of Prof Pierre Simon at Pitié-Salpêtrière (University of Paris). During the modafinil licensing agreement, he closely collaborated with the Cephalon Research team. He has authored more than 20 papers on modafinil and adrafinil and presented on this topic numerous communications in international congresses on pharmacology and sleep. In 1991, he was awarded the Galien Prize for Drug Innovation conferred to Laboratoire Louis Lafon research team and in 1994 the Science & Defense Prize (with Prof Michel Jouvet and the French Air Force Medical Research teams). After 40 years of research efforts dedicated to the discovery new drugs, 30 years of which involved modafinil and its derivatives, he retired at the end of 2004.

Jean François Hermant was born in 1958 in Rouen (France) and studied Pharmacy at the University René Descartes – Paris, where he obtained his diploma (Pharm D) in 1985. He joined the Laboratoires Louis Lafon in 1984 where he worked in the Central Nervous System Group under the direction of Francis Rambert for more than 20 years. His current research interest is focused on wake promotion.

Dominique Schweizer was born in 1951 in Paris (France) and studied Chemistry at the University Jussieu and at ESCOM (Ecole Supérieure de Chimie Organique et Minérale) – Paris, where she obtained her diploma as Chemical Engineer in 1975. She joined the Laboratoires Louis Lafon in 1976 where she worked in the Medicinal Chemistry Department. Under the direction of Dr Roger Gombert, she performed the synthesis of modafinil in 1976. Today, she is involved in the synthesis of new chemical entities related to central nervous system diseases.

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8.13 Zyvox

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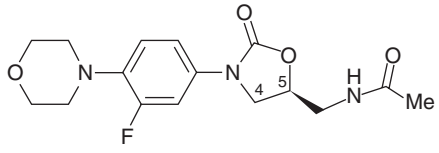
8.13.1 Introduction

The era referred to as the 'Golden Age of Antibiotics' in the mid-twentieth century was one of unprecedented impact on the practice of medicine. In times prior to the availability of these life-saving antibiotics, the outcome of an infection pivoted solely on the ability of the individual's immune system to keep the pathogen from overwhelming the host. In all but the past six decades in the entire span of our existence, humans have known the harsh realities of serious infection: violent coughing with production of blood, or high fever with discharge of pus from an inflamed and infected wound, was recognized as an omen of death. This reality was particularly evident with the battlefield wounded, most of whom died as a result of blood poisoning with a streptococcal or staphylococcal infection, or from tetanus, prior to the 1940s, when the synthetic sulfonamides and penicillin became available.[1] With the advent of antibiotics, this previously bleak prognosis of the outcome of an invasive infection was dramatically transformed. Physicians suddenly were given the means to affect a cure of most bacterial infections, saving those for whom previously there would have been little recourse.

Sulfanilamide had its origins in Gerhard Domagk's 1932 discovery of the curative properties of a dye, red prontosil, which was found to be metabolized to the active antibacterial agent.[2] In actuality, the genesis of the sulfonamide class of antibacterial drugs can be linked back to Paul Erlich, the father of antimicrobial chemotherapy, as his work inspired Domagk. Erlich was the first to systematically screen for anti-infective microbe-selective toxins among various dyes that were absorbed by living tissues, and found that trypan red could cure horses afflicted with sleeping sickness.[3] The introduction of penicillin into medicine proved to be an epic watershed event, launching a very aggressive and extensive search for additional antibiotics that could be extracted from fermentations of soil microorganisms. At pharmaceutical companies and academic institutes around the globe, researchers isolated, tested, and identified thousands of new antibiotics. Yet very few of these classes of antibiotics would prove to have the utility and acceptable safety profiles to ultimately be commercialized, either as the parent natural product (e.g., penicillin G and erythromycin A), or, more typically, as a semisynthetic derivative. Among the class of β-lactam cell wall biosynthesis inhibitors, large numbers of modified penicillins and four generations of cephalosporin derivatives would reach the market, and lesser numbers of others (e.g., carbapenems and monobactams). These massive antibiotic research efforts also led to the discovery and

development of several important classes of bacterial protein synthesis inhibitors – the tetracycline, streptogramin, lincosamide, chloramphenicol, macrolide, and aminoglycoside antibiotics. (The oxazolidinones also inhibit protein synthesis, but in a completely different manner, by inhibiting the initiation process, and thus do not have cross-resistance with these other classes.) Other natural products of utility were the glycopeptides vancomycin and teichoplanin, which target the inhibition of bacterial cell wall synthesis by a different mechanism than the β-lactams. In the USA, vancomycin has proved to have great utility against the problematic Gram-positive pathogens such as β-lactam-resistant *Staphylococcus aureus*. The last family of antibiotics discovered in the Golden Age was a series of synthetic quinolones, of which nalidixic acid was the progenitor, and which led to a number of very potent fluoroquinolones, antibacterial agents that kill bacteria by inhibiting DNA topoisomerases.[4]

Prior to the US Food and Drug Administration (FDA) and other regulatory agency approvals of the oxazolidinone antibacterial agent linezolid (**1**, Zyvox) in 2000, no other distinctly novel class of antibiotics had been brought to the marketplace since the discovery of nalidixic acid, representing a span of over 35 years.[5] This was followed by the approval in the USA in 2003 of another member of a new class, the lipopeptide antibiotic daptomycin (Cubicin, Cubist Pharmaceuticals).[6]



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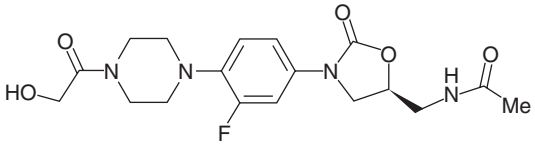
This case history discusses key aspects of the discovery and development of the first marketed oxazolidinone antibiotic, linezolid. The primary focus of this review is to present a personal perspective on the many challenges confronted by the research team that discovered linezolid, and the strategies that proved successful for dealing with those challenges. In addition, key aspects are discussed of both the delineated structure–activity relationships (SARs) and structure–toxicity relationships (STRs) that led this team to the successful identification of linezolid and the progenitor drug candidate, eperezolid (**2**, U-100592).



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The research activities described herein commenced at The Upjohn Company, and continued (through a series of corporate mergers and an acquisition) at Pharmacia & Upjohn, Inc., and then the Pharmacia Corporation. In 2003, Pharmacia was acquired by Pfizer Inc., at which post-approval clinical studies with linezolid have continued. The reader interested in a review of the clinical study outcomes and details of the biological profile of linezolid will find a more comprehensive discussion of those and other aspects presented elsewhere (*see* 7.23 Oxazolidinone Antibiotics).

8.13.2 The Medical Need for New Antibiotics: Multi-Drug Resistance

As a consequence of the selective pressure of antibiotic therapy on bacterial populations, the evolution of bacterial resistance to antibiotics is an expected and natural phenomenon.[7] Resistance results from survival and multiplication of those bacteria having a mutation or acquired resistance determinant that allows those organisms to remain viable in the presence of the antibiotic. The problem of cross-resistance presents a formidable hurdle that complicates the difficult objective of designing new structural features into older classes, as a means of treating resistant infections. In contrast, a member of a completely novel drug class having a new mode of action is a priori unlikely to demonstrate cross-resistance with other established antibiotics. The development of cross-resistance is due to bacterial mechanisms that can subvert the action of all members of a given family of antibiotics, undermining their usefulness against those particular strains, and potentially other organisms as well, if there is interspecies genetic transfer of the resistance determinant.

By the early 1990s, a sudden and dramatic rise in the incidence of multidrug resistance in a number of important human pathogens had reached a stage where, in some cases, there were few or no approved antibiotics that remained effective in treating those serious infections. Such multidrug resistant (MDR) bacteria have developed a both remarkable and disconcerting variety of mechanisms for subverting the killing effects of antibiotics.[8] Genetic resistance determinants can be widely shared among bacterial populations, mediated by the promiscuous transfer of plasmids and transposons through the process of conjugation, where one species' genes for resistance can be passed on to another, even those of a different genus.[9]

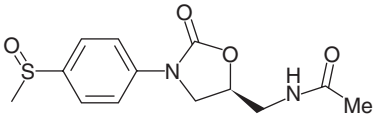
Over the past 15 years, MDR strains have become highly prevalent among important Gram-positive pathogens, particularly in those isolates taken from patients with hospital-acquired infections in intensive care units (ICUs).[7] The continually escalating incidence of methicillin-resistant strains of *Staphylococcus aureus* (MRSA) in many European countries now exceeds 25%[10]; in the USA[11] and Japan,[12] this figure currently approaches 60% and 70%, respectively. Of concern, new clones of MRSA have been isolated from outbreaks in healthy people within community settings.[13]

At the time that our work on the oxazolidinones initiated at Upjohn in late 1987, a new MDR strain of a Gram-positive enterococcal pathogen was just on the cusp of emergence – but not yet recognized as the problem pathogen it has now become. Our interest at that time in the oxazolidinones was based on attributes of two lead compounds described by DuPont.[14] Researchers there had demonstrated in animal models of infection the promise of their leads to potentially meet the challenges of treating the increasingly prevalent MRSA infections.[14] These MRSA strains were also resistant to a broad collection of other drugs, but were still susceptible to 'the agent of last resort' – vancomycin – that was seeing significant use for treatment of these infections. In 1988, the first reports of the newcomer strains of vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis* (VRE) appeared.[15,17] In the following 4 years, the incidence of VRE would skyrocket in US hospital ICUs.[18] Today, VRE strains have become resistant to virtually all known antibiotics, and are associated with high mortality rates among VRE-infected hospitalized critically ill patients, particularly the immunocompromised.[16]

At the time of this writing, the most problematic hospital-acquired pathogens remain those seen in the 1990–2000 decade: MRSA, MDR *Streptococcus pneumoniae* (MDR-SP), and the VRE – all MDR Gram-positive bacteria.[11] Of considerable concern are the newer community-acquired MRSA infections, and a few sentinel strains of vancomycin-intermediate-resistant (VISA) and vancomycin-resistant strains of *Staphylococcus aureus* (VRSA),[18a–18c] with three confirmed clinical cases of the latter reported in the USA.[19] Linezolid has been found to have excellent potency against all of the above problematic sensitive and MDR Gram-positive pathogens, and has been approved for treatment of MDR-SP, MRSA, and VRE infections.

8.13.3 Genesis of the Upjohn Oxazolidinone Program

In October 1987, there was an appealing disclosure by DuPont scientists of two novel oxazolidinone antibacterial agents as clinical candidates, at the Interscience Conference on Antimicrobial Agents and Chemotherapy meeting, held in New York City. There, Andrew Slee and his co-workers[14] disclosed preclinical data on DuP-105 (**3**) and DuP-721 (**4**), two totally synthetic compounds having potent antibacterial activity against Gram-positive bacteria, including MRSA, and good pharmacokinetic (PK) properties in rodents that included high oral bioavailability. Ranger[20] has reviewed in significant detail the origin of these DuPont leads.



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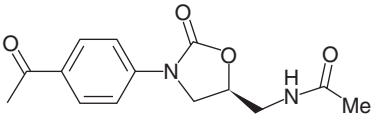

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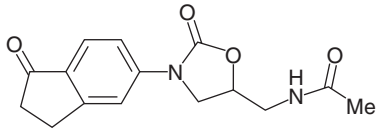
Intrigued by the attributes of these compounds, in particular the more active DuP-721, I soon thereafter initiated a small exploratory oxazolidinone project at Upjohn. This was sanctioned by a formal Upjohn company policy that allowed scientists to devote up to 10% of their time in efforts directed at exploring scientific pursuits of personal interest outside their principal project accountabilities. Working with my associates Peter Manninen and Debra Allwine née Ulanowicz, we began rapidly exploring a variety of novel oxazolidinones in racemic form. At this early project stage there were two principal biology laboratories at Upjohn engaged in the evaluation of antibiotic compounds, and I was able to easily gain their enthusiastic support in testing these new compounds. The microbiology laboratory, led by Gary Zurenko, and comprising his associates Rhonda Schaadt, Betty Yagi, and John Allison, evaluated our exploratory

compounds for in vitro antibacterial activity through the measurement of dipped-disk zones of inhibition, and the more standard minimum inhibitory concentration (MIC) determinations. Selected compounds with attractive in vitro potency were then submitted to the laboratory led by Charles Ford (with his associates Judith Hamel, Douglas Stapert, Judy Moerman, and Debbie Wilson), where they carried out the in vivo efficacy evaluations in a number of precedented mouse models of Gram-positive infections. Before long, we had discovered several proprietary and potent oxazolidinone series.[21–23]

8.13.4 Demonstration of a Structure–Toxicity Relationship: A Strategy for Lead Progression

Fairly early on in our exploratory project we had identified one particularly interesting compound that demonstrated very good in vitro potency and oral in vivo efficacy comparable to DuP-721, and was found to have similar PK properties in the rat. This was the indanone oxazolidinone (±)-U-82965 (**5**, PNU-82965),[24,25] one of two cyclic ketones we had targeted in order to explore the consequence of restricting the rotational conformers of the DuP-721 methyl ketone, via constraint in a five- or six-membered ring. Not long after U-82965 was in hand, we became aware of sketchy information that DuPont had dropped their oxazolidinone program, putatively on the basis of toxicological findings of their clinical candidates.[20,25] This surprising news obviously represented a very critical juncture for our project, for should we have been unable to rapidly identify an active oxazolidinone that could be differentiated from the DuPont lead (i.e., with a significantly improved toleration profile), our continued work in this area would likely be short lived.



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As will be seen, it would turn out to be most fortuitous that at that time we had identified U-82965. It was no less auspicious that we also happened to establish a working relationship with Richard Piper, an Upjohn pathologist, who offered excellent leadership and assistance in developing a strategy for potentially moving the project past this significant hurdle. Furthermore, we also benefited considerably from the expert opinion and guidance on this matter and one concerning the potential for oxazolidinone inhibition of monoamine oxidases, which was provided by our resident infectious diseases clinical expert, Donald Batts.

Piper graciously volunteered the efforts of his laboratory, staffed by his associates John Palmer and Thomas Platte; this was also enabled under the Upjohn 10% free-time policy. Piper's laboratory proceeded to design and carry out a protocol for an exploratory 1 month duration comparative toxicology study in the rat, with a side-by-side evaluation of (±)-U-82965 and (±)-DuP-721. John Greenfield conducted the PK determinations that provided decisive data supporting this comparative study, by establishing that both compounds had similar exposure levels upon oral administration in the rat.

The drug-sparing 30-day toxicology protocol designed by Piper required that my laboratory need only prepare 8–12 g of each test compound. The use of such limited drug quantities was feasible, as the protocol would involve the dosing of only three rats per sex. As the rationale of the study was to enable the expeditious identification of oxazolidinones having at least a 10-fold therapeutic index in the rat, the test compounds were dosed orally, twice daily (b.i.d.), at a dosage level 10-fold the ED_{50} (the effective dose ($mg\,kg^{-1}$) that protected 50% of the mice from death after an injection of a lethal dose of *S. aureus*). As the comparative study progressed, the toxicologists reported several distinct toxicological findings that were readily apparent by clinical observation in the group of rats dosed orally with (±)-DuP-721 at $100\,mg\,kg^{-1}\,day^{-1}$. Those findings included alopecia, severe anorexia, ataxia, and the death of one of the six animals; another two animals observed in a moribund state were euthanized prior to the end of the study. On histopathological examination there was evidence of bone marrow toxicity and terminal circulatory failure.[26] In contrast, we were elated to find that all six animals treated with U-82965 at the same dose fared very well; the compound was tolerated very well over the course of the 30 day trial.[26]

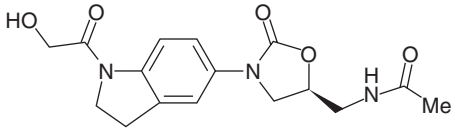
Following our filing of the Upjohn patent application on the promising U-82965 compound, we learned of a subsequently published DuPont US patent[27] (which had been filed earlier than our application) that claimed this indanone compound. Hence, while this was disappointing, we nevertheless had gained through the study of U-82965

invaluable insight and knowledge concerning the vastly different toleration in a rodent species of two closely related ketones – one cyclic, the other an acetophenone – which clearly established that an STR existed for this series.

The outcome of this study had a major impact on our project – one that would definitively play a significant role in our eventual successful discovery of eperezolid and linezolid. The importance of Piper's role in that success cannot be over-emphasized. First, a principal initial objective of the study was met in substantiating the rumored toxicological problems with the lead oxazolidinone. Second, the protocol had identified an equipotent analog of DuP-721 of close structural similarity with a clean toxicological profile. Third, Piper's study design had clearly demonstrated the utility of the drug-sparing protocol, thereby allowing us to plan on using this for expedited subsequent lead oxazolidinone toxicology evaluations. Finally, it shaped what would become our enabling strategy, allowing us to proceed. That strategy pivoted on the need to establish an understanding of STRs as a means of acquiring confidence in the selection of our advanced leads for further progression to drug candidate status. The necessity of conducting multiple, early, multiday toleration studies presented obvious additional hurdles for our team, obstacles that were considerably above those normally encountered in a typical SAR-driven program at that time. The strategy we instituted may arguably be one of the earliest of the few projects in the industry that succeeded in delivering a first-in-class drug to market, from a research program heavily reliant on early toxicological evaluation as a means to vet numerous, promising lead compounds.

Another series of compounds we focused on following the successful outcome with U-82965 would add substantially to our understanding of the STRs, and led to the identification of structural features that would eventually be incorporated into our drug candidates. We had chosen to examine various fused-ring heterocyclic oxazolidinone derivatives[22,23] for the explicit purpose of replacing the indanone ketone of U-82965, while retaining a five-membered benzo-fused ring. This interest included a series of active indazoles[22] and 5′-indolines, among others. With the principle of locating a carbonyl with an orientation proximate to that of the ketone in U-82965 or DuP-721, we prepared a series of amides attached to a 3-(5′-indolinyl)-5-acetamidomethyl-2-oxazolidinone core. This series of 5′-indolinyl amides[23] had superior activity to the isomeric 6′-indolinyl analogs.

Two of these active 5′-indoline amides, U-97456 (**6**) and U-85910 (**7**), were tolerated extremely well in the 30 day rat toxicology protocol.[28] Those results thereby established for the first time that a nitrogen atom substituted at the oxazolidinone phenyl *para* position could lead to compounds with improved toleration profiles. The thiophene amide **7** was of interest in that it was determined by James Kilburn and Suzanne Glickman at the Centers for Disease Control and Prevention (CDC) to have potent in vitro activity against *Mycobacterium tuberculosis*.[29] While the Gram-positive antibacterial potency of U-97456 was slightly below our targeted profile desired for a clinical candidate, the excellent safety profile of this compound and **7** laid the foundation for the eventual synthesis of many other oxazolidinone series similarly substituted with nitrogen-containing heterocycles at the *para*-phenyl position. This includes our first drug candidate, the piperazinyl fluorophenyl eperezolid, and the morpholinyl fluorophenyl analog linezolid.



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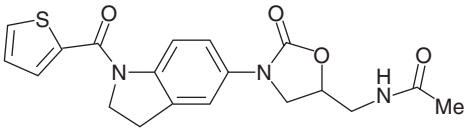

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U-97456 realized another important SAR finding. When the optimal *N*-hydroxyacetyl substituent found on U-97456 was incorporated into the piperazinyl fluorophenyl oxazolidinone (giving eperezolid), it also engendered very high potency, and excellent oral in vivo efficacy comparable to vancomycin subcutaneously (s.c.), as well as an excellent 30 day toleration profile. This same moiety has subsequently been singled out by other researchers as bringing about optimal activity in additional oxazolidinone and oxazolidinone-surrogate series (i.e., where other heterocycles replace the oxazolidinone).[30,30a,31,31a]

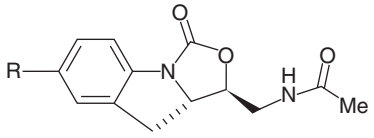
8.13.5 Insights Gained from Rigid Fused-Ring Oxazolidinones

An area of major interest in my laboratory involved a series of novel tricyclic-fused oxazolidinones. Contrary to prior SAR conclusions that had been reported in the literature,[24] we demonstrated that substitution could indeed be tolerated at both the *ortho*-phenyl and the oxazolidinone C-4 positions, provided these loci were connected with a short alkyl bridge in a *trans* orientation, relative to the 5-acetamidomethyl oxazolidinone side chain. My associate Peter Manninen initially synthesized the racemic *trans*-[6,5,5]-tricyclic-fused oxazolidinone analog (±)-**8a** corresponding to DuP-721.[32] Later,

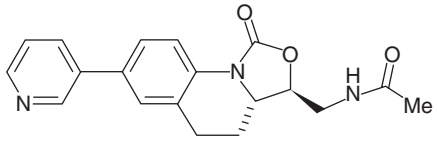
Mark Gleave, a postdoctoral researcher in my laboratory, carried out an asymmetric Sharpless epoxidation followed by an intramolecular version of the Manninen cyclization (vide infra), to give (+)-**8a** in 98.8% ee, and this compound demonstrated good potency, with only a twofold reduction in in vitro and in vivo activity compared with DuP-721.[33]

Based on a report from DuPont researchers[34] in which the methyl ketone of DuP-721 was replaced by aromatic or heteroaromatic rings to good effect, we proceeded to examine this type of substitution on our [6,5,5]-tricyclic oxazolidinones.[35] Many of those compounds demonstrated compelling in vitro Gram-positive activity. The most active derivative was the (±)-3-pyridyl-[6,5,5]-tricyclic-fused oxazolidinone U-92300 (**8b**),[25,35] which demonstrated excellent in vitro potency, and in vivo oral efficacy commensurate with vancomycin, dosed s.c. Upon advancement of this compound into the rat toxicology protocol, however, it was disappointing to find that U-92300 elicited toxic effects in the rat when dosed twice daily at 100 mg kg^{-1} day^{-1}. Thus, this particular example is illustrative of the value afforded the team in conducting these early multiday toxicology studies.

Based on the high potency of U-92300, Gleave[35] also went on to synthesize the racemic des-fluoro-tricyclic-fused version of linezolid **8c**. Surprisingly, compound **8c** was found to be 16- to 64-fold less active in vitro than linezolid. In a similar fashion, the complexities of correctly predicting the suitability of such rigid analogs will be also illustrated by the following example of earlier exploits at attempting to design more potent compounds, based on the hypothesis-driven modification of these rigid frameworks. On the basis of computational considerations of a conformational feature we believed could influence the activity of these tricyclic compounds, our colleague Douglas Rohrer had predicted that the corresponding ethylene-bridged [6,6,5]-tricyclic fused analogs would be more active, in that this structural motif more closely approached the three-dimensional structural arrangement of the two ring systems found in the lowest-energy conformation of DuP-721.[36] Debra Allwine completed the synthesis of the 3-pyridyl-[6,6,5]-tricyclic oxazolidinone **9**, corresponding to the highly potent homologue [6,5,5] analog **8b**, only for us to find that **9** demonstrated an eightfold reduction in in vitro activity relative to **8b**.[35] It was hypothesized that the disappointing weak activity of **9** could be indicative that the [6,6,5] template, with its larger central ring than that in the [6,5,5] congener, is not as well accommodated within the oxazolidinone binding site.



8a R = COMe
8b R = 3-pyridyl
8c R = morpholinyl



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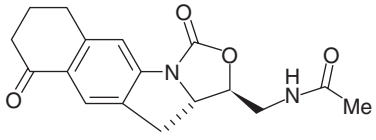
This hypothesis would be in keeping with generalizations put forth by Brittelli and co-workers,[24] which they derived from studies of multi-substitution about the oxazolidinone phenyl ring. From that work it was suggested that the oxazolidinone binding site was narrow, with limited space in what would be the general region of our tricyclic template central ring. They also noted that this narrow binding site appeared to possess a small pocket on one (undetermined) side of the linear axis (extending through the aryl and oxazolidinone rings) that could accommodate a small *meta* substituent.[24] We subsequently prepared a series of six different rigid [6,6,5,5]-tetracyclic-fused oxazolidinones,[25] which we considered might add further insights and refinement to this DuPont model of the (then completely unknown) binding site, by taking advantage of the fixed orientation of *meta* substituents affixed by nature of the rigidity of these templates. Examining the biological activity of the pairs of congeners, each designed to fit into one of the two possible *meta*-positioned clefts, we observed modest, but significant, differences in the measured in vitro activity favoring isomers such as **10**. These data best supported the positioning of the cleft in the DuPont binding site model at the *meta* position, which is proximal to the oxazolidinone carbonyl.



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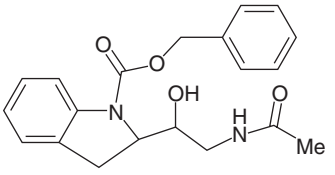
One of the several synthetic pathways we used to construct our various tricyclic-fused oxazolidinones would come to play an important role in finding a viable solution to the need for an alternative route to optically active oxazolidinones.

This chemistry involved a route to the racemic [6,5,5]-tricyclic oxazolidinone nucleus that was developed with a summer student, Kristine Lovasz. We demonstrated that in mixtures of the *threo* and *erythro* indolinyl benzylcarbamates **11**, having an appendant alcohol at C-2, only the *threo* isomer would rapidly (0.5 h) undergo smooth intramolecular cyclization by simply treating with the mild base K_2CO_3 in refluxing CH_3CN, giving *trans*-**12**. Reaction of the *threo* isomer required 24 h to cyclize to the *cis* tricyclic oxazolidinone.[35]



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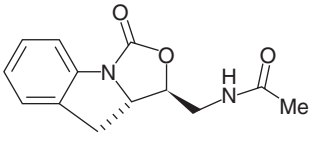

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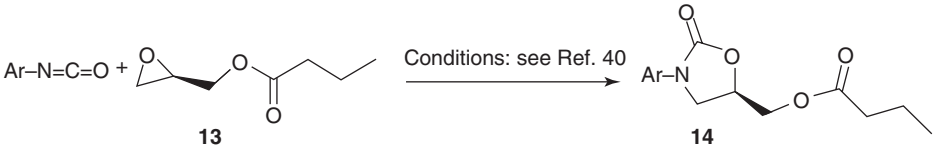
8.13.6 The Development of a Viable Synthesis of Oxazolidinones with High Enantiomeric Purity

At the beginning of our oxazolidinone exploratory project, we had elected to work with racemic compounds as a means of enabling the rapid SAR exploration and discovery of proprietary oxazolidinone series. The route we chose to the racemic oxazolidinones exploited an iodocyclocarbamation reaction, first developed by Fraser-Reid,[37] and subsequently employed by others[38,39] in a more closely related sense to our work. Our use of this iodine-mediated cyclization of *N*-allyl carbamates was the first to apply it to the preparation of 3-aryl- and 3-heteroaryl-oxazolidinones. For the heteroaryl analogs, we found addition of excess pyridine and iodine was needed to prevent an undesired side reaction caused by a Friedel–Crafts-type alkylation of the heteroaryl ring.[36] Overall, the iodocyclocarbamation served us well in allowing rapid generation of a very wide range of racemic novel aryl- and heteroaryl-oxazolidinones, and was used widely by our oxazolidinone chemistry team.

However, our success in finding compounds of great interest soon required us to identify a viable approach to the preparation of optically active 5-(*S*)-acetamidomethyl-2-oxazolidinones for our more extensive PK, pharmacological, and toxicological profiling of the best leads. Additionally, we would need a robust route to high optical purity material to support an investigational new drug application and manufacture clinical bulk supplies.

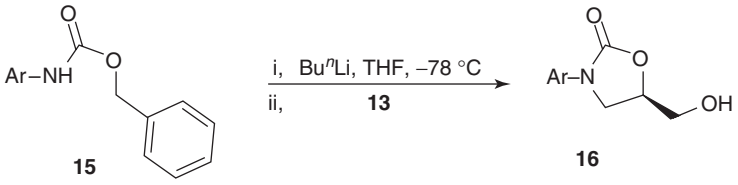
The DuPont group[40] had chosen to employ a cyclization that had first been described in a racemic fashion decades earlier,[41,42] and involved the high-temperature cyclization of an optically enriched epoxide, (*R*)-glycidyl butyrate (**13**), with an aryl isocyanate, and provided an oxazolidinone butyrate ester intermediate **14** that was used for the synthesis of DuP-721 (**Scheme 1**). For our purposes, we recognized there would be some substantial limitations to our use of this approach. For the broad variety of oxazolidinone series in which we had interest, that sequence would have required our in-house preparation of many noncommercially available isocyanates. This typically involves treatment of an aryl amine with phosgene, and is complicated by the low extent of conversion to the isocyanate, as the reaction is stalled by concomitant formation of the aniline hydrochloride salt. Our most overriding concern was the significant potential safety hazards represented by frequently working with substantial quantities of phosgene in a discovery research laboratory setting. On that basis, my laboratory began searching for a new approach in earnest.

In thinking about the broader utility of exploiting the carbamate as an internal acylating agent as seen in the tricyclic-fused work above, the general idea developed to an approach for a possible new enantioselective synthesis of 5-(*S*)-acetamidomethyl oxazolidinones. This was envisioned as involving deprotonation of an aryl-NHCBZ (**15**) with a suitable base, alkylation of the resulting carbamate anion with epoxide **13**, and then cyclization of the nascent alkoxide by closure onto the CBZ carbonyl. When I first attempted this sequence using NaH as the base, the reaction proceeded to afford a prolific mixture of products – it clearly appeared that it was time to go back to the drawing board. Unbeknownst to me, Peter Manninen had proceeded, completely on his own initiative, to get this failed transformation



13 **14**

Scheme 1



Scheme 2

to work. There followed a memorable 'eureka' moment in July 1992, when he brought forth the news that he had discovered the right conditions: the key was in using Bu^{n}Li as the base. We subsequently demonstrated that the presence of lithium was absolutely critical in this reaction.[43] As an added bonus, the product he isolated in very high yield was the 5-(*R*)-hydroxymethyl oxazolidinone **16**, not its butyrate ester **14** (as obtained in the isocyanate route). This was ideal, as the alcohol is a key intermediate for conversion to the active 5-(*S*)-acetamidomethyl oxazolidinones, and Manninen's reaction conditions provided this compound in very high enantiomeric excess, typically >99%.[44] The entire expanded chemistry team (vide infra) subsequently found the Manninen reaction was very reliable and general in scope, as has now been validated by its widespread use by many of the numerous researchers who have reported work in this field (Scheme 2).

This innovative contribution by Manninen had an invaluable impact on the success of our program, both in enabling the ready synthesis of oxazolidinones in high yield and optical purity, and by greatly facilitating the scale-up of initial multikilogram bulk quantities of eperezolid and linezolid. Having a viable route to the synthesis of a large range of optically active 3-aryl oxazolidinones accelerated not only our discovery efforts but also the entire time-line of progression to human trials for our two clinical candidates. It was gratifying to see that essentially the same route developed in our discovery laboratory[45] was used during the first 100 kg scale preparation of good manufacturing practices (GMP) clinical supplies of eperezolid and linezolid, carried out with only minor variations, by David Houser. After considerable efforts by a number of colleagues, the current production route for linezolid employs a more significant variation of the Manninen reaction, where instead of (*R*)-glycidyl butyrate, 2-(*S*)-3-chloro-1,2-propane-diol is employed.[28,46]

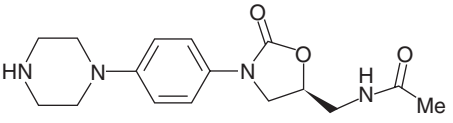
8.13.7 Formation of the Oxazolidinone Working Group

The successful outcomes of the U-82965, U-97456, and U-85910 toxicology studies (as well as the poor performance of U-92300), gave us considerable confidence in our ability to use the 30 day toxicology protocol to identify oxazolidinone series most worthy of continued pursuit – and those unworthy of advancement due to an unacceptably low therapeutic index. The establishment of this knowledge basis contributed directly to the eventual increased support of the oxazolidinone project with the allocation of additional chemistry and biology resources. In May of 1990, the chemistry laboratory headed by Douglas Hutchinson, and comprising his associates Raymond Reid and Stuart Garmon, later joined by Robert Reischer, was assigned to this project. In early 1991, Michael Barbachyn also joined the effort, along with his associates Kevin Grega and Dana Toops, and, later, Susan Hendges. With the tripling of the size of our original chemistry team, I was appointed the oxazolidinone chemistry team leader, and asked to chair a larger, interdisciplinary team of scientists called the 'Oxazolidinone Working Group.' The latter team added considerably more biology resources, to allow in-depth study of the then poorly understood mechanism of action (MOA), as well as other pharmacology of the oxazolidinones, and provide designated absorption, distribution, metabolism, excretion and toxicology (ADMET) evaluation resources. Robert Yancey's laboratory also helped profile the in vivo activity of the oxazolidinones.

The MOA studies were led initially by Keith Marotti, working with Jerry Buysse, and Dean Shinabarger, together with their associates William Demyan and Donna Dunyak. Following the PK evaluation of early compounds by John Greenfield, Mary Lou Sedlock and Robert Anstadt were added to the team in this capacity. Later, all PK and ADME work on this series was transferred to a group of colleagues led by Robert Ings at the Upjohn laboratories in Crawley, UK, including the laboratories of Martin Howard, Iain Martin, Peter Daley-Yates, Phil Jeffries, William Speed, Mark Ackland, and Neil Duncan. In Michigan, the toxicology studies on eperezolid were conducted by Richard Piper's laboratory, along with those of John Lund and Robert Denlinger; and colleagues in Upjohn's Tsukuba, Japan, laboratories would carry out the toxicological evaluation of linezolid. This latter group consisted of S Koike, H Miura, R Nakamura, and K Chiba, and James Moe.

8.13.8 Key Structure–Activity Relationship Refinements

Following the establishment of the Oxazolidinone Working Group, over the next two and a half years (leading up to the discovery of linezolid), the team identified a number of proprietary and safe oxazolidinone series. The Hutchinson laboratory initially focused on examining the effect of an additional methyl substitution at C-5, or on the C-5-acetamidomethyl side chain itself in the indanyl oxazolidinone template,[21] but both resulted in complete loss of activity.[25] This laboratory then proceeded to examine various heterocyclic amines appended at the *para*-phenyl position of the oxazolidinone, which included the piperazine **17**, homopiperazine, and pyrrolidine rings. The genesis of the idea to install the piperazine ring was inspired by several considerations[45]:



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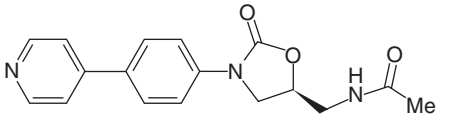
- the 3-(4-pyridyl)-phenyl oxazolidinone reported by DuPont, E-3709 (**18**),[47] had potent activity, and its pyridyl nitrogen was positioned in the general vicinity of the distal piperazine nitrogen;



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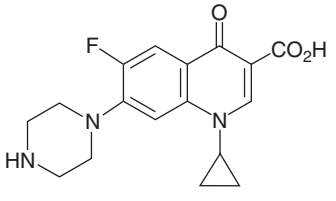
- the earlier demonstration within the 5′-indolinyl oxazolidinone series of the improved toleration profile of compounds having a nitrogen directly attached at the *para*–phenyl position; and
- a cognizance of the fluoroquinolone antibiotic SAR, where a piperazine moiety installed at the C-7 quinolone position resulted in the successful drug ciprofloxacin (**19**).[48]



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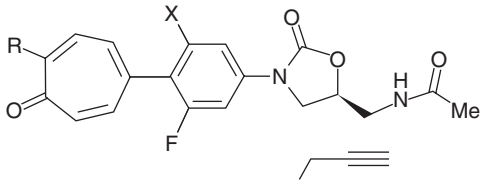
Later, following the identification of eperezolid and linezolid in early 1993, the chemistry laboratories of Mikio Taniguchi, Kiyotaka Munesada, and Hiroyoshi Yamada from Upjohn's laboratories in Tsukuba, Japan, would join the effort, and they focused on further exploration of the SARs of pyrrolidinyl and piperidinyl oxazolidinone derivatives.[49]

Another key SAR advancement was instituted in the Barbachyn laboratory. This concerned the demonstration that installation of one or two fluorine atoms substituted on the oxazolidinone phenyl ring improved in vitro activity and in vivo oral efficacy. The origin of this advance was also influenced by prior experience in the fluoroquinolone area, where addition of fluorine was known to have made substantial improvements in the antibacterial activity of the quinolones.[50] Barbachyn's team initially demonstrated this potentiating effect in a series of tropone-substituted mono- and difluorophenyl oxazolidinones.[51] The propargylamino-substituted (**20a**) and methoxy-substituted (**20b**) troponyl phenyl oxazolidinones with one or two fluorines, respectively, had an in vitro potency equal to or fourfold improved compared with vancomycin, and demonstrated equivalent efficacy to this benchmark antibiotic in vivo against *S. aureus* in a mouse model of infection. In contrast to these Upjohn findings was a report from DuPont[24] that described, for the case of DuP-721, addition of fluorine at the same *meta*-phenyl position resulting in a twofold reduction of activity.

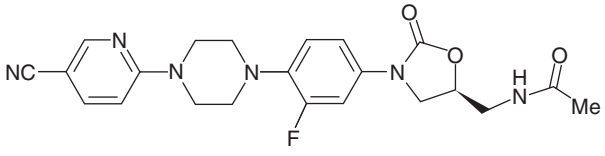


20a X = H, R = HN
20b X = F, R = OMe

8.13.9 The Identification of Eperezolid (U-100592), and Mechanism of Action Studies

In late 1992, our team decided to focus all of our SAR exploration efforts on only one of the three most interesting lead oxazolidinones series (i.e., 5′-indolines, piperazines, and tropones), in order to maximize our probability of success in identifying a drug candidate. After assessing the overall attributes and issues associated with each of the three series, the team decided to focus on the piperazinyl oxazolidinone series. While difluoro substitution in the troponyl and piperazinyl series did provide improved activity over the corresponding monofluorophenyl congeners, ultimately the team found that the piperazinyl monofluorophenyl oxazolidinone template provided the best overall profile of all properties desired for a drug candidate, including PK properties and solubility. As an example, for the series of analogs corresponding to eperezolid wherein there are no, one, or two fluorine atoms in the *meta*-phenyl positions, the solubilities in pH 7 phosphate buffer were 1.1, 4.2, and 2.6 mg mL^{-1}, respectively. As we desired both intravenous (i.v.) and oral dosage forms, the solubility advantage seen with the monofluorinated phenyl oxazolidinone was important, and facilitated the development of the i.v. formulation.

As we were interested in exploring a large number of substituents on the distal piperazine nitrogen atom, we planned for success, and requested a kilogram of the monofluorophenyl piperazine oxazolidinone template from our scale-up group, long before the first drug candidate, eperezolid, was identified. David Houser and the late Thomas Fleck delivered this sizeable quantity of oxazolidinone template in high optical purity and in a timely manner, taking advantage of the new Manninen process. In retrospect, this strategic decision significantly facilitated the rapid progression of the project. With this substantial quantity of template in hand, the discovery team chemists were more easily able to prepare the gram quantities needed for toleration and efficacy studies of several compounds in support of the selection of the first drug candidate. Following the identification of eperezolid and our realization of its overall promising properties, having this bulk material also enabled us to seek additional assistance. Here, we sought and received support from our chemistry management, Richard Thomas and Paul Aristoff, in requesting help from all available medicinal chemists at the research site to rapidly synthesize the numerous novel analogs that could be obtained from this template. One of the more fruitful new subseries of piperazines to come from this was proposed by our colleague John Tucker. He and the team went on to examine a large series of six-membered heteroaromatic rings directly appended to the piperazine (e.g., **21**), which had potent in vitro activity and in vivo efficacy comparable to linezolid.[52,52a]



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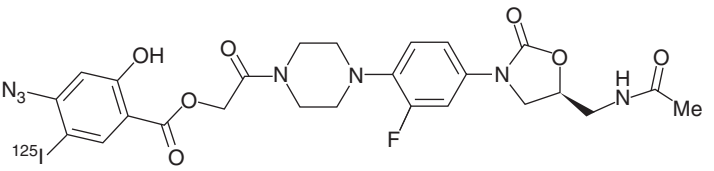
As noted, eperezolid (**2**) was the first Upjohn oxazolidinone drug candidate, a piperazinyl monofluorophenyl-5-acetamidomethyl oxazolidinone with a hydroxyacetyl moiety on the piperazine distal nitrogen.[45] While this is the identical group that had proved optimal in the 5′-indolinyl series (with U-97456), the sequence of events leading to the identification of eperezolid was somewhat more roundabout than may be obvious. At an early point in the piperazinyl project, the Hutchinson laboratory had installed the hydroxyacetyl group on the racemic, nonfluorinated piperazinyl phenyl oxazolidinone template. The resultant compound did not particularly distinguish itself from other analogs on the basis of its level of in vitro activity, and thus was not pursued further at that point. It was only later, after we had access to and exclusively worked with the optically active compounds (which provided a twofold increase in potency, as the 5-(*R*)-acetamidomethyl oxazolidinone enantiomers are inactive), and the monofluorophenyl template (which also elevated potency over the des-fluoro analog by twofold), that the beneficial properties of this moiety would become

evident. One of the piperazinyl substitutions my laboratory had prepared was that having an acetoxyacetyl group on the distal nitrogen. Upon evaluation by our biology colleagues in the Ford laboratory, this compound particularly stood out, demonstrating excellent oral in vivo activity, with an ED_{50} considerably better than its in vitro MICs would have predicted. Working on a hypothesis that it was likely this acetoxyacetyl could be functioning as an oral ester prodrug (i.e., undergoing esterase-mediated cleavage of the acetate in vivo to release the true active component), we immediately targeted the synthesis of the presumed ester cleavage product. As surmised, this hydroxyacetyl piperazine fluorophenyl oxazolidinone (U-100592) proved to be of outstanding interest, and went on to become our first drug candidate, eperezolid.

The Zurenko laboratory demonstrated that eperezolid had excellent antibacterial potency against all of the MDR Gram-positive strains of interest, including vancomycin-resistant enterococci.[53] Likewise, our colleagues in the Ford laboratory extensively evaluated this compound, and also determined that U-100592 had the properties we had been seeking for a drug candidate, with excellent oral efficacy in a number of models of sensitive and resistant Gram-positive infection.[54] Oral eperezolid performed very well in a model of infection with vancomycin-resistant *E. faecium* ($ED_{50} = 12.5\,mg\,kg^{-1}$) in immunocompromised mice, was more active than vancomycin (dosed s.c.) against MRSA, and showed exceptional oral activity ($ED_{50} = 2.0\,mg\,kg^{-1}$) against penicillin-and cephalosporin-resistant *Streptococcus pneumoniae*.[54] Eperezolid dosed orally was very well tolerated in 30 day toxicology studies in the rat and dog; for both species, the no-observed-adverse-effect-level (NOAEL) was $25\,mg\,kg^{-1}\,day^{-1}$.[55] Upon the endorsement of eperezolid as our first drug candidate in May 1994, the early clinical development team charged with the planning and executing of Phase I clinical trials was formed, and chaired by Susan Speziale.

As our first oxazolidinone clinical candidate, eperezolid would come to be the compound with which a number of important MOA studies were conducted, in an effort to add to a more detailed understanding of where the oxazolidinones bind on the bacterial ribosome. While DuPont scientists reported that the oxazolidinones had a unique MOA, inhibiting an early event in bacterial protein synthesis,[56] the details of the MOA were not fully understood. One particularly compelling aspect of the oxazolidinones was the difficulty with which resistant mutants could be raised in the laboratory. Using serial passages (20, over 7 weeks) of a sensitive *S. aureus* strain on a spiral gradient plater, Zurenko was able to raise a stable eperezolid-resistant mutant, which had utility in MOA studies.[57] Keith Marotti, Jerry Buysse, Dean Shinabarger, and their colleagues Robert Murray, Alice Lin, Earline Melchior, Steve Swaney, Donna Dunyak, and William Demyan along with Alex Mankin (University of Chicago), determined that the resistance was associated with a mutation in the peptidyl transferase region of 23S ribosomal RNA.[58] This was supported by their demonstration that eperezolid binds to the 50S ribosomal subunit at a site overlapping chloramphenicol and lincomycin.[59,60] Some of the most revealing mechanistic work would come later from this group working together with Lisa Thomasco, Jerry Colca and Robert Gadwood.[61] They prepared a radioactive photosensitive probe that was directly attached to eperezolid through its alcohol, to give **22**, and then photolyzed this compound in growing *S. aureus* cells. They determined that **22** cross-linked to several components, most importantly tRNA and the universally conserved nucleotide at position A-2602 (*E. coli* numbering) in the peptidyl transferase center of 23S rRNA. This work extensively advanced the understanding of the location of the oxazolidinone binding site on the 50S bacterial ribosomal subunit, suggesting that it is located in the vicinity of the ribosomal peptidyl transferase center near the P site, where the peptide bond is constructed by the ribosome. Additional work conducted later included nuclear magnetic resonance (NMR) studies by Brian Stockman and Casey Zhou,[62] who studied the binding of eperezolid to *E. coli* bacterial ribosomes, using ^1H NMR transferred nuclear Overhauser enhancement, and demonstrated that it bound only to the 50S ribosomal subunit, not the 30S.



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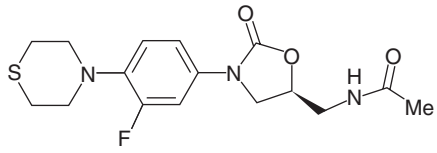
8.13.10 The Identification of Linezolid (U-100766) and U-100480, a Potent Antimycobacterial

Within the chemistry team, upon having consolidated all of our medicinal chemistry efforts on the piperazinyl series, one of the concepts that came forth from one of many brainstorming group sessions (Hutchinson and Barbachyn) was to

also examine isosteres of the piperazine ring, notably the morpholine and thiomorpholine rings. Prosecution of this work in my laboratory led directly to the morpholinyl compound linezolid (**1**, U-100766, PNU-100766),[45] and the interesting thiomorpholinyl oxazolidinone U-100480 (**23**, PNU-100480)[63] was made in the Barbachyn laboratory. An interesting aspect, from a discovery time-line perspective, is that the very first samples of eperezolid and linezolid were synthesized within only 2 days of each other, in the spring of 1993.



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U-100480 is notable due to its highly potent activity against *M. tuberculosis*.[63] U-100480, linezolid, and eperezolid were evaluated at the CDC by James Kilburn and Suzanne Glickman, and all were shown to have MICs of $<0.125\,\mu g\,mL^{-1}$ for *M. tuberculosis* H37Rv, comparing exceedingly well with the benchmark anti-TB drug isoniazid ($MIC = 0.2\,\mu g\,mL^{-1}$).[45,63] Against a panel of five drug-resistant TB strains, U-100480 was the most active of the three oxazolidinones, inhibiting in the 0.125–0.50 $\mu g\,mL^{-1}$ concentration range. Michael Cynamon and Sally Klemens at the Veterans Affairs Medical Center (and SUNY Syracuse) showed that a dose of $100\,mg\,kg^{-1}$ of U-100480 in a mouse model of TB infection had comparable efficacy to isoniazid at $25\,mg\,kg^{-1}$.[63,64] U-100480 was very well tolerated in a 30-day rat toxicology study.

Linezolid was evaluated by our biology colleagues, and was immediately recognized as another excellent oxazolidinone with very compelling antibacterial activity. The Zurenko and Ford laboratories demonstrated linezolid had excellent antibacterial potency against all of the MDR Gram-positive strains of interest, including vancomycin-resistant enterococci.[53,54] Overall, the in vitro activity of linezolid was slightly less than eperezolid. Like eperezolid, linezolid also performed very well in a model of infection with vancomycin-resistant *E. faecium* ($ED_{50} = 24\,mg\,kg^{-1}$) in immunocompromised mice, was equipotent with vancomycin (dosed s.c.) against MRSA, and showed exceptional oral activity ($ED_{50} = 2.7\,mg\,kg^{-1}$) against penicillin-and cephalosporin-resistant *S. pneumoniae*.[54]

Linezolid proved to have exceptional PK properties in the rat and in humans, compared with eperezolid; in the dog, the oral bioavailabilities for both compounds were very similar. Linezolid dosed orally was very well tolerated in 30 day toxicology studies in the rat and dog; the NOAEL was $20\,mg\,kg^{-1}\,day^{-1}$ for both species; and doses of 50 and $40\,mg\,kg^{-1}\,day^{-1}$ were well tolerated with only mild effects for the rat and dog, respectively.[65]

With the submission of two drug candidate investigational new drug applications came the need to propose nonproprietary names for these oxazolidinones to the US Adopted Name (USAN) Council. The early development team headed by Speziale met several times to brainstorm around deriving a suitable moniker for the stem that would represent the oxazolidinone pharmacophore core. As with the 'floxacin' core used with all fluoroquinolone drugs, we desired a truncated terminology that would easily identify by name that a compound was a member of the oxazolidinone class. The choice of 'ezolid' (as a variant of the 'azolid' portion of 'oxazolidinone') was sanctioned by the USAN Council. The suffixes were derived from components of the distinguishing structural features of the differing heterocycles (i.e., for 'eperezolid,' the suffix was derived from the piperazine moiety; for 'linezolid,' the suffix was formed from the morpholine substituent). Ranbaxy has recently reported on their Phase I oxazolidinone candidate RBX-7644 that is named ranbezolid,[66] employing the same stem.

Donald Batts and Charles Wajszczuk conducted the first Phase I clinical studies with eperezolid, and Steve Pawsey (at the Upjohn laboratories in Crawley, UK) the Phase I clinical studies with linezolid. Dennis Stalker, Greg Slatter, Gail Jungbluth, and their co-workers carried out the PK studies. The Phase I clinical trials with oral dosing of eperezolid commenced on 10 October, 1994 at the Jasper Clinic in Kalamazoo, just across the street from the research laboratories where the drug discovery had occurred. The linezolid oral formulation Phase I clinical trials began on 20 April, 1995 in the UK.

Some of the outstanding PK profile features of linezolid relative to eperezolid were its 100% oral bioavailability,[67] and the fact that it is not metabolized by cytochrome P450 (CYP) isozymes, nor does it inhibit or induce CYP enzymes.[68] The steady state PK data obtained with a 625 mg dose of linezolid given twice daily showed that blood serum levels exceeded the MIC_{90} for all of the targeted Gram-positive pathogens for the entire dosing period. As a result, the exposure obtained for linezolid in humans was considerably improved over eperezolid, and, on this basis, it was selected to proceed into Phase II clinical trials. Overall, the entire development time-line for linezolid was a relatively fast ~54 months from first-in-humans to New Drug Application submission.

Linezolid also has an excellent aqueous solubility of 3.7 mg mL^{-1}, which, like eperezolid, greatly facilitated the i.v. formulation development; as Zyvox, it is available as a 2.0 mg mL^{-1} solution. The PK characteristics have made the switch from i.v. to oral therapy with linezolid particularly easy for physicians, with no dosage adjustments necessary. This capability can provide advantages over competing agents. It also is advantageous in allowing patients continuing on oral therapy to leave the hospital earlier than if they were maintained on i.v. therapy, which can result in cost benefits.[69]

The editors explicitly requested that case histories be written as personal perspectives. In that respect, the most singularly fulfilling aspect of this work – one I am certain must surely resonate with my colleagues – has come from hearing the uplifting testimonials of patients who benefited from treatment with linezolid during the clinical trials, particularly those for whom all other therapies had failed. From the period late 1997 to mid-2000, several hundred clinical investigators enrolled over 700 particularly ill patients in open-label, compassionate-use basis trials with linezolid, for the treatment of significant, antibiotic-resistant Gram-positive bacterial infections. One of the more striking accounts to come from one of these studies concerned a very seriously ill infant struggling to survive an infection with an MDR strain of VRE. As the attending physician had exhausted all treatment options, the prospects for the patient's survival were dim. Emergency arrangements were made, with the FDA's approval, to acquire linezolid from Pharmacia & Upjohn in order to treat this child on a compassionate-use basis. Because an i.v. access could not be established at that point, the drug powder was formulated with saline and dosed orally. Within 48 h after initiation of linezolid therapy, the infant was alert and sitting up, and subsequently fully recovered.[70] The reported assessment of the overall cure rate with linezolid (dosed in adults at 600 mg b.i.d. for a minimum of 10 days) observed in the entire compassionate-use basis program was 90.5%.[71]

On April 17, 2000, the FDA approved linezolid for treatment of susceptible and resistant Gram-positive infections in an initial set of indications that subsequently was broadened following additional approvals. The indications currently approved in the USA are for the treatment of hospital- and community-acquired pneumonia caused by *S. aureus* (methicillin-susceptible or MRSA) or *S. pneumoniae* (penicillin-susceptible or MDR strains), and VRE *E. faecium* (including concurrent bacteremias). Linezolid is the only approved drug for treatment of hospital-acquired MDR *S. pneumoniae*, and the first oral agent ever approved for the treatment of VRE infections.[72,72a] Linezolid has also been approved for treatment of complicated skin and skin structure infections, including diabetic foot infections without concomitant osteomyelitis, which are caused by methicillin-susceptible *S. aureus* and MRSA, *S. pyogenes*, or *Streptococcus agalactiae*. Linezolid has been approved for use in children and newborns against Gram-positive infections.

In summary, for the foreseeable future, the significant medical need presented by growing multidrug resistance will remain the impetus to continue the search for new antibacterial agents. Novel drugs such as linezolid that have a unique MOA can avoid cross-resistance to agents already in use, and may slow the rate of resistance development. The FDA approval of linezolid was the culmination of a 12 year research program that faced significant hurdles with the need to establish STRs, as well as SARs. Linezolid has established itself as an important antibiotic for the treatment of susceptible and MDR Gram-positive infections in the practice of medicine.

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Biography

Steven J Brickner, PhD, is a Research Fellow in Antibacterials Chemistry, at Pfizer Global Research and Development in Groton, CT. He graduated from Miami University (OH) with a BS in chemistry with honors in 1976. He received his MS and PhD in organic chemistry from Cornell University (NY), and was an NIH Postdoctoral Research Fellow at the University of Wisconsin-Madison. Brickner is a medicinal chemist with over 20 years of research experience in the pharmaceutical industry, all focused on the discovery and development of novel antibacterial agents. He is an inventor/co-inventor on 21 US patents, and has published numerous scientific papers within the areas of oxazolidinones and novel azetidinones. Since 1997, Brickner has been a member of the Forum on Microbial Threats at the Institute of Medicine (National Academy of Sciences), and is a member of the Editorial Advisory Board for *Current Pharmaceutical Design*. He was named the 2002–03 Outstanding Alumni Lecturer, College of Arts and Science, Miami University, and was a co-recipient of the 2003 American Chemical Society's 31st Northeast Regional Industrial Innovation Award. Prior to joining Pfizer in 1996, he led a team at Pharmacia and Upjohn that discovered and developed Zyvox (linezolid).

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8.14 Copaxone

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8.14.1 Introduction

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) that leads to myelin destruction and axonal loss. It is the most common, nontraumatic, disabling neurological disorder in young adults. While the etiology of MS remains unknown, its pathogenesis involves autoimmune reactivity to various myelin antigens such as myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), and other myelin minor components. MS is often characterized by relapsing episodes of neurological impairment followed by remissions. This type of disease is termed relapsing-remitting (RR). In about one-third of MS patients this disease evolves into a progressive course, termed secondary progressive MS.[1]

Currently approved immunomodulatory therapies for the treatment of RR-MS include glatiramer acetate (GA; Copaxone) and the recombinant β-IFNs, Avonex (IFN-β1a), Rebif (IFN-β1a), and Betaseron (IFN-β1b). All modify the course of the disease, reduce the number of relapses, and slow the accumulation of disability.[2] The clinical efficacy

of the four drugs appears to be similar; however, GA is distinct from the IFNs as it is a more specific immunomodulator and also combines in its effect neuroprotection.[3] It also has milder side effects and better tolerability.

Copaxone (GA), formerly known as Copolymer 1 or Cop1, is a synthetic polymer of four amino acids L-alanine, L-lysine, L-glutamic acid, and L-tyrosine.[4] This is a novel and unique drug: it is the first drug based on antigen-specific suppression of an autoimmune disease.[5] It is also the first case in which a synthetic polymeric substance comprises the main ingredient of a drug.[6] We are familiar with the use of biopolymers, for packing a drug, for slow and controlled release, and for many other uses, but never as an active ingredient against a disease.

In the following we intend to describe the path of discovery of this drug and its development into a drug against RR-MS. We will discuss its mechanism of action, making it a prototype for therapeutic vaccines against autoimmune diseases.[7]

8.14.2 Preclinical Studies

8.14.2.1 The Beginning

It all began as basic research into the mechanisms involved in the induction and suppression of EAE, which is the primary animal model for MS. EAE is an acute neurological autoimmune disease, induced by the injection in complete Freund's adjuvant of brain- or spinal cord-derived substances which constitute the encephalitogenic antigens. These include several proteins such as MBP, PLP, MOG, and others. The disease is mediated by CD4+ autoreactive T cells, which recognize the encephalitogenic antigen(s) in association with major histocompatibility complex (MHC) class II molecules. These autoreactive cells migrate into the CNS and mediate the pathogenic process.[8] When we started our research in 1967, the only encephalitogenic material identified in the CNS was the MBP, and the only information available about it was its overall amino acid composition. Also the basic understanding of immunology and its role in EAE was in its infancy.

Our approach to the study of EAE was the synthetic one, using copolymers of amino acids. Research with amino acid polymers was pioneered by Ephraim Katchalski.[9] These synthetic protein-like molecules were shown to be useful models to study the structure–function relationship of proteins, the structural basis of antigenicity, and other immunological processes.[6,10] Employing these amino acid polymers, immune response to a large variety of antigenic determinants including nonprotein moieties such as sugars and lipids could be induced. Of special interest was the immune response to lipid components, which was not easy either to elicit or investigate because of solubility problems. However, conjugates, in which synthetic lipid compounds were attached on to synthetic copolymers of amino acids, elicited a specific response to lipids such as cytolipin H,[11] which is a tumor-associated glycolipid, or sphingomyelin.[12] Furthermore, we demonstrated that both the sugar and lipid components of such molecules contributed to their immunological specificity. The resultant antilipid antibodies were capable of detecting the corresponding lipids both in water-soluble systems and in their physiological milieu. This was fascinating because it gave us a glimpse into some disorders involving lipid-containing tissue and consequently led to our interest in demyelinating diseases, namely, disorders in which the myelin sheath, which constitutes the lipid-rich coating of all axons, is damaged, resulting in various neurological dysfunctions.

Our working hypothesis was that EAE induced by MBP might actually be caused by demyelinating antilipid immune response and that the positively charged MBP might serve only as a carrier for an acidic lipid (e.g., phospholipids). In order to test this hypothesis we synthesized several positively charged copolymers of amino acids whose composition resembled to a certain extent that of natural MBP, of which Cop1 was the simplest one, and tested their capacity to simulate the MBP ability to induce EAE. However, efforts over the course of more than a year led to negative results. None of these synthetic polymers possessed any encephalitogenic activity.[4] Furthermore, even the conjugation of sphingolipid moiety – which could potentially enhance the antisphingomyelin response and consequently the demyelination process – did not endow these polymers with any encephalitogenic activity whatsoever. At that time we became aware of the work of Elizabeth Roboz Einstein, who was among the first to show that MBP, its modifications as well as other nonencephalitogenic basic proteins, can inhibit EAE.[13] We proceeded therefore to test the copolymers for a possible inhibitory effect on the induction of EAE. The results of the inhibition experiments showed that all the tested copolymers (Cop1, Cop2, and Cop3) showed efficacy in suppressing EAE in guinea-pigs, the most active among the series being Cop1. Thus, we had started out by trying to design a molecule that could cause EAE and ended up with one that suppressed EAE. Over the next years the study of Cop1 has proceeded along two tracks: (1) its clinical development for the treatment of MS patients; and (2) the scientific research to understand how it affects the immune system. The latter advanced in parallel with the increased knowledge of immunology and the increased sophistication of the research tools and methods.[14]

8.14.2.2 The Chemistry of Copaxone

Copaxone (Copolymer 1) is a synthetic amino acid copolymer which is prepared by polymerization of the monomers, *N*-carboxy α-amino acid anhydrides. These anhydrides are obtained by reacting the respective amino acids with phosgene in dioxane. The anhydrides may be readily polymerized to form amino acid polymers.[9] The polymerization is usually carried out in inert solvent such as dioxane, in the presence of suitable initiator amines or strong bases. In this type of polymerization a growing chain always reacts with a monomer, leading to a narrow, Poissonian distribution of molecular weights.

The length of the polymer depends on the ratio between the monomer and the initiator, which is usually a primary or secondary amine. Keeping this ratio constant leads to high reproducibility of molecular size in different batches of the polymers. Furthermore, the rate of polymerization is an intrinsic property of the different *N*-carboxyanhydride derivatives, and hence, different samples of a polymer with the same composition of amino acids, although of random sequence in their nature, will be very similar in their physical and chemical properties.

In the case of Copolymer 1, the *N*-carboxyanhydrides used for polymerization were those of tyrosine, alanine, γ-benzyl glutamate, and ε, *N*-trifluoroacetyl lysine. The γ-benzyl and ε,*N*-trifluoroacetyl protective groups were deblocked after polymerization, yielding a water-soluble polymer with a residue molar ratio of 4.2 L-alanine : 3.4 L-lysine : 1.4- L-glutamic acid : 1.0 L-tyrosine. The original polymer was of average molecular weight 23 kDa.[4] Currently, the molecular weight range of the polymer constituting Copaxone and termed GA by the Food and Drug Administration (FDA) is of 4700–13 000 Da.

8.14.2.3 Studies in Experimental Animal Models

GA was demonstrated to suppress EAE induced by MBP in a variety of species: guinea-pigs, rabbits, mice, and two species of monkeys – rhesus monkeys and baboons. In contrast to rodents, where GA inhibits the onset of the disease, in primates it was treatment of the ongoing disease. A remarkable degree of suppression of EAE by GA was demonstrated in all species studied, even though different encephalitogenic determinants of MBP are involved in disease induction in the different species. Furthermore, GA was effective in suppressing the chronic relapsing EAE, a disease which shows a closer resemblance to MS, that can be induced by either spinal cord homogenate or encephalitogenic peptides derived from PLP and MOG.[5] Thus, the suppressive effect of GA in EAE is a general phenomenon and is not restricted to a particular species, disease type, or the encephalitogen used for EAE induction. More recent studies have demonstrated that, in addition to the parenteral route of administration used in all the studies described so far, oral administration of GA is also effective in suppressing EAE in rats, mice, and primates. Furthermore, oral GA was more effective than oral MBP in suppressing the disease.[15,16]

The suppressive effect of GA in EAE is a specific one, since GA lacked any suppressive effect on the immune response in several systems – humoral and cellular immune responses to a variety of antigens and vaccination against various induced infections. GA treatment also did not suppress other experimental autoimmune diseases, including myasthenia gravis, thyroiditis, diabetes, and systemic lupus erythematosus.[5,17] However, it has been reported to inhibit another autoimmune disorder, namely experimental uveoretinitis,[18] a disease interrelated with MBP and EAE. Recently, GA was also shown to be effective in the case of experimental colitis.[19] In addition, GA also had an effect on a murine model for graft-versus-host disease, as well as in three systems of graft rejection.[20]

The specific effect of GA in EAE may be explicable in terms of immunological specificity. Indeed, marked cross-reactivity was demonstrated between GA and MBP, both at the cellular and the humoral levels of the immune response. Thus, using monoclonal antibodies, we could demonstrate clearly that several monoclonal anti-MBP antibodies reacted with GA and vice versa.[21] At the cellular level, cross-reaction was observed both in vitro and in vivo.[22] Of interest is the very good correlation between the extent of cellular immunological cross-reactivity and the suppressive effect on EAE of various synthetic copolymers, and of particularly interest is the observation that a polymer resembling GA in all parameters, except that it is built of D-amino acids rather than L-amino acids, does not cross-react with MBP and has no EAE-suppressing activity whatsoever.[23]

8.14.3 Clinical Investigations

Several comprehensive review articles[24–28] dedicated almost exclusively to this subject have described in detail the various clinical trials that led to the approval of GA as a drug for the treatment of MS, and its evaluation. In the following we will relate to these clinical studies briefly and focus on additional findings that were reported more recently.

8.14.3.1 Early Clinical Trials

In view of the putative resemblance between EAE and MS[8] and based on the efficacy demonstrated by GA in suppressing EAE in all species including primates, both rhesus monkeys and baboons, the next step was testing it in MS patients. We first conducted some basic toxicological studies in our laboratory which included acute and subchronic administration to mice, rats, rabbits, and beagle dogs, uptake studies and Ames test (mutagenicity test). GA was found to be nontoxic and eligible for phase I clinical trial. Two early clinical trials were conducted, one in Israel[29] and the other in the US.[30] The former, in which only 4 patients participated, receiving the same, relatively low dose (2–3 mg, 2–3 times a week for 6 months), indicated possible slight improvement in disability, but mainly no apparent adverse affect of GA. The latter, conducted in 16 patients with RRMS or chronic progressive MS, was actually a phase I trial, using increasing dosage, and led to the definition of the optimal dose, 20 mg GA daily, administered subcutaneously. While efficacy could not be evaluated in this early trial, GA treatment was well tolerated in all patients, with no toxicity noted and no adverse effects recognized in the clinical disease.

8.14.3.2 Clinical Studies Leading to Food and Drug Administration Approval

8.14.3.2.1 Bornstein study

The results of the phase I trials paved the way for a phase II double-blind, randomized, placebo-controlled pilot trial conducted by Bornstein *et al*.[31] The whole trial was executed without the backup of a pharmaceutical company. It was a National Institutes of Health-supported trial and the GA batches used in this trial were prepared and characterized in our laboratory. The study involved 50 patients with RRMS treated for 2 years by daily subcutaneous injections of either 20 mg GA or placebo. The results demonstrated a remarkable effect on two primary outcome measures: (1) the relapse rate (75% reduction); and (2) the proportion of relapse-free patients.

8.14.3.2.2 Phase III studies

Following the publication of these results, in 1985 TEVA Pharmaceutical Industries of Israel licensed the rights to produce and market GA. TEVA undertook a drug development program and started producing the copolymer in a chemically defined manner with consistent performance in bioassays. This substance was used in two phase III clinical studies. The first was an open-label trial, involving 271 patients conducted in four medical centers in Israel.[32] The clinical results obtained were similar to those reported in the double-blind phase II trial, namely 73% reduction in relapse rate. Since this was an open-label study, the results could not be used for regulatory purposes. Another double-blind phase III clinical trial was required to get FDA approval. To this end, a multicenter study involving 11 centers in the USA and 251 patients was designed in which patients were treated with either 20 mg GA or placebo for 2 years (core study). Results at 24 months[33] showed 29% reduction relative to placebo in relapse rate (the primary endpoint) in favor of GA ($P = 0.007$). The original core study has been extended for a totally blinded and placebo-controlled observation period up to 35 months.[34] By the end of this phase there was a 32% reduction in mean relapse rate. Secondary endpoint results showed that the proportion of patients improved by $\geqslant 1$ expanded disability status (EDSS) steps from entry favored GA (27.2% versus 12.0%; $P = 0.001$) and the mean EDSS score improved by -0.11 in the GA group and worsened by $+0.34$ in the placebo group ($P = 0006$). On the basis of the above-described results, the FDA approved GA (Copaxone) for the treatment of patients with RRMS. Copaxone is now approved in 44 countries worldwide, including the US, Canada, Australia, Israel, and all the European countries.

8.14.3.3 Recent Clinical Studies

8.14.3.3.1 Open-label extension of the American phase III trial

The American phase III trial had an additional phase – an open-label extension in which patients who received placebo crossed over to active treatment with GA and patients who received GA during the double-blind phase continued to receive GA. The open-label extension phase is ongoing and now in its 13th year, and data are available from the 6-, 8-, and 10-years time points.[35–37] The annualized relapsed rate of patients treated from the beginning of the study dropped each year and was 0.23 in the sixth year compared to a 1.52 pretrial relapse rate. This low rate was also maintained after 8 and 10 years of GA treatment. In patients who were on placebo and switched to GA, although their relapse rate was significantly higher during the placebo controlled phase, it began to equalize to that of the GA group in the third year. However, EDSS analysis showed that mean EDSS levels during 10 years increased from randomization by 0.48 steps for patients always on GA and 0.8 steps for those switching from placebo to active treatment (**Figure 1**). In addition, comparing the proportion of patients with confirmed disability progression showed that patients treated

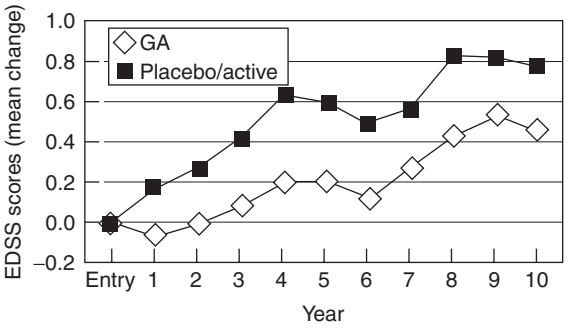

Figure 1 Yearly EDSS change from randomization for patients always on GA versus placebo/active patients. Yearly data are derived from the number of patients starting each specific year. (Adapted from Ford, C.; Johnson, K.; Brooks, B.; Goodman, A.; Kachuck, N.; Lisak, R.; Myers, L. W.; Panich, H. S.; Pruitt, A.; Rizzo, M. *et al. Mult. Scler.* **2003**, *9*, S120.)

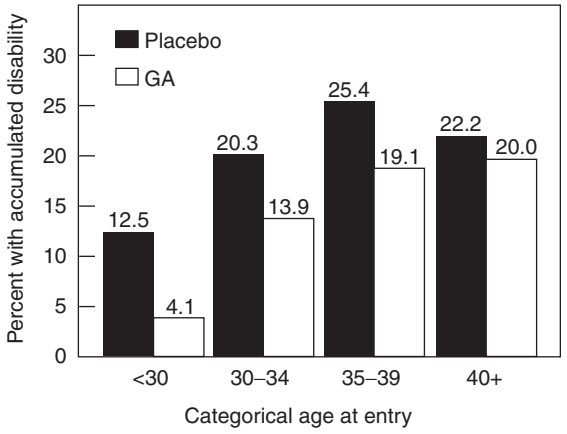

Figure 2 Effect of age at initiation of GA therapy on accumulated disability in RRMS. Patients were stratified by age roughly into quartiles and the proportion with progression defined as $\geqslant 1$ unit change in EDSS sustained $\geqslant 90$ days calculated for each age category. (Reproduced from Wolinsky, J. S. *Exp. Opin. Pharmacother.* **2004**, *5*, 75–891.)

with GA since randomization had significantly lower risk to progress than those taking placebo during the double-blind phase of the trial.[37] It may thus be concluded that GA continues to be effective and safe after a decade of use in a large proportion of RRMS patients. The results validate the importance of long-term compliance with GA therapy, and they also support the growing conviction that early and extended treatment offers the best outcomes in RRMS.[28]

8.14.3.3.2 Meta-analysis of the double-blind, placebo-controlled clinical trials

A meta-analysis using pooled data from 540 patients in three randomized, double-blind, placebo-controlled trials was recently published.[38] It was designed to investigate whether the treatment effect varied according to disease-related variables at baseline. Regression models were developed to estimate the annualized relapse rate, total number of on-trial relapses, and time to first relapse. Also explored were the effect of GA on accumulated disability and the potential role of clinical variables as predictors of relapse rate and treatment efficacy. There was a 28% reduction in the average annualized relapse rate in the GA-treated group; a 36% reduction in on-trial relapses occurred in the GA group. Drug assignment ($P = 0.04$), baseline EDSS score ($P = 0.02$), and the number of relapses during the 2 years prior to study entry ($P = 0.002$) were significant predictors of on-trial relapse rate. A beneficial effect of GA on slowing accumulated disability was also found (risk ratio: 0.6; $P = 0.02$).[28] In GA-treated patients the time to confirmed progression was also doubled when compared to placebo patients (ratio estimate 1.88; $P = 0.02$). Two other factors unfavorably influencing the accumulation of disability were the number of on-trial relapses and patients' age (Figure 2). The analysis suggests that the risk of accumulating new disability increases with age and that this risk is best curtailed by the early initiation of GA therapy. This finding underscores the importance of early initiation of MS therapy to maximize its effectiveness.

8.14.3.3.3 Magnetic resonance imaging studies

MRI offers a noninvasive estimate of some of the pathological changes that are ongoing within the CNS. Preliminary retrospective or single-center studies reported a reduction in the frequency of gadolinium (Gd)-enhancing lesions in GA-treated patients compared to treatment-naive subjects. MRI evaluation made 6 years after randomization in the open-label extension phase of the US trial showed that the odds of finding Gd-enhancing lesion were 2.5 times higher for the group originally randomized to placebo compared with the GA group.[28]

A large European–Canadian multicenter double-blind, placebo-controlled study was conducted specifically to address the onset, magnitude, and durability of the effect of GA on MRI-monitored disease activity.[39]

For the primary outcome measure, patients in the GA-treated group demonstrated 29% fewer Gd-enhancing CNS lesions (areas of acute inflammation representing disruption of the blood–brain barrier) than patients in the placebo group. For secondary MRI outcomes, GA showed significantly greater lesion reductions (ranging from 30% to 82.6%) than placebo. Although this 9-month trial period was considered too short to demonstrate a significant reduction in the volume of hypointense T1 lesions (representing areas of demyelination and axonal loss), further analysis of these scans has shown that, after 8 months, the proportion of new T2 lesions evolving into these hypointense T1 lesions (black holes) in patients receiving GA was half that shown in patients receiving placebo (P value < 0.002). These results are of significance since black holes are indicators of more severe and permanent tissue disruption and strong correlations have been found between the extent of black holes in the brain and MS-related disability. A recent post hoc analysis of this trial showed some nonsignificiant reduction in brain tissue loss in the GA group during the double-blind phase, which became significantly lower in the open-label phase for patients treated with GA since randomization.[40]

8.14.3.3.4 Study of primary progressive multiple sclerosis

In addition to the phase II clinical trial in RRMS, Bornstein and co-workers[41] also conducted a double-blind trial, in two centers, in New York and Texas, which included 106 patients suffering from chronic progressive MS. The primary outcome measure of this trial was confirmed progression of disability by full-grade change in the EDSS. Out of 23 patients that fulfilled this criterion, 9 were in the GA-treated group and 14 in the placebo group, which did not manifest a statistically significant difference. Progression rates at 12 and 24 months were higher for the placebo group, with a 2-year probability of 29.5% compared to 20.4% for the treated groups ($P = 0.088$). The difference in the 2-year progression of 0.5 EDSS units ($P = 0.03$) was significant.

The patients in this trial categorized as chronic progressive included patients that now would have been considered to have secondary progressive as well as primary progressive disease types.

A double-blind, placebo-controlled study of primary progressive MS patients was started in 1999 in the US, France, and the UK. More than 900 patients in more than 50 centers participated in this trial, that was given the code name Promise. The primary endpoint of the study was to determine whether GA slows confirmed disease progression in the absence of relapses. A data safety-monitoring committee for the trial in interim analysis concluded that it was improbable that the study would reach statistical significance. All patients were taken off study medication in an organized fashion and offered entry into a natural history study. A full intent-to-treat analysis of all trial data is in progress.[28]

8.14.3.3.5 Oral study

Studies in laboratory models of EAE showed that oral administration of GA is effective in suppressing EAE in both rodents and primates in acute as well as chronic relapsing disease.[15,16] In view of these results a double-blind and placebo-controlled study was initiated in RRMS patients. In this trial, given the code name Coral, two doses of oral GA, 5 and 50 mg respectively, or placebo, were given daily for 14 months, to 1650 MS patients enrolled from 158 sites in 18 different countries. The results of this trial showed that, even though the drug was safe when administered as an oral formulation, it failed to show clinical or MRI evidence of an effect at either dose. It is not clear whether the discrepancy between the animal model and humans reflects some aspect of trial design, e.g., dosing, formulation, or site of drug release. A limited clinical trial testing a high dose of oral GA is now in progress.

8.14.3.3.6 Comparative studies

The relative efficacy of the disease-modifying therapies currently approved for use in RRMS, the three β-IFNs and GA, is a matter of great interest. Direct randomized controlled trials comparing these agents pose substantial methodological logistical and cost problems and so far no such trials have been performed. Few open-label prospective and retrospective observational studies have been conducted[28] that suggest differences in efficacy among the different treatments and somewhat larger treatment responses for GA. A study that just appeared[42] describes a 24-month

comparison of immunomodulatory treatments – a retrospective open-label study in 308 patients treated with β-IFNs or GA. The reduction of relapse rate was significantly higher for patients treated with GA compared with all β-IFNs (-0.71, $P < 0.001$). In addition, the discontinuation rate within 24 months was significantly lower for GA (8.9% versus 24%).

8.14.3.4 Safety and Tolerability

Safety data accumulated from >3500 MS patients treated with GA in controlled and uncontrolled studies indicate withdrawal from therapy for adverse experience in 8.4%.[28] The most frequent reasons recorded for treatment withdrawals were dyspnea and vasodilation (2% for each). The most commonly reported adverse experiences are local injection site reactions which generally decline over time. They consist of erythema, pain, inflammation, pruritus, and swelling, but no skin necrosis. Localized lipoathrophy occurs in some areas after ⩾1 year of GA treatment.

Approximately 10–15% of GA-treated patients report a postinjection systemic reaction that includes flushing, chest tightness, palpitations, dyspnea, tachycardia, and anxiety. Symptoms were generally transient and resolved spontaneously without sequelae. Controlled studies demonstrated that GA does not provoke hematological abnormalities, elevation of hepatic enzymes, flu-like symptoms, depression, or abnormalities of blood pressure.

It may thus be concluded that GA has a favorable side-effect profile, with excellent patient compliance and long-term acceptance of therapy. Based on the above it was concluded in several review articles that GA is a valuable first-line treatment option for RRMS patients.[26,28]

8.14.4 The Immunopharmacology of Glatiramer Acetate

Extensive studies conducted during the last decade in both the animal model of EAE and in humans have demonstrated several immunological properties of GA and elucidated its mechanism of action. These studies were recently summarized in several review articles.[20,25,27,43] In the following we will relate to these studies briefly and describe more recent findings.

8.14.4.1 Immunological Properties of Glatiramer Acetate

Several immunological properties of GA are thought to contribute to the effects of GA.

8.14.4.1.1 Binding to major histocompatibility complex molecules

GA exhibits a very rapid, high, and efficient binding to many different MHC class II haplotypes on living murine and human antigen-presenting cells (APCs).[44] GA was also shown to interact with purified human leukocyte antigen (HLA)-DR molecules – DR1, DR2, and DR4 – with high affinity.[45] As a result of its high and efficient binding to MHC class II molecules, GA is capable of competing for binding with MBP and their myelin associated proteins, such as PLP and MOG, and even displace them from the MHC binding site.

8.14.4.1.2 Inhibition of T-cell responses by glatiramer acetate

It has been demonstrated that GA can competitively inhibit the immune response to MBP of diverse MBP-specific murine and human T-cell lines (TCLs) and clones, which have different MHC restrictions and respond to different epitopes of MBP.[46–48] GA also inhibited the response of TCLs reactive with PLP and MOG peptides. These results suggest that the observed inhibition was due to competition between GA and nominal antigen for the MHC peptide-binding site. This mechanism may be less specific, and indeed GA was shown also to inhibit in vitro some other immune responses.[48,49] In addition to the relatively nonspecific MHC-blocking, GA was shown to inhibit the response to the immunodominant epitope of MBP peptide 82–100 in a strictly antigen-specific manner by acting as T-cell receptor (TCR) antagonist.[50]

8.14.4.1.3 Induction of antigen-specific T-regulatory cells

In vivo studies have demonstrated that GA-treated animals (either by subcutaneous injections or by oral administration) develop GA-specific T suppressor (Ts) cells in the peripheral immune system. These cells can adoptively transfer protection against EAE.[15,51] Furthermore, Ts cell hybridomas and lines that inhibited EAE in vivo could be isolated from spleen cells of mice rendered unresponsive to EAE by GA.[52] These Ts cells were characterized

as Th2/3-type cells secreting anti-inflammatory cytokines such as interleukin (IL)-4, IL10, and transforming growth factor (TGFβ), but not Th1 cytokines, in response to both GA and MBP. Other myelin antigens such as PLP, MOG and αβ crystalline could not activate the GA Ts cells to secrete Th2 cytokines. Yet the disease induced by PLP and MOG can be suppressed by these Ts cells, probably due to a bystander suppression mechanism.[53,54] More recently, it has been demonstrated that these GA-specific Th2 suppressor T cells which were induced in the periphery by either injection or oral treatment accumulate in the brain.[55,56]

The GA-specific cells accumulated in the CNS demonstrated in situ extensive expression of the anti-inflammatory cytokines IL10 and TGFβ and the brain-derived neurotrophic factor (BDNF), but not the inflammatory cytokine IFN-γ. Furthermore, the GA-induced cells infiltrating the brain induced bystander expression of IL10 and TGFβ by resident astrocytes and microglia.[57] These findings clearly indicate that the GA-specific cells which penetrate the CNS function in vivo as regulatory cells and mediate the therapeutic effect of GA in the target organ.

It was recently suggested that, in addition to the induction of GA-specific Th2 cells, GA also led to the conversion of CD4 + CD25– T cells to CD4 + CD25 + regulatory T cells through activation of transcription factor Foxp3.[58] The induction of Foxp3 by GA was mediated through its ability to produce IFN-γ and, to a lesser extent, TGFβ These findings were demonstrated both in MS patients treated with GA and in wild-type B6 mice, but not in IFN-γ knockout mice.

8.14.4.1.4 Effect of glatiramer acetate on antigen-presenting cells

Several groups have recently reported on the effects of GA on various types of APC. Thus GA blocked lipopolysaccharide-mediated induction of several activation markers of human monocytes and the release of tumor necrosis factor (TNF-α) and IL12. On the other hand, it induced increased production of IL10 by the monocytes.[59,60] Similarly, GA inhibited production of IL2 and TNF-α by in vitro-generated human dendritic cells (DC). DC exposed to GA induced IL4-secreting Th2 cells and enhanced the level of IL10.[61] There is also evidence that GA treatment modifies in vivo the properties of APC. Thus, the spontaneous and triggered release of IL10 was enhanced in monocytes from GA-treated patients whereas the stimulated secretion of IL12 was reduced.[60] It is not clear, however, whether in vivo GA affects the monocytes directly or indirectly by TH2 cytokines secreted by GA-specific T cells. It seems that APC deviation into APC favoring Th2 differentiation may be an additional contributing factor to the therapeutic effect of GA.

8.14.4.1.5 Neuroprotective effects of glatiramer acetate

Recent studies have revealed an additional aspect of GA activity – neuroprotective effects that might also be relevant to MS. It was demonstrated that, similarly to MBP, active immunization with GA as well as adoptive transfer of T cells reactive to GA can inhibit the progression of secondary degeneration after crush injury of the rat optic nerve.[62] The GA-specific T cells secreted significant amounts of BDNF,[62] a neurotrophin that plays a major role in neuronal survival. Furthermore, vaccination with GA protected neurons against glutamate cytotoxicity,[63] and aggregated beta-amyloid-induced toxicity.[64]

GA treatment also increased survival time and improved motor function in a mouse model of amyotrophic lateral sclerosis.[65] Adoptive transfer of GA-specific T cells was effective in protecting dopaminergic neurons in a mouse model of Parkinson disease.[66] Taken together, these results show that GA may have neuroprotective functions in human neurodegenerative diseases.

Several lines of evidence suggest that GA also has a neuroprotective effect in EAE and MS. The effect of GA was studied in MOG-induced EAE, which is considered to be a model that simulates neurodegeneration more than inflammation.[67] It was demonstrated that GA immunization attenuates both inflammation and associated neuronal axonal damage. In the murine model of Theiler's virus-induced demyelinating disease, it was demonstrated that polyreactive antibodies to GA promoted myelin repair.[68]

As indicated before, we have demonstrated that adoptively transferred GA reactive cells migrate to the CNS and also produce in situ BDNF in addition to anti-inflammatory cytokines.[57] In this regard it should be noted that the BDNF receptor trkB is expressed in neurons and astrocytes in MS lesions.[69] Therefore, BDNF secreted by GA-specific cells in the CNS could exert neurotrophic effects directly in the MS target tissue.

Human GA-specific T cells, of both TH1 and Th2 type, are capable of producing BDNF.[70] Studying BDNF production by 73 GA and 33 MBP reactive short-term TCLs, it was found that the mean BDNF level for the GA cell lines was significantly higher than that for MBP lines.[71]

There are also limited clinical data pointing to the neuroprotective effects of GA therapy. Thus, in the European Canadian MRI study, it was demonstrated that GA produced a 50% reduction in the proportion of new MS lesions evolving into persistent black holes[72] (i.e., lesions where severe tissue disruption has occurred). In another study, *N*-acetylaspartate (NAA), which is a reliable marker of neuronal and axonal injury, was measured using magnetic

resonance spectroscopy.[73] In patients treated with GA for 2 years, the ratio of NAA to creatinine increased from 1.96 at baseline to 2.17, while it declined from 2.01 to 1.83 in untreated patients. These results, as well as the results described earlier on the effect of GA on brain atrophy,[40] indicate that GA affects positively three different MRI surrogate markers of neuroprotection (black holes, NAA level, and brain atrophy).

8.14.4.2 Immunological Effects of Glatiramer Acetate in Multiple Sclerosis Patients

8.14.4.2.1 Antibody response

Evaluation of the immunological responses to GA in MS patients revealed that all patients treated with GA developed anti-GA antibodies, whereas placebo-treated patients were negative.[74] The antibody level peaked at 3 months after initiation of treatment and reached a level of 8–20-fold above baseline. It decreased at 6 months and remained low. The anti-GA-reactive antibodies were of immunoglobulin G (IgG) type, with IgG_1 levels two- to threefold higher than those of IgG_2 at all time points examined. IgG4 anti-GA antibodies were also shown to be frequently produced.[75] The anti-GA antibodies did not interfere with the GA activity in vitro – they did not inhibit its binding to MHC molecules and T-cell stimulation, nor did they inhibit the Th2 cytokine secretion of a human GA-specific clone. Furthermore, the patients' sera with the highest GA antibody titer did not affect at all the capacity of GA to block EAE symptoms.[76]

Most significantly, these anti-GA antibodies are nonneutralizing and they do not interfere at all with the therapeutic effect of GA, nor do they correlate with the reported side-effects of GA. Moreover, relapse-free patients at 18 and 24 months of therapy had significantly higher antibody titer.[74]

In another study 42 patients who were treated with GA for 1–5 years were tested for anti-GA antibodies and their blocking effects. Six serum samples had an inhibitory effect in vitro on GA-induced proliferation of GA-specific TCLs.[77] Although these results are not conclusive they warrant further studies in long-term GA-treated patients.

8.14.4.2.2 T-cell response to glatiramer acetate in naive multiple sclerosis patients

Several studies have demonstrated the presence of GA-reactive T cells in peripheral blood mononuclear cells (PBMC) of both untreated MS patients and normal individuals.[74,78–80] The proliferative response to GA in naive MS and normal individuals could be inhibited by anti-DR but not anti-DQ antibodies.[74,79] Another study reports that class I restricted T cells are also involved in this reactivity.[81] These results indicate that the proliferation induced by GA is mediated by the TCR and is MHC-restricted. Thus, there is compelling evidence that GA is recognized as a conventional antigen and not as a mitogen or superantigen.

8.14.4.2.3 T-cell response to glatiramer acetate in treated multiple sclerosis patients

The proliferative responses to GA, MBP, and purified protein derivative of tuberculin (PPD) were followed up for 2 years in 86 patients participating in the phase III open-label study in Israel.[74] Following an initial, slight increase, the response to GA was markedly and gradually reduced as a function of time in the trial. On the other hand, the response to the nonrelevant antigen, PPD which was high at baseline, did not change during the trial. Recent results from several research groups[79,80–82] confirm these observations. The decline in the proliferative response to GA may reflect an antigen-induced cell death due to the repetitive stimulations, energy, or a shift to a Th2 type of response, as discussed herewith.

Different lines of evidence suggest that GA treatment induces a shift from Th1 to Th2 response: (1) such a shift is indicated by the pattern of the anti-GA antibody isotypes, namely, higher IgG1 than IgG2[74] and production of IgG4[75]; (2) treatment of MS patients with GA led to an elevation of TGFβ, IL10, and suppression of TNF-α mRNA from PBMC[83]; and (3) recent reports by several groups on short-term and long-term GA-specific TCLs demonstrate that TCLs from untreated MS patients and healthy controls are predominantly of the Th1 type, secreting IFN-γ and TNF-α. On the other hand, TCLs derived from GA-treated patients are predominantly Th2 cells, secreting IL4, IL5, and IL13.[79,80,82,84]

Using an automated enzyme-linked immunospot (ELISPOT) assay it was demonstrated that there is increase of GA-reactive T cells producing IL4 or IFN-γ The elevated IFN-γ response was partially mediated by CD8+ T cells after stimulation with very high concentrations of GA.[80] The induction of these IFN-γ-secreting cells in the periphery seems to correlate with a positive clinical response.[85] This was recently corroborated in a study demonstrating that, whereas GA-induced CD4+ T-cell responses are comparable in healthy individuals and MS patients, CD8+ T cells are significantly lower in untreated MS patients. Treatment with GA resulted in upregulation of these CD8+ responses with restoration to levels observed in healthy individuals.[86] Both CD4+ and CD8+ GA-specific responses are HLA-restricted.

It has been shown that the Th2-biased immunological response to GA is sustained over long-term treatment (6–9 years) and is partially cross-reactive with MBP and MBP 83–99, as measured by proliferation and cytokine release assays.[87] It was also demonstrated that, with increasing duration of treatment, the surviving GA-reactive T cells become more degenerate and respond to an increasing number of components from a combinatorial peptide library.[79] However, this response still led to the secretion of Th2 cytokines. Treatment with GA also results in increased apoptosis of a substantial percentage of activated CD69+ CD4+ T cells.[88]

The cross-reactivity between GA and MBP demonstrated before in animal studies was confirmed in the human system.[82,84,88] Thus, it was shown in many cells lines that GA and MBP cross-stimulate human T cells at the level of cytokine secretion. It was also reported that two GA-specific TCL could be stimulated to produce IFN-γ with another myelin antigen MOG.[84]

It is unknown whether GA-specific T cells migrate into the CNS in treated MS patients, but such an effect was clearly demonstrated in the EAE system in mice. Furthermore, human Th1 as well as Th2 GA-reactive T cells were able to migrate across an artificial blood barrier in vitro.[89]

8.14.4.2.4 Proposed mechanism of action of glatiramer acetate-specific immunomodulation

As emerges from the cumulative experimental results, GA affects MS at various levels of the immune response involved, which differ in their degree of specificity. Its binding to the MHC class II molecules, which is the least specific step, is a prerequisite for its effect by any mechanism. Following this interaction, three mechanisms were clearly shown to be effective:

1. GA can compete for binding to MHC class II with several myelin-associated antigens, e.g., MBP, PLP, MOG, and αβ-crystallin, resulting in inhibition of antigen-specific T-cell effector functions (i.e., proliferation, interleukin secretion, and cytotoxicity). This mechanism is by its nature antigen-nonspecific, as MHC blockage may also lead to interference with other immune responses, depending on the strength of TCR MHC/peptide engagement.

2. TCR antagonism and competition at the level of TCR between the complex of MBP-derived peptides with class II MHC antigen, and the complex of GA with class II antigen. This is a specific mechanism since it involves interaction with a specific TCR. By engaging the specific TCR, GA can also act as altered peptide ligand and induce energy or Th2 shift of the pathogenic T cells.

The two above activities, however, do not necessarily play an essential role in the modulation of MS since GA is degraded in the periphery and thus it is not likely to reach the CNS and compete with the relevant myelin antigens in situ.

3. GA binding to the relevant MHC leads to peripheral activation of the regulatory/suppressor cells, which are activated by shared suppressive determinants between MBP and GA, to secrete Th2-suppressive cytokines. These GA-specific Th2 cross the blood–brain barrier. Local reactivation of these cells in the brain by MBP stimulates the release of anti-inflammatory cytokines which downregulate the autoaggressive response to MBP as well as to other myelin antigens (e.g., PLP and MOG), which are colocalized with MBP, due to bystander suppression (**Figure 3**). It is currently believed that Th2-regulatory T cells play a major role in the mode of action of GA, and bystander suppression is a central element in it.

In addition to the in situ release of anti-inflammatory cytokines, the GA-specific Th2 cells were also shown to release BDNF in the CNS, which may explain the neuroprotective effects recently attributed to GA.

Other mechanisms recently suggested for GA, such as induction of APC favoring Th2 differentiation of T cells, or induction of CD4+CD25+ regulatory T cells, may also contribute to the mechanism of GA activity.

8.14.5 Concluding Remarks

This review article describes the path of the development of Copaxone (GA, Copolymer 1) from basic research and clinical studies to the bedside. It is a synthetic polymer of amino acids, and has a specific effect on the immune process involved in EAE and MS.

As for the chemistry angle of this drug, it is of interest that Copaxone is the first drug of a polymeric nature approved for treatment of disease. It is a macromolecular preparation obtained by polymeric techniques, in which probably no two molecules are completely identical. The macromolecular nature of GA, combined with its microheterogeneity, could actually contribute to its effectiveness by leading to its binding to MHC class II of many genetic backgrounds.

It is worth mentioning that GA is a drug that is effective against MS, probably because of its chemical and immunological resemblance to MBP. Indeed, GA can be considered the prototype of an autoantigen-directed, autoantigen-derived selective agent. This illustrates the concept of specificity in treating autoimmune diseases, similarly to vaccines against infectious diseases, where nobody expects to have one vaccine against all diseases.

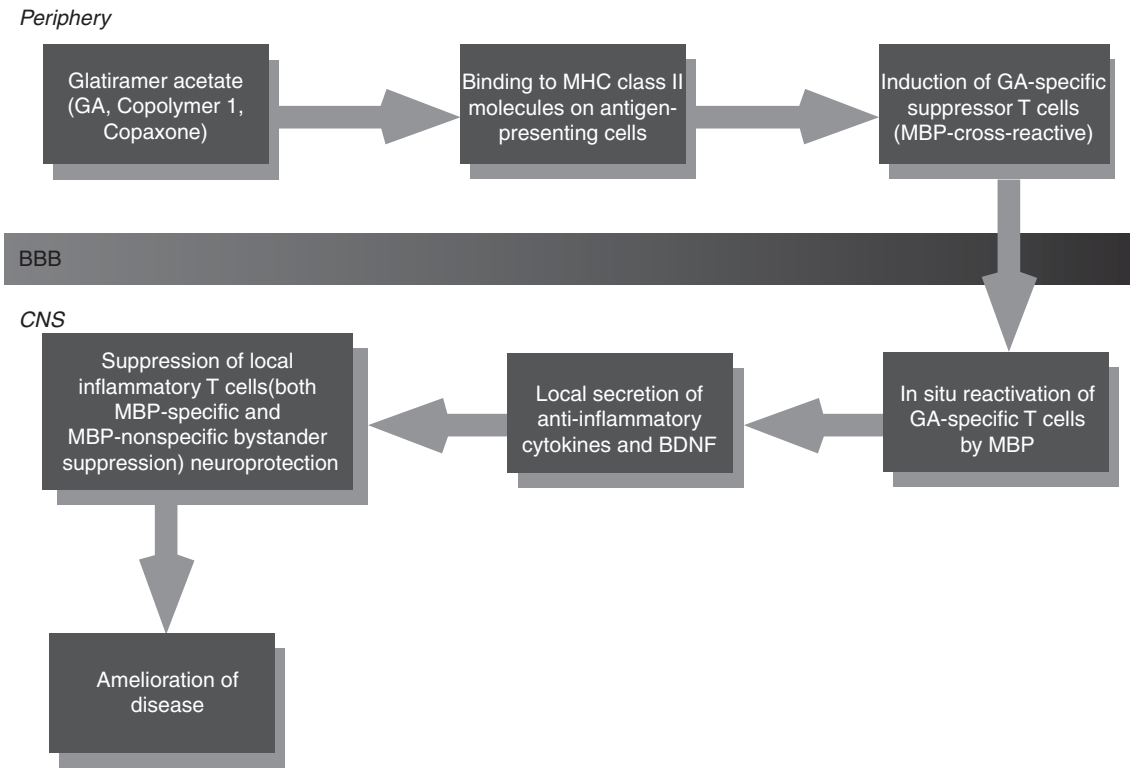

Figure 3 Mechanism of action of glatiramer acetate (GA, Copaxone, Copolymer 1) in EAE and MS.

GA is thought to act by inducing a population of regulatory (Th2-type) T cells that migrate to inflammatory sites in the CNS, where they are activated by cross-reacting myelin antigens to exert their beneficial bystander effect.

In addition to bystander suppression, GA may also confer neuroprotection, as indicated by both animal and human studies. The latter activity may be of relevance for both MS and for neurodegenerative diseases. Furthermore, in accord with its capacity to induce Th1 to Th2 shift, GA has recently been shown to have an ameliorating effect in a few additional autoimmune disorders as well.

As illustrated in this review, the number of publications describing studies on GA, whether in experimental animal models or clinical studies, in vitro and in vivo, grew exponentially in recent years, and it is hoped that in the not too distant future we shall understand even better both the mechanism of action of this drug, and, most importantly, we shall be able to evaluate the long-term impact and health improvement of the MS patient.

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8.15 Ritonavir and Lopinavir/Ritonavir

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8.15.1 Introduction

In the 25 years since the recognition of acquired immunodeficiency syndrome (AIDS) and the subsequent identification of its causative pathogen, the human immunodeficiency virus (HIV), the pandemic from the most deadly infectious disease in human history has grown to monumental proportions. According to recent estimates, nearly 40 million people worldwide are infected with HIV, with 4.9 million new infections and 3.1 million AIDS-related deaths in 2004.[1] The epidemic is particularly acute in sub-Saharan Africa but is also growing at alarming rates in portions of southern and eastern Asia. Along with the expansion of the epidemic has come an extraordinary effort toward the identification of chemotherapeutics to treat HIV infection, and the success of highly active antiretroviral therapy (HAART) based on combinations of these agents ranks as one of the hallmarks of modern rational drug discovery. This is especially evident in the discovery of HIV protease inhibitors providing an example of progression from identification of a molecular therapeutic target to the approval of drugs that changed the course of the epidemic in the developed world in a few short years. This chapter outlines key observations and inventions leading to two currently used protease inhibitors, ritonavir and lopinavir/ritonavir.

8.15.2 Design of Symmetry-Based Human Immunodeficiency Virus Protease Inhibitors

The discovery of HIV protease inhibitors began with one of the first applications of modern genomics in medicine. Shortly after the isolation and identification of HIV as the causative agent for AIDS, its entire retroviral genome was sequenced and homology modeling[2] revealed a genetic motif (Asp-Thr-Gly) suggestive of an aspartic proteinase (HIV protease). HIV encodes its structural and enzymatic proteins in the *gag* and *pol* genes, respectively, and translation provides large *gag* and *gag–pol* polyproteins that require specific proteolytic processing by HIV protease for production of mature viral particles. Early experiments demonstrated the essential nature of HIV protease in the viral replication cycle by showing that viral mutants in which the protease was disabled by mutations in the catalytic aspartic acid, assembled and were released from cells but retained an immature morphology and were rendered completely noninfectious.[3] Unlike higher organisms, HIV, like other lentiviruses, encodes only half of the amino acids required for a functional aspartic protease molecule (including only one of two essential catalytic Asp-Thr-Gly triads common to all proteases of this class). Formation of a catalytically active enzyme requires homodimerization to a complex containing a single active site. The presumed C_2-symmetric structure of this active site, later confirmed in crystallographic studies,[4] became one of the defining features of the inhibitor series leading to ritonavir and lopinavir.

The initial design of HIV protease inhibitors was widely influenced by efforts in the early 1980s toward discovering inhibitors of the human aspartic protease renin, an enzyme involved in the processing of angiotensinogen to the potent pressor molecule angiotensin I.[5] Extensive studies had established that noncleavable peptide isosteres substituted into

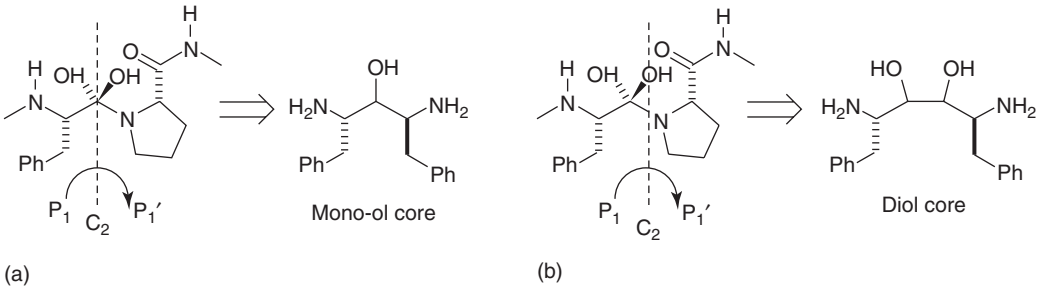

Figure 1 Design of C_2-symmetric HIV protease inhibitor cores. (a) Axis of symmetry placed through the carbonyl carbon; (b) axis of symmetry placed through the middle of the carbon–nitrogen bond undergoing proteolytic cleavage. (Reprinted with permission from Kempf, D. J.; Norbeck, D. W.; Codacovi, L.; Wang, X. C.; Kohlbrenner, W. E.; Wideburg, N. E.; Paul, D. A.; Knigge, M. F.; Vasavanonda, S.; Craig-Kennard, A. *J. Med. Chem.* **1990**, *33*, 2687–2689 © American Chemical Society.)

the substrate cleavage site sequence led to potent, reversible renin inhibitors. Key to the potency of most inhibitors were the tetrahedral geometry of the isosteric peptide replacement, which presumably reflected the geometry of the transition state leading to the tetrahedral intermediate formed by enzyme-catalyzed addition of a molecule of water across the peptide bond, and a secondary hydroxyl group capable of both donating and accepting a hydrogen bond to and from the catalytic aspartate residues at the center of the active site. Following the identification and characterization of a protease in the HIV genome, these isosteric strategies were widely applied to the various HIV protease substrate sequences, and in many cases led to potent inhibitors.[6] The highly symmetric structure of HIV protease provided additional opportunity for innovation (**Figure 1**). In a three-step conceptual process, an axis of symmetry was overlaid on the tetrahedral intermediate for cleavage of an asymmetric peptide substrate, the C-terminal half of that substrate was removed, and the N-terminal half was duplicated and rotated around the C_2-axis to produce 'N-to-N' configured, symmetric, or pseudosymmetric mono-ol and diol core fragments.[7] Placement of one or two hydroxyl groups completed the unique symmetry-based dipeptide isosteres. Elongation of these symmetric cores with P2 and P3 amino acids produced potent inhibitors that bound to the HIV protease active site in a highly symmetric or nearly symmetric orientation.[8] Corresponding 'C-to-C' core fragments also provided unique inhibitors, as did replacement of the secondary hydroxyl group(s) with other functionalities (e.g., difluoroketone) capable of strong hydrogen bonding interactions with the catalytic aspartates.[9]

8.15.3 Structure–Activity Studies and the Struggle with Oral Bioavailability

Like renin and a number of other nonviral aspartic proteinases, the active site of HIV protease is lined principally with hydrophobic amino acids and encompasses approximately six amino acids (subsites P3 to P3′). Initial structure–activity relationship (SAR) studies with symmetry-based inhibitors indicated that the highest binding affinity could be achieved when all six subsites were occupied with hydrophobic amino acid side chains. Consequently, early inhibitor structures were large, hydrophobic, and insoluble. It is therefore unsurprising that the major hurdle in all HIV protease inhibitor discovery programs was not the identification of potent inhibitor structures, but the modulation of structure to introduce sufficient aqueous solubility and other properties to achieve good oral bioavailability. In the case of symmetry-based inhibitors, an early x-ray crystal structure of prototype inhibitor A-74704 (**Figure 2**) revealed that the terminal phenyl groups were solvent-exposed.[8] Substitution with pyridyl groups greatly improved solubility and in one case (2-pyridyl substitution) produced a compound with approximately 20% oral bioavailability in rats.[10] The potency of this inhibitor derived from the 'mono-ol' core (**Figure 1**) was approximately 10-fold lower than those derived from the three diasteromeric diol core structures. Unfortunately, similar 2-pyridyl substitution in the diol series produced compounds that, while highly potent, displayed uniformly poor bioavailability. However, because no convenient animal efficacy model of HIV infection was available, a representative from this inhibitor series, A-77003 (**Figure 2**), with substantially improved aqueous solubility, was advanced into the initial studies in HIV-infected volunteers in a proof-of-concept study.[11] In vitro, A-77003 inhibited a variety of laboratory and clinical HIV isolates, including an AZT-resistant strain, with EC_{50} values of 300 nM or less.[10] In humans, A-77003 was administered by continuous intravenous infusion at one of four doses, and antiviral efficacy was monitored by changes in HIV-1 p24 antigen. Importantly, the clearance of A-77003 in humans was found to be exceedingly high (average 62 L h^{-1}), and target plasma concentrations based on the EC_{50} in HIV tissue culture assays could only be maintained at the highest dose, which produced severe infusion site phlebitis. The concentrations did not exceed the EC_{90}, and no evidence of antiviral activity was observed.[11] On this basis, further studies on A-77003 were abandoned.

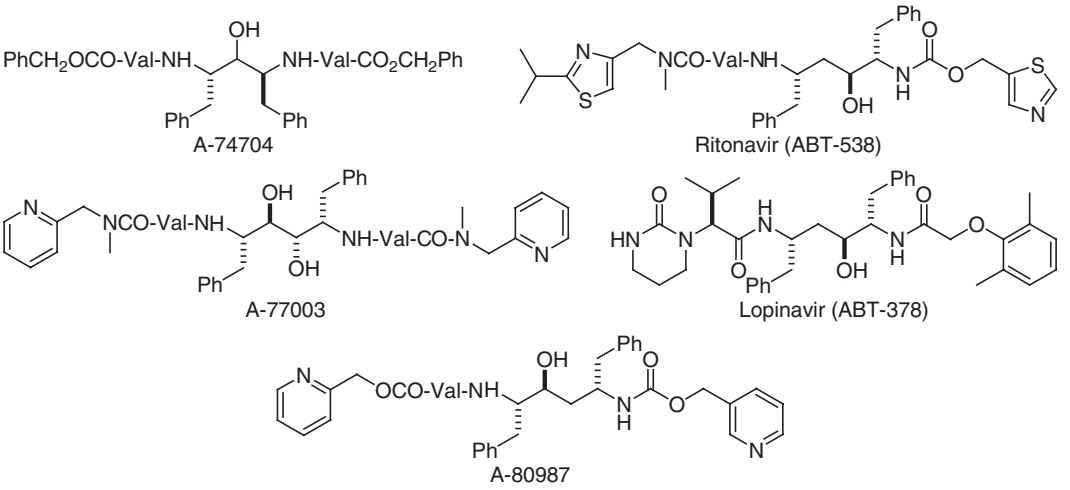

Figure 2 Structures of symmetry-based HIV protease inhibitors A-74704, A-77003, A-80987, ritonavir (ABT-538), and lopinavir (ABT-378).

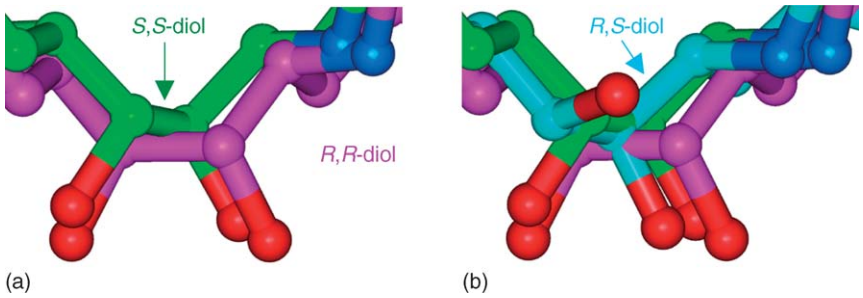

Figure 3 Binding of diastereomeric diols in the HIV protease active site. (a) Overlap of the central portions of *S*,*S*-diol (A-76928, green) and *R*,*R*-diol (A-76889, magenta). Hydroxyl groups (red) are projected down toward the catalytic aspartates. (b) Overlap of the above inhibitors with the *R*,*S*-diol (A-77003, blue), showing the shift from symmetry to allow the *R*-hydroxyl group to interact with both aspartates. (Reprinted with permission from Hosur, M. V.; Bhat, T. N.; Kempf, D. J.; Baldwin, E. T.; Liu, B.; Gulnik, S.; Wideburg, N. E.; Norbeck, D. W.; Appelt, K.; Erickson, J. W. *J. Am. Chem. Soc.* **1994**, *116*, 847–855 © American Chemical Society.)

Although the proof-of-concept study with A-77003 was unsuccessful in demonstrating antiviral activity, this endeavor, along with other preclinical results with this series of inhibitors, provided valuable insights. Most notably, careful analysis of the x-ray crystal structures of A-77003 (*R*,*S*-diol core) and its two diasteromers (A-76889, *R*,*R*-diol and A-76928, *S*,*S*-diol) bound to HIV protease revealed three different modes of binding.[12] Whereas the *R*,*R*-diol adopted one gauche orientation across the carbon–carbon bond adjacent to the central carbon–carbon bond, the *S*,*S*-diol assumed the alternate gauche orientation, which allowed both isomers to project both hydroxyl groups toward the two enzyme aspartate residues (**Figure 3a**). Interestingly, while the *S*,*S*-diasteromer A-76928 bound perfectly symmetrically in the active site, the *R*,*R*-diol A-76889 was slightly shifted from a symmetric position. The *R*,*S*-diol A-77003 adopted a gauche orientation similar to the *R*,*R*-diol but was observed to shift even more from a symmetric binding mode (approximately one-half bond length) to allow the *R*-hydroxyl group to lie on the symmetry axis and hydrogen bond to both catalytic aspartate residues (**Figure 3b**). Because of the relatively weak interaction of the *S*-hydroxyl group of this isomer with enzyme active site residues, removal of this hydroxyl to provide the 'deoxy-diol' core analog (A-78791) resulted in increased affinity compared to A-77003. The deoxy-diol bound in an identical fashion to the *R*,*S*-diol, shifted in position from a purely symmetrical orientation with respect to the axis of symmetry of the enzyme.[12]

The implications of the above structural observations to the ongoing discovery process for this series were several fold. First, it was apparent from both structural analysis and ongoing SAR studies that the carbon framework constituting the diol cores placed adjoining amino acids groups in an optimal position for binding the P2–P3 and P2′–P3′ subsites, compared to the 'mono-ol' core (**Figure 1**). This observation is consistent with the fact that in the asymmetric substrates of HIV protease, amino acid α-carbons are separated by two atoms. Second, the asymmetric orientation of the *R*,*S*-diol and deoxy-diol with respect to the enzyme C_2-axis suggested that the contributions of

adjacent groups attached to the two ends of these core groups may differ, a prediction borne out by the activities of pairs of inhibitors functionalized with nonidentical adjacent acyl groups. Most importantly, the increased potency of the deoxy-diol core, compared to the diols, allowed the investigation of truncated compounds containing functionality binding to only five subsites (P3–P2′ or P2–P3′) rather than six.[13] Optimization of the initial series of truncated inhibitors provided A-80987 (Figure 2), with similar in vitro potency to the longer inhibitor A-77003. Importantly, whereas the oral bioavailability of A-77003 in rats was 0.7%,[10] A-80987 provided significant plasma levels in rats and dogs after oral dosing (26% and 23% bioavailability, respectively).[14] A-80987 was the second compound in this series to be advanced to human studies and, while demonstrating oral bioavailability in HIV-infected subjects, still displayed high clearance due to rapid metabolism.

8.15.4 The Discovery of Ritonavir (Norvir)

A significant improvement on the rapid clearance of A-80987 was achieved in subsequent SAR studies. In vitro metabolism studies in human liver microsomes indicated that N-oxidation of the pyridyl groups of both A-77003 and A-80987 occurred rapidly to produce the major metabolites. Systematic studies in which each of the two pyridyl groups were independently replaced with thiazolyl groups, which were more stable to oxidation, suggested that further improvements in the pharmacokinetics of A-80987 were possible. Coincidentally, alkyl groups on the P3-thiazolyl group were shown to improve in vitro potency.[14] Subsequent crystallography studies revealed a hydrophobic contact between this alkyl group and the side chain of the valine at position 82 in the enzyme active site.[15] Combining the above observations led to the discovery of ritonavir (ABT-538), which represented a substantial improvement over A-80987.[14] Thus, in MT4 cells (an immortalized T-cell-derived cell line amenable to HIV infection) the average EC_{50} of ritonavir against a panel of typical wild-type laboratory strains of HIV was 23 nM (approximately 10-fold more potent than A-80987 and A-77003). Furthermore, the oral bioavailability of ritonavir in rats, dogs, and monkeys exceeded 70%, and plasma levels remained above the in vitro EC_{50} for >6–8 h after a 10 mg kg^{-1} oral dose in all three species. On the basis of these attributes, ritonavir was advanced into human trials, and in single-dose studies, plasma concentrations >14-fold higher than those observed with A-80987 were observed.[14]

Significant advances in the synthetic routes to this series of symmetry-based HIV protease inhibitors allowed the clinical examination of the above three inhibitors. The initial synthesis of the protected diol core proceeded via a cumbersome McMurray pinacol coupling of Boc-phenylalaninal to give a mixture of *R*,*R*-, *S*,*S*-, and *R*,*S*-isomers, which were subsequently separated and identified.[7] Significant improvements were realized using a Pedersen coupling of Cbz-phenylalaninal, which produced almost exclusively the *R*,*R*-diol isomer.[16] Selective protection of one hydroxyl group and activation of the other as the corresponding mesylate, followed by stereochemical inversion via internal cyclization of one of the Cbz carbonyl groups provided the *R*,*S*-diol core required for A-77003 (Figure 4). Activation with α-acetoxyisobutyryl bromide produced the corresponding inverted bromoacetate, which could be debrominated to yield the deoxy-diol core. Although this route enabled the discovery of A-80987 and ritonavir, both of which contain this core unit, it was clearly too inefficient for production of supplies for toxicological and clinical studies. A key improvement of the synthesis was realized in a sequence in which the enaminoketone intermediate derived from the sequential addition of acetonitrile anion and benzyl Grignard to protected phenylalanine benzyl ester was stereoselectively reduced in a one-pot set of reactions (initial 1,4-reduction with sodium borohydride/methanesulfonic acid followed by carbonyl reduction with sodium trifluoroacetoxyborohydride) to the protected deoxy-diol core.[17] This synthesis has been scaled up to produce metric tons of both ritonavir and lopinavir, the latter of which also contains this common symmetry-based core.

Initial single-dose studies of ritonavir in healthy human volunteers confirmed its excellent pharmacokinetic profile, and four doses of ritonavir were studied as monotherapy in HIV-infected subjects.[18] In stark contrast to A-77003 and A-80987, plasma HIV RNA (initially measured with a relatively insensitive assay with a 10 000 copies mL^{-1} lower limit of quantitation) immediately plummeted upon the initiation of therapy. The rapid decline in viral load was unprecedented in clinical studies of antiretroviral agents (which up to that point consisted primarily of nucleoside reverse transcriptase inhibitors), and enabled the first quantitative estimates of HIV production and turnover in vivo.[19] The startling results, which indicated that, on average, 1–10 billion HIV particles per day are produced in an untreated, infected individual, overturned the prevailing notion of a 'latent' phase of HIV infection prior to the appearance of the opportunistic infections that define AIDS as a syndrome. These findings also began to redefine the goal of antiretroviral therapy to not only delay the progression of symptomatic AIDS, but to lower viral load to undetectable levels.

Subsequently, the clinical efficacy of ritonavir was established in an innovative phase III study. In a double-blind, placebo-controlled study, ritonavir was added to existing standard-of-care therapy (consisting of combinations of nucleosides) in individuals at high risk for developing AIDS (the median CD4 level in this patient population was 18 cells mm^{-3}). Within a few months, ritonavir treatment was shown to produce a highly statistically significant

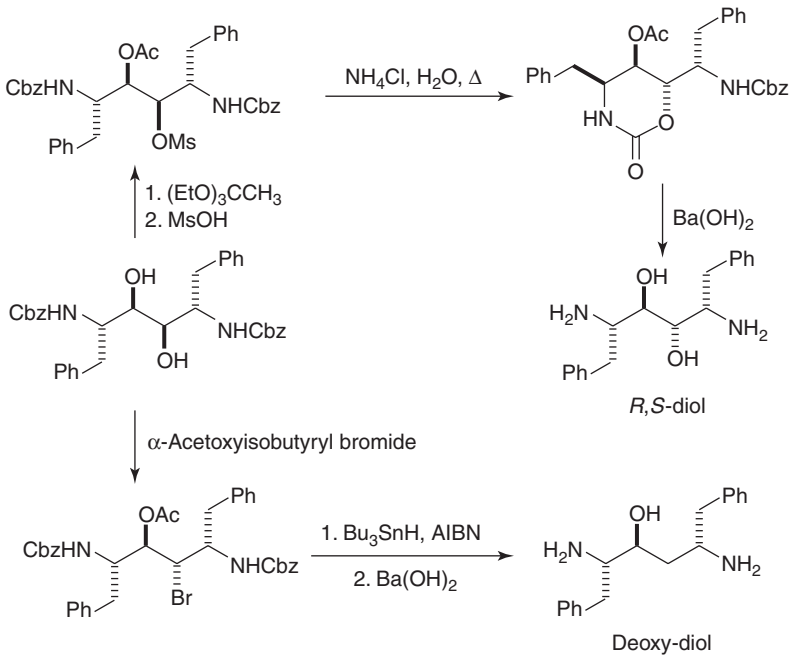

Figure 4 Initial syntheses of the *R*,*S*-diol and 'deoxy-diol' core diamines of A-77003 and A-80987. (Reprinted from Kempf, D. J.; Marsh, K. C.; Fino, L. C.; Bryant, P.; Craig-Kennard, A.; Sham, H. L.; Zhao, C.; Vasavanonda, S.; Kohlbrenner, W. E.; Wideburg, N. E. *Bioorg. Med. Chem.* **1994**, *2*, 847–858, with permission from Elsevier, and Kempf, D. J.; Sowin, T. J.; Doherty, E. M.; Hannick, S. M.; Codacovi, L.; Henry, R. F.; Green, B. E.; Spanton, S. G.; Norbeck, D. W. *J. Org. Chem.* **1992**, *57*, 5692–5700 © American Chemical Society.)

prolongation of the time to either death or AIDS-defining illness.[20] These results established ritonavir as the first protease inhibitor to demonstrate clinical efficacy, and the compound was licensed under the brand name Norvir in early 1996. This study also influenced the acceptance of plasma HIV RNA quantitation (viral load) as a surrogate marker for clinical efficacy, enabling the rapid development of all subsequent antiretroviral drugs.

8.15.5 Lessons from the Ritonavir Development Program

Several key findings from the development and clinical programs with ritonavir had a major impact on the subsequent HIV protease inhibitor discovery effort, leading ultimately to the discovery of lopinavir. The first was the characterization of drug resistance during ritonavir monotherapy in phase II studies. Longitudinal assessment of plasma samples from patients who initially responded to therapy with a drop in viral load, but whose plasma HIV RNA rebounded over time, revealed the stepwise accumulation of specific mutations in the HIV protease gene. Viruses isolated from the blood of these patients displayed reduced phenotypic susceptibility to ritonavir, as well as to some other protease inhibitors. Importantly, the rate at which the mutations appeared inversely correlated with the plasma trough levels of ritonavir in different patients.[21] Thus, those patients with lower drug levels experienced the evolution of drug resistance at a higher rate than those with higher drug levels. The rate at which rebound occurred also inversely correlated to the degree of suppression of viral load, indicating that ongoing replication allows the production and emergence of resistant variants.[22] These key findings led to the articulation of a hypothetical pharmacokinetic–pharmacodynamic (PK/PD) model for the emergence of drug resistance to protease inhibitors. Since protease inhibitors are reversible inhibitors of HIV protease, and, in general, penetrate into and egress from cells relatively quickly (in contrast to nucleosides, which are trapped intracellularly in mono-, di-, and/or triphosphate forms), protease inhibitor trough plasma levels are likely to be reasonable temporal surrogates for minimum intracellular drug levels. If, during a dosing cycle (prior to the next dose), drug concentrations decline to a level that is incompletely suppressive, allowing significant viral replication to begin, preexisting mutants in the HIV quasispecies have a replication advantage in the presence of drug. Preferential replication of these mutants results in the accumulation of additional mutations. With reduced susceptibility to drug, these multiple mutants begin replicating at even higher drug concentrations, providing increased opportunity for the evolution and selection of even more mutations. Ultimately, combination mutants with

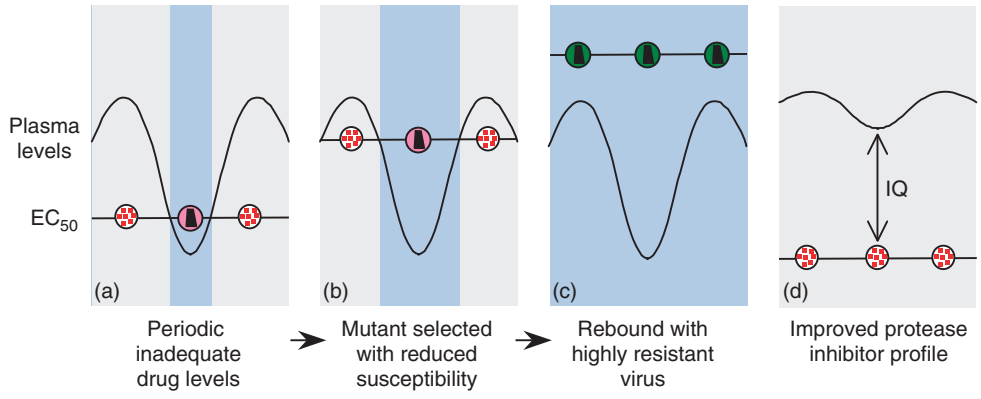

Figure 5 (a)–(c) PK/PD model for resistance development for HIV protease inhibitors; (d) idealized PK/PD profile with high inhibitory quotient.

sufficient resistance to overcome all drug concentrations encountered in a dosing cycle are produced, the drug loses its antiviral effect, and viral load rebounds to pretherapy levels (Figure 5a–c). This PK/PD resistance model suggested that resistance could be delayed or prevented if (a) the drug was substantially more potent (i.e., much lower EC_{50}), and/or (b) the drug maintained higher plasma concentrations (particularly trough concentrations: Figure 5d). Both of these goals were realized with the development of lopinavir/ritonavir (see below).

A second key finding from the ritonavir development program was the realization that this compound, like many protease inhibitors, is highly bound to human serum in vivo, both to serum albumin and α-acid glycoprotein. The effect of this high protein binding was assessed in vitro by adapting the HIV tissue culture system to tolerate the presence of 50% human serum. Upon the addition of human serum, the EC_{50} of ritonavir increased approximately 20-fold, suggesting that its potency in vivo was substantially compromised from that observed in standard tissue culture assays.[23] This finding had several important consequences. First, the average protein-adjusted EC_{50} for ritonavir for several wild-type viral strains was approximately 1 μM (as opposed to approximately 50 nM in the absence of human serum). At full dose, average trough plasma concentrations of ritonavir are only four to five times higher than this value. Thus, the PK/PD resistance model described above, wherein replication occurs to select resistance as drug levels decline to the trough, is consistent with the development of resistance to ritonavir by most patients receiving monotherapy (particularly since many patients experiencing rapid evolution were assigned to lower, investigational doses of ritonavir and had trough levels significantly lower than the full-dose average). This ratio between plasma trough levels and the human serum-adjusted EC_{50}, later to become known as the inhibitory quotient (Figure 5), was estimated to be four or less for all first-generation protease inhibitors. Subsequent studies reveal that a fourfold or greater decrease in viral susceptibility (i.e., ≥fourfold higher EC_{50} and thus average inhibitory quotient of one or less) significantly impacted the virologic response to those protease inhibitors, even in combination with other antiretrovirals, suggesting that the inhibitory quotient model as articulated above has clinical relevance.

One enigma remained with respect to the serum binding of ritonavir. While the EC_{50} increased by 20-fold upon the addition of 50% human serum, the free fraction of ritonavir in human plasma was found to be <1% (suggesting that serum binding should have an even greater effect on the EC_{50}). This issue was clarified in a recent study showing that even in the absence of human serum, ritonavir (as well as lopinavir) is relatively highly bound to the 10% fetal bovine serum present in the tissue culture antiviral assay media. In fact, the EC_{50} and free fraction in the tissue culture media were proportional under a variety of low- and high-serum conditions, allowing the calculation of both serum-free and 100% human serum-adjusted EC_{50} values.[24] The latter value for both ritonavir and lopinavir is closely approximated by the EC_{50} determined in the presence of 10% fetal bovine serum plus 50% human serum. The recognition of the importance of serum binding led to the routine screening of all new protease inhibitor analogs both in the absence and presence of 50% human serum, a change in paradigm that enabled the discovery of lopinavir (see below).

The third key finding from the ritonavir development program was the recognition of its potential as a pharmacokinetic booster by virtue of potent inhibition of the 3A isozyme of cytochrome P450 (CYP3A). CYP3A, the most predominant metabolizing enzyme in the liver and intestine, is the primary route of metabolic transformation and clearance of virtually all HIV protease inhibitors. Ritonavir was found to produce a Type II spectral perturbation in the CYP absorbance spectrum in human liver microsomes, suggesting direct binding of its unhindered 5-thiazolyl group to the CYP heme iron atom.[25] In vitro, ritonavir potently inhibited not only standard CYP3A substrates, but also the metabolism of other protease inhibitors in both rat and human liver microsomes. In rats, coadministration with ritonavir

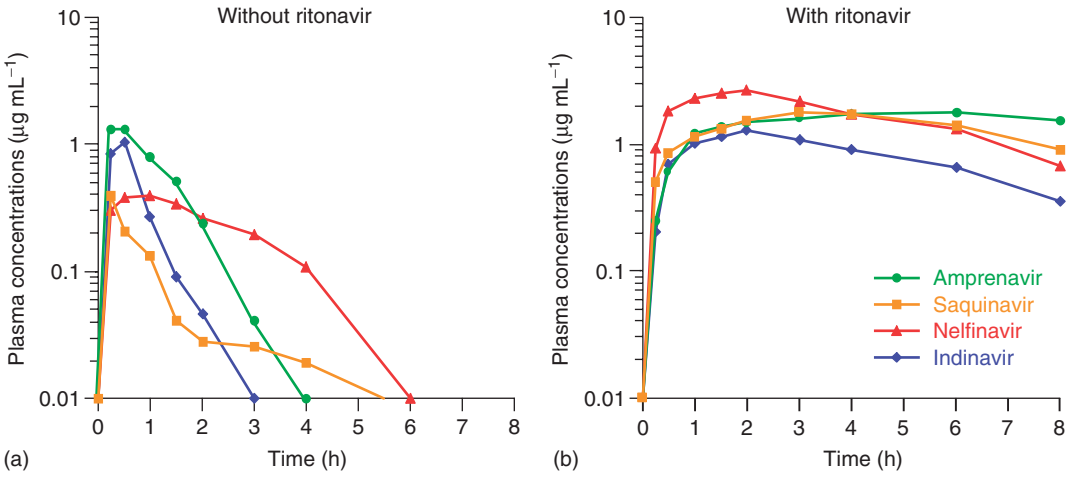

Figure 6 Pharmacokinetic boosting by ritonavir in rats. HIV protease inhibitors were dosed orally at 10 mg kg^{-1} without or with a concomitant 10 mg kg^{-1} dose of ritonavir. (Reprinted with permission from Kempf, D. J.; Marsh, K. C.; Kumar, G.; Rodrigues, A. D.; Denissen, J. F.; McDonald, E.; Kukulka, M. J.; Hsu, A.; Granneman, G. R. *Antimicrob. Agents Chemother.* **1997**, *41*, 654–660 © American Society for Microbiology.)

increased the plasma levels of other protease inhibitors by 8- to 46-fold and substantially increased the serum half-life (Figure 6). Similar enhancements were observed in humans. Given the relatively low inhibitory quotient values for these first-generation protease inhibitors, enhancements of plasma trough levels by ritonavir codosing significantly improved potency, and the use of ritonavir-boosted protease inhibitors is now recommended as the preferred method for use of this drug class in most HIV treatment guidelines. Importantly, a combination of ritonavir and saquinavir was shown to durably suppress viral replication in most patients even without the concomitant use of nucleoside therapy, providing an example of a potent class-sparing regimen.[26]

One study using ritonavir enhancement is worthy of special note with respect to validation of the inhibitory quotient PK/PD model. Although the inhibitory quotient model is normally based on trough levels, there is generally high correlation between peak levels (C_{max}), overall exposure (area under curve, AUC), and minimum concentrations; thus, the most relevant pharmacokinetic parameter to be utilized in a PK/PD model had not been established. In this study, patients failing therapy with indinavir (800 mg three times daily) plus nucleosides were switched to indinavir/ritonavir (400 mg twice daily each) without a change in backbone nucleoside therapy. Viral isolates from most patients were at least partially resistant to both indinavir and ritonavir prior to the switch. Because of the 67% decrease in total indinavir dose, the C_{max} of indinavir in combination with ritonavir was lower than that produced by indinavir 800 mg three times daily alone (the AUC was approximately the same). However, indinavir C_{trough} increased by 6.5-fold due to the increase in half-life from ritonavir boosting. Three weeks after the change from indinavir to indinavir/ritonavir, 58% of patients experienced an incremental virologic response.[27] The indinavir inhibitory quotient following the change to indinavir/ritonavir (based on indinavir C_{trough}) was the best predictor of response, providing further validation of the inhibitory quotient PK/PD model and, in particular, indicating that the C_{trough} (or C_{min}) rather than the C_{max} or AUC is the most appropriate PK parameter to include in the calculation of inhibitory quotient.

8.15.6 The Discovery of Lopinavir and the Development of Kaletra (Lopinavir/Ritonavir)

As mentioned previously, the evaluation of the antiviral potency of new protease inhibitors in the discovery program was expanded to include assays in the presence of 50% human serum, to best estimate 'in vivo potency.' Following the observation of substantial boosting by ritonavir, the preclinical pharmacokinetic screening protocol was also modified to include evaluation in rats and dogs, both alone and following codosing with ritonavir. The goal for an advanced generation protease inhibitor was twofold: improved potency in the presence of human serum and improved pharmacokinetics through ritonavir boosting. A third key element of the design of lopinavir was the incorporation of structural data on the resistant mutants isolated during ritonavir monotherapy. The primary mutation, which occurred in most individuals early after viral rebound, was an amino acid change from valine at position 82 to alanine (V82A), phenylalanine (V82F), or threonine (V82T). As mentioned earlier, valine 82 is positioned in the active site of HIV

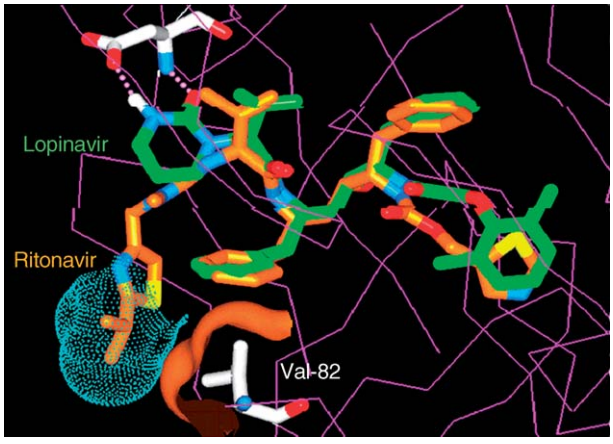

Figure 7 Overlay of ritonavir (orange) and lopinavir (green) in the HIV protease active site, illustrating the interaction of the P3-isopropylthiazolyl group of ritonavir with the side chain of valine 82. (Reprinted from Stoll, V.; Qin, W. Y.; Stewart, K. D.; Jakob, C.; Park, C.; Walter, K.; Simmer, R. L.; Helfrich, R.; Bussiere, D.; Kao, J. *et al. Bioorg. Med. Chem.* **2002**, *10*, 2803–2806, with permission from Elsevier.)

protease (P3 and P3′ subsites) and interacts via nonbonded hydrophobic interactions with the isopropyl substituent on the P3-thiazolyl group of ritonavir. Modeling of the V82A, V82F, and V82T mutant proteases suggested that in each case this hydrophobic interaction would be lost upon viral mutation, lowering the affinity of ritonavir for the enzyme. In order to minimize loss of binding to the position-82 mutants, the ritonavir structure was truncated to remove the P3 isopropylthiazolyl group. Initial analogs were much less potent, but the incremental decrease in activity upon addition of 50% human serum was less than threefold. A key finding was that the terminal urea following truncation could be cyclized, affording >10-fold improved potency.[28] Finally, systematic studies to replace the 5-thiazolyl group remaining from ritonavir produced lopinavir (ABT-378) (**Figure 2**), which, in the presence of human serum, was 10-fold more active than ritonavir.[23] Crystallographic analysis[15] confirmed that the interaction with valine-82 was lessened, compared to ritonavir (**Figure 7**), and the K_i of lopinavir against the V82A, V82F and V82T protease increased only slightly ($\leq$fourfold), whereas the K_i of ritonavir was increased by up to 50-fold. The average resistance of multiply mutant clinical HIV isolates to lopinavir was also substantially lower (approximately threefold) than to ritonavir. Combined with the 10-fold higher potency of lopinavir, the EC_{50} of lopinavir against highly resistant isolates remained similar to that of ritonavir against wild-type HIV.[28]

The metabolism of lopinavir occurred almost exclusively via CYP3A in rat and human liver microsomes, and was inhibited at very low concentrations of ritonavir (IC_{50} 0.036 and 0.073 μM, respectively). The concentrations required to inhibit saquinavir metabolism were significantly higher, suggesting that lopinavir would be exquisitely sensitive to ritonavir boosting. In rats, oral dosing of lopinavir alone produced very low exposures, and in dogs and monkeys, no plasma concentrations were detected due to exceedingly rapid metabolic clearance. By contrast, coadministration of lopinavir with ritonavir produced high and sustained plasma levels.[28] In dogs, the AUC increased by >350-fold, and lopinavir levels remained stable for >12 h at concentrations >64-fold above the human serum-adjusted antiviral EC_{50}. Thus in lopinavir, the dual goals of improving potency and pharmacokinetics over ritonavir had been achieved, along with improved activity against resistant virus. Lopinavir, enhanced by ritonavir, was advanced into clinical studies as the first protease inhibitor regimen designed to be pharmacokinetically boosted.

Initial pharmacokinetic studies of lopinavir/ritonavir in humans confirmed the exquisite sensitivity of lopinavir to ritonavir pharmacokinetic enhancement, providing high plasma levels of lopinavir. At steady-state, a twice-daily regimen of 400 mg lopinavir with low-dose ritonavir (100 mg) produced trough levels of lopinavir >75-fold above its serum adjusted EC_{50} (inhibitory quotient >75). This regimen eventually became the approved clinical dose and has been written lopinavir/r to signify that the low-dose ritonavir is present merely as a pharmacokinetic booster and is unlikely to elicit significant antiviral activity, in contrast with earlier dual protease inhibitor regimens using higher, efficacious doses of ritonavir. In the initial Phase II study, lopinavir/r was studied as monotherapy for 3 weeks prior to the addition of nucleosides. A mean decline in plasma HIV RNA of 1.85 log copies mL^{-1} was observed at week 3,[29] and after nearly 7 years, 95% of patients remaining on study had <50 copies mL^{-1} of HIV RNA in their plasma.[30] In the same group of patients, the average increase in CD4 levels was 511 cells mm^{-3}, demonstrating substantial and prolonged immune restoration. Because of its high inhibitory quotient, lopinavir/r was also active in patients who had

previously failed therapy with other protease inhibitors and whose viruses were drug-resistant. In one study in multiple protease inhibitor-experienced patients, activity indistinguishable from that in treatment-naive patients was observed in subjects whose baseline (study entry) viruses displayed up to 10-fold reduced susceptibility to lopinavir in vitro[31] and up to five mutations associated with reduced susceptibility to lopinavir.[32] Evidence of partial activity in patients with baseline strains with up to 40- to 60-fold reduced lopinavir susceptibility provided confirmation of the high inhibitory quotient erected by this regimen.[33] The statistically significant correlation between virologic response and individual inhibitory quotient values in these patients also served to validate the inhibitory quotient as an appropriate PK/PD model for protease inhibitor efficacy.[34]

In a large phase III study, lopinavir/r was compared in a placebo-controlled, double-blind fashion to another protease inhibitor (nelfinavir) in combination with two nucleosides (stavudine and lamivudine). The virologic response in lopinavir/r-treated patients was statistically significantly superior to the response in nelfinavir-treated subjects.[35] In addition, analysis of the viral isolates from patients in both study arms with HIV RNA >400 copies mL^{-1} revealed a startling difference in the evolution of resistance.[36] Thus, 43/96 (45%) of nelfinavir-treated patients demonstrated genotypic resistance to nelfinavir and 79/96 (82%) displayed resistance to lamivudine. In contrast, none of the 51 patients treated with lopinavir/ritonavir for whom genotypes were available demonstrated resistance to lopinavir (or any other protease inhibitor). Furthermore, the rate of lamivudine resistance was also significantly lower (19/51, 37%) than in nelfinavir-treated patients. This study revealed a substantial barrier to resistance erected by lopinavir/r in previously untreated patients that was not present with earlier protease inhibitors. On the basis of this phase III study, lopinavir/r was licensed in the USA in 2000 under the brand name Kaletra. Only recently have the first cases of evolution of resistance to lopinavir/r in treatment-naive patients been documented, attesting to the high barrier to resistance provided by this boosted protease inhibitor regimen.[37,38]

The high pharmacological barrier to resistance of lopinavir/r is consistent with its unique pharmacokinetic profile as a boosted protease inhibitor. Because of its high inhibitory quotient, drug concentrations are unlikely to enter the 'zone of highest selective pressure' (the concentration range just above the EC_{50} for wild-type HIV where any preexisting mutants in the HIV quasispecies with low-level reduced susceptibility have a maximal replication advantage over the major susceptible population) with normal dosing frequency.[36] Furthermore, if doses are missed, the clearance of lopinavir increases over time as drug concentrations continue to fall due to the decline in the inhibitory effects of ritonavir on hepatic CYP3A. Consequently, there is a large difference in lopinavir plasma half-life between the first 12 h following dosing ($t_{1/2}$ 8 h) compared to 24 h after a dose ($t_{1/2}$ 2.2 h), the time at which drug levels are estimated to reach the zone of highest selective pressure (**Figure 8**).[39] Since lopinavir passes rapidly through this zone and decays further to concentrations that are no longer selective for resistance, overall evolution of the multiple mutations required for resistance is disfavored, even during periods of imperfect adherence when significant viral replication is expected to commence. This non-log-linear decay is not observed for protease inhibitors unboosted by ritonavir, since hepatic clearance remains relatively constant.

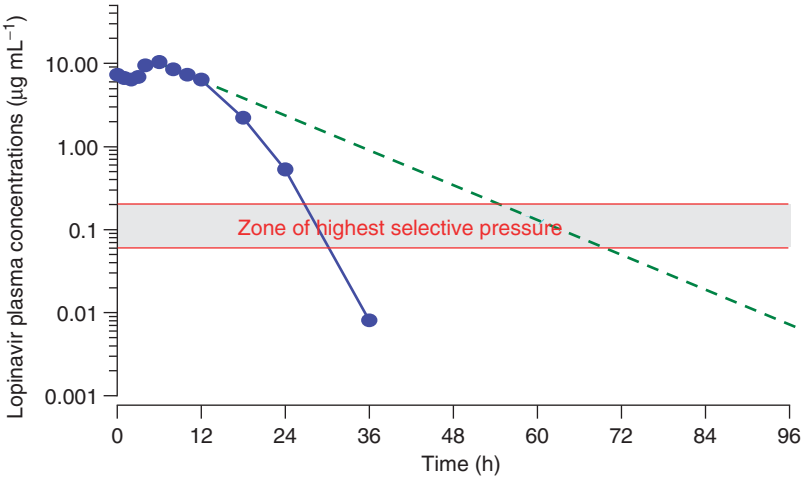

Figure 8 Estimated decay of lopinavir plasma levels through the zone of highest selective pressure following missed doses of lopinavir/r (dosed at 400 mg twice daily in healthy human volunteers at steady state). Blue line: mean plasma levels; green dashed line: extrapolated plasma concentration based on the half-life observed between 6 and 12 h following the final dose.

8.15.7 Conclusion

The high potency and generally good tolerability of lopinavir/r has prompted its wide use, particularly in the most difficult-to-treat patient populations (e.g., children, patients presenting with very high viral loads or failing other regimens, individuals coinfected with hepatitis C), and lopinavir/r has become the most widely prescribed protease inhibitor worldwide since its launch in 2000. Meanwhile, ritonavir, while no longer widely used as an active protease inhibitor for inhibiting HIV, is universally employed as a pharmacokinetic booster for the protease inhibitor class. A key element of the ritonavir and lopinavir/r discovery programs was the iterative integration of lessons learned in the development phase back into the discovery process. Structural information on resistant mutants allowed the design of analogs retaining high potency against these mutants and contributed to the erection of a high barrier to resistance with lopinavir/r. The development of a quantitative PK/PD understanding of this class enabled the optimization of characteristics contributing to high virologic efficacy. Finally, the opportunistic use of the profound drug–drug interactions of ritonavir, normally viewed unfavorably in drug development, to enhance efficacy created a new paradigm for the use of the protease inhibitor class and spurred the development of lopinavir/r. Each of these innovations ultimately contributed to improved patient care, allowing persons with HIV to live normal productive lives and providing hope for the future.

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Biography

Dale J Kempf is a Distinguished Research Fellow and Director of Antiviral Research at Abbott Laboratories. He received his PhD in organic chemistry from the University of Illinois, Urbana–Champaign, in 1982, and, following 2 years of postdoctoral research at Columbia University, began his career in drug discovery at Abbott. Over the last 21 years, he has worked extensively in the areas of aspartic protease inhibitors and antiviral agents, including more than 15 years of experience in HIV-related research. He is a coinventor of ritonavir and the method of boosting pharmacokinetics by coadministration with ritonavir. Dr Kempf has more than 100 scientific publications and 58 US patents to his credit, and has received international recognition for his work in HIV protease inhibitors, including Inventor of the Year from the Intellectual Property Owners Association (1997), Discoverer's Award from the Pharmaceutical Research and Manufacturers of America (1999), and the Heroes of Chemistry Award from the American Chemical Society.

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8.16 Fosamax

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8.16.1 Introduction

8.16.1.1 Osteoporosis and Fracture Risk

Osteoporosis is a reduction in bone mass and bone microarchitecture leading to increased bone fragility and fracture risk. The most common cause of osteoporosis is increased bone turnover with excessive bone resorption (destruction) that exceeds bone formation. Among women, this is often caused by estrogen deficiency following menopause. A second large and independent contributor is glucocorticoid use. Later in life, a combination of vitamin D insufficiency, reduced $1,25(OH)_2$-vitamin D_3 production and inadequate calcium nutrition contribute to bone loss in both men and women. Both menopause and glucocorticoid use cause an imbalance between the processes of bone resorption (removal) and formation, leading to bone loss. A woman can experience a loss of up to 5% of her bone mass per year during the first half decade postmenopause. There exists a correlation between the reduction in bone mineral density[1–4] and/or increased bone turnover[5–7] with increased fracture risk.

Incidence of fracture increases with age, and associated increased risk of trauma with falls, which is an independent contributor. The most common fractures occur in the spine, and their frequency increases progressively in women and men beginning in the sixth and seventh respective decades of life. The most serious fractures are of the hip. The incidence of these increases steadily, reaching a rate of about 5% per year in the ninth decade of life. Approximately 70–75% of all hip fractures occur in women, likely due to their earlier and more dramatic bone loss, gender-based differences in bone mass, and greater longevity. Men reach the fracture threshold about a decade later than women.

With the continued increase in life expectancy due to medical and other advancements it is projected that the incidence of osteoporotic fractures will reach epidemic proportions within the next couple of decades if effective means to combat them are not implemented.

*Deceased

8.16.1.2 Clinical Use of Alendronate (Fosamax)

Alendronate (ALN) has had the most extensive clinical use to date in terms of the number of patients, over 4 million, and duration of monitored treatment, over 10 years. Its ability to reduce hip and other fractures is documented in large randomized placebo-controlled clinical trials, and 10 years of follow-up data are available from the extension of phase III ALN clinical trials.[8] ALN is widely used for the treatment and prevention of osteoporosis in postmenopausal women and glucocorticoid-treated patients of both genders.[9–16] ALN has been proven effective in significantly reducing the incidence of both vertebral and nonvertebral fractures, including those of the hip. The reduced risk of vertebral fracture is also associated with less height loss,[17] as well as a significant reduction in the number of days where patients experience disability.[18] Because ALN acts via a nonhormonal pathway, it has also been effectively used to increase bone mass associated with a number of different diseases, including Paget's disease of bone, and bone loss associated with hyperparathyroidism, human immunodeficiency virus (HIV) infection (treatment-associated), and cystic fibrosis.[19–25] Thus the clinical utility of ALN as an antiosteoporotic, antifracture agent is very broad.

In postmenopausal osteoporosis, bone turnover increases an average of 2–3 standard deviations above mean premenopausal levels.[26,27] In high-turnover states such as menopause average mineralization decreases because new bone tissue is remodeled again before it can become fully mineralized.[28] Following initiation of ALN treatment, bone turnover is returned to premenopausal levels within a few months.[29] By reducing the rate of turnover, there are fewer remodeling sites at any given time and therefore fewer potentially weak areas (stress risers) in trabecular bone. ALN treatment therefore allows secondary mineralization of bone to be completed, which increases inherent bone strength.[30,31] At an effective dose, ALN treatment is associated with increases in bone mineral density (BMD) and bone mineral content (BMC), which occur rapidly during the first 6 months to 1 year. This initial, rapid BMD increase has been attributed to 'filling of the remodeling space.' It refers to the continuation of bone formation and subsequent mineralization process, which proceed for months and years, respectively, at existing remodeling sites that were initiated prior to the biphosphate treatment-induced reductions in turnover.

Interestingly, the increase in BMD at some skeletal sites with ALN (10 mg daily) treatment continues for up to 10 years with mean increases (versus baseline) in BMD of 13.7% at the lumbar spine, 10.3% at the trochanter, 5.4% at the femoral neck, and 6.7% at the total proximal femur.[8] The ultimate goal for reducing bone turnover and increasing bone BMD is the reduction of fractures, and ALN has proven effective in cutting in half the risk of fractures of the spine, as well as nonvertebral fractures, including those of the hip.[9–11,17,29,32,33]

8.16.2 Bisphosphonate Properties

8.16.2.1 Structure

Bisphosphonates (BPs) are analogs of pyrophosphate (P–O–P) in which the geminal oxygen has been substituted by carbon (**Figure 1**). No known enzyme can cleave the P–C–P bond, which minimizes the possibility for metabolism, and none has been detected for ALN in pharmacokinetic studies.[34,35] A main feature of the P–C–P backbone is that, by adhering to the hydroxyapatite component of bone, it localizes these compounds in the target tissue. While the affinity for human bone is low (K_D in the 60–120 μM),[127] the skeleton has a large surface area and virtually an unsaturable capacity for the binding of these compounds. Substituents of the geminal carbon of the bisphosphonate have been shown to enhance both affinity for bone and efficacy in suppressing bone resorption. In particular, the presence of a hydroxyl at R^1 may increase BP affinity to bone,[36] while the presence of a nitrogen atom in the R^2 attachment approximately 3–4 positions from the geminal carbon greatly enhances antiresorptive potency. The nitrogen component and its relative positioning within the molecule does not alter in any significant way the affinity of these compounds for binding to human bone,[127] although it does alter affinity for inhibition of the key intracellular target, as discussed later in this chapter. Because of the critical nature of this nitrogen atom, the BPs can be divided into two classes, the N-BPs and the non-N-BPs, ALN being a member of the former.

8.16.2.2 Pharmacokinetics

The P–C–P backbone of ALN endows it and the entire BP class with several common properties, especially regarding pharmacokinetics. The highly charged phosphonate moieties of ALN limit absorption in the gut to around 0.6%, when administered fasting and formulated as the trihydrate monosodium salt of alendronic acid, with similar values seen for other N-BPs. Entry into the bloodstream is by paracellular transport, and renal excretion is the only route for elimination in part by glomerular filtration and by a secretory process that remains to be elucidated.[37] The

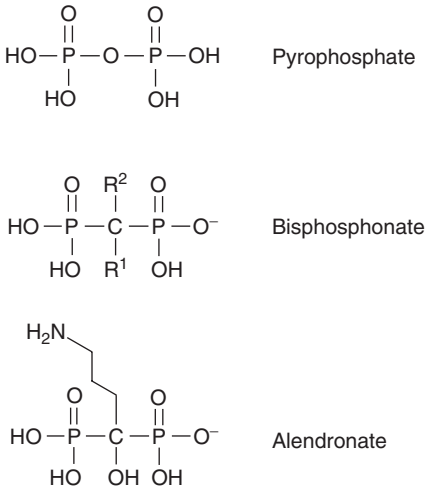

Figure 1 Structures of pyrophosphate, a basic bisphosphonate, and alendronate. Note that in the basic structure, substitutions at R^1 are generally considered to affect binding to bone, while substitutions at R^2 affect potency.

hydrophilic phosphonate moieties limit the penetration of BP molecules through cellular lipid bilayer membranes to undetectable levels, so that distribution is limited to an extracellular compartment and, one day after a dose, is essentially limited to the surface of bone. The bioavailable ALN is thus rapidly cleared from the circulation with an end result of about 50% binding to the hydroxyapatite bone mineral and the remainder being excreted in the urine.[21] This half-life in the circulation is approximately 1–2 h. In humans, the bulk of the ALN not retained on the bone surface is excreted within the first 24 h during the first elimination phase. Intermediate elimination phases exist, whereby the calculated half-lives are days to weeks. In the final elimination phase, ALN has a half-life of about 10 years in humans. Both the intermediate and the terminal half-lives exclusively represent the ALN that is released from the bone.

On bone ALN binds to the mineral surface with no known interactions with the protein matrix. Because the resting surfaces of bone are covered with cells (osteoblasts and lining cells), the most exposed sites are those undergoing active bone resorption. It was shown that these are the preferential sites for ALN uptake in bone at pharmacologically relevant doses.[38–40] At suprapharmacological doses (far above those used to treat humans), the ALN is more or less evenly distributed over the bone surface. Before bone formation is initiated at a given site, the ALN can be readily released from the surface as a result of bone resorption. Before ALN or any other BP can inhibit resorption, it must be ingested by the osteoclast. ALN release from bone is facilitated by acidification of the surface, which takes place during resorption.[38] The removal of mineral and protein from the resorption lacuna beneath the osteoclast occurs through a process of transcytosis.[41,42] This is hypothesized to release not only calcium and phosphate into the blood stream, but also ALN.[43] As time proceeds, the concentration of ALN on the surface of bone increases to steady-state level related to its half-life on the surface of bone, and the ALN inhibition of osteoclast function would gradually increase. This has been documented to occur over 3–6 months in osteoporotic women. Meanwhile, bone formation would proceed to sequester the ALN, as discussed below. Based on these facts, one would predict that, following a single dose, osteoclast-mediated release of bone-associated ALN into the bloodstream would be quite rapid at first, but it should then decline over time. Consistent with this model, the first three of four half-lives for ALN release in clinical testing were calculated as 0.80 days (days 4–7), followed by 6.6 days (days 9–16), and then 35.6 days (days 30–180).

The calculated terminal half-life for ALN is measured in years rather than days. The ALN released during this phase mostly includes BP previously buried beneath the bone surface. This is because ALN preferentially labels the bone resorption surface, and the resorption cycle is always followed by a subsequent cycle of bone formation at the same site. The ALN localized on the resorption surface is therefore covered by de novo synthesized bone, as has been demonstrated in the rat.[40] The bone formation process itself takes 3–4 months, and it can be years before a new resorption cycle reinitiates at a given site. The buried ALN remains pharmacologically inert until it is released back into the circulation as a result of normal bone turnover. Rates of turnover from both cortical and cancellous bone determine not only the subsequent release of BPs but also the relative uptake and distribution of BPs when administered. The cancellous bone takes up a relatively larger proportion of the absorbed BP than the cortical bone,

since cancellous bone is subject to substantially higher turnover. Accurate assessments of terminal half-life in pharmacokinetic analysis therefore required a substantial follow-up, since the curve for elimination from bone is nonlinear for many months.[21] The average terminal elimination half-life of ALN from the skeleton, estimated by urinary excretion in an 18-month follow-up study, is about 10 years. A similar half-life is estimated from modeling of bone turnover at the various compartments, and the total body burden of ALN after 10 years of treatment with an averaged daily dose of 10 mg orally is 75 mg.[44] Although no other BP has been studied in a clinical pharmacokinetic trial long enough to establish a terminal elimination half-life, all other BPs should theoretically exhibit a similar half-life after incorporation into bone. Clinical benefits of bone retention of ALN can be seen after discontinuation whereby bone loss is gradual in comparison to the rapid loss seen after estrogen therapy withdrawal.[45]

8.16.3 Mechanism of Action

8.16.3.1 Alendronate Action at the Molecular Level

Although tested for clinical use since the mid-1980s, the molecular target for ALN, along with other N-BPs, was not identified until 1999. Over the years, BPs were shown to affect several biochemical pathways. For example, ALN and numerous other BPs were found to inhibit the activity of several protein tyrosine phosphatases.[46–50] These actions occurred usually at the upper range of pharmacologically relevant concentrations and failed to correlate with the pharmacological potency of these agents. Although these phosphatase inhibitory activities could be involved in the mechanism of action of some BPs, more compelling proof was obtained for a different molecular target responsible for BP inhibition of osteoclastic bone resorption, as described below.

8.16.3.2 Nitrogen-Containing Bisphosphonate Inhibition of the Cholesterol Biosynthetic Pathway

Over 15 years ago, it was shown that certain BP derivatives (isoprenoid (phosphinylmethyl) phosphonates) weakly inhibit the cholesterol biosynthetic enzyme squalene synthase.[51] The search for more potent inhibitors that might block cholesterol production revealed that the N-BPs incadronate (YM175) and ibandronate potently inhibit squalene synthase.[52] Subsequent studies examined the structure–activity relationship (SAR) for inhibition of squalene synthase.[53–55] In vivo testing showed that certain compounds suppressed serum cholesterol in rodents.[53] Other cholesterol-lowering bisphosphonates were shown to trigger degradation of hydroxymethylglutaryl coenzyme A (HMG-CoA).[56–58] In the same context, utility of squalene synthase inhibition by bisphosphonate was also used for the development of an assay to measure zoledronate levels in animals and clinical serum samples.[59] Although cholesterol itself is important for osteoclast signaling and survival, the osteoclast relies on low-density lipoprotein (LDL) as an external source rather than synthesis through internal pathways.[60,61] Therefore, although ALN, like another N-BP, pamidronate, has been shown to inhibit cholesterol synthesis, this is through inhibition of an enzyme other than squalene synthase. Restoration of cholesterol in the ALN-treated osteoclast does nothing to interfere with its inhibitory action on bone resorption.[62] This then lead to a search for other possible enzymes that could account for its antiresorptive effects.

8.16.3.3 Farnesyl Diphosphate Synthase as the Molecular Target of Alendronate

The ability of ALN to inhibit sterol biosynthesis upstream of squalene synthase[52] suggested inhibition of an enzyme upstream of squalene synthase in the mevalonate pathway,[63] as was indeed identified (**Figure 2**). In subsequent studies, the key enzyme inhibited by ALN was found to be farnesyl diphosphate (FPP) synthase.[60] The reason for continuing to search within the cholesterol biosynthetic pathway for a target enzyme, despite lack of evidence that ALN's effect on cholesterol synthesis would be important to its effects on the osteoclast, was based on the observation that restoration of a branch pathway (leading to protein geranylgeranylation) was sufficient to block all effects of ALN or other N-BPs on osteoclastic bone resorption,[62,64] as discussed below. Modeling of the interaction between ALN and FPP synthase suggests binding to the geranyl diphosphate site,[65] where it acts as a transition-state analog. Enzymological studies suggest that inhibition of FPP synthase is indeed complex.[66] Both competitive and noncompetitive inhibition is reported, depending on the substrate used in the assay, isopentenyl diphosphate or geranyl diphosphate, respectively. Other studies have centered on the SAR for N-BP inhibition of FPP synthase. Modeling using the N-BP risedronate showed that modifications (e.g., addition of a methyl group) to the structure of the side chain can give rise to analogs with markedly less potent inhibition of FPP synthase, making them less effective inhibitors of bone resorption in vivo.[67]

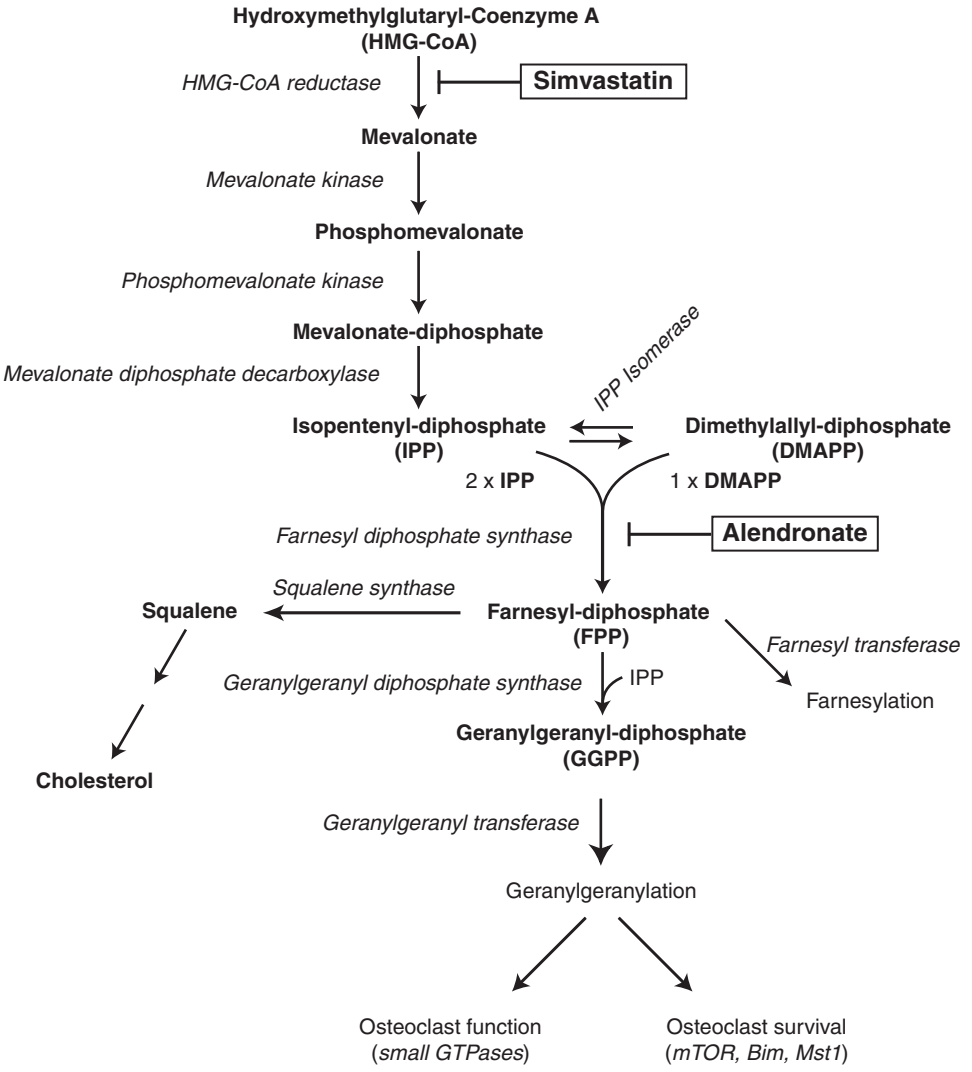

Figure 2 Schematic of the mevalonate pathway. All enzymes are listed in *italics*, while metabolites are in bold. The target of inhibition (farnesyl diphosphate synthase) for alendronate is enclosed within a box, as is the target for the statin, simvastatin, which is shown for reference.

The variable that confers potency against FPP synthase relates to the position of the nitrogen group relative to the phosphonate groups. Interestingly, a modification in one of the phosphonate groups of risedronate, while drastically reducing FPP synthase inhibition, gave rise to a new compound with new activity against type II geranylgeranyl transferase.[68] This derivative has substantially less antiresorptive activity than risedronate in vivo, likely due to reduced binding to bone.[69] Other modifications of risedronate can confer specificity for isopentenyl diphosphate isomerase in addition to FPP synthase.[70] It remains unclear, as yet, whether equivalent modifications to ALN or other N-BPs would confer similar changes in enzyme specificity.

8.16.3.4 Inhibition of Farnesyl Diphosphate Synthase Blocks Protein Isoprenylation and Sterol Synthesis

FPP synthase is responsible for the production of isoprenoid lipids FPP (15 carbon) and geranylgeranyl diphosphate (GGPP) (20 carbon). While FPP, formed by the condensation of three isopentenyl diphosphates (or isomers), is primarily used to synthesize cholesterol, it also can be used for protein isoprenylation. FPP can also be condensed with a fourth isopentenyl diphosphate to form GGPP. The blockade in synthesis of GGPP, albeit through indirect effects on

FPP synthase, is critical for N-BP effects on suppressing osteoclastic bone resorption[62] and inducing osteoclast apoptosis.[71] GGPP, like FPP, is a substrate for protein isoprenylation, and both isoprenoids exhibit specificity in the proteins to which they can be coupled. Isoprenylation involves the transfer of a farnesyl or geranylgeranyl lipid group onto a cysteine amino acid residue in characteristic C-terminal (e.g., CAAX) motifs.[72,73] Most of the isoprenylated proteins identified to date are small guanosine triphosphatases (GTPases) that are geranylgeranylated, and specific CAAX motifs are responsible for directing which lipids are attached to each respective protein.[72] Geranylgeranylated signaling proteins are important for the regulation of a variety of cell processes required for osteoclast function, including cytoskeletal regulation, formation of the ruffled border and regulation of apoptosis.[74–77]

The ability of ALN and other N-BPs to inhibit the cholesterol biosynthetic pathway and protein isoprenylation was actually first demonstrated in J774 macrophages.[52,63] The relevance of this model relates to the fact that J774 cells come from the same lineage as osteoclasts, and that these cells, like osteoclasts, undergo apoptosis in response to N-BP treatment. In these early studies, it was recognized that N-BP inhibition of the cholesterol biosynthetic pathway and isoprenylation was important.[63] Using a more relevant system, it was later discovered that ALN inhibits incorporation of [^{14}C]mevalonate into either isoprenylated proteins or sterols in purified murine or rabbit osteoclasts.[60,78] The relevance of this effect was proven through the ability of a GGPP precursor, geranylgeraniol (GGOH) to block ALN effects on the osteoclast,[62,71,79] as discussed in detail below.

8.16.3.5 Evidence for Molecular Mechanisms In Vivo

The molecular actions of the N-BPs, described above, have been confirmed in vivo using surrogate markers.[80,81] In one study, the well-documented feedback regulation of HMG-CoA reductase expression by cholesterol biosynthetic intermediates was examined.[80] ALN and other N-BPs, but not those lacking a nitrogen, suppressed expression of HMG-CoA reductase in osteoclasts from the proximal tibia. While ALN induced changes in HMG-CoA reductase expression in the osteoclast, no changes were seen in other bone- or marrow-associated cells, which is consistent with the observed targeting of ALN to the osteoclast.[38,40] This effect in the osteoclast appeared to be mediated by the accumulation of metabolites upstream of FPP synthase rather than those lying downstream. The loss of HMG-CoA reductase expression along with inhibition of FPP synthase in the osteoclast could potentially have additive effects on the mevalonate–cholesterol biosynthetic pathway. It may also prevent accumulation of too many upstream metabolites that might otherwise compete with ALN binding to FPP synthase. In the second study, osteoclasts were examined for the in vivo actions of ALN on protein geranylgeranylation.[81] In osteoclasts purified (by immunoadsorption onto magnetic beads) after ALN treatment, geranylgeranylation of the small GTPase Rap1A was suppressed. In this regard, Rap1A was used as a marker for ALN action, although there was no specific link made between the inhibition of this small GTPase and any loss of osteoclast function. For comparative purposes, clodronate was also tested, and this nitrogen-free BP had no effect on geranylgeranylation.

8.16.3.6 Mechanism of Action at the Cellular Level

The relationship between molecular action and antiresorptive effects has been documented for BPs lacking and containing nitrogen. For the non-N-BPs, which are intracellularly metabolized to form toxic analogs, the mechanism is accepted based on the ability of the toxic analogs to reproduce the effects of the parent BPs when administered to the osteoclast.[82] Perhaps the best documentation for a cause–effect relationship has been established for ALN and the other N-BPs, where inhibition of FPP synthase and consequential effects on the osteoclast (loss of resorption, induction of apoptosis) can be overcome simply by reintroducing the critical lost metabolite. Among the downstream metabolites that could specifically restore the three major processes leading to cholesterol synthesis, farnesylation or geranylgeranylation, only geranylgeraniol (GGOH), a lipid alcohol that can replenish GGPP, prevents the ALN effect.[62] Other metabolites downstream of FPP synthase that feed into farnesylation or sterol synthesis are without effect. The observation that farnesol, which is readily metabolized to form FPP, cannot restore osteoclast survival or function was unexpected.[62,83] FPP, like GGPP, is sufficient to block N-BP-induced macrophage apoptosis.[63] The reasons for farnesol not being metabolized to GGPP during BP (or statin) treatment remain to be elucidated. Interestingly, the upstream metabolite, mevalonate, can also partially rescue inhibition of resorption, although this effect disappears with increasing concentration of ALN.[62,63] This is consistent with very recent data suggesting, in part, competitive inhibition of FPP synthase by N-BPs.[66] By this token, lower concentrations of N-BP may show a disproportionate loss of activity, since upstream metabolite accumulation could result in more effective competition for binding sites within FPP synthase. In the context of the in vivo finding that ALN can also suppress HMG-CoA reductase expression,[80] this

feedback mechanism might serve as a secondary method to increase efficacy by preventing excessive accumulation of such metabolites.

In addressing the downstream cellular mechanisms related to suppression of bone resorption, substantial evidence has accumulated to link loss of geranylgeranylation to induction of osteoclast apoptosis, disruption of the actin cytoskeleton, and altered membrane trafficking.[71,78,79,84,85] The original observation that osteoclasts undergo apoptosis in response to ALN and other N-BP treatment[86] remained for several years the primary model for BP action in vitro and in vivo. The apoptotic action of both N-BPs, like ALN, and BPs lacking nitrogen results from intracellular action within the osteoclast, as opposed to other indirect actions that could be mediated via osteoblasts, which in turn could control osteoclast survival.[71] That N-BPs cause apoptosis by interfering with geranylgeranylated proteins in osteoclasts was demonstrated by blocking the effect simply by restoring GGPP levels in the osteoclast through the addition of GGOH. Induction of osteoclast apoptosis by ALN and risedronate, but not BPs lacking nitrogen, can be blocked by addition of GGOH, but not farnesol. For reasons unknown, farnesol feeds only into the farnesylation pathway and cannot restore geranylgeranylation.[71] This effect of farnesol was unexpected, since it feeds into the metabolic pathways downstream of the site of inhibition and upstream of the step required for synthesis of GGPP. In contrast to the osteoclast, both FPP and GGPP can prevent ALN- or N-BP-induced apoptosis in other cell types, perhaps suggesting easier conversion of FPP to GGPP in these cells.[63,87–89]

The signaling pathways involving geranylgeranylated small GTPases that are affected by bisphosphonates and that lead to osteoclast apoptosis remain to be determined. Perhaps most proximal to the GTPases is the mammalian target of rapamycin (mTOR)/ribosomal protein S6 kinase (S6K) signaling pathway.[90] Signaling through this path is suppressed when geranylgeranylation is blocked in the osteoclast (**Figure 2**). Furthermore, specific inhibition of mTOR by rapamycin causes induction of osteoclast apoptosis over a similar time course to that of the N-BPs. Signaling through mTOR represents a relatively novel pathway downstream of receptor activator of NFκβ (RANK), tumor necrosis factor alpha (TNF-α), and interleukin-1 (IL-1) signaling in the osteoclast.[91,92] Downstream of phosphoinositol-3 kinase, signaling through the Akt kinase to mTOR was originally implicated in maintaining osteoclast survival, putatively through the regulation of protein translation, which itself was shown to be critical for osteoclast differentiation and survival. More recent evidence suggests that Akt is actually dispensable for survival, whereas mTOR, and its signaling to the Bcl-2 family member Bim, form the critical pathway required for the survival of the osteoclast.[92] Bim is a proapoptotic mammalian regulator of cell death. Akt, in turn, is critical for differentiation, which ALN and other N-BPs can also inhibit. Not only can ALN suppress signaling from survival cytokines such as TNF-α and RANK ligand, to mTOR,[91] but also specific inhibition of protein geranylgeranylation with a geranylgeranylation inhibitor and/or the withdrawal of cholesterol from the osteoclast[61] can lead to both suppression of mTOR signaling and the induction of osteoclast apoptosis. This illustrates both the importance of this signaling pathway as well as its reliance on both protein isoprenylation and cellular cholesterol content for proper functioning. The caveat here is that FPP is critical for osteoclast isoprenylation alone, whereas LDL is critical for maintaining cholesterol levels in the osteoclast.

Downstream consequences of ALN inhibition of mTOR signaling include induction of (proapoptotic) Bim expression and suppression of protein translation. Independent effects of translation inhibitors such as cyclohexamide on the osteoclast do include the rapid induction of caspases, leading to osteoclast apoptosis.[90] With regard to Bim, selective interfering RNA, used to suppress expression of the Bim protein, can increase osteoclast survival after macrophage colony-stimulating factor withdrawal.[92] Although not shown to cause apoptosis in response to ALN treatment, increased Bim expression can cause caspase activation. Caspase 3 is the major effector caspase activated in osteoclasts undergoing apoptosis following treatment with a range of bisphosphonates in vitro.[89] A downstream effector of the caspases is Mst1 kinase, which acts as both a substrate for caspases 3, 7, and 9 and as an activator of these caspases.[90,93,94] Indeed, Mst1 was identified as a proapoptotic signaling intermediate downstream of the bisphosphonates that is activated during apoptosis by ALN and other N-BPs, and clodronate.[71] Caspase cleavage of Mst1 results in the formation of an unregulated, highly active kinase species, shown to cause nuclear condensation.[95] High Mst1 activity also leads to caspase 3 and 9 activation, thus creating a sort of proapoptotic cycle.[90] What lies downstream of Mst1 in the osteoclast, other than its feedback activation of the caspases, remains unknown.

While induction of apoptosis will lead to a decrease in the number of osteoclasts and in turn suppress resorption, this effect is usually seen in vivo only after extended treatment with bisphosphonate. An observation that defines the N-BPs as a class is actually the increase in osteoclast number found in vivo within about 48 h after treatment with ALN, ibandronate, and risedronate, but not clodronate or etidronate (the latter two lacking a nitrogen atom).[80] Previous studies reported that ALN treatment increased osteoclast number and increased bone surface,[96,97] but, the more recent work suggested an early increase in osteoclast number was seen as soon as inhibition of bone resorption occurred

(i.e., before bone surface increased).[80] The finding that suppression of resorption is seen prior to reductions in osteoclast number suggests direct inhibition of osteoclast function by the bisphosphonate is responsible, rather than osteoclast apoptosis. Decades ago it was reported that BP administration causes the osteoclasts to change morphology and appear inactive.[98] The changes in the osteoclast are numerous[99] and include disruption of the cytoskeleton, including actin and vinculin, as well as disruption of the ruffled border.[38,98,100,101] The actin cytoskeleton is required for adhesion of the osteoclast to the bone surface, cell migration, and formation of the ruffled border. The ruffled border is a highly convoluted membrane structure situated above the resorption lacuna that is responsible for excretion of acid and proteases onto the bone surface. The ruffled border is also the point of invagination, whereby membrane vesicles form to engulf the released bone mineral and peptides as a first step in the transcytosis process. Consistent with direct inhibition of the osteoclast as the key to resorption inhibition, ALN was shown to disrupt the actin cytoskeleton, a marker for disrupted function, prior to induction of apoptosis.[79] On the other hand, with etidronate, a non-N-BP that acts primarily via induction of osteoclast apoptosis, the two effects were simultaneous. In separate studies, electron microscopic examination revealed apoptotic osteoclasts associated with resorption inhibition by clodronate, while with ALN, morphology was altered (retracted cells, loss of microvilli from the ruffled border) without substantial evidence of apoptosis.[84] Importantly Z-VAD-FMK, a caspase inhibitor that can suppress induction of apoptosis, can reduce osteoclast apoptosis induced by ALN in vitro, but it cannot suppress ALN inhibition of bone resorption.[79] However, for clodronate and etidronate, interference with the induction of apoptosis was sufficient to significantly and substantially increase bone resorption. Therefore, while a postapoptotic osteoclast would be incapable of bone resorption, it is more likely that ALN inhibits osteoclast function first. Apoptosis may come later, as a result of osteoclast inactivity, or at supraphamacological doses.

Based on these observations, other mechanisms of suppressing osteoclastic bone resorption seem more likely for ALN when administered at clinically relevant doses. As mentioned above, all BPs are rapidly taken up by the skeleton and localize preferentially on exposed mineral at bone resorption surfaces. Osteoclasts, the bone-resorbing cells, attach to the exposed mineral and start the bone resorption process. The result of the intracellular action of N-BPs, shown for pamidronate and ALN,[38,102] is disappearance of the ruffled border, while osteoclast morphology shifts toward the generation of large and plump cells.[80] These plump cells contain a higher than usual number of nuclei (the osteoclast being a multinuclear cell to begin with), and the nuclear morphology is normal (i.e., nonapoptotic). As noted above, the ruffled border is a convoluted membrane, which faces the bone surface and is a hallmark of active osteoclasts. Ruffled border formation is a process that is highly dependent on cytoskeletal function, strongly regulated by geranylgeranylated GTP-binding proteins, such as Rac, Rho, etc. Moreover, the vesicles normally located above the ruffled border (which disappear after N-BP treatment) are needed for the formation of the ruffled border itself, and the trafficking of these vesicles is largely under the control of the Rabs, which are also geranylgeranylated proteins. Disappearance of the ruffled border in the absence of signs of apoptosis, therefore, provides morphological evidence for mechanism-based osteoclast inactivation and could explain the lack of acid extrusion caused by ALN in isolated osteoclasts.[103]

During resorption osteoclasts internalize the content of the resorption lacunae via the ruffled border and translocate it through the cell by a process of transcytosis.[41] This process was already discussed as a likely means of releasing bone-associated ALN into the bloodstream. A second function of transcytosis relates to ALN uptake into the cytoplasm, thus enabling access to FPP synthase. Roughly two decades ago it was documented by microradiography that following administration of radioactive ALN in vivo, the BP can be detected inside the osteoclast 4 h later,[38,40] consistent with the recently shown transcytotic uptake. Other studies have pointed to a requirement for cellular BP uptake for its ultimate effect. It was shown in vitro that osteoclasts that have lost the ability to take up material from their surroundings, due to a mutation (e.g., osteoclasts from the *oc/oc* mouse) do not respond to tiludronate.[101] A response could be produced, however, by microinjecting this BP into the cells. Ruffled border is not required, however, for incadronate (YM175) to induce osteoclast apoptosis when injected at high dose ($1\,mg\,kg^{-1}$) into *oc/oc* mice.[104] It is possible, therefore, for bisphosphonates to enter the osteoclast via a second, and as yet unidentified, route. Finally, slime mold growth inhibition by BPs is reduced when uptake by pinocytosis is inhibited,[105] and the non-N-BP clodronate can be used to suppress N-BP effects on isoprenylation in J774 macrophages, suggesting that uptake is mediated through an active transport mechanism.[106] Taken together, uptake of ALN via ruffled border seems to be required for the intracellular action of this BP. It provides for the formation of transcytotic vesicles that can engulf ALN into vesicles, and these may contain membrane proteins that can facilitate the entry of ALN into the cytoplasm. However, since ALN suppression of protein geranylgeranylation subsequently suppress the formation and function of this cellular structure, inhibition of the transcytosis process not only results in suppression of osteoclastic bone resorption, but may also limit intracellular exposure to the N-BP. This then might reduce exposure and the likelihood that the osteoclast undergoes apoptosis.

8.16.3.7 Mechanism of Action at the Tissue Level

Osteoporosis and other types of bone loss are associated with increased bone turnover and elevated levels of bone resorption. Osteoclastic bone resorption is a 2-week process that begins the bone remodeling process. Resorption itself can be effectively slowed or controlled by inhibiting osteoclast generation, reducing osteoclast activity, or both. ALN is one of the most effective inhibitors of bone resorption. ALN improvement of mechanical strength, reflected in a reduction in fracture risk, is caused by an increase in bone mass and mineralization (discussed above) as well as by an improvement in architecture, attributable to a reduction in bone turnover. A higher number of bone remodeling sites, where excessive osteoclastic destruction of bone takes place, leads to loss of bone tissue, formation of areas of stress concentration, and increased fracture risk. By reducing turnover, bisphosphonates reverse this condition. Effects on bone turnover can be estimated by measuring either cross-linked C-terminal or N-terminal bone collagen degradation products or deoxypyridinoline (formed from type 1 bone collagen) in the urine or in the blood. These degradation products come as a result of proteolytic activity within the resorption lacuna, followed by their release during transcytosis and subsequent extraskeletal metabolism. ALN-induced suppression of these markers can be detected within days, and maximal effects are reached within a few weeks whereupon levels stabilize and remain reduced at a stable level for the duration of treatment, followed up to 10 years for ALN so far.[8] Bone formation is also reduced, albeit about 3 months later than resorption, as part of the reduction in bone turnover, reaching a nadir at 3–6 months. This is a reflection of the so-called 'coupling' between resorption and formation whereby, through mechanisms that have not been fully elucidated, changes in resorption engender changes in formation in the same direction. Another mechanism for increased bone strength is the increase in mineralization associated with lower bone turnover.[28,30,31] This has been described in ALN-treated baboons[28] and, more importantly, in osteoporotic women.[30,31] Lower turnover lengthens the lifespan of the bone remodeling BMU (basic multicellular unit), thus permitting it to mineralize more completely and increase mineral content. This is a process that can take years to fully complete. The effect is to reduce the proportion of incompletely mineralized, recently formed bone. The mineralization of mature bone is not increased. BMD or BMC measure the combined BP effects on bone mass and mineralization. The initial rise in bone mass measured by dual-beam x-ray absorptiometry is caused by the continued rebuilding of preexisting BMUs that were initiated prior to ALN treatment. BPs subsequently reduce the number of new BMUs, and at individual BMUs, they act by decreasing the depth of resorption and possibly increasing wall width during the formation phase.[107] A continuous increase in spinal BMD was observed during 10 years treatment of postmenopausal women with ALN.[8] Increases in BMD and mineralization are associated with improvements in bone strength. Increased bone strength following BP treatment has been documented in experimental animals by ex vivo biomechanical testing[108–112] and is reflected in the reduction in fracture risk observed in clinical trials.

Very high doses of ALN (six times above clinical dosing), like risedronate, when administered for a period of 1 year, were reported to suppress bone turnover in dogs by up to 95% and cause accumulation of microcracks in both cortical and cancellous bone.[113] In this study, microcracks are defined by the presence of microscopic streaks in bone sections that stain with basic fuchsin. Interestingly, the amount of microcracks in dog bones was not associated with any extrinsic biomechanical property, although it was associated with an increase in compressive strength. The clinical relevance of these findings with suprapharmacological doses of ALN are uncertain. The best comparator for this type of modeling comes from the 10-year data for ALN, which show no increase in nonvertebral fracture risk when years 8–10 are compared to years 1–3.[8] This suggests either absence of microcrack accumulation at the usual osteoporosis treatment dose of ALN or a lack of relevance of microcracks to fracture risk. Consistent with the latter, untreated elderly women with and without femoral neck fractures were found to have the same degree of microcrack accumulation,[114] suggesting that microcracks themselves are not predictors of fracture risk.

8.16.4 Relationship between Mechanism of Action and Toxicology

Toxicological animal studies have been published on ALN, clodronate, etidronate, incadronate, pamidronate, and tiludronate. When bisphosphonates are administered subcutaneously, local toxicity can occur, with local inflammation and necrosis. This is especially the case for the N-BPs. Clinical evidence of upper gastrointestinal irritation is observed in some patients.[115,116] The risk of this problem is reduced through dosing instructions designed to avoid both esophageal tablet retention and reflux of acidic stomach contents in patients with gastroesophageal reflux disease (GERD). Moreover, all oral bisphosphonate products are soluble salts rather than less soluble free acids.

To examine the mechanism of esophageal adverse events after oral administration of N-BPs,[117] the effects of oral BPs were examined in special studies in animals. ALN, given orally to rats at suprapharmacological doses, has been reported to induce gastric and esophageal erosions and ulcerations and delay healing of indomethacin-induced gastric

erosions. These effects were not attributable to changes in gastric acid secretion, or prostaglandin synthesis, but are thought to be due to a topical irritant effect. No esophageal or gastrointestinal effects are observed with intravenously administered bisphosphonates. From analyses in dogs, the pH of the BP solution at pharmacologically relevant doses does play a critical role in the response, since there was no irritant effect at a pH of 3.5 or higher.[118] In consideration that osteoclast uptake of the BPs occurs via the acidified compartment within the resorption lacuna, this may suggest that BP penetration into cells is more or less restricted to acidic conditions. Whereas the gastric mucosa has many physiologic mechanisms that resist damage due to both low pH and proteolytic enzymes, the esophageal lining is not ordinarily exposed to acid, except under conditions of gastric reflux (e.g., when a patient with GERD lies down after dosing). Similar irritant effects in animals have been reported for risedronate, and the non-N-BPs etidronate and tiludronate, when given at pharmacologically relevant doses.[119–121] Thus it is advised that with all BPs, patients do not lie down during the period between dosing and the first ingestion of food.

The effect of BPs does not appear due to be primarily due to an extracellular physicochemical effect on the mucosa. To address the issue of intracellular mechanism, recent studies have examined apoptosis and suppression of cell growth in in vitro models of the esophageal stratified epithelium and the large intestine.[122–125] In CACO-2 intestinal epithelium cells and in Ch1.Es esophageal fibroblasts, the N-BPs induced apoptosis, which could be blocked by the addition of GGOH.[122,124,125] This suggested that N-BP inhibition of protein geranylgeranylation was instrumental in the apoptotic response. In normal human epidermal keratinocytes[123] and, as a second phenotype, in CACO-2 cells, growth suppression was observed in response to N-BP treatment. In the keratinocyte, used as a model for stratified squamous epithelium lining the esophagus, this was associated with both suppression of cholesterol biosynthesis and protein geranylgeranylation (**Figure 2**). Reduced cell growth was linked to a block in regulation of proteins (cyclin-dependent kinases) that control the cell cycle.[123] These recent in vitro studies suggest that N-BP-induced gastrointestinal irritation and/or delayed repair of damage produced by acid reflux are mediated by inhibition of FPP synthase in the affected tissues. These findings have recently been validated in vivo, where irritation at the site of subcutaneous injection was blocked by coadministration of an agent that causes accumulation of isoprenoids (e.g., FPP and GGPP).[125]

Ultimately, it is the randomized, placebo-controlled trials that provide the highest level of evidence with regard to the upper gastrointestinal safety of the BPs. Interestingly, an analysis of numerous clinical trials found no detectable increase in upper gastrointestinal adverse events, suggesting that the increase in risk is small.[126] This included evidence that patients who discontinued taking BPs could be randomized to blinded retreatment with either a BP or placebo, and a great majority of these patients were able to continue treatment, with no difference in adverse events between the bisphosphonate and placebo groups.

8.16.5 Conclusion

In conclusion, recent data have identified the mevalonate pathway enzyme FPP synthase as the primary molecular target of ALN. Inhibition of this enzyme reduces the isoprenylation of regulatory proteins in osteoclasts, thus reducing bone resorption. The specific loss of protein geranylgeranylation, and not farnesylation, is responsible for osteoclast inactivation. Because of the low bioavailability and short circulating half-life of ALN, the osteoclasts are the only nongastrointestinal cells exposed to high enough concentrations to allow ALN inhibition of FPP synthase. As such, these are the only bone-associated cells that respond to ALN treatment, as documented in their morphological and functional changes, as well as their selective loss of FPP synthase activity. The gastrointestinal mucosal irritation occasionally produced by N-BPs is a function of their very high local concentration immediately following an oral dose and appears to be related to effects on the same metabolic pathway. No other cells (briefly) exposed to circulating ALN display any similar response. In retrospect, the targeting of FPP synthase in the osteoclast provides an excellent mechanism for inhibition of osteoclast activity. Of note, the mechanism was identified when Fosamax had already been marketed for several years.

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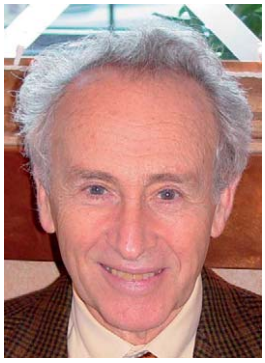
Biographies

Alfred A Reszka earned his PhD degree from the University of Illinois, Champaign–Urbana in 1992. His thesis research focused on mapping out the sites of interaction between β1 integrins and the actin cytoskeleton. He then went on to conduct his postdoctoral research at the University of Washington as a recipient of a Jane Coffin Childs fellowship, where he characterized the physical and regulatory interactions between MAP kinases, ERKs 1 and 2, and the microtubule cytoskeleton. Dr Reszka joined Merck & Co. in 1997 where he focused on elucidation of the mechanism of action of Fosamax. His scientific contributions at Merck include the identification of farnesyl diphosphate synthase as the target enzyme inhibited by Fosamax in the osteoclast and the identification of the geranylgeranylation pathway as the critical path in the osteoclast inhibited by Fosamax. He has also identified key osteoclast signaling pathways downstream of prosurvival cytokines that are required for osteoclast survival (mTOR and S6 kinase) as well as characterizing Mst1 as a proapoptotic kinase involved in osteoclast cell death. He is the author of many peer-reviewed scientific articles, book chapters, and reviews. He is a member of the American Society for Bone and Mineral Research and the Endocrine Society.



Gideon A Rodan received his MD and PhD degrees from the Hebrew University and the Weizman Institute of Science, respectively. He was professor and head of the Department of Oral Biology at the University of Connecticut, moving to Merck & Co. in 1985 where he established the Department of Bone Biology and Osteoporosis Research. His scientific contributions include establishing transformed osteoblastic cell lines, regulation of bone cell metabolism by hormones and cytokines, mediation of mechanical stimulation by cAMP, cloning of alkaline phosphatase, establishing the role of $\alpha v\beta 3$ integrin in osteoclast function, and coauthoring a hypothesis that osteoblasts activate osteoclasts. He was responsible for fostering the development of the first bisphosphonate, alendronate (Fosamax) for the treatment of osteoporosis providing insight into alendronate inhibition of bone resorption in vivo and elucidating the mechanism of action of bisphosphonates. He was an author of numerous peer-reviewed scientific articles, book chapters, invited editorials, reviews, and perspectives and the coeditor of *Principles of Bone Biology*. He served as President of the Gordon Conference on Bones and Teeth, the American Society for Bone and Mineral Research, and the International Bone and Mineral Society and is the recipient of numerous awards. He was an Adjunct Professor at the University of Pennsylvania School of Medicine. He died on January 1, 2006.

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8.17 Omeprazole

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8.17.1 My Early Years in Chemistry

My father was a mechanical engineer but had a special passion for chemistry. He conveyed this interest to me with such enthusiasm that, by the age of 13, when I had my first chemistry lessons at school, I knew that I wanted to be a chemist. On completion of my first degree in chemical engineering at the University of Technology in Lund, Sweden, in 1969, I therefore continued my studies there as a graduate student in the Organic Chemistry Department. I still regard my thesis work on the toxic principle of the mushroom *Coprinus atramentarius*, supervised by Professor Börje Wickberg, as my most important piece of work, which set the tone for the rest of my career.

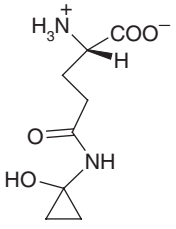
8.17.1.1 My Thesis Work

The goal of my thesis work was to isolate and identify the toxic principle of the gray inky cap mushroom *C. atramentarius*. It had been known for some time that this mushroom was edible and palatable, but if eaten along with alcohol it caused flushing, nausea, vomiting, palpitation, and increased blood pressure. Numerous scientific publications had appeared in the literature going back to the beginning of the twentieth century, describing attempts to isolate the active principle, but without success. Börje Wickberg therefore made it clear at the outset that this project might present a challenge for a graduate student. He was right. It took a year of hard work before I had any breakthrough on how to monitor the isolation work, which we believed to be a prerequisite for future success.

As it was thought that the reaction with the mushroom and concomitant alcohol was caused by inhibition of the liver aldehyde dehydrogenase, I began by testing mushroom extracts for their ability to inhibit the partially purified enzyme from bovine liver, but with no success. After fruitless attempts to establish an efficient collaboration with pharmacologists at the local university, I also introduced my own animal testing (in the chemistry laboratory) and started working with mice, again without success. In my thesis[1] you can find the following footnote: "In a more-or-less desperate experimental situation during present attempts to find the toxic principle, the author ate 300 g of boiled

C. atramentarius and then, on the following day, took 20 cL of ethanol (40%), but no uncomfortable effects were experienced." Luckily, however, I finally developed a test method in rats, in which per oral administration of mushroom extracts dissolved in water was followed by a large dose of alcohol 6 h later. If the extract contained the active compound, the rats developed a tremendous facial edema some 12 h later. Using this test method to monitor the success of extraction and separation techniques, I was able to isolate the active compound in a couple of months.[1,2]

Elucidation of the structure of the active compound was performed in the classical way, and various degradation reactions finally revealed N^5-(1-hydroxycyclopropyl)-glutamine (coprine; **1**), the first (and probably still the only) natural product to be isolated that contains a cyclopropanone equivalent. After initial synthesis of the important fragment, 1-hydroxycyclopropylammonium chloride (**2**) (the free base being unstable), from cyclopropanone, concentrated ammonia and concentrated hydrochloric acid, I was able to synthesize the compound via an efficient photolysis reaction. By acylation of 1-hydroxycyclopropylamine by means of N^1-phthaloyl-glutamic acid anhydride, coprine could be synthesized in good yield.[1,2]



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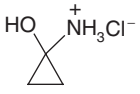

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8.17.1.2 My First Pharmaceutical Project

In 1974, Börje Wickberg and I, along with Professor Arvid Carlsson (Nobel Laureate in Medicine in 2000) of the Department of Pharmacology, University of Göteborg, established an industrial collaboration, supported by the Swedish Board for Technical Development (STU) and sponsored by the Swedish pharmaceutical companies Astra and Kabi, with the aim of developing a new alcoholic deterrent with fewer side effects than disulfiram (Antabuse, the alcoholic deterrent used most commonly at the time).[3] An additional chemist, Rolf Bergman, was employed and worked under my supervision in this program. Our work focused on making analogs in a structure–activity relationship program and on the development of a large-scale synthetic pathway to the cyclopropanone moiety of coprine (i.e., compound **2**) and hence to coprine itself, which we eventually synthesized in a 250 g scale for toxicity studies. Our effort was aided on the biochemical side through collaboration with Dr Olof Tottmar (and his graduate student Hans Marchner[4]: in 1979, Hans Marchner defended his thesis on the mode of action of coprine and 1-aminocyclopropanol) at the University of Uppsala. Tottmar was an expert on liver aldehyde dehydrogenase and inhibition of this enzyme by Antabuse and other chemicals.[5]

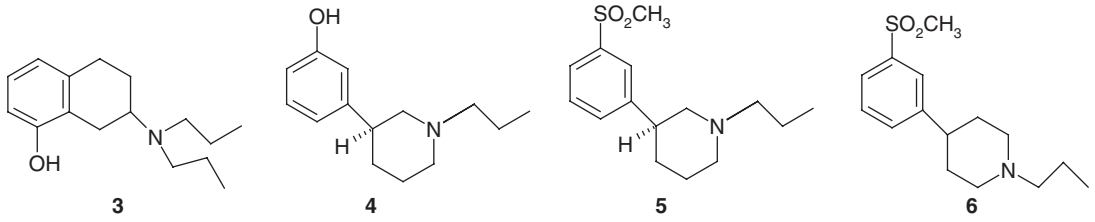
The pharmaceutical project was discontinued in 1977 due to the unacceptable chronic toxicity (testicular lesions) of coprine in both rats and dogs.[6] *C. atramentarius* has since been considered as toxic in mushroom handbooks, leading one to speculate about how many edible and palatable mushrooms are chronically toxic without giving any acute symptoms.

8.17.1.3 Moving to Göteborg to Work at the Department of Pharmacology

At the beginning of 1976 I was offered a job as Head of the Organic Synthesis Unit in the Department of Pharmacology at the University of Göteborg, and started the new post in June 1976, more than a year before my dissertation. My role was to lead the synthetic chemistry group, in which Håkan Wikström was a graduate student. From the outset, we collaborated closely with Professor J Lars G Nilsson and Uli Hacksell, graduate student, in Uppsala. Lars Nilsson (who, after a single meeting at a symposium and a recollection that I was able to give an interesting talk on my lack of results in my early *Coprinus* work, was instrumental in my recruitment to Göteborg and at the same time initiated the fantastic Göteborg–Uppsala network collaboration) was a major influence on me and from him I learned a lot about leadership and the important factors for a creative climate.

Our joint work resulted in the identification of several central nervous system (CNS)-active compounds with novel and highly interesting pharmacological profiles. Some of these compounds have since become important and widely used pharmacological tools and reference compounds. For example, 8-OH-DPAT (**3**), a selective 5-HT_{1A} agonist, has

been mentioned in more than 5000 publications to date,[7] and structural modifications to (–)-3-PPP (**4**), a selective dopamine autoreceptor agonist,[8] during the past 10 years have led to the development of the so-called dopamine stabilizers, such as (–)-OSU 6162 (**5**),[9] and ACR 16 (**6**).[10]



We were granted substantial financial support from STU, enabling the group to expand on both the chemistry (Dr Domingo Sanchez in Göteborg and Lars-Erik Arvidsson, graduate student, in Uppsala) and pharmacology sides (Stephan Hjorth and Kjell Svensson, graduate students). The medicinal chemistry work received strong support from Arvid Carlsson, was highly productive, and resulted in numerous publications. Furthermore, in collaboration with Astra, we filed about 10 patent applications on various groups of compounds within the dopamine and 5-hydroxytryptamine areas.

8.17.2 My Employment at Hässle

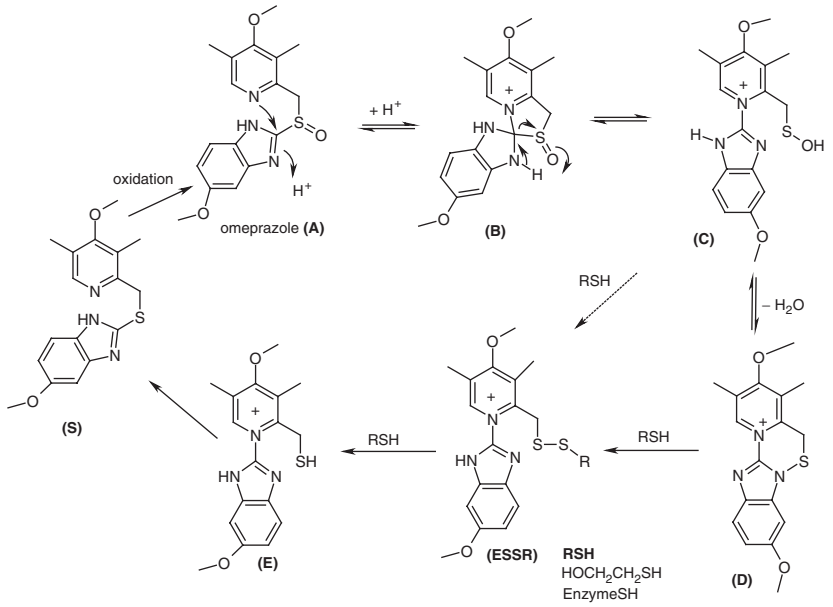
In 1982, the opportunity arose for me to join Hässle AB (within the Astra group) as one of the section heads of the Organic Chemistry (Medicinal Chemistry) Department, where I took on responsibility for the chemistry related to the recently synthesized acid secretion inhibitor omeprazole. In addition to chemical work, I initiated a project in 1983 to elucidate the mechanism of action of omeprazole at a molecular level, to add to the knowledge of its biochemistry and chemical reaction kinetics. In this work, a fruitful collaboration was developed with Björn Wallmark, biochemist, and Arne Brändström, medicinal chemist. Björn Wallmark, with his outstanding scientific knowledge and enormous interest in new ideas, having a generous and humble attitude and a direct and consistent management style, demonstrated personally that it is possible to inspire respect in a whole organization and create stability by mere presence. Arne Brändström showed me how useful it can be to introduce kinetic data in mechanistic thinking and how much fun we could have working on the mechanism of action of omeprazole.

8.17.2.1 The Unique Action of Omeprazole

The success of omeprazole in the clinic could be ascribed to the very effective inhibition of gastric acid secretion achieved through specific inhibition of the gastric H^+,K^+-ATPase, which constitutes the gastric acid (or proton) pump. In whole-body autoradiography in mice, using ^{14}C-labeled omeprazole, the radiolabel was confined to the gastric mucosa, and further studies showed that omeprazole only binds to the H^+,K^+-ATPase in the gastric mucosa.

The elucidation of the mechanism of action of omeprazole became a thrilling task, spurred on by competition with many other pharmaceutical companies. Omeprazole was known to be unstable in acid, with a half-life of 2 min at pH 1 but about 20 h at pH 7. Of crucial importance was the simplification of the decomposition by adding β-mercaptoethanol to the acid before the addition of omeprazole (**A**) (**Scheme 1**). This caused only two compounds to form: the sulfide S and an adduct with β-mercaptoethanol. X-ray analyses of this adduct, as well as an unstable intermediate, eventually revealed their structures as the disulfide **ESSR** and the sulfenamide **D**, respectively. As the H^+,K^+-ATPase inhibition was associated with modification of mercapto groups in the enzyme, the disulfide adduct (**ESSR**) was considered as a model of the enzyme–inhibitor complex, and the sulfenamide (**D**), or possibly the sulfenic acid (**C**), as the active inhibitor, binding covalently to cysteine residues in the H^+,K^+-ATPase. The identity of the active inhibitor remains a topic of debate, with Professor George Sachs of the University of California, Los Angeles, US, one of our most important scientific consultants, coming down strongly in favor of the sulfenic acid.[11]

By April 1984, after x-ray investigations we had the necessary knowledge to propose this inhibition mechanism, as well as the reaction mechanism for the acid transformation of omeprazole (**A**) to the sulfenamide isomers **D**, as outlined in Scheme 1.[12,13] The uniqueness of omeprazole is that it is inactive per se, essentially stable at neutral pH, and accumulates in the acid space of the parietal cell, where it is rapidly transformed into the active inhibitor close to its target enzyme, via an acid-catalyzed reaction. The active inhibitor reacts rapidly with mercapto groups on the H^+,K^+-ATPase, forming a covalent inhibitor complex (**Figure 1**).[14,15] Largely as a result of our work on the



Scheme 1 Mechanism for the transformation of omeprazole in acid. Formation of the active sulfenamide (or, alternatively, the sulfenic acid), the inhibitory action with the enzyme, and cleavage of the enzyme–inhibitor complex by the mercaptan (the 'Omeprazole Cycle').[12,13]

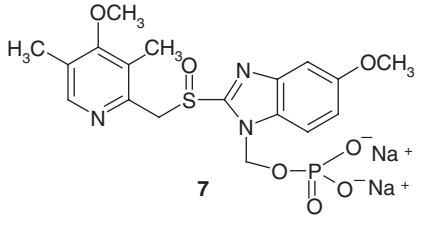
elucidation of the mechanism of action of omeprazole, Arne Brändström, Björn Wallmark, and I were jointly awarded the Wilhelm Westrups Prize in Lund, Sweden, in 1993.

We filed a patent application on the sulfenamides in June 1984. Dramatically, at a medicinal chemistry symposium in Cambridge, UK, in September 1985, Björn Wallmark and I gave a joint oral presentation on the omeprazole mechanism[16,17] and, at the following poster session, Byk Gulden and SmithKline Beecham presented a poster with essentially the same mechanism.[18]

Omeprazole was launched as Losec in Europe in 1988 and as Prilosec in the US in 1990. The product rapidly became a success under the guidance of Enar Carlsson, 'Mr omeprazole,' with his broad knowledge, enormous feeling for what is important, tremendous sense of good judgment, and ability to create an unforgettable climate in the Gastrointestinal (GI) Management Team in Astra. In 1996, omeprazole became the world's biggest selling ever pharmaceutical and, by 2004, over 800 million patients worldwide had been treated with the drug.

8.17.2.2 Omeprazole Prodrug for Parenteral Use

In the mid-1980s a special project was devoted to the development of a prodrug of omeprazole (itself being a prodrug) for parenteral administration, both intravenously and intramuscularly. The challenge was to make the compound much more water-soluble (the entire dose for humans soluble in 1–2 mL), while ensuring its chemical stability. We established a close collaboration with one of the best-known scientists in the prodrug area, the late professor Hans Bundgaard of the Farmaceutiske Höjskole in Copenhagen, and, during discussions with him, the idea of making a phosphate ester prodrug of omeprazole was suggested. After about 1 year of effort we were successful in synthesizing a disodium phosphate prodrug of omeprazole (H229/29; compound **7**).[19] This fulfilled all the criteria we had sought, including extremely high water solubility, high chemical stability, rapid and quantitative in vivo conversion to the parent compound omeprazole, and with only two endogenous compounds (the phosphate and formaldehyde) formed in addition.



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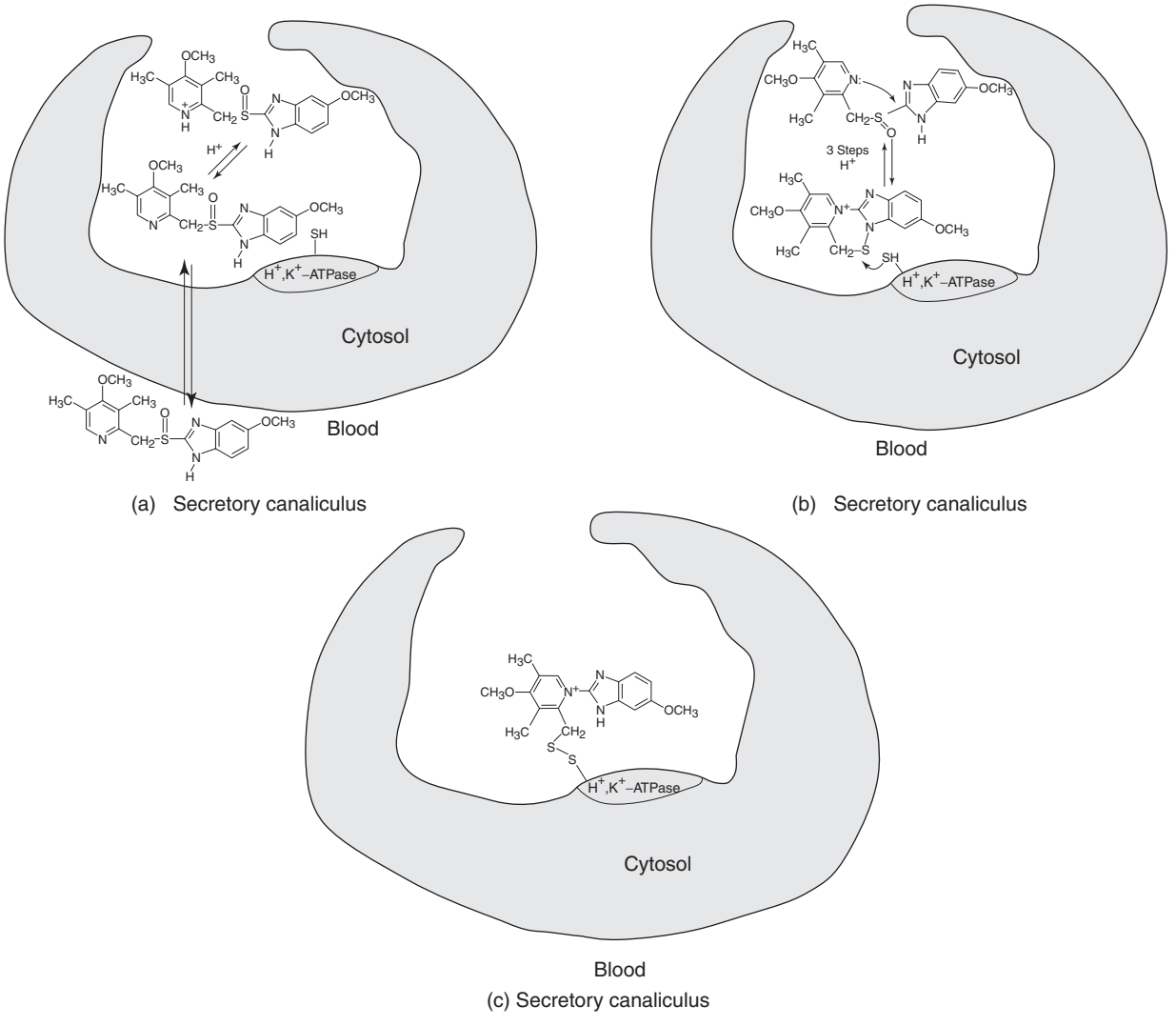
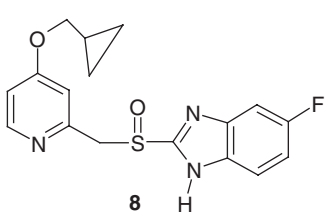


Figure 1 Events leading to inhibition of gastric acid secretion by omeprazole within the parietal cell. (a) The concentration of the protonated form of omeprazole in the acidic compartment; (b) the transformation of omeprazole to the sulfenamide and the inhibitory reaction; (c) the structure of the enzyme–inhibitor complex. (Reprinted from Lindberg, P.; Brändström, A.; Wallmark, B. *Trends Pharmacol. Sci.* **1987**, *8*, 399–402, with permission from Elsevier.)

8.17.2.3 Esomeprazole – The Follow-Up

Although omeprazole provided more effective control of acid secretion than previous therapies, it was not equally effective in all patients. Our next goal was therefore to find a compound with improved pharmacokinetic and metabolic properties that exhibited increased bioavailability, and reduced the interindividual variation in effectiveness observed for omeprazole. A focused research program began in 1987 to find new protein pump inhibitors (PPI) that fulfilled these requirements. After initial investigations, we decided to keep the basic structural framework of omeprazole, and to vary the substituents on the pyridine and the benzimidazole rings, with the aim of altering the metabolic pathways or decreasing metabolism relative to omeprazole and thereby increasing bioavailability and effectiveness. Under the leadership of Gunnel Sundén, who confirmed my expectation that one can become an excellent leader of a project without having a doctor's degree and gave me inspiration to try new things in my role as the leader of GI Medicinal Chemistry, H 259/31 (**8**) and H 326/07 (**9**) became two promising candidates with high bioavailability, and both were tested in humans. Finally, however, considering all relevant parameters, only one compound exceeded omeprazole. It was H 199/18 (**10**), the *S*-(–)-enantiomer of omeprazole or esomeprazole (initially named perprazole) as alkaline salt.[20–22]



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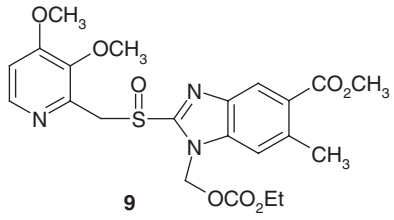

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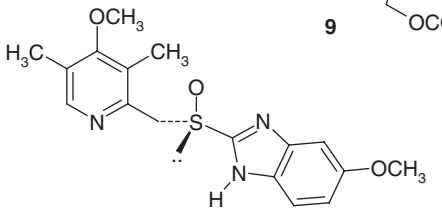

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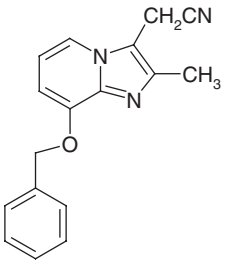
But why had we not considered the isomers of omeprazole earlier at the start of the follow-up program? Our knowledge of the mechanism of acid inhibition had led us to predict that both isomers of omeprazole would have exactly the same effect, and that acid-catalyzed conversion of either isomer to the same active nonchiral sulfenamide would occur at the same rate. Also, as we had earlier observed racemization of the isomers in vitro, we believed that the single enantiomers would racemize too easily. Furthermore, only milligram quantities of the partially purified isomers of omeprazole had been available, so this did not seem a very attractive option.

Still, I and pharmacologist Lars Weidolf sought to explore possible differences in pharmacokinetics between the two isomers. Using a new type of chromatographic separation, one of my co-workers, Sverker von Unge, was able to isolate hundreds of milligrams of the single isomers, and we also found that alkaline salts of the isomers were stable against racemization. On testing such salts in the rat, the *R*-isomer showed higher bioavailability than the *S*-isomer. However, to our great surprise, it was the *S*-isomer that gave the highest bioavailability in humans, with an area under the plasma concentration curve (AUC) 4–5 times greater than with the *R*-isomer and about double that of the racemate. Thus, the alkaline salt of the *S*-isomer fulfilled our aim to identify a compound with significantly higher bioavailability than omeprazole. In addition, it showed higher AUC and oral potency than that of omeprazole, which was an extra bonus.[23–26] Furthermore, the *S*-isomer showed much less variability in AUC between poor and extensive metabolizers (the polymorphism which emerged to be the major reason for the interpatient variability observed with omeprazole) than either the *R*-isomer or the racemate, omeprazole. Based on these findings, the *S*-isomer of omeprazole (in its alkaline salt form) was chosen as a candidate drug (CD), and was subsequently demonstrated to provide a significant clinical advance over omeprazole and other PPIs in direct comparative clinical studies.[27–32] Interestingly, in later studies in the dog, no significant difference in efficacy could be detected between the two isomers. If the initial in vivo experiments had been performed solely in dogs, we would probably have stopped further work with the isomers.

Esomeprazole magnesium salt (Nexium) received approval in Sweden in August 2000, in the rest of the European Union during the fall of that year, and was approved and launched in the US early in 2001. It quickly became a blockbuster, with total sales of more than US$4 billion.

8.17.2.4 Reversible H^+,K^+-ATPase Inhibitors

Our interest in acid control also led us into work on a reversible inhibitor of H^+,K^+-ATPase. In 1982, the year I came to Hässle, Schering Plough reported effective inhibition of gastric acid secretion in humans with a non-H_2-blocker, SCH 28080 (**11**), a compound with an imidazopyridine skeleton. Based on our current understanding of the importance of the sulfoxide group for the action of omeprazole, we concluded that this compound must have a different mechanism of action. However, in our efforts to synthesize SCH 28080 (**11**), we failed in the final step, the conversion to the 3-cyanomethyl substitution. If, at that time, we had tested the final intermediate, which had a 3-hydroxymethyl group, we would have revealed the potent, reversible, and K^+-competitive, H^+,K^+-ATPase-inhibitory effect of these compounds more than 1 year earlier than we did. Instead, it was not until we received a sample from Schering Plough (late in 1984) that we obtained this knowledge and it became clear to us that they were well ahead of us in this research. We therefore initiated contacts with Schering Plough, only to discover that they had discontinued their research in the area and were open to collaboration. There followed a very fruitful and positive exchange of knowledge and material, especially with Dr Jim Kaminski, a medicinal chemist at Schering, US. Having evaluated the imidazolpyridine/imidazopyrazine compounds from both pharmacology–toxicology and business perspectives, we decided to continue in the area of reversible PPIs, but based on alternative structural templates, such as benzimidazoles (**12**) and 7-azaindoles (**13**).



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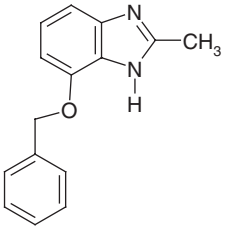

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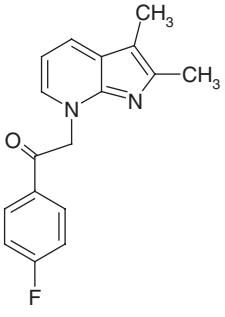

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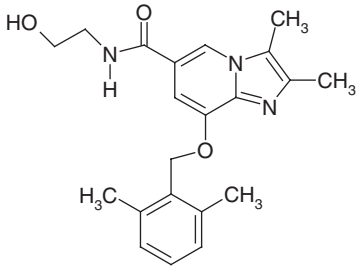
This project formed an important part of the research within my department[33] and I put considerable effort into it during the subsequent years. A number of CDs were selected but all of them were discontinued for potency, toxicological, or related reasons. In the mid-1990s, a few years after I left the Medicinal Chemistry Department, the unusually creative medicinal chemistry team of Ingemar Starke and co-workers returned to imidazolpyridines once more. The important, unexpected, 10-fold increase in potency from a 2,6-disubstitution in the benzyl moiety, and the observation that a substituent in the 6-position had a propensity to reduce certain liver-related side effects (a common problem with these imidazolpyridines), paved the way for selection of a number of CDs of the imidazopyridine class.

In my current role as senior scientific advisor, I am partly involved in this project again, 20 years after its start, and can see the promise of these compounds,[34] now named potassium-competitive acid blockers (p-CABs), as extremely efficient acid secretion inhibitors in the human. Linaprazan (**14**) is an example of a p-CAB. The dual impact of the efficient protonation of the 1-nitrogen of the imidazolpyridine nucleus (with a pK_a of about 6) – by providing both the protonated active species and the 'superaccumulation' of this species in the extracellular acid space of the parietal cell – may explain the very efficient inhibition of acid secretion with these compounds. This differentiates the p-CABs from the PPIs, where the protonated and accumulated forms are not themselves the active species.



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8.17.2.5 Preclinical Alliances Group and Scientific Patent Support

In 1993 I took up a new position within the company, with responsibility for the Preclinical Alliances Group, covering both the gastrointestinal and cardiovascular areas. It was recognized that there was a need to map the external scientific community in a more systematic way and to bring more innovative projects and ideas into our exploratory preclinical research, both from universities and industry. The aim was to establish new scientific collaborations, which involved contacts with external and internal scientists, business representatives and lawyers, evaluation of the ideas and their patent situations, and participation in business negotiations, and during which I had the honor to collaborate with the former Head of Clinical Research at Hässle, Professor Gillis Johnsson, who taught me a lot. The job was interesting and stimulating and I am sure that I would still be working in this group if not 'drafted,' in 1998, into the Scientific Patent Support Team for the worldwide omeprazole litigation. This team included both chemists and pharmacists to help the local litigation teams and to educate and support the lawyers in relevant areas.

8.17.3 Learning Points

8.17.3.1 Creativity

My experience has shown that a prerequisite for success in medicinal chemistry is creativity, which is facilitated by a creative environment and fueled by enthusiasm. There are two kinds of creativity: (1) the ability of an individual to generate ideas (personal creativity); and (2) group creativity, when two or more people work together to generate ideas. Both are important in pharmaceutical research. The first has much to do with personal history and the ability to make associations and apply previous experience to current tasks or problems. I believe that changing jobs during your career may have a positive influence on personal creativity, by enriching your personal background. Although the way that ideas from personally creative people are handled within an organization is very important, it is group creativity that is far more sensitive to organizational factors. The mere nature of industrial pharmaceutical research, which involves scientific networks of specialists in various disciplines and highly interesting, exciting, and challenging work, does have the inherent power to provide excellent opportunities for superior group creativity. However, a prerequisite is to achieve a creative climate within the group of people and, as discussed below, such a climate is extremely sensitive and easy to destroy.

During my career, I have had great pleasure to be part of a number of creative collaborations. I would specifically like to mention two people: firstly, Håkan Wikström (as a graduate student) during my 6 years with Arvid Carlsson; and secondly, Arne Brändström during my first 10 years with Astra. I had daily early-morning discussions with these two people, who were both extremely creative and, in most cases, I took on the moderating role. Wikström was especially creative when it comes to the small, narrow, and, with hindsight, simple ideas, and we had many exciting and productive discussions, the majority with chirality and the stereochemistry of dopamine and serotonin agonists as the theme. During our unusually exciting work on the elucidation of the mechanism of action of omeprazole, Brändström, with his tremendous knowledge in all areas of chemistry, came up with new results and thoughts on the reaction-kinetics more or less every day, and I tried to fit these together with possible molecular mechanisms. When Brändström is stimulated by questions and provocations, he becomes immensely productive.

I believe that most people who have been in research experience moments in their lives that, retrospectively, have been of particular importance. I remember, with some amusement, a couple of episodes when I became particularly excited about chemical structures. One is from 1977, early in my time with Arvid Carlsson, when I was walking along the seaside with a paper that included the stereostructure of the serotonin agonist lysergic acid diethylamide (LSD) on top of the baby carriage in which my newborn daughter was sleeping. It became clear to me, by imagining the structures in front of me (with the sea in the background), that the aminotetralin moieties in LSD and apomorphine, a dopamine agonist, actually have opposite stereochemistries.[35] This had probably been realized by many other chemists before me (although perhaps not communicated). However, it became an important and exciting starting point for the novel way our research group regarded our newly synthesized 5-, 6-, 7-, and 8-hydroxy-2-dipropylaminotetralines (all as racemates), of which the latter (8-OH-DPAT; **3**) had just been proven to be an extremely potent serotonin agonist (see above), while the other three were dopamine agonists of similar potencies (!). Of course, it also had a great impact on the way we looked at the 3-phenylpiperidines, such as 3-(3-hydroxyphenyl)-*N*-propylpiperidine (3-PPP; **4**), and we decided to resolve all active compounds synthesized, which so far had been unresolved.[36]

The other episode is from 1983, at the start of our work on the mechanism of action of omeprazole, when I had brought home with me the available nuclear magnetic resonance (NMR) spectra of sulfoxides, sulfides, and mercaptoethanol adducts, all with substituents corresponding to each other. When, late in the evening, I compared how

the NMR shifts for the various analogs moved when going from the sulfide to the corresponding adduct, I suddenly realized that our internally well accepted theory of how the structure of the important mercapto-adduct (understood by us at that time to be a model for the enzyme–inhibitor complex) must be wrong, and that the pyridine moiety must be more heavily involved than we earlier thought. I poured out a 'wee dram' (of Scotch whisky), started to draw new possible reactions and structures, and became more and more excited. After a couple of hours (and drams) late at night, I thought I had the solution and would refer to this as 'the wonderful night.' The next morning, I started to write all the reactions on my white board and when Arne Brändström came into my room (as usual), I went through it and he accepted it. Fine! However, it subsequently turned out to be wrong again. This was not discovered until 6 months, and then my 'spiro' intermediate proposal (later published by others[37]) happened to be close enough for us to be able to circumvent the vast stability problems with the crystals of the intermediate, so we were eventually able to obtain crystals good enough for an x-ray study.

8.17.3.2 Creative Climate

My experiences of working with a number of creative people have led me to speculate about the determining factors that are the most important for cultivating a creative climate within a group of people. In my opinion, these are a suitable leadership (if there is a group leader), direct information, no hierarchy, no 'stolen' ideas, a high degree of openness, honesty, generosity, lack of prestige, and, finally, a sense of humor. Thus, it is important that all relevant information reaches the team members simultaneously, so that no one feels handicapped compared with the others. The leader of the group should convey enthusiasm for new ideas coming from the other team members, have a rewarding attitude, and provide room for mistakes. The innovator should always be named the first time his or her idea is mentioned by somebody else to an uninitiated person or a group. The leader may even teach this need for openness and generosity, in order to prevent any feelings that ideas can be 'stolen.' A flat organization within the group, without hierarchy, will facilitate direct information flow and ensure that ideas are dealt with positively. This provides a good climate for immediate release of new ideas, which is important for speeding up cross-fertilization within the group. It is therefore possible that the modern Anglo-American bonus model, which prioritizes only personal benefits, may have a negative impact on group creativity and provide incentives to conceal brilliant ideas for some time. The old Swedish model, with equal reward-sharing across the group, may have advantages here.

A creative climate is, of course, advantageous in all research organizations but it would be no exaggeration to state that creativity is compulsory for medicinal chemists in the pharmaceutical industry, where those involved must take full responsibility for generating new ideas concerning chemical structures. As the compounds they generate must be patentable, they also need to be inventive. Most of the ideas and creative work leading to new inventions are focused on problem-solving. This is reflected in the way a patent application is built up: a problem is presented, followed by information on the invention that provides a solution to the problem. A more difficult creative act, however, is to define the problem, and this can often be the great invention! Mats Sundgren has recently completed his thesis work on organizational creativity in pharmaceutical research and development.[38] Based on the interviews he conducted at pharmaceutical industries in Sweden, the UK, and the US, creativity is the most important thing for securing success, but there is hardly anyone who talks about this. The demands concerning effectiveness in drug development projects have increased markedly, and this has led to more and more detailed project planning. Sundgren concluded, however, that planning for what is going to happen comes from the generation of ideas, and the effectiveness comes from creativity. He therefore stressed the need for a balance between effectiveness and organizational creativity.

8.17.3.3 Enthusiasm

In the pharmaceutical industry a core activity is to synthesize compounds and test them in biological models. While there are many novel techniques available today compared with 10–20 years ago, these are most relevant to the very early phases of discovery. When progressing towards registration, there are few shortcuts. Indeed, the demands during the late phases are much tougher than before and, ultimately, it is the patent-protected compounds that are registered as new drugs that count, not the techniques that were used. If we are not developing compounds and testing them, there will be no new drugs. The laboratory work, which is very time-consuming, has to be done, but is also sensitive to disturbances.

In the pharmaceutical industry of today, you can spend much of your time reading and sending e-mails, informing yourself via the intranet and internet, and going to meetings, seminars, symposia, and courses. As a result, many people feel stressed and frustrated, yet most of these activities are, in themselves, useful and necessary. We cannot blame the surrounding world, but must decide for ourselves how our time should be used. We all know this, but what can we do?

In my view, the only thing that can meet and counteract this 'development' is increased enthusiasm to achieve the required goals. Prohibitions, rules, and limitations belong to the past.

A prerequisite for the generation of enthusiasm is that your manager (or evaluating person) shows interest in the work that you do and notices the results you achieve. Other important factors include the degree of participation, immediate information on results without having to wait for a meeting, the feeling of belonging to a group, and a feeling of urgency. For scientists, participation in scientific symposia may be a positive factor in generating enthusiasm, enabling you to meet with your competitors and perhaps become aware that you know things that they don't.

8.17.3.4 Patents

Scientists, especially medicinal chemists, should also take an enthusiastic interest in the patent work. Patents can be as important for the company as the compounds and drugs, and I believe that the effort put into the 'patent work' is not always as optimal as it should be.

Patent work in pharmaceutical research has two quite different aspects: the patent professional (attorney) and the scientific. A comparison can be made with the structure–activity relationship work, which also involves two sides: the biological and the chemical. In both cases, a close collaboration between the two, involving a 'bridging over' of competence, is important in determining a positive outcome. From the chemical side, this has even led to the creation of the special discipline of medicinal chemistry. As far as patent work is concerned, the attorney's basic education has generally included science, but the scientists involved do not have the corresponding education about patents. My impression is that there is normally a nonoptimal balance of bridged input in patent work in pharmaceutical research. It is therefore important for scientists, mainly medicinal chemists, to increase their patent knowledge and to show enthusiasm for patents. Furthermore, patent work and initiatives should be driven from the scientific side, which would, no doubt, provide more optimal discussion partners for the attorneys.

8.17.4 Future Perspectives of Medicinal Chemistry

8.17.4.1 Visions for the Future

Unfortunately, I am not optimistic about the future of industrial pharmaceutical research. As well as escalating demands on safety and the introduction of reference price systems, which result in increased costs and decreased profitability, there is a continuing decline in productivity, measured as new drugs coming to the market. Moreover, if a drug is successful in getting to the market, there is an increased threat of challenges to, and invalidations of, the patents. It also seems likely that reference price systems for drugs will change the basis of research in the pharmaceutical industry, which has for many years been dominated by analog-based drug design that aims to improve on already-existing medical principles. Effort will now need to switch to pioneer or first-in-class drug research, which requires a high level of innovation. However, I believe that there is a general decline in the innovative climate in the major pharmaceutical companies.

I fear that the general decrease in productivity may be related to an increasing focus on molecular biology-driven, target-oriented drug discovery, in which valuable and important techniques are frequently being used out of perspective. The approach has been heavily criticised, as highlighted by the following citations:

> The elegance of these techniques is seductive – so much so that I believe they are taken too readily as valid models of disease for evaluating drugs[39]
>
> …we are whole animals and if you do your experiments on isolated cells and the tissues can't talk, then you tend to get results that may not be representative of the whole animal[40]
>
> …the complexity of the in vivo situation cannot be mimicked. Also, cell phenotypes that develop outside the body (i.e., in vitro) might exist exclusively in the test tube[41]
>
> But what the in vitro system cannot do is construct a functional and valid in vivo biochemistry. And that is potentially a fatal flaw. For in most human diseases it is the functional biochemistry and not the anatomical biochemistry which goes wrong[42]
>
> A receptor molecule is a very dynamic molecule, built to undergo conformational change with lightning speed. That it can look like the same after removal, grinding and suspension of the tissue is a preposterous belief.[43]

The industry has invested heavily in high-throughput chemistry, computational chemistry and various enabling in vitro techniques, including HTS, supported by vast substance libraries with special delivery facilities. Furthermore, there has been a concomitant reorganization of scientists into specialist functions along the generally accepted drug discovery

time-line, not unlike assembly lines in car production years ago. I believe this is a research trap which it is not easy to escape and, in the meantime, resources are probably being misspent.

During my recent discussions with Arvid Carlsson, he presented convincing arguments in favor of in vivo screening in drug research. Carlsson Research Company has synthesized compounds, such as (–)-OSU 6162 (**5**) and ACR 16 (**6**), which are so-called dopamine stabilizers. If the release of dopamine is too high, these compounds will decrease it, and if the release is too low, they will increase it to an acceptable level. Thus the same compounds can either block or stimulate dopamine receptors in the CNS. In test studies in patients seriously ill with Parkinson's disease and Huntington's chorea, (–)-OSU 6162 has shown close to miraculous effect, as well as clear positive effects in schizophrenic patients. However, a fundamental characteristic of these compounds is that they have low, or even zero, affinity for the receptors in classical binding models, and would therefore be considered inactive and uninteresting in an HTS screen!

These findings have more general implications for in vitro versus in vivo testing. Cloning may be used to provide a chemically homogeneous form of a particular dopamine receptor, for example, although this receptor may not be functionally homogeneous in vivo. According to Arvid Carlsson, receptors in vivo have a fabulous ability to adapt themselves functionally to the concentration of their relevant transmitter substances, in this case dopamine, which may, perhaps, vary 1000-fold within the synaptic area. This adaptation is not a matter of receptor density but is functional and may have an evolutionary background, when the original concentration of the transmitter may have been zero. In the presence of different concentrations of the transmitter, the same receptor is now able to act either as a stimulator or as an inhibitory autoreceptor. Binding of antagonists to these receptors with different functions also appears to be different.

In my view, it would therefore not be surprising to find that results from HTS screening to identify micromolar affinities for a particular dopamine receptor may not transfer well to an in vivo situation. Similarly, gene knockout animals and cell lines as models for diseases have frequently proven to create misleading artifacts. Furthermore, there are known paradoxical pharmacological effects, which may have similar background, that result in the same compound acting as a full and partial agonist and antagonist at different functional states of the 'same' receptor. For example, from my own experience, the potent 5-HT_{1A} agonist 8-OH-DPAT (**3**) showed a strong stimulatory effect on the sexual behavior of rats.[44] This was surprising, since stimulation of serotonin neurons normally inhibits sexual behavior, as in the case of selective serotonin reuptake inhibitors, for example, where sexual disturbances are among the common side effects.

Some of the most successful medicines are remarkably weak or nonselective, and many are active on several receptors. I believe there is a low probability that we will identify optimal drugs that are active only on one receptor, particularly for CNS-active compounds. Such compounds are, of course, excellent pharmacological tools, but most diseases are related to unbalanced multilocalized abnormal biochemical patterns. However, HTS screening of today would not rate weakly binding, nonspecific compounds as 'hits.' Ironically, omeprazole, one of the world's biggest selling drugs, is probably one of the best examples of a drug that would not have been discovered by using HTS screening. As a targeted prodrug that is only slowly converted to the active species at about neutral pH, omeprazole would not have been potent enough to become a 'hit' when applied to an H^+,K^+-ATPase enzyme-screening model.

8.17.4.2 Innovation

The risk is that the current decline in productivity in the big pharma industry is putting more and more pressure on effectivity, with research dominated by targets for delivery of numbers of synthesized compounds, CDs, and patent applications at predestined deadlines. Although high productivity in the laboratory is important, such benchmarking may be at the expense of new thinking and innovation. I believe that organization of drug discovery work according to time-lines (e.g., hit identification, hit to lead, lead optimization, etc.), with different people becoming specialized in each phase, is detrimental for the development of chemists and may lead to decreased enthusiasm. Broad competence in medicinal chemistry has, in the past, been important not only in the pioneering structure discovery and optimization of potency and selectivity, but also for the generation of new, important, 'simple' ideas, and in the difficult tasks of improving pharmacokinetics, metabolism, and bioavailability and avoiding toxicological problems. Most experienced medicinal chemists know that the efforts required to progress from the first CD in a project to a final, useful CD may be tremendous, and that the timeframe cannot easily be scheduled. For example, in 1984, we were already aware that SCH 28080, mentioned above, was an H^+,K^+-ATPase inhibitor. Thus, we knew its target, site of action, mechanism of action, and that it had a potent and desired effect in humans. In addition, we had several lead compounds and a full setup of test models, both in vivo and in vitro. Despite all these enabling prerequisites, it has taken 20 years to get to where we are today, with a CD under development.

My hope for the future is that soon the pendulum will swing back, and we will once again focus on biological activity and in vivo screening. In the meantime, we need to safeguard the classical medicinal chemistry discipline and to improve it with better knowledge about patents and increased understanding of the factors of importance for nurturing creativity and enthusiasm.

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Biography

Per Lindberg graduated in organic chemistry from the University of Technology Lund, Sweden, in 1977 and was appointed Associate Professor of Organic Chemistry at the Chalmers Institute of Technology, Gothenburg, Sweden, in 1982. After working for several years with Prof Arvid Carlsson (Nobel Prize winner in Medicine, 2000) in the Department of Pharmacology, University of Gothenburg, he joined Hässle AB (today AstraZeneca R&D Mölndal, Sweden) in 1982 as Head of Medicinal Chemistry GI and held that position until 1993. He was involved in several projects, including the development of the first proton pump inhibitor omeprazole (Losec) and elucidation of its mechanism of action, as well as the follow-up project to develop esomeprazole (Nexium). From 1993 to 1998 he was Director of the Preclinical Alliances Group. In 1998, he became Scientific Advisor in the Scientific Patent Support Team directed to patent litigation of omeprazole and, since April 2002, has been Senior Scientific Advisor within the Gastrointestinal Therapeutic Area. He has been the Scandinavian representative in the IUPAC Medicinal Chemistry Section since 1990, and is author of numerous publications in medicinal chemistry and many inventorships, including co-inventorship of esomeprazole.

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8.18 Calcium Channel α_2–δ Ligands: Gabapentin and Pregabalin

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8.18.1 Early Preclinical Work Leading to the Development of Gabapentin

Gabapentin was conceived as part of a drug discovery program to treat neurological diseases, including epilepsy, spasticity, multiple sclerosis, and other central nervous system (CNS) disorders. This program began in the early 1970s at the German company, Goedecke, A.G., in Freiburg, Germany, which was a part of Warner-Lambert (now incorporated into Pfizer). The history of this project included chemical attempts to inhibit γ-amino-butyric acid (GABA) degradation in brain with compounds that inhibited the catalytic pyridoxylphosphate of GABA-transaminase. It had already been known for some time that GABA was a key inhibitory neurotransmitter, and that experimental chemical impairment of GABA systems could cause seizures in experimental animals. The GABA transaminase project at Goedecke had progressed a compound to phase I clinical trials, but these were halted because of safety concerns. The chemical matter developed within the GABA transaminase project had no direct relationship to the chemical matter that led to gabapentin, although both had a similar conceptual approach based on GABA.

8.18.1.1 Selection of γ-Amino-Butyric Acid as a Mimetic Compound to Target ($GABA_B$ Receptors)

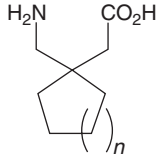
In early 1973, Gerhardt Satzinger of Goedecke conceived a series of about 25 derivatives of GABA that were later synthesized. This project was an attempt to design GABA mimetic drugs acting at GABA receptors that (unlike GABA) would penetrate the blood–brain barrier. At this early time, GABA was only recently acknowledged as an inhibitory neurotransmitter, and there were only a few known GABA agonists, of which baclofen (a selective $GABA_B$ agonist) was one example.[1] Most of the compounds that were later synthesized turned out to be inactive in a $GABA_B$ radioligand-binding assay, but nevertheless several prevented seizures in mice when given systemically against chemical challenge with the GABA synthesis inhibitors mercaptopropionic acid or thiosemicarbazide. The mechanism of action of these anticonvulsant drugs was not known at the time, but there were already several known differences between the Goedecke 3-GABA derivatives and baclofen. Many of the original pharmacological studies compared the effects of gabapentin and related compounds to those of baclofen[2] but there were many differences, particularly a complete lack of gabapentin effects on $GABA_B$ receptors.

8.18.1.2 The Discovery and Structure–Activity Relationship (SAR) of Gabapentin: Serendipity Leads to the Discovery of a New Drug Target

As it is sometimes the case in drug discovery, the discovery of gabapentin was made through a rather indirect path. Since GABA does not penetrate the blood–brain barrier, Satzinger and colleagues directed attention to increasing the oral bioavailability of GABA, by raising the log *P* of its analogs through the incorporation of lipophilic groups on the carbon backbone.[1–3] Although compounds described in Satzinger's original work did possess anticonvulsant activity (as did centrally administered GABA), it was ultimately found that none of these compounds affected either metabotropic or ionotropic GABA receptors, nor fluxed through the blood–brain barrier by passive diffusion. However, it was from these fortuitous studies that (1-aminomethyl-cyclohexyl)-acetic acid (**2**) (gabapentin, Neurontin) emerged as a potent and efficacious anticonvulsant in the thiosemicarbazide-induced tonic convulsion model in mice (**Table 1**). Compared to other analogs in this series, gabapentin clearly had greater activity. Several years later, its anticonvulsant properties were confirmed in the low-intensity mouse electroshock model, where again it proved to be the most potent member within this series. Notably, this early SAR study, driven largely through in vivo work, hinted at the optimal size of the cycloalkyl ring for anticonvulsant activity, with the cyclohexyl (**2**) and cycloheptyl (**3**) moieties most preferred.

As interest in the anticonvulsant properties of gabapentin grew, efforts to identify the molecular target in the CNS were initiated by several research groups. This culminated in the discovery of the calcium channel α_2–δ subunit as the molecular target of this compound.[4] A retrospective study of Satzinger's original work identified gabapentin as the most potent amino acid with regard to affinity for this protein. When studied much later in a radioligand-binding assay,[5] the cyclopentyl (**1**) and cyclooctyl (**4**) analogs showed a two- and 15-fold drop respectively in binding affinity at pig brain membrane α_2–δ sites (measured with [^{3}H]-gabapentin) when compared to unlabeled gabapentin. Thus, this

Table 1 Effect of varying ring size on α_2–δ binding affinity and anticonvulsant action



Compound	*n*	*TSCZ ED_{50} ($mg\,kg^{-1}$ IP)*	*Low-intensity electroshock ED_{50} ($mg\,kg^{-1}$ IP)*	*α_2–δ binding affinity (IC_{50}, nM)*
1	1	<31	NT	260
2	2	5	4.5	140
3	3	<63	~60	110
4	4	>250	NT	1810

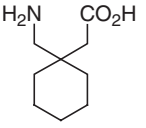
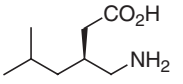
TSCZ, thiosemicarbazide.

serendipitous finding by Satzinger eventually led to the identification of a new drug target and to two new effective therapies for the treatment of human disease.

When compared to commonly used criteria to signal oral druglike properties, the physical characteristics of gabapentin, and its clinical successor pregabalin, are quite unique (Table 2). Both of these amino acids differ, particularly in their Clog*P* values as well as physical state at physiological pH when compared to most other pharmaceuticals. In the case of gabapentin and pregabalin, however, these unusual properties confer advantages, such as no interaction with enzymes of the cytochrome P450 system, no interaction with the hERG cardiac potassium channel (human ether-a-gogo related gene), and no evidence of in vivo metabolism in humans.

Since the original discovery of gabapentin by Satzinger and colleagues, numerous SAR studies have focused on the relatively constrained substructure of the parent amino acid, which possesses only eight carbon atoms within an overall molecular weight of 171. Researchers investigating the SAR of this class of molecules have striven hard to improve upon the in vitro and in vivo profile of gabapentin. Most of this successful work has retained the amino acid functionality within the molecule. To date, only Merck has reported nonamino acid ligands that bind to the α_2–δ protein.[6–8] Since 1988, publications emanating from the α_2–δ arena have grown steadily over time (Figure 1). Most of the SAR work has originated from the laboratories of the former Parke-Davis Research Division (now Pfizer Global Research and Development) and has focused upon understanding the chemical space within amino acids that target the α_2–δ binding site. This work has not only mapped the SAR but has also had the added benefit of enforcing the hypothesis of the α_2–δ as the target protein underlying the mechanism of action.

Table 2 Structure and chemical properties of gabapentin and pregabalin

Property	*Gabapentin*	*Pregabalin*
Structure	 **2**	 **5**
Physical state at physiological pH	Zwitterionic	Zwitterionic
Clog*P*	− 0.66	− 0.92
log P^a	− 1.25	− 1.35
Polar surface area	63.6	63.6
Aqueous solubility	100 mg mL^{-1}	32 mg mL^{-1}
Molecular weight	171.2	159.2
Primary mechanism of absorption	LAT-1 amino acid transport and paracellular	LAT-1 amino acid transport and paracellular

[a] log of *n*-octanol/0.05 M phosphate buffer partition coefficient, pH 7.4.

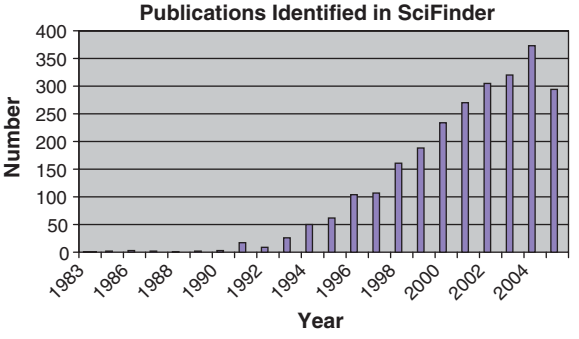


Figure 1 Publications identified in SciFinder including the searched term 'gabapentin.'

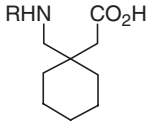
In addition, collective analysis of the SAR reported to date indicate several distinguishing features of this area, including the observation that very subtle changes to the carbon skeleton of amino acids that target this protein result in drastic changes to in vitro and in vivo properties and that the stereochemical disposition of functionality plays a profound role in the SAR.

Examples pertaining to the above points as well as an overall overview of the SAR in this area are presented below.

Satzinger originally conceived a series of functionalized amino acids. This included simple N-alkylation of the amine motif with methyl (**6**), isopropyl (**7**) and *n*-butyl (**8**) groups that led to a two- to 10-fold drop in binding affinity (Table 3). Complete loss of binding was observed with the larger aryl motifs (**9–11**). In addition, the lack of binding following amidation of the amine (**9**) and carboxyl groups (data not shown) indicates a possible need for effective salt bridges between the amine and carboxyl groups with the α_2–δ protein or alternatively reflect the effect of amidation on pK_a.

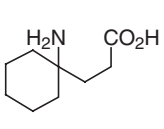
Transposition of the cyclohexyl substituent along the GABA backbone provided further evidence that binding at the α_2–δ protein was sensitive to modification to the parent, gabapentin (Table 4). Shifting the cyclohexyl ring to the 4-position of the GABA backbone gave 3-(1-amino-cyclohexyl)-propionic acid (**12**) which abolished affinity for the α_2–δ subunit. The corresponding 3,4-(**13**) or 2,3-amino acid (**14**) also resulted in a loss of affinity for the α_2–δ subunit by seven- and 40-fold respectively.

Table 3 Binding affinity at α_2–δ of some alkylated analogs of gabapentin

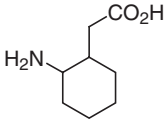


Compound	*R*	α_2–δ *binding affinity* (IC_{50}, *nM*)
2	H	140
6	Me	270
7	*i*-Pr	290
8	*n*-Bu	1070
9	PhCO	>1000
10	Bn	>1000
11	Ph	340

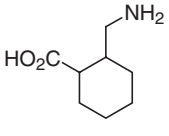
Table 4 Effect of transposition of the cyclohexyl group on the α_2–δ binding affinity of gabapentin



12



13



14

Compound	α_2–δ *binding affinity* (IC_{50}, *nM*)
2	140
12	>100 000
13	890
14	5900

8.18.2 Additional Structure–Activity Relationship at the Calcium Channel α_2–δ Site

8.18.2.1 Studies of Gabapentin Derivatives

Since the original work of Satzinger and colleagues, little was reported from this area until the late 1990s when publications detailing the SAR of gabapentin were disclosed. Researchers at the former Parke-Davis Neuroscience Research Centre in Cambridge, UK, were pioneers as this was the only group publishing SAR from this area at the time.

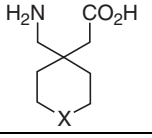
This work mapped out preferred binding conformations of analogs,[9–12] the effect of ring constraints upon affinity for the α_2–δ subunit,[12] the inclusion of alkyl motifs within the cyclohexyl ring,[13] and the effect of incorporating heteroatoms in the cyclohexyl ring (Table 5).[13] Heteroatoms studied included nitrogen, oxygen, and sulfur at the 3′-position of gabapentin. None out of pyran (**15**), thiopyran (**16**), or piperidine (**17**) analogs showed enhanced affinity for the α_2–δ protein when compared to the parent carbon analog (**2**). The Parke-Davis group postulated that, with the increasing polarity of the heteroatom, the affinity for the binding site decreases. This suggested that the 3′-position of gabapentin may access a hydrophobic domain within the α_2–δ subunit.

In the late 1990s, the group significantly expanded knowledge of the preferred binding conformations of gabapentin and its analogs using a thorough assessment of the preferred binding conformation of the cycloxhexyl ring and aminobutyric acid moiety. This work, detailed in a series of papers between 1997 and 1999,[9–12] shed light on how certain conformations of analogs affected binding to the α_2–δ protein. With chair conformations for the two lowest energy conformations of gabapentin, they proposed that the solution conformation for gabapentin contains the carboxylate group in the preferred axial position (Figure 2).[10,11]

This conclusion was reached through the stereoselective synthesis of analogs that were locked in specific conformations. Hence, both *cis* and *trans* versions of 4-methyl and 3,5-dimethyl gabapentin analogs were prepared and evaluated at the gabapentin binding site. As shown in Table 6, the two conformers with the aminomethyl group in the axial position (**19** and **20**) were less potent in displacing [^{3}H]-gabapentin from isolated pig brain membranes than were the conformers with the aminomethyl group in the equatorial position (**18** and **21**). The authors inferred that gabapentin also adopts a preferred equatorial aminomethyl group when binding to the α_2–δ subunit.[11]

This insight led to a more thorough examination of this hypothesis, through the application of further low-temperature nuclear magnetic resonance (NMR) work in deducing the conformations of analogs of gabapentin.[11] This included the synthesis of analogs containing substituents at the 3-position of the cycloalkyl group, which presumably lock the conformation of the ring in the desired orientation. As an example, both *cis* and *trans* isomers of (1-aminomethyl-3-methyl-cyclohexyl)-acetic acid were prepared (**22** and **23** respectively) (Table 7). Upon examination by ^{1}H NMR at –80°C, both isomers were found to exist in a single conformation (>99%), with both

Table 5 Effect of the incorporation of heteroatoms at the 4-position of gabapentin



Compound	*X*	α_2–δ *binding affinity* (IC_{50}, *nM*)
2	C	140
15	O	2,380
16	S	385
17	NH	>10 000

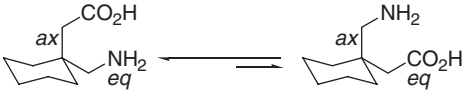
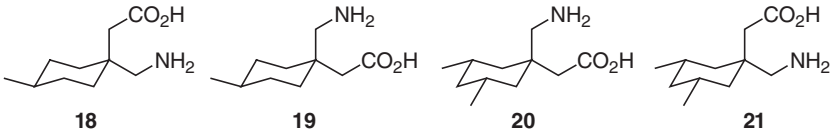


Figure 2 Proposed solution conformations of gabapentin (**1**).

Table 6 Binding affinity of axial and equatorially dispersed analogs of gabapentin (**1**)

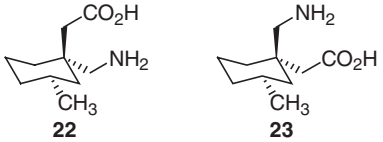


18 **19** **20** **21**

Compound	α_2–δ *binding affinity* (IC_{50}, *nM*)
2	140
18	420
19	17% at 10 μM
20	38% at 10 μM
21	143

Note: conformations of compounds were determined by NMR spectroscopy.

Table 7 Affinity of 3-methyl substituted analogs of gabapentin for the α_2–δ protein



22 **23**

Compound	α_2–δ *binding affinity* (IC_{50}, *nM*)
2	140
22	42
23	>10 000

methyl groups in the equatorial position, and with the aminomethyl group axial in **23**, but equatorial in the isomer (**22**). As expected, the isomer with the aminomethyl group nestled in the equatorial position proved to be significantly more potent than the corresponding axial isomer (**23**). (Note that compounds **22** and **23** were first identified by Satzinger as their racemates in earlier work.)

Further studies using gabapentin and either (1*S*,3*R*) 3-methyl-gabapentin (**22**) or (1*R*,3*R*) 3-methyl-gabapentin (**23**) as chemical probes were instrumental in elucidating the influence of stereochemistry and affinity for the α_2–δ protein on in vivo activity as well as providing a strong correlation between affinity for α_2–δ and in vivo activity. (1*S*,3*R*) 3-Methyl-gabapentin blocked the maintenance of static allodynia in the rat streptozocin and Chung models of neuropathic pain in a dose-dependent manner.[14] (1*R*,3*R*) 3-Methyl gabapentin however, failed to prevent either static or dynamic allodynia in the streptozocin model. These differences in in vivo activity were attributed to the different affinities for displacing [^{3}H]-gabapentin from α_2–δ. Other studies showed that both gabapentin and (1*S*,3*R*) 3-methyl-gabapentin inhibited tactile allodynia in the spinal nerve ligation (SNL) assay as well as in the second phase of the formalin model.[15] Interestingly, in the SNL model, this publication[15] suggested that the analgesic response of gabapentin, but not (1*S*,3*R*) 3-methyl-gabapentin, was blocked by the $GABA_B$ receptor antagonist CGP52432. Furthermore, (1*S*,3*R*)-3-methyl-gabapentin was studied in parallel to gabapentin in the thiosemicarbazide-induced seizure model and showed similar anticonvulsant activity to gabapentin (Table 7).[13]

With the latitude of effective binding of conformations of the cyclohexyl ring established, attention was turned to understanding the role of conformations of the aminobutyric acid motif in affinity for α_2–δ. To enable this, several conformationally restricted spirocyclic and fused carboxylic acids were prepared (Table 8).[11,12] In this series of

Table 8 Constrained analogs of gabapentin (**1**)

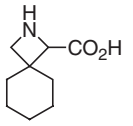
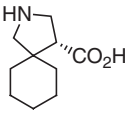
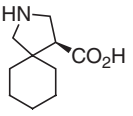
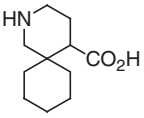
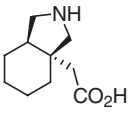
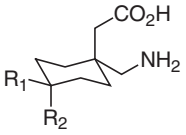
Compound	 **24**	 **25**	 **26**	 **27**	 **28**
α_2–δ binding affinity (IC_{50}, μM)	1.5	0.12	>10	>10	>10

Table 9 The effect of substitution of the 4-position of gabapentin (**1**) on binding affinity



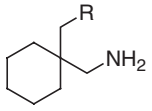
Compound	R_1	R_2	α_2–δ *binding affinity* (IC_{50}, *nM*)
2	H	H	140
29	H	Me	>10 000
30	Me	H	440
31	Et	H	700
32	iPr	H	>7020
33	Me	Me	1312

compounds, the 2-aza-spiro[45]decane-4-carboxylic acid (**25**) was the most potent ligand that displayed similar activity to gabapentin in the reversal of carrageen-induced thermal hyperalgesia. The enantiomers **25** and **26** were overlaid with low-energy conformations of gabapentin determined through geometry optimization. Six low-energy conformations of gabapentin were obtained, and both enantiomers overlaid quite well with two of these. However, only the (*R*)-isomer (**25**) displayed potent affinity for the α_2–δ protein and therefore this work pointed to a preferred binding conformation of gabapentin at the α_2–δ-binding site.

Further SAR studies by the former Parke-Davis group showed that substitution at the 4-position of the cyclohexyl ring of gabapentin was not well tolerated (Table 9).[13] This held true even when appropriate substituents to maintain the preferred conformation were used. Together with the data observed for the heterocyclic analogs (**15–17**), this indicated that the region of the α_2–δ protein that interacts with this portion of the molecule is rather intolerant to steric and electronic changes.

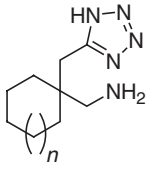
Following an exhaustive examination of the effect of incorporating alkyl motifs to the cyclohexyl ring of gabapentin, researchers next explored additional modifications of the aminobutyric acid motif. Satzinger touched on this area earlier but a more thorough examination took place in subsequent years, primarily through the examination of bioisosteric replacement of the carboxylate group. Bioisosteric replacement of functional groups is a commonly used strategy in medicinal chemistry.[16] This strategy often works well with carboxylic acids where subtle changes in pK_a can be achieved through suitable biosteric replacement. Carboxylate isosteres were studied in the gabapentin SAR.[17] Sulfinic acid (**34**), sulfonic acid (**35**), phosphonic acid (**36**), and tetrazoles **37** and **38** were all studied as bioisosteric replacement of the carboxylate group in gabapentin (Table 10). The sulfinic (**34**), sulfonic (**35**), and phosphonic acids (**36**), as well as the *N*-1-methyl-tetrazole (**38**), were inactive when compared to the parent molecule (**2**). But with the acidic functionality present in tetrazole (**37**), potency at isolated pig membranes was restored. The tetrazole moiety in particular closely mimics the parent acid in pK_a.[18]

Table 10 The influence of bioisosteres on the ability to displace [^{3}H]-gabapentin from the α_2–δ protein



Compound	*R*	α_2–δ *binding affinity* (IC_{50}, *nM*)
2	CO_2H	140
34	SO_2H	>10 000
35	SO_3H	>10 000
36	PO_2H	>10 000
37	Tetrazole	100
38	*N*-1-Me-tetrazole	>10 000

Table 11 The α_2–δ, anticonvulsant and pharmacokinetic properties of a series of tetrazole analogs compared to gabapentin (**1**)



Compound	*n*	α_2–δ *binding affinity* (IC_{50}, *nM*)	*DBA/2 (30 mg kg*$^{-1}$, *PO; % of mice protected)*	*Cl (mL min*$^{-1}$ *kg*$^{-1}$*)*	*%F*	$t_{1/2}$
2	n/a	140	80	6.0	76	1.5
39	2	100	100	10.6	85	1.0
40	3	2357	0	–	–	–

DBA/2, percent of mice protected from audiogenic tonic-extensor seizures; Cl, pharmacokinetic clearance after oral administration (5 mg kg^{-1}) to rats; %*F*, percent oral bioavailability; $t_{1/2}$, pharmacokinetic half-life of drug in plasma.

The effect of ring size was studied in the aminotetrazole series and it was found that the cycloheptyl analog (**39**) was similar to gabapentin in binding affinity to pig brain membranes (Table 11). Also, tetrazole (**39**) was found to be equipotent to gabapentin on a dose-for-dose basis in the DBA/2 seizure model.

8.18.2.2 A Critical Piece of the Puzzle: Transport of Amino Acids Across Membrane Barriers

An important aspect of the pharmacology of amino acids that target the α_2–δ protein is the need for ready flux of drugs across cell membranes of the gut and particularly across the blood–brain barrier. As discussed above, the chemical properties of amino acids such as gabapentin and pregabalin prevent the 'typical' path of drug absorption, namely passive diffusion across the lipid bilayer. Therefore, penetration of membrane barriers had to be enhanced to allow for in vivo efficacy. In a manner similar to that of amino acids taken in by the gut from foods, several exogenous amino acids that target the α_2–δ protein are taken across membrane barriers by active transport systems. Preclinical and clinical studies with gabapentin and pregabalin have revealed some significant differences in the absorption of these two compounds but active transport is a critical component of the central activity of both compounds. In humans,

gabapentin, but not pregabalin, undergoes dose-limiting absorption from the gut. Similarly, in rats, pregabalin (but not gabapentin) shows oral absorption that is linear with dose. Both compounds flux across cell membranes via sodium-independent mechanisms shared by the endogenous branched-chain amino acids. This system of transporters, termed 'system L,' is largely mediated by the LAT-1 protein.[19] It is likely that the more linear oral absorption of pregabalin observed in vivo derives from its lower affinity at system L, which makes it less prone to saturation of transporters in the gut. Taken together, studies indicate that the system L amino acid transporter is the principal transporter for the uptake of pregabalin and gabapentin in cells. Within the pregabalin and gabapentin SAR, interaction of analogs with the L-type amino acid transporter, and subsequent transport have been found necessary for in vivo activity in preclinical models of epilepsy and anxiety (Table 11).

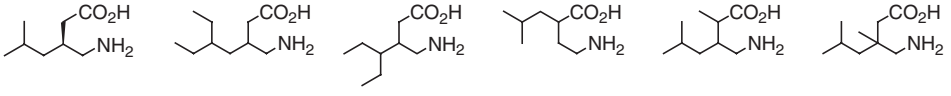
8.18.2.3 The Discovery and Structure–Activity Relationship of Pregabalin: A Second Fortuitous Discovery

Much like gabapentin, pregabalin was identified through a somewhat circuitous path. R. Silverman (Northwestern University) developed a series of 3-alkyl-4-aminobutyric acids as part of a program to develop activators of glutamic acid decarboxylase (GAD), a pyridoxyl 5′-phosphate-dependent enzyme involved in the synthesis of GABA in the brain.[20,21] In this work, racemic 3-isobutyl-γ-aminobutyric acid was found to be a weak activator of GAD but the most potent compound in the prevention of tonic extensor seizure in mice.[22,23] Yuen went on to synthesize both enantiomers of 3-isobutyl-γ-aminobutyric acid using an oxazolidinone approach,[24] and further studies showed that (*S*)-isobutyl-γ-aminobutyric acid (also known as PD 0144723, CI-1008, pregabalin, Lyrica) dose-dependently inhibited the binding of [^{3}H]-gabapentin to pig brain membrane whereas the corresponding *R*-isomer had 12-fold less affinity for binding and no activity in a seizure model in vivo.[25] This report supported other findings suggesting that binding affinity at α_2–δ was a critical component of the pharmacology of this class of compounds.

One of the first large reports of the SAR of pregabalin was published by Belliotti *et al.* (Table 12).[26] This study examined the effects of modifying the pregabalin backbone at the 2-, 3-, and 4-positions of the butyric acid backbone and the 1-position of the isobutyl side chain. These studies looked more comprehensively at preclinical activities in animal models of seizure, analgesia, and anxiety as well as activity at the LAT-1 amino acid transporter. For the first time, preliminary observations on the divergence in SAR between affinity for the α_2–δ protein and affinity for system L transport were reported.

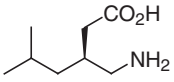
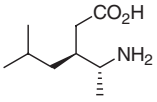
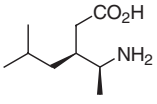
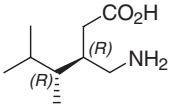
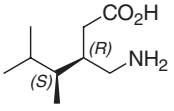
Much like the SAR seen to date with compounds related to gabapentin, subtle changes in the arrangement of the carbon skeleton drastically altered the affinity of pregabalin for the α_2–δ subunit. For instance, in the SAR seen in 2- and 3-analogs of pregabalin, transposition of the side chain to the 2-position (**43**) greatly reduced binding affinity. Similarly, incorporation of the methyl moiety at the 2-position (**44**) and 3-position (**45**) reduced binding considerably. It is also interesting to note the tolerance to structural changes shown by the system L transporter. Rearrangement of the alkyl side chain did not greatly shift the affinity of compounds for system L transport; the only major structural

Table 12 The effect of substitution at the 2- and 3-position on the ability to displace [^{3}H]-gabapentin from the α_2–δ protein



Compound	**5**	**41**	**42**	**43**	**44**	**45**
α_2-δ binding affinity (IC_{50}, nM)	80	1330	203	2060	>10 000	3300
System L (IC_{50}, μM)	158	142	146	>10 000	117	89
DBA/2 (30 mg kg^{-1}, PO; % of mice protected)	100	0	20	0	0	0

Table 13 Stereospecific analogs of pregabalin and their in vitro and in vivo properties

	α_2–δ binding affinity (IC_{50}, nM)	*System L (IC_{50}, μM)*	*DBA/2 (30 mg kg^{-1}, PO; % of mice protected)*
 **5**	80	158	100
 **46**	480	> 10 000	0
 **47**	39	> 1000	0
 **48**	21	157	100
 **49**	1700	28	20

change that altered affinity for transport was the transposition of the isobutyl side chain from the 3- to the 2-position (**43**). Again, considering the physical properties of zwitterionic molecules, the ability of these compounds to interact with the system L amino acid transporter (or possibly other membrane-bound influx transporters) was found to be critical in ensuring translocation of the molecule across the gut and blood–brain barriers. Assuming that affinity of compounds **41–45** at the system L transporter with IC_{50} values less than about 200 μM confers permeability, the lack of antiseizure activity of several compounds with low affinity α_2–δ binding (e.g., **46**, **49**) in the DBA/2 mouse model of epilepsy strongly supports the identity of the α_2–δ protein as the molecular target for these compounds.[26]

Modifications of the 4-position as well as modifications of the isobutyl side chain gave additional possibilities for enhancing the affinity of pregabalin for binding at the α_2–δ subunit (Table 13).

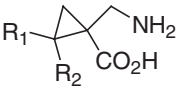
Both the (*R*)- and (*S*)-4-methyl isomers (**46**) and (**47**), respectively, afforded affinity for the α_2–δ protein with the (*S*)-isomer (**47**) being twofold more potent than pregabalin in binding. The most potent compound from this series was (3*R*,4*R*)-3-aminomethyl-4,5-dimethyl-hexanoic acid (**48**). This compound had relatively high affinity for both the α_2–δ protein and the system L transporter and, as a consequence, afforded complete protection against sound-induced seizures in DBA/2 mice at a dose of 30 mg kg^{-1} PO.

8.18.2.4 Beta Amino Acids

Evidence that activity at both the system L transporter and also at the α_2–δ protein was needed for in vivo activity was again provided by a series of cyclopropyl-based α-substituted β-amino acids reported by the Pfizer group in 2005.[27] This disclosure was notable in being the first report of the SAR of β-amino acids with affinity for α_2–δ (Table 14).

In this study, several potent ligands for the α_2–δ protein were found, but none were substrates of the system L transporter, as measured by inhibiting the uptake of [^{3}H]-leucine in Chinese hamster ovary (CHO) cells. Thus, after oral dosing of 1-aminomethyl-spiro[25]octane-1-carboxylic acid (**53**), no anticonvulsant activity was observed in the

Table 14 Cyclic β-amino acids and their affinity for α_2–δ and system L



Compound	*R_1*	*R_2*	*α_2–δ binding affinity (IC_{50}, nM)*	*System L (IC_{50}, μM)*
5	n/a	n/a	80	158
50	$CH(CH_2CH_3)_2$	H	56	>100
51	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$	200	>100
52	$-(CH_2)_4$		23	>100
53	$-(CH_2)_5$		13	>100
54	$CH(CH_3)_2$	H	340	>100
55	$CH_2CH(CH_3)_2$	H	330	>100
56	c-C_3H_5	H	200	>100
57	H	$CH_2CH(CH_3)_2$	37	>100
58	H	$CH(CH_2CH_3)_2$	630	>100
59	H	H	>10 000	>100

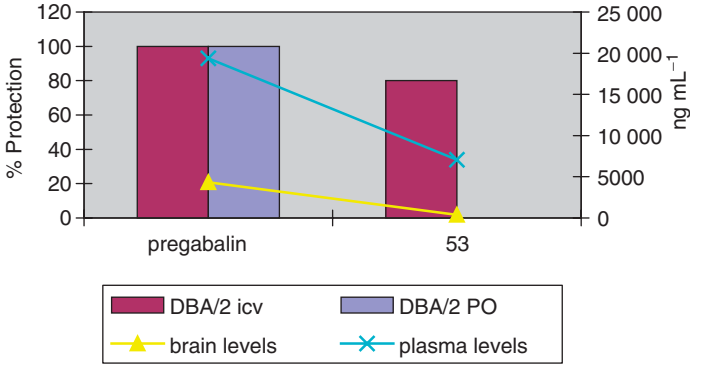


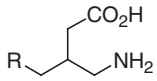
Figure 3 Biological and exposure data for amino acid **53** compared to that of pregabalin **5**. Compound **53** potentially inhibits [^{3}H]gabapentin binding but is not active in the DBA/2 seizure model with oral dosing (blue bars). This is related to the lack of activity of compound **53** at the system L amino acid transporter, resulting in very low exposures of **53** to brain (yellow symbols). Despite this, direct administration of **53** by ICV injection prevents seizures in DBA/2 mice (red bar). (The graph is from [27].)

DBA/2 mouse test. However, once the blood–brain barrier was circumvented by direct intracerebroventricular administration, the spirocarboxylic acid (**53**) gave 80% protection against seizures. Further analysis of the plasma exposure of both pregabalin and the acid (**53**) after oral administration to mice (Figure 3) showed a clear difference, indicating low absorption of **53** from the gut. Furthermore, extremely low brain levels were measured with **53**, further indicating poor brain penetration. Presumably, the levels of **53** obtained in the brain were not enough to elicit antiseizure activity, despite potent binding at α_2–δ.

8.18.2.5 Aliphatic Side-Chain Replacements

Schelkun and coworkers examined the effect of replacement of the isobutyl side chain of pregabalin with heteroaromatic, aromatic, and heterocyclic groups in order to expand the scope of SAR (Table 15).[28] Binding affinity in this series trended with the following rank order: 2-furan ~3-furan>3-thiophene>2-thiophene. Overall, though, modifications in this study

Table 15



Compound	*R*	*Stereochemistry*	*α_2–δ binding affinity (IC_{50}, nM)*	*System L (IC_{50}, μM)*	*DBA/2 (30 mg kg^{-1}, PO; % of mice protected)*
5	iPr	*S*	80	158	100
60		–	>10 000		0
61		–	421	10 000	20
62		–	518	1422	0
63		–	2140	2936	40
64		–	832	7034	60
65		*S*	178	10 000	40
66		*R*	1460	10 000	0

led to compounds with lower affinity for α_2–δ measured by displacement of [^{3}H]-gabapentin from pig brain membranes than pregabalin. The 2-furyl analog **61** was the most potent ligand in this class. Enantiomers of **61** were stereoselectively synthesized using the oxazolidinone strategy. Consistent with the stereodivergence seen with enantiomers of pregabalin **5** and 3-Me gabapentin **22**, the (*S*)-enantiomer **65** was eightfold more potent at α_2–δ than the corresponding (*R*)-enantiomer **66** and only the more potently binding enantiomer was active in vivo. Besides reducing affinity for α_2–δ binding, incorporation of heteroatoms in the amino acid framework resulted in decreased affinity for blocking [^{3}H]-leucine uptake into CHO cells. It is thought that reduced affinity for both the system L amino acid carrier and the α_2–δ protein led to diminished in vivo activity as measured by both the Vogel conflict model for anxiolytic-like action (not shown) and also the DBA/2 seizure model with this heterocyclic class. However, DBA/2 activity with compounds **63**, **64** and **65** suggests the untested idea that these compounds traverse membrane barriers by mechanisms other than System L transport.

8.18.3 Clinical Development of Gabapentin

8.18.3.1 Early Clinical Studies

Gabapentin was studied in animal toxicology at Goedecke from about 1980 to 1982, and was first studied for tolerance, safety, and pharmacokinetics in healthy human subjects in a study contracted from Goedecke in 1982.[29] Clinical phase I single-dose and multiple-dose tolerance studies showed elimination with a plasma half-life of about 6 h and dose-proportional absorption.[29]

Clinical phase II studies with gabapentin began in 1983 and continued in 1984 and 1985 with several quite small studies against Huntington's disease, hemiplegia, and spasticity (single-blind placebo-controlled) with dosages of 200 and 400 or 600 and 900 mg day^{-1} given q.i.d. or t.i.d. In early 1984, spasticity was studied in open-label clinical trials at three medical centers, each with relatively low dosages of gabapentin and very small numbers of patients (total $n = 10$–65

patients per study; one study used rising dosages up to 1800 mg day^{-1} given t.i.d. for 1 week). Unfortunately, due to the low number of patients, lack of parallel placebo groups, and heterogeneity of spasticity diagnoses and severity, no conclusions could be drawn from these studies other than a relatively benign side-effect profile for gabapentin.

8.18.3.2 Development of Gabapentin for Epilepsy

The first clinical study of gabapentin in epilepsy showing efficacy was conducted with 25 patients in 1985 as a placebo-controlled add-on crossover study in refractory partial seizures (Bernd Schmidt *et al.*, Goedecke, unpublished data), unpublished data. All patients were maintained on their prior medications, but were then crossed over between four different study groups with the addition of placebo, 300, 600, or 900 mg gabapentin per day. The 900 mg dose group showed a significant difference from placebo in weekly seizure frequency (overall 45% decrease compared to placebo). In addition, there was a 43% responder rate (number of patients with more than 50% decline in seizure frequency) in this study with 900 mg day^{-1}, versus 14% responder rate at 300 mg day^{-1}. These results started the serious clinical development of gabapentin, which was done by a planning team at first chaired by Schmidt, and run from Goedecke.

Later (in mid-1986) a global planning team for gabapentin was formed, and although still run from Goedecke, it included regulatory and clinical personnel from Parke-Davis Research in Ann Arbor, Michigan. Subsequent to 1986, the pivotal clinical studies of gabapentin for epilepsy were mainly coordinated by Jan Wallace and Kent Schellenberger (Parke-Davis, Ann Arbor) and after 1990 by Elizabeth Garofalo and colleagues[30,31] (Parke-Davis, Ann Arbor). Positive results in the pivotal clinical efficacy studies with gabapentin treatment as add-on for refractory partial seizures were eventually filed with the US Food and Drug Administration for the New Drug Application (NDA) for gabapentin in 1992. These studies were coordinated by many outside investigators, including Dennis Chadwick in the UK,[32,33] and Michael McLean,[34] Eugene Ramsey,[35] BJ Wilder, Ahmad Beydoun[36] (and others) in the US. Three months before the US Food and Drug Administration (FDA) advisory committee meeting for the epilepsy approval in December 1992, Mark Pierce (Parke-Davis, now Pfizer Ann Arbor, recently retired) joined the clinical group from his prior position at Abbott, and defended the clinical efficacy and safety information before the FDA epilepsy advisory committee. Approval of the gabapentin epilepsy NDA was achieved after final label wording negotiations on December 31 of 1993. Neurontin was launched by Parke-Davis for the US epilepsy market in February 1994.

After the gabapentin product launch, gabapentin was used extensively by physicians for treating epilepsy. Because of a relatively benign adverse event profile and few drug–drug interactions, it was also prescribed for off-label indications, including neuropathic pain, anxiety, and other psychiatric indications, essential tremor, spasticity, postsurgical pain, and prevention of postmenopausal hot flashes. None of these additional indications were supported in the gabapentin product labeling or approved by regulatory agencies until the FDA approved a supplemental NDA for gabapentin to treat postherpetic neuralgia in July 2001 (*see* Section 8.18.3.3, below).

8.18.3.3 Development of Gabapentin for Neuropathic Pain

Lakhbir Singh and Mark Field performed initial studies of gabapentin for use as an analgesic in animal models in 1992–1993 at the Parke-Davis Cambridge (UK) Research Centre. They found that gabapentin was active in several rat models of antihyperalgesic action (formalin test, carrageenan test),[37,38] although at rather high dosages. Gary Bennett obtained a sample of gabapentin in 1993, and tested it in his model of neuropathic pain from sciatic nerve ligation in rats.[39] These results from animal models were presented by Bennett at a national meeting in 1994, and at about the same time, the first case reports of gabapentin use for neuropathic pain appeared in the literature.[40,41] Subsequently, a large number of investigators found gabapentin to be active in animal models of pain states[14,38,42–61] and also in several clinical studies.[62–65]

In 1995 and 1996, Parke-Davis began two large placebo-controlled parallel group studies of gabapentin for treating neuropathic pain. Clinical trials for diabetic peripheral neuropathy were supervised by Elizabeth Garofalo (Parke-Davis, Ann Arbor), and postherpetic neuralgia studies by Leslie Magnus-Miller (Parke-Davis, Morris Plains). Both studies were done with virtually identical protocols. These studies were both resoundingly positive in comparison to placebo treatment, and the results were published side by side in the *Journal of the American Medical Association* in 1998.[66,67]

In 1998 and 1999, based on the published *Journal of the American Medical Association* clinical studies, the first regulatory approvals were obtained for gabapentin to treat neuropathic pain in Asia and Latin America, then later in Europe. In early 2001, it was decided to submit a supplementary NDA (sNDA) application in the US with gabapentin for neuropathic pain, based upon the recently obtained clinical data. The submission was completed in August 2001 and the FDA approved the sNDA and provided revised labeling for gabapentin for treatment of postherpetic neuralgia on May 24, 2002. The commercial launch of gabapentin for postherpetic neuralgia was in August of 2002.

8.18.4 Studies of the Mechanism of Action of Gabapentin and Pregabalin

The original animal pharmacology studies of gabapentin from Goedecke (1981–1984: W. Reimann, G. Bartoszyk, and others, unpublished data) included studies of anticonvulsant action in mice, rats, and monkeys and antispasticity effects in animal models with mice, rats, and anesthetized cats. Early mechanism of action work centered on electrophysiological changes in spinal reflexes (W. Steinbrecher, 1982, unpublished data) and reduced monoamine neurotransmitter release from brain tissue slices in vitro (W. Reimann, E. Schlicker).[2,68] Also in 1984, a group of pharmacologists at Parke-Davis (P. Boxer, R. Anderson *et al.*, Ann Arbor, MI, unpublished data) studied gabapentin in several preclinical models of spasticity in mice. Gabapentin reduced muscle rigidity and increased locomotor agility (apparently by reducing spinal polysynaptic reflexes) at dosages of 10–100 mg kg^{-1} IP. The results with gabapentin compared favorably with other experimental treatments for spasticity (e.g., baclofen, diazepam). Slightly later, mechanism studies focused on transport of gabapentin by the system L amino acid transporter (which proved to be the main mechanism of gabapentin entry across the gut and also across the blood–brain barrier[69,70]). However, the lack of pharmacological activity with several system L transporter substrates that lacked high affinity for α_2–δ (data not shown) soon turned attention to other potential sites of action.

8.18.4.1 Discovery of the Calcium Chemical α_2–δ Binding Site

Important mechanism of action work began in about 1991 in the Parke-Davis Cambridge Research Centre. This work lead to the identification of a specific [^{3}H]gabapentin-binding site in rat brain tissues by David Hill, Nirmala Suman-Chauhan, and colleagues.[5,71] They showed that the site was distributed heterogeneously in rat brain, with high densities of binding in regions that were also rich with synaptic endings. Later, Nicholas Gee and Jason Brown of the Cambridge Unit used protein biochemistry techniques to purify solubilized protein fractions from pig brain that bound with high affinity to [^{3}H]gabapentin. After four stages of column purification, and sequencing of a short peptide fragment from the purified protein, the high-affinity site was identified as the α_2–δ type 1 protein, a subunit of voltage-gated calcium channels.[4] Recombinant production of α_2–δ protein in mammalian cells showed [^{3}H]gabapentin-binding properties essentially identical to those of pig or rat brain membranes, confirming the identity of the binding site.[4]

8.18.4.2 Drug-Induced Changes in Calcium Channel Function?

Building upon previous findings, David Dooley and colleagues (Pfizer Ann Arbor) showed that gabapentin reduces calcium influx and neurotransmitter release from rat and human brain tissues.[72–78] Alexander McKnight and YP Maneuf (Parke-Davis Cambridge) found that gabapentin reduced glutamate release in a manner suggesting relevance for analgesia.[79,80] Electrophysiologists in Cambridge and at Aberdeen University in the UK demonstrated changes in calcium channel and synaptic function that were presumed to be caused by gabapentin action at the α_2–δ site.[81–85] These findings together with SAR described in Sections 8.18.2.3–8.18.2.5 provided a strong circumstantial argument that binding at to the α_2–δ site was important to the pharmacology of gabapentin and pregabalin.

8.18.4.3 Studies Relating to γ-Amino-Butyric Acid Concentrations and $GABA_B$ Receptors

At about the same time as the α_2–δ publications, reports appeared indicating that gabapentin in humans caused an elevation of brain GABA concentrations measured by molecular resonance spectroscopy (MRS; O.A. Petroff, R. Mattson *et al.*, Yale University.[86–91] Other studies suggested that gabapentin increased nonsynaptic release of GABA from brain tissues (Jeffery Kocsis, George Richerson, Yale University.[92–95] Furthermore, with prolonged treatment, both pregabalin and gabapentin increased the number of GABA transporters present on neuronal cell membranes (Michael Quick, University of Alabama Birmingham).[96] However, the changes in whole-brain GABA concentration reported with gabapentin were smaller in magnitude than after treatment with the GABA-transaminase inhibitor vigabatrin.[88,89,97] Furthermore, other reports showed increases in MRS brain GABA signals caused by topiramate and lamotrigine, two very different antiepileptic drugs that are not known to alter brain GABA metabolism or degradation.[97–99] In addition, neither gabapentin nor pregabalin altered GABA concentrations in rat brain,[100] despite the robust pharmacological action of both gabapentin and pregabalin in rat models of seizures, pain, and antianxiety-like actions. It is somewhat confusing to reconcile these apparently conflicting data. However, MRS studies of whole-brain GABA measure almost exclusively the large pool of GABA contained within inhibitory neurons, and not the much smaller extracellular GABA

pool that is available to bind to inhibitory neuronal receptors. Therefore, the relevance of the whole-brain GABA findings to drug pharmacology is unclear. Finally, electrophysiological studies of both gabapentin and pregabalin in anesthetized rats suggest that rapid GABA synaptic action in rat brain is reduced, rather than enhanced, by drug treatment.[101] Therefore, any potential contribution of changes in brain GABA concentration to the anticonvulsant action of gabapentin and pregabalin remains far from clear.

To complicate the picture further, several publications from a group at the Merck-Frosst Research Center in Canada[102–104] implicated certain subtypes of the $GABA_B$ receptor as targets for gabapentin. However, several other laboratories were unable to replicate these findings,[105–110] and therefore, actions of gabapentin at $GABA_B$ receptors are not generally accepted to be relevant. Furthermore, neither pregabalin nor gabapentin displace radioligand binding to subtypes of $GABA_B$ receptors, including recombinant heteromeric $GABA_{BR1a}/GABA_{BR2}$ receptors, heteromeric $GABA_{BR1b}/GABA_{BR2}$, and $GABA_B$ receptors from native rat brain membranes (unpublished data, not shown). Therefore, consistent and reproducible actions of this class of drugs at $GABA_B$ receptors have not been observed.

8.18.4.4 Mutation and Deletion Studies of α_2–δ Protein

More recent studies of gabapentin and pregabalin mechanisms relating to the α_2–δ binding site were initiated based on findings by Ti-Zhi Su and James Offord (Pfizer Molecular Sciences, Ann Arbor) and Jason Brown and Nicolas Gee (Parke-Davis Research Centre, Cambridge) in about 1998–1999. This work began with experimental deletions and mutations to the DNA sequence for α_2–δ type 1, expressed in recombinant cell systems in vitro.[111,112] Work with mutant mice lacking α_2–δ proteins was hindered by the fact that a global knockout of α_2–δ type 1 caused lethality (J. Offord, unpublished data) and mouse mutations that cause a functional knockout of α_2–δ type 2 protein caused extreme behavioral abnormalities and early lethality.[113–115]

Subsequently, Offord and colleagues began work to produce genetically altered mice that selectively expressed a single amino acid mutation within the coding sequence of α_2–δ type 1. This single change to recombinant α_2–δ proteins in vitro had previously been shown to diminish greatly drug binding affinity for [^{3}H]gabapentin and [^{3}H]pregabalin.[112] When homozygous genetically altered mice incorporating the same single amino acid change were obtained (called R217A mutants because of a substitution of alanine for the wild-type arginine at position 217 of the α_2–δ type 1 protein), it was confirmed that these mice had reduced drug binding for gabapentin and pregabalin selectively to portions of brain and spinal cord.[116–118] Furthermore, this mutation did not cause untoward effects such as ataxia or seizures in whole animals. Finally, studies of pregabalin and gabapentin given systemically to wild-type and R217A mutant mice showed that drug actions in models of analgesia, anticonvulsant actions, and antianxiety-like effects were selectively reduced in R217A mutant mice, while the actions of other drugs, such as morphine, amitriptyline, and phenytoin, remained unchanged.[118,119] These findings strongly support the independent findings from structure–activity studies (*see* Section 8.18.2, above) to indicate that high-affinity binding to the α_2–δ type 1 protein is required for pharmacological effects of both pregabalin and gabapentin in vivo. It is interesting that preliminary findings with R217A mice showed a less marked effect of the mutation on anticonvulsant action of pregabalin than on analgesic or antianxiety-like actions of pregabalin. This suggests the possibility that pregabalin binding to α_2–δ type 2 proteins (that were not altered by the R217A mutation) may be important for anticonvulsant actions. However, additional experiments will be required to test this idea.

8.18.4.5 Mechanism of Action: Beyond the Drug-Binding Site

As can be appreciated from the preceding paragraphs, the mechanism of action of gabapentin (and also pregabalin) has been the subject of some debate. Despite that, quite a lot is now known about their pharmacology. Neither compound is active at a wide variety of radioligand-binding sites associated with the actions of some other antiepileptic drugs, including batrachotoxinin sites on voltage-gated calcium channels, GABA receptors ($GABA_A$, $GABA_B$, $GABA_C$, benzodiazepine, or GABA transporter sites), or glutamate receptors (*N*-methyl-d-aspartate, strychnine-insensitive glycine, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate or mGluR1, mGluR5 glutamate receptors). Furthermore, both compounds are remarkably silent even at high concentrations in a wide array of other radioligand-binding assays for common drug and neurotransmitter receptors. This is probably due to the unusual chemical nature of the molecules, that are both relatively small, hydrophilic, and possess strong amine and acid moieties. Both compounds, in contrast, are high-affinity ligands for α_2–δ type 1 and α_2–δ type 2 proteins, labeled with [^{3}H]gabapentin[5] or [^{3}H]pregabalin[117,120] or [^{3}H]L-leucine.[121]

8.18.4.6 Decreases in Neurotransmitter Release

How does binding at the α_2–δ site produce anticonvulsant effects? Numerous studies have examined voltage-gated calcium channel function with gabapentin or pregabalin, and these studies have produced conflicting results. Several studies report that gabapentin reduced current through voltage-clamped calcium channels on neuronal cell body membranes.[82,83,85,122–125] However, other studies with neuronal cell bodies[126,127] or recombinant cell systems expressing calcium channels with α_2–δ subunits[128] have shown no change in calcium currents after application of gabapentin. Despite these conflicting data, studies with calcium influx measured with fluorescent probes in synaptosomes from rat or human neocortex[75,78,127] show that both gabapentin and pregabalin reduce calcium influx measured at presynaptic terminals. In addition, studies measuring the synaptic release of glutamate,[73,79,80,129–133] norepinephrine, serotonin, or dopamine from neocortical tissues,[73,74,77] or the release of sensory peptide neurotransmitters substance P or calcitonin gene-related peptide (CGRP)[134] have shown subtle but reproducible reductions in calcium-dependent neurotransmitter release. In some systems, pretreatment in vivo with inflammatory agents or in vitro with neuropeptides or protein kinase activators are required before clear drug effects are seen.[80,134]

Recently, several studies have indicated that not only calcium-dependent release of neurotransmitters, but also asynchronous (miniature potentials – calcium-independent) release of vesicles is reduced by drug treatment. These experiments include the release of vesicles from glutamate synapses in spinal cord slices,[81,129] entorhinal cortex slices,[135] cultured rat hippocampal neurons,[141] and the release of cholinergic vesicles from mouse neuromuscular junction.[142] Miniature synaptic potentials or asynchronous release is reduced in each of these preparations by treatment with gabapentin or pregabalin. These results suggest that the actions of α_2–δ ligands to reduce neurotransmitter release may not always require inhibition of calcium influx. And so, drug actions might also be mediated by an interaction between α_2–δ and synaptic proteins in addition to calcium channel α subunits that are involved in the release or trafficking of synaptic vesicles.

8.18.4.7 Mechanisms of Action – Summary

The question remains whether these actions of pregabalin and gabapentin on α_2–δ proteins to reduce neurotransmitter release can fully account for all of the pharmacological actions of these drugs. Could other pharmacological sites of action contribute to the pharmacological actions of pregabalin? It is impossible to rule out contributions from other unknown drug targets. However, based on the great similarity in pharmacology in many different animal models between gabapentin, pregabalin, and several other α_2–δ ligand compounds that are earlier in development, it does not appear necessary to postulate additional sites of action to account for the pharmacology of this drug class. Furthermore, no other candidate molecular drug targets for these compounds have yet been identified. The potential drug targets of glutamic acid dehydrogenase,[136] branched chain amino acid aminotransferase,[137–139] GABA-transaminase,[140] and the system L transporter[19] have mostly been ruled out. This is because they are not affected similarly by gabapentin and pregabalin, and none of these sites show sufficient affinity for the known compounds to account for their clinically relevant drug actions. Conversely, for GABA transaminase and system L transport mechanisms, there are known compounds that act at these sites that do not share the pharmacology of gabapentin and pregabalin. Therefore, because of the lack of other potentially relevant molecular targets, and with known actions of both gabapentin and pregabalin ascribed to binding at the α_2–δ site, other potential sites of drug action remain speculative.

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Biographies

Andrew J Thorpe, PhD, is a Director in the Department of CNS and Inflammation Chemistry, Pfizer Global Research and Development, Ann Arbor, MI, USA. He obtained his BSc in Medicinal Chemistry from University College London in 1990 and a PhD in organic synthesis from the University of Exeter in 1993, working under the guidance of Prof Stanley M Roberts, where his focus was on the synthesis of novel nucleoside analogs. From Exeter he moved to a Postdoctoral Research Associate position with Prof Tomas Hudlicky at Virginia Polytechnic Institute and State University and later at the University of Florida, where he studied the chemistry of cyclohexadiene-*cis*-diols. After his research in the Hudlicky group, he moved to Eli Lilly and Company where he served as a Postdoctoral Research Fellow under the mentorship of Tony Shuker in Endocrine Research. He then joined Parke-Davis Research (now Pfizer Global Research and Development) in Ann Arbor, Michigan in the CNS department where he studied the chemistry and pharmacology of α_2–δ ligands. His research experience is extended beyond CNS and incorporates that of inflammatory diseases.

Charlie P Taylor, PhD, is a Research Fellow in the Department of CNS Biology, Pfizer Global R&D, Ann Arbor, MI, USA. He obtained a BA in Zoology from University of Texas, Austin in 1975. He received a PhD in Neurobiology at

University of California, Berkeley in 1980. He served as an NIH Postdoctoral Fellow for two years to study electrophysiology of epilepsy using brain slices in vitro with FE Dudek at Tulane University in New Orleans. This fellowship resulted in eight publications and two book chapters describing mechanisms of neuronal synchronization in epilepsy.

He continued research in epilepsy at Parke-Davis in Ann Arbor in 1982. Early drug discovery focused on blockers of voltage-gated sodium channels. He was a member of the gabapentin (Neurontin) development team with Parke-Davis since 1985. He studied experimental treatments for cerebral stroke for several years including glutamate antagonists and sodium channel blockers, and worked in a collaborative program with Neurex (California) to discover nonpeptide calcium channel blockers.

He worked on the discovery and development of pregabalin (Lyrica) since 1990, whose anticonvulsant activity was discovered in collaboration with R Silverman (Northwestern University). In 1995, Charlie was an early collaborator (between Parke-Davis groups in Ann Arbor and Cambridge, England) to discover new drugs and mechanisms related to gabapentin and pregabalin. Charlie continues to work with scientists inside and outside Pfizer to better understand the mechanism of α_2–δ drugs such as gabapentin and pregabalin.

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