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INDUCTION OF DRUG-METABOLIZING ENZYMES: A Path to the Discovery of Multiple Cytochromes P450*

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■ **Abstract** This article provides a personal account of the discovery of the induced synthesis of drug-metabolizing enzymes and of subsequent research that led to the discovery of multiple cytochromes P450 with different catalytic activities. The manuscript also emphasizes the role of environmental factors (in addition to genetic polymorphisms) in explaining person-to-person and day-to-day differences in rates and pathways of drug metabolism that occur in the human population.

EARLY DAYS

I was a depression baby born in 1930 in Chicago where my father was a pharmacist managing a Liggett's drugstore. In 1937, my father opened an independent pharmacy in Winnetka, Illinois, and we moved into a small house on the less expensive west side of Winnetka where my next door neighbor was Donald Rumsfeld (current Secretary of Defense). My early childhood was marked by much good fortune—loving and hardworking parents with strong ethical values, a pharmacy named Conney's Pharmacy (which still retains the name today) that was a center of our family life, and an outstanding school system that was (and still is) among the best in our nation. Graduates from Winnetka's New Trier High School included such notables as Jack Steinberger (Nobel Laureate, physics), Bruce Alberts (President, National Academy of Sciences, U.S.A.), Donald Rumsfeld (Secretary of Defense), Ralph Bellamy (actor), and Archibald McLeish (poet).

*This manuscript is dedicated to the memory of my graduate school mentors James A. Miller and Elizabeth C. Miller and to the memory of my friends and former colleagues Alvito Alvares, James Gillette, and Shu-jing (Caroline) Wei.

I started working in my father's pharmacy at age 10 for 10 cents an hour and all the ice cream that I could eat. I enjoyed my interactions with the people that came into the store, and I learned much from my father about the use of drugs for the treatment of diseases. My early years were marked by a love of my academic studies, music (both jazz and classical), and competitive sports (table tennis and baseball). I played flute and piccolo in a number of concert bands and orchestras during my early years and also later in college where I was a member of the concert band and the marching band that performed at football games.

COLLEGE DAYS

In 1948, I entered the School of Pharmacy at the University of Wisconsin-Madison with the intention of pursuing a career in pharmacy. The School of Pharmacy and the basic science departments at the University had an exceptionally strong research-oriented faculty, and they provided me with a strong education in the basic sciences and the practice of pharmacy (including instructions on how to make suppositories during the heat of summer). The faculty in the School of Pharmacy provided their students with opportunities for doing research, and I took a part-time job as a research assistant helping Professor Louis Busse in studies on the properties of powders that would be helpful in preparing tablets. My first "independent" research project was with Professor Takeru Higuchi who was a leader in physical pharmacy. He had me work on the development of a silica gel chromatography system that would allow the specific analysis of chloramphenicol.

After completion of pharmacy school, I passed the Pharmacy State Board Examination in Illinois and became a registered pharmacist. Although I could have started a career in pharmacy in my father's drugstore, I chose to pursue further education in preparation for a career in research after also considering the possibility of entering medical school. I inquired about opportunities as a graduate student in a number of biochemistry-oriented departments on the Madison campus. Again, I had good fortune in meeting with Dr. Harold Rusch, director of the McArdle Laboratory for Cancer Research. Dr. Rusch spent a great deal of time with me describing the oncology graduate program and the many interesting research projects that were ongoing at McArdle. After this meeting, I was convinced that I wanted to enter the oncology Ph.D. program at McArdle, and Dr. Rusch introduced me to Drs. James and Elizabeth Miller who discussed their research on chemical carcinogenesis and carcinogen metabolism with me and accepted me as their graduate student. There were no laboratory rotations in the early 1950s, and a simple introduction by Dr. Rusch and an agreement between the student and professor was all that was needed to start the graduate student-mentor relationship.

MY FAILURE AS A SYNTHETIC CHEMIST

I joined the Miller laboratory in September, 1952, and my first project was to synthesize 1-hydroxy-2-aminonaphthalene, a suspected carcinogenic metabolite of β-naphthylamine (a bladder carcinogen). After many attempts to synthesize

and purify the β-naphthylamine derivative during a two-month period failed to yield pure product and also resulted in two explosions, the Millers realized that I did not have much future as a synthetic chemist; I believe they were also worried about the destruction of their laboratory. These concerns led to a change in my research project. My failure as a synthetic chemist was extremely fortunate because it led me into a new field—the induced synthesis of microsomal drug-metabolizing enzymes—which was to become my major area of research for the next several decades.

THE JOYS OF DISCOVERY: ENZYME INDUCTION BY POLYCYCLIC AROMATIC HYDROCARBONS

After my failure as a synthetic chemist, the Millers and I discussed earlier research by H.L. Richardson and his colleagues indicating that administration of 3-methylcholanthrene inhibited the hepatocarcinogenic activity of 3′-methyl-4-dimethylaminoazobenzene in rats (1). We discussed the possibility that treatment of rats with the protective hydrocarbon might alter the metabolism of carcinogenic aminoazo dyes, and in late 1952 or early 1953 I started studies on the effects of treating rats with 3-methylcholanthrene and other polycyclic aromatic hydrocarbons on the hepatic N-demethylation and azo-link reduction of aminoazo dyes—metabolic pathways that resulted in noncarcinogenic products. Almost immediately, I experienced the joys of discovery by finding that treatment of rats with a single i.p. injection of 3-methylcholanthrene caused a rapid and many-fold increase in azo dye N-demethylase activity [M.S. thesis (2)]. A stimulatory effect of dietary 3-methylcholanthrene on azo dye N-demethylase activity was also observed during these early studies (2). While I was studying the effects of 3-methylcholanthrene on hepatic azo dye N-demethylase activity in rats, Raymond Brown (another Miller graduate student) found that livers from mice fed a Friskies dog chow diet for two weeks had about twice as much hepatic azo dye N-demethylase activity as the livers of mice fed a purified diet (3). Brown then focused his attention on constituents in the crude diet that could increase azo dye N-demethylase activity when fed in the purified diet (3).

My first full-length publication provided strong evidence that treatment of rats with a single i.p. injection of 3-methylcholanthrene induced the synthesis of hepatic aminoazo dye N-demethylase and azo-link reductase (4). The induction of hepatic azo dye N-demethylase and azo-link reductase activities by 3-methylcholanthrene is shown in Figure 1. These studies on the induced synthesis of azo dye-metabolizing enzymes were early examples of enzyme induction in mammals. Curiously, my first full-length publication started on page 450 of *Cancer Research* (Vol. 16) (4). The coincidence of this paper starting on page 450 and the later development of the field of multiple cytochromes P450 was striking. Our early research indicated that polycyclic aromatic hydrocarbons that inhibited aminoazo dye-induced liver cancer (1, 5) were potent inducers of azo dye N-demethylase activity (2, 4), whereas other polycyclic hydrocarbons that did not influence azo

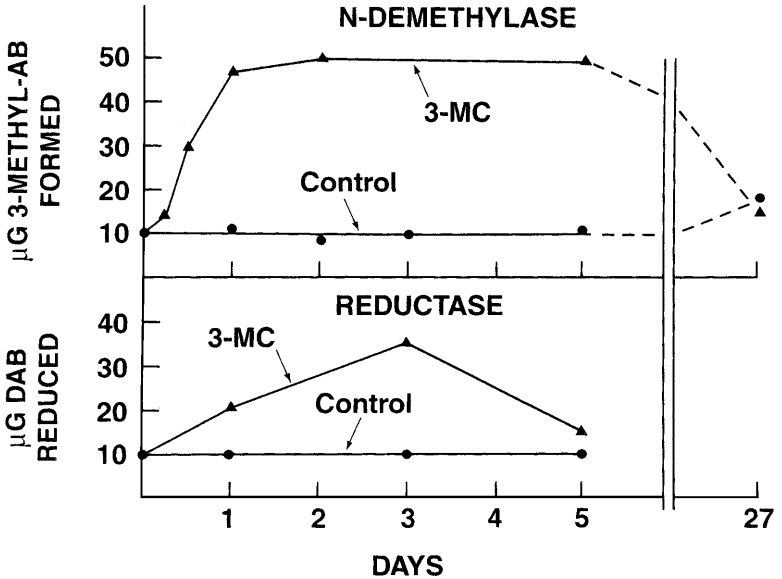

Figure 1 Induction of hepatic aminoazo dye N-demethylase and reductase activities. Rats (50 g) were injected once with 1 mg of 3-methylcholanthrene (3-MC). N-Demethylase activity was determined in fortified liver homogenates by measuring the metabolism of 3-methyl-4-monomethylaminoazobenzene to 3-methyl-4-aminoazobenzene (3-methyl-AB). Reductase activity was determined by measuring the reduction of the azo linkage of 4-dimethylaminoazobenzene (DAB). Demethylase activity is expressed as μg of 3-methyl-AB formed per 50 mg of liver per 30 min. Reductase activity is expressed as μg of DAB reduced per 30 mg of liver per 30 min. Each point is the average of the activities for two or three rats. Taken from Ref. (4).

dye-induced carcinogenesis (5) had little or no effect on azo dye metabolism (2, 4). Our research on the induction of azo dye-metabolizing enzymes provided a mechanistic explanation for the inhibitory effects of polycyclic hydrocarbons on azo dye carcinogenesis and was an early example of mechanisms of cancer chemoprevention. These studies also placed into perspective the meaning of safety and benefit/risk ratio for the fields of pharmacology and cancer chemoprevention. If I were a rat in an environment of carcinogenic aminoazo dyes that would with great certainty cause liver cancer and all that was available for protection was 3-methylcholanthrene (a poor carcinogen when administered orally), I would ingest the hydrocarbon because it would save my life. Clearly, safer chemopreventive agents would be more desirable, and having a mechanistic understanding of chemoprevention led to a search for safer and more effective chemopreventive agents.

In 1955, the Millers and I initiated research to determine whether polycyclic aromatic hydrocarbons that stimulated azo dye N-demethylase activity could also

stimulate their own metabolism. In 1957, we reported that treatment of rats with benzo[a]pyrene (BP), 3-methylcholanthrene, or several other polycyclic hydrocarbons induced the synthesis of hepatic BP hydroxylase (6). In later studies after leaving Madison, my colleagues and I found that induction of BP hydroxylase was paralleled by increased in vivo metabolism of BP and other polycyclic aromatic hydrocarbons. The increased level of BP hydroxylase activity in BP-pretreated rats was reflected in vivo by decreased blood and tissue concentrations of this carcinogen and enhanced biliary excretion of its metabolites (7, 8). The stimulatory effect of BP on its own metabolism was illustrated by a decreased tissue concentration of this compound that occurred when it was administered chronically. At 24 h after a single oral dose of 1 mg of [^{3}H]BP to adult rats, the concentration of the hydrocarbon in fat was 249 ng/g, whereas 24 h after seven daily doses of [^{3}H]BP, its concentration in fat was only 24 ng/g (7).

In 1966–1967, we demonstrated that treatment of rats with certain polycyclic hydrocarbons or aromatic azo derivatives stimulated the in vitro and in vivo metabolism of 7,12-dimethylbenz[a]anthracene (9, 10), which provided a mechanistic explanation for earlier research indicating an inhibitory effect of these compounds on 7,12-dimethylbenz[a]anthracene-induced mammary cancer and adrenal toxicity in rats (11–15). These early studies on mechanisms of inhibition of azo dye and polycyclic hydrocarbon carcinogenesis as well as subsequent studies suggested that induction of carcinogen detoxifying enzymes may be a useful strategy for cancer chemoprevention [reviewed in Refs. (16–18)].

ENZYME INDUCTION BY DRUGS

After receiving my Ph.D. degree, I worked in my father's drug store for a few months as a pharmacist before pursuing a full-time career in research. I was intrigued by the possibility that polycyclic aromatic hydrocarbons may induce the synthesis of drug-metabolizing enzymes and also by the possibility that drugs might induce the synthesis of drug-metabolizing enzymes. The last experiment that I did before leaving Madison in 1956 indicated that treatment of rats with aminopyrine (an analgesic drug metabolized by N-demethylation) stimulated hepatic aminoazo dye N-demethylase activity. This study (which was never published) was an early indication of a stimulatory effect of a therapeutically useful drug on hepatic drug metabolism. In 1957, I met with Dr. Bernard Brodie, whose Laboratory of Chemical Pharmacology in the Heart Institute at the National Institutes of Health was at the forefront of drug metabolism research in the 1950s, and I discussed with him the possibility that drugs may induce increased levels of microsomal drug-metabolizing enzymes. I wanted to obtain a postdoctoral position in Brodie's laboratory to study the pharmacological significance of microsomal enzyme induction. Although Brodie was not able to take me into his laboratory, he introduced me to Dr. John Burns who was interested in the stimulatory effects of drugs on ascorbic acid synthesis, and we wondered whether the drugs that stimulated ascorbic acid synthesis would also enhance drug metabolism. John Burns

obtained a $6000/year fellowship stipend from McNeil Laboratories for me to study the fate of zoxazolamine (Flexin) and chlorzoxazone (Paraflex), which were muscle relaxant drugs produced by McNeil Laboratories.

In 1957, I moved with my wife and infant son from the drugstore in Winnetka to Bethesda, Maryland, and started working with John Burns at the National Institutes of Health. I isolated and identified metabolites of zoxazolamine and chlorzoxazone from my urine (19, 20), and I also studied microsomal enzyme induction part time. We found that treatment of rats with several drugs and polycyclic aromatic hydrocarbons stimulated both ascorbic acid synthesis and hepatic aminoazo dye N-demethylase activity (21). The reasons for this intriguing relationship and the mechanisms responsible for the stimulatory effect of drugs on ascorbic acid synthesis are still unknown. We then found that treatment of rats with phenobarbital, barbital, aminopyrine, phenylbutazone, orphenadrine, or benzo[a]pyrene caused a marked increase in the activity of liver microsomal enzymes that metabolized several drugs (22). Evidence was presented in our early studies that administration of phenobarbital induced the synthesis of drug-metabolizing enzymes and also stimulated liver microsomal protein synthesis (22, 23). A young Alfred Gilman provided considerable help with these studies during a summer internship prior to his interest in G proteins (23). Administration of several drugs individually enhanced the ability of liver microsomes to metabolize the same or a closely related compound. Thus, treatment of rats with phenylbutazone, aminopyrine, benzo[a]pyrene, or phenobarbital increased the ability of liver microsomes to metabolize the compound administered or a closely related compound (22). At the time we were studying the effects of drugs and polycyclic aromatic hydrocarbons as inducers of microsomal monooxygenases at the National Institutes of Health, Drs. Herbert Remmer and Ryuichi Kato, working independently, also demonstrated a stimulatory effect of barbiturates and other drugs on drug metabolism (24–27).

SELECTIVE ENZYME INDUCTION BY BENZO[A]PYRENE: EVIDENCE FOR MULTIPLE DRUG-METABOLIZING ENZYMES

In 1959, we reported that treatment of rats with benzo[a]pyrene had a selective stimulatory effect on the hydroxylation and N-demethylation of some foreign compounds but not others. Treatment of rats with benzo[a]pyrene had a large stimulatory effect on the hydroxylation of benzo[a]pyrene, acetanilide, and zoxazolamine, but there was little or no effect on the hydroxylation of chlorzoxazone (Table 1) (28). Similarly, treatment of rats with benzo[a]pyrene markedly stimulated the N-demethylation of 3-methyl-4-monomethylaminoazobenzene (3-Me-MAB) but had a much smaller stimulatory effect on the N-demethylation of N-methyl-aniline, and this treatment inhibited the N-demethylation of diphenhydramine (Benadryl®) and meperidine (Table 1) (28). These studies suggested a family of monooxygenases with individual members that were under separate regulatory control. In additional studies, we found that treatment of rats with benzo[a]pyrene stimulated

TABLE 1 Selective induction of liver microsomal monooxygenase activities by benzo[a]pyrene

		Metabolism (nmol per gm liver/hr)		
Reaction	**Substrate**	**Control**	**Benzo[a]pyrene**	**Activity ratio**
Hydroxylation	Benzo[a]pyrene	0.42	5.1	12.0
	Acetanilide	0.48	3.1	6.5
	Zoxazolamine	1.3	4.9	3.8
	Quinoline	0.20	0.41	2.0
	Chlorzoxazone	2.2	3.0	1.4
N-Demethylation	3-Me-MAB	1.9	10.0	5.3
	N-Methyl-aniline	0.41	0.82	2.0
	Benadryl	0.46	0.32	0.70
	Meperidine	0.38	0.12	0.32

Immature male rats were injected once with corn oil or with 1 mg of benzo[a]pyrene in corn oil, and the animals were sacrificed 24 h later. 3-Me-MAB is 3-methyl-4-monomethylaminoazobenzene. Taken from Ref. (28).

the hydroxylation of the muscle relaxant drug zoxazolamine to an inactive product, stimulated the in vivo metabolism of zoxazolamine, and shortened its duration of action from 730 min to 17 min (22). In contrast to these observations, treatment of rats with benzo[a]pyrene did not stimulate the liver microsomal metabolism of hexobarbital or shorten its duration of action (22). Although benzo[a]pyrene had a selective stimulatory effect on the metabolism of only certain drugs, administration of phenobarbital or the administration of several other drugs stimulated the metabolism of many drugs (22). Selectivity for enzyme induction by polycyclic aromatic hydrocarbons such as benzo[a]pyrene and 3-methylcholanthrene and the broadness of inducing activity for phenobarbital towards many substrates gave rise to the notion during the 1960s of two kinds of enzyme inducers for xenobiotic metabolism—polycyclic aromatic hydrocarbons (such as benzo[a]pyrene and 3-methylcholanthrene) that were selective inducers and phenobarbital-like drugs that stimulated the metabolism of many drugs.

In 1970, I visited Hans Selye in Montreal to give a Claude Bernard Lecture, and we discussed Selye's observation that treatment of rats with certain steroids inhibited the toxicity of a large number of foreign chemicals. Pregnenolone-16α-carbonitrile was one of Selye's most active compounds, and I suggested the possibility that this steroid was an inducer of microsomal detoxifying enzymes. In 1972, we identified pregnenolone-16α-carbonitrile as a new type of inducer of xenobiotic-metabolizing enzymes in liver microsomes (29). Treatment of rats with pregnenolone-16α-carbonitrile induced a different profile of xenobiotic-metabolizing enzymes than occurred after treatment of rats with either phenobarbital or 3-methylcholanthrene (29). The cytochrome(s) that were induced by pregnenolone-16α-carbonitrile were later identified as members of the CYP3A family, a major cytochrome P450 family in human liver. Later studies indicated that

treatment of rats with 3-methylcholanthrene, phenobarbital, pregnenolone-16α-carbonitrile, ethanol, dioxin, polychlorinated biphenyls, isosafrole, or clofibrate each induced a different profile of xenobiotic metabolism and a different profile of cytochromes P450. In additional studies, Takemori & Mannering showed that addition of 10^{-5} M SKF 525-A to a mouse liver 9000 $\times$ g supernatant fraction inhibited the demethylation of morphine and codeine by about 50%, but the N-demethylation of 3-methyl-4-monomethylaminoazobenzene was unaffected (30). The selective effects of microsomal enzyme inducers and inhibitors on the oxidative metabolism of several drugs suggested multiple enzyme systems for the metabolism of these compounds, and stronger evidence for this concept would come from studies on factors that regulated the metabolism of a single substrate that was hydroxylated in multiple positions.

SPECIFICITY IN THE REGULATION OF 6β-, 7α-, AND 16α-HYDROXYLATION OF TESTOSTERONE: FURTHER EVIDENCE FOR MULTIPLE MONOOXYGENASES

In early studies, we found that treatment of rats with phenobarbital or certain other inducers of xenobiotic metabolism also increased the level of liver microsomal enzymes that metabolized testosterone, $^{4}\Delta$-androstene-3,17-dione, estradiol, estrone, progesterone, and deoxycorticosterone, and this treatment decreased the action of these steroids (31–35). During the course of this research, we developed paper chromatography methods that allowed us to measure the metabolism of ^{14}C-labeled testosterone to 6β-, 7α-, and 16α-hydroxytestosterone and to study factors that regulated these reactions. We found that the three hydroxylation reactions were selectively modulated during the development of rats with age (first 13 weeks of life), during enzyme induction, after castration, during the storage of frozen microsomes, and after the addition of chlorthion or carbon monoxide (CO) (36).

Different patterns for the development of the 6β-, 7α-, and 16α-hydroxylation of testosterone were observed during the first 13 weeks of life in male rats (Figure 2) (36). The 16α-hydroxylation of testosterone was low at birth and remained low for four weeks, but increased markedly during the next several weeks. In contrast to the 16α-hydroxylation activity, 6β-hydroxylation activity increased during the first week of life, remained relatively constant during the next six weeks, and increased moderately during the next three weeks. The 7α-hydroxylation of testosterone was low at birth and increased by one week. This activity remained constant for an additional three weeks and then decreased during the following three weeks.

Storage of frozen rat liver microsomes for 20 or 48 days at -15°C resulted in a progressive decrease in enzyme activity for the 6β- and 16α-hydroxylation of testosterone, but 7α-hydroxylation activity was stable (36). The in vitro addition of 10^{-4} M chlorthion almost completely inhibited the 16α-hydroxylation of testosterone by rat liver microsomes but only inhibited the 6β- and 7α-hydroxylation reaction by 31 and 14 percent, respectively (36, 37).

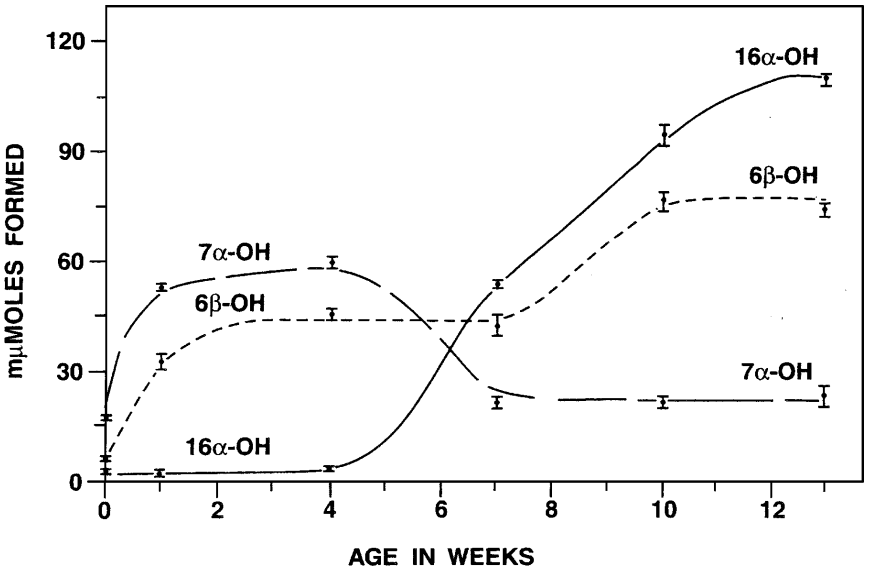

Figure 2 Effect of age on the 6β-, 7α-, and 16α-hydroxylation of testosterone by rat liver microsomes. Liver microsomes equivalent to 333 mg of wet-weight liver from male rats were incubated with 700 mμmoles of testosterone-4-C^{14} in a final volume of 5.7 ml for 7.5 min at 37°C in the presence of an NADPH-generating system. Formation of 6β-hydroxytestosterone (6β-OH), 7α-hydroxytestosterone (7α-OH), and 16α-hydroxytestosterone (16α-OH) was measured. Each value represents the average $\pm$ SE of three to six values where each value was obtained with the pooled livers from three to six rats. Taken from Ref. (36).

Treatment of immature male rats with phenobarbital for three days increased the 6β-, 7α-, and 16α-hydroxylation of testosterone by liver microsomes to different degrees (36). The 16α-hydroxylation reaction was stimulated several-fold, whereas the 6β- and 7α-hydroxylations were stimulated to a smaller extent. In contrast to these results, the administration of 3-methylcholanthrene had little or no stimulatory effect on the 6β- or 16α-hydroxylation of testosterone by liver microsomes but caused a significant increase in the 7α-hydroxylation reaction.

Early studies by Klingenberg (38), Garfinkel (39), and Omura & Sato (40, 41) demonstrated the presence of a unique liver microsomal pigment with a carbon monoxide difference spectrum with a peak at 450 nm (after reduction of the hemoprotein with dithionite). This hemoprotein was named cytochrome P450 by Omura & Sato (40, 41). In 1963 and 1965, Estabrook, Cooper, Rosenthal, and their colleagues demonstrated the functional role of cytochrome P450 for the oxidative metabolism of steroids and drugs—a key discovery (42, 43), and there was considerable debate during the middle to late 1960s about the number of liver microsomal cytochromes P450 that participated in the oxidative metabolism of drugs and

steroids. Some thought there was only a single cytochrome P450 that could exist in multiple interchangeable forms, whereas our laboratory favored the concept of multiple cytochromes P450 that were under separate regulatory control. Alvares and his colleagues in my laboratory presented evidence for the presence of at least two CO-binding cytochromes in liver microsomes (44–46). Whereas treatment of rats with phenobarbital increased the concentration of a CO-binding pigment with a reduced CO difference spectrum maximum at 450 nm, treatment of rats with 3-methylcholanthrene increased the concentration of a CO-binding pigment with a reduced CO difference spectrum maximum at 448 nm (44–46). Because this effect of 3-methylcholanthrene on the reduced CO difference spectrum maximum was prevented by treating the rats with ethionine or actinomycin D, we believed that it represented the synthesis of a previously unidentified cytochrome P450 (44–46). Other spectral evidence for the induction of different microsomal cytochromes by phenobarbital and 3-methylcholanthrene was presented by Sladek & Mannering (47).

Carbon monoxide inhibited the 6β-, 7α-, and 16α-hydroxylation of testosterone by rat liver microsomes to different extents. A CO/O_2 ratio of 0.5 inhibited the 7α-, 6β-, and 16α-hydroxylation reactions by 14%, 25%, and 36%, respectively, and the ratio of CO/O_2 needed for 50% inhibition of testosterone hydroxylation in the 16α-, 6β-, and 7α-positions was 0.93, 1.54, and 2.36, respectively (36, 48). Studies on the photochemical action spectrum revealed that CO inhibition of the three hydroxylation reactions was maximally reversed by monochromatic light at 450 nm, but there were differences in the shape of the photochemical reactivation spectra for the 6β-, 7α-, and 16α-hydroxylation reactions (36, 48). The data from our laboratory summarized above and at the First International Symposium on Microsomes and Drug Oxidation in 1968 pointed to multiple cytochromes P450 with different catalytic activities that were under separate regulatory control (36, 45, 46), and we indicated that the actual number of cytochromes that participate in the multiple hydroxylation reactions must await the solubilization and purification of the microsomal system (36). The use of different inducers of liver microsomal monooxygenases caused selective increases in the concentration of specific cytochromes P450 in liver microsomes that greatly facilitated the isolation and purification of these hemoproteins.

SOLUBILIZATION AND PURIFICATION OF MULTIPLE CYTOCHROMES P450

In 1957, we reported on the use of the detergent deoxycholate for the solubilization of aminoazo dye N-demethylase, and we also reported an inhibitory effect of CO on aminoazo dye N-demethylase activity (49). In a key study in 1968, Lu & Coon described the deoxycholate-dependent solubilization and resolution of a liver microsomal fatty acid ω-hydroxylation system into three components by column chromatography, and they were able to reconstitute catalytic activity by combining the three fractions (50). The three fractions were identified as cytochrome P450,

NADPH cytochrome P450 reductase, and a lipid fraction or phosphatidylcholine (51, 52). The reconstituted hydroxylation system was then shown to metabolize a large number of drugs, carcinogens, and steroid hormones. These studies paved the way for subsequent studies on the purification and characterization of cytochrome P450.

In 1970, Anthony Lu joined my laboratory and we initiated studies on the purification and catalytic activities of cytochrome P450 obtained from liver microsomes of control rats, phenobarbital-treated rats, and 3-methylcholanthrene-treated rats. We anticipated finding different cytochromes P450 with different catalytic activities in liver microsomes from animals treated with the different inducers, and we found that the reconstituted hydroxylation enzyme system from rats treated with phenobarbital exhibited high catalytic activity for benzphetamine N-demethylation in vitro but had very low activity for benzo[a]pyrene hydroxylation (Table 2, Experiment 1) (53). Replacement of the cytochrome P450 fraction by the cytochrome P448 fraction from rats treated with 3-methylcholanthrene resulted in a marked decrease in benzphetamine N-demethylation and a large increase in benzo[a]pyrene hydroxylation (Table 2, Experiment 1) (53). Conversely, the reconstituted system from 3-methylcholanthrene-treated rats showed high benzo[a]pyrene hydroxylase

TABLE 2 Effect of different P450 fractions on drug metabolism in reconsitituted systems

	Experiment 1		
	PB P450	**3-MC P448**	
Substrate	**nmoles metabolized**		
Benzo[a]pyrene	0.07	1.13	
Benzphetamine	3.23	0.97	
	Experiment 2		
	Control P450	**PB P450**	**3-MC P448**
Substrate	**nmole metabolite formed**		
Pentobarbital	0.10	1.00	0.06
Benzo[a]pyrene	0.33	0.23	1.29
Chlorcyclizine	1.02	2.26	1.58

Rats were treated with vehicle (control), phenobarbital (PB), or 3-methylcholanthrene (3-MC). Cytochrome P450, lipid, and reductase fractions were prepared and reconstituted. The reductase and lipid fractions were prepared from PB-treated rats. No hydroxylation activity was detected when hemoprotein was omitted from the reaction mixture. In Experiment 1, benzo[a]pyrene metabolism was measured by formation of fluorescent phenolic metabolites, and benzphetamine metabolism was measured by the rate of benzphetamine-dependent NADPH oxidation. In Experiment 2, the metabolism of pentobarbital, benzo[a]pyrene, and chlorcyclizine was measured by product formation. Experiment 1 was taken from Ref. (53) and Experiment 2 was taken from Ref. (55).

activity, which was greatly decreased when the cytochrome P448 fraction was replaced by the cytochrome P450 fraction from rats treated with phenobarbital (Table 2, Experiment 1) (53). The reductase and lipid fractions obtained from phenobarbital- or 3-methylcholanthrene-treated rats were interchangeable in supporting benzo[a]pyrene and benzphetamine metabolism. This study and subsequent studies in our laboratory indicated that the substrate specificity for the hydroxylation of drugs and steroids resided in the cytochrome P450 or P448 fractions rather than in the other components (53, 54). In additional studies, we found that the cytochrome P450 fraction from liver microsomes of control, phenobarbital-treated, or 3-methylcholanthrene-treated rats each had its own distinct substrate specificity when reconstituted with reductase and lipid (Table 2, Experiment 2) (55). These studies confirmed our prediction of the presence of multiple cytochromes P450 with different catalytic activities in rat liver microsomes. Work on the purification and properties of the multiple cytochromes P450 in rabbit liver by Minor Coon and Eric Johnson; in rat and human liver by Fred Guengerich; and in rat liver by Anthony Lu, Wayne Levin, and Ronald Kuntzman continued for the next several years and greatly advanced our understanding of the properties of these enzymes. The use of phenobarbital, 3-methylcholanthrene, pregnenolone-16α-carbonitrile (or dexamethasone), polychlorinated biphenyls, dioxin, isosafrole, isoniazid (or ethanol), and clofibrate as selective inducers to increase the levels of different liver microsomal cytochromes P450 greatly facilitated their purification, isolation, and characterization.

In 1978, Anthony Lu left my laboratory to head a drug metabolism group at Merck, and Wayne Levin and his colleagues continued studies on the purification and characterization of the multiple cytochromes P450 in rat liver microsomes. Levin and his colleagues purified to homogeneity 11 different cytochromes P450 from rat liver microsomes (56–61), and they characterized these proteins with respect to their minimum molecular weights, electrophoretic properties, spectral properties, chromatographic properties, immunological properties, primary structures, and catalytic activity profiles with a large number of xenobiotic and steroid substrates. In 1979, Levin and his colleagues demonstrated different partial amino acid sequences for different forms of cytochrome P450 (cytochromes P450a, P450b, and P450c; CYP2A1, CYP2B1, and CYP1A1, respectively) (62). This report on the partial structures of three purified cytochromes P450 demonstrated that different forms of cytochrome P450 possessed different primary structures and that these different forms of cytochrome P450 were separate gene products. In an additional early study, Haugen & Coon showed different amino acid compositions and a different COOH-terminal amino acid for cytochromes $P450_{LM2}$ and $P450_{LM4}$ from rabbit liver microsomes (63).

Paul Thomas, Wayne Levin, and their colleagues utilized their highly purified cytochromes P450 to pioneer in the development and use of monospecific antibodies (polyclonal and monoclonal) for studies on the structure, function, and regulation of multiple cytochromes P450. This research provided early reports on the immunoquantitation of specific cytochrome P450 isozymes and early reports

on the effects of age, sex, and inducers on the levels of these proteins in microsomes (64–66). By 1980, the presence of multiple liver microsomal cytochromes P450 as separate gene products that were under separate regulatory control was well established (67).

MULTIPLE MONOOXYGENASES IN HUMAN LIVER: STUDIES WITH ACTIVATOR FLAVONOIDS

An approach for determining the presence of multiple monooxygenases in rat liver microsomes was the use of chemicals that selectively affected certain monooxygenase activities but not others. For example, 7,8-benzoflavone (α-naphthoflavone) markedly inhibited the hydroxylation of benzo[a]pyrene by liver microsomes from 3-methylcholanthrene treated rats or by a cytochrome P448-dependent reconstituted enzyme system (68, 69), but there was no effect or only a small stimulatory effect of 7,8-benzoflavone on benzo[a]pyrene metabolism in livers from untreated rats (68).

In the mid-1970s, we initiated studies with 7,8-benzoflavone to determine whether there were multiple monooxygenases that metabolize xenobiotics in human liver and also to determine whether livers from different individuals had different profiles of the monooxygenases. Although we expected to find inhibitory effects of 7,8-benzoflavone on xenobiotic metabolism by human liver, we were surprised to observe a dramatic stimulatory effect of added 7,8-benzoflavone (50–100 μM) on the hydroxylation of benzo[a]pyrene by human liver homogenates or microsomes (up to an 11-fold increase in catalytic activity) (70). 7,8-Benzoflavone also increased the rates of hydroxylation of zoxazolamine and antipyrine by human liver (70) and stimulated the metabolic activation of benzo[a]pyrene 7,8-dihydrodiol and aflatoxin B_1 to mutagens (71, 72). In contrast to these results, addition of 7,8-benzoflavone to homogenates of human liver had little or no effect on the rates of oxidative metabolism of 7-ethoxycoumarin, coumarin, or hexobarbital (70). The selective stimulatory effect of 7,8-benzoflavone on the metabolism of some substrates but not others indicated the presence of multiple monooxygenases in human liver. Marked individuality for the activating effects of 7,8-benzoflavone was observed in different liver samples, and we pointed out that individuality for activation may result both from the presence of multiple monooxygenases in varying amounts and proportions in the different liver samples and from a selective effect of 7,8-benzoflavone on certain of the monooxygenases (70). Although 7,8-benzoflavone is a synthetic flavonoid, examples of naturally occurring flavonoids that are activators of monooxygenases in human liver include flavone, tangeretin, and nobiletin (72). Several-fold activation of benzo[a]pyrene metabolism by 7,8-benzoflavone was observed in liver microsomes from rabbits, hamsters, and humans, but little or no activation was observed in liver microsomes from rats or guinea pigs (73).

In mechanistic studies with cholate-solubilized human or rabbit liver microsomes, 7,8-benzoflavone decreased the Km for cytochrome P450 reductase,

increased the Vmax for benzo[a]pyrene hydroxylation, and stimulated the NADPH-dependent reduction of cytochrome P450 either in the presence or absence of benzo[a]pyrene (73). The results suggested that 7,8-benzoflavone stimulated the hydroxylation of benzo[a]pyrene in liver microsomes at least in part by enhancing the interaction between cytochrome P450 and cytochrome P450 reductase (73).

In two partially purified cytochrome P450 fractions (isolated by column chromatography) from rabbit liver microsomes, flavone had a specific stimulatory effect on one of the reconstituted partially purified cytochrome P450 systems, but an inhibitory effect on the other reconstituted cytochrome P450 system (73). In studies with highly purified cytochrome P450 isozymes from rabbit liver, benzo[a]pyrene metabolism was stimulated more than fivefold by the addition of flavone to a reconstituted monooxygenase system containing cytochromes $P450_{LM3c}$ or cytochrome $P450_{LM4}$ (74). In contrast, an inhibitory effect of flavone was observed when cytochromes $P450_{LM2}$, $P450_{LM3b}$, or $P450_{LM6}$ was used in the reconstituted system (74). Addition of 7,8-benzoflavone (50–100 μM) had a strong stimulatory effect on cytochrome $P450_{LM3c}$-mediated benzo[a]pyrene hydroxylation, but $P450_{LM6}$-mediated benzo[a]pyrene hydroxylation was strongly inhibited (74). Our results demonstrated that the activating and inhibiting effects of 7,8-benzoflavone on benzo[a]pyrene metabolism depended on the type of cytochrome P450 used in the reconstituted monooxygenase system (74). Later studies demonstrated that the activator flavonoids were potent activators of human CYP3A4 (75, 76). Other examples of activation of CYP3A4-dependent reactions included 7,8-benzoflavone-mediated stimulation of the 6β- and 16α-hydroxylation of progesterone, the 10,11-epoxidation of carbamazepine, and the 1′-hydroxylation of midazolam (77–79). Studies on the activation of xenobiotic metabolism by flavonoids were recently reviewed (80).

IN VIVO ACTIVATION OF DRUG METABOLISM

We investigated whether the activator flavonoids could activate the metabolism of drugs in vivo. In preparation for in vivo activation studies, we evaluated the effects of several naturally occurring and synthetic flavonoids on the metabolism of zoxazolamine to 6-hydroxyzoxazolamine in a five-day-old rat model. Flavone, nobiletin, tangeretin, and 7,8-benzoflavone (50–250 μM) stimulated the hydroxylation of zoxazolamine by liver microsomes obtained from five-day-old rats (81, 82). Evidence was obtained indicating that flavone decreased the apparent Km for zoxazolamine at high but not at low substrate concentrations, and the Vmax value for zoxazolamine hydroxylation was increased (82). The i.p. injection of 5 μmol of flavone together with or 90 min after a 3 μmol dose of zoxazolamine immediately stimulated the total body metabolism of zoxazolamine to 6-hydroxyzoxazolamine in five-day-old rats (81, 82). Although an i.p. injection of 5 μmol of flavone caused an immediate three- to fivefold stimulation in the in vivo metabolism of 740–3000 nmol of zoxazolamine, flavone had little or no stimulatory effect when a

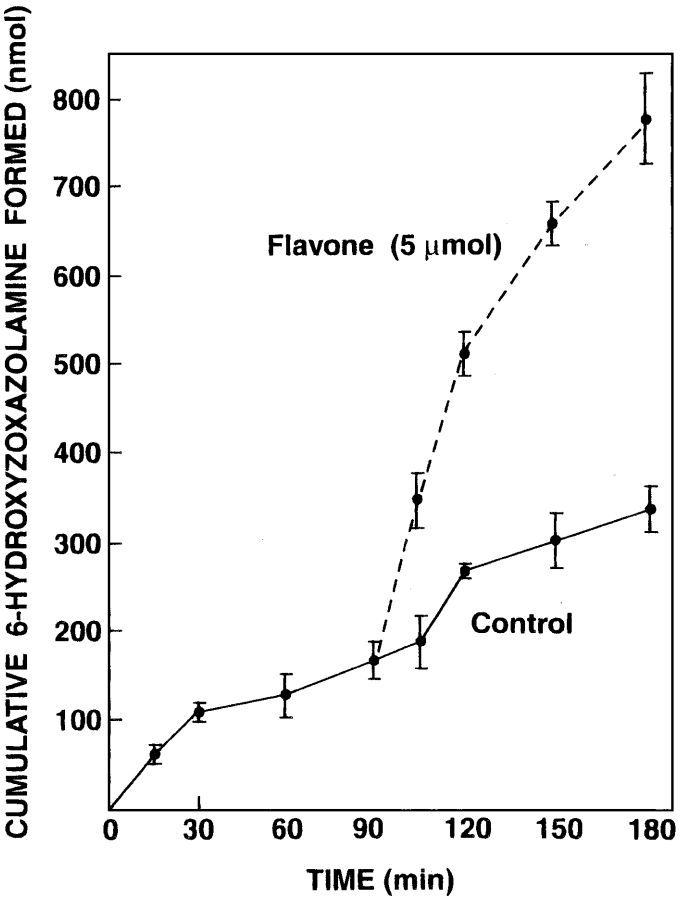

Figure 3 In vivo activation of zoxazolamine metabolism by flavone. Neonatal rats were injected with 3 μmol of [4,6-^{3}H]zoxazolamine followed 90 min later with an injection of 5 μmol of flavone or vehicle. Total body homogenates were made at the indicated times, and ^3H_2O was measured and expressed as 6-hydroxyzoxazolamine formed. Each point represents the average $\pm$ SE from four animals. Taken from Ref. (82).

much lower 74 nmol dose of zoxazolamine was administered (82). The immediate activating effect of flavone on the in vivo metabolism of zoxazolamine is shown in Figure 3.

A second example of in vivo activation of drug metabolism was recently reported by Wei Tang and his associates (83). The in vitro addition of quinidine to monkey liver microsomes or hepatocytes stimulated the cytochrome P450 3A4-mediated metabolism of diclofenac to 5-hydroxydiclofenac (83). Although quinidine had little or no effect on the Km for diclofenac metabolism by monkey

liver microsomes, the Vmax was increased 2.5-fold in the presence of quinidine. The intravenous infusion of diclofenac alone or together with quinidine in rhesus monkeys indicated that quinidine rapidly stimulated the clearance of diclofenac presumably by the activation of cytochrome P450 3A4 (83). There are now many examples of drugs that activate xenobiotic metabolism in vitro, and possible mechanisms have been described (73, 80, 82). However, the possibility of in vivo activation of foreign compound metabolism by these drugs has not been well explored. Enzyme induction and enzyme inhibition are well-recognized major mechanisms of drug-drug interactions in humans. The possibility of drug-induced activation of drug-metabolizing enzymes in humans is another potential mechanism of drug-drug interactions that is worthy of further investigation.

INDUCTION AND INHIBITION OF DRUG METABOLISM IN HUMANS

Effects of Drugs on Drug Metabolism

Many drugs are selective inducers of drug metabolism in humans and examples include phenobarbital (inducer of the CYP2B and 3A families), rifampicin (inducer of the CYP3A and 2C families), clotrimazole (inducer of CYP3A4), omeprazole (inducer of the CYP1A family), phenytoin (inducer of the CYP2C family), and ethanol (inducer of CYP2E1). Recent reports indicate that self-medication with the herbal antidepressant St. John's wort enhances the metabolism of the HIV protease inhibitor indinavir, the immunosuppressant cyclosporin, and oral contraceptives that are metabolized by CYP3A4 (84–88). The stimulatory effect of St. John's wort on drug metabolism explains the rejection of heart transplants in two patients treated with cyclosporin who also self-medicated with St. John's wort (88). Treatment of primary human hepatocytes with an extract of St. John's wort or with hyperforin (a major antidepressant constituent of St. John's wort) induces the expression of CYP3A4 (89). Hyperforin was shown to stimulate drug metabolism by functioning as a ligand for the pregnane X receptor (PXR) that regulates the expression of CYP3A4 (89). These studies with St. John's wort point out potential hazards of interactions between herbal remedies and prescription drugs.

Examples of drugs that inhibit drug metabolism in humans include bishydroxycoumarin, chloramphenicol, phenyramidol, sulfaphenazole (inhibitor of CYP2C9), cimetidine (inhibitor of several P450s), ketoconazole (inhibitor of the CYP3A family), itraconazole (inhibitor of the CYP3A family), and quinidine (inhibitor of CYP2D6). The antihypertensive drug mibefradil (potent inhibitor of the CYP3A family) is an example of a drug that was removed from the market shortly after its introduction because of serious interactions with statins and other drugs. Drug-drug interactions and interactions between drugs and herbal remedies are important problems during drug therapy and many clinically important examples have been described.

Effects of Cigarette Smoking on Drug Metabolism

Polycyclic aromatic hydrocarbons are selective inducers of cytochromes P4501A1 and 1A2 in the rat, and these hydrocarbons are ubiquitous environmental contaminants formed as products of incomplete combustion (90) that very likely contribute to interindividual differences in the metabolism of xenobiotics in humans. Because cigarette smoke contains substantial amounts of polycyclic aromatic hydrocarbons, we studied the effects of cigarette smoking on xenobiotic metabolism in experimental animals and humans. Investigations on the effects of cigarette smoking on the oxidative metabolism of drugs, carcinogens, and steroid hormones in human placenta at full term revealed that cigarette smoking markedly stimulated benzo[a]pyrene hydroxylase, aminoazo dye N-demethylase, zoxazolamine hydroxylase, and estradiol 15α-hydroxylase activities (91–94), and CYP1A1 levels were also elevated (95). Cigarette smoking had a smaller stimulatory effect on the O-dealkylation of 7-ethoxycoumarin (96) and did not change the hydroxylation of estradiol in the 2-position (94) or the oxidative aromatization of Δ^4-androstene-3,17-dione to estradiol and estrone (97). These results indicated the presence in human placenta of several monoxygenases that are under different regulatory control. Among the subjects who smoked 15 to 20 cigarettes per day, placental benzo[a]pyrene hydroxylase activity varied over a 70-fold range (97). The expression and regulation of drug metabolism in human placenta was recently reviewed (98).

Studies in rats identified pyridine and acetone as major constituents of cigarette smoke that synergistically induce hepatic and extrahepatic xenobiotic metabolism and increase the levels of CYP1A1 and CYP1A2 in liver and CYP1A1 in the lung (99). Research with human lung explants revealed induction of CYP1A transcripts by pyridine, 2-hydroxypyridine (a metabolite of pyridine), and acetone in some but not all samples (100). Additional studies on the relative inducing activities of polycyclic aromatic hydrocarbons, pyridine, and acetone alone or in combination are needed. It is likely that the stimulatory effect of cigarette smoking on human drug metabolism results from exposure to a complex mixture of inducers in tobacco smoke.

Because the metabolism of phenacetin to its major metabolite, acetaminophen, occurs by a polycyclic aromatic hydrocarbon-inducible enzyme system in rat liver and intestine (CYP1A family) (101), we investigated the effect of cigarette smoking on the in vivo metabolism of phenacetin in humans. We found that cigarette smoking lowered the plasma levels of orally administered phenacetin without changing its plasma half-life or the plasma levels of total acetaminophen (102, 103). The ratio of the concentration of total acetaminophen in plasma to that of phenacetin in plasma was markedly increased in cigarette smokers, which suggests that cigarette smoking stimulated the metabolism of phenacetin to acetaminophen in the gastrointestinal tract and/or during its first pass through the liver. Although cigarette smoking stimulated the metabolism of phenacetin in most subjects studied, some cigarette smokers did not have enhanced phenacetin metabolism.

Additional studies revealed that cigarette smokers have shorter plasma half-lives of antipyrine (104), theophylline (105), and caffeine (106) than do nonsmokers, but cigarette smoking did not stimulate the metabolism of phenytoin, meperidine, or nortriptyline, indicating selective effects of smoking on the induction of some but not other monooxygenases. The clinical significance of the effects of cigarette smoking on the metabolism and action of drugs was recently reviewed (107, 108).

Effects of Diet on Drug Metabolism

Studies in collaboration with Drs. Attallah Kappas, Alvito Alvares, and Karl Anderson at Rockefeller University and with Dr. Eugene Pantuck at Columbia University showed that several dietary factors influenced the metabolism of drugs in humans. Charcoal-broiled beef, a food that contains high concentrations of polycyclic aromatic hydrocarbons, is eaten by large numbers of people. Feeding a charcoal-broiled beef diet for several days enhanced the CYP1A1- and CYP1A2-dependent oxidative metabolism of phenacetin, theophylline, and antipyrine, but the conjugation of acetaminophen was not altered (109–111). In these studies, feeding charcoal-broiled beef for four days markedly lowered the plasma levels of orally administered phenacetin and increased the ratio of acetaminophen to phenacetin in the plasma. Marked interindividual differences occurred in the plasma concentrations of phenacetin among the nine subjects who had been fed the control diet, and there were also large individual differences in the responsiveness of the subjects to the charcoal-broiled beef diet. Switching from the control diet to a charcoal-broiled beef diet resulted in a decreased area under the plasma concentration of phenacetin versus time curve for seven of the nine subjects. The two subjects who did not respond to charcoal-broiled beef feeding had very low plasma concentrations of phenacetin while on the control diet. The reason(s) for these low plasma concentrations in two of the subjects throughout the study is unknown, but a low concentration of phenacetin in plasma may have resulted from genetic and/or environmental factors. One of the two subjects worked as a carpenter and may have been exposed to volatile oil inducers of drug metabolism that are present in certain soft woods.

Increasing the ratio of protein to carbohydrate in the diet stimulated the oxidative metabolism of antipyrine and theophylline in humans, but changing the ratio of fat to carbohydrate had no effect (112–114). Although the average half-lives of antipyrine and theophylline increased 63% and 46%, respectively, when six subjects were shifted from a high-protein, low-carbohydrate diet to an isocaloric high-carbohydrate, low-protein diet, there was considerable individuality in response to this alteration. The increase in antipyrine half-lives among the six subjects studied ranged from no change in one subject to 111% in another subject. The increase in theophylline half-lives ranged from 14% to 71% in the different subjects. Other studies have shown that switching people from their home diet to a semisynthetic diet caused a 57% decrease in the cytochrome P450-dependent oxidative

dealkylation of 7-ethoxycoumarin in the intestinal mucosa, but NADPH-cytochrome c reductase and 1-naphthol glucuronyltransferase activities were not affected (115). These results indicate that certain cytochrome P450-dependent oxidations in the intestinal mucosa are very sensitive to dietary changes.

Ingestion of cabbage and brussels sprouts for 10 days enhanced the oxidative metabolism of phenacetin and antipyrine and stimulated the glucuronidation of acetaminophen in humans (116, 117). The glucuronidation of oxazapam, however, was not enhanced (117), indicating selectivity of cabbage and brussels sprouts administration for the induction of glucuronidation of different drugs. In an additional study, feeding a brussels sprouts and broccoli–containing diet for 12 days enhanced the metabolism of caffeine (CYP1A2 probe) and decreased the urinary excretion of unchanged 2-amino-3,8-dimethylimidazo(4,5-f)quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo-(4,5b)-pyridine (PhIP) ingested in a cooked meat meal, suggesting that the vegetable diet may have enhanced the metabolism of these heterocyclic amines (118).

Ingestion of watercress, which contains high levels of phenethyl isothiocyanate, increased the urinary levels of 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol (NNAL) and its O-glucuronide [metabolically inactivated metabolites of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)] in smokers (119). This treatment also increased the glucuronidation of cotinine and *trans*-3′-hydroxycotinine in smokers, and the glucuronidation of *trans*-3′-hydroxycotinine correlated with the glucuronidation of NNAL (120). In other studies, ingestion of watercress inhibited the oxidative metabolism of acetaminophen and chlorzoxazone, suggesting an inhibitory effect on the activity of CYP2E1 (121, 122). Watercress administration, however, had no effect on acetaminophen glucuronidation (121). These results indicate that ingestion of watercress has a selective stimulatory effect on some but not all UDP-glucuronosyltransferase enzymes and that ingestion of watercress also inhibits CYP2E1. It is apparent from these studies that the effects of watercress ingestion on xenobiotic metabolism are complex.

A single glass of grapefruit juice increased the oral bioavailability of felodipine, nifedipine, and several other drugs that are metabolized by CYP3A4 presumably by inhibiting the first pass metabolism of these drugs in the gastrointestinal tract and/or liver (123, 124). It was shown that administration of grapefruit juice three times a day for six days increased the area under the plasma concentration–time curve for felodipine and caused a 62% decrease in the concentration of CYP3A4 in the small bowel epithelium without influencing the concentration of CYP1A1 or CYP2D6 in the small bowel or the hepatic CYP3A4 activity as measured by the [^{14}C-N-methyl] erythromycin breath test (124).

Alcoholics, when sober, show a tolerance for drugs to which they are highly sensitive when inebriated. The tolerance to drugs that is observed in alcoholics when they are sober may be explained in part by an induction of CYP2E1 and other cytochromes P450 in human liver after chronic ingestion of ethanol and by the enhanced rates of drug metabolism measured in vivo in alcoholics (125). In contrast to the stimulatory effect of chronic ingestion of alcohol on drug metabolism, the

administration of large amounts of ethanol immediately before administration of meprobamate or pentobarbital increased the plasma half-lives of these two drugs by two- to four-fold (125). This inhibitory effect of acute ethanol administration on human drug metabolism in vivo helps explain the dangerous and synergistic central depression that has been observed when ethanol and a sedative or hypnotic drug are ingested together.

The results of our studies on enzyme induction in humans indicated marked person-to-person differences in the response of individuals to enzyme-inducing substances. Some individuals were markedly induced whereas others were refractory. In addition, the extent of activation of xenobiotic metabolism in human liver by 7,8-benzoflavone was also variable and depended on the liver sample studied. Studies in twins indicated that the magnitude of the stimulatory effect of phenobarbital administration on the in vivo metabolism of antipyrine varied in different twin pairs, but much greater concordance was observed among monozygotic twins than among dizygotic twins (126), indicating that variability for induction of drug metabolism in different individuals is under genetic control. Examples and possible explanations for variability in the induction and inhibition of drug metabolism was recently reviewed by Lin & Lu (127).

INTRAINDIVIDUAL VARIABILITY IN DRUG METABOLISM

Both genetic and environmental factors control the rates and pathways of xenobiotic metabolism in humans. Early studies indicated that interindividual variations in the oxidative metabolism of certain drugs in the absence of known inducers or inhibitors are greater in dizygotic than in monozygotic twins (128–130). The importance of genetic factors in the regulation of human drug metabolism has also been emphasized by molecular genetics studies. There are now many studies demonstrating polymorphisms in genes that code for specific drug-metabolizing enzymes, and mutations in these genes can lead to impaired drug metabolism and altered drug action in patients (131). In addition, there are examples of gene duplication or multiple copies of a gene that codes for a drug-metabolizing enzyme, and these individuals metabolize the drug more rapidly than the general population (131). Although studies in subjects with genetic mutations and multiple gene copies help explain person-to-person differences in drug metabolism, environmental factors also have an important impact in explaining person-to-person differences in rates of drug metabolism.

Variability in the in vivo metabolism of a prototype drug that occurs when it is given to an individual on several occasions is an approach that we have used for assessing the influence of environment and life style on the metabolism of xenobiotics in humans (132, 133). In these studies, we found that the amount of day-to-day variation in the oxidative metabolism of three prototype drugs (phenylbutazone, antipyrine, and phenacetin) by seven healthy volunteers who

TABLE 3 Intraindividual variations in the metabolism of phenacetin as measured by AUC values

Subject	Area under phenacetin plasma concentration-time curve, 0→7 h, AUC (μg · min · ml^{-1})						% Difference min–max*
	10/21	12/2	1/13	2/24	4/6	Mean AUC	
A	250	174	156	261	115	191	127
B	40	19	31	122	19	46	542
C	266	421	768	358	234	409	228
D	480	267	414	457	1110	546	316
E	390	71	515	610	257	369	759
F	35	41	122	237	124	112	577
G	461	501	336	250	160	342	213

Phenacetin (900 mg) was administered orally between 8 and 9 a.m. to seven healthy volunteers on five occasions at approximately six week intervals. Breakfast was withheld for 2 h after each dose of drug. Plasma concentrations of phenacetin were measured, and areas under the plasma concentration-time curves (AUCs) were determined. Taken from Ref. (133).

*The percent difference between the maximum and minimum AUC of each subject $= \frac{\max}{\min} - 1 \times 100$.

were not taking any medications but who were allowed to pursue their normal life styles and to eat unrestricted diets depended on both the drug and the subject studied (132, 133). Substantial intraindividual variations in the areas under the phenacetin plasma concentration–time curves (AUCs) were observed in subjects given an oral 900 mg dose of phenacetin—a drug that undergoes extensive first pass metabolism (Table 3). When phenacetin was administered to seven individuals before breakfast on five occasions at approximately six week intervals, the percent difference in the areas under the plasma concentration of phenacetin-time curves (from the minimum to the maximum AUC) on the five occasions varied over a 127% range for subject A and over a 759% range for subject E (Table 3). Intraindividual differences for the metabolism of caffeine and dextromethorphan were recently evaluated and some individuals exhibited substantial intraindividual differences when they were studied on several occasions (134, 135). Kalow has also emphasized the use of studies on intraindividual variations in drug metabolism as an approach for assessing the role of environmental and life style factors in regulating drug metabolism (136–138).

It is important to point out that the use of intraindividual differences in drug metabolism as an approach for studies on the role of environment on drug metabolism tends to underestimate the role of environment in regulating human drug metabolism because the presence of a potent environmental modifier of drug metabolism would remain undetected unless the degree of exposure to the modifier changed during the study. Many of us lead a rather routine life, eat similar foods from day-to-day, and don't vary our life-styles dramatically from day to day. Others, however, may have a more variable life style that could influence the metabolism and therapeutic or toxic action of drugs during therapy. Environmental

factors that influence the metabolism of xenobiotics include ingestion of medicinal agents or herbal remedies, cigarette smoking, alcohol ingestion, dietary factors, viral infections, and exposure to environmental chemicals that influence the levels and activities of the multiple cytochrome P450 enzymes.

CONCLUDING REMARKS

Our studies started 50 years ago by asking why 3-methylcholanthrene administration inhibited the formation of liver cancer by carcinogenic aminoazo dyes in rats? In answering that question, we found that 3-methylcholanthrene induced the synthesis of liver microsomal enzymes that metabolized the dyes to noncarcinogenic products. These studies then led to further research indicating that polycyclic aromatic hydrocarbons had selective inducing effects on the oxidative metabolism of some foreign compounds but not others, indicating a family of xenobiotic-metabolizing monooxygenases with members that were under different regulatory control. In addition, treatment of animals with different microsomal enzyme inducers resulted in different profiles of catalytic activity for the metabolism of foreign compounds. Selective modulation of the liver microsomal 6β-, 7α-, and 16α-hydroxylation of testosterone was observed during the development of the animals with age and by the use of enzyme inducers and CO as an inhibitor. These results and the selective induction of liver microsomal CO-binding hemoproteins with different spectral properties in animals treated with phenobarbital or 3-methylcholanthrene indicated that multiple cytochromes P450 catalyzed the hydroxylation of testosterone in the 6β-, 7α-, and 16α-positions. Treatment of animals with different inducers of the monooxygenases selectively increased the levels of certain cytochromes P450 that facilitated their purification and characterization. Solubilization, purification, and characterization of the microsomal monooxygenase system confirmed the presence of multiple cytochromes P450 with different amino acid sequences and different but often overlapping substrate specificities. The number of cytochromes P450 identified in rats and humans has grown markedly in recent years and is now estimated to approach 60 in each species. The pharmacological significance of the induction of cytochromes P450 for the action of drugs and other foreign compounds depends on which ones are induced and the action and profile of metabolites that are produced by the induced cytochromes P450.

Modulation of the cytochrome P450 enzymes at the portals of entry of chemicals into the body (gastrointestinal tract, lung, and skin) and in extrahepatic target cells at or near drug receptors are particularly important areas for further research. Changes in the level or activity of a drug-metabolizing enzyme in the gastrointestinal tract may influence the amount of drug that reaches the systemic circulation. Changes in the level or activity of a drug-metabolizing enzyme at or near a receptor may change the drug's pharmacological activity without a corresponding change in the blood level.

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PROTEIN FLEXIBILITY AND COMPUTER-AIDED DRUG DESIGN

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■ **Abstract** Although computational techniques are increasingly being used in computer-aided drug design, the effects due to protein flexibility are still ignored in many applications. This review revisits rigorous statistical mechanical methods for predicting binding affinity, discusses some recent developments for improving their speed and reliability, and examines faster approximate models for facilitating virtual screening and lead optimization.

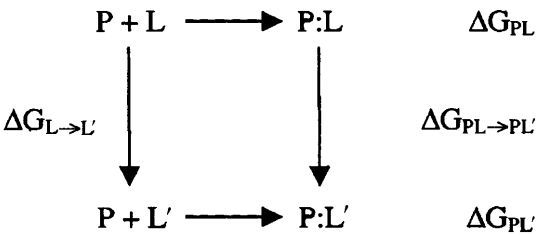
INTRODUCTION

The rapid advance of computer technology and the development of new modeling software have made computer-aided drug design an increasingly useful tool. This review focuses on addressing the role of protein flexibility in drug discovery, as this remains one of the most challenging problems in computer-assisted drug development and many modeling efforts still assume proteins to be rigid. The first molecular dynamics simulation of a protein, bovine pancreatic trypsin inhibitor (1), revealed in atomic details large structural fluctuations that were previously unexpected. The possible significance of these effects on determining protein functions and molecular recognition was quickly recognized and has triggered the development of methods for including receptor flexibility in modeling protein-ligand interactions. For example, rigorous simulation methods for calculating binding free energy were introduced in the early 1980s (2–8). Although these simulations were limited to several tens of picoseconds in the early stages, they moved a step beyond the fixed-conformation picture that dominated many scientists' thinking at the time. Despite this advance, an issue rapidly came up concerning the length of simulations required to generate representative ensembles for reliable predictions. Although the simulation length required to gain good statistics depends on the specific problem at hand, many applications of practical interest demand a large amount of computer time, preventing these modeling techniques from being used

routinely in day-to-day drug discovery. However, progress has been made in developing new algorithms to accelerate these calculations, in employing implicit rather than explicit solvent models to facilitate solute conformational samplings, and in introducing approximate methods to screen many derivatives of a drug lead more efficiently. Here, we briefly review some earlier work and then discuss various encouraging recent developments.

FREE ENERGY CALCULATIONS

In 1984 (2), the thermodynamic cycle-perturbation method was introduced to help compare the binding affinity of a group of similar inhibitors. This method is based on constructing a thermodynamic cycle relating two binding processes:



Because free energy is a state function,

$$\Delta\Delta G = \Delta G_{PL'} - \Delta G_{PL}, \qquad 1.$$

which compares the binding affinity of two ligands, L and L′, to a protein P, can also be obtained from

$$\Delta\Delta G = \Delta G_{PL\rightarrow PL'} - \Delta G_{L\rightarrow L'}. \qquad 2.$$

This trick converts the difficult problem of calculating $\Delta G_{PL'}$ and ΔG_{PL} into evaluating $\Delta G_{L\rightarrow L'}$ and $\Delta G_{PL\rightarrow PL'}$. $\Delta G_{PL'}$ and ΔG_{PL} are usually more difficult to calculate directly because they involve simulating the displacement of a large number of water molecules before the binding between P and L/L′ can occur. $\Delta G_{PL'}$ and ΔG_{PL} are often much easier to calculate, especially when the two ligands are similar, because they involve relatively small changes in chemical functional groups. One can use special simulation methods, such as Zwanzig's perturbation theory (9), to calculate the free energy differences, $\Delta G_{L\rightarrow L'}$ and $\Delta G_{PL\rightarrow PL'}$. For the Helmholtz free energy, Zwanzig's perturbation theory reads

$$\Delta A = -RT \ln \langle \exp(-\Delta H/RT) \rangle_r, \qquad 3.$$

where RT is the gas constant times the absolute temperature, $\Delta H = H_p - H_r$ in which H_p and H_r are the classical Hamiltonians of the perturbed and reference systems respectively, and $\langle \ldots \rangle_r$ represents an ensemble average over the reference

state. (The quantities ΔG and ΔA are virtually the same in many cases.) This formula is exact, although it is hard to obtain a reliable estimate of ΔA when the difference between the reference and perturbed system is large. One way to alleviate this problem is to change the reference system into the perturbed one in N steps, rather than in one single step, and use the perturbation formula N times to estimate ΔA from

$$\Delta A = -RT \sum_{i=1}^{N} \ln \langle \exp(-\Delta H_i/RT) \rangle_i, \qquad 4.$$

where $i = 1$ refers to the reference state, $i = \mathrm{N} + 1$ corresponds to the final state, and $\Delta H_i = H_{i+1} - H_i$. These calculations can be very expensive to do when large modifications are made because many windows are required. Consequently, these calculations are usually only practical to use at the late-stage refinement of an already good lead. However, methods to further speed up free energy calculations are constantly being introduced and some recent developments are discussed here.

Locally Enhanced Sampling

Protein side chains or ligand functional groups can adopt multiple conformations, and the barriers separating these conformations may be rather high such that the transitions among them are difficult. Thus, it may require a long simulation to reliably estimate a free energy change if more than one conformation contributes significantly to binding. To improve the sampling of these conformations in free energy calculations, Verkhivker et al. (10) utilized the locally enhanced sampling (LES) method (11). The essence of this method is to replace a side chain or a functional group by N copies that do not interact with each other, and each copy only interacts with itself and its surrounding with $1/\mathrm{N}^{\mathrm{th}}$ of the original strength. The use of multiple copies and the reduction of the interaction potentials can significantly enhance the sampling of the conformations of the side chain or the functional group. This requires two additional perturbation calculations: one in changing from the single-copy representation of the reference state to the multiple-copy representation and the other in going from the multiple-copy representation of the perturbed state into the single-copy representation. But Verkhivker et al. (10) demonstrated that the benefits of adopting a multiple-copy representation outweighed the additional costs of introducing two more perturbation calculations. More recently, Simmerling et al. (12) applied this method to study the $\alpha \rightarrow \beta$ anomerization of glucose and found that the free energy calculations converged an order of magnitude faster than with the single-copy method.

λ Dynamics Method

The λ dynamics method (13, 14) is another technique introduced to speed up free energy calculations. In this method, multiple ligands are placed in the binding site of their receptor at once with the interaction potential of each ligand reduced from its full strength. The fraction, λ_i^2, of the interaction potential of each ligand

is determined dynamically during a simulation, with λ_t treated as a particle with a fictitious mass. Because the interaction potential of each ligand is reduced, the barriers for conformational transitions are lower. The reduced barriers can enable a ligand to explore different orientations and conformations more easily. Also, the ranking of the ligands can emerge quickly during the simulation as λ_t^2 can increase rapidly for the winners at the expense of the losers. The identification of the strong binders can therefore be much quicker than by doing many free energy perturbation calculations including only one ligand at a time. This method was able to quickly distinguish strong benzamidine inhibitors of trypsin from weaker ones (15).

Systematic Sensitivity Analysis

One does not always need to aim for highly accurate binding constants to be productive in drug design. It is already useful to generate rules or constraints from computational studies to guide the design of chemical libraries for high-throughput screening and to direct the optimization of a drug lead. To this end, one does not necessarily make physical modifications, but nonphysical ones that probe the relative significance of different features of functional groups in affecting binding. For example, by sequentially turning off the atomic partial charge or dipole moment of every relevant functional group in a lead compound, one can determine which charges or dipole moments are important to keep in optimizing a drug lead and which charges or dipole moments should be turned off to improve binding affinity. For this more modest goal, one can use mathematical tricks to carry out many free energy difference calculations simultaneously.

One way to do this is to adopt an approach that has been used by engineers for a long time (16, 17) and has recently been applied to study molecular and biomolecular systems (18–28). In order to identify the key model parameters determining system properties, engineers calculate the derivatives, $\frac{\partial O}{\partial \lambda_i}$s, of a property, O, of the system with respect to the model parameters, λ_is, to measure the sensitivity of the observable to parameter changes. (The dimensionless logarithmic derivatives $\frac{\partial \ln O}{\partial \ln \lambda_i} = \frac{\partial O}{\partial \lambda_i}\frac{\lambda_i}{O}$s are also often calculated to facilitate comparison among different types of parameters.) Parameters that do not affect system properties yield negligible $\frac{\partial O}{\partial \lambda_i}$s. On the other hand, important parameters would yield large $\frac{\partial O}{\partial \lambda_i}$s. Analytical expressions can be worked out for calculating these derivatives based on the dynamical behavior of a single reference system. Because the calculation of the derivatives of a number of observables with respect to all the parameters of a model only adds a small fraction to the costs of doing dynamical simulations on the reference system, it is not difficult to systematically compare the role of all the parameters in a model so that no important parameters are overlooked. This idea can be generalized to include higher-order derivatives so that a Taylor's series expansion,

$$\Delta O = \sum_i \frac{\partial O}{\partial \lambda_i}\Delta\lambda_i + \frac{1}{2}\sum_{i,j}\frac{\partial^2 O}{\partial \lambda_i \partial \lambda_j}\Delta\lambda_i\Delta\lambda_j + \ldots, \qquad 5.$$

can be used to predict the influence of larger parameter changes on system properties. The Taylor's series expansion also permits the effects of many different combinations of parameter changes to be examined. However, it is more difficult to calculate higher-order derivatives, and the convergence of the Taylor's series can be slow, if it converges at all, when parameter modifications are large. Different strategies have been introduced to deal with somewhat larger perturbations.

One-Step Application of Zwanzig's Perturbation Theory

The single-window Zwanzig perturbation theory (9) described above can provide quick estimates of free energy changes when parameter perturbations are sufficiently small. In fact, this strategy was used in earlier free energy calculations when computers were much less powerful. For example, an early free energy perturbation study focuses on examining the effects of making conservative modifications on free energy changes (3). In one case, benzamidine was modified into parafluorobenzamidine and the effects on trypsin binding were examined. In this calculation, only simulations on the reference systems, benzamidine and the trypsin-benzamidine complex, were performed, and the single-window Zwanzig perturbation formula was used to calculate $\Delta G_{L \to L'}$ and $\Delta G_{PL \to PL'}$ directly.

By focusing on small perturbations, a single-window Zwanzig formula (9) can be used to provide initial estimates of the effects of making many physical or nonphysical changes on binding affinity, without carrying out expensive molecular dynamics simulations for all the perturbed systems. Only simulations of the reference system are needed. This technique, or its close cousin in which the Helmholtz free energy change is obtained by expanding ΔA in terms of ΔH and keeping up to second-order term,

$$\Delta A = \langle \Delta H \rangle_r + \frac{1}{2RT} \left\langle (\Delta H - \langle \Delta H \rangle_r)^2 \right\rangle_r, \qquad 6.$$

has been used to examine the effects of adding or removing protons (29, 30) or of changing molecular charge distribution on free energy changes (31). In drug design applications, focusing on a small chemical subspace for which a single-window perturbation formula can be used to study many modifications should already be useful for finding better derivatives of a drug lead. One can then use a few of the promising derivatives for further single-window perturbation calculations to enlarge the chemical subspace for identifying other drug candidates. Although this full-blown molecular dynamics–based method has not yet been applied extensively to drug-design applications, an implicit solvent model has already been used in pilot studies on protein kinases to develop pharmacophore models for mining new drug leads from small-molecule libraries, to generate constraints for designing focused chemical libraries for specific targets, and to produce guiding principles for optimizing a drug lead (32, 33).

The range of application of this idea can be extended by using soft-core potentials in reference simulations (34). A single perturbation formula does not work well when larger atoms or atomic groups are added or deleted because the

reference simulation does not adequately sample the configuration states relevant to the modified systems. If a large atom or atomic group is going to be deleted, the reference simulation may not have sampled well the space, allowing the surrounding solute or solvent atoms to get closer. If a large atom or atomic group is going to be created, the reference simulations may have many configurations that create unfavorable steric clashes at the modified sites. To alleviate this problem, Liu et al. (34) utilized soft-core potentials. For example, a modified Lennard-Jones potential of the form

$$V(r_{ij}) = 4\varepsilon_{ij}\left[\frac{\sigma_{ij}^{12}}{\left(\alpha\sigma_{ij}^{6} + r_{ij}^{6}\right)^{2}} - \frac{\sigma_{ij}^{6}}{\alpha\sigma_{ij}^{6} + r_{ij}^{6}}\right] \qquad 7.$$

can be used at selected sites in a reference simulation so as to create more space where atoms are going to be added and to allow atoms to get closer to sites where atoms are going to be deleted. In the above equation, ε_{ij} and σ_{ij} are the Lennard-Jones parameters between atoms i and j, r_{ij} is the distance between the two atoms, and α is a softening parameter that prevents the potential from diverging as $r_{ij} \rightarrow 0$. This approach was able to predict well the free energy differences among a number of para-substituted phenols in water solvent (34). There is a limit to which this approach works well. Mordasini & McCammon (35) later examined the range of applicability of this single-reference approximation by introducing increasingly larger modifications. They found that this model could still yield reasonable qualitative scoring when functional groups involving up to three atoms were deleted.

Combining Explicit and Implicit Solvent Models

Recently, explicit and implicit solvent models have been combined to facilitate free energy calculations (36–40). This approach uses explicit solvent molecular dynamics simulations to relax crystal structures to solution ones, and then uses the simulated solution structures in implicit-solvent calculations to obtain free energy. Using implicit-solvent models eliminates the extensive simulation time required for sampling solvent configurations. This approach assumes that the free energy of a system can be obtained by averaging the potential of mean force, obtained from an implicit solvent model, of dynamics snapshots generated from explicit-solvent models. Entropy contributions of the solute can be estimated from the harmonic or quasiharmonic model. These approximations appear to work well. Vorobjev et al. (36) used one such approach successfully in distinguishing correctly folded protein conformations from misfolded ones. In their study, Vorobjev et al. (36) ran a quick molecular dynamics simulation of a protein for approximately 50–100 ps and used an implicit-solvent model to calculate the free energy of the correctly and incorrectly folded protein. The free energy was found to be lower for the correctly folded protein. The implicit solvent model included contributions from the gas-phase energy of the solute, the energy of cavity formation, the solute-solvent interaction energy, and the solvent electrostatic polarization energy. The energy of

cavity formation was assumed to be proportional to the solvent accessible surface area of the solute. The solvent electrostatic polarization energy was obtained by solving the Poisson-Boltzmann equation and the other terms were obtained from a molecular mechanics force field.

A similar MM/PBSA (molecular mechanics/Poisson-Boltzmann-surface area) approach was used to study protein-ligand interactions. For example, Kuhn & Kollman (38) obtained encouraging results by applying this method to predict the binding affinity of seven ligands to avidin and streptavidin. They obtained a correlation coefficient of 0.92 between the calculated and the experimental binding affinities. The root-mean-square difference between calculated and experimental results, which covered a range of approximately 16 kcal/mol, was on the order of 1.7 kcal/mol. In these calculations, the length of the molecular dynamics simulation used for the averaging was 300 ps. No simulations on the separated protein and ligands were done. Instead, the protein and ligands were assumed to adopt the same conformation as that in the molecular dynamics simulations of the complexes. They also used the harmonic approximation to calculate the entropy change upon binding using six quenched dynamics snapshots. This approximation introduced a relatively large uncertainty in calculating entropy changes; the discrepancy among results from the six snapshots could amount to 5 kcal/mol in the worst case that they studied. So the solute entropy contributions remain a challenge to calculate.

To further speed up this approach, one can replace the expensive explicit-solvent simulations with implicit ones. Statistical mechanical theory gives the Helmholtz free energy A, apart from the scaling constant of the classical partition function that cancels out in binding energy calculations, as

$$A = -RT \ln \iint \exp(-\beta H(u, v)) du\, dv, \qquad 8.$$

where R is the gas constant, T is the absolute temperature, $\beta = {}^{1}/_{RT}$, and $H(u, v)$ is the classical Hamiltonian expressed in terms of the solute coordinates u and the solvent coordinates v. Integrating over the solvent coordinates gives

$$A = -RT \ln \int \exp(-\beta W(u)) du, \qquad 9.$$

where $W(u)$ is the potential of mean force of the solute with conformation defined by u and the kinetic energy term for the solute is ignored because it cancels out in binding energy calculations. $W(u)$ can be estimated by a continuum solvent model as in the previous examples. If one can calculate the atomic forces resulting from $W(u)$, one can carry out a molecular dynamics simulation to generate an ensemble of structures for calculating the Helmholtz free energy according to Equation 9, although it is known to be difficult to obtain a free energy from this equation directly. If one adopts similar approximations as in previous MD/PBSA calculations, one can calculate the internal energy E of a system via

$$E = \langle W(u) \rangle, \qquad 10.$$

where $\langle \ldots \rangle$ represents an ensemble average over snapshots obtained from an implicit solvent simulation and the Helmholtz free energy can be obtained from

$$A = E - TS. \qquad 11.$$

Again, one can use the harmonic or quasiharmonic approximation to estimate the entropy term, although a more realistic implicit solvent model can be used here. Most of the previous MM/PBSA calculations employed more approximate distance-dependent dielectric models.

Several methods have already been introduced to use the relatively sophisticated Poisson-Boltzmann model to calculate electrostatic forces during molecular dynamics simulations (41, 42). A method for calculating forces resulting from solvent accessible surface area–dependent hydrophobic term have also been developed (43). However, it is still expensive to evaluate Poisson-Boltzmann forces on the fly during molecular dynamics simulations. An alternative is to use the significantly cheaper generalized Born model. Dominy & Brooks (44) have parametrized the generalized Born model by Qui et al. (45) for the CHARMM force field (45a) and found that the model performed quite well in reproducing molecular solvation energy and conformational free energy. And using this model in molecular dynamics simulations of a 56-residue protein yielded results agreeing well with corresponding explicit-solvent simulations. These results are encouraging as they demonstrate that this type of model can make it much easier to include protein flexibility, via molecular simulations, in modeling protein-drug interactions. This model has also been incorporated into the UHBD program (46, 47) to use together with constrained Brownian dynamics simulations (48). The constrained Brownian dynamics simulation algorithm can use a larger time step than molecular dynamics simulation algorithms to speed up the conformational sampling of small molecules. This is useful for improving the calculation of the free energy of floppy ligands in solution. As mentioned before, many earlier MM/PBSA simulations assumed the ligand conformational distribution in solution to be the same as that in a protein-ligand complex. This approximation may not serve well for floppy ligands and for ligands that adopt very different conformations in the bound and unbound states.

Chemical-Scanning Computational Experiments

The explicit/implicit solvent approach just described requires doing at least one simulation for each protein-ligand complex. Therefore, it is still difficult to examine the binding of a large number of compounds to a receptor. However, if one focuses on a small subset of chemical space around a lead compound, one can adopt the same approximations as described earlier in free energy calculations so that simulations on the reference systems alone can be used to predict the effects of making many modifications on a lead compound. In these calculations, no molecular dynamics simulation needs to be performed on the derivatives of a lead compound. Instead, snapshots of the reference simulations are modified

to change different functional groups of the lead compound into new ones. For example, Kuhn & Kollman (49) were able to predict a derivative that binds stronger than biotin to avidin by changing different C–H groups of biotin into C–F groups. This approach has also been applied to cases where larger modifications are made. For example, Massova & Kollman (50) performed a computational alanine scanning experiment and were still able to obtain qualitative agreement with experiments when studying protein-protein interactions even though the chemical modifications were rather large (they involved changing non-alanine amino acids into alanines).

Semi-Empirical Linear Response Theory

Another way to facilitate the comparison of the binding affinity among a number of rather different ligands is the semi-empirical linear response approach (51–56). This method assumes the binding affinity, ΔG, between a ligand-receptor pair to be approximated by the relation

$$\Delta G = \alpha\left(\left\langle U_{electrostatic}^{Bound}\right\rangle - \left\langle U_{electrostatic}^{Unbound}\right\rangle\right) + \beta\left(\left\langle U_{Lennard\text{-}Jones}^{Bound}\right\rangle - \left\langle U_{Lennard\text{-}Jones}^{Unbound}\right\rangle\right) + \gamma\left(\left\langle \Omega^{Bound}\right\rangle - \left\langle \Omega^{Unbound}\right\rangle\right), \quad 12.$$

where $\langle U_{electrostatic}^{Bound,\,Unbound}\rangle$ is the averaged ligand-surrounding electrostatic interaction energy, $\langle U_{Lennard\text{-}Jones}^{Bound,\,Unbound}\rangle$ is the averaged ligand-surrounding Lennard-Jones interaction energy, and $\langle \Omega^{Bound,\,Unbound}\rangle$ is the averaged solvent-accessible surface area of the complex or the uncomplexed molecule(s). α, β, and γ are empirical parameters determined by a least-square fit of the experimental binding free energy of a number of inhibitors to the ensemble averaged quantities of Equation 12 obtained from molecular simulations. Once α, β, and γ are determined, they can be used to predict the binding affinity of inhibitors whose binding affinity has not been measured. Encouraging results have appeared in a number of applications. For example, a recent application of this approach to study the binding of the tetrahydroimidazo[4,5,1-jk][1,4]benzodiazepine-2(1H)-thione and -one class of compounds to HIV reverse transcriptase (55) yielded a root-mean-square deviations of less than 1 kcal/mol from experimental results when the observed range of binding affinity was ∼4 kcal/mol. The Åqvist group did not use the solvent-accessible surface area term in their semi-empirical model but still obtained good correlation with experimental data in a number of applications (57).

Dynamic Pharmacophore Method

Most receptor-based pharmacophore models have been developed by using one crystal, NMR, or model structure. Pharmacophore models based on a single receptor structure could fail to identify inhibitors that bind to structures that are somewhat different from the experimental or model structure but that are still readily accessible at physiological temperatures. To address this issue, Carlson et al. (58–60) developed a dynamic pharmacophore model in which a number of

snapshots from molecular dynamics simulations were used to construct the model. For each snapshot, they determined components of a pharmacophore model by identifying favorable binding sites of chemical functional groups using the multiunit search for interacting conformers (MUSIC) program available in the BOSS program (61). The MUSIC procedure identifies favorable binding sites of probe molecules by simultaneously energy refining a large number of probe molecules, which do not interact with other, in the potential field of a drug target. Strong binding sites tend to cluster many probe molecules in well-defined orientations and locations. By carrying out MUSIC calculations on a number of dynamic snapshots, one can identify strong binding sites consistently appearing in many rather than only one or a few snapshots. These sites can form important components in a pharmacophore model. This approach can also uncover useful binding sites that are not presented by the initial starting structure. By using methanol as probe molecules, Carlson et al. (58–60) developed dynamic pharmacophore models that perform better than the single conformation model in identifying potent inhibitors of HIV-1 integrase. Unfortunately, the dynamic model also increased the number of false positives.

Relaxed Complex Methods

Recently, another computational approach has been described to discover ligands that may bind with "induced fit" of their target molecules (62, 62a). The new methods, which are called "relaxed complex methods," are inspired by two successful experimental methods for rapid discovery of ligands that bind strongly to a receptor, namely the "SAR by NMR" method (63) and the "tether method" (64). These methods recognize that ligands may bind to conformations that occur only rarely in the dynamics of the receptor, and that strong binding often reflects multivalent attachment of the ligand to the receptor. The new computational approach includes a single ligand method and a double ligand method.

The basic element of these new methods is the automated docking of small libraries of compounds to a diverse selection of target conformations. The first phase of the approach involves generating the target conformations. This might make use of a long molecular dynamics simulation of the unliganded target molecule, an ensemble of short molecular dynamics simulations, or some other way of generating target conformations. The second phase involves the rapid docking of mini-libraries of candidate inhibitors to the conformational snapshots of the target. In this phase, a relatively simple scoring algorithm is used to allow fast docking. The third phase attempts to improve the scoring of the best complexes found in the docking calculations by use of a slower but more accurate algorithm for estimating the standard free energies of binding.

The scheme described above represents the single-ligand method. The double-ligand variant recognizes that two ligands with relatively low binding affinities to the target can be linked to form a high-affinity ligand. Because the binding of the first ligand could introduce unfavorable interactions for the binding of the second

ligand, the combination of the best-ranked ligands for respective binding sites does not necessarily produce the best composite compound. Continuing from the previous single-ligand studies, the first ligand is therefore treated as part of target, and the docking simulations of the second ligand are repeated in a limited search space, based on the allowable lengths of linkers. Again, the binding of the second ligand is subsequently rescored by other more accurate approaches.

The first applications of the relaxed complex methods have focused on an experimentally well-characterized system, FKBP (FK506 binding protein) (62, 62a). A long molecular dynamics calculation was used to sample the FKBP conformations and the AutoDock software (65–67) was used for the initial docking. The rescoring was done using the MM/PBSA routines from the Amber software (68) and APBS evaluation of the electrostatic energies (69). The results to date encourage the further development and application of these methods.

CONCLUSIONS

Although protein flexibility can play an important role in determining molecular recognition and drug design, most modeling efforts ignore these effects because they are costly to include. However, with the rapid advance of computer technology and algorithmic development, it should become feasible to take these effects into account more frequently in practical drug discovery applications. More expensive but rigorous free energy calculations can be used in the later stage of a lead optimization process. Approximate but faster methods relying on single-reference states, such as sensitivity analysis and chemical scanning, can be used to quickly identify productive and nonproductive features of a lead compound in molecular recognition. The identification of these features can help decide how a lead compound should be modified to improve binding affinity, by pointing out features that are profitably kept and those that should be modified. These features can also help to construct pharmacophore models for mining new drug leads from small-molecule libraries and generate constraints for designing combinatorial chemical libraries targeted towards the desired receptors. Intermediate between these two extremes are methods such as MM/PBSA and semi-empirical linear response approach that can further screen out less promising compounds suggested by the single-reference-state models before more rigorous free energy calculations are carried out. Sophisticated implicit solvent models may also play an important role in speeding up conformational sampling so that the effects of protein flexibility can be accounted for in the earlier stages of a drug design process.

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RETINOID RECEPTORS AND THEIR COREGULATORS

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Key Words retinoic acid, coactivators, corepressors, gene transcription, chromatin

■ **Abstract** Retinoids regulate gene transcription by binding to the nuclear receptors, the retinoic acid (RA) receptors (RARs), and the retinoid X receptors (RXRs). RARs and RXRs are ligand-activated transcription factors for the regulation of RA-responsive genes. The actions of RARs and RXRs on gene transcription require a highly coordinated interaction with a large number of coactivators and corepressors. This review focuses on our current understanding of these coregulators known to act in concert with RARs and RXRs. The mechanisms of action of these coregulators are beginning to be uncovered and include the modification of chromatin and the recruitment of basal transcription factors. Challenges remain to understand the specificity of action of RARs and RXRs and the formation of specific transcription complexes consisting of the receptors, coregulators, and other unknown factors.

INTRODUCTION

Retinoids, a group of small lipophilic molecules, are essential for a variety of biological processes. The indispensability of retinoids in animals was first demonstrated in a controlled study by Hale (1), which showed that deficiency in vitamin A (retinol) induced congenital malformations in pigs. Following this pioneering work, Warkany's group demonstrated congenital abnormalities in vitamin A-deficient (VAD) rat dams, such as those affecting the eyes, myocardium, aorticopulmonary septum, diaphragm, and the respiratory and urogenital systems (2). Subsequent studies showed that vitamin A was also indispensable in postnatal life for survival, reproduction, vision, and maintenance of epithelial tissues (3, 4). The first indication that retinoic acid (RA) was the active ingredient of vitamin A came from the observation that RA could repair most of the defects caused by VAD, except defects in the visual system, which specifically required retinaldehyde (4, 5).

In the late 1980s, the cloning of the nuclear RA receptor α (RARα) (6, 7) opened an avenue for the dissection of the RA signaling pathways. Subsequently, the detection of endogenous RA in animals substantiated the notion that RA could be the physiologically active component of vitamin A (4). This was further supported

by two studies that showed the rescue of VAD embryos by RA at specific stages of embryogenesis (5) and the teratogenic effects of pharmacological doses of RA during embryogenesis (4). It was then proposed that RA could play an essential role in many aspects of life, such as development, growth, reproduction, vision, and cancer prevention. With the help of molecular genetic tools, a direct role for RA in many of these biological processes was unambiguously demonstrated in mutant animals that were deficient in specific functional receptors (8–10). These studies have provided the ultimate proof that RA (specifically all-*trans* and 9-*cis* RA) constitutes the active ingredient of vitamin A and its nuclear receptors are ligand-activated transcription factors responsible for regulating the expression of RA-responsive genes. However, it remains highly challenging to dissect the complex RA signaling pathways because the specificity of the actions of retinoids in different gene systems cannot be accounted for by the actions of the receptors alone. This puzzle began to unravel through the discovery of a large number of receptor coregulators (11–15) that are now known to act in concert with the receptors. Identification of these coregulators marked the beginning of a new era for the dissection of the complicated mechanisms underlying specific actions of RA in different gene systems.

This review focuses on our current understanding of coregulators of the nuclear RA receptors, as well as the working mechanisms underlying their actions. I begin with a summary of the features and functions of these receptors followed by a review of the identification and the properties of their coregulators (coactivators and corepressors). Finally, a discussion is provided on the problems that challenge future studies of the action of vitamin A, their receptors, and their coregulators.

NUCLEAR RECEPTORS FOR RA AND THE PRINCIPLE OF THEIR ACTIONS

Cloning of Nuclear Receptors for RA

It was a long-standing and challenging task to address the pleiotropic effects of a group of structurally simple molecules like retinoids. Biochemical and ligand-binding studies, as well as the demonstration of genome-wide changes triggered by these small molecules, suggested the existence of intracellular receptors for these retinoids (16, 17). It was the discovery and cloning of the RARα from the groups of Chambon (6) and Evans (7) that marked the beginning of an era of molecular biology leading to our understanding of hormone nuclear receptors. It is now known that RARs and retinoid X receptors (RXRs) belong to the superfamily of nuclear receptors that transduce signals to the gene transcription machinery, resulting in changes of gene expression (8, 18–20).

The RA receptor family consists of RARα, RARβ, RARγ, RXRα, RXRβ, and RXRγ, as well as their isoforms. RARs bind to all *trans*- and 9-*cis* RA, whereas RXRs bind specifically to 9-*cis* RA. From molecular and genetic studies, it has become clear that the actions of RA are closely tied to the functions of these receptors. The most convincing evidence comes from combinatorial genetic

studies where mutant animals were made defective in the function of one or two of these receptors. These mutant animals essentially recapitulated the entire spectrum of VAD (10). Single mutant mice lacking the function of a single RAR or RXR gene were viable and displayed one or some of the many aspects of postnatal VAD syndrome. However, double mutants defective in a pair of isotypes such as RARβ/RARγ, RARα/RARβ, and RARα/RARγ (10) all died in utero or at birth, suggesting essential roles for these receptors in animal survival and some functional redundancy of RARs. The physiological functions of RXRs were less conclusive based upon these genetic studies. However, it seemed that RXRα was the most critical receptor, whereas RXRβ and RXRγ appeared to be dispensable. In vitro structural studies as well as molecular biological approaches all suggested an RAR/RXR heterodimer as the physiologically functional unit (21–25). This notion was supported by the genetic studies of compound mutants that were defective in one RXR and one RAR (4, 10).

The complexity of vitamin A signaling pathways was suggested from studies that detected many types and isoforms of these receptors, as well as the discovery of many similar yet distinct RA response elements (RAREs) present in the regulatory regions of RA target genes (20, 26). Most studies have supported the notion that the functional unit of these receptors is a dimer consisting of one RAR and one RXR. Therefore, a large reservoir of distinct receptor dimers can potentially be generated from the different receptor types and their isoforms. The dimeric receptor pair binds to a specific RARE located in the proximity of a gene promoter's regulatory regions. As a result, the activity of vitamin A target genes can be dictated by a combination of various receptor pairs that bind to different RAREs in the context of specific promoters. Questions remain as to what determines the specificity of these receptor dimers for these very similar RAREs of different genes and how the structurally similar molecular entities (receptor dimers) are able to elicit variable actions on different gene promoters. The recent discovery of "coregulators" for RAR and RXR provides a clue for another level of control that can potentially contribute to the diverse actions of these dimeric receptor units.

Domain Features of RARs and RXRs

All the nuclear receptors, including RARs and RXRs, share common modular domains (A to F) (27–29) that can be interchanged without loss of function (Figure 1). The N-terminal A/B domains are the least conserved and contain an autonomous activation function, named AF-1. The C domain is the most highly conserved domain and contains two zinc finger modules responsible for DNA binding, and is thus named the DNA-binding domain (DBD). This domain spans approximately 60–70 amino acid residues among which 8 of the 9 conserved cysteines tetrahedrally coordinate two zinc ions, resulting in the formation of two compact finger-like structures that bind DNA. The region at the base of the first finger is named the D box and is responsible for discriminating the DNA sequence, whereas the region at the base of the second finger participates in receptor dimerization.

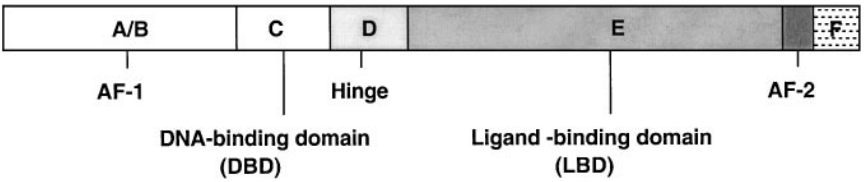

Figure 1 Major functional domains of RAR and RXR. The N-terminal *A/B* segment encodes the activation function-1 (AF-1) domain. The *C* segment encodes the DNA-binding domain (DBD). The *D* segment encodes a flexible hinge region. The *E* segment encodes the ligand-binding domain (LBD) and the activation function-2 (AF-2) domain. The C-terminal tail, *F* domain, is present in some, but not all, of the receptors.

The D domain, or hinge region, is variable and connects the zinc finger DBD to the variable E region, which encompasses the ligand-binding domain (LBD). It is also known that the hinge region contains a nuclear localization signal and is probably responsible for the interaction with certain nuclear receptor coregulators (30, 31). Our recent studies using fluorescence correlation spectroscopy showed a dramatically reduced diffusion rate of a hinge region dissected from orphan receptor TR2, indicating potentially extensive protein interaction involving this region (L.-N. Wei, unpublished observations).

The LBD is a multifunctional domain that is responsible for ligand binding, receptor dimerization, and interaction with other proteins such as coactivators. Crystallization studies have shown a canonical structure for the LBD of all nuclear receptors, including RARs and RXRs (28, 29). This structure is formed by folding 12 conserved α-helical regions named H1 to H12, with a conserved β-turn connecting H5 and H6. These helices are folded into a three-layer antiparallel helical sandwich in which a pocket is created for ligand binding. The cognate agonist makes a stereospecific, high-affinity interaction with the pocket of the LBD, inducing a conformational change in the receptor. The most C-terminal F domain is found in certain members and participates in the action of a second activation domain named AF-2. The AF-2 is composed of an amphipathic α helix that is also highly conserved and is responsible for the agonist-induced conformational change of receptors. Upon agonist binding, the receptor undergoes a conformational change and the structure becomes much more compact. This conformational change results in changes in protein interaction and stimulates the recruitment of coactivators, leading to the activation of gene transcription (see details in the section on Coregulators of RARs and RXRs). It has become apparent that these receptors can also be activated by signals other than the retinoid ligands, such as those involving protein kinases and metabolic products (32, 33). Presumably, modification of receptors by these enzymes also induces a conformational change resulting in the recruitment of coactivators. Evidence has been provided that extracellular signals are able to modulate the activity of nuclear receptors in certain pathways (34–37).

Receptor Dimerization

A large number of studies have demonstrated heterodimerization of one RAR with one RXR (21–25, 38), and it is suggested that the functional receptor unit is a heterodimer. Dimerization is mediated primarily by the interacting surface of the LBD on each receptor. It is believed, at least in the in vitro situation, that receptors are present in oligomeric states and ligand binding induces dissociation of receptor oligomers and facilitates the formation of heterodimers (39–42). It is the heterodimeric receptors that bind a specific RARE with a high affinity. Additionally, occupancy of either receptor by an agonist is sufficient to induce a conformational change of the AF2 domain in the dimeric receptor unit, thereby recruiting associate proteins and leading to the activation of the transcription machinery. The combinations of receptor pairs, as a result of pairing different RAR and RXR types and isoforms, provide a rich reservoir of potential functional receptor dimers. The specificity of transcriptional activation of a gene in a particular cell or tissue type is partially dictated by the action of different receptor pairs that are cell specific. Furthermore, the specific sequence of an RARE and the context of a specific gene regulatory region, i.e., the sequences adjacent to the RARE, can also contribute to specificity of receptor action. Recently, it has become apparent that the regulatory mechanism orchestrated by RAR/RXR is further complicated by the recruitment of receptor associate proteins (coregulators) expressed in specific cells.

COREGULATORS OF RARs AND RXRs

The cloning of numerous proteins that interact with RARs and/or RXRs suggested the existence of a large number of coregulatory proteins for RARs and RXRs. It has become increasingly clear that these associate proteins of RAR and RXR play important roles in the ultimate control of the activity of a gene promoter regulated by RA. Currently, various working models are proposed to address the complexity and specificity of the actions of RA, which are based upon the principle of cofactor recruitment by receptors, i.e., corepressor recruitment by apo-receptors (gene silencing in the absence of hormones) and coactivator recruitment by holo-receptors (gene activation in the presence of hormones) (9, 43–47) (Figure 2). Several coregulators (both coactivators and corepressors) are known to bind to an overlapping surface on the LBD of the receptors. Mutational and structural studies have suggested that in the absence of ligands, or upon binding by antagonists, the receptors recruit corepressors to a hydrophobic surface groove formed by helices 3, 4, 5, and 6 of the LBD. In the presence of an agonist, the AF-2 domain at the C-terminal tail of the receptor changes its position, releasing corepressors and, together with helices 3, 4, and 5, forms a cleft that recruits coactivators onto the receptor pairs. Recent studies have attempted to map the cofactor-interacting surface of the receptors, and have proposed a leucine-rich "LXXLL" signature motif for the p160 coactivator families (48, 49) and a related "L/IXXI/VI" motif for certain corepressors such as N-CoR and SMRT (50–52). It is proposed that these

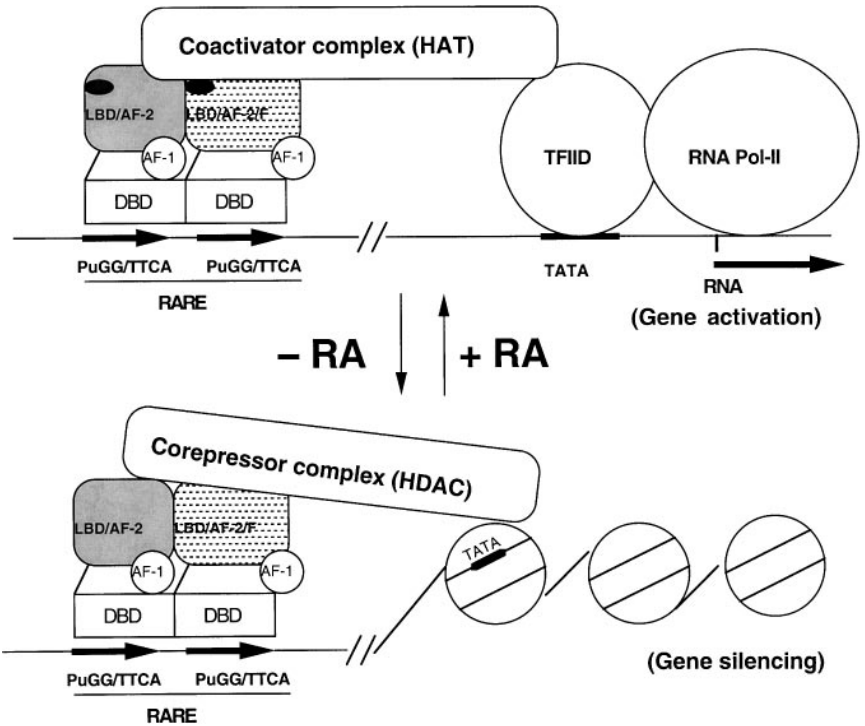

Figure 2 A model for RAR/RXR acting in concert with coactivator or corepressor complex for gene activation or silencing. In the presence of ligands (+RA), the holo-receptor pair binds to the RA response element (RARE) and recruits coactivator complex, which encodes histone acetyl transferase (HAT) activity. HAT acetylates histone proteins, opens up the chromatin, and allows the transcription machinery to act on the promoter for active gene transcription. In the absence of ligands (−RA), the apo-receptor pair binds to the RARE and recruits corepressor that encodes histone deacetylase (HDAC) activity, inducing histone deacetylation, chromatin condensation, and gene silencing.

complexes occupy an overlapping hydrophobic groove on the receptor surface. Although this seemingly simple change in receptor conformation is able to explain the mutually exclusive nature of receptor interaction with either coactivators or corepressors, it is not sufficient to account for the complexity of gene regulation, such as the variable level of induction and silencing, or the combinatorial control of gene expression by many signals acting in concert. As such, it is tempting to speculate that further highly specific mechanisms are adopted by different cells for the same hormonal signals to be variably transduced in a wide variety of tissue/gene systems, most likely through orchestrating extensive protein-protein interactions and multiprotein complex formation in a very specific manner.

An increasing number of coregulators of transcription factors, including that of nuclear receptors, are being identified. Several of these coregulators are shared by a large number of transcription factors and some are highly specific to certain members. By a strict definition, some of these coregulators do not fall into the category of either coactivator or corepressor. For the sake of simplicity, the coregulators reviewed here are categorized as coactivators or corepressors according to the outcome of their actions on the RAR- and RXR-systems.

Coactivators of RARs and RXRs

The ultimate target of RA is the genome of the cells, and the principle of its action is to induce a conformational change of the receptor pairs that interact with the transcription machinery, resulting in altered gene transcription rates. As such, components of the transcription machinery, including the enzymes and the substrates, can all potentially be involved in the interaction with RARs and RXRs. Broadly speaking, coactivators of a transcription factor can be divided into two major classes: adapters that recruit transcriptional apparatus and components that are involved in chromatin-remodeling or modification. Although some of the coactivators of RARs and RXRs are known for their apparent functions, the roles of many remain to be determined or are complicated by their presence in overlapping complexes. By definition, a coactivator must fulfill several criteria: an agonist-dependent direct interaction with holo-receptors and the ability to interact with the components of the basal transcription machinery and enhance the basal transcriptional activity. Because of the ambiguity of several members that participate in gene activation by RARs and RXRs, the coactivators are not categorized by using this stringent definition. Rather, the coactivators of RAR and RXR, as reviewed here, are grouped based upon their effects on RAR- and RXR-mediated gene expression and the most commonly adopted nomenclature and/or their apparent molecular features.

SRC1/p160, CBP/p300, AND p/CAF COMPLEXES The steroid receptor coactivator (SRC) family consists of three subfamilies, i.e., SRC-1, TIFII/GRIP1/p160, and RAC3/ACTR/pCIP/AIB-1 (49) (Figure 3*A*). SRC-1 was first cloned in a yeast two-hybrid screening with the progesterone receptor (PR) as the bait (53), and was demonstrated as a common coactivator for all steroid receptors tested. It was later shown to encode a new member of the basic helix-loop-helix-PAS domain family and interact with the RARs and the p300/Phospho-CREB binding protein (CBP)/p300/CBP-associated factor (P/CAF) complex, as discussed later. A coactivator complex containing SRC-1/p300/CBP/PCAF was then proposed as a component that mediates the activating function of RARs (54). Subsequently, evidence was presented that SRC-1 contained intrinsic histone acetyltransferase (HAT) activity specific to H3 and H4 histones, an activity encoded by the C-terminal region of SRC-1 (55). This provided the first evidence for a role of a hormone nuclear receptor's coactivator in modifying chromatin by facilitating the

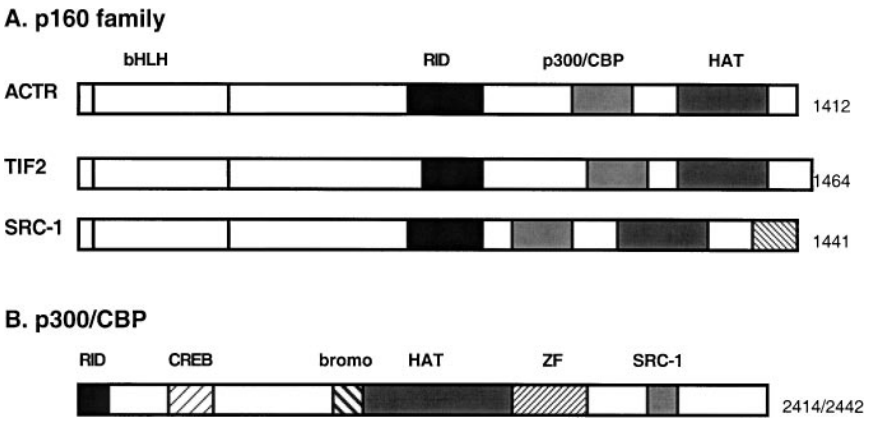

Figure 3 Domain features of the SRC and p300/CBP coactivators. (*A*) The three SRC coactivator families, ACTR, TIF2, and SRC-1, are highly homologous, with characteristic bHLH and HAT domains. The receptor interacting domain (RID) and p300/CBP-interacting domain (p300/CBP) are depicted. (*B*) Domain features of p300/CBP. RID: receptor interacting domain; CREB: CREB-binding domain; bromo: bromo domain; HAT: histone acetyl transfease; ZF: zinc-finger domain; SRC-1: SRC-1 interacting domain.

acetylation of histone proteins, thereby opening the chromatin and enhancing the formation of a stable preinitiation complex.

p300 was first identified as a 300-kDa nuclear protein and a cellular target for the adenovirus E1A oncoprotein transcription factor (56). It was shown to be responsible for a variety of the effects of E1A in host cells (57). The CBP was found to interact with the *trans*-activating domain of CREB and to mediate its activating function in response to cAMP (58, 59). It was also shown that CBP interacted with the basal transcription factor TFIIB and RNA polymerase II itself, and also possessed HAT activity (60), further supporting its role as a coactivator (59). Because of their interchangeable properties in many aspects, p300 and CBP are considered functional homologues. Their connection to the nuclear receptors was first demonstrated in a study where the LBDs of multiple nuclear receptors, including RARs, were shown to interact directly with p300/CBP, which also interacted with the SRC-1/p160 family. In addition, numerous transcription factors were also known to interact with p300/CBP. Structurally, p300/CBP contains discrete domains responsible for their diverse functions, including a bromodomain, a HAT domain, an SRC1 domain, and a receptor interacting domain at the N terminus (Figure 3*B*). Thus, it was proposed that p300/CBP could play a role in coordinating many signaling pathways and serve as a platform for the actions of different transcription factors including nuclear receptors (61–63).

P/CAF was identified that competed with E1A for interaction with p300/CBP. It was also demonstrated that P/CAF possessed intrinsic HAT activity (64). Later,

P/CAF was found to interact directly with liganded RAR/RXR dimer at the LBDs of these receptors (65) and was also found in a complex consisting of more than 20 polypeptides that include TATA-binding protein (TBP)-associated factors (TAFs) and some of the TFIID components. As the binding of P/CAF to RAR/RXR is independent of the interaction of RAR/RXR with p300/CBP, it is suggested that P/CAF and p300/CBP represent two independent activating pathways for nuclear receptors (65), both involving HAT activity to modify the chromatin. The identification of gene-specific coactivator complexes remains a challenge for future studies.

GRIP1/TIF2/p160 With the LBD of glucocorticoid receptor (GR) as the bait, a GR interacting protein (GRIP1) was cloned from a mouse embryo cDNA library and shown to act as a transcription coactivator (66). A 160-kDa human nuclear protein called transcriptional mediators/intermediary factor II (TIFII) was later isolated that appeared to be the homologue of GRIP1 and exhibited typical coactivator properties such as agonist-dependent interaction with receptors and an autonomous transcription activation activity (67). The family of GRIP1/TIFI2/p160 exhibits a partial sequence homology to SRC-1 and possesses a single nuclear receptor-interacting domain that is composed of three LXXLL-containing segments, as well as two autonomous activation domains (AD1 at the N terminus and AD2 at the C terminus). The activity of the AD1 appeared to be mediated by its interaction with the CBP complex, whereas the activity of the AD2 was CBP-independent (68). It was later found that the AD2 of GRIP1/TIFII/p160 acted on the AF-1 of androgen receptor (AR), but the principle of its activation function remains to be determined. Thus, GRIP1/TIFII/p160 can potentially mediate both of the two signal outputs of nuclear receptors, the AF-1 and AF-2.

ACTR/RAC3 AND p/CIP A receptor-associated cofactor 3 (RAC3) was cloned as a coactivator for the liganded receptor LBD (69) and found to be identical to another coactivator named ACTR, which was known to possess intrinsic HAT activity (70). Sequence comparison revealed a homology to the SRC-1 and TIFII members (69); therefore, it was also categorized in the SRC family. A closely related coactivator, the p300/CBP cointegrator associated protein (p/CIP), was found to interact with CBP and was required for the actions of CBP-dependent transcription factors (71). These members represent the third SRC family that functions as coactivators for RARs and RXRs, and possesses HAT activity.

TIF1 The mouse transcriptional intermediary factor I (TIFI) was originally found to interact with RAR/RXR in a ligand-dependent manner and was able to enhance AF-2 mediated activation of receptors (72). A structurally and functionally related protein was later isolated and named TIFIα. TIFI appeared to also interact with heterochromatin protein 1 (HP1) (73), suggesting that the coactivating function of TIF1 was related to its effects on chromatin structure.

TRAP220 The thyroid hormone receptor (TR) associated protein 220 (TRAP220) was identified in immunopurified complexes from thyroid hormone–treated cells and later shown to also interact with many hormone receptors including RARs and RXRs (74). Later, in an RXR/PPARγ heterodimer system, TRAP220 was shown to be selectively recruited by holo-PPARγ, whereas p160 was selectively recruited by holo-RXR, suggesting that cooperative effects of PPARγ and RXR could be due to selective coactivator recruitment by each receptor of the heterodimer (75). TRAP220 also contains multiple LXXLL motifs that mediate its interaction with the LBDs of holo-receptors and is known to stimulate thyroid hormone–activated gene transcription (76).

TRIP1/SUG1 The human TR interacting protein I (TRIP1) was originally cloned in yeast two-hybrid screening experiments with TR and RXR as the baits. TRIP1 is a homologue of a component of yeast transcription mediator, SUG1, which interacts with transcription factor TBP and TFIIB (77). Mouse SUG1 was subsequently isolated with RAR as the bait and was shown to interact with hormone receptors including RAR, RXR, vitamin D receptor (VDR), estrogen receptor (ER), and TR in a ligand-dependent manner. This interaction involved the AF-2 domain of nuclear receptors (78). It was shown that this transcription mediator was able to confer an eightfold greater activity in basal transcription and a 12-fold greater efficiency of phosphorylation of RNA polymerase II (79). Human TRIP1 was also shown to be able to functionally substitute for yeast SUG1. Thus, TRIP1/SUG1 functions as a coactivator for the RAR/RXR heterodimer by facilitating interaction with the basal transcription machinery.

SW1/SNF The yeast SWI/SNF genes were shown to be involved in positive transcriptional regulation of the *HO* and the *SUC2* genes (80, 81). A connection of nuclear receptors to these gene products was first demonstrated for GR (82). In this study, it was found that activation by GR required its interaction with SWI proteins at an early step, prior to the formation of the initiation complex. Later, the human homologue, hSWI/SNF, was shown to mediate the ATP-dependent disruption of nucleosomes (83). A direct ligand-dependent interaction of hormone receptors with SWI2/SNF2 was later demonstrated (84). Therefore, the SWI/SNF coactivators, upon interacting with holo-receptors, remodel chromatin structure to facilitate the binding of transcription factors.

Ada3 A family of transcription factor coregulators, named alteration/deficiency in activation (Ada), was first identified in yeast. In yeast, Ada3 mutation resulted in slower growth and temperature sensitivity. In addition, selection of initiation sites by general transcription machinery in vitro was altered in this mutant, suggesting that Ada3 was a component of the general transcription machinery (85). Later, it was found that the Ada proteins were a part of the yeast Spt-Ada-Gcn5-Acetyltransferase (SAGA) activator complex and that Ada3 protein interacted with the LBDs of RXR and ER in yeast, resulting in holo-receptor activation

(86). Using yeast genetics, a trimeric transcriptional complex consisting of holo-TR/p160/GCN5 was shown to interact with the Ada/SAGA adaptor complex and to mediate activation of target genes by thyroid hormones (87).

PGC-1 A tissue-specific coactivator for PPARγ, named peroxisome proliferator-activated receptor-gamma coactivator-1 (PGC-1) (88), also appeared to be a coactivator for other hormone receptors. PGC-1 was shown to interact with a number of hormone receptors including T3R, RAR, RXR, ER, and GR in a ligand-dependent manner (89, 90), which required the LXXLL motif-containing sequence of PGC-1. A number of studies have suggested a physiological function of PGC-1 in gluconeogenesis by augmenting the activity of phosphoenolpyruvate carboxylase and glucose-6-phosphatase via coactivation of GR and transcription factor HNF-4α (91). Recently, it was demonstrated that PGC-1 and TIF2 synergistically activated RXRα-mediated reporter gene activity (90). It is suggested that PGC-1 plays a role in a wide range of physiological processes that involve nuclear receptors using RXR as the obligate partner.

NSD1 The mouse NR-binding SET-domain-containing protein 1 (NSD1) was isolated in yeast two-hybrid screening experiments and found to encode several distinct domains, including a SET domain and multiple PHD fingers (92). This protein was found to interact with both apo- and holo-forms of many nuclear receptors including RAR and RXR. Interestingly, NSD contains separate activation and repression domains, which may define a new class of bifunctional transcriptional intermediary factors (93). Distinct roles in the presence and absence of hormones were proposed for this protein. Recent genetic studies have identified a gene fusion of the *NSD1* gene to the *NUP98* gene in childhood acute myeloid leukemia (94) and amplification of the NSD gene in human breast cancer cell lines (95), implicating a potential role for NSD1 in human malignancy.

FKHR The human FKHR, a forkhead homologue in rhabdomyosarcoma, was cloned from a tamoxifen-resistant MCF-7 cDNA library by using the LBD of ER as the bait (96). This gene belongs to the hepatocyte nuclear factor 3/forkhead homeotic gene family, and the protein was found to interact with ER ligand dependently but with RAR and TR ligand independently. In transient transfection assays, FKHR stimulated RAR- and TR-mediated gene activation, but it repressed ER-mediated transcription. Therefore, FKHR also appears as a bifunctional intermediary protein that can either activate or repress hormone receptor-mediated gene transcription, depending upon the receptors involved.

PNRC Two proline-rich nuclear receptor coregulatory proteins (PNRC1 and PNRC2) were cloned from a mammary gland cDNA library (97, 98). These proteins are relatively small, with molecular weights of 16 kDa and 35 kDa, respectively. Both PNRCs interacted with hormone receptors in a ligand-dependent manner, mediated by an activation function domain that contains an NR box-like

sequence (LKTLL) and an SH3 domain binding motif (SEPPSPS). These proteins enhanced transcriptional activation of both hormone receptors and orphan receptors, and their interaction with nuclear receptors required a functional AF2 domain in the nuclear receptors. The exact mechanism of their activation functions remains undetermined.

CRABPII Two forms of cellular retinoic acid binding proteins (CRABPs) have been identified, CRABPI and CRABPII. These are cytosolic binding proteins for RA and are primarily involved in the synthesis and degradation of RA, thereby controlling RA homeostasis (3). Recently, CRABPII was shown to be directly involved in RAR/RXR-mediated gene activation (99–102). CRABPII was able to enter the nuclus and interact directly with RAR/RXR in a ligand-independent manner (99, 101). Activation of RA-responsive reporter was enhanced by CRABPII; thus, CRABPII can act as a specific coactivator for the RAR/RXR families. It is suggested that CRABPII interaction with RAR/RXR facilitates the formation of holo-receptors, thus enhancing transcriptional activity (100).

ASC-2 The activating signal cointegrator-2 (ASC2) was isolated with the LBD of RXR as the bait and was shown to interact with many nuclear receptors in a ligand-dependent manner (103). In addition, it also interacted with a number of other nuclear factors, such as basal transcription factors TFIIA and TBP, coactivators CBP/p300 and SRC-1 (103), serum response factor (SRF), activating protein-1 (AP-1), and nuclear factor-κ B (NF κ B) (104). ASC2, either alone or in conjunction with CBP/p300 and SRC-1, was able to stimulate ligand-dependent activation of nuclear receptors; the AF-2 domain of nuclear receptor was required for this activation. Its interaction with nuclear receptors involved its two NR boxes, whereas its interaction with CBP involved two of its three activation domains. Its activation function was blocked by E1A, a CBP-neutralizing factor. It is proposed that ASC-2 acts by binding to nuclear receptors and recruiting CBP for nuclear receptor-mediated gene activation (105).

p120 p120 encodes 920 amino acids with a molecular mass of 120 kDa. The cDNA of p120 was originally isolated as a cofactor for TR, and its T3-dependent interaction was shown to require the AF-2 domain of TR (106). Recently, p120 was also shown to enhance PPARγ/RXR-mediated transactivation in the presence of either PPARγ- or RXR-specific ligands, but the interaction appeared to be mediated by RXR. It is proposed that p120 can be a specific coactivator for RXR in PPAR/RXR-activated pathways (107).

CARM1 CARM1 (coactivator-associated arginine methyl-transferase I) was shown to methylate nucleosomes and potentiate transcriptional activation by RAR/RXR (108). However, CARM1 was also able to methylate CBP/p300, which blocked CREB activation by disabling the interaction between CBP/p300 and CREB. Therefore, it appears that CARM1 can be a coactivator for hormone

receptors and at the same time a corepressor for transcription factors transducing cAMP signals.

Corepressors of RARs and RXRs

Activation of gene expression by hormones has been the central dogma of a long-standing view on hormonal control of gene regulation. Hormone receptors were believed to function only in the presence of hormones. However, the discovery that basal transcription of a gene could be further repressed in the absence of hormones motivated the field to re-examine the potential roles of hormone receptors in the absence of ligands. A negative role for nuclear receptors in gene expression was first implicated in two early studies of thyroid response element (TRE)-containing promoters. In one study, the v-erb-A oncoprotein abolished thyroid hormone responses of a TRE in the Moloney murine leukemia virus long terminal repeat (109). Another study showed that apo-TR was able to repress the activation of a TRE responsive to RAR-epsilon (110). These observations initially suggested a "passive" repression mechanism, where the apo-receptors repress transcription by competing for DNA binding. Later, a potentially "active" repression, or silencing activity, of receptors was suggested because both RAR and TR were found to contain a transferable, functional silencing domain at the C terminus of the receptor (111). Based upon these observations, the hypothesis was proposed that nuclear receptors could recruit corepressors to actively silence genes. Three major criteria for a corepressor are interaction with apo-receptors and dissociation from receptors upon the binding of agonists, potentiation of repression by receptors, and possession of intrinsic repressor activity. The first two bona fide corepressors were identified in 1995 (112, 113). A number of corepressors were later cloned that were shown to facilitate gene repression and were classified as corepressors (114). However, some of these members were found to interact with both liganded and unliganded receptors. Furthermore, several coregulators function as either a coactivator or a corepressor, depending upon the receptors involved. Studies of the working mechanisms of these dichotomous coregulators are being actively pursued. For the sake of simplicity, the coregulators shown to exert suppressive activities on the RAR/RXR system are categorized here as corepressors of RARs and RXRs.

SMRT AND N-CoR The nuclear receptor corepressor (N-CoR) was identified as a bona fide corepressor of RAR and TR (112). At the same time, another bona fide corepressor was cloned, named silencing mediator for retinoid and thyroid-hormone receptors (SMRT) (113). It was later found that N-CoR and SMRT are related in terms of function, domain structure, and primary sequence (Figure 4*A*). Both N-CoR and SMRT contain multiple repressive domains and receptor interacting domains and they interact with the same components of other nuclear factors such as TFIIB and Sin3 [reviewed in (114–116)].

Their repressive activity was attributed to two mechanisms. One mechanism involved their direct interaction with the corepressor mSin3, originally identified

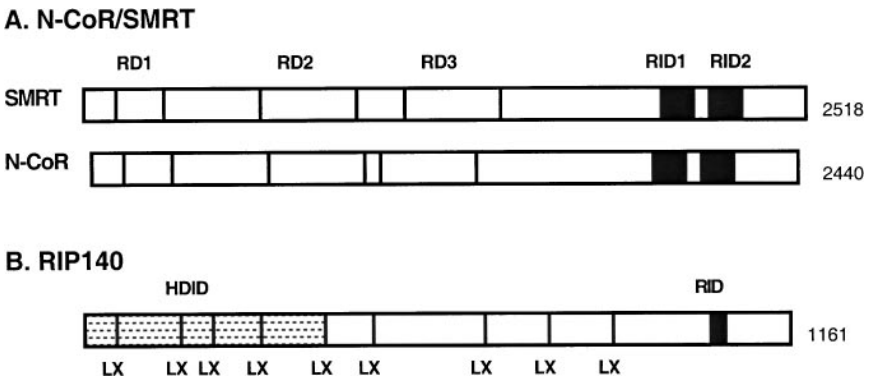

Figure 4 Domain features of N-CoR/SMRT and RIP140 corepressors. (*A*) Alignment of N-CoR and SMRT, with three repressive domains (RD1, RD2, and RD3) and two receptor interacting domains (RID1 and RID2) depicted. (*B*) Nine LXXLL (LX) sequences scattered throughout the entire RIP140 molecule. The histone deacetylase-interacting domain (HDID) is present at the N-terminal portion and a novel LYYML-containing ligand-dependent receptor interacting domain (RID) is present at the C-terminal portion, which is devoid of a canonical LXXLL sequence.

for Mad-Max and Mxi-Max transcription repressors (117, 118). The formation of complexes containing N-CoR/SMRT, mSin3, and HDAC was later demonstrated (119–121). A model was proposed where N-CoR and SMRT served as the platform adapters to recruit Sin3-HDAC complex as well as other Sin3-associated proteins to form a repressive complex, which was then recruited by the apo-receptors to the DNA (116, 122). However, recently it was found that both SMRT and N-CoR could directly interact with the class II HDACs (123), suggesting that a Sin3-independent recruiting pathway could also be adopted by N-CoR and SMRT. For both Sin3-dependent and Sin3-independent pathways, recruitment of histone modifying enzymes appears to be one common underlying mechanism for the corepressive activity of N-CoR and SMRT.

The second mechanism of repression was postulated to be mediated by their direct interaction with certain basal transcription factors, thereby precluding the formation of the basal transcription complex. Before the identification of corepressors, an early study had in fact demonstrated an inhibitory effect of unliganded TR on preinitiation complex assembly (124). Later, unliganded TR was found to directly contact TBP (125). Recently, a direct contact of N-CoR and SMRT with transcription factors such as TFIIB (126, 127), TAFII32, and TAFII70 (127) was demonstrated. Although a growing number of reports have supported the mechanism involving direct or indirect chromatin modification by these repressive complexes, the exact identities of the components present in these complexes remain to be established in more rigorous reconstitution experiments.

RIP140 The human receptor interacting protein 140 (RIP140, or newly renamed as NRIP1) (128) was first cloned with the LBD of ER as the bait (129) and was reported to act as a coactivator for ER functions. Mouse RIP140 was cloned from a testis cDNA library with the LBD of orphan receptor TR2 as the bait (130) and found to function as a corepressor. Most recent studies have shown a repressive role for RIP140 in many other gene systems, including those regulated by nuclear receptors and other transcription factors. For instance, RIP140 was reported as a corepressor for Pit-1, TR, ER (131), PPAR/RXR (132), GR (133), and RAR/RXR (134). In contrast to the first study proposing that the LXXLL motif of RIP140 mediated its ligand-dependent interaction with receptors (135), recent studies demonstrated that ligand-dependent interaction of RIP140 with receptors could be mediated by a region devoid of the LXXLL motifs (136, 137). Furthermore, RIP140 could directly interact with histone deacetylases (HDACs) (138), and tetramolecular complexes consisting of RAR/RXR/RIP140/HDAC were formed in the presence of RA, resulting in the suppression of reporter gene expression in the presence of RA (136). In the presence of RIP140, the RAR/RXR-targeted promoter region was highly deacetylated and the expression of the reporter was also repressed. We have proposed a model where hormone target genes can be selectively suppressed by complexes consisting of ligand-bound RAR/RXR, RIP140, and HDACs. RIP140 was also found to interact with the carboxyl terminal binding protein (CtBP) and to be acetylated by p300/CBP (139), raising the possibility that HDAC recruitment by RIP140 could modify RIP140 itself. Like the studies of many coregulators, the biological activity of RIP140 was examined by either transient transfection of mammalian cells or by yeast reporter assays, where complication by unknown nuclear factors from the mammalian or yeast cells cannot be prevented. Direct evidence for the activity of RIP140 and many coregulators should ensue from studies of in vitro reconstituted systems where defined components are provided in the reactions. We have begun to address the biological activity of RIP140 in RA regulation of its target genes by using assembled chromatin of the reporter as the template for in vitro transcription. Our preliminary results have demonstrated that in the presence of RIP140, RA-induced transcription was inhibited. Furthermore, the inhibition was likely to be a result of the competition of RIP140 with coactivators, like P/CAF, in the binding to holo-receptors (L.-N.Wei, unpublished). We have further employed a quantitative method to obtain kinetic data on these molecular interactions, based upon plasmon surface analyses on a BIAcore machine. In these studies, the real-time kinetic analysis of molecular interaction between RIP140 and liganded RAR/RXR was conducted using highly purified proteins. These kinetic data showed a substantial competition between P/CAF and RIP140 for binding to the RAR/RXR heterodimer, further substantiating the strong competitive nature of RIP140 with other coactivators, such as P/CAF, for interacting with receptors. One indication of this result is the significance of dynamic interactions among these nuclear factors that act in concert to ultimately control specific gene expression. This presents one of the most challenging issues to be addressed in future studies.

Therefore, both in vivo and in vitro transcription studies have supported a corepressor role for RIP140 in nuclear receptor-mediated gene expression. The underlying mechanisms could be its competition with coactivators for binding to holo-receptors and/or its ability to directly recruit HDAC to the gene promoter.

SUN-CoR A small (16 kDa) nuclear protein, small unique nuclear receptor corepressor (SUN-CoR), was isolated by using the unliganded LBD of TR as the bait (140). This protein was found to function as a corepressor for nuclear receptors, including RAR, and to interact with N-CoR and SMRT in vitro. It is suggested that SUN-CoR may be a component of the N-CoR/SMRT complexes. However, the exact mechanism of its repressive activity remains undetermined.

TRUP In a yeast two-hybrid screening experiment, a protein named thyroid hormone receptor uncoupling protein (TRUP) was identified with the bait prepared from the unliganded LBD of TR (141). TRUP was later found to interact with both TR and RAR, but not ER, in a hormone-independent manner through the hinge and N-terminal portions of their LBDs. Its repressive activity is attributed to its interference with DNA binding of TR and RAR.

CALRETICULIN Two studies independently reported that a calcium-binding protein, calreticulin, interacts directly with the DBD of many nuclear receptors including RAR/RXR (142, 143). Although calreticulin was found primarily in the endoplasmic reticulum, it was also found in the nucleus (142). Expression of calreticulin inhibited the activation of RAR and AR by their hormones (143). However, it remains to be determined if blocking receptors' DNA binding via interacting with their DBD is the principal mechanism of the regulatory action of calreticun.

PSF A polypyrimidine tract-binding protein-associated splicing factor (PSF) was isolated by using a biochemical approach to identify proteins interacting with apo-receptors (144). PSF was found to interact with the DBD of apo-receptors and to recruit Sin3A/HDAC complexes. It is suggested that PSF acts as a corepressor through a Sin3A-mediated pathway.

CONCLUSION AND FUTURE DIRECTIONS

The discovery of numerous associate proteins for nuclear receptors, including those for RARs and RXRs, suggests that a complicated regulatory network governs the actions of nuclear receptors. Because many of these associate proteins interact with an overlapping spectrum of target proteins that can be nuclear receptors or other transcription factors, it is likely that different cells have evolved to utilize a rich reservoir of coregulators in order to achieve a high level of cellular

specificity at the transcriptional control of gene expression. Alternatively, some of these coregulators may have been retained, despite their redundant nature, in order to achieve a homeostatic control of a variety of essential physiological processes. As coregulators of nuclear receptors continue to be discovered, a number of issues will have to be addressed in the future.

The first challenge is to address the specificity of these coregulators. To this end, the tissue-, cell-, and stage-specific expression patterns of these coregulators have to be examined and compared to that of RARs and RXRs. Subsequently, the relative affinity of each coregulator to the receptors has to be determined. Theoretically, a high-affinity coregulator would be considered more efficient in terms of its ability to regulate gene transcription. However, these coregulators very often act in concert with other proteins. Therefore, it is tempting to speculate that the ultimate control of transcription would lie in the unique combination of components in a transcription complex consisting of nuclear receptors, coregulators, and other proteins. As such, it would also be critical to determine the exact contents of a specific transcription complex and how a coregulator may affect the formation of a particular complex in the context of a specific gene's regulatory region.

The second challenge is to delineate the pathways and to determine the mechanisms of action of these coregulators. Currently, most of the known actions of coactivators are related to their roles in histone acetylation, and most corepressors are shown to recruit histone deacetylases. However, studies have been presented that reveal substrates of these enzymes, including not only DNA, but also transcription factors themselves. Thus, acetylation/deacetylation of transcription factors could also contribute to the activity of coregulators. More recently, the action of coregulators in another form of covalent DNA modification, methylation, has also been demonstrated (108). However, it remains to be seen whether this is common to different coregulators. Another proposed mechanism of coregulator's action is the sequestration of transcription factors. In theory, this can be commonly adopted by various coregulators. Our recent study of RIP140 competition with P/CAF for interaction with holo-RAR/RXR would support such a hypothesis. To effectively adopt such a mechanism of action, the transcription complexes or subcomplexes are probably more likely to be formed in solution before they are recruited to the target DNA. However, studies to address transcription complexes formed either in solution or on target DNA are scarce. Furthermore, evidence has yet to address this issue in the context of chromatin for many of these coregulators. Toward this end, one is presented with a challenging technical problem that has been vigorously tackled in the field, i.e., the use of appropriate chromatin template for the analysis. Ideally, one would use the chromatin of a natural gene, at least in terms of the nucleosomal organization of the regulatory region of the gene, for the analysis of DNA-protein interaction in order to examine the recruitment of transcription complexes to their targets. However, it is not a simple task to determine the nucleosomal structure of a natural gene, not to mention reconstituting a piece of chromatin that mimics, biochemically and thermodynamically, a natural gene.

The third problem is the ambiguity of ligand-dependency in terms of coregulator interaction with holo-receptors. Although it is demonstrated, in most cases, that coactivators interact with hormone receptors ligand dependently, recently evidence has suggested a complication in the nature of ligand-dependency for the interaction of the coregulators with the receptors. The rule of LXXLL-motif as a ligand-dependent NR signature has been challenged recently by studies of several coactivators and corepressors. For instance, RIP140 was initially demonstrated as a ligand-dependent coactivator and the receptor interaction motif was mapped to the LXXLL-containing regions (134). However, we have demonstrated a suppressive role for RIP140 in genes regulated by the RAR/RXR, and numerous later studies have also shown suppressive effects of RIP140 in many other gene systems. Moreover, we have recently discovered an LYYML motif in the carboxyl terminus of RIP140, which turns out to be a strong ligand-dependent receptor interaction motif for interacting with RAR and RXR (133, 135). This combination of a suppressive role for RIP140 and its ligand-enhanced interaction with RAR/RXR has suggested an unusal but testable hypothesis that retinoid hormones can directly act as a negative signal for the transcription of certain genes, and that it involves negative coregulators such as RIP140. This hypothesis challenges the current dogma of hormonal action. If this hypothesis is substantiated, one important question will be to address the physiological implication of positive versus negative gene regulation that is directly triggered by the hormones. Furthermore, the concept of the LXXLL-motif as a required ligand-dependent NR signature motif has also been challenged during the re-examination of RIP140 interaction with RAR and RXR using different methods. Similar problems have been encountered in the study of other coregulators such as PGC-1, which was initially shown to be a ligand-dependent coactivator for the PPARγ (88, 89) and later demonstrated to interact with receptors in a ligand-independent manner. Therefore, it is tempting to speculate that the effect of ligands, either agonists or antagonists, on receptors' interaction with coregulators may not be as simple as once thought. Currently, most studies rely on assays where complications from unknown cellular factors cannot be avoided. In the future, structural data, as well as interaction studies using more stringent methods like the BIAcore method to allow real-time kinetics to be determined in a highly purified system, will be required to address this issue.

In summary, the cloning of RARs and RXRs has allowed the signaling pathways underlying the action of retinoids to be dissected. The recent discovery and cloning of numerous receptor coregulators, including coactivators and corepressors, has marked the beginning of a new era in the studies of the action of retinoids. In future studies, the specificity of gene expression regulated by the retinoid, which requires highly coordinated protein-protein interaction and variegated complex formation, will present one of the most challenging subjects in this field. The direct connection of the action of these RAR and RXR coregulators to the components of the transcription machinery, including the substrate (chromatin) and the enzymes (transcription complexes), will provide ample opportunities to address the issue of the pleiotropic effects of retinoids.

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NOVEL PHARMACOLOGICAL APPROACHES TO MANAGE INTERSTITIAL LUNG FIBROSIS IN THE TWENTY-FIRST CENTURY

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■ **Abstract** Pharmacological agents currently in use to treat interstitial lung fibrosis are either ineffective or too toxic in humans. This review addresses mechanistically based novel approaches that have the potential to minimize the accumulation of collagen in the lung, a hallmark of lung fibrosis. These approaches include maintaining the intracellular levels of NAD^+ and ATP, blocking the biological activities of TGF-β and integrins, evaluating the effectiveness of PAF-receptor antagonists and NOS inhibitors, and developing a new generation of cysteine pro-drugs with an adequate degree of bioavailabilty. A critical analysis of each approach as it relates to management of IPF in humans is presented.

INTRODUCTION

Interstitial lung fibrosis (ILF) is the end stage of a heterogenous group of disorders characterized by the excessive and deranged accumulation of extracellular matrix (ECM) proteins in the lungs. It is a potentially lethal and chronic response of the lung to injury resulting from a wide range of causes (1). The known causes of ILF encompass a wide variety of systemic, iatrogenic, occupational, and lifestyle–related diseases (2), including bacterial infection, inhalation of organic and inorganic dusts, radiation, trauma, and drugs (3). However, there are a number of diseases in which the causes of lung fibrosis remain elusive, as in idiopathic pulmonary fibrosis (IPF, also known as cryptogenic fibrosing alveolitis), eosinophilic granuloma, and sarcoidosis and some diseases affecting multiple-organ systems such as rheumatoid arthritis and systemic sclerosis. Regardless of the origin, ILF is invariably associated with fibrosing alveolitis characterized by inflammation and an overexuberant repair process preceded by an excess number of fibroblasts (4), an absolute increase in lung collagen content, and abnormality in the ultrastructural appearance and spatial distribution of collagen types (5, 6). Accumulation of collagen-rich ECM in the lung interstitium and peripheral air spaces causes a

derangement of the alveolar wall and loss of functional capillary units. IPF, the most common interstitial lung disease in humans, typically affects individuals aged 40 through 70, with slight male predominance, and shares some of the histological and biochemical features of ILF of known etiological origins, as described above. Until recently, it was thought that IPF affects only 5/100,000 persons in the United States per year. However, more recent data indicate that the rate of incidence is much higher (7).

During the past 25 years, the use of a diverse range of animal models for lung fibrosis has enhanced our understanding of the pathogenic mechanisms of this disease. It is now widely accepted that an initial and potentially reversible inflammatory phase leads to an irreversible fibrotic phase of the lung in response to injurious agent. The inflammatory event results in the generation of proteinaceous and cellular infiltrate in the distal airways, followed by the release of soluble mediators from the activated inflammatory cells and from the injured resident cells. These mediators then activate either fibroblasts/myofibroblasts or local resident cells within the alveolar wall to secrete mediators that induce fibroblasts to migrate, proliferate, and subsequently produce an excess amount of collagen. The earliest events, detected by electron microscopy of the lung from patients at the early stages of IPF, include endothelial and epithelial injury and regeneration, followed by edema, inflammation, and fibrosis (8). The damaged and regenerating endothelial and epithelial cells and infiltrating cells such as neutrophils, macrophages, lymphocytes, monocytes, and eosinophils release a number of mediators that modulate the fibroblast function in favor of increased production and excess deposition of collagen in the lung interstitium. Clinically, a patient with IPF will present with dyspnea on exertion, fatigue, and cough; patients with advanced IPF usually have a rapid shallow breathing pattern as compared with normal (9). In the advanced stages of IPF, there is also a reduction in lung volumes, lung compliance, and diffusion capacity (9). There may be hypoxemia at rest and frequently during exercise. The natural history of IPF generally involves gradual progression of the disease, usually culminating in corpulmonale and respiratory failure; death occurs within three to six years after onset. The standard treatment of IPF includes corticosteroids with or without cytotoxic agents such as colchicine, cyclophosphamide, or azothioprine. Treatment with corticosteroids results in objective improvement in only 10%–20% of patients, probably reflecting both the advanced stage of fibrosis at which the patients are brought to medical attention and the inability of corticosteroids to alter the noninflammatory phase of the fibrotic processes (10). The combined treatment with corticosteroid and cytotoxic agents failed to affect the survival rate and appears to be invariably associated with overt systemic toxicity. Therefore, it is of utmost importance to develop new drugs that can be safely used for management of ILF. Rather than attempting an exhaustive survey of the voluminous literature on lung fibrosis in experimental models, this review briefly discusses existing drugs developed based on their ability to interfere with collagen biosynthesis at various steps, highlights recent advances in the molecular-based mechanisms for the genesis of lung fibrosis, and discusses emerging, mechanistically based strategies for

pharmacological interventions to manage lung fibrosis. The rationale, critical evaluation, and potential applicability to IPF in humans for each emerging therapeutic modality is provided from the perspective of an investigator in pulmonary research.

COMPOUNDS INTERFERING WITH COLLAGEN BIOSYNTHESIS

Because an excess accumulation of collagen is a hallmark of ILF, various strategies have been developed to interfere with the synthesis and degradation of collagen in the lungs. The biochemical pathways of collagen synthesis and degradation have been extensively reviewed (11, 12). A variety of compounds have been developed that interfere with collagen synthesis at the transcriptional, translational, and post-translational levels and with its degradation. The effectiveness of these compounds in experimental models of lung fibrosis has been reviewed elsewhere (13). Most of these compounds were considered either ineffective or too toxic for use in humans with IPF.

MOLECULAR-BASED MECHANISMS AND PHARMACOLOGICAL INTERVENTIONS

Bleomycin-Promoted Lung Injury: An Experimental Model of ILF

Our understanding of the pathogenesis of ILF has been enhanced considerably by the widespread use of a rodent lung fibrosis model that involves treatment with bleomycin (BL). This model, which has enabled investigators to study the underlying cellular and biochemical mechanisms for the pathogenesis of lung fibrosis, induces morphological and biochemical changes of the animals' lungs that resemble those observed in human IPF [reviewed in (13)]. This review focuses first on the cellular biochemical mechanisms responsible for BL-induced lung injury that eventually culminates in lung fibrosis. Next, the various strategies that interfere with those mechanisms and abrogate the development of lung fibrosis are discussed. Overall, use of an animal model in which fibrosis is chemically induced has provided excellent basic information regarding the pathogenic mechanisms responsible for fibrosis. In addition, this approach has had some limited success in developing novel drugs of potentially therapeutic significance to treat patients with IPF, even though the animal model of lung fibrosis does not always accurately reflect IPF morphologically.

The processes governing the development of BL-induced lung fibrosis fall into three categories: acute injury including cell death; inflammatory response of varying degrees depending on dose and route of administration, characterized by direct injury to capillary endothelium, basement membrane, and pneumocyte type I epithelial cells; and chronic response featured by hypercellularity and increased deposition of ECM proteins (14). Infiltration of the lungs by neutrophils is an

early event in BL-induced lung fibrosis in animal models (15). After adhering to endothelial cells, neutrophils migrate into tissue and release a variety of mediators, including reactive oxygen species (ROS), that can have potent deleterious effects on the lung (16–18). In addition, neutrophils contain the enzyme, myeloperoxidase, which oxidizes halides Cl^-, Br^-, or I^- to their corresponding hypohalous acid in a reaction involving H_2O_2 (18). The biocidal property of hypochlorous acid (HOCl) in oxidizing various vital constituents of cells and its ability to deplete cellular NAD^+ and ATP are well established (19). Furthermore, BL generates ROS under aerobic conditions after binding to intracellular DNA and Fe^{2+} (20). This complex functions as a minienzyme, catalytically reducing molecular oxygen leading to the generation of various types of ROS that cause prominent DNA strand scission (21). Various agents that damage DNA ultimately result in strand breakage, which induces nuclear enzyme, poly (ADP-ribose) polymerase (PAP) activity. In fact, broken double-strand DNA stimulates PAP activity directly (22). PAP uses NAD^+ as a substrate for modification of nuclear proteins at free carboxyl groups by polymerization of ADP-ribose moieties (23). This response may facilitate repair of DNA damage or cause acute cell injury by NAD^+ depletion (24, 25). A number of drugs and chemicals have been found to activate PAP activity secondary to DNA damage, subsequently leading to intracellular depletion of NAD^+ levels (26–28). This is consistent with the observations that the intratracheal (i.t.) administration of a fibrogenic dose of BL in hamsters increases the activity of lung PAP concomitantly with intracellular depletion of NAD^+ levels (26). The activation of PAP activity and intracellular depletion of NAD^+ levels have been well-documented following exposure to other DNA-damaging agents including dimethyl sulfate, H_2O_2 (27, 28), and alkylating and ionizing radiation (29). The intracellular depletion of NAD^+ precedes the ATP loss (27, 28), which if extensive, may lead to cell death. These two processes may not have to be coupled together (28). Hyot & Lazo found a significant NAD^+ depletion by direct exposure of lung slices to BL from BL-sensitive $C57B_1/6N$ mice (30). Although ATP depletion may be a prerequisite for oxidant-induced cell death, it is not the sole determinant (31). Thus, depletion of NAD^+ will compromise the cell's viability since NAD^+ plays a specific role in the maintenance of cell integrity independently of its energy status (30), and depletion of ATP will likely lead ultimately to a perturbation in cellular energy-requiring processes. The disruption of normal epithelium-fibroblast interaction resulting from necrosis of epithelial cells and a delay in the repair of epithelial cells due to inadequate availability of NAD^+ and ATP may be a stimulus sufficient to promote fibroblast growth and deposition of excess collagen (32). This suggestion is based on the hypothesis that proliferation of fibroblasts in the lung is normally hampered by intact epithelial cells because, under normal conditions, the lung injury is repaired (33); however, if the injured epithelial cells do not repair, fibroblast proliferation would be triggered, leading to an excess synthesis and deposition of collagen in the lung (34, 35). This process is consistent with the results that niacin (nicotinic acid), a B vitamin and an established precursor of NAD^+ and NADP (36), prevents cytotoxicity and DNA damage by maintaining the NAD^+

level of cells (37). This property explains the protective effects of niacin against BL-induced lung fibrosis (38). Furthermore, daily treatment with niacin not only replenishes the BL-induced depletion of NAD^+ and ATP, it also significantly attenuates the development of lung fibrosis at 10 and 14 days after i.t. instillation of BL in hamsters (39).

Taurine, a naturally occurring sulfur-containing amino acid, traps HOCl (40) and possesses antioxidant and membrane-stabilizing properties. Because of these beneficial properties, taurine has been used successfully against lipid peroxidation (41), ozone-induced lung injury (42), and BL-induced lung fibrosis (43). Because taurine and niacin produce their antifibrotic effects by different mechanisms, it was hypothesized that the combined treatment with these two compounds would be more effective against lung fibrosis than treatment with either compound alone. In fact, this indeed turned out to be the case. Combined treatment with taurine and niacin completely ameliorated the BL-induced lung fibrosis in hamsters (44). This treatment decreased the BL-induced increases in collagen accumulation in lungs as measured by hydroxyproline content. In addition, the combined treatment also decreased BL-induced increases in lung lipid peroxidation, and superoxide dismutase, PAP, and prolyl hydroxylase activities and raised the lung content of NAD over the group treated with BL alone (44). The beneficial effects of the combined treatment with taurine and niacin at the biochemical level was complemented by morphological and morphometric studies that show the presence of fewer inflammatory cells in bronchoalveolar lavage fluid (BALF) and lung interstitium, less epithelial necrosis, decreased pulmonary vascular permeability, increased lung volume, and reduced fibrotic lung lesions (45).

Transforming Growth Factor-β

Many alterations in lung structure and function associated with chronic interstitial lung diseases are considered to be a consequence of the activation and persistence of inflammatory cells within the lower respiratory tract (46). The presence of macrophages in the areas of chronically inflamed lung is thought to play a central role in orchestrating the fibrotic response. Treatment with BL using a regimen that produces lung fibrosis stimulates alveolar macrophages to release growth factors (cytokines), which are believed to promote fibrosis by stimulating chemotaxis, fibroblast proliferation, and synthesis of ECM proteins (47). Results from a wide body of work indicate that the underlying mechanism for fibroproliferative diseases involves dysregulation and overproduction of certain cytokines (48–50). Among a number of cytokines investigated, a continued overproduction of TGF-β appears to be at the heart of the molecular mechanism in the genesis of lung fibrosis: (*a*) TGF-β is a potent modulator of a number of genes involved in organogenesis, tissue regeneration, and fibrosis, including genes for the ECM (51); (*b*) TGF-β increases the production and/or activity of connective tissue growth factor (52) and it stimulates biosynthesis of type I collagen (53, 54), fibronectin (53), and proteoglycans (55); (*c*) TGF-β inhibits the expression of ECM protease; and (*d*) TGF-β promotes the

expression of tissue inhibitor of metalloproteinase (56). These actions of TGF-β on the metabolism of ECM result in an excess accumulation of ECM proteins, a hallmark of fibrosis. The role of TGF-β in fibrosis is supported by a number of studies. For instance, significant elevations in TGF-β gene expression preceded the perturbations in the expression of genes of ECM proteins in two different models of BL-induced lung fibrosis (57, 58). More importantly, the elevated levels of TGF-β gene expression were coordinately regulated with increased mitogenesis and DNA synthesis in hamster lungs (57). These findings in animal models of lung fibrosis are consistent with the findings reported in humans with IPF with respect to an abundant expression of TGF-β mRNA in alveolar macrophages (59) and a marked and consistent increase in TGF-β production in epithelial cells and macrophages in lung sections from patients with advanced IPF (60). Besides the lungs, the role of TGF-β has also been demonstrated in other organs undergoing fibrotic changes (61). Despite a growing body of evidence suggesting that TGF-β is a cytokine vital to tissue repair, TGF-β-induced deposition of ECM proteins at the site of injury invariably leads to scarring and fibrosis that could be fatal if the scarring is confined within lungs. Furthermore, the ability of TGF-β to induce its own production may be the key to the scarring and fibrosis that develop in chronic and progressive conditions, leading to obliteration of the normal architecture (62). The pleiotropic actions of TGF-β and its ability to regulate the production of other cytokines in a paracrine fashion (63, 51), combined with its profound influence on ECM protein metabolism, put TGF-β on the target list for therapeutic intervention. The following strategies have been developed to minimize the BL-induced lung fibrosis that is predominantly mediated by an excess release of TGF-β in the lung.

TAURINE AND NIACIN A multifaceted approach has been launched to blunt the fibrogenic effects of TGF-β in the BL-rodent models of lung fibrosis as a preclinical assessment of possible therapeutic benefits against lung fibrosis in humans. Combined treatment with taurine and niacin against BL-induced lung fibrosis also depends for its antifibrotic effect on the ability of this combination to downregulate the BL-induced overexpression of both type I and type III procollagen mRNAs (64). In view of the findings that increases in the levels of TGF-β_1 precede increases in type I and type III procollagen mRNAs in BL-induced lung fibrosis, it was found that taurine and niacin treatment also blocked the BL-induced overexpression of TGF-β_1 mRNA. This blockade was the result of decreased TGF-β_1 gene transcription, as ascertained by nuclear run-off assays (65). The down-regulation of TGF-β mRNA correlated with decreases in TGF-β protein in the BALF and decreases in the lung collagen content in BL-treated hamsters receiving taurine and niacin (65). The experimental evidence indicates that combined treatment with taurine and niacin ameliorates BL-induced lung fibrosis by down-regulating the BL-induced overexpression of TGF-β_1 mRNA at the transcriptional level.

The expression of fibrogenic cytokine genes including TGF-β occurs in response to activation and translocation of nuclear factor-κB (NF-κB) into the

nucleus where this transcription factor binds to the promoter region of cytokine genes containing the NF-κB motif, thereby stimulating their expression (66, 67). According to prevailing theory, NF-κB is an oxidant-sensitive transcription factor (68) that is activated in some cell lines in response to elevated levels of reactive oxygen species (ROS) (69). Because BL causes oxidative damage by generating ROS, it is highly likely that it activates NF-κB and thus stimulates the expression of fibrogenic cytokine genes, including TGF-β. Recent findings showed that i.t. instillation of BL in mice progressively increased the activation of NF-κB in lung, followed by expression of the TNF-α, IL-1α, and TGF-β genes, and correspondingly elevated levels of these cytokines in the BALF (67). Treatment with taurine and niacin minimized the BL-induced lung fibrosis by suppressing BL-induced increased activation of lung NF-κB, followed by downregulation of the above cytokine genes and their gene products in the BALF (67). This was not surprising since taurine, like other compounds with antioxidant properties, blocks the ROS-induced activation of NF-κB and thus minimizes the tissue damage in response to oxidants (67, 70). Even though taurine and niacin are proven to be safe and are used therapeutically in humans, their use in combination has unfortunately not been emphasized by pulmonologists for the management of patients with IPF.

PIRFENIDONE Pirfenidone is an investigational drug synthesized by Marnac, Inc., (Dallas, TX), registered under the trademark Deskar® in the United States. The antifibrotic effect of pirfenidone has been demonstrated in several animal models of fibrosis for different organs. For example, the dietary intake of pirfenidone ameliorated BL- and cyclophosphamide-induced lung fibrosis in hamsters and mice (71–73). Subsequently, it was reported that dietary intake of pirfenidone starting after the second dose in a three-dose bleomycin model also minimized the lung fibrosis in hamsters (72). This finding has therapeutic significance since treatment with pirfenidone not only prevents but also can retard the progression of the lung fibrosis once it has started. The beneficial effects of pirfenidone against BL-induced lung fibrosis were demonstrated not only at the biochemical level but also at the functional level by a significant improvement in the quasistatic compliance and total, vital, and inspiratory capacities of the lungs (74). Interestingly, Raghu et al. reported the beneficial effects of pirfenidone against IPF in an open clinical trial in humans at advanced and end stages of fibrosis (75). A recent editorial comment in *Lancet* proposed, "Pirfenidone thus seems to be a promising new drug that deserves a well-designed and randomized controlled trial to establish its efficacy and safety" (76). The clinical efficacy of pirfenidone in IPF patients is being evaluated in double-blind and randomized control trials in Japan and Mexico, with plans underway to conduct similar clinical trials in this country.

In a series of studies, several molecular mechanisms for antifibrotic effects of pirfenidone have been established. As discussed earlier, BL induces oxidative damage in lungs by stimulating the generation of ROS (20), which are responsible for various stages of the inflammation and lipid peroxidation followed by fibrosis.

The beneficial effect of pirfenidone against BL-induced oxidative damage of the lung is corroborated by the following findings: First, it has been demonstrated both in vitro (77) and in vivo (78) that pirfenidone directly scavenges ROS including $O2^{\cdot -}$, H_2O_2, and $^{\cdot}OH$, resulting in the inhibition of lipid peroxidation in a dose-dependent manner. Second, pirfenidone has anti-inflammatory effects in the BL-hamster model of acute lung inflammation (79). Third, pirfenidone suppresses the production of fibrogenic cytokines such as TNF-α (80), TGF-β (81), and platelet-derived growth factor (PDGF) (82). In fact, an exaggerated release of PDGF by alveolar macrophages from patients with IPF is a characteristic feature of this disease, as demonstrated by Martinet et al. (83). Since pirfenidone is an effective scavenger of ROS, this compound very likely blocks the activation of NF-κB resulting from BL-generated ROS in a manner similar to that of other compounds with antioxidant properties. The inhibition of NF-κB activation by pirfenidone explains the ability of this compound to down-regulate the BL-induced overexpression of TGF-β mRNA in the lungs and TGF-β (81) and PDGF (82) proteins in the BALF. Collagen accumulation in the lungs is subsequently reduced by downregulation of BL-induced overexpression of the procollagen mRNAs (84).

NF-κB ANTISENSE OLIGONUCLEOTIDES The role of NF-κB in the pathogenesis of BL-induced lung fibrosis was further confirmed by the finding that treatment with antisense oligonucleotides to this transcription factor attenuated BL-induced lung fibrosis in mice (85). These findings indicate that NF-κB is required for maximal transcription of many proinflammatory cytokines including TGF-β and any strategies that block the activation of this transcription factor would have potential therapeutic benefits in many lung diseases including IPF. However, the drawbacks to antisense oligonucleotide therapy include limited stability due to rapid degradation by intracellular endonucleases, need for parenteral route of delivery, and systemic toxicity.

INTERFERON GAMMA Interferon gamma (INF-γ) is a potentially attractive target molecule for therapeutic control of fibrosis because of its ability to regulate the functions of both macrophages and fibroblasts. INF-γ diminishes the expression of insulin-like growth factor, a profibrogenic growth factor produced by macrophages, and mast cells. In addition, it also inhibits fibroblast growth factors and a variety of neutrophil-derived cytokines, thereby suppressing fibroblast proliferation and collagen synthesis (86, 87). The antifibrotic effects of IFN-γ or inducers of interferon in BL-rodent models of lung fibrosis have been well documented (88, 89). Recently, the mechanistic basis for the antifibrotic effect of IFN-γ at the molecular level has been established by the finding that it downregulates BL-induced overexpression of TGF-β and procollagen genes in the lung (90). The beneficial effects of IFN-γ in combination with a low dose of prednisolone on pulmonary function and oxygenation were recently demonstrated in a randomized and double-blind trial of 20 patients with IPF, compared with patients treated with prednisolone alone

who showed deterioration (91). However, the findings reported in this trial are controversial [discussed by Baughman & Alabi (92)]. Although a multicenter trial is currently underway to establish the clinical efficacy of IFN-γ in IPF patients, this drug is unlikely to be therapeutically desirable for a long-term treatment. There is a possibility of inducing chronic obstructive pulmonary disease with progressive emphysema, enhanced lung volumes, and macrophage and neutrophilic inflammation of the lung, as was found in the transgenic mice overexpressing IFN-γ in the lung (93). Second, the drug must be administered either intramuscularly or subcutaneously, which will influence patient compliance. Third, a flu-like syndrome is a common side effect of administration of IFN-γ.

ANTI-TGF-β ANTIBODY, TGF-β SOLUBLE RECEPTOR, AND DECORIN Experimental approaches that block the biological activities of TGF-β attenuate the degree of fibrosis in BL-rodent models of lung fibrosis. For example, antibodies to TGF-β significantly reduced the experimental lung and kidney fibrosis (94, 95) and a receptor antagonist to this cytokine also decreased accumulation of lung collagen induced by BL (96). Repeated i.t. instillation of TGF-β soluble receptor starting after the second BL or third BL dose in a three-dose BL model of lung fibrosis in hamsters also significantly reduced the BL-induced lung fibrosis, as evaluated by both biochemical and histopathological endpoints (97). These findings indicate the beneficial effects of even delayed treatment with TGF-β soluble receptor in attenuating the progression of ongoing fibrotic process and suggest its potential therapeutic application in the management of lung fibrosis in humans, once an acceptable delivery system for this protein in the distal airways has been worked out. Although treatment with antibody to TGF-β and its soluble receptor minimized the lung fibrosis in animal models, the therapeutic efficacy for long-term treatment is limited in controlling a progressively advancing chronic disease such as fibrosis. The body may develop its own immune reaction against the antibodies; and second, extreme reductions in the TGF-β level may lead to auto-immune-like illness, such as that seen in TGF-β_1 gene knockout mice that die soon after birth (98). To circumvent these problems, the antifibrotic potential of decorin in the BL-hamster model of lung fibrosis was evaluated. Decorin is a small proteoglycan that binds and reduces biological activities of all isoforms of TGF-β (99, 100). Repeated i.t. instillation of decorin minimized the BL-induced lung fibrosis as evaluated by biochemical, histopathological, immunohistochemical, and morphometric studies (101). The antifibrotic effect of decorin was confirmed later by a transient overexpression of the decorin gene in a murine model of lung fibrosis induced by overexpression of TGF-β gene (102). Although the i.t. instillation route for drug delivery is the least desirable, this route has nevertheless uncovered a potentially novel antifibrotic compound. The use of decorin may have clinical application provided that the drug can be delivered in an aerosol form. In addition, decorin is a natural human compound that can be produced as a recombinant molecule and used for treatment of fibrotic conditions of any organs with little risk of initiating adverse immunological reactions, unlike TGF-β antibodies.

ANTAGONIST OF TGF-β-MEDIATED SIGNALS Recent studies have identified the signal-transduction events involved in TGF-β-mediated biological effects. TGF-β signals from membrane to nucleus using somitabun mothers against decapentaplegic (*Smad*) proteins (103, 104). The activated TGF-β receptors induce phosphorylation of *Smad2* and *Smad3*, which form hetero-oligomeric complexes with *Smad4*. These complexes then translocate to the nucleus and regulate transcriptional responses (103–106). Recently *Smad7* has been shown to act as an intracellular antagonist of TGF-β signaling and an inhibitor of TGF-β-induced transcriptional responses. *Smad7* associates with activated TGF-β receptors and interferes with the activation of *Smad2* and *Smad3* by preventing their receptor interaction and phosphorylation (107). These findings led Nakao et al. (108) to examine the effect of exogenous *Smad7*, introduced by a recombinant human type 5 adenovirus vector into the lung, on BL-induced lung fibrosis in mice. These investigators clearly demonstrated the antifibrotic effect of *Smad7* in BL-treated mice, as reflected by suppression of type I procollagen mRNA, reduced hydroxyproline content, and lack of fibrotic lung lesions as compared with BL-treated mice receiving adenovirus carrying *Smad6* DNA. However, this approach is not of clinical significance because of low efficiency of gene transfer, adenovirus vector-mediated inflammatory and immunological responses of the lungs (109), and undesirable consequences resulting from long-term elimination of the biological effects of TGF-β.

Anti-Integrin Antibodies

The traffic and state of activation of leukocytes are modulated by various surface proteins such as the integrins. Cell-cell interactions as well as cell-ECM interactions are critical for the pathogenesis of pulmonary fibrosis. A consistent finding both in patients with active pulmonary fibrosis and in animal models of fibrotic lung diseases is the accumulation of increased numbers of immune and inflammatory cells in areas undergoing fibrosis (110). The α4 integrin subunit CD49d associates with either the β1 (CD29) or β7 subunit to form the integrin heterodimers called very late antigen (VLA)-4 (α4β1; CD49d/CD29) and α4β7 (111). The α4 integrins are heterodimeric leukocyte cell surface molecules with cell and matrix adhesive properties. In addition, integrin αvβ6 activates latent TGF-β in lung and skin, and this is involved in acute lung injury (112). Studies in vivo using blocking mAbs have established a role for CD49d in leukocyte recruitment in a range of inflammatory and immune disorders, and it appears to play an important role in inflammatory cell recruitment particularly in allergic disorders (113). Evidence for a central role for the integrins α4β1 and α4β7 in leukocyte pathophysiology is accumulating rapidly. mAbs specific for the leukocytic integrins CD-11a or CD-11b has been shown to prevent lung fibrosis in a BL-mouse model (114). Recently, Wang et al. (115) demonstrated the antifibrotic effect of integrin α4 antibody against BL-induced lung fibrosis in mice. This finding suggests that the integrin molecules are critical in both the normal physiology and pathology of the

lung diseases and that the use of their antibodies offers a therapeutic potential for management of lung diseases including IPF in humans.

Platelet-Activating Factor Receptor Antagonists

Platelet-activating factor (PAF) is a membrane-derived phospholipid involved in a range of inflammatory conditions of lungs including bronchopulmonary dysplasia resulting from chronic lung injury that gradually progresses to airway obstruction and interstitial fibrosis (116). Interaction of PAF with specific PAF receptors activates heterotrimeric GTP-binding proteins, and this triggers the activation of various protein kinases, leading to mobilization of intracellular free calcium (117). Signal transduction initiated by PAF mediates diverse cellular effects including activation of monocytes/macrophages to produce inflammatory mediators, such as eicosanoids, TNF-α, IL-1, and IL-6 and upregulation of adhesion molecules on neutrophils (118). According to a number of studies, PAF appears to play a significant role in acute and chronic lung injury (118). PAF receptors are upregulated in rat alveolar macrophages by ozone inhalation (119), which produces lung fibrosis after chronic exposure (120). The findings that the PAF-receptor antagonist, WEB 2086, attenuated both BL- and amiodarone-induced lung fibrosis suggested the involvement of PAF in the pathogenesis of lung fibrosis (121, 122). These findings were later corroborated by studies showing an upregulation of functional PAF-receptors in alveolar macrophages from hamsters developing fibrosis in response to BL treatment (123). Since intracellular Ca^{2+} mobilization acts as a second messenger of PAF-induced signal transduction in PAF-responsive cells and the mobilization of Ca^{2+} is responsible for PAF-induced stimulation of cytokine production, it is highly likely that the upregulation of functional PAF-receptors in alveolar macrophages after BL treatment might stimulate these cells to produce excess amounts of various fibrogenic cytokines in response to endogenous PAF. This hypothesis may explain why alveolar macrophages from BL-treated hamster lungs were more sensitive to the Ca^{2+}-releasing effect of PAF than those from saline-treated control lungs (123). Therefore, any strategy, including the use of PAF-receptor antagonists, that interferes with the mobilization of free calcium from the internal calcium pools may have therapeutic significance in IPF patients. Unfortunately, this line of research has received little attention despite the availability of a host of new-generation PAF-receptor antagonists. These agents warrant further investigation.

Nitric Oxide Synthase Inhibitors

The cellular injury in lung fibrosis is initially mediated by ROS produced by infiltrating inflammatory cells. Activated macrophages produce both nitric oxide (NO) and peroxynitrite (124). The latter is a potent oxidant that is produced rapidly in a reaction of NO with superoxide radicals (125). Neutrophil- and macrophage-derived nitrogen free radicals are suggested to play an important role in cytotoxicity and in the pathogenesis of many lung diseases. For example, increased levels

of exhaled NO are associated with fibrosing alveolitis (126), asthma (127), and bronchiectasis (128). Furthermore, increased production of NO is also linked with hepatic fibrosis (129).

As discussed earlier, BL-induced lung fibrosis results initially from an inflammatory reaction of the lungs mediated, in part, by neutrophils and macrophages that generate ROS such as hydroxy radicals, superoxide ions, and hydrogen peroxide. The ROS are believed to damage the lung parenchyma, and this damage subsequently progresses to lung fibrosis. In addition, BL itself is capable of generating hydroxy radicals and superoxide ions. Thus, the ability of BL to generate superoxide radicals in vivo increases the possibility of an enhanced production of peroxynitrite, a potent oxidant involved in inflammatory diseases of the lungs including IPF (130). Recently, we have demonstrated an increased level of NO in the BALF and overexpression of inducible nitric oxide synthase (iNOS) mRNA and NOS protein in the lungs during the course of the development of BL-induced pulmonary fibrosis in mice (131). This is not surprising since iNOS gene is transcriptionally regulated by NF-κB (132), which is activated in the lungs in the experimental model of BL-induced lung fibrosis (67). The inducible activation of NF-κB stimulates the transcription of the iNOS gene leading to an increase in NO production (132). The combined treatment with taurine and niacin that attenuated BL-induced pulmonary fibrosis also blocked the BL-induced increased production of NO in the BALF, as well as overexpression of iNOS mRNA and NOS protein (131). These findings are in agreement with recently reported data showing that NO produced via iNOS also plays a critical role in ozone-induced lung inflammation (133), and an excess production of NO was linked with lung injury leading to fibrosis. This led us to hypothesize that any strategy that minimizes the production of unphysiological levels of NO will have beneficial effects against BL-induced lung fibrosis. This turned out to be the case since aminoguanidine, a specific inhibitor of iNOS (134), abrogated BL-induced lung fibrosis in mice without producing any systemic toxicity (135). This is a significant finding because it provides the impetus for exploring and developing this class of compounds into novel antifibrotic drugs of potential clinical significance for managing patients with IPF.

Eicosanoids and Gamma-Linolenic Acid

Extensive research has focused on defining the role of eicosanoids in the pathogenesis of lung fibrosis. Important findings from this line of investigation include the stimulatory effects of arachidonic acid metabolites on collagen synthesis in general, with the exception of prostaglandin E (PGE). For instance, leukotrienes have been reported to directly stimulate proliferation of mesenchymal cells and fibroblasts and collagen synthesis (136). This is consistent with the finding of a constitutive activation of 5-lipoxygenase responsible for generating LTB4 and peptido LTC4 from arachidonic acid in the lungs of patients with IPF (137). In fact, the lung homogenate from these patients contained 15-fold more LTB4 than homogenate from nonfibrotic lungs. Conversely, PGE is a potent inhibitor of fibroblast proliferation (138) and collagen synthesis (139). It is normally present

in the lung at much higher concentrations than in plasma (140) and may play an important role in maintaining normal lung ECM homeostasis. The role of PGE as an endogenous antifibrotic agent was further confirmed by two additional experiments. Cyclooxygenase-2 (COX-2) deficiency resulted in a loss of antiproliferative response to TGF-β in vitro arising from inadequate production of PGE2 in fibroblasts derived from fibrotic as compared to nonfibrotic human lungs. Mice deficient in COX-2 exhibited a greater fibrogenic response to BL than the wild-type mice (141). This supports an earlier finding that the cultured lung fibroblasts isolated from IPF patients had diminished ability to synthesize PGE2 and express COX-2 (142). Furthermore, PGE levels in the epithelial lining fluid (ELF) of individuals with pulmonary fibrosis were 50% lower than those of normal individuals (143). Attempts have been made to increase the PGE1 levels in the ELF by intravenous infusion of PGE_1. Although this treatment decreased early neutrophil traffic to the airways and reduced injury to the lung permeability barrier in the BL-hamster model of lung fibrosis, it failed to significantly alter the course of development of fibrosis (144). However, dietary intake of gamma-linolenic acid (GLA) contained in evening primrose oil (EPO) increased the synthesis of antiproliferative eicosanoid, PGE_1, decreased the synthesis of proinflammatory eicosanoid, LTB4, in lungs and attenuated BL-induced lung fibrosis in hamsters (145). The rationale for this novel strategy was that dietary GLA is elongated by elongase present in the lungs to dihomo gamma linolenic acid (DGLA), which in turn is metabolized by cyclooxygenase into PGE1 and by 15-lipoxygenase into 15-hydroxyeicosatrienoic (15-HETrE) acid. Both metabolites are antiinflammatory and antiproliferative, and 15-HETrE also inhibits 5-lipoxygenase and reduces the synthesis of a proinflammatory eicosanoid, LTB4 (146, 147). The beneficial effects of dietary intake of GLA-containing oil against BL-induced lung fibrosis correlated well with the increased tissue levels of DGLA, PGE_1, and 15-HETrE acid and decreased level of LTB_4 in BL + EPO group as compared to control BL + CO (corn oil) group (145). The findings reported in this paper agree with the beneficial effects of GLA-containing oils (EPO and borage) against other inflammatory conditions such as endotoxin-induced acute lung injury in pigs (148) and rheumatoid arthritis in humans (149, 150). The dietary intake of highly purified GLA-containing oils or sufficient intake of constituent polyunsaturated fatty acid of these oils may very likely be useful in alleviating inflammation and excess accumulation of collagen in the lungs of IPF patients. The important biological activity of GLA combined with the fact that this agent has negligible side effects support the need for additional clinical studies.

Cysteine Pro-Drugs

Alveolar lining fluid contains higher concentrations of glutathione (GSH) than found in most extracellular fluids (151). The concentration of GSH in BALF from patients with IPF is 23% of normal (152). Depletion of GSH may place the alveolar space at increased risk of additional injury caused by ROS generated by the influx of inflammatory cells during the inflammatory phase of the development

of lung fibrosis. Therefore, increasing the tissue levels of non-protein sulfhydryl (NPSH), particularly GSH, has been suggested as a potential strategy to protect lungs and other organs against free radical–induced injury (153, 154). Since exogenous administration of GSH is relatively ineffective against chemically-induced organ toxicity owing to the inability of the intact tripeptide to enter the cells and rapid hydrolysis (155), cysteine pro-drugs have been used to achieve the same objective. Cysteine pro-drugs are thought to be resistant to hydolysis and to provide a constant source of cysteine for intracellular synthesis of GSH. In this context, N-acety-L-cysteine (NAC), which promotes the production of GSH by furnishing L-cysteine (156), was found to protect the lung against polymorphonuclear leukocyte-mediated cytotoxicity (157). Many reports support the ability of NAC to sustain the tissue levels of GSH (153, 154), a finding that has led to clinical uses of NAC in acetaminophen poisoning (158) associated with the depletion of hepatic GSH (154), followed by death. The effects of NAC against BL-induced lung fibrosis are not without controversy. Although i.t. instillation of NAC was effective (159), subcutaneous and intraperitoneal (i.p.) routes of NAC administration were not effective in preventing BL-induced lung fibrosis in experimental models (160, 161). This discrepancy was attributed to a difference in the bioavailabilty of cysteine after different routes of administration. The observation that i.p. administration of cysteine pro-drug Z2196 (2RS, 4R-2-methylthiazolidine carboxylic acid), which allowed a greater bioavailability of cysteine than other cysteine pro-drugs, attenuated BL-induced lung fibrosis (162) supports the significance of the bioavailability of cysteine in offering protection against chemically induced toxicity of organs. The recent finding that the administration of aerosolized NAC attenuated BL-induced lung fibrosis in mice provides additional support (163), and this result was attributed to an optimal level of NAC bioavailability at the site of injury. Although studies of therapeutic efficacy of NAC in IPF patients are currently underway, there is an urgent need to design and develop a new generation of cysteine pro-drugs that can be administered orally and that will make cysteine readily bioavailable at the site of injury in an adequate amount to elicit desirable therapeutic effects.

CONCLUSIONS

Interstitial lung fibrosis of known or unknown etiology is a crippling disease that has defied any therapeutic modality to date. Generally, the standard treatment for IPF includes synthetic glucocorticoids with or without cytotoxic drugs like colchicine, cyclophosphamide, or azothioprine. This line of therapy has failed to improve the survival rate and appears to be invariably associated with overt systemic toxicity. A number of compounds developed over several decades, based on their ability to interfere with the synthesis of collagen at the strategic sites of transcription, translation, posttranslation, secretion, and cross-link formation in the experimental models of lung fibrosis, have proven to be either ineffective or too toxic for use in humans. Our understanding of the molecular mechanisms for

the pathogenesis of lung fibrosis has improved considerably with the use of the BL-rodent model of lung fibrosis. This model has opened up an unprecedented range of mechanistically based possibilities for novel pharmacological interventions to manage IPF. These include maintenance of intracellular levels of NAD^+ and ATP; evaluation of various strategies to interfere with the biological activities of TGF-β and integrins; evaluation of a new generation of PAF-receptor antagonists; evaluation of nitric oxide synthase inhibitors; evaluation of a polyunsaturated fatty acid, such as gamma-linolenic acid; and the development and evaluation of a new generation of cysteine pro-drugs. Basic research conducted by investigators worldwide using experimental models of lung fibrosis has substantially advanced our knowledge of mechanisms of inflammation and fibrosis in the lung. It is hoped that this knowledge can be translated into treatment for patients with inflammatory and fibrotic lung diseases. Effective therapy for management of IPF in the twenty-first century is a realistic prospect.

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NITRIC OXIDE–RELEASING DRUGS

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■ **Abstract** Pharmacological compounds that release nitric oxide (NO) have been useful tools for evaluating the broad role of NO in physiology and therapeutics. NO deficiency has been implicated in the genesis and evolution of several disease states. Both medical needs and commercial opportunities have fostered attempts to modulate NO in the human body for therapeutic gain. Strategies for NO modulation encompass antiinflammatory, sexual dysfunction, and cardiovascular indications. Apart from newly developed drugs, several commonly used cardiovascular drugs exert their beneficial action, at least in part, by modulating the NO pathway. This review discusses the fundamental pharmacological properties and mechanisms of action of NO-releasing drugs. Some of these compounds may enter in the clinical arena providing important therapeutic benefits in human diseases.

INTRODUCTION

A gaseous nitrogen monoxide radical, nitric oxide (NO), is a ubiquitous signaling molecule able to diffuse readily across cell membranes, modulating a plethora of physiological responses including gene regulation, cytostasis, apoptosis, platelet function, vascular smooth muscle cell (VSMC) relaxation and proliferation, neurotransmission, memory, and immune stimulation [reviewed in (1–3)]. Since the discovery of NO, an increasing number of studies have attempted to address its chemistry and its connection with the biology of this unique mediator. Unlike other biological molecules, the chemistry of NO determines its biological properties. NO is produced by the basic semiessential amino acid L-arginine (2-amino-5-guanidinovaleric acid) in a reaction catalyzed by a family of nitric oxide synthases (NOSs) (1–3). The classical physiological role of NO generally reflects direct activation of guanylate cyclase to generate cGMP, followed by kinase-mediated signal transduction (1–3). However, several other NO-related activities are cGMP-independent. NO-derived reactive nitrogen oxide species (RNOS) can

modify bioactivity of certain molecules such as proteins, lipids carbohydrates, and nucleic acids (4). Indeed, oxygen radicals can enhance the bioreactivity of NO through RNOS formation (1–4). However, reaction of NO with superoxide radical, although antioxidant in the sense that a superoxide radical is removed from the system, can damage cell membranes and cell function by producing peroxynitrite, a highly oxidizing agent capable of perturbing bioactivity, or by consuming regulatory NO (1–4). Detailed consideration of general NO biology and chemistry is obviated by recent presentations elsewhere (1–3, 5). Therefore, appreciation of this "double-edged sword" aspect of NO under conditions supporting mammalian life plays a pivotal role to any attempts at therapeutic NO modulation. Distinction between a beneficial and an adverse body response to NO at the physiological level could be related to tissue redox status, tissue NO concentration, tissue NOS activity, presence of oxygen radicals, formation of RNOS, site of exogenous NO production or scavenging, autooxidation of NO, and interaction of NO with cellular constituents. Moreover, the nature of a given biological response to NO need not influence its ultimate therapeutic gain. Indeed, NO can limit cell proliferation by acting as reversible cytostatic agent (pro-apoptotic effect) or as a nonspecific rank cytotoxin (6).

NO-releasing drugs are pharmacologically active compounds that, in vivo or in vitro, release NO. For a detailed overview of the general role of NO-donors (e.g., direct NO-donors, NO-donors requiring metabolism, and bifunctional NO-donors), the reader is referred elsewhere (7). There are some diseases that result from quantitative or functional NO deficiency. A NO insufficiency may be characterized by a net tissue NO deficit, enhanced NO inactivation, impaired NO availability, or altered NOS catalysis. In all these states, a NO deficiency would limit NO-dependent signal transduction pathways to the detriment of normal cellular function. For example, dysfunction of the normally protective endothelium is found in several cardiovascular diseases, including atherosclerosis, hypertension, heart failure (HF), coronary heart disease (CHD), arterial thrombotic disorders, and stroke (1, 2, 8). Endothelial dysfunction leads to NO deficiency (1, 2, 8), which has been implicated in the underying pathobiology of many of these disorders. In the case of the gastrointestinal tract, NO is a critical mediator of mucosal defense and repair (9). Finally, NO is an essential mediator of penile erection, which is fundamentally a hemodynamic process (10).

Pro-inflammatory effects of NO include vasodilation, edema, cytotoxicity, and the mediation of cytokine-dependent processes that can lead to tissue destruction [reviewed in (11)]. Conversely, the production of NO by endothelial cells may serve a protective, or antiinflammatory, function by preventing the adhesion and release of oxidants by activated neutrophils in the microvasculature (11). In light of this multifaceted pathophysiological scenario, replacement or augmentation of endogenous NO by exogenously administered NO-releasing drugs has provided the foundation for a broad field of pharmacotherapeutics.

Here, we review our current understanding of NO-releasing drugs and of commonly used cardiovascular drugs that modulate the bioactivity of NO.

SODIUM NITROPRUSSIDE

In sodium nitroprusside (SNP), NO is coordinated as a nitrosyl group liganded to iron in a square bipyramidal complex (12, 13) and is released spontaneously at physiological pH from the parent compound. SNP remains an effective, reliable, and commonly used drug for the rapid reduction of significant arterial hypertension regardless of the etiology, for afterload reduction when blood volume is normal or increased, and for intraoperative-induced alterations of blood pressure (12, 13). SNP has been used effectively for decades for the treatment of hypertension and HF (12, 13). The use of SNP is limited by the need to administer it parenterally, tolerance, and the potential for the development of thiocyanate toxicity with prolonged administration (in rhodanase-deficient individuals). Careful attention to infusion rates, particularly in patients at risk for depleted thiosulfate stores, is mandatory, and the use of other drugs in conjunction with or instead of SNP should always be considered. Despite its toxicity, SNP is popular because it is often the most effective drug in some difficult clinical circumstances.

Mutagenic effects of some NO donors have also been demonstrated. Birnboim & Privora, using a very sensitive detection system, showed that glyceryl trinitrate and SNP appear to promote mutagenesis in a glutathione-dependent manner; in this study, N-acetylcysteine and oxothiazolidine-4-carboxylate paradoxically reduced mutagenicity of the NO donors (14).

NITROVASODILATORS: ORGANIC NITRATE AND NITRITE ESTERS

The classic nitrovasodilators, organic nitrate and nitrite esters, including nitroglycerin, amyl nitrite, isosorbide dinitrate, isosorbide 5-mononitrate, and nicorandil, have been used for many years in the treatment of cardiovascular diseases (15–17). Indeed, the organic nitrates are a safe and effective choice for the management of ischemic syndromes related to coronary heart disease (CHD). Their principal action is vasorelaxation, mediated by guanylyl cyclase activation and by direct inhibition of nonspecific cation channels in VSMCs. As such, these agents represent the prototypical form of NO-replacement therapy. In addition to their well-established venodilative activity, nitrates are now known to cause vasorelaxation of coronary arteries, coronary stenoses, and coronary collateral vessels and to prevent episodic coronary constriction. A direct antiplatelet effect has also been investigated. Notwithstanding these shortcomings, a careful and controlled use of these agents represents the mainstay of therapy for patients with CHD. The first reports of the clinical use of organic nitrates and nitrite esters was derived from the seminal work of Brunton in 1857 showing the clear benefits of these compounds in the treatment of angina pectoris (18). The rapid- but short-acting nitrate preparations are useful in arresting and preventing acute attacks of angina pectoris, whereas longer-acting oral and transdermal formulations are indicated for the

relief of chronic symptomatic and asymptomatic ischemia (15). The intermittent nitrate dosing regimens introduced in recent years have reduced the likelihood of tolerance, which greatly limited the usefulness of long-acting nitrates in the past. Intravenous infusion of nitroglycerin is particularly appropriate for the management of unstable angina and the early complications of myocardial infarction (MI) (15). In patients with acute MI, intravenous nitroglycerin lowers left ventricular (LV) filling pressure and systemic vascular resistance. At lower infusion rates (less than 50 μg/min) nitroglycerin is principally a venodilator, whereas at higher infusion rates more balanced venous and arterial dilating effects are seen. Patients with HF demonstrate increased or maintained stroke volumes, whereas patients without HF show a decrease in stroke volume (15). Longer-term infusions (24–48 hours) have resulted in myocardial preservation and positive effects on remodeling, as assessed by global and regional LV function and laboratory indices of infarct size. Comparison of intravenous nitroglycerin and SNP reveals increased intercoronary collateral flow with nitroglycerin, in contrast to a decrease with SNP, compatible with a "coronary steal" (15). Short-term administration of intravenous nitroglycerin with or without chronic administration of long-acting nitrates has been found both to reduce short-term mortality and to have long-term beneficial effects on LV remodeling in patients with anterior transmural MI (15). Current clinical practice would utilize intravenous nitroglycerin as initial therapy for patients receiving intravenous thrombolytic therapy and/or acute percutaneous transluminal coronary angioplasty within four to six hours of the onset of symptoms of acute MI, in order to optimize intercoronary collateral flow until reperfusion can be accomplished. Patients reaching the hospital more than 6 hours but less than 14 hours after symptom onset can still benefit from intravenous nitroglycerin administered for 24–48 hours.

The limitations of this class of agents are well known and include potentially adverse hemodynamic effects, drug tolerance, lack of selectivity, and limited bioavailability. All of the organic nitrate esters are prodrugs requiring enzymatic metabolism to generate bioactive NO. The major enzyme system involved is located within microsomal membranes, has an estimated apparent molecular mass of 160 kDa, and manifests enhanced activity in the presence of reducing equivalents (19). Although the enzyme has not been more specifically characterized, growing evidence suggests that the cytochrome P450 system is required for the linked metabolic processes of denitration and reduction of organic nitrate esters to authentic NO (16, 20–23). Importantly, thiols potentiate the action of organic nitrate esters (16, 20–24).

Tolerance limits the clinical use of organic nitrite and nitrate esters; it is associated with increased angiotensin II (ANGII)-dependent vascular production of superoxide radical from NAD(P)H oxidase and endothelial NOS (eNOS) (25, 26). The superoxide radical generated by these enzymes reacts with NO derived from the NO-donor to form peroxynitrite, as indicated by the finding of increased urinary 3-nitrotyrosine in nitrate-tolerant patients (27). Importantly, nitrate tolerance is also associated with cross-tolerance to endothelium-derived NO (28), both by

the oxidative inactivation of this endogenous NO to peroxynitrite and by "uncoupling" of eNOS activity (29). Low-molecular-weight thiols, ascorbate, L-arginine, tetrahydrobiopterin, hydralazine, angiotensin converting enzyme (ACE) inhibitors, and folate have been successfully used to reverse or prevent nitrate tolerance (30).

S-NITROSOTHIOLS

Early studies have demonstrated that S-nitrosothiols represent a source of circulating endogenous NO, and may have potential as donors of NO, distinct from currently used agents (16, 21–23). Their stability is influenced by the properties of the R group, heat, light, the presence of transition metal ions (in particular copper), and the presence of other thiols. S-nitrosothiols participate in transnitrosation reactions in which the NO group is transferred to another thiol to form a more stable compound (16, 21–23). Thus, the stability of these compounds in vivo is difficult to predict. Potential interactions of S-nitrosothiols include that with ascorbic acid (vitamin C), which enhances the ability of copper to catalyze the degradation of S-nitrosothiols. S-nitrosothiols, offer advantages over the existing drugs because they do not share the drawbacks of organic nitrates and SNP, including a limited capacity for inducing oxidant stress and tolerance in vascular cells (31). Initial small clinical studies suggest that they may be of benefit in a variety of cardiovascular disorders (32). S-nitrosothiols are a class of naturally occurring NO-donating compounds that spontaneously release NO and nitrosonium (NO^+); they may also gain access to the intracellular compartment by the catalytic action of plasma membrane-bound protein disulfide isomerase and associated transnitrosation reactions (33). However, the action of these compounds is linked to NO because NO^+ does not activate guanylate cyclase (21–23). Members of this class of agents include S-nitroso-glutathione, S-nitroso-N-acetylpenicillamine, and S-nitroso-albumin (34) (Figure 1). Investigators have chosen to modify existing pharmacological agents with these functional groups in an effort to exploit some

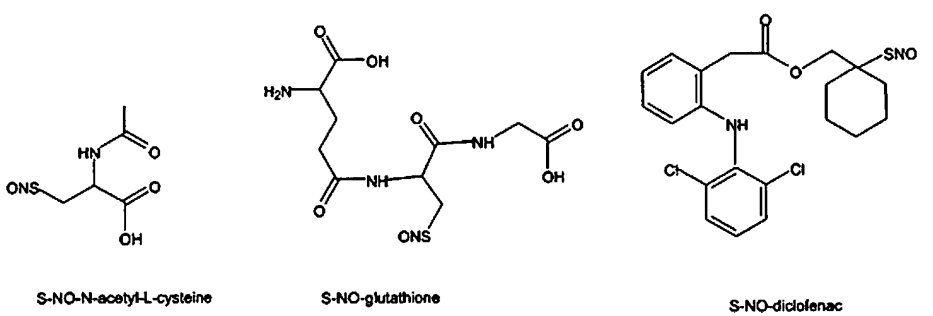

Figure 1 Structures of selected S-nitrosothiols.

of the beneficial effects of NO without limiting the pharmacological effect of the parent compound. Early work with S-nitroso-captopril represents one such effort (35, 36). A theoretical role of S-nitrosothiols has been suggested in the treatment of asthma and in their potential to be used as agents to treat infectious diseases ranging from the common cold to immunodeficiency diseases.

NONSTEROIDAL ANTIINFLAMMATORY DRUGS (NSAIDS) AND THE DEVELOPMENT OF NO-NSAIDS

Nonsteroidal antiinflammatory drugs (NSAIDs) are among the most commonly used medications. They are prescribed largely for their antiinflammatory, antipyretic, and analgesic properties. Moreover, low-dose aspirin is increasingly used on a long-term basis for its well-documented antithrombotic effects (37, 38). The major limitation for the use of NSAIDs is their ability to cause gastrointestinal toxicity. Indeed, even low-dose aspirin maintains its ability to damage the gastric mucosa by inducing bleeding ulcers and/or erosions (38, 39). The efficacy and toxicity of NSAIDs appears to be closely related to cyclooxygenase (COX) activity inhibition. The discovery of a second inducible isoform of COX (COX-2) has given a new boost to NSAID research in the attempts to develop COX-2 selective inhibitors. These drugs have been developed in an effort to improve gastrointestinal tolerability and efficacy. However, questions remain about the effectiveness and safety of this new class of drugs (40). Because some biological effects are mainly driven by COX-1 products, as in thrombosis (41), COX-2 inhibitors cannot replace aspirin in antithrombotic therapy because by definition they do not inhibit COX-1, the constitutive form of the enzyme, present in platelets.

Prostaglandins and NO are thought to play a major role in maintaining mucosal integrity. Indeed, NO has cytoprotective properties that derive from its ability to increase local blood flow and to scavenge highly reactive free radicals in the stomach and other organs (42–45). In particular, NO exhibits many of the same actions in the stomach as prostaglandins, such as stimulation of mucus secretion (46) and maintenance of mucosal blood flow (47). These overlapping physiological properties at the gastric level have prompted efforts to design an aspirin-like compound that can release NO, counterbalancing the negative effect due to prostaglandin inhibition at this level. Indeed, local delivery of NO could be a surrogate for prostaglandin effect, restoring the imbalance between destructive and protective factors caused by COX-1 inhibition in the gastrointestinal tract. In addition, because NO plays an important role in pathophysiological conditions where platelets, endothelial, and other blood cells are actively involved and in the inflammatory process, these compounds also have an intense antiinflammatory effect.

New classes of NSAIDs that release NO, called NO-NSAIDs, have been discovered and early clinical evidences suggests that these compounds could be safe and effective alternatives to conventional NSAIDs. These agents comprise two classes of compounds, one that contains a nitrate ester functionality (48) and one that contains an S-nitrosothiol functionality (49).

NO-RELEASING ASPIRINS

NO-releasing aspirins are nitrate-ester compounds and include 2-acetoxybenzoate 2-(2-nitroxy-methyl)-phenyl ester (NCX-4016) and 2-acetoxybenzoate 2-(2-nitroxy)-butyl ester (NCX-4215) (Figure 2). NCX-4016 is a stable compound that requires enzymatic hydrolysis to liberate NO, and the kinetics of this metabolic processing leads to durable production of NO released at a constant rate (48). Following intragastric administration of NCX-4016, levels of NO are elevated both in gastric content and plasma (48). NO generation by NCX-4215 has also been evaluated by using human platelets and measuring NO generation by chemiluminescence (48). To establish whether NCX-4215 could spontaneously liberate NO, the compound was incubated in the absence or presence of platelets. A significant release of NO was observed only in presence of platelets. The biological activity of NCX-4016 has been evaluated in a variety of experimental models to characterize its antiinflammatory and antithrombotic effects (48).

The antiplatelet activity of nitroaspirins NCX-4215 and NCX-4016 was compared in vitro to aspirin with comparable results for maximal inhibition of arachidonic acid-stimulated platelet aggregation (50). Furthermore, NCX-4016 was more efficient in inhibiting platelet activation induced by thrombin (48, 50). This was reversed by oxyhemoglobin and methylene blue acid, further supporting the NO-related inhibitory effect. NCX-4016 was shown to possess greater antiinflammatory and analgesic activity (51, 52). The antithrombotic activity of NCX-4016 was also present in vivo (53) and reduced pulmonary thromboembolism

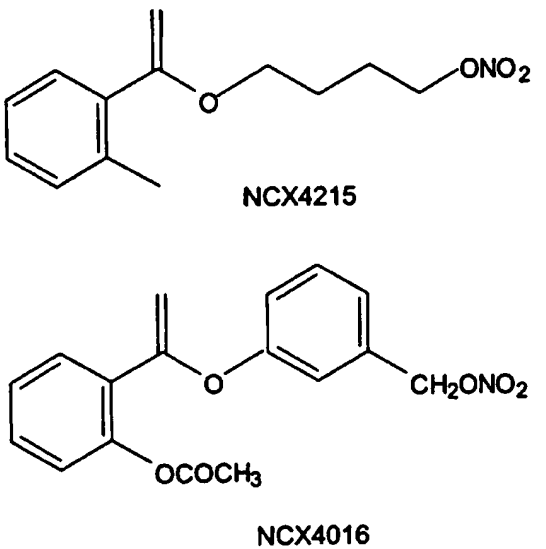

Figure 2 Structures of nitro-aspirin derivatives.

in several platelet dependent and -independent animal models (54). The compound also has greater protective activity than aspirin in focal cerebral ischemia in the rat (55). NCX-4016 and diethylenetriamine/NO, a NO-donor, raised myocardial production of prostacyclin and thromboxane during MI (56). Interestingly, NCX-4016 also reduces infarct size after myocardial reperfusion injury (57, 58).

Inhibitory effects on caspases 1 and 3 (59, 60), T lymphocyte activation (60), cytokine release (60), stimulation of apoptosis (60), and IL-1Bβ converting enzyme (61) have been described for NCX-4016. In very recent studies, NCX-4016 reduces the degree of restenosis after arterial injury in low-density lipoprotein-receptor-deficient mice (62) and in aging rats (63). This protective effect was associated with reduced VSMC proliferation and macrophage deposition at the site of injury. This response appears to agree very well with the potent inhibitory properties on VSMC proliferation possessed by NCX-4016 (64). Thus, a NO-releasing–aspirin derivative could be an effective drug in reducing restenosis following PTCA, especially in the presence of hypercholesterolemia or advancing age.

Another therapeutic goal of NO-aspirin was to achieve lower gastrointestinal toxicity in comparison to aspirin (48). NCX-4215 does not produce macroscopically visible or histological damages in the rat stomach when administered up to 300 mg/kg, whereas 100 mg/kg aspirin produces widespread hemorrhagic damage (65, 66). These protective effects were also seen in the stomach of aged rats treated with NCX-4016 (63). NCX-4016 produced an equipotent inhibition of mucosal PGE_2 generation in the stomach as compared with aspirin (67), suggesting that gastrointestinal damage is not linked to a lack of effect on COX enzymes.

Leukocyte adherence is not affected by pretreament with NCX-4016, whereas aspirin causes a fivefold increase in neutrophil adherence (68). Furthermore, whereas aspirin has no effect on human umbilical vascular endothelial cell (HUVEC) apoptosis induced by TNFα, NCX-4016 causes a concentration-dependent inhibition of TNFα-induced apoptosis (59). The hypothesis that nitroaspirin is also able to positively modulate change in gastrointestinal damage was challenged by testing the ability of NCX-4016 to prevent gastric damage in a rat model of shock (68). Oral administration of NCX-4016 or glyceryl trinitrate or depletion of circulating neutrophils with antineutrophil serum significantly reduced the extent of gastric damage induced by hemorrhagic shock, whereas aspirin had no effect. Recent data also showed the lack of gastric toxicity of NCX-4016, but not aspirin, in the stomach of diabetic rats (69).

The effects of NCX-4016 and aspirin on the release of thromboxane; TNF-α; interleukin-6; and expression and activity of tissue factor (TF) in stimulated, adherent human monocytes were recently investigated (70). These data showed that NCX-4016 inhibits thromboxane generation, cytokine release, and TF activity in human monocytes via NO-dependent mechanisms. Moreover, NCX-4016 also reduces blood pressure in hypertensive rats, not simply through the direct vasodilatory actions of the NO released by this compound, but also through possible interference with endogenous pressor compounds (71). These properties, added to its antithrombotic effects, suggest that NCX-4016 may be a safer alternative to aspirin for use by hypertensive patients.

S-NITROSO-NSAIDS

S-nitroso-diclofenac (Figure 1), as a prototype of the S-nitrosoester class of NSAIDs that release NO, possesses peculiar properties (49). This agent is orally bioavailable as a prodrug, producing significant levels of diclofenac in plasma within 15 min after oral administration to mice. In addition, S-nitroso-diclofenac has equipotent antiinflammatory and analgesic properties as diclofenac, but is gastric-sparing compared with the parent NSAID. Thus, S-nitrosothiol esters of diclofenac and other NSAIDs [reviewed in (72)] comprise a novel class of NO-donating compounds with antiinflammatory and analgesic properties but a markedly enhanced gastric safety profile. The use of NO-NSAIDs for general analgesic and antiinflammatory purposes or for primary or secondary cardiovascular prevention awaits the results of ongoing long-term clinical trials.

NO-DELIVERY SYSTEMS

There has been considerable long-term interest in developing NO delivery systems that can be used to target drug action and modulate the kinetics of drug release. Drug-eluting vascular stents with a variety of coatings, including fibrin, heparin, and multiple polymers that contain NO-donors, have been tested with variable effects (73, 74). NO-containing crosslinked polyetheleneimine microspheres that release NO (half-life of 51 hours) have been applied to vascular grafts (75) in order to prevent thrombosis and restenosis. Similarly, the [N(O)NO] group has been incorporated into polymeric matrices synthesized to modulate the time-course of NO release (76). This approach shows potent antiplatelet activity in baboons (76).

Bovine serum albumin (BSA) can be modified covalently to bear multiple S-NO groups, which possess vasodilatory and antiplatelet properties (77). Poly-S-nitrosated BSA applied locally to a site of vascular injury reduced restenosis in a rabbit model (78). Local delivery of poly-S-nitrosated BSA induced a 50%–70% reduction in platelet attachment and surface activation, together with a 40% reduction in neointimal area when compared to BSA (79). The advantages to the use of this agent include the avidity of the subendothelial matrix for albumin; its long half-life in vivo; and its ability to serve as a local depot of NO, requiring trans-S-nitrosation reactions (thiol-S-nitrosothiol exchange) to deliver NO via low-molecular-weight S-nitrosothiol intermediates (80).

CARDIOVASCULAR AGENTS MODULATING THE NO-PATHWAY

Calcium Channel Blockers

1,4-Dihydropyridine calcium channel blockers (CCBs) have been used for many years in the treatment of angina pectoris and hypertension (81). Their mechanism of action is based on inhibition of the smooth muscle L-type calcium current, thereby

decreasing intracellular calcium concentration and inducing smooth muscular relaxation. 1,4-Dihydropyridine CCBs (nifedipine, nitrendipine, and lacidipine) can also induce the release of NO from the vascular endothelium (81). CCBs counteract the effects of ANG II and endothelin-1 at the level of vascular smooth muscle by reducing Ca^{2+} inflow and facilitating the vasodilator effects of NO. Indeed, these compounds can reverse impaired endothelium-dependent vasodilation in different vascular beds, including the subcutaneous, epicardial, and peripheral arteries of the forearm circulation. In the forearm circulation, nifedipine and lacidipine can improve endothelial dysfunction by restoring NO availability (81). Furthermore, in several experimental preparations, including micro- and macrovascular studies, the sensitivity of the vasorelaxing effect of the 1,4-dihydropyridines to inhibitors of NOS, such as L-N^G-nitroarginine or L-N-nitro-argininemethylester, has been clearly demonstrated. These studies show that the NO-releasing effect is not unique to nitrendipine but is a class phenomenon shared by 1,4-dihydropyridine CCBs and several nondihydropyridine CCBs (81). More importantly, 1,4-dihydropyridine CCBs also have a potent antioxidant activity in vivo (82). Thus, the above effects on NO could also be due to this property. Indeed, the underlying mechanism of NO release evoked by these drugs is not entirely clear but may also involve modulating endothelial membrane potential via a myo-endothelial interaction (83), upregulating eNOS expression (84), increasing activity of endothelial superoxide dismutase(s) (85), and enhancing flow-mediated release of endothelial NO via VSMC relaxation and vasodilation. These dual modes of action, i.e., the direct relaxing effect of inhibiting smooth muscle L-type calcium current and the indirect relaxing effect of releasing NO from vascular endothelium, may help explain the beneficial antihypertensive effect of the 1,4-dihydropyridine CCB class.

Ace-Inhibitors and ANGII Type 1 Receptor Antagonists

ANGII and bradykinin levels within the vascular wall are controlled by ACE (86). ACE degrades bradykinin (87) and generates ANGII; in turn, bradykinin stimulates the endothelium to release vasodilating substances, in particular, NO. Thus, by potentiating bradykinin, ACE-inhibitors may promote the release of endothelial NO (88). Indeed, ACE-inhibitors exert some of their beneficial pharmacological effects by increasing vascular NO activity (86, 89, 90). Moreover, due to the significant constitutive expression of NOS in the juxtaglomerular apparatus, NO appears to act as a tonic enhancer of renin secretion via cGMP-dependent inhibition of cAMP degradation [reviewed in (91)]. This effect may also revert to an inhibitory effect compatible with the inhibition of renin secretion by cGMP-dependent kinase(s). Moreover, ANGII can stimulate superoxide production, which reduces the bioavailability of NO (92), an event that can be blocked by ACE inhibitors.

In patients with high cardiovascular risk, chronic ACE inhibition improves endothelial function (86, 88). This may explain why patients treated with ACE inhibitors experience a greater cardiovascular benefit than is attributable to the decrease in blood pressure. Indeed, ACE inhibitors improve endothelial function

in the subcutaneous, epicardial, and renal circulation, but are ineffective at potentiating the blunted response to acetylcholine in the forearm of patients with essential hypertension [reviewed in (86)]. In addition, ANGII type 1 (AT-1) receptor antagonists can restore endothelium-dependent vasodilation to acetylcholine in the subcutaneous tissue, but not in the forearm microcirculation (90). Treatment with an AT-1 antagonist can improve basal NO release and decrease the vasoconstrictor effect of endogenous endothelin-1 (90). Thus, drugs interfering with the renin-angiotensin-aldosterone pathway may affect NO signaling by several mechanisms; however, the molecular mechanisms involved in the relationship between ACE inhibitors and the NO-pathway via bradykinin are still unclear (93).

There is mounting evidence that (*a*) ACE efficiently catabolizes kinins; (*b*) ANG-derivatives such as ANG-(1-7) exert kinin-like effects; (*c*) kallikrein probably serves as a prorenin-activating enzyme; (*d*) the protective effects of ACE inhibitors are at least partly mediated by a direct potentiation of kinin receptor response on bradykinin stimulation; and (*e*) studies on AT-1 antagonists, which do not directly influence kinin degradation, and studies on ANG-receptor transgenic mice have revealed additional autocrine interactions among the NO, kinins, prostaglandins, cyclic GMP, and ANGII receptor effects. The beneficial effects of ACE inhibitors or AT-1 antagonists are reportedly mediated by NO in HF. It was recently hypothesized that in the absence of eNOS, both LV dysfunction and myocardial remodeling would be more severe after MI, and the cardioprotective effect of ACE inhibitors or AT-1 antagonists would be diminished or absent in mice with HF after MI (94). eNOS knockout mice and wild-type C57BL/6J mice were subjected to MI by ligating the left coronary artery. One month after MI, each strain was treated with vehicle, enalapril or valsartan, for five months (94). ACE inhibitors improved cardiac function and remodeling in wild-type mice, as evidenced by increased LV ejection fraction and LV shortening fraction, and decreased diastolic LV dimension, mass, myocyte cross-sectional area, and interstitial collagen fraction, but these benefits were absent or diminished in eNOS knockout mice. Interestingly, AT-1 antagonists had benefits similar to those of ACE-inhibitors. These interesting data suggest that the absence of NO does not alter the development of HF after MI; however, it significantly decreases the cardioprotective effects of these drugs.

β-Blockers

Some β-blockers may also interfere with the NO-pathway. For example, nebivolol, a β1-blocker and a racemic mixture of (S,R,R,R) and (R,S,S,S) enantiomers (88, 95), was found to induce endothelium-dependent arterial relaxation in dogs in a dose-dependent fashion (96). However, its hemodynamic effects differ from those of classical β-adrenoceptor antagonists as a result of a vasodilating action. Indeed, the endothelium-dependent relaxation induced by nebivolol is abolished by L-NAME, an inhibitor of NOS.

Nebivolol and atenolol have been compared in phenylephrine preconstricted dorsal hand veins of healthy men (97, 98). Nebivolol caused venodilation, which

was antagonized by N(G)-monomethyl-L-arginine (LNMMA), whereas atenolol did not, suggesting that such a mechanism could also operate in human veins. Venodilation could be functionally important in reducing cardiac pre-load. β2-adrenoceptor stimulation increases forearm blood flow (FBF) by activating the L-arginine/NO pathway, but nebivolol lacks direct β2-adrenoceptor agonist activity. Resistance vessel function has been studied by measuring FBF by venous occlusion plethysmography in healthy men during brachial artery infusions of racemic nebivolol and its enantiomers, atenolol, carbachol (a stable analogue of acetylcholine that vasodilates this vascular bed, in part, by activating the L-arginine/NO pathway), SNP, and LNMMA (97, 98). Nebivolol increased FBF by 91 $\pm$ 18% ($p < 0.01$), whereas an equimolar dose of atenolol had no significant effect. LNMMA inhibited responses to nebivolol and carbachol to a significantly greater extent than it reduced responses to SNP. Antagonism of nebivolol by LNMMA was abolished by L-arginine. The (S,R,R,R) and (R,S,S,S) enantiomers caused similar increases of FBF.

To determine whether brachial artery infusion of nebivolol causes vasodilation in the forearm resistance vasculature of patients with essential hypertension and to investigate the possible involvement of the L-arginine/NO pathway, healthy volunteers with uncomplicated essential hypertension were also studied (99, 100). Nebivolol caused similar vasodilatation as in normotensive subjects, and these responses were sensitive to inhibition by LNMMA. If acute effects of nebivolol on the L-arginine/NO pathway persist during chronic treatment of patients with hypertension or HF, this could reduce cardiac after-load as well as pre-load, improve organ perfusion, and reduce atherogenesis and thrombosis.

Finally, experimental studies have established that nipradilol, another NO-releasing beta-adrenergic blocker, enhances postischemic recovery and limits infarct size (100a). Moreover, there is a clear involvement of NO in the ocular hypotensive action of nipradilol (100b). These studies should be carried out in humans before a conclusion is made.

Hydroxymethylglutaryl-CoA Reductase Inhibitors

The efficacy of the widely prescribed hydroxymethylglutaryl (HMG)-CoA reductase inhibitors (statins) in decreasing the incidence of cardiac events and mortality is likely enhanced by their possible antioxidant and other unknown properties (101).

In 1997, it was demonstrated that statins prevent hypoxia-induced down-regulation of eNOS in normocholesterolemic cells by stabilizing eNOS mRNA, leading to an increase in NO production by endothelial cells (102). Subsequently, it was shown that statins exert their salutary effects on eNOS expression predominantly by posttranscriptional mechanisms that are mediated by blocking geranylgeranylation of the small GTP-binding Ras-like protein Rho due to inhibition of the biosynthesis of geranylgeranylpyrophosphate (GGPP) (103). By inhibiting L-mevalonate synthesis, statins also reduce the synthesis of farnesylpyrophosphate (FPP) and

GGPP inducing important posttranslational modification of a variety of proteins, including eNOS and Rho. Inhibition of Rho results in a threefold increase in eNOS expression and nitrite generation, and the effects of statins on eNOS expression are reversed by GGPP, but not by FPP or LDL cholesterol (103). Thus, an important non-cholesterol-lowering effect of statins is the upregulation of eNOS expression via inhibition of Rho. A recent study sheds light on additional mechanisms involved in the statin-induced upregulation of eNOS activity by demonstrating that simvastatin rapidly activates the serine/threonine kinase AKT in endothelial cells, which in turn leads to phosphorylation of eNOS, resulting in an increase in its activity and enhanced NO production (104). This may serve as an additional beneficial mechanism in individuals with atherothrombotic disease. The potential clinical importance of these observations was underscored by the finding that prophylactic treatment of normocholesterolemic mice with statins increased cerebral blood flow, reduced cerebral infarct size, and improved neurological function via a NO-mediated mechanism (105). Other experimental studies in animal models (106, 107) and human cells (108) have confirmed the statins' lipid-independent ability to upregulate eNOS expression. Notably, the increase of eNOS protein in endothelial cells in response to statin therapy was established to be associated with enhanced release of NO (109). Moreover, statins prevent the downregulation of eNOS induced by TNF-α (110). A very recent study also demonstrated that statins preserve the structure of coronary adventitial vasa vasorum in experimental hypercholesterolemia independent of lipid lowering (111). These effects may play an important role in the setting of chronic statin therapy for the primary and secondary prevention of CHD. Furthermore, simvastatin preserves endothelial function in experimental porcine hypercholesterolemia in the absence of any lipid lowering effect (101). In accordance with these results, statins improved coronary endothelial function in cynomolgus monkeys, which were pretreated with an atherogenic diet for two years, independent of serum lipoprotein concentrations (112). These experimental data are also associated with beneficial effects of statins on endothelial function in patients with CHD (8, 113). Thus, the statins can now be considered as agents that both enhance the bioactivity of NO and improve endothelial function in patients with coronary plaques.

Antioxidants and L-Arginine

Atherogenic lipids, particularly oxidized low-density lipoprotein (oxLDL), are responsible for a wide range of cellular dysfunctions within the arterial wall (114, 115). Oxidative modification of LDL plays a pivotal role in human early atherogenesis (116, 117). Concerning the regulation of vascular tone, oxLDL may disturb relaxation or act directly against vasodilating substances [reviewed in (115)]. Native and oxLDL can uncouple eNOS (118, 119). OxLDL may also induce a decreased uptake of L-arginine (119). Interestingly, physiological differences can affect arterial segments from different regions. For example, oxLDL impairs contraction and endothelium-dependent relaxation in carotid but not in basilar

arteries (120), suggesting that intracranial arteries may be relatively protected from atherosclerosis via endothelial resistance to oxidative injury. NO produced by inducible NOS in VSMCs inhibits oxidation of LDL (121). Thus, NO release via inducible NOS action induced by cytokines in VSMC may play a protective role during LDL oxidation.

Although antioxidants and L-arginine are not considered classical drugs but perhaps as dietary supplements, the L-arginine hypothesis and increased oxidative stress may actually fit together and are not mutually exclusive. L-arginine administration partially restores endothelium-dependent vasodilation in hypercholesterolemia (122, 123) and dilates coronary stenoses in patients with CHD (124). L-arginine supplementation for six months also improves coronary small-vessel function in association with a significant improvement in symptoms (125). Therefore, L-arginine administration could be a therapeutic option for patients with endothelial dysfunction and nonobstructive CHD (125, 126). The effects of intracoronary administration of L-NMMA and L-arginine were studied in patients with normal angiograms and in patients with CHD (127). L-arginine reversed the effect of L-NMMA and caused greater dilation of the diseased arteries, indicating that there is a deficiency of L-arginine. Moreover, there was a significant clinical improvement in more than 70% of patients, which was associated with a significant decrease in proinflammatory cytokines (128). Thus, L-arginine may have clinical benefits in patients with intractable angina. Furthermore, L-arginine improved myocardial perfusion during exercise in patients with angina and normal coronary arteries (129). The value of L-arginine as adjunctive therapy to improve endothelial function in patients with advanced CHD maintained on medical therapy has also been investigated (130). Oral L-arginine therapy did not improve NO bioavailability in patients with CHD and thus may not benefit this group of patients. However, it is possible that a more prolonged period of L-arginine treatment and fewer coronary lesions are necessary in order to see clinical improvement in CHD patients. The effect of exogenous L- and D-arginine on coronary stenosis vasomotion in relation to stenosis morphology in patients with CHD and stable angina was also examined (131). During intracoronary infusion of L-arginine, but not D-arginine, a larger proportion of complex stenoses than smooth stenoses dilated by 10% ($p < 0.01$). Irrespective of the type of morphology, there was a positive correlation ($p < 0.01$) between the severity of stenoses and the magnitude of vasodilatation to L-arginine. This finding is consistent with a deficiency of L-arginine at the site of coronary stenoses (131).

A corollary of the oxidation hypothesis of atherogenesis is that antioxidants may reduce the progression of the disease (114). Antioxidants present in LDL, including alpha-tocopherol, and antioxidants present in the extracellular fluid of the arterial wall, including ascorbic acid (vitamin C), inhibit LDL oxidation (132), and this action is extended to multiple oxLDL-mediated signaling pathways (133). Vitamin C may potentiate NO activity and normalize vascular function in patients with CHD and classical risk factors (132). Thus, NO may restore endothelial dysfunction and ameliorate vascular remodeling in several clinical correlates to experimental

models (1–5). The hypothesis that the plasma concentration of alpha-tocopherol is associated with the preservation of NO-mediated endothelium-dependent vasomotion was tested in humans (134). Patients who were not taking vitamin supplements were studied using coronary angiography. Coronary endothelium-dependent and -independent vasomotion was assessed by intracoronary infusions of acetylcholine and nitroglycerin. Plasma alpha-tocopherol was significantly correlated with the acetylcholine response but not the nitroglycerin response. Thus, alpha-tocopherol may preserve endothelial vasomotor function in patients with coronary atherosclerosis. However, the results of clinical intervention trials using antioxidants have been contradictory. The CHAOS trial carried on patients with CHD (135) and the SPACE trial designed for patients with severe end-stage renal diseases (136) showed clinical beneficial effects of antioxidants. However, two clinical trials (GISSI-Prevenzione and HOPE trials) (137, 138), using adequate doses of vitamin E, demonstrated no effect on a composite endpoint of nonfatal infarction, stroke, or death from cardiovascular causes. The experimental data on which these trials were based deal primarily with the evaluation of early atherosclerotic lesions (fatty streaks). This pathophysiological scenario does not necessarily provide a rational basis for predicting what antioxidant intervention will do in patients with advanced atherosclerotic lesions, particularly when the end-points used relate to unstable atherosclerotic plaques and fatal thrombosis. Moreover, the same antioxidants (and doses) used successfully in animals may not be effective in humans. Negative clinical trials with antioxidants, in patients with advanced CHD and lasting only a few years, should not be taken as refutation of the oxidation hypothesis of atherogenesis. Conversely, results from several observational and experimental studies consistently support an effect of vitamin E supplementation on reducing risk of cardiovascular events [reviewed in (139, 140)]. The evidence suggests that the major effect, if any, is found at supplemental intake levels at or greater than 100 IU/day. Moreover, oxLDL is already present in early atherosclerotic lesions in human fetuses and children (116, 117). Perhaps different types of human trials are needed, trials in which the development of newly formed lesions is measured, in order to test whether antioxidants can decrease the rate of initiation and progression of atherosclerosis as they do in experimental models (140). In this regard, the Physicians' Health Study-II (PHS II) is a randomized, double-blind, placebo-controlled trial (141), testing alternate day beta-carotene, alternate day vitamin E, daily vitamin C, and a daily multivitamin in the prevention of total and prostate cancer, cardiovascular diseases, and the age-related eye diseases. The PHS-II trial is the only primary prevention trial testing the balance of benefits and risks of vitamin E on cancer and cardiovascular diseases in apparently healthy men. However, by age 55 there are already advanced atherosclerotic lesions in apparently healthy men. Thus, in this study the impact of antioxidants on early lesion formation and major cardiovascular events could be missed and the benefits of the approach underestimated.

Whereas low concentrations of vitamin E may improve endothelial function, high concentrations may worsen endothelial function (140). If confirmed in

further long-term trials done in young adults [or at earlier age in order to adverse in utero programming events, reviewed in (142)], the net benefit of antioxidant supplementation among populations with existing CHD may be substantial, although the current clinical results are insufficient to warrant a change in broad policy recommendations.

Phosphodiesterase Inhibitors

Sildenafil is a selective inhibitor of phosphodiesterase type-5 that is orally effective in the treatment of erectile dysfunction. Its pharmacological actions are a consequence of prolonging the signaling actions of NO because this drug prevents cGMP hydrolysis by inhibition of a cGMP phosphodiesterase V subtype enriched in penile smooth muscle (1, 10, 143).

Diabetic men have a more than threefold increased prevalence of erectile dysfunction compared with nondiabetic men (1). Erectile function is primarily a vascular phenomenon, triggered by neurologic controls and facilitated by appropriate hormonal and psychological components (1). Recent advances in the understanding of the physiology of penile vasculature and its role in male sexual performance have influenced the clinical approach to erectile dysfunction (1). The pathophysiological alterations leading to impotence in diabetic men include vasculogenic, neurogenic, and hormonal etiologies. A clinical work-up, including a thorough history and physical examination, is an important aspect of erectile dysfunction management. Oral medications acting through phosphodiesterase type-5 inhibition in penile vasculature have revolutionized the treatment of impotence. The long-term safety and efficacy of vacuum-constriction devices, intraurethral suppositories, intracavernosal injections, and other therapies are still under investigation. However, in patients with stable angina coadministration of sildenafil with isosorbide mononitrate or nitroglycerin produces significantly greater reductions in blood pressure than nitrates alone (144). This issue was further investigated in a randomized, double-blind, placebo-controlled crossover trial conducted at a ambulatory-care referral center among 105 men with a mean age of 66 years who had erectile dysfunction and known or highly suspected CHD (145). All patients underwent two symptom-limited supine bicycle echocardiograms separated by an interval of one to three days after receiving a single dose of sildenafil (50 or 100 mg) or placebo one hour before each exercise test. In men with stable CHD, sildenafil had no effect on symptoms, exercise duration, or presence or extent of exercise-induced ischemia, as assessed by exercise echocardiography (145). However, in men with chronic HF, sildenafil citrate reduces vagal modulation and increases sympathetic modulation, probably through its reflex vasodilatory action (146). The autonomic system changes induced with sildenafil citrate could alter QT dynamics. Both changes could favor the onset of lethal ventricular arrhythmias (146). Therefore, before prescribing sildenafil for erectile dysfunction in patients with known cardiac disease or multiple cardiovascular risk factors, physicians should discuss the potential cardiac risk of sexual activity and perform a complete medical assessment.

To date, the actions of sildenafil in vascular disorders distinct from that of erectile dysfunction have yet to be studied adequately. For example, oral sildenafil is an effective and specific pulmonary vasodilator in patients with pulmonary arterial hypertension (146a).

On the other hand, an alternative, NO-based approach for erectile dysfunction therapy has recently been suggested by evidence that pathways inhibiting erection and favoring smooth muscle contraction are mediated by adrenergic nerves (147). S-nitrosated-α-adrenergic receptor antagonists have been developed that contain an S-nitrosothiol functionality linked to an α-adrenergic receptor antagonist (yohimbine and moxisylyte) by an inert, organic-ester tether. The rationale behind the development of this agent is that it prompts early (immediate) vasodilation, whereas the α-adrenergic blocker maintains the vasodilator effect. Pharmacological demonstration of these NO-donor properties in relaxing human penile smooth muscle, their α-adrenergic antagonism, and their ability to induce erection in laboratory animals (148) suggest that NO-releasing adrenergic receptor antagonists may be useful as bifunctional agents for local treatment of erectile dysfunction. However, experimental models to study the effect of agents on penile erection usually include in vitro models (poor models when transferred to in vivo conditions) and electrical stimulation of peripheral nerves in anesthetized animals combined with systemic or intracavernous injection of drugs. In contrast, conscious rabbits can be used as a simple and quantitative model for the assessment of compounds that show potential for the treatment of erectile dysfunction (149). Erection was assessed by measuring the length of uncovered penile mucosa before and after the intravenous administration of agents. Animals did not require anesthesia during the course of the study. The phosphodiesterase type-5 inhibitors vardenafil and sildenafil were given intravenously, and measurements were taken for 0–5 hours (149). The effects of phentolamine and milrinone were also evaluated. Vardenafil induced dose-dependent penile erections in conscious rabbits. The efficacy of vardenafil was potentiated, and the minimal effective dose was reduced significantly to 0.01 mg/kg by simultaneous administration of SNP. Administration of the NO-synthase inhibitor L-NAME abolished the effect. Sildenafil was also effective in this model. Phentolamine induced erections with a slower tmax compared with vardenafil and sildenafil. Intravenous administration of milrinone was less effective than vardenafil (149). Further studies are warranted in this model.

CONCLUSIONS

NO-releasing drugs can elicit beneficial actions relevant to cardiovascular disorders. Figure 3 shows pathways by which these drugs can interfere with NO release. Although more than 20 years have passed since the identification of NO as an endogenous substance produced by the cardiovascular system, attempts toward developing accepted therapeutic approaches for modulating endogenous NO activity have progressed slowly until recently. For example, recent data have contradicted the notion that NO acts solely as a negative inotrope and have shown positive inotropic activity in both isolated rodent and human ventricular myocytes

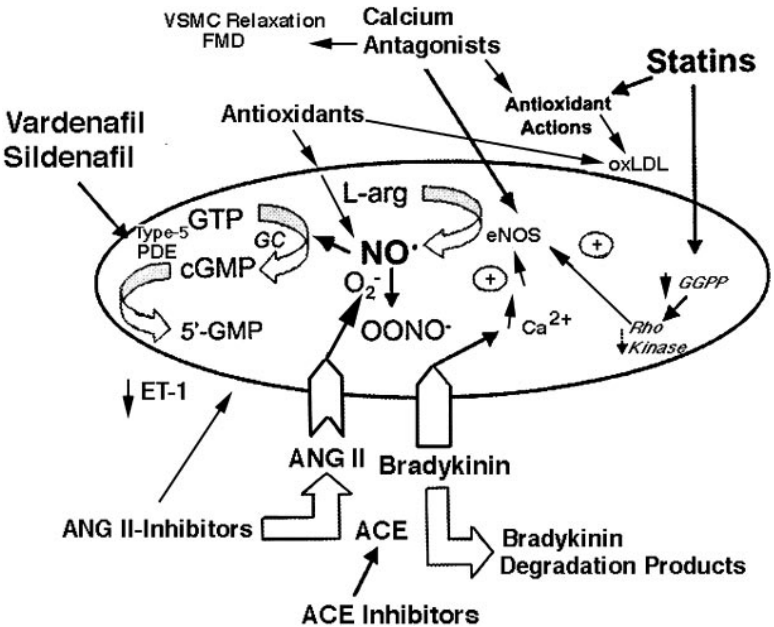

Figure 3 Mechanisms of cardiovascular agents that indirectly modulate endogenous NO activity.

in response to a range of NO donors [reviewed in (150)]. Recent advances in gene transfer technology and the cloning of the inducible NOS gene have led to the development of strategies for gene therapy to increase NO production for the treatment of disorders ranging from vascular restenosis to impaired wound healing. Different NO donors or NO-releasing drugs have different NO release kinetics and may generate a range of nitrogen monoxide species that may interact at a number of subcellular targets. In the case of cardiac in vitro preparation, the observed response to a NO donor represents the net effect of activation of different effector targets and may explain the contradictory reported effects of NO (150). The use of inhaled nitric oxide (INO) allows selective pulmonary vasodilatation and it is effective in the acute management of reversible pulmonary hypertension and is also useful in assessing the pulmonary vasodilator capacity in patients with chronic pulmonary hypertension [reviewed in (151)]. The clinical use of INO in cardiac failure, postoperative cardiac patients, patients with congestive cardiac failure, or congenital heart disease can be also hypothesized (151). However, to realize the complete therapeutic potential of NO requires specific targeting at the subcellular level. Understanding the complex pathophysiological role of NO and its transduction responses, developing new targeted therapies for delivery of NO, and choosing the optimal adjunctive therapies that potentiate the benefits of NO-releasing drugs are issues that require additional preclinical and clinical controlled studies.

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2,5-HEXANEDIONE-INDUCED TESTICULAR INJURY

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Key Words microtubule, testis, Sertoli cell, apoptosis, toxicant

■ **Abstract** Now in its third decade of mechanistic investigation, testicular injury caused by 2,5-hexanedione (2,5-HD) exposure is a well-studied model with a rich database. The development of this model reflects the larger changes that have moved biology from a branch of chemistry into the molecular age. Critically examined in this review is the proposed mechanism for 2,5-HD-induced testicular injury in which germ cell maturation is disrupted owing to alterations in Sertoli cell microtubule-mediated functions. The goal is to evaluate the technical and conceptual approaches used to assess 2,5-HD-induced testicular injury, to highlight unanswered questions, and to identify fruitful avenues of future research.

INTRODUCTION

This review is divided into sections that roughly parallel the temporal progression of the experiments and the changing nature of the field. The first section, Background, covers the time frame of approximately 1975–1985 and focuses on chemistry, clinical observations, and histopathology. In the second section, The Tubulin Hypothesis, extending from 1985 to approximately 1990, the experimental approach is dominated by biochemical techniques. The sections Microtubule-Dependent Transport is a Target and Apoptosis, Stem Cell Factor/c-Kit, and Irreversible Injury reflect the advances in cell biology techniques in the early 1990s and their use in defining mechanisms of toxicity. In a final part, Future Directions, unanswered research questions and problems are highlighted and new approaches and molecular techniques are proposed to further refine our mechanistic understanding of this model.

BACKGROUND

n-Hexane is a volatile organic solvent widely used in industry and commerce. Low level environmental exposure is ubiquitous because *n*-hexane is a

component of gasoline (1). *n*-Hexane is metabolically converted to the γ-diketone, 2,5-hexanedione (2,5-HD), via sequential cytochrome p450–dependent ω-1 hydroxylation and oxidation (2, 3) (Figure 1). 2,5-HD can combine with primary amines, such as protein lysyl ε-amines, to form substituted 2,5-dimethylpyrroles (4). Pyrroles form and accumulate on tissue proteins during in vivo exposure to 2,5-HD as a required step in the induction of both testicular and nervous system injuries (5–8). Subsequent oxidation and cross-linking of these tissue-bound heterocyclic aromatic compounds result in a complex array of products, including pyrrole dimers (9, 10).

Toxicologically significant human exposure to *n*-hexane has been reported mainly in occupational settings, although chronic intentional inhalation of consumer products containing *n*-hexane ("glue sniffing" or "huffing") has produced disease (11). The clinical manifestations of "hexacarbon" or γ-diketone-induced toxicity are those of a peripheral polyneuropathy [(12); for review see (13)]. A well-studied example of γ-diketone-induced toxicity occurred among workers in an Ohio fabric printing plant in 1973. Shortly after substituting methyl *n*-butyl ketone as a cleaning agent, a sudden outbreak of polyneuropathy developed among the fabric printing workers who were repeatedly exposed to the solvent via inhalation and skin contact. The distribution of nerve involvement, severity of the deficits, and temporal course correlated with the amount of exposure (14). Sensory symptoms and signs predominated in mild cases; however, both motor and sensory deficits occurred in severe cases. Although the distribution was characteristically distal, proximal involvement occurred in severe cases. Onset was gradual without prominent systemic symptoms and neurological involvement was symmetrical. The symptoms progressed for several months after cessation of exposure and recovery was slow.

These clinical characteristics of 2,5-HD-induced neurotoxicity are most compatible with an axonopathy affecting primarily long fibers. The histopathological manifestations of *n*-hexane polyneuropathy include distal axonal swellings filled with neurofilaments proximal to the nodes of Ranvier. This injury is sometimes followed by Wallerian-like degeneration of the axon distal to the swelling, resulting in muscle atrophy. 2,5-HD-induced neurofilament cross-linking has been proposed as the molecular mechanism of this neurotoxicity (15) based on the chemical reactivity of 2,5-HD and histopathological manifestations, although alternative models have been suggested (16–21).

Initial animal studies demonstrated a close correlation between the blood levels of 2,5-HD, the ultimate toxicant, and neurological symptoms (22). In addition, these initial animal studies identified the testis as a target organ of exposure to *n*-hexane and its metabolites. 2,5-HD targets Sertoli cells, resulting in germ cell apoptosis and testicular atrophy (23, 24). The assignment of the Sertoli cell as the target cell for testicular injury is based on the early histopathological alterations in this cell following exposure (described below). To appreciate the testicular injury, one must understand Sertoli cell biology and the dependence of germ cells on Sertoli cells for structural and trophic support.

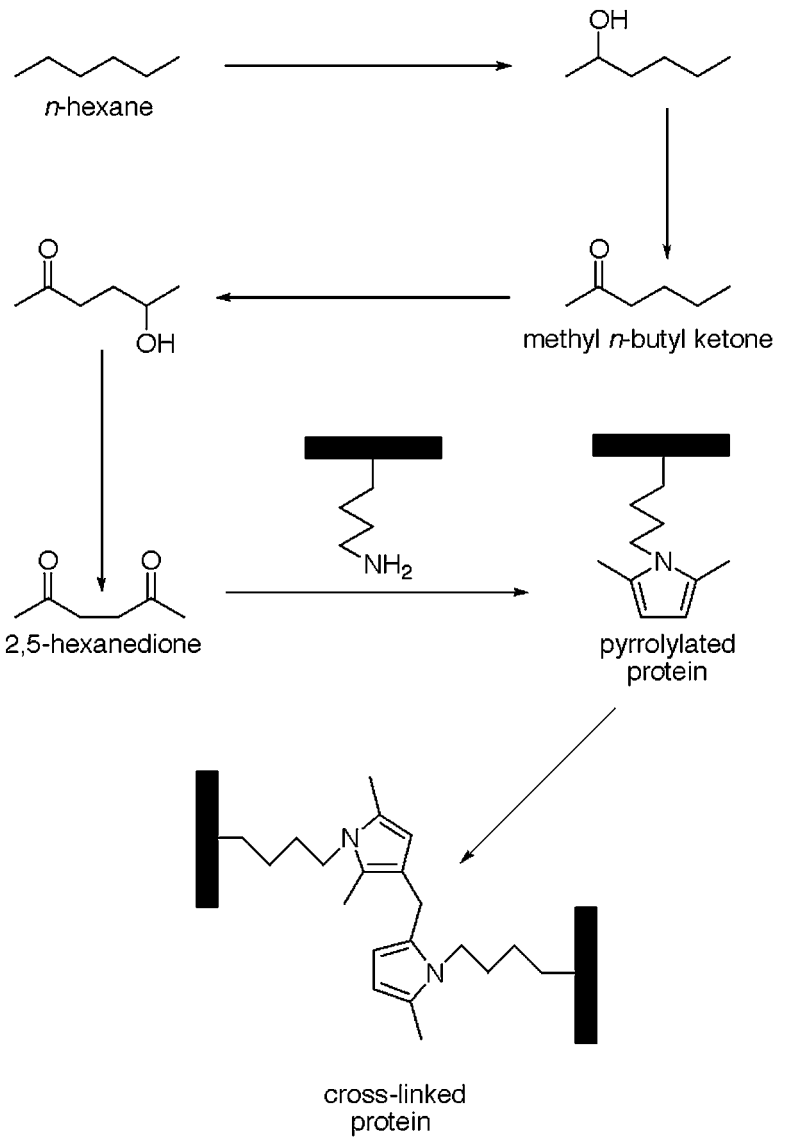

Figure 1 The common solvents *n*-hexane and methyl *n*-butyl methane are converted by ω-1 hydroxylation and oxidation to the ultimate toxicant, 2,5-hexanedione (2,5-HD). 2,5-HD reacts with lysyl ε-amines of proteins (*black rectangle*) to form pyrrolylated proteins, which undergo intra- and intermolecular cross-linking reactions, including dimer formation.

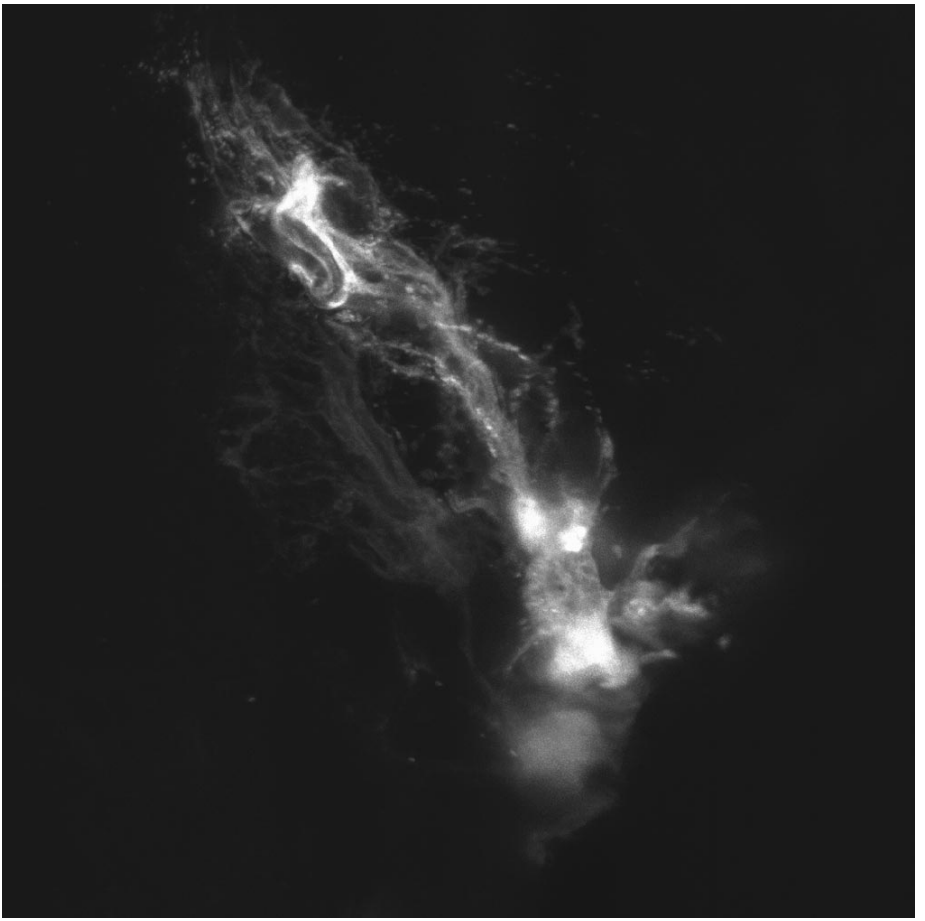

Figure 2 The Sertoli cell extends from the base of the seminiferous tubule (*bottom right*) to the lumen (*upper left*) and has numerous cytoplasmic projections that surround and support germ cells. In this maximum intensity projection of 13 series confocal images, a single Sertoli cell in an intact seminiferous tubule is visible because of green fluorescent protein expression following adenovirus infection. The Sertoli cell nucleus is visible in the basal cytoplasm surrounded by bright fluorescence. The overall length of this Sertoli is approximately 100 μm.

The Sertoli cell, a highly elongated cell type, is found in seminiferous tubules with its base adhering to the basal lamina and its apex extending to the lumen (Figure 2; see also Figure 4). These cells form numerous lateral cytoplasmic processes that surround developing germ cells. Sertoli cells contain a well-developed cytoskeletal network composed of actin filaments, intermediate filaments, and microtubules. Adjacent Sertoli cells are bound together by tight junctions, forming the blood-testis barrier. This barrier, which separates the seminiferous tubule into

a basal and an adluminal compartment, is important in protecting the advanced germ cells from blood-born products, including immune cells. The blood-testis barrier allows Sertoli cells to create the necessary microenvironment for germ cell development by secreting a seminiferous tubule fluid that contains nutrients and proteins, including transport and binding proteins, proteases, antiproteases, and growth factors that regulate proliferation and differentiation. Sertoli cells have many fundamental roles in spermatogenesis; thus, their dysfunction would result in disruption of the normal process of spermatogenesis.

The most commonly used animal model for studying 2,5-HD-induced testicular injury has involved exposure of rats to 2,5-HD as a 1% drinking water solution for three to five weeks (24, 25). Beginning two weeks after exposure, assembly of purified testis tubulin was altered followed by a decrease in seminiferous tubule fluid formation by three weeks (26, 27). Large, basally located Sertoli cell vacuoles in stages I, XII, XIII, and XIV were the first histopathological signs of cellular injury at all doses, followed by sloughing and loss of germ cells by four to five weeks (24, 28). The cycle of the seminiferous epithelium was altered with a decreased prevalence of certain stages and a concomitant increase in other stages (24, 29). The seminiferous tubules were devoid of differentiating germ cells by 8–12 weeks after a 3–5 week 2,5-HD exposure but contained proliferating spermatogonia (28). This "irreversible" atrophic state continued for greater than 70 weeks after exposure in the rat (30).

In the rat, testicular atrophy associated with 2,5-HD exposure can occur at cumulative exposure levels below those that produce clinical neurotoxicity. High-level exposure for relatively brief periods produced testicular injury without clinical evidence of distal polyneuropathy (28), whereas chronic low-level exposure produced clinical evidence of distal polyneuropathy without testicular injury (31). In fact, the testicular injury was dose-rate sensitive, whereas the extent of nervous system toxicity was related to the total dose over a range of dose-rates (22, 32). These tissue-selective pharmacokinetic effects may, in part, explain the predominance of neurotoxicity in human exposures to 2,5-HD precursors. In addition, the clinical manifestations of neurotoxicity are obvious, whereas those of testicular injury are subtle.

THE TUBULIN HYPOTHESIS

Because of the selective injury to the axon by γ-diketones and the known importance of microtubules to axonal structure and function, this cytoskeletal element was considered as a molecular target for γ-diketone neurotoxicity. Because the Sertoli cell has axon-like characteristics (33), tubulin was considered an attractive molecular target in testicular injury as well.

The core proteins of the microtubule are α- and β-tubulin, which combine to form a 100-kDa heterodimer. Tubulin heterodimers join end-to-end to form protofilament chains that, through lateral interactions, wrap into a tube-like structure—the microtubule. Because tubulin dimers are oriented within protofilaments,

microtubules are polar structures with fast-growing (+) ends and slow-growing (−) ends. The assembly of microtubules from soluble tubulin heterodimers consists of three main phases: (*a*) a slow nucleation phase in which tubulin subunits are organized into seeds for microtubule growth, (*b*) a fast elongation phase during which tubulin subunits are readily added to the growing protofilament chains, and (*c*) a steady state phase during which the addition and removal of tubulin subunits is in equilibrium and the aggregate microtubule length remains constant [reviewed in (34–37)] (Figure 3). Many factors are known to influence the kinetics of assembly and the maintenance of microtubules, including temperature, solvent conditions, GTP concentration, and microtubule-associated proteins (MAPs). MAPs serve many functions, including nucleation of assembly and stabilization of the

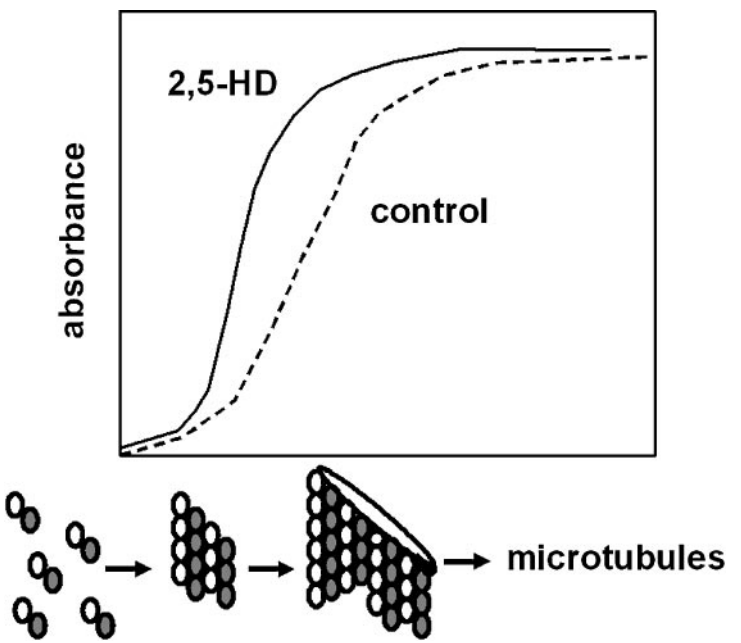

Figure 3 A typical microtubule assembly reaction is initiated by warming a solution of ice-cold tubulin dimers to 37°C in the presence of GTP. Tubulin dimers (*adjacent white and gray circles*) slowly form nucleating seeds (*heptameric tubulin aggregate*), which catalyze a rapid phase of microtubule elongation (*growing microtubule*) enroute to a steady state condition of microtubule formation and destruction. The assembly reaction is monitored by measuring the change in absorbance at 350 nm. In vitro incubation of microtubules with 2,5-HD or in vivo exposure of animals to 2,5-HD followed by tubulin purification yields pyrrolylated tubulin with altered assembly behavior. 2,5-HD-modified tubulin quickly forms numerous seeds, resulting in more rapid assembly into greater numbers of shorter microtubules compared to the control.

microtubule. Presumably, these modifying factors provide a means by which a cell can coordinate microtubule-mediated events in response to different environmental cues and stimuli.

2,5-HD did not alter the proportion of tubulin that polymerized into microtubules when chronically administered to rats (38) or when added to in vitro assembly assays without preincubation (39). However, 2,5-HD exposure did have a dramatic effect on the kinetics of microtubule assembly (25). A detailed evaluation of the 2,5-HD-induced alterations in microtubule assembly behavior followed this initial observation. Brain and testis tubulin isolated from 2,5-HD-exposed rats had a shortened nucleation phase, a more rapid rate of elongation, and contained high-molecular-weight bands on denaturing polyacrylamide gel electrophoresis (SDS-PAGE) that consisted of cross-linked tubulin (25). Tubulin isolated from exposed rats displayed a high level of pyrrole adducts as compared to controls (26). This altered tubulin had remarkable "pro-assembly" characteristics, readily forming microtubules in the presence of calcium and at low temperatures. When rats were exposed to the same total dose of 2,5-HD in the drinking water at dose-rates varying from two to six mmol/kg/day, the increased dose-rate was associated with progressively more severe histopathological alterations in the testis. In concert with these progressive histopathological alterations, the kinetics of microtubules assembled from testicular tubulin purified from the exposed animals were altered. These data positively correlated the extent of histopathological injury with the extent of microtubule assembly abnormality.

These initial observations of 2,5-HD-induced alterations in microtubule assembly led to the articulation of a tubulin-based hypothesis for 2,5-HD-induced testicular injury as follows: "[1] intoxication with 2,5-HD alters microtubule assembly kinetics, [2] altered assembly produces changes in the number and length of Sertoli cell microtubules which compromises Sertoli cell function, and [3] malfunctioning, nonsupportive Sertoli cells disrupt germ cell maturation resulting in testicular atrophy" (25).

The results using tubulin purified from treated animals were confirmed and extended with microtubules treated in vitro with 2,5-HD (40). In vitro incubation with high concentrations of 2,5-HD generated a markedly altered tubulin that could assemble in the absence of added GTP, could readily nucleate the assembly of control tubulin, and was resistant to cold-induced disassembly. The induction of these 2,5-HD-induced assembly alterations required that the incubation take place with assembled microtubules. Negative-stain electron microscopy showed that 2,5-HD incubation followed by assembly led to shorter microtubules than control assemblies, a result explained by the treatment-related induction of numerous nucleating seeds.

Sioussat & Boekelheide (41) explored the biochemical nature of the 2,5-HD effect on tubulin in greater detail. Isolated bovine brain tubulin was treated with 100 mM 2,5-HD for 16 h at 37°C and subjected to three cycles of microtubule assembly with the assembly temperature of each cycle progressively lower, making microtubule assembly progressively restrictive. This procedure effectively concentrated a component of 2,5-HD-treated tubulin with strong nucleating features.

This highly nucleating tubulin preparation had (*a*) a lowered rate of tubulin dissociation from the microtubule polymer, (*b*) a 19-fold decrease in the critical concentration for assembly (the concentration of tubulin required for nucleation to occur), and (*c*) the ability to copolymerize with and seed untreated tubulin assembly at concentrations below that typical for spontaneous nucleation in vitro (39).

The reaction between 2,5-HD and assembled tubulin produced numerous derivatized and cross-linked products visualized as monomers, cross-linked dimers, and higher multimers on SDS-PAGE. The progressively stringent cycling that concentrated the nucleating elements did not change the pattern of bands by SDS-PAGE, indicating that dimers and high multimers were not the nucleating factor. By using limited proteolysis and immunoblotting, the native conformation of the tubulin subunits was probed. Concentration of the 2,5-HD-induced nucleating element by stringent cycling resulted in an altered α-tubulin with a more open structure susceptible to selective tryptic and chymotryptic digestion. With these findings, a biochemical explanation for the assembly alterations was formed; namely, that 2,5-HD treatment of assembled microtubules fixes the tubulin heterodimer into a pro-assembly conformation through a specific intramolecular modification of the α-subunit (41).

The ability of 2,5-HD-modified tubulin generated in vitro to alter microtubule function in an in vivo model system was verified using sea urchin zygotes. Microinjection of 2,5-HD-treated tubulin into normal sea urchin zygotes before the first mitotic cycle caused obvious abnormalities, including small spindles, abnormal chromosomal movement at anaphase, and poor cytokinesis. Depending on the protocol used, mitosis was either grossly disrupted or simply slowed (42).

Having identified microtubule function as an in vivo molecular alteration induced by 2,5-HD, the next phase of the investigation examined unique characteristics of microtubule-dependent activity in Sertoli cells, the testicular target for toxic injury, which were susceptible to disruption.

MICROTUBULE-DEPENDENT TRANSPORT IS A TARGET

In order to maintain the proper microenvironment within the seminiferous tubule, Sertoli cells actively secrete a seminiferous tubule fluid. The elongated structure of Sertoli cells and their ability to target products to different cohorts of germ cells imply a delivery system that is both dynamic and provides exquisite specificity.

Microtubule networks promote targeted secretion in a number of polarized cell types, including MDCK cells (43, 44) and Caco-2 cells (43). Sertoli cell microtubules are oriented parallel to the long axis of the cell and, like those of many polarized cells, arise from nucleation centers located in the apical aspect of the cell (45, 46), not from the basally located centrosome (47).

MAPs include families of motor proteins—kinesins and cytoplasmic dyneins—that use microtubules as a scaffold for transport [reviewed in (48)]. Kinesins and cytoplasmic dyneins convert energy derived from ATP cleavage into movement along the length of the microtubule, mediating the transport of molecules and

vesicles bound to them. Originally, kinesins and cytoplasmic dyneins were believed responsible for transport of cargo to opposite poles of the microtubule, with kinesins directing transport to the (+) end and cytoplasmic dyneins directing transport toward the (−) end; more recent information has made the picture more complex (48).

As predicted by its "minus-end-up" microtubule orientation and active secretion, Sertoli cells express the minus-end microtubule-dependent motor cytoplasmic dynein at a high level. Cytoplasmic dynein consists of a large complex of at least 10 proteins (33, 49). The dynein heavy chain isolated from testis shares features with other cytoplasmic dyneins: It binds microtubules tightly in the absence of ATP, releases from microtubules in the presence of ATP, exhibits microtubule-dependent ATPase activity, is sensitive to inhibitors in a similar manner to that of other dynein species, and is sensitive to vanadate-mediated photocleavage (33). Localization of testicular cytoplasmic dynein to Sertoli cells has been demonstrated by immunofluorescence. Throughout development, and in all stages of spermatogenesis, dynein is observed in a diffuse, granular pattern throughout the Sertoli cell cytoplasm, consistent with its proposed role as a cytoplasmic transport molecule (50, 51). During stages IX–XIV, a more intense Sertoli cell pattern is observed, as well as an intense pattern associated with ectoplasmic specialization regions of step 9 and 10 spermatids (50). Sertoli cell ectoplasmic specializations have been proposed to have a number of roles. They bind microtubule networks and are thought to be involved in spermatid head shaping (50, 52) and in positioning and translocating spermatids in the seminiferous epithelium (46). Ectoplasmic specializations are also thought to be the site at which some Sertoli cell secretory products accumulate (50). This notion was supported by the observation that microtubule-associated cisternae of endoplasmic reticulum coassociate with the Sertoli cell membrane at this point via a thick, hexagonal array of actin filaments (52). Recent work has verified the association of microtubule motors with ectoplasmic specializations in support of the hypothesis that these motors are responsible for the elongate spermatid movements that occur during germ cell maturation (46, 53–55).

Kinesin participates in vesicle transport in a number of cell types, including fast axonal transport in neurons (56). In Sertoli cells, kinesin has been observed to localize to the *trans* Golgi network, a location suggesting involvement in membrane trafficking within the cell (57). In addition, kinesin is localized to ectoplasmic specializations where it may be involved in the movement and positioning of elongate spermatids within the seminiferous epithelium (55).

Alterations in the distribution of microtubule-associated motor proteins occurred progressively with 2,5-HD exposure. The pattern of Sertoli cell cytoplasmic dynein staining changed from an intense signal associated with ectoplasmic specializations of elongate spermatids to a diffuse cytoplasmic signal (58). 2,5-HD treatment had similar effects on Sertoli cell kinesin distribution, which was tightly associated with the highly organized Golgi network and became more diffuse with treatment-induced Golgi disruption (58). Microtubules treated in vitro with 2,5-HD exhibited functional differences in their ability to support microtubule-dependent transport. Microtubules treated with either 2,5-HD or glutaraldehyde

(a generalized protein cross-linker) exhibited diminished rates of kinesin-based transport in vitro compared with untreated microtubules. In comparison, tubulin treated with acetyl 2,5-HD, a noncross-linking pyrrole-forming agent, supported rates of kinesin-based transport similar to those of untreated microtubules (59). This suggests that 2,5-HD treatment itself is capable of altering microtubule-based transport and that it is the cross-linking activity of the 2,5-HD that promotes those changes (59).

Synthesizing the observed effects on microtubule assembly in vivo and microtubule-dependent transport in vitro led to the hypothesis that disruption of microtubule-dependent vesicle transport from the endoplasmic reticulum to the plasma membrane underlies the seminiferous tubule fluid alterations observed following 2,5-HD exposure (60) (Figure 4). Microtubule-dependent vesicle transport involves the movement of secretory vesicles from the endoplasmic reticulum to the plasma membrane, and these vesicles have been demonstrated to associate with microtubules in polarized cells (61, 62). However, direct assessment of the effect of microtubule disruption on vesicle movement and seminiferous tubule fluid formation is difficult. Measurement of seminiferous tubule fluid production in isolated tubules, however, has revealed that the classic microtubule disrupter, colchicine, as well as the inhibitor of intracellular membrane transport, brefeldin A, cause reduced rates of fluid production (27). Similarly, seminiferous tubules isolated from rats exposed to 2,5-HD for three or four weeks exhibited reduced rates of seminiferous tubule fluid formation compared to those isolated from untreated rats (27). Importantly, the deficit in seminiferous tubule fluid formation preceded the histopathological alterations in germ cells, indicating an etiologic relationship. The mechanism by which this deficit is translated into germ cell loss is considered in the next section.

APOPTOSIS, STEM CELL FACTOR/C-KIT, AND IRREVERSIBLE INJURY

Although the Sertoli cell is the target of 2,5-HD injury, the end result is a depletion of germ cells. This raises several questions: How does Sertoli cell dysfunction translate into germ cell loss? Is the atrophy the result of the loss of a supportive factor normally produced by the Sertoli cell or is it the consequence of a death signal produced by the Sertoli cell? Is the depletion due to a decrease in stem cell commitment to the differentiating germ cell pool or increased germ cell death? In this section, we consider germ cell apoptosis, growth factors [specifically stem cell factor (SCF)], and the "irreversible" nature of the germ cell loss after 2,5-HD exposure.

Apoptosis

Apoptosis, a controlled and highly ordered cell death, occurs during spermatogenesis to regulate germ cell production (63). In the rat, an important control point

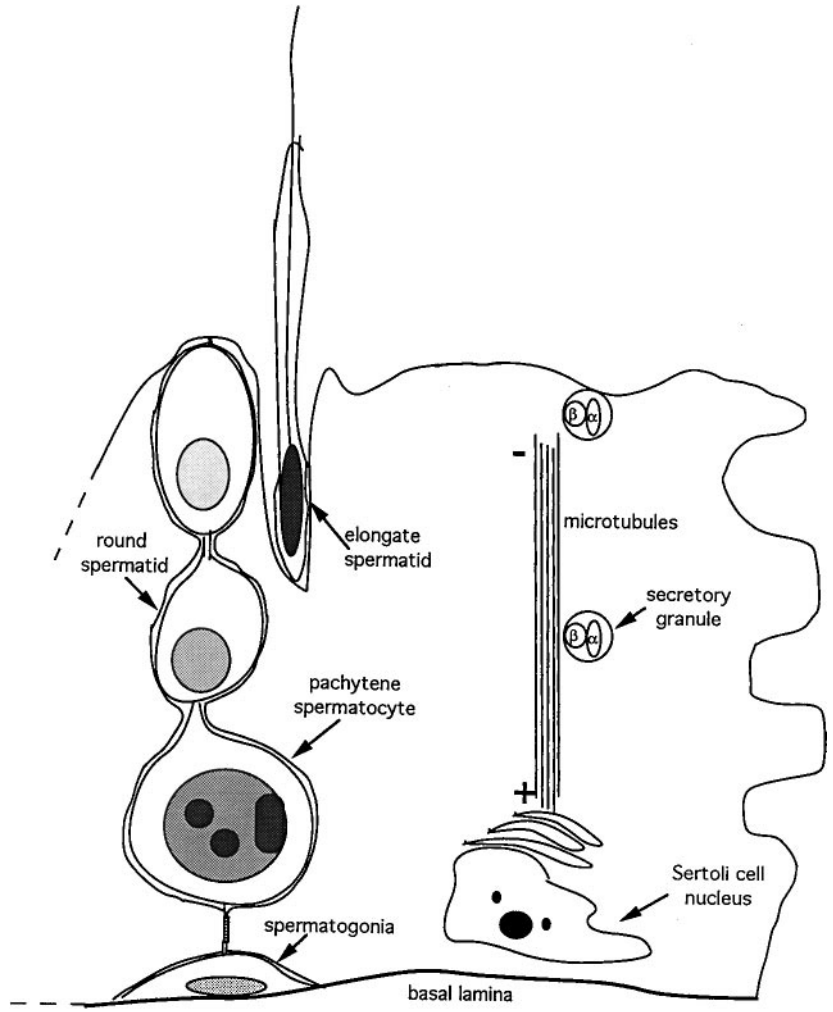

Figure 4 The Sertoli cell provides trophic factors to various germ cells (spermatogonia, pachytene spermatocyte, round spermatid, and elongate spermatid) by secreting a seminiferous tubule fluid. Microtubule-dependent transport facilitates this supportive role, using microtubule motors to move vesicles along the abundant, radially oriented, Sertoli cell cytoplasmic microtubules. In this model, a secretory granule is moving toward the microtubule (−) end, translocating from the perinuclear Golgi apparatus to the seminiferous tubule lumen. Cytoplasmic dynein, an abundant Sertoli cell protein (33), is the presumptive motor to catalyze this basal-to-lumenal microtubule-dependent transport.

involves type A_{2-4} spermatogonia; a large portion of these germ cells normally undergo apoptosis (64, 65). Germ cell apoptosis also can be upregulated massively in response to hormonal or cytokine deprivation or in response to injuries, such as heat, radiation, or toxicant exposure (66–69). Initiators of apoptosis include intracellular "stress sensors" or external signals, which trigger apoptosis via ligand binding to cell surface receptors. The Fas system is a paracrine pro-apoptotic signaling pathway; in the testis, Fas ligand is expressed by Sertoli cells and induces apoptosis in germ cells expressing Fas receptor (70–73). p53 is a transcription factor with an important pro-apoptotic role in the testis that modulates the expression of bcl-2 family members, the Fas receptor, and other elements of the apoptotic machinery (74, 75).

The apoptotic signal, whether intracellular or extracellular in origin, results in activation of a caspase cascade that disassembles the cell (76–78). This controlled destruction includes the cleavage of cytoskeletal proteins, changes in the lipid membrane, and DNA fragmentation evident as a DNA ladder by gel electrophoresis. Blanchard et al. (79) established that the mechanism of germ cell death in response to 2,5-HD exposure is indeed apoptosis. Using gel electrophoresis to visualize DNA fragmentation, ladders were most prominent five weeks into the toxicant exposure, and in situ DNA end-labeling showed an increase in germ cell apoptosis as early as two weeks into treatment.

How the apoptotic system is activated depends on the mechanism of injury and how the cells sense the injury. Because the Sertoli cell is the target of 2,5-HD-induced injury, germ cell death is secondary to Sertoli cell dysfunction and could be the result of a paracrine death signal initiated by the Sertoli cell or the lack of a survival or proliferation factor produced by the Sertoli cell. The possibility that the Sertoli cell actively signals germ cell death is supported by the concomitant increases in Fas ligand and Fas mRNA expression with the onset of germ cell apoptosis (71, 80). Mutant mice lacking functional Fas ligand have less germ cell apoptosis than wild-type controls following exposure to the model Sertoli cell toxicant, mono-(2-ethylhexyl)phthalate (81). An intellectually satisfying model is one in which Sertoli cells send a death signal to the population of dependent germ cells they can no longer support, thereby explaining the increased Fas system mRNA.

Stem Cell Factor/c-Kit

Whatever the signals are that trigger germ cell apoptosis, the initiating event could be a deficiency of a survival factor due to failure of the normal Sertoli cell microtubule-dependent formation of seminiferous tubule fluid. One important survival factor made by Sertoli cells and required by germ cells is stem cell factor (SCF) (82).

SCF is a ligand that is expressed on Sertoli cells. A proteolytic cleavage site within exon 6 results in two isoforms of SCF, a soluble isoform (sSCF) and a membrane-bound isoform (mSCF). c-Kit, the SCF receptor, is a 145-kD transmembrane tyrosine kinase expressed on germ cells and Leydig cells, the

testosterone-producing cells in the testicular interstitium. Structurally, c-kit belongs to the platelet-derived growth factor receptor superfamily (83, 84).

Two well-studied mutants of SCF and c-kit are Steel factor (Sl) and dominant white spotting (W) mice, respectively. Sl and W mice are deficient in melanogenesis, erythropoiesis, and gametogenesis (85). During embryogenesis, SCF and c-kit are essential for maintenance and proliferation of primordial germ cells (86, 87). Fertility is restored upon transplantation of W mice with Sl or wild-type germ cells, but not by transplantation of W germ cells into Sl mice (88). In another genetic complementation experiment, injection of mSCF-expressing replication-deficient adenovirus into the seminiferous tubule lumens of Sl mice resulted in Sertoli cell infection and restoration of spermatogenesis (89). Immunolocalization with an anti-c-kit antibody showed expression on type A, In, and B spermatogonia along with Leydig cells and preleptotene spermatocytes. Use of an anti-c-kit antibody to block SCF binding in vivo led to a loss of type $A_{2–4}$ spermatogonia (90). These data implicate binding of Sertoli cell-derived SCF to germ cell c-kit as an important regulatory mechanism controlling survival of spermatogonia.

The seminiferous tubule atrophy induced by 2,5-HD treatment persists long after the exposure has ended (30). Spermatogonia were present in reduced (91), albeit relatively constant, numbers following toxicant-induced injury (28). Interestingly, the remaining spermatogonia were actively proliferative but failed to repopulate the atrophic testis (92). Spermatogonial modeling in 2,5-HD-treated rats with irreversible injury has shown a large increase in apoptosis of type A_3 and A_4 spermatogonia (92). As discussed above, this block in spermatogenesis at type $A_{3–4}$ spermatogonia correlates with the germ cell developmental stage requiring SCF/c-kit activity for survival.

Because 2,5-HD is a Sertoli cell toxicant and SCF is expressed by Sertoli cells, alterations in its function after toxicant exposure could contribute to the persistence of testicular atrophy. Interestingly, SCF was present in testis after exposure; however, the ratio of sSCF to mSCF was altered (91). Normally in adult rats, twofold more mSCF is expressed than sSCF. Exposure to 2,5-HD led to a preferential expression of sSCF, which correlated temporally with the onset of atrophy (91). Reversal of the atrophic state with GnRH agonist therapy (see below) resulted in a significant increase in the proportion of mSCF that was expressed, returning the expression pattern toward normal (93). In a pharmacologic test, exogenous SCF was administered to the atrophic testes of 2,5-HD-treated rats. This therapy was associated with an increase in the percentage of seminiferous tubules with large clones of proliferating germ cells (91), suggesting that manipulations of the in vivo level of SCF could affect proliferation and survival of type $A_{2–4}$ spermatogonia in 2,5-HD-induced irreversible injury.

The spontaneous mouse mutant called Steel-Dickie (Sl^d), which expresses only the soluble form of SCF, provides a molecular model of deficient mSCF expression. In the Sl^d mutant, a 4-kb intragenic deletion of the SCF sequence leads to expression of only sSCF, resulting in anemia, white coat color, and infertility (94, 95). This mutant has normal migration of primordial germ cells to the genital ridge but fails to maintain normal spermatogenesis. In vitro, germ cells exhibit a

diminished capacity to bind Sl^d Sertoli cells, a defect corrected by expression of recombinant mSCF by Sertoli cells (96). Notably, the Sl^d mouse mutant and the 2,5-HD-injured atrophic rat testis share in common a deficiency of mSCF expression and a failure of the seminiferous epithelium to support germ cell development beyond spermatogonia.

The apoptotic machinery involved in cell death following SCF deprivation has not been well characterized. However, germ cell apoptosis in c-kit-deficient mice has been shown to be p53 dependent—that is, germ cell apoptosis in W mice is reduced if functional p53 is missing (97). Additionally, decreases in bax and other pro-apoptotic bcl-2 family members and increases in anti-apoptotic bcl-2 family members have been demonstrated in response to SCF (98).

Irreversible Injury

The reason for the persistence of 2,5-HD-induced testicular atrophy is unknown. Both the accumulation of testicular pyrroles and the alteration in testicular microtubule assembly that occur during treatment with 2,5-HD return to baseline levels soon after exposure ends (26).

Treatment with a gonadotropin releasing hormone (GnRH) agonist can successfully reverse the "irreversible" 2,5-HD-induced testicular atrophy. In rats treated with 2,5-HD for 23 days and given depot GnRH agonist therapy for 10 weeks immediately after toxicant exposure, greater than 90% of seminiferous tubule cross-sections showed signs of germ cell repopulation, with approximately 80% of the seminiferous tubules containing mature spermatids. In contrast, toxicant-treated controls repopulated only 1% of their seminiferous tubules (93). A similar reversal of testicular atrophy has been observed following treatment of testis-irradiated LBNF1 rats with GnRH agonists, GnRH antagonists, or testosterone (99, 100). Testicular atrophy induced by the chemotherapeutic agent procarbazine is also reversed by GnRH agonist therapy (101).

The ability of GnRH agonist therapy to stimulate spermatogenesis in atrophic testes is apparently mediated by a suppression of intratesticular testosterone levels (102). It is widely accepted that spermatogenesis is dependent upon gonadotropin support, mediated by complex feedback through the hypothalamic-pituitary-gonadal axis, and that withdrawal of testosterone and FSH results in a stage-specific loss of germ cells. However, the ability of testosterone-suppressing therapies to stimulate spermatogenesis in the atrophic testis suggests that testosterone may be inhibitory to the differentiation of type A spermatogonial cells in cases of testicular atrophy, perhaps by enhancing apoptosis among spermatogonial cells. Although the evidence supporting the involvement of intratesticular testosterone suppression in the reversal of testicular atrophy is quite compelling (103), ablation of Leydig cells in atrophic testes from 2,5-HD-treated rats failed to stimulate a recovery of spermatogenesis. Acute exposure to ethane dimethane sulphonate (EDS) suppressed intratesticular testosterone levels, yet regardless of whether EDS was administered alone or in combination with GnRH agonist therapy, failed to reverse

2,5-HD-induced testicular atrophy (104). These results suggest that Leydig cell factors, in addition to a lowered intratesticular testosterone level, may be important to the reversal of 2,5-HD-induced testicular atrophy.

FUTURE DIRECTIONS

Considerable effort has been applied to investigating 2,5-HD-induced testicular injury, both to enhance our understanding of pathways susceptible to disruption and to identify those complex mechanisms that govern spermatogenesis. As described in this review, the proposed pathogenic sequence for 2,5-HD-induced testicular injury is as follows: 2,5-HD-induced cross-linking of tubulin leads to altered microtubule assembly, which results in altered microtubule-dependent transport; in Sertoli cells, this altered microtubule-dependent transport is manifested as decreased seminiferous tubule fluid formation and a failure to provide adequate support to germ cells causing them to undergo apoptosis. In developing this mechanistic hypothesis, many unanswered issues have been raised. A brief listing of some of these issues follows in the hope that their articulation will pique the interest that they deserve:

- Additional structural studies could further elucidate the biochemical basis of the 2,5-HD-induced alteration in microtubule assembly, but progress in this area is limited by the poorly understood chemistry of γ-diketone adducts and cross-links.
- The underlying basis for the selective nervous system and testicular injury resulting from 2,5-HD exposure is unknown, but this pattern of toxicity suggests either a similar molecular target or a shared architectural vulnerability in the two tissues.
- Although indirect evidence supports the assertion that 2,5-HD alters Sertoli cell microtubule-dependent transport and inhibits seminiferous tubule fluid formation, the molecular connections between these processes remain to be elucidated.
- Because deficiencies in the p53 and Fas system pathways protect against germ cell loss, effective pharmaceuticals resulting from a better molecular understanding of the apoptotic machinery could ameliorate the consequences of toxicant-induced testicular injury.

In a sense, the very general chemical reactivity of 2,5-HD is both its greatest strength and greatest weakness as an investigative tool. As a blunt instrument, 2,5-HD has been useful for identifying microtubule-dependent transport as a vulnerable, failure-prone pathway within the Sertoli cell, but the lack of specificity in its reactivity has made it difficult to progress from correlation to causation. As described below, future experiments using molecular techniques are being designed to specifically disrupt Sertoli cell microtubule-dependent processes and examine subsequent germ cell effects.

Historically, insight into the role of Sertoli cell microtubules in promoting spermatogenesis has been gleaned from analyzing the actions of known microtubule disrupters in the testis. Both colchicine and taxol lead to defects in spermiation and residual body elimination, activities attributed to Sertoli cells (105–107). Because microtubules are present in all cells, however, these studies cannot rule out potential actions on other cellular targets, particularly on the germ cells themselves.

A true test of this model requires selective access to Sertoli cell microtubule networks in vivo, independent of direct effects on germ cells. Such a mechanism precludes toxicant or pharmacologic manipulations of microtubules, which exert effects globally. Cell-type specificity can only be afforded by molecular interventions that are capable of being either delivered or expressed in subsets of cells.

To date, few cell-specific interventions have been devised. Progress has been made with tissue-specific transgenics, which allow for expression of proteins in subsets of cells (108); however, these constructs are difficult to make and validate. Moreover, effects of ectopic gene expression on spermatogenesis in adult animals may not be easily separable from developmental effects, unless expression is tightly controlled.

Recent success has been achieved with the use of adenoviral vectors in the testis (22, 109–111). Adenoviral vectors are capable of expressing high-levels of transgene and may be used both in atrophic testes and in testes with intact spermatogenesis (22, 109–111). They have been demonstrated to be effective therapeutic agents to correct defective Sertoli gene products, resulting in partial restoration of spermatogenesis (22). Their utility in targeting adult Sertoli cells in testes with intact spermatogenesis makes them particularly useful for studying Sertoli–germ cell interactions in adult animals.

To address the question of whether selective disruption of Sertoli cell microtubule networks could impair spermatogenesis, it would be necessary to deliver a gene product that was capable of promoting microtubule polymerization or stabilization to Sertoli cells in intact, adult testes. γ-Tubulin, a microtubule nucleating protein localized to the centrosome of dividing cells, has been observed to disrupt microtubule networks and leads to mitotic arrest when overexpressed in mitotically active cells (112–115). Similar to 2,5-HD or taxol treatment of tubulin, γ-tubulin promotes microtubule assembly by serving as a seed or template for microtubule elongation (116–118). Delivery of an adenoviral vector that overexpressed γ-tubulin would be expected to perturb the Sertoli cell microtubule network similar to treatment with 2,5-HD, without direct effects on germ cells, which do not express adenoviral transgenes (109). Indeed, overexpression of γ-tubulin was observed to alter the distribution of tubulin immunoreactivity in infected Sertoli cells (data not shown) and to inhibit spermatid release and residual body processing (Figure 5), processes presumed to be dependent upon normal microtubule function.

This model also has the potential to address more fundamental questions of Sertoli cell microtubule organization and Sertoli–germ cell interdependence. The mechanism of microtubule nucleation in any polarized epithelial cell is presently

unknown. The apical orientation of the microtubule organizing center of Sertoli cells has been well documented (47); however, no information is available about the underlying structures that determine the distribution of microtubules. That Sertoli cell microtubule networks are important for spermatogenesis is suggested by their highly dynamic nature throughout the seminiferous epithelial cycle and their close apposition to associated germ cells (107, 119). This implies that a precise mechanism of microtubule organization is in place to facilitate restructuring of the seminiferous epithelium by accommodating changes in germ cell position and shape.

Of perhaps greater importance for understanding the nature of the Sertoli–germ cell interaction is determining the degree to which germ cells depend upon Sertoli cell secretory products. Given the elongated structure of the Sertoli cell and the elaborate architecture of its cytoplasmic associations with germ cells, it is likely that microtubule disruption would impair timely delivery of secretory products to their appropriate germ cell destinations. Inhibition of Sertoli cell secretory pathways would provide insight about whether germ cells could undergo normal differentiation in the absence of a functional Sertoli cell secretory system, and would provide insight into the roles that Sertoli cells perform in maintaining spermatogenesis.

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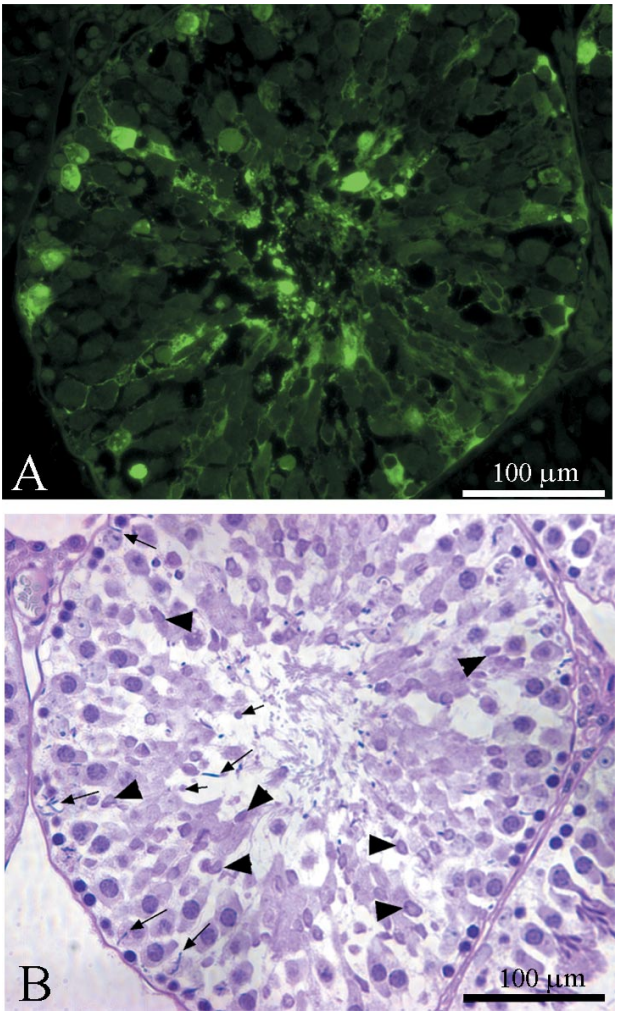

Figure 5 γ-tubulin overexpression in Sertoli cells in vivo leads to disruption of spermatogenesis. An adenovirus co-expressing γ-tubulin and green fluorescent protein was injected into the rete testis and back-perfused into seminiferous tubule lumens. After 72 h, the infected testis was embedded and serially sectioned. Many Sertoli cells in this seminiferous tubule cross section were infected and are expressing green fluorescent protein (*A*). Although structurally intact, the seminiferous epithelium is significantly disrupted with retention of advanced spermatids (*long arrows*) after the next generation of spermatids has begun elongating (*arrow heads*). Sertoli cells in infected tubules also failed to eliminate residual cytoplasm appropriately from elongate spermatids (*short arrows*). Staining (*B*) was performed with periodic acid Schiff's reagent and hematoxylin.

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HUMAN EXTRAHEPATIC CYTOCHROMES P450: Function in Xenobiotic Metabolism and Tissue-Selective Chemical Toxicity in the Respiratory and Gastrointestinal Tracts*

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■ **Abstract** Cytochrome P450 (CYP) enzymes in extrahepatic tissues often play a dominant role in target tissue metabolic activation of xenobiotic compounds. They may also determine drug efficacy and influence the tissue burden of foreign chemicals or bioavailability of therapeutic agents. This review focuses on xenobiotic-metabolizing CYPs of the human respiratory and gastrointestinal tracts, including the lung, trachea, nasal respiratory and olfactory mucosa, esophagus, stomach, small intestine, and colon. Many CYPs are expressed in one or more of these organs, including CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2A13, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP2F1, CYP2J2, CYP2S1, CYP3A4, CYP3A5, and CYP4B1. Of particular interest are the preferential expression of certain CYPs in the respiratory tract and the regional differences in CYP expression profile in different parts of the gastrointestinal tract. Current research activities on the characterization of CYP expression, function, and regulation in these tissues, as well as future research needs, are discussed.

OVERVIEW

Cytochrome P450 (CYP) enzymes, particularly those in the *CYP1*, *CYP2*, and *CYP3* gene families (1), catalyze the biotransformation of a wide variety of xenobiotic compounds. The organ that expresses the highest levels of CYP is the liver,

*Abbreviations used in text: CYP, cytochrome P450; HMPA, hexamethylphosphoramide; NDEA, N-nitrosodiethylamine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; CPR, NADPH-cytochrome P450 reductase; RT-PCR, reverse transcriptase-polymerase chain reaction; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; AFB1, aflatoxin B1; BaP; benzo(a)pyrene; G.I., gastrointestinal.

which plays the dominant role in the first-pass clearance of ingested xenobiotic compounds and controls the systemic level of drugs and other substrate chemicals. Extrahepatic tissues, especially those that are the portals of entry for foreign compounds, such as the respiratory and the gastrointestinal tracts, also express xenobiotic-metabolizing CYPs. In these tissues, CYPs not only contribute to the first-pass clearance but may also influence the tissue burden of foreign compounds or bioavailability of therapeutic agents.

CYP-mediated drug metabolism can also lead to altered drug efficacies through inactivation of an active drug or activation of a prodrug. Because most drugs have their targets in extrahepatic tissues, the extent and characteristics of target-tissue drug metabolism may have a significant impact on effectiveness of treatment. By the same token, toxic compounds may be detoxified following CYP-catalyzed biotransformation, and inert xenobiotics, including drugs, may be activated to become toxicants. Most xenobiotic compounds require metabolic activation by CYPs to form ultimate carcinogens or toxicants. The reactive intermediates resulting from CYP-catalyzed metabolic activation are often unstable and therefore are unlikely to be transported from the liver to other tissues to exert toxicity. Thus, chemical toxicity in extrahepatic tissues frequently results from in situ metabolic activation mediated by CYPs in the target organ, and the toxicity of a given compound is tightly linked to its metabolic fate in the target tissue.

Each tissue has a unique profile of CYP enzymes that, by and large, determine the sensitivity of that organ to a given xenobiotic compound. Although most CYPs expressed in extrahepatic tissues are also present in the liver, and often at higher levels, at least some of them may be regulated differently in different tissues, therefore leading to a tissue-selective response to chemical exposure. Furthermore, some CYPs are expressed preferentially in extrahepatic tissues, which may lead to unique extrahepatic metabolites and tissue-specific consequences in cellular toxicity and organ pathology. Risk assessment of potential human toxicants is currently based primarily on data obtained from animal bioassays and on knowledge of the mechanism of toxicity derived from experimental animals. However, because of the well-known species differences in biotransformation, a detailed characterization of the expressed CYP enzymes in human target tissues is crucial for a more accurate prediction of human risk.

Tremendous progress has been made in recent years in the characterization of extrahepatic-tissue CYP expression, function, and regulation. The scope of this review does not allow discussion of all extrahepatic tissues or studies on all species. We have chosen to focus on the human respiratory and gastrointestinal (G.I.) tracts because of their importance as portal-of-entry organs in xenobiotic metabolism and because of our active research in these areas. Human *CYP* genes expressed in different parts of the respiratory and G.I. tracts are summarized in Table 1.

TABLE 1 Human cytochrome P450 genes expressed in different parts of the respiratory and gastrointestinal tracts[a]

Organ	CYPs detected[b]
Nasal mucosa	2A6, 2A13, 2B6, 2C, 2J2, 3A
Trachea	2A6, 2A13, 2B6, 2S1
Lung	1A1, 1A2, 1B1, 2A6, 2A13, 2B6, 2C8, 2C18, 2D6, 2E1, 2F1, 2J2, 2S1, 3A4, 3A5, 4B1
Esophagus	1A1, 1A2, 2A, 2E1, 2J2, 3A5
Stomach	1A1, 1A2, 2C, 2J2, 2S1, 3A4
Small intestine	1A1, 1B1, 2C9, 2C19, 2D6, 2E1, 2J2, 2S1, 3A4, 3A5
Colon	1A1, 1A2, 1B1, 2J2, 3A4, 3A5

[a]See text for references.

[b]Either mRNA or protein.

CYTOCHROME P450 IN THE RESPIRATORY TRACT

Introduction

Tissues of the respiratory tract, which are exposed to both inhaled and blood-borne xenobiotic compounds, are important targets for environmental toxicity. The nasal mucosa is a "metabolic hot spot" in animals. Nasal tumors and other nontumor toxic effects are readily induced in experimental animals following inhalation or systemic exposure to a variety of industrial chemicals, environmental pollutants, therapeutic agents, and cigarette smoke-associated chemicals including the tobacco-specific nitrosamines [for a review, see (2)]. The incidence of human nasal tumors is generally low, although it is a common cancer in parts of China. An increased incidence of nasal tumors is found in smokers and in persons occupationally exposed to wood dust, chromate, and other chemicals (2), and extensive DNA damage is found in the nasal epithelium of children exposed to urban pollution (3). Numerous compounds have also been found to cause toxicity in the lung [for reviews, see (4, 5)]. In humans, lung cancer is the leading cause of cancer-related death in the United States, and cigarette smoking is the most important contributing factor to lung cancer (6, 7). In addition, possible links between respiratory tract xenobiotic metabolism and the etiology of asthma as well as multiple chemical sensitivity are important topics for exploration. Thus, characterization of biotransformation enzymes in human nasal mucosa and lung is important for risk assessment of potential respiratory tract toxicants.

Many cell types within the lung, such as the bronchial epithelial cells, Clara cells, type II pneumocytes, and alveolar macrophages, are capable of metabolizing xenobiotics. In rodents and rabbits, however, Clara cells and type II cells are the

most active [for reviews, see (8, 9)]. Accordingly, these cell types are also more susceptible than other cell types to toxicities resulting from the metabolic activation of xenobiotics. In humans, the Clara cells may be less important as a target tissue for metabolic activation of xenobiotics because they seem to lack smooth endoplasmic reticulum (4). In the nasal mucosa, the microsomal CYPs are expressed in non-neuronal cells, including the sustentacular cells in the olfactory epithelium and cells of the Bowman's glands in the submucosa (10).

Most studies on xenobiotic-metabolizing CYPs in the respiratory tract focus on the role of these enzymes in metabolic activation and toxicity. Little is known of the roles of human respiratory tract CYPs in the disposition or efficacy of therapeutic agents despite an increased interest in using the nose as an alternative or even preferred routed of drug delivery. Nevertheless, the knowledge gained from the basic characterization of the respiratory tract CYPs and the biological models established for studying the role of these CYPs in metabolic activation and portal-of-entry organ xenobiotic toxicity will facilitate future studies on their possible roles in local drug clearance and efficacy.

The subject of respiratory tract CYPs has been covered in several previous reviews [e.g., (2, 11–13)] and by a comprehensive monograph (14). Therefore, only recent advances are reviewed here, with an emphasis on CYPs expressed preferentially in the respiratory tract. Unlike the following section on CYPs in the G.I. tract, this section is not divided according to anatomical parts; the nasal mucosa, lung, and trachea all seem to share a common subset of CYPs that are expressed preferentially in the respiratory tract.

Expression of Cytochrome P450 in the Respiratory Tract

Many microsomal CYPs have been detected in human lung, including CYP1A1, 2B6, 2E1, 2F1, 3A4, 3A5, 4B1 (13), CYP1A2 (15), 1B1 (16, 17), 2A6 (18, 19), 2A13 (20), 2C (18, 21), 2D6 (22), 2J2 (23), and 2S1 (24). Most of these enzymes are expressed in the lung at levels much lower than in liver, but several, including CYP2A13 (20), 2F1 (25), 2S1 (24), 3A5 (26), and 4B1 (27), are preferentially expressed in the lung. CYP2A6, 2A13 (20), 2B6 (28), and 2S1 (24) have also been detected in the trachea.

Several studies have examined the expression of CYPs in human nasal mucosa. CYP2A6 (29), 2A13 (20, 30), 2C, and 3A (31) have been detected in adult nasal mucosa, and CYP2A6, CYP2A13, CYP2B6, and CYP2J2 have been detected in fetal nasal mucosa (32). Additional CYPs are expected to be present in human nasal mucosa because more than 10 different microsomal CYPs, including CYPs of the 1A, 2A, 2B, 2C, 2E, 2G, 2J, 3A, 4A, and 4B subfamilies, have been identified in the nasal mucosa in various animal species (33). Notably, CYP content in the nasal mucosa is among the highest of all extrahepatic tissues in many mammalian species, although apparently not in humans (2, 12).

Interestingly, all functional *CYP* genes in a *CYP2* gene cluster on chromosome 19, including *CYP2A6*, *2A13*, *2B6*, *2F1*, and *2S1* (34), are expressed in the

respiratory tract. Moreover, whereas CYP2A6 is expressed primarily in the liver [e.g., (20)] and CYP2B6 is expressed in liver and many other tissues (28), CYP2A13, 2F1, and 2S1 are preferentially expressed in the respiratory tract. These latter genes, which are particularly interesting because of their likely roles in tissue-selective chemical toxicity, are described in more detail below. Notably, the *CYP2G1* gene, which is expressed only in the olfactory mucosa and is functional in other mammals, has apparently been inactivated in humans (35), and the *CYP2B7P* gene, which is expressed in the lung, is also nonfunctional.

Three full-length genes are known in the human *CYP2A* subfamily (34): *CYP2A6*, *CYP2A7*, and *CYP2A13*. CYP2A6 has been detected in human nasal mucosa (29) and lung (18, 19). CYP2A7, which is nonfunctional (34), was not detected in human nasal mucosa or lung (19, 30). CYP2A13 mRNA is expressed at its highest levels in the nasal mucosa, followed by the trachea and lung (20). The level of CYP2A13 mRNA is much higher than that of CYP2A6 in the respiratory tract.

The expression of CYP2A protein in human lung was reported in an immunoblot study in which a polyclonal anti-CYP2A6 antibody was used (18); this antibody most likely cross-reacts with the highly homologous CYP2A13. More recent studies involving in situ hybridization suggest that CYP2A13 is expressed in both bronchial and alveolar epithelia (J. Guo & X. Ding, unpublished data). Abundant expression of CYP2A proteins in both olfactory and respiratory nasal mucosa was demonstrated in an earlier immunohistochemical study (10). A CYP2A13-specific antibody is not yet available. Heterologously expressed CYP2A13 is active toward many compounds (20), such as 2′-methoxyacetophenone, 2,6-dichlorobenzonitrile, hexamethylphosphoramide (HMPA), *N*,*N*-dimethylaniline, *N*-nitrosodiethylamine (NDEA), and *N*-nitrosomethylphenylamine. CYP2A13 also appears to be the most efficient CYP enzyme in the metabolic activation of a well-known tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK).

There is only one functional gene in the human *CYP2F* subfamily. CYP2F1 was originally cloned from a human lung cDNA library (25). Substrates for CYP2F1 include ethoxycoumarin, propoxycoumarin, and pentoxyresorufin, but not ethoxyresorufin (25). Heterologously expressed CYP2F1 had only modest activity in the metabolic activation of 4-ipomeanol, a pulmonary toxin (36), but it was efficient in the metabolic activation of several other lung toxins, including 3-methylindole (37), naphthalene (37), and styrene (38). The relative rates of metabolic activation of 3-methylindole by CYP2F1 and CYP2A13 have not been examined, but CYP2F1 was approximately three times more active than was CYP2A6 in this reaction (39).

CYP2S was recently identified through a bioinformatics approach; the full-length cDNA was obtained by reverse transcription-polymerase chain reaction (RT-PCR) (24). CYP2S1 mRNA appears to be highly expressed in trachea and lung, and CYP2S1 protein was also detected in human lung by Western blot analysis using an antiserum against the C terminus of the enzyme. However, the activity of the enzyme was not reported. Of interest, CYP2S1 was inducible

by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in a human lung epithelial cell line (40).

Because of potential vulnerability of fetuses to developmental toxicity resulting from transplacental xenobiotic exposure, several studies have examined the expression of CYPs in various fetal tissues. CYP1A1 mRNA was detected in fetal lung at gestational days 55 to 145 (41). CYP1B1 mRNA and proteins immunochemically related to CYP3A were also detected in fetal lung (42). Expression of several CYPs, including CYP2A6, CYP2A13, CYP2B6, and CYP2J2, was detected in human fetal nasal mucosa in a recent study (32). The expression of CYP2A13, an avid enzyme for metabolic activation of NNK, in human fetal nasal mucosa may have significant implications in developmental toxicology because NNK can be transferred transplacentally from women who smoke cigarettes to fetuses (43).

Several studies have compared the expression level of CYPs in tumor versus nontumor tissues in the lung [e.g., (44)]. These data may provide useful information for cancer chemotherapy, but may not indicate a role of these CYPs in the initiation of tumorigenesis. The CYP expression levels may change at different stages of tumor development.

Role of Individual Cytochromes P450 in Microsomal Metabolism

Studies on the roles of various human CYPs in the metabolic activation of xenobiotics in the respiratory tract are of paramount importance for understanding the mechanisms of chemically induced nasal and pulmonary toxicities, yet only limited progress has been made. Although CYP-dependent xenobiotic-metabolizing activities have been detected in human nasal microsomes [e.g. (45)], the role of individual CYPs has not been examined. The very limited availability of human nasal tissues for metabolic studies remains a major obstacle. In contrast, human lung microsomes are now readily available for in vitro studies, though these microsomes usually have very low metabolic activity. It is difficult to assess the extent to which in vitro data reflect activities in intact lung in vivo because the level and activity of pulmonary CYPs may be highly sensitive to the surgical conditions to which most lung biopsy or autopsy tissues were exposed. Nevertheless, in vitro studies are still valuable, particularly when combined with other approaches, including the quantification of the levels of enzyme expression in the same microsomal preparations used for metabolic studies and kinetic analysis of heterologously expressed CYPs.

CYP1A1 plays a major role in the metabolic activation of many aromatic hydrocarbons in human lung, such as 5-methylchrysene and 6-methylchrysene (46). Several CYPs, including CYP2F1, CYP4B1, and CYP2A6, may be involved in the metabolic activation of 3-methylindole in human pulmonary microsomes (47). The metabolic activation of aflatoxin B1 (AFB1) in human lung may involve CYP3A4 (48). The metabolism of 1,1-dichloroethylene to a reactive epoxide by human lung microsomes may be mediated by CYP2E1 (49).

Human lung microsomes are active in the metabolism of NNK. However, the major CYPs responsible for NNK activation have not been identified. In lung microsomes, CYP2A6 or a related enzyme was implicated in NNK activation; lipoxygenases and lipid hydroperoxides may also be involved (50). The high efficiency of CYP2A13 in NNK metabolic activation and the preferential expression of CYP2A13 in the respiratory tract (20) suggest that CYP2A13 may play a major role in the metabolic activation of this important respiratory tract procarcinogen. However, direct evidence for its involvement has not been demonstrated at the microsomal level.

NNK metabolism has not been examined in human nasal mucosa. However, human nasal microsomes were found to have relatively high activities in the N-deethylation of NDEA (45). Metabolic activation of NDEA has also been demonstrated in human lung cell lines (51). However, the CYPs responsible for the metabolic activation of NDEA in human nasal mucosa and lung have not been identified. Human nasal mucosa is also active in the metabolic activation of HMPA, a known rodent nasal carcinogen (45). Both CYP2A6 and CYP2A13 are active in this reaction, with CYP2A13 being the more active of the two (20).

Biological Models for Studying Function of Respiratory Tract Cytochromes P450 in Xenobiotic Metabolism and Chemical Toxicity

Metabolic studies using biological models are important for determining the significance of specific enzymes or pathways in toxicity. For example, in an interesting series of studies (52), murine lung tumors were found to have diminished CYP expression and activities, and they were also found to be resistant to cytotoxicity induced by xenobiotics, thus supporting a role of local CYP in metabolic activation. However, to date no studies have directly evaluated the in vivo role of human respiratory tract CYPs in xenobiotic metabolism or toxicity. Limited studies have been done with animal models or cell lines. For example, Mace and coworkers (53) transfected human CYP1A2 into a human lung cell line and demonstrated increased cytotoxicity following exposure of the cells to AFB1. The abilities of specific lung cells to metabolize toxicants were also demonstrated for isolated lung cells of different types (54). Other models include microdissected airway cultures, which maintain their differentiated status and CYP expression (55).

Studies correlating the levels of benzo(a)pyrene (BaP)-DNA adduct in human lung with *CYP1A1* genotype have also been reported (56). Although these studies do not necessarily indicate the role of lung CYPs in metabolic activation, a recent study did confirm the role of CYP1A1 induction in BaP-DNA adduct formation in two human lung tumor cell lines (57). The potential correlation between genetic polymorphisms in *CYP* genes, such as *CYP1A1*, *CYP2D6*, or *CYP2E1*, and lung cancer incidence has also been studied [e.g., see (7)]. However, a clear and conclusive correlation has not been found with any of the *CYP* genes studied. An interesting development along this line is the somewhat controversial finding that

reduced hepatic clearance of nicotine, as a consequence of genetic polymorphisms in the *CYP2A6* gene, is associated with a lower incidence of lung cancer in certain populations (58). Notably, the *CYP2A13* gene is also highly polymorphic [(59); X. Zhang & X. Ding, unpublished data]. Thus, individuals defective in both *CYP2A6* and *CYP2A13* genes may be further protected from cigarette smoking-induced respiratory toxicity.

An increasing number of animal studies, utilizing *CYP*-knockout mice or utilizing chemical inhibitors, have dealt with the role of CYPs in respiratory toxicity. Again, studies measuring in vivo systemic clearance generally do not provide data regarding specific extrahepatic organ contribution. Although several *CYP*-knockout and transgenic mouse models have been generated [for a review, see (60)], mice with tissue-specific *CYP* gene deletion or respiratory tract–selective expression of human *CYP* transgene have not been reported. However, the *Cyp1a1*-null mice are suitable for conditional gene targeting (61) and therefore should be useful for studying specific roles of CYP1A1 in extrahepatic tissues. Other models, including mice with tissue-specific NADPH-cytochrome P450 reductase (*CPR*) gene deletion, under development in this (X. Ding, unpublished data) and other (62) laboratories may also be highly useful, as they will allow analysis of the combined roles of all microsomal CYPs in a given organ. A potential problem with in vivo studies in mice, however, is the existence of the well-known, yet poorly characterized, strain-related differences in gene expression and function. Furthermore, potential complications resulting from the genetic manipulations involved in the production of a mutant mouse strain may also lead to unexpected results. For example, the expression of the neighboring *Cyp2a5* gene was suppressed in the liver of *Cyp2g1*-null mice (X. Zhuo & X. Ding, unpublished data).

Two studies have examined the effects of *CYP* gene deletion on nasal xenobiotic toxicity. Whereas systemic deletion of *Cyp2e1* protected nasal mucosa from chloroform toxicity (63), deletion of *Cyp1a2* did not protect the nose from acetaminophen toxicity (64). In the latter case, acetaminophen activation by nasal mucosa-selective CYP2A5 and 2G1 was thought to be responsible for the local toxicity. The same may apply to many other compounds known to cause nasal mucosa-selective toxicity, such as coumarin, 2,6-dichlorobenzonitrile, and HMPA. Clearly, knockout models for these nasal mucosa-predominant CYPs should be invaluable for determining whether the nasal CYPs are responsible for tissue-selective toxicity. In the case of CYP2G1, for which humans do not have a functional ortholog, the *Cyp2g1*-null mice also represent a humanized mouse model that may be useful for risk assessment.

Regulation of Cytochromes P450 in Human Respiratory Tract

Several CYPs, including CYP1A1, 1A2, 1B1, 2E1, and 3A5, may be inducible in human lung. For example, CYP1A1, which is normally expressed in the lung at trace levels, is induced significantly by cigarette smoking [e.g., (65)]. Induction of CYP1A1 and 1A2 by TCDD and other compounds was also suggested by a recent

study using human lung explant culture system (66). Hukkanen and coworkers (67) observed that in the human alveolar type II cell-derived A549 adenocarcinoma cell line, CYP1A1 and 1B1 mRNAs were induced 56-fold and 2.5-fold, respectively, by TCDD; CYP3A5 mRNA was induced 8-fold by dexamethasone and 11-fold by phenobarbital, whereas CYP3A4 was not detected. The mRNAs of several other CYPs, including CYP1A2, 2A6, 2A7, 2A13, 2F1, and 4B1, were also not detected in this study. The pulmonary inducibility of CYP2A6, 2B6, 2C, and 3A4, which are all inducible in hepatocytes, has not been documented. Inducibility of CYPs has not been examined in human nasal mucosa, although it has been suggested that potential induction of nasal CYPs by tobacco smoke may enhance resistance to xenobiotics implicated in parkinsonism and other neurologic diseases (68).

Studies correlating levels of CYP expression (mostly of mRNA) with amount of cigarette smoking have been reported, but these are complicated by issues including incomplete accounting of patient exposure and medical history (such as the extent of use of a respirator during surgery), specimen quality (postmortem time and specimen storage condition), heterogeneity of the tissues assayed in which different cell populations may undergo different responses, and potential genetic polymorphisms in *CYP* gene regulation. In vivo studies using noninvasive marker substrates have limited application for extrahepatic tissues due to difficulties in determining the organ distribution of metabolism, but they may hold promise for CYPs expressed preferentially in the respiratory tract, such as CYP2A13. In this regard, it should be feasible to detect and quantify volatile metabolites from inhaled CYP2A13 substrates via techniques such as real-time gas chromatography–mass spectrometry of exhaled air. For some CYPs, such as CYP2E1, the inducibility in extrahepatic tissues can be predicted according to the mechanisms of the CYP's induction, but the extent of its induction will be affected by the concentration of the inducers in the respiratory tract.

The tissue-restricted inducibility of some CYPs, such as CYP2A (69) and CYP2B (11), has been demonstrated in animal models. From a physiological point of view, each CYP may have unique, yet unknown, functions in a tissue, and thus there may be a need for the tissue to maintain a relatively constant level of its expression. Alternatively, some CYPs in the respiratory tract may not respond to ingested or systemically administrated inducers because the level of inducers and duration of exposure cannot be maintained. In addition, an important issue to consider when studying CYP induction in the respiratory tract is that many inducers may cause tissue-selective toxicity, including inflammatory responses, leading to the loss of CYP expression. Thus, a lack of observed induction may not necessarily indicate the existence of tissue-specific transcriptional or posttranscriptional regulatory mechanisms.

The tissue-selective expression of several *CYP* genes in the respiratory tract has been explored in order to identify mechanisms of regulation, primarily through the use of animal models. For *CYP2A*, a nuclear factor I-like element (called NPTA element) is present in the proximal promoter region; it is conserved in rodent and

human *CYP2As* (70). The intriguing possibility that the *CYP2A*, *2B*, *2F*, and *2S* genes, which are part of the chromosome 19 *CYP2* gene cluster, may share common regulatory mechanisms for selective expression in the respiratory tract remains to be explored. The lack of cell lines that normally express these CYPs renders it very difficult to perform traditional promoter analysis. It is also challenging to perform in vitro DNA-binding studies with nuclear extracts from human tissues. A transfection experiment with human lung cells (71) showed that C/EBP factors are needed for differentiation-dependent expression of rat 2B1 in A549 cells; this experiment used a construct previously shown to lead to lung-specific expression in transgenic mice.

The promoter region of human *CYP2F1* is being explored through in vitro methods, with nuclear extracts from human lung (G. Yost, personal communication). An apparently lung-selective binding site was identified within a 31-bp sequence (−152 to −182) in the *CYP2F1* promoter that does not match any known regulatory motif. The same group also evaluated the regulation of the human *CYP4B1* gene and identified two regulatory enhancer domains in the proximal region of the *CYP4B1* promoter, which appear to be unique, previously uncharacterized regulatory elements.

Several transgenic mouse studies have attempted to map important promoter regions for selective CYP expression in lung or nasal mucosa. In one, a 1.3-kb fragment of the rat *CYP2B1* promoter was able to confer tissue-selective expression of a reporter gene in lung and liver (72). Similarly, a rat *CYP2A3* transgene with 3.4-kb 5′-flanking sequence was selectively expressed in the nasal mucosa, as well as in other tissues known to express the endogenous mouse *Cyp2a5* gene (73). However, in both cases, the level of transgene expression was quite low. In another study, a 3.6-kb mouse *Cyp2g1* promoter was used to drive the expression of a reporter gene (74). The transgene was expressed only in the olfactory mucosa, consistent with the idea of tissue-specific expression of the *Cyp2g1* gene. Nevertheless, within the olfactory mucosa, the reporter gene was expressed primarily in the duct cells of Bowman's gland, whereas the endogenous *Cyp2g1* gene is expressed primarily in the supporting cells and Bowman's gland. The results from these studies suggest the need to use large DNA inserts to avoid effects of integration site on transgene expression. Other approaches, such as the use of viral vectors, are being explored (X. Ding, unpublished data); these are less time-consuming, although they are often restricted in the amount of DNA sequence that can be included.

CYTOCHROMES P450 IN THE GASTROINTESTINAL TRACT

Introduction

Elucidation of the roles for CYPs in G.I. tract xenobiotic metabolism has been a slow process, particularly in humans. The capability of the small-intestinal mucosa to metabolize xenobiotics, such as tetrahydrocannabinol (75), flurazepam (76), ethoxycoumarin (77), and aryl hydrocarbons (78), was reported as early as

the mid-to-late 1970s. By the late 1980s and early 1990s, the expression of CYP2C, 2D6, and 3A, as well as CPR, in the human small intestine had been reported (79–83). Ethoxycoumarin *O*-deethylase and aminopyrine *N*-demethylase activities were detected in both the colon and the ileum of older patients (84). The early status of CYP-mediated G.I. tract metabolism was summarized in a review (85), and that of small-intestine metabolism was described in a review from our laboratory (86).

Several factors have contributed to the slow rate of progress of research into G.I. tract xenobiotic metabolism. Principal among these are the low levels of expression of the CYPs, particularly relative to hepatic expression levels, and the difficulties in determining functions for the expressed CYPs. Possible roles in protection of the body against orally ingested xenobiotics through limitation of systemic uptake have been proposed, as have roles in carcinogenesis through target-organ bioactivation.

In recent years, interest in G.I. tract metabolism, particularly small-intestinal metabolism, has exploded. The resultant studies over the past 10 years are reviewed here.

Anatomy and Physiology of the Gastrointestinal Tract

For the purposes of this review the G.I. tract is defined as comprising the esophagus, stomach, small intestine (subdivided into the duodenum, jejunum, and ileum), and the colon. The tract serves as the portal of entry for orally ingested xenobiotics, including therapeutic drugs and nutrients.

The gross structures of the luminal surfaces of the G.I. tract components are essentially similar, covered with a layer of columnar epithelial cells, goblet cells, and endocrine cells (87). However, the cell types vary among organs. In the stomach, the cells are specialized for the secretion of acid, pepsinogen, gastrin, and intrinsic factor. In the small intestine, the epithelial cells are sited along the villi and microvilli and contain digestive enzymes, transport mechanisms, and metabolic enzymes, including CYPs (see below). The colonic epithelial cells function primarily to absorb fluid and electrolytes. The average lengths and absorbing surface areas of the G.I. tract organs, determined post mortem, are: esophagus, 25 cm and 0.02 m^2; stomach, 20 cm and 0.11 m^2; duodenum, 25 cm and 0.09 m^2; jejunum, 300 cm and 60 m^2; ileum, 300 cm and 60 m^2; and colon, 150 cm and 0.25 m^2, respectively (88, 89). The bacterial floral counts in the various organs show considerable variation: stomach, 0–5 $\log_{10}$ number of viable organisms per g wet weight; proximal small intestine, 0–5; distal small intestine, 6–7; and large intestine, 7–10 (90). Because the presence of gut flora can confound metabolic studies of the G.I. tract, these variations in content are important for investigations of G.I. tract CYP function.

Cytochrome P450 Expression in the Esophagus

The human esophagus is a target organ for cancer, and this has potentiated searches for metabolic bioactivators of tobacco-smoke carcinogens in the organ. Human

esophageal microsome preparations, obtained from individuals in the United States and China, all activated *N*′-nitrosonornicotine (91), as did esophageal cultures (92). Rates of bioactivation were higher in tissue from those Chinese patients who resided in an esophageal cancer high-risk area. This activity was decreased by 20%–26% in the esophageal microsomes by troleandomycin, a CYP3A inhibitor (91). A role for CYP2A6 was ruled out because of the lack of coumarin inhibition found. Additionally, for esophageal squamous-cell carcinomas, levels of what was presumed to be CYP3A4 were 30%–50% decreased, relative to the levels in the surrounding noncancerous tissue. Low levels of CYP2E1 were also detected in these microsomes through immunoblotting. Another nitrosamine, *N*-nitrosomethyl-*N*-amylamine, was also metabolized by esophageal CYPs (93).

The expression of CYP3A in the esophagus was confirmed at the mRNA level (94). Subsequently, in a comprehensive study of esophageal CYP expression, it was resolved by RT-PCR that only CYP3A5, and not CYP3A4, was expressed in all of 25 non-neoplastic surgical samples (95). This preference for expression of one or the other of the two CYP3As is an interesting feature of the G.I. tract and is discussed in more detail below. In the same study, a combination of RT-PCR and immunoblots using specific antibodies revealed the expression of CYP1A, 2E1, and 4A in the esophagus (95). CYP4B1 mRNA was also detected, as was a protein that cross-reacted with anti-CYP2A. CYP1A2 mRNA was detected in 11 of 19 samples, but expression of the protein was not confirmed. CYP1A1 was detected by immunoblot analysis and enzyme assays in all of 41 samples from squamous-cell cancer patients (96). Tumor tissue expressed higher levels of CYP1A1 protein than did normal tissue, but levels were apparently not influenced by the smoking behavior of the patients.

The detection of expressed CYP2E1 in the esophagus prompted a study to determine whether the C1/C1 variant *CYP2E1* genotype would affect the susceptibility of an individual to esophageal cancer (97). This genotype, when studied jointly with the glutathione transferase GSTM1 non-null genotype, showed an odds ratio of 8.5, suggesting that CYP2E1 plays a role in development of esophageal cancer. However, a subsequent study in China did not detect any relationship between the *Rsa* I homozygous *CYP2E1* genotype and esophageal cancer (98). The recently identified CYP2J2 is expressed most prominently in the esophagus among all G.I. tissues (99). The enzyme, which is an arachidonic acid epoxygenase, is speculated to be involved in neuropeptide release.

Cytochrome P450 Expression in the Stomach

There is very limited evidence for CYP expression in the human stomach. Furthermore, it is difficult to propose any function for gastric CYPs because the gastric epithelium secretes rather than absorbs. However, the potential of those CYPs expressed in the stomach to play roles in stomach cancer has been investigated in cases of intestinal metaplasia of the stomach. Intestinal metaplasia of the stomach, which involves the replacement of the gastric mucosa with a small intestine-like epithelium (100), is considered to be a precancerous lesion (101). A combination

of immunohistochemistry, immunoblotting, and RT-PCR have identified CYP3A4 in the foveolar or pitted epithelium of the stomach and in the pyloric gland when intestinal metaplasia is present, but not in its absence (31, 102). These authors also detected expression of CYP2C, by immunoblotting, in gastric fundic glands.

Similar results were obtained with immunoblot and RT-PCR probes for CYP1A1 and 1A2 (103). CYP1A1 and 1A2, as well as CPR, were reported to be detected in human gastric mucosa with intestinal metaplasia and in pyloric gland cells. Microsomes prepared from these cells activated BaP and 2-amino-2-methylimidazole [4,5-f]quinoline.

In view of the precancerous nature of intestinal metaplasia, it has been postulated that the coincident expression of various CYPs in the gastric mucosa of such patients plays a role in the bioactivation of gastric carcinogens. In a comparison of normal stomach tissue with stomach cancer, it was determined that in the normal tissue, no CYPs were detected, whereas in the case of stomach cancer, CYP1A and 3A were detected in 51% and 28% of cases, respectively (104). The presence of intestinal metaplasia was not noted in this study.

Other studies have reported expression of CYPs in human stomach mucosa. The recently identified CYP2S1 mRNA was detected by dot blot analysis (24), and CYP2J2 was detected by immunoblot (99).

Cytochrome P450 Expression in the Small Intestine

The significant xenobiotic absorptive function of the human small intestine provides the framework for an enhanced metabolic role for this organ's expressed CYPs, relative to their roles in other G.I. tract organs. Small-intestinal CYP-mediated metabolism can serve as a barrier to the systemic uptake of xenobiotics, including drugs, by facilitating excretion to the lumen of the intestine or by bioactivation of the xenobiotics, with consequent binding to enterocyte macromolecules. Covalently bound xenobiotics will be removed with the sloughed-off enterocytes, which have very short half-lives. These metabolic activities of the small intestine can produce a detoxification by diminishing systemic uptake of toxicants. This barrier activity of small-intestinal CYPs is greatly facilitated, in the case of CYP3A4 substrates, by the multidrug efflux pump P-glycoprotein, which is expressed in the small intestine and functions in accord with CYP3A4 (105–107). It is probable that small-intestinal CYP3A4 contributes substantially to the first-pass metabolism of high-turnover CYP3A4 substrate xenobiotics (108).

The most extensive characterization of human small-intestinal CYP expression was conducted recently using enterocytes eluted from 10 small intestines by an EDTA-containing buffer. This method of enterocyte preparation produces only villous enterocytes, without crypt cell contamination (109). RT-PCR of enterocytes revealed the expression of CYP1A1, 1B1, 2C, 2D6, 2E1, 3A4, and 3A5 mRNAs. Not detected were CYP1A2, 2A6, 2A7, 2B6, 2F1, 3A7, and 4B1 mRNAs. However, when probed by immunoblots, only CYP3A4, 1A1 (in two of eight intestines tested), and 2C proteins were detectable, and CYP1B1, 2E1, 2D6, and 3A5 proteins were not detectable. In a broader study of 33 small intestines, no

CYP3A5 protein was detected (L.S. Kaminsky, unpublished data). The variability of expression determined for CYP1A1 is consistent with the conflicting reports of its expression (110–112). This expression of small-intestinal CYP1A1 is probably inducible rather than constitutive, to judge from its reported induction by omeprazole, as determined through duodenal biopsies. Total microsomal protein content decreased markedly as a function of distance along the intestine, from the duodenum to the ileum. Total CYP content increased slightly in proceeding from the duodenum to the jejunum and then decreased sharply toward the ileum (109). Our observations that CYP3A4 is the predominant small-intestinal CYP3A expressed and that CYP3A5 protein expression is not detectable are at odds with observations of some other investigators. In a study of 20 enterocyte preparations, a band on immunoblots in four of the samples was indicated to represent CYP3A5, but no positive identification was provided (113). In an earlier study, 14 of 30 patients were reported to express just-detectable levels of CYP3A5 in intestinal biopsies, which were probed with an antibody that was claimed to be specific for CYP3A5; however, no data were presented (114). A clear conclusion is that, at the very least, CYP3A4 expression greatly predominates over that of CYP3A5 in human small-intestinal enterocytes.

The determination of CYP2C protein expression in the small intestine (109) confirmed the results of an earlier study (82). In a subsequent study, metabolic activities were assayed to determine which forms of CYP2C were expressed in the human small intestine and to assess the interindividual variability in expression levels (115). Expression of CYP2C9 and that of CYP2C19 were demonstrated by activities of diclofenac 4′-hydroxylase and mephenytoin 4′-hydroxylase, respectively. Interindividual variability for the 10 intestines investigated was 18-fold for CYP2C9 and 17-fold for CYP2C19. On the basis of 6β-testosterone hydroxylase activity, CYP3A4 activities varied sevenfold for these 10 small-intestinal preparations, although in larger populations much greater variability has been observed. The basis for such variability probably resides in the pathways of regulation of *CYP3A4*, rather than in genetic polymorphisms leading to structural CYP protein variants (116, 117).

Several other CYPs are reported to be expressed in the human small intestine. These include CYP2S1 (24); CYP4F12, which catalyzes the antihistaminic ebastine's metabolism (118); and CYP2J2, which catalyzes arachidonic acid metabolism (99).

Observations that one or more constituents of grapefruit juice can decrease the metabolic function of human small-intestinal CYP3A4 have provided an approach to investigate this function in vivo. The original observation was that grapefruit juice, when administered together with either of the calcium antagonists nifedipine or felodipine, increases the plasma concentration of the drug (119). Subsequently, it was demonstrated that the flavonoids naringenin, quercetin, and kaempferol in grapefruit juice inhibit CYP3A4, which could explain the previous observation (120). Quercetin was later excluded as a possible inhibitor (121), and narignin and naringenin were shown not to be the primary inhibitors in grapefruit juice (122). The metabolism of coumarin, cyclosporine, ethinylestradiol, midazolam,

terfenadine, and verapamil [for reviews, see (123, 124)], as well as that of saquinavir (125, 126) and erythromycin (127), were also shown to be decreased by grapefruit juice.

The grapefruit-mediated decrease in substrate metabolism was determined to be through a mechanism-based inactivation of enterocyte CYP3A4, possibly by a furancoumarin constituent of grapefruit juice (128). Through the use of small-intestinal biopsies and an erythromycin breath test, it was determined that orally ingested grapefruit juice did not affect hepatic CYP3A4 activity but did decrease small-intestinal CYP3A4 levels by 62%, without any corresponding change in the enterocyte CYP3A4 mRNA levels (110). These results are consistent with the proposed mechanism. A recent study showed that at least six furano-coumarins in grapefruit juice contribute to the inhibition of CYP3A4 and that a combination of competitive and mechanism-based inhibition occurs (129).

Cytochrome P450 Expression in the Colon

Interest in expression of CYPs in the colon has been stimulated by the prominence of the colon as a target organ for cancer. Despite this, few recent studies have been reported, and most studies on CYP regulation have been conducted in cell preparations.

No exhaustive studies of CYP expression profiles in the colon have been published. The most prominent CYP expressed is CYP3A, and there is some disagreement concerning which members of this subfamily are actually expressed. As indicated previously, the test systems, e.g., immunoblots or RT-PCR, must be capable of differentiating between the forms to resolve whether CYP3A4 or 3A5 is being expressed. Furthermore, the detection of mRNA for one or the other of the two CYP3As does not necessarily imply that the corresponding protein will be expressed at high-enough levels to be detectable by immunoblot analysis.

Testing by RT-PCR of biopsy tissue from five colons identified the expression of CY3A3 (considered to be an artifact and to be indicative of CYP3A4), CYP1A1, and CYP1A2 mRNAs (130). This contrasts with an earlier study, in which the CYP1A subfamily was not detected (131). However, in this early study, CYP3A was detected at the mRNA level in some individuals and not in others. When CYP3A mRNA was detected in an individual colon, it was observed throughout the length of the organ. In a study of 11 human colons, Northern blot analysis revealed marked interindividual variations in CYP3A mRNA levels, and two species of mRNA were detected in some individuals. CYP3A4 was detected in 5 of the 11 colon samples, and CYP3A5 in 6 of the 11 colon samples (111). In a carefully controlled study in which isoelectric focusing was used to resolve the various CYP3A members, it was determined that CYP3A5 was the "main" form expressed in the human colon at both the mRNA and protein levels (132). In this study, patients were treated with rifampicin. The arachidonic acid epoxygenase, CYP2J2, is also expressed in the colon and presumably plays roles in vascular tone and motility (99).

It has also been reported that CYP1B1 is expressed at high frequency in colon tumors but not in normal colon tissue (133). This result needs to be confirmed

because the same authors have made similar claims, which were not verified, for breast tissue.

Assessment of induction of human colon CYPs in vivo using biopsy tissue has not been reported. An alternative approach, which uses xenografts of human colon tumor in mice, has revealed that CYP2A, 2B, 2C, 3A, and 4A family and subfamily members are inducible at the transcriptional levels by the prototypic CYP inducing agents 3-methylcholanthrene, β-naphthoflavone, clofibrate, dexamethasone, and phenobarbital (134). Colon mucosal tissue obtained by endoscopic biopsy was used to assess the effects of repeated grapefruit juice ingestion on CYP3A expression levels in the colon (110). Colon levels of CYP3A5 were not affected by the grapefruit juice. This contrasts with the marked decrease in small-intestinal CYP3A4 protein previously discussed.

Human Colon Cell Lines

Human colon adenocarcinoma LS-174 and Caco-2 cells, when treated with low doses of natural indoles such as ascorbigen, show up to 21-fold increases in CYP1A1 levels (135). Natural isothiocyanates did not produce any comparable induction, although they induced CY1A1 mRNA. When benzanthracene, pyrazole, or phenobarbital was added to these cells, each induced CYPs, as determined by analysis for *O*-6-methylguanine DNA adducts with 1,2-dimethylhydrazine (136).

CYP3A4 can be induced in the colon cancer cell line Caco-2 by 1,25-dihydroxyvitamin D-3 (137), and this induction is suppressed by nitric oxide but not by a guanylate cyclase inhibitor or by 8-bromo cGMP (138). These results were interpreted to indicate that the nitric oxide suppression is possibly not mediated by a guanylate cyclase pathway. These cells also express CYP1A1, 2E1, and 3A proteins (139). In a carefully conducted study, the major CYP3A in both Caco-2 and HT29, a human colonic cell line, was reported to be CYP3A5 (132).

The colon carcinoma cell line LS180 is unusual because it can be readily induced for CYP1A2 mRNA and protein by TCDD, 3-methylcholanthrene, and benz[a]anthracene (140). Both CYP1A1 and 1B1 were similarly induced in these cells. In this same cell line, CYP3A4 was reportedly induced by rifampin, phenobarbital, clotrimazole, reserpine, and isosafrole. CYP3A5 expression in these cells was not affected by most of these agents, but it was upregulated by reserpine and clotrimazole (141).

In summary, the major CYP expressed in the human colon is CYP3A5, but CYP3A4 also appears to be expressed in some individuals. Very little information is available on the in vivo regulation of colonic CYPs, although inducing agents have been identified in cell systems.

FUTURE PERSPECTIVE

Despite the recent surge in research activities, we are just beginning to understand the potential for the CYPs in the respiratory and G.I. tracts to play significant roles in xenobiotic metabolism and chemical toxicity. The application of molecular and

genomics approaches, as well as the availability of genetically modified animal models, will tremendously increase our capability for generating data, but it will still be an enormous challenge to use these data to understand the complexity of in vivo situations in humans. In this regard, exciting developments are underway in the development and characterization of various humanized mouse models, but it remains to be seen to what extent we can use these mouse models to study human extrahepatic CYPs, particularly because we are still far from understanding how different humans are from laboratory animals in terms of extrahepatic xenobiotic metabolism. Ultimately, we need to know the extent to which human extrahepatic tissues contribute to drug clearance, and we need to determine the relative importance in chemical toxicity of hepatic clearance versus extrahepatic target tissue metabolic activation.

It will not be long before most of the genetic polymorphisms in human *CYP*s are identified. This resource will be invaluable for determining the role of human CYPs in individual differences in xenobiotic metabolism and chemical sensitivity. However, some issues may prove to be more difficult to resolve, including how to interpret the roles of genetic polymorphisms that affect both hepatic and extrahepatic metabolism, how to phenotype individuals for genetic polymorphisms of extrahepatic *CYP*s, and how to determine the functional consequences of regulatory region SNPs for *CYP* genes expressed predominantly in extrahepatic tissues. To that end, more studies with human tissues and human subjects are clearly needed to determine which enzyme(s) is important for human extrahepatic microsomal metabolism in vitro and in vivo and to understand the extent of xenobiotic inducibility of human extrahepatic CYPs.

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HORMESIS: The Dose-Response Revolution

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Key Words U-shaped, J-shaped, biphasic, risk assessment, stimulation

■ **Abstract** Hormesis, a dose-response relationship phenomenon characterized by low-dose stimulation and high-dose inhibition, has been frequently observed in properly designed studies and is broadly generalizable as being independent of chemical/physical agent, biological model, and endpoint measured. This under-recognized and -appreciated concept has the potential to profoundly change toxicology and its related disciplines with respect to study design, animal model selection, endpoint selection, risk assessment methods, and numerous other aspects, including chemotherapeutics. This article indicates that as a result of hormesis, fundamental changes in the concept and conduct of toxicology and risk assessment should be made, including (*a*) the definition of toxicology, (*b*) the process of hazard (e.g., including study design, selection of biological model, dose number and distribution, endpoint measured, and temporal sequence) and risk assessment [e.g., concept of NOAEL (no observed adverse effect level), low dose modeling, recognition of beneficial as well as harmful responses] for all agents, and (*c*) the harmonization of cancer and noncancer risk assessment.

INTRODUCTION

Toxicology, as defined by Gallo & Doull (1), is the "study of the adverse effects of xenobiotics." This perspective is consistent with the later definitions of toxicology by Furst & Fan (2), Hayes (3), and others. The key term in this definition is "adverse." The term adverse is typically employed by regulatory agencies (e.g., U.S. EPA) in critical risk assessment related concepts, such as the no observed adverse effect level (NOAEL) and lowest observed adverse effect level (LOAEL). This most evident manifestation of the toxicological concept of adverse implies that toxicology is an above NOAEL discipline because it is the study of "adverse effects." It also implies that either there are no effects below the NOAEL or that they are not relevant to and/or part of toxicology.

Over the past five years, we have demonstrated that there are numerous responses to chemical/physical agent exposures that occur below the traditional NOAEL (4–11). These findings may also have profound effects on the health of the individual. Such findings challenge not only how we design experiments, integrate data, and apply biostatistical extrapolation models, but also how we define toxicology itself. In fact, these emerging data on the dose response strongly suggest

that the earlier definitional paradigms of toxicology that have guided the field for so long should be amended to the study of the "entire dose-response continuum." Besides suggesting that the definition of toxicology itself should be changed, where is the dose-response revolution leading us?

WHAT IS THE DOSE-RESPONSE REVOLUTION?

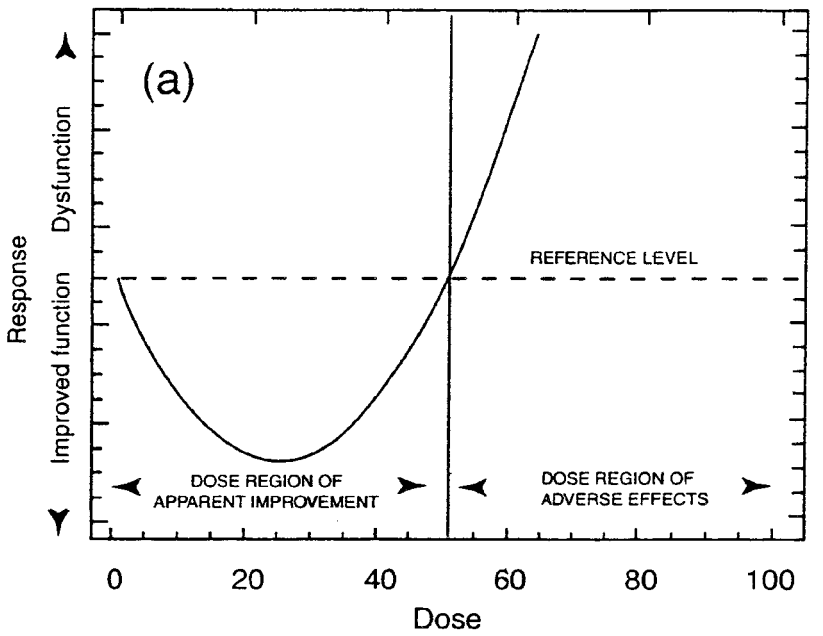
The dose-response revolution is the changing perception that the fundamental nature of the dose response is neither linear nor threshold, but U-shaped. The field of toxicology was lulled into the belief that these two ruling dose-response paradigms (i.e., threshold/linear) were universally valid in their respective domains and the only broadly applicable models relevant to federal risk assessment. The threshold assumption was steeped in common experiences of physical (e.g., melting and boiling points) and biological observations (i.e., vast numbers of studies assessing responses at high doses, constant hazard assessment preoccupation with NOAEL/LOAEL derivation, and the use of biostatistical models that were either emphasizing LD_{50} estimation or their application to extrapolate findings far beyond the observable range). In addition, endpoints, such as serum enzymes and hematological parameters, that are easily and reliably measured were emphasized in which thresholds were the dominant observation. This would also be the case with animal models in which background disease incidence was negligible for most organs in short-term studies (up to 13 weeks in duration). In the case of low-dose linearity, with cancer risks approaching 10^{-4} to 10^{-7}, this is a public health-motivated, theoretically based, biostatistical construct that is impossible to prove in any conceivable practical experimental setting. Despite its validation limitations, the assumption of low-dose linearity has become accepted and continues to dominate the actions of public health and environmental agencies.

The dose-response revolution argues that the toxicology community, including those in the regulatory-risk assessment domain, recognize the existence of U-shaped dose responses not only as real in specific cases but also as broadly generalizable. However, acceptance that hormetic-like U-shaped dose responses are widespread and real has been difficult to achieve. The reasons for this are many, but in general include the following. First, the field of toxicology has become progressively and insidiously dependent on the role of government to set the national (and international) toxicological agenda. This agenda translates into designing and interpreting studies to fit into current risk assessment paradigms. That is, in the case of noncarcinogens, regulatory agencies design hazard assessment methodology to provide a NOAEL, whereas in the case of carcinogens, the study needs data that can be employed to estimate low-dose cancer risk. Such NOAEL and/or low-dose evaluations are dominating concerns. These controlling governmental regulatory perspectives have provided a seductive focus on toxicological thinking, providing the flow of financial resources and forcing private-sector and academic institutions to respond to such initiatives. Second, there is fear among many within the regulatory community that acceptance of hormesis as a toxicological dose-response

principle implies that low doses of at least some, but most likely most, toxic substances may produce a beneficial effect at low doses, such as enhanced longevity or decreased disease incidence. This fear can result in a powerful emotional bias that can stifle objective assessment of toxicological data. Third, the belief in the universality of biological thresholds became firmly established and accepted by the scientific community and government public health/regulatory agencies during the early to mid decades of the twentieth century. These beliefs became codified in risk assessment/management procedures of the U.S. FDA and U.S. EPA and by comparable governmental organizations of other countries. These threshold beliefs were expanded in the 1970s to accommodate the acceptance of low-dose linearity for carcinogens. This codified governmental risk assessment procedure created inflexibility in dealing with challenges, and not just rare exceptions to the government's established paradigms. That is, once a procedure is established, it often takes an extraordinary amount of effort and data to effect a change by governmental agencies. This appears to be especially the case if the change has the support of the industrial sector. For example, it took over a decade of consistent findings for the EPA to accede to industrial pressure that chemically induced kidney tumors in the male rat due to chemically induced hyaline droplets were species/gender-specific and could not be reliably extrapolated to humans. The extraordinary and massive amount of research on this issue resulted in an agent-specific response victory but relatively minor conceptual concession by the EPA. The point is, once agencies fix a procedure it is nearly impossible to effect a change, even in the face of overwhelming data. Fourth, despite the above statements about hormesis being broadly generalizable and real, it is not actually seen too often. Its assessment requires stringent and powerful study designs with a large number of doses, above and below the NOAEL, properly spaced, and often with a temporal component. Put quite simply, such studies are in a small minority, thus explaining its low visibility. In fact, only 1%–2% of toxicological studies over the past 30 years have satisfied the needed rigorous entry criteria to even begin to assess whether hormesis exists or not (12). Fifth, the low-dose stimulation is quite modest, being at maximum only approximately 30%–60% greater than controls (4, 5, 12). When one combines the inherent bias against hormesis that denies its existence or rejects its implications with the fact that most studies cannot even study hormesis, it simply reinforces the initial bias.

EVIDENCE FOR THE EXISTENCE OF HORMESIS

In 1996, we received a grant from the Texas Institute for Advanced Chemical Technology (TIACT) at Texas A&M to assess whether the hormesis hypothesis was toxicologically credible. We set forth to make initial judgments on the existence of hormesis based on the conformity of published dose responses to the hormetic β-curve (Figure 1). In order to assess this in an objective manner, we developed a priori criteria based on study design features, quantitative characteristics of the dose response, statistical power, and reproducibility of experimental findings. These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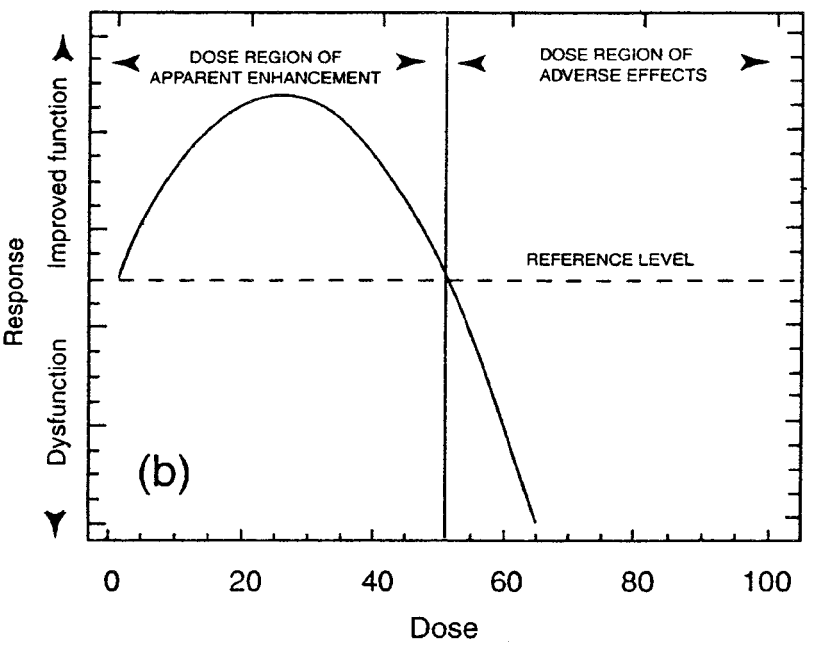
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Dose

concepts were transformed into a mathematical algorithm and then applied to thousands of toxicology investigations (4, 5). We determined that a large number of toxicology studies expressed dose-response relationships of an hormetic-like biphasic nature. These findings revealed that such effects were not only common but seen across chemical class and physical stressor, animal model, age/gender of subject, and biological endpoint, and therefore, broadly generalizable (Figure 2). We also discerned the quantitative features of the hormetic dose response. The amplitude of the hormetic response was inherently modest, almost never exceeding a factor of twofold greater than the control, but usually no greater than 130%–160% of the control. The width of the low-dose stimulatory range was approximately 10-fold, being contiguous with the NOAEL. In general, approximately 70% of the several thousand examples were equal to or less than a factor of 20, whereas 95% were within a 100-fold range. On rare occasions (∼2%), the width of the stimulatory range did exceed 1000-fold (Figure 3) (30, 31).

Although this information was important in establishing the toxicological reality of hormesis and some of its dose-response features, it was legitimately criticized by Crump (32) for not providing a frequency estimate of hormetic responses in the toxicological literature. As a result of these initial limitations, we established rigorous a priori entry and evaluative criteria to assess the frequency of hormesis in the toxicological literature. Over 20,000 articles were evaluated from the mid-1960s to the late 1990s, with only 1.5%–2.0% of studies being able to satisfy entry criteria to assess hormesis as an hypothesis (12). However, of those that did pass the entry criteria (i.e., having an appropriate study design), approximately 40% satisfied the evaluative criteria (i.e., the functional definition of hormesis).

In addition to satisfying entry and evaluative criteria for hormesis, a complementary perspective on the issue of whether the low-dose stimulation could have occurred by random process was devised. Of the nearly 1800 doses below the NOAEL, an assessment was made of the proportion of responses that statistically significantly differed from the control in the direction of hormesis or in the opposite direction. If the responses were random, one would expect that the response would vary similarly for either possibility. However, responses displaying statistical significance in the hormetic direction occurred 32 times more frequently than the opposite! Thus, these findings strikingly support the conclusion that the hormetic responses cannot be explained by random processes (12).

Further extending the evidence on the occurrence of hormesis is that we have recently completed an assessment of the occurrence of hormesis within the NTP

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Figure 1 (*a*) General form of U-shaped dose-response curve showing response relative to a reference level, with a region of apparent improvement (e.g., reduction in dysfunction) as well as a region of toxic or adverse effects. (*b*) Reciprocal of the same curve showing a region of apparent enhancement (e.g., increase above normal level of function) as well as a region of toxic or adverse effects. From Davis & Svendsgaard (13).

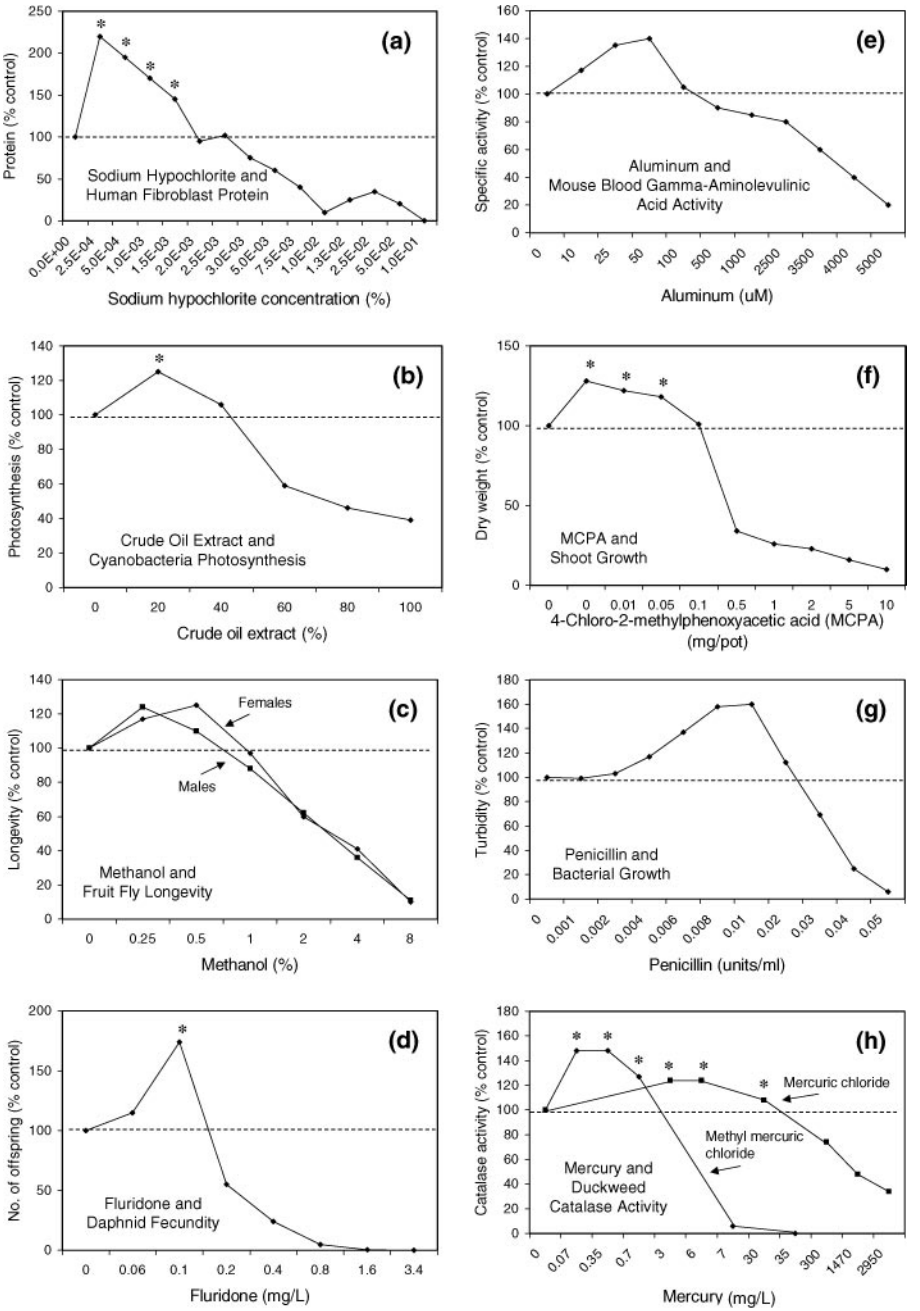

Figure 2 Representative examples of inverted U-shaped dose-response relationships displayed by a variety of experimental models and chemical agents. The asterisks indicate statistically significant data ($^{*} = P \leq 0.05$, $^{**} = P < 0.01$, $^{***} = P < 0.005$). Absence of statistical significance denotes studies that did not perform statistical analyses on their data. Sources of data for (*a*)–(*p*) are References 14–29, respectively.

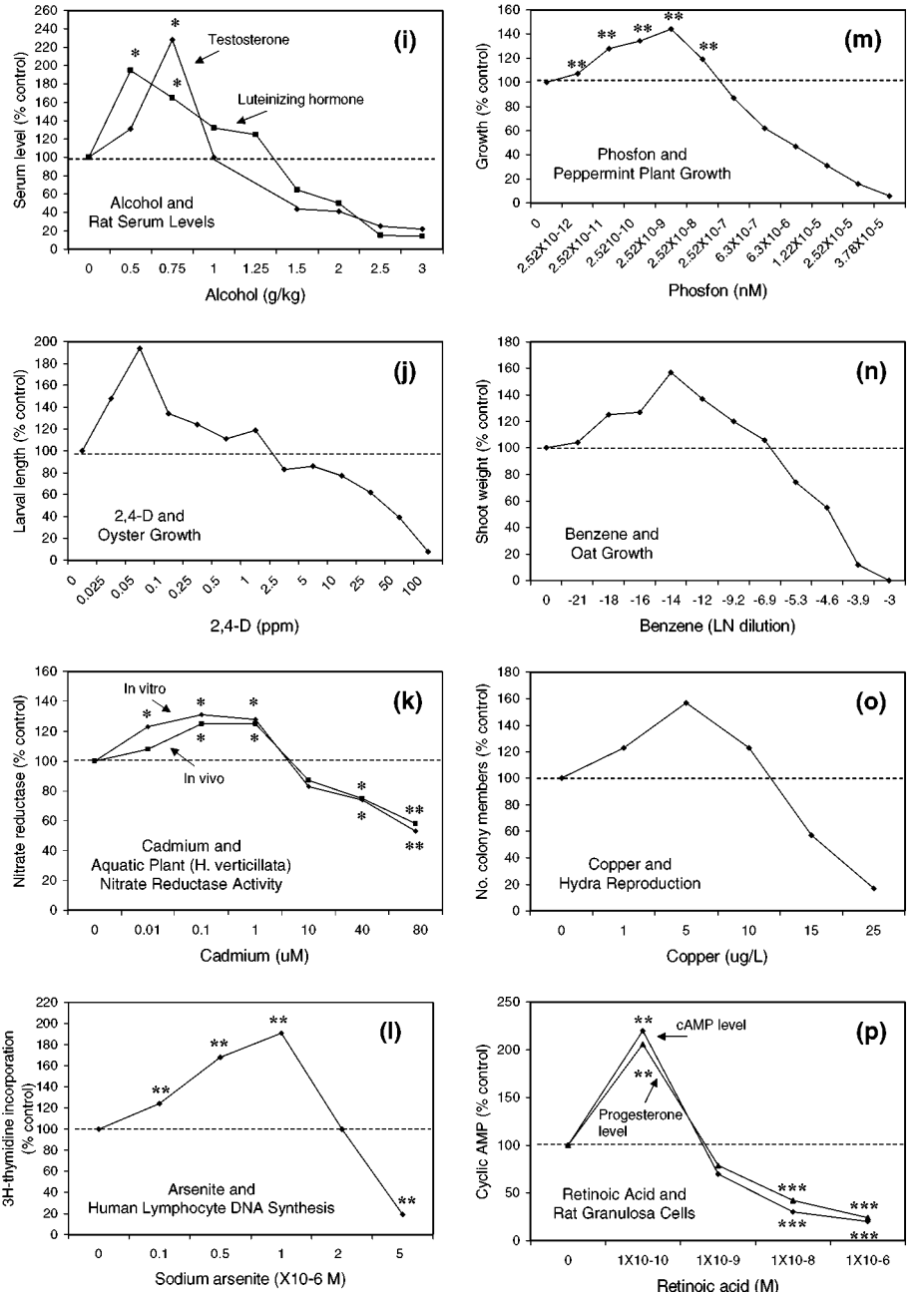

Figure 2 (*Continued*)

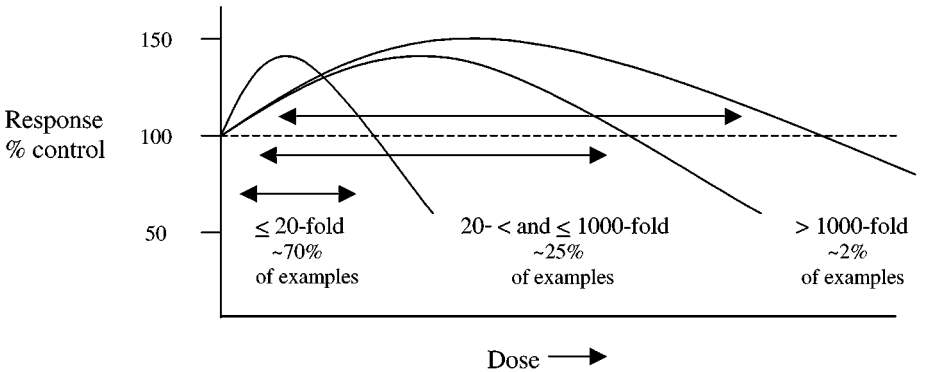

Figure 3 Stylized dose-response curves reflecting the relative distribution of stimulatory dose ranges. Note the maximum stimulatory response is usually 130%–160% of the control value regardless of the width of the stimulatory dose range; the inverted U-shaped curve was used for illustrative purposes only, whereas examples in the hormesis database include both inverted U- and U-(J-) shaped curves depending upon the endpoint measured. Modified from Calabrese (30).

dose-range finding studies (E.J. Calabrese & L.A. Baldwin, in preparation). In this assessment, hormetic responses satisfying our previously employed a priori evaluative criteria (4, 5) revealed hormetic responses in over 60% of studies involving male mice and over 40% involving female mice. These observations are particularly significant because they represent findings from the extensive, carefully overseen and reviewed U.S. government toxicological testing program.

The next criticism affecting the acceptance of hormesis was then put forth by Klaassen (33), who indicated the need to demonstrate underlying mechanism(s) in order for hormesis to gain credibility. To this end, we had obtained evidence, especially in the pharmacological literature, that provides mechanistic explanations to account for many hormetic biphasic dose responses. More specifically, we have evidence accounting for hormetic responses at least to the receptor level, but frequently at levels of further complexity, for nearly 30 different receptor systems (Table 1). In these investigations, we find that investigators typically made use of synthetic agonists/antagonists to dissect and then reconstruct their reported biphasic dose responses (34). It is important to note that such dismantling of the dose response within experimental pharmacology has rarely been reported in the toxicological literature. Thus, the mechanism argument against the hormetic hypothesis, like that of the frequency issue, is no longer tenable. The key conclusion revealed by the numerous mechanistically oriented investigations is that there is no single hormetic mechanism. Each endpoint considered in an hormetic evaluation may be affected by a different receptor system (or by interacting receptor systems). What each mechanism does have in common is the quantitative feature of the dose-response curve; that is, the amplitude and range of the

TABLE 1 A partial listing of receptor systems displaying biphasic dose-response relationships

Receptor systems displaying biphasic dose-response relationships	
Adenosine	Neuropeptides[1]
Adrenoceptor	Nitric Oxide
Bradykinin	NMDA
CCK	Opioid
Corticosterone	Platelet-derived growth factor
Dopamine	Prolactin
Endothelin	Prostaglandin
Epidermal growth factor	Somatostatin
Estrogen	Spermine
5-HT	Testosterone
Human chorionic gonadotrophin	Transforming growth factor β
Muscarinic	Tumor necrosis factor α

[1]For example, substance P and vasopressin.

Abbreviations: CCK, cholecystokinin; 5-HT, 5-hydroxytryptamine (serotonin); NMDA, N-methyl-p-aspartate.

low-dose stimulatory response and its relationship to the NOAEL are strikingly alike regardless of agent, model, and endpoint. This strongly supports the conclusion that the hormetic process represents a common strategy for resource allocation when systems need to respond to low-level metabolic perturbations. Thus, we believe that continuing to search for/demand a single molecular explanation (i.e., toxicological Holy Grail) to account for hormesis is a belief in an incorrect paradigm.

The next argument employed against accepting hormesis (at least as broadly generalizable) is that there is unconvincing evidence that it is operational for mutations and cancer (35, 36). Although there are unique challenges facing hormesis in terms of these endpoints (e.g., typically very high doses in cancer bioassays, limited number of dosages, use of models with very low background tumor incidence), there are a substantial number of cases in the literature that document hormetic responses for the various stages of the process of carcinogenesis, including tumor formation. This is the case for both chemical- and radiation-induced tumors (37, 38). These findings indicate that the concept of hormesis is compatible with the dose response for chemical- and radiation-induced tumorgenicity. The examples discussed here are not trivial cases, but ones that are reported from highly experienced and respected laboratories, passing rigorous peer review in the most highly regarded journals. Especially in the case of radiation, the experiments have been particularly robust, often having many hundreds, sometimes up

to several thousands, of animals/dose, sample sizes far exceeding those employed in the U.S. NTP bioassays. In fact, in the case of Ullrich & Storer (39), 15,562 mice were employed over seven treatments plus control in assessing the effects of gamma radiation of lung tumor incidence in the female RFMf/Un mouse [see (38), Table 1, p. 331].

WHEN IS ENOUGH EVIDENCE ENOUGH?

Although we believe that the accumulated evidence is overwhelmingly sufficient to establish hormesis a secure place in toxicology, including its ample presence in basic toxicology texts, a critical issue is what role should hormesis play in risk assessment. The principal question is whether hormesis should be proven on a case-by-case basis or should it be accepted as a default assumption. To establish hormesis on a case-by-case basis would require a substantial change in how hazard assessment is conducted. It would affect the number of animals/treatment, selection of endpoints to be measured, as well as the specific animal model. It would also affect the need to demonstrate replication of critical findings because hormetic responses are generally modest. The establishment of a case-by-case approach for the acceptance of hormesis for regulatory purposes, while appearing quite rational and the proper path to proceed, would essentially derail the hormesis concept for widespread practical use in risk assessment. The evidence supporting the generalizability of the hormetic model is sufficiently convincing. Of particular importance to the current discussion is that we have demonstrated that the hormetic model occurs with significantly greater frequency as compared with the traditional threshold model, thereby arguing for its acceptance as the principal dose-response default option. This conclusion is further emphasized in practice because biostatistical approaches cannot preferentially distinguish among possible dose-response models for most individual experiments given the limitations in experimental design. Thus, it is necessary to consider data from the broad body of available studies to derive toxicology-based default assumptions. On scientific grounds, therefore, the hormetic model should strongly prevail over its rivals.

In discussion of high-profile carcinogens, such as dioxin and arsenic, the concept of hormesis is often raised. Although there is evidence supporting hormesis in both cases [dioxin (40, 41) and arsenic (42–44)], the more appropriate and defensible position is to require detailed consideration of the broad-based findings on hormesis. To limit the argument for hormesis to a simplified agent only restricts the use of available data and inevitably forces a decision based on a more limited and insecure foundation. Second, the default should incorporate the concept of the most biologically plausible toxicological outcome rather than a philosophy of minimization. As is now being seen in multiple dimensions of the biomedical community, minimization is giving way to optimization for endpoints, such as cholesterol, blood pressure, body weight, exercise, as well as bilirubin and the theoretically important domain of reactive oxygen species (45–47).

THE CONCEPT OF HORMESIS IS STRENGTHENED BY ITS OCCURRENCE IN NONTOXICOLOGICAL FIELDS

The significance of a biological concept is often judged by its generalizability and the extent to which it may affect related disciplines. In this case, the hormetic concept provides numerous applications in multiple areas of the biological sciences as well as providing a basis for theoretical foundations within the broad evolutionary, biological-toxicological-medical continuum. Several examples illustrate the rich generalizability of the hormesis concept.

Experimental Psychology

A well-known "law" in experimental psychology, the Yerkes-Dodson Law, describes a dose-response relationship that is similar in its qualitative and quantitative features to hormesis. Robert Yerkes, the famous Harvard psychologist after whom the Yerkes Primate Center in Atlanta was named, and his graduate student, John Dodson, reported in 1908 that learning performance by rodents was optimized by a modest amount of stress but diminished at either too low or excessive stress (48). Furthermore, these investigations altered the quantitative features of the dose response (i.e., width of the stimulatory enhancing zone) by changing the complexity of the task. This observation is of considerable interest because it provides an experimental model to assess and manipulate a quantitative dimension of the dose response. The Yerkes-Dodson phenomenon has been repeatedly observed over the past century and has been routinely discussed as a general phenomenon in numerous introductory psychology texts (49). More recent investigations have explored the suggestion that such behavior may be related to endogenous alterations in corticosterone concentrations and have reconstructed the biphasic dose response of the more descriptive studies in relationship to endogenous biphasic changes in corticosterone levels (50). The implications of the Yerkes-Dodson Law are substantial, affecting optimal workplace strategies, learning environments, accuracy of eyewitnessing at different levels of stress, and numerous other aspects.

Plant Biology

ALLELOPATHY This area of plant biology studies the effects of root exudates on the surrounding microorganisms and plants. Numerous experimental studies have revealed that the effects of such exudates on a wide variety of species displays hormetic-like biphasic responses (51–53). These observations are noteworthy because they suggest that hormetic effects may be instrumental in affecting the occurrence of primary and secondary succession of ecological systems. Furthermore, numerous investigations have begun to utilize the concept of hormesis within the context of allelopathy to develop natural product-based herbicides. However, in this case the focus would be on the upper (i.e., toxic) end of the dose response.

SYNTHETIC HERBICIDES Numerous synthetic herbicides induce hormetic effects in target plant species. Large-scale screening of chemical agents has consistently revealed the capacity of herbicidal agents to induce biphasic dose-response relationships of an hormetic nature (19, 54–56). The findings that low doses of herbicides can stimulate plant growth have important implications concerning herbicidal drift and their effects on adjacent crops.

Chemotherapy

Numerous chemotherapeutic agents display hormetic responses. These include antibacterials, antivirals, antitumor, and antiangiogenesis agents, as well as agents such as minoxidil, which may stimulate hair growth. The quantitative and temporal features of the dose response for chemotherapeutic agents are similar to that reported for both chemicals and radiation. The clinical recognition of the significance of the hormetic features of the dose response of therapeutic agents has been greatly underappreciated, typically due to the focus on the high-dose functions of the drug, ignoring the low-dose enhancing on the virus, bacteria, fungal, or tumor growth.

The implications of hormetic effects for chemotherapeutics also extend to the domain of peptide biology and its relationship to the human genome. Numerous hormetic-like biphasic dose responses exist for peptides, further displaying the broad generalizability of the hormetic concept. Recent assessments of both chemotherapeutic (57) and peptide (58) examples of hormetic effects have been completed.

RISK ASSESSMENT

In this section, the implications of hormesis are explored in the area of environmental health/toxicology as well as within the more broadly based biomedical sciences.

Hazard Assessment

If hormetic effects are an evolutionary/biological/toxicological expectation, then it implies that hazard assessment strategies include a protocol to assess its possible occurrence. This has practical importance because hormetic effects may affect both the concept and derivation of the NOAEL. The derivation of the NOAEL could change if the low-dose stimulation were determined to be an adverse effect. The hormetic dose-response continuum in this instance (i.e., both the increase at low doses and the decrease at high doses from the control) could be viewed as adverse. However, if the low-dose stimulation were deemed as beneficial, it would have little direct effect on the concept of the NOAEL, but could affect how the traditional NOAEL is derived (59) as well as challenging the basic goal of the risk assessment process from the exclusive focus on the avoidance of potential harm to also include the concept of benefit.

Within a traditional public health framework, beneficial effects may be reasonably and unambiguously identified. For example, such responses could include:

- increased average lifespan
- reduced incidence of tumors
- reduced incidence of birth defects
- reduced incidence of various diseases and illnesses
- enhanced learning and other positive behavior performances.

Adverse health effects would simply be responses opposite to those established as beneficial. However, there would be a number of responses that would be difficult to resolve and classify as beneficial or adverse without more detailed assessment. Such responses could include, but not necessarily be limited to, increased organ weight, increased body weight gain, increased fecundity, and increased immune responsiveness [see (60) for a more detailed discussion of these endpoints in relation to hormesis].

The capacity of assessing hormesis within the context of these endpoints and their public health meaning requires the careful selection of animal model along with appropriate study design. In general, current models and experimental protocols are generally ill equipped to accomplish this essential task.

Although it may be desirable to demonstrate the existence of hormesis for each agent tested for all endpoints of concern, this may be an infeasible objective with respect to time, money, and model limitations. If this were the case, it is recommended that one consider the possibility of accepting the hormetic expectation as a default assumption (60).

Agencies like the EPA commonly employ default assumptions in exposure, hazard assessment, and risk assessment assumptions. In most of these matters, the amount of available evidence is far less than that available for hormesis. Furthermore, our collective information confirms that among the available toxicological models, the hormetic one is the most predominant.

The recognition of the impact of hormesis on the risk assessment process has the potential to broaden study goals of the hazard assessment process. That is, besides defining the upper end of the dose-response continuum [NOAEL-LOAEL-FEL (Frank Effect Level)], additional doses could be directed to defining the subNOAEL response zone. In addition, because hormetic responses are likely to be modest (i.e., no greater than 130%–160% of the control), this would have important implications for sample size and statistical power issues. Likewise, in order to affirmatively address the possibility of hormesis, it is necessary to consider the issue of background disease incidence and animal model selection. In practice, it has been desirable to use models that are reasonably susceptible to develop agent-induced disease while having a low background incidence. Assessing hormesis has only been possible for a few selected endpoints in chronic bioassays, that is, when the background incidence is high (e.g., testicular cancer in the F344 male rat, mammary tumors in the Sprague-Dawley female rat).

Risk Characterization

The risk assessment implications of hormesis are varied, complex, and may be seen whether the context is evaluating noncarcinogens or carcinogens.

NONCARCINOGEN RISK ASSESSMENT In the case of noncarcinogen risk assessment, the current EPA methodology is limited to NOAEL derivation, the application of uncertainty factors (UFs) to derive a reference dose (RfD), and the application of a relative source contribution. The standard derivation process uses a risk management plan that ensures that a regulated agent would not have a harmful effect on the general public as well as most members of the high-risk subsegment of the population. The hormesis concept provides a series of new risk management options to decision makers. In this case, if low-dose stimulatory responses were assumed to be beneficial, the decision maker could view hormesis as adding potential benefit to society and could estimate an optimized population-based exposure standard. Although this could be a complex and, indeed, controversial approach, it is of more than academic interest. It would seek to estimate not a de-minimus risk, but an optimized population-based beneficial dose. The de-minimus risk option that has guided essentially all environmental regulatory agencies places its entire emphasis on avoiding harm, lacking consideration of affirmative benefit. The optimized benefit approach would be based on the concept of hormesis and integrate data from the range of risks and benefits to estimate an exposure standard. A methodology to estimate a so-called optimized benefit dose would need to quantitatively integrate information on the dose-response continuum for the normal and high-risk subsegments of the population, their relative proportions in populations, and the cost-benefit relationship for the relevant endpoints at each dose. Even if this approach were not employed, it would be critical for decision makers to be aware of such information.

CARCINOGEN RISK ASSESSMENT In the area of carcinogen risk assessment, hormesis could have a very significant practical impact. Because the hormetic concept assumes the existence of thresholds at doses higher than the hormetic zone, the acceptance of hormesis could change the current practice of cancer risk assessment. However, it is important to recognize that current EPA cancer risk assessment methodology assumes that the human and animal models are equally susceptible. This is in contrast to the noncancer assessment process in which humans are assumed to be 10-fold more sensitive. Acceptable exposures in the case of noncarcinogens are derived by UFs, whereas in the case of carcinogens, acceptable exposures are derived by conservative low-dose extrapolation modeling. Sielken & Stevenson (61) have provided a detailed consideration of how quantitative risk assessment for carcinogens could be made responsive to the concept of hormesis. Such changes are summarized in Table 2. The fact that hormesis infers thresholds for both cancer and noncarcinogen endpoints and that they display similar quantitative features of the hormetic dose-response continuum

TABLE 2 How quantitative risk assessment could be made responsive to the concept of hormesis [adapted from Sielken & Stevenson (61)]

Recommendations for incorporating hormesis into risk assessment
Dose-response models should have greater flexibility to fit the observed shape of the dose-response data; such models should not be constructed to be forced to always be linearly decreasing at low doses
Hazard assessment evaluations should incorporate greater opportunity to identify the hormetic portion of the dose-response relationship
New dose metrics should be used that incorporate age or time dependence on the dose level rather than a lifetime average daily dose or its analog for a shorter time period
Low-dose risk characterization should include the likelihood of beneficial effects and the likelihood that a dose level has reasonable certainty of no appreciable adverse health effects
Exposure assessments should fully characterize the distribution of actual doses from exposure rather than just the upper bounds
Uncertainty characterizations should include both upper and lower bounds
Risk should be characterized in terms of the net effect of a dose on health instead of a single dose's effect on a single disease endpoint (i.e., total mortality rather than a specific type of fatal disease)

indicates a toxicologically based means to harmonize cancer and noncancer risk assessment.

USE OF HORMESIS TO HARMONIZE CANCER AND NONCANCER RISK ASSESSMENT

The differences in risk assessment methodologies to assess risk from exposure to carcinogens and noncarcinogens are striking. The basic assumption underlying the differences in risk assessment approaches for carcinogens and noncarcinogens is that they display fundamentally different dose-response relationships—one being linear at low doses, the other acting via a threshold. An assessment of the hormesis literature indicates that the dose-response relationship of chemical/radiation-induced cancer responses (Figure 4) and that of noncancer responses (Figure 2) are fundamentally U-shaped. Furthermore, the quantitative features of the dose-response in both instances are similar [e.g., amplitude and range of response, relationships to the zero equivalent point (i.e., ZEP, the highest dose showing a response equal to the control response)]. These quantitative features are also independent of the specific toxicological/pharmacological mechanisms. This observation has been essentially overlooked during the wide-ranging discussions concerning the cancer/noncancer harmonization process. If the fundamental dose-response relationship unity via the hormetic paradigm had been recognized in the mid-1970s, it is likely that an integrated risk assessment framework could have been constructed. Nonetheless, if harmonization is to effectively proceed, regulatory scientists need to address the issue of hormesis.

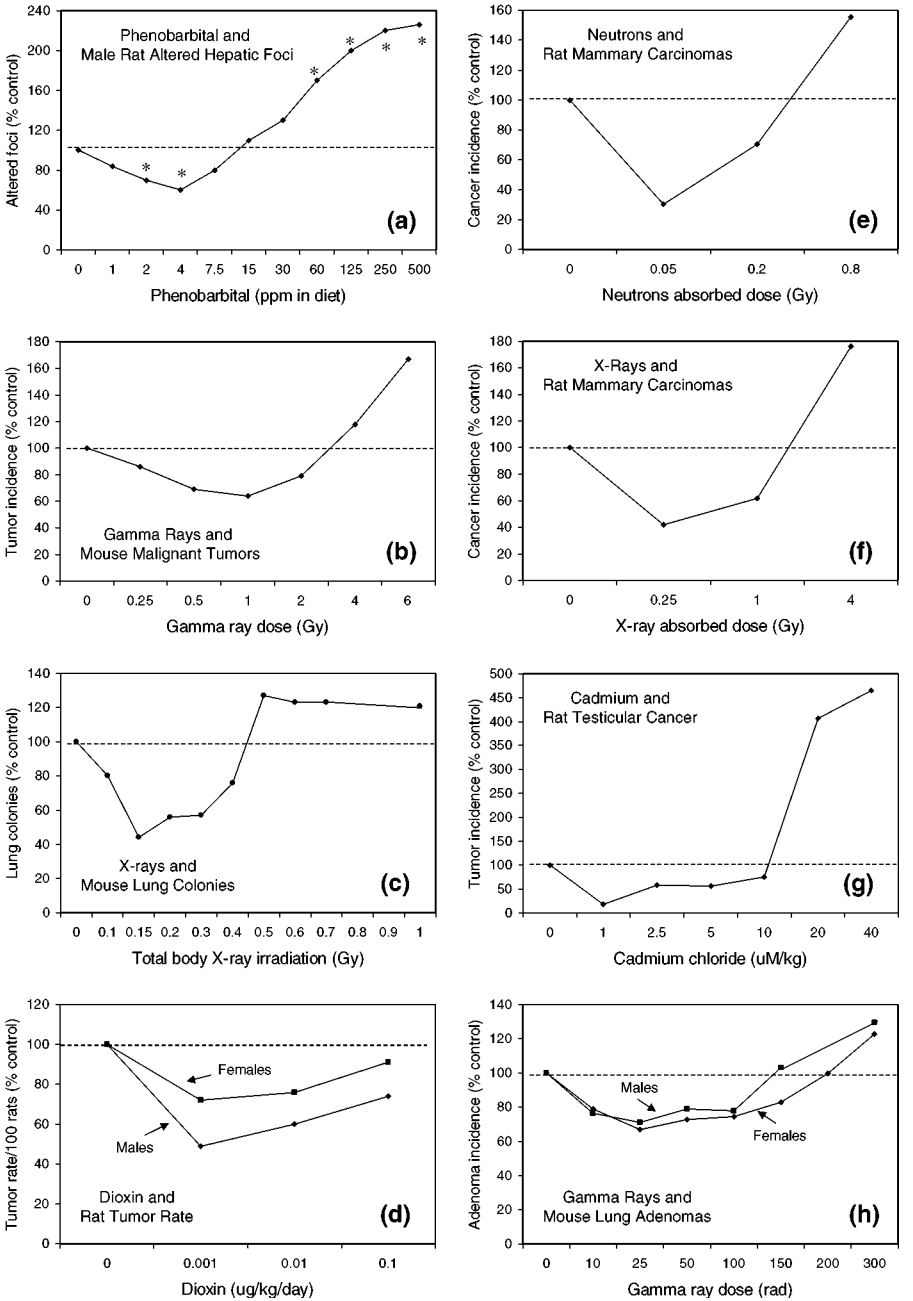

Figure 4 Representative examples of U- (or J-) shaped dose-response relationships of cancer responses induced by various radiation sources. The asterisks indicate statistically significant data ($^{*} = P \leq 0.05$, $^{**} = P < 0.01$). Absence of statistical significance denotes studies that did not perform statistical analyses on their data. Sources of data for (*a*)–(*d*) are References 62–65; for (*e*) and (*f*), Reference 66; for (*g*), Reference 67; for (*h*), Reference 39; and for (*i*)–(*l*), References 68–71.

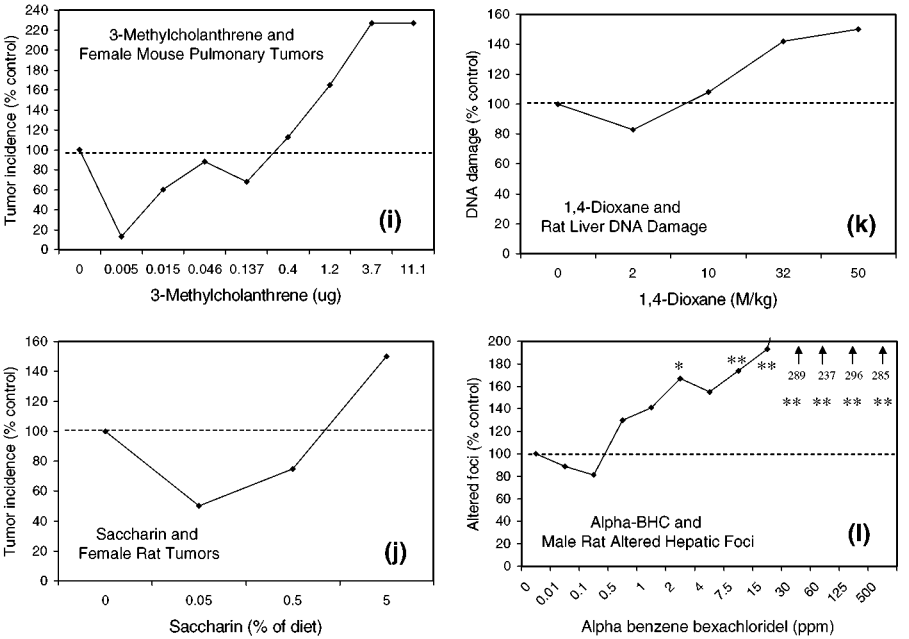

Figure 4 (*Continued*)

Risk Communication

Hormesis presents unique challenges for the discipline of risk communication (72, 73). This is particularly the case when the low-dose stimulatory response is viewed as beneficial (e.g., reduced disease incidence, enhanced longevity). Over the past 30 or more years, the goal of public health and environmental health education has emphasized concepts of nonthreshold for carcinogen responses, thresholds for noncarcinogens using the NOAEL, and no such thing as a beneficial effect from a nonnutritive pollutant. In the case of hormesis, each of these central, public, and environmental health dogmas are turned upside down. Such radical changes in low-dose risk assessment are likely to pose an enormous challenge to the acceptance of hormesis. Acceptance of hormesis will be difficult, therefore, because:

- Agencies will need to accept the possibility (actually, the likelihood) that toxic substances, even the most highly toxic (e.g., cadmium, lead, mercury, dioxin, PCBs, etc.) can cause beneficial effects at low doses.
- Hormesis will likely be seen as self-serving for the chemical industry.
- Elected administrations, whose EPA accepts the concept of hormesis as central to hazard/risk assessment, will be strongly attacked. This recently happened to John Graham in his senate confirmation hearings when he suggested that dioxin may exhibit such characteristics.

- The public may be very confused because the entire educational/public media on environmental issues had always characterized pollutants as harmful.
- Many industries thrive on environmental fears, such as companies featuring asbestos and radon remediation. This would also be the case of soil and other types of remediation technology-driven industries.

Despite these impediments to the acceptance of hormesis (even in the presence of compelling data), there are activities that suggest that the hormesis concept could be embraced by society. The widely accepted and well-established observation that ingestion of a daily glass or two of red wine reduces the risk of cardiovascular disease may have preconditioned society to consider the hormetic hypothesis for pollutants and radiation. In addition, the recognition that anticancer agents can both stimulate and inhibit tumor growth via an hormetic dose response may enhance the clinical interest in this concept.

Legal Implications

The legal implications emerging from the concept of hormesis remain to be more fully explored. However, the concept of hormesis has begun to be addressed by legal scholars (74). Of particular interest has been an exploration of the potential means to incorporate the concept of pollutant-induced beneficial effects in the risk assessment and cost-benefit process. Within this general framework, the District Court forced the EPA to recognize the beneficial effects of ozone pollution on the risk of UV-induced skin cancer in its overall assessment of the health effects of ozone (75, 76).

DISCUSSION

Hormesis is a toxicological concept that has been marginalized for over the past 70 years by several generations of toxicologists, although there is distinct evidence that this is changing. Although there are multiple reasons for this marginalization, the principal explanation results from the emphasis on high-dose testing in the historical and recent past, and the inadequacy of the vast majority of toxicological study designs to assess sub-NOAEL (ZEP) responses. Like most ideas, hormesis will become adopted only if it offers an improved explanation or means to solve problems. Whereas interest in low-dose stimulation in the 1920s, especially for radiation, was often generated by the search for medical elixirs, interest in hormesis in the 1990s was in response to governmental cancer risk assessment methods and policies that adopted the use of low-dose linearity and the linkage of such risk estimates to extremely expensive environmental clean-up standards. Hormesis has been seen as a direct challenge to low-dose linearity because it asserts the existence of thresholds. However, the significance of hormesis involves much more than cancer risk assessment, for it can affect how hazard assessment is performed (e.g., study design, sample size, dose selection, model selection, and which endpoints

are measured). It can also affect noncancer risk assessment, especially with respect to the concept and derivation of the NOAEL.

Hormesis is also influencing much larger biological/ecological and biomedical domains, the extent to which is grossly under-recognized. For example, hormetic-like biphasic dose-response relationships are also seen in essentially all pharmacological receptor systems (Table 1). Recognition that endogenous and exogenous agonists/antagonists display hormetic dose-response relationships may affect not only pharmacological experimentation but also clinical practices. Numerous examples exist that indicate that agents that are antagonists at high doses may become partial agonists at lower concentrations following an hormetic dose response. This implies that the same agent used to treat tumors at high concentrations may enhance their growth at lower concentrations. These dose-response features are critical to recognize. As hormetic-like biphasic dose responses become progressively more recognized and appreciated, they will improve research methods in toxicology, risk assessment procedures, chemotherapeutic methods, and drug development, as well as fundamental insights to evolutionary biology. We believe that the substantial and mounting data in support of the hormetic perspective are in the early stages of affecting such a profound series of changes in the biomedical/toxicological sciences that it will be seen as a true dose-response revolution, affecting a tidal-shift in toxicological perceptions, principles, and activities.

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SIGNAL TRANSDUCTION–DIRECTED CANCER TREATMENTS*

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Key Words protein kinase, ansamycin, farnesyltransferase, phospholipid, monoclonal antibody

■ **Abstract** The pathogenic mechanisms giving rise to cancer frequently involve altered signal transduction pathways. Therefore therapeutic agents that directly address signal transduction molecules are being explored as cancer treatments. Inhibitors of protein tyrosine and threonine kinases including STI-571, ZD-1839, OSI-774, and flavopiridol are ATP-site antagonists that have completed initial phase I and phase II evaluations. Herceptin and C225 are monoclonal antibodies also directed against signaling targets. Numerous other kinase antagonists are in clinical evaluation, including UCN-01 and PD184352. Alternative strategies to downmodulate kinase-driven signaling include 17-allyl-amino-17-demethoxygeldanamycin and rapamycin derivatives, and phospholipase-directed signaling may be modulated by alkylphospholipids. Farnesyltransferase inhibitors were originally developed as inhibitors of *ras*-driven signals but may have activity by affecting other or additional targets. Signal transduction will remain a fertile basis for suggesting cancer treatments of the future, the evaluation of which should include monitoring effects of the drugs on their intended target signaling molecules in preclinical and early clinical studies.

INTRODUCTION

An emerging understanding of the molecular basis of neoplastic cell behavior recognizes that cancer is a signaling disease. Many oncogenes are altered forms of cellular proto-oncogenes, whose expressed proteins normally participate in signal transduction pathways. Negatively acting tumor suppressor genes frequently act to directly modify signaling pathways or are actual substrates for the action of signaling pathways. Thus, it is intellectually satisfying as well as mechanistically well founded that therapeutic interventions taking account of this biology might

have as primary targets the respective signal transduction molecules contributing to the pathogenesis of cancer.

Numerous drugs have been selected to act as potential signal transduction modulators. This article focuses on those drugs that have already advanced to the clinic and have completed at least one phase I evaluation. Reference is made only in passing to biological functions of the signal transduction pathways affected, as these have been addressed in reviews elsewhere. Figure 1 illustrates an overview of the relationships between signaling systems activated in cancer cells and illustrates where many of the agents to be discussed here have been posited to act.

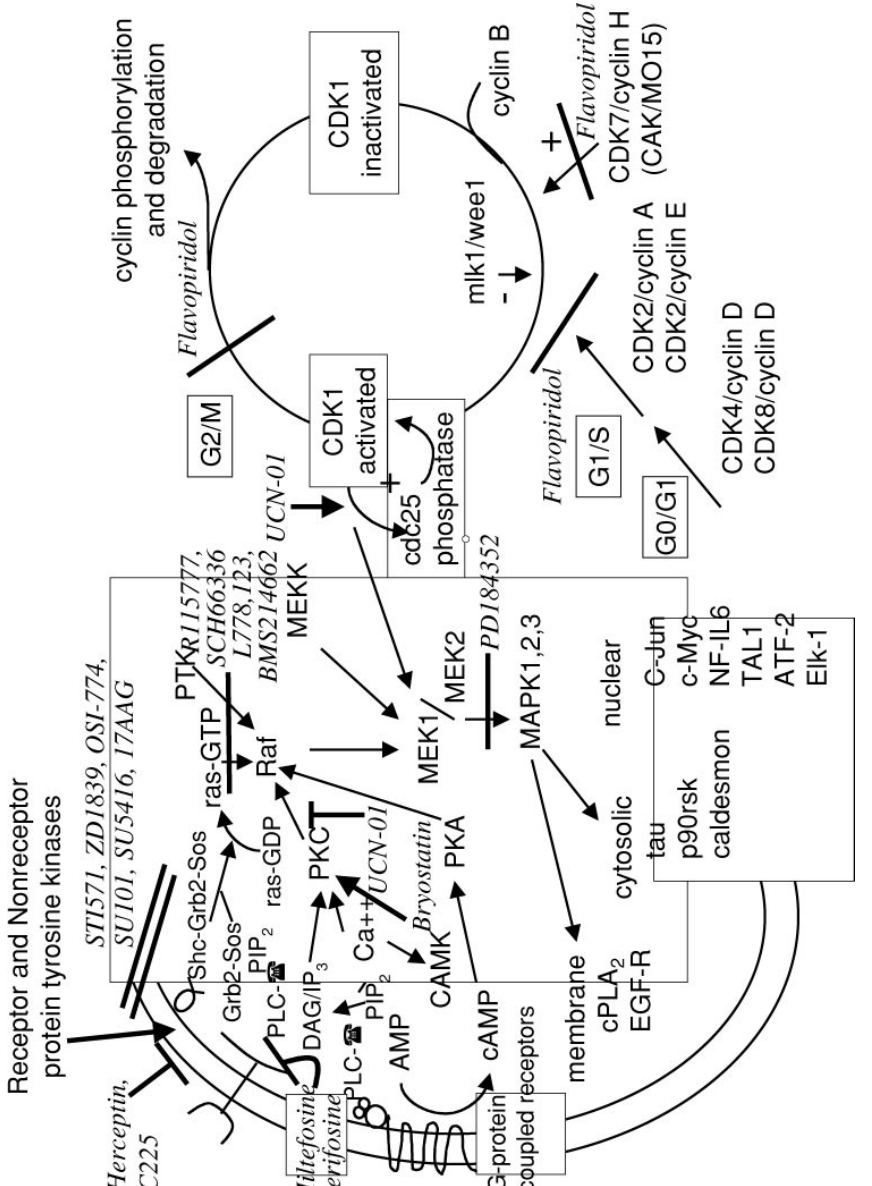
TYROSINE KINASE INHIBITORS

STI-571 (CGP57148B, GLEEVEC®, Imatinib Mesylate)

STI-571 was selected for platelet-derived growth factor (PDGFR) inhibition and later optimized as a potent and specific inhibitor of Abl tyrosine kinase from a series of phenylaminopyrimidines (1). The presence of constitutively active Bcr-Abl tyrosine kinase in chronic myelogenous leukemia (CML) patients and its importance in the pathogenesis of CML provided a convincing rationale to support continued development of the compound. STI-571 inhibited substrate phosphorylation and cellular tyrosine phosphorylation in vitro (at 0.025 μM and 0.25 μM, respectively) (2). Abl (all forms), PDGFR, and c-kit tyrosine kinases are the only kinases known so far to be potently inhibited by STI-571 (3, 4). STI-571 differentially inhibited growth of p210 $^{Bcr\ Abl}$CML and p185 $^{Bcr\ Abl}$ containing acute lymphoblastic leukemia cells (2), without effect on normal marrow cells. Optimal inhibition of tumor growth in animals required continuous exposure (5).

The efficacy of STI-571 was validated in a remarkable series of clinical trials. A mean maximal concentration of 4.6 μM at steady state and a 24-h trough concentration of $\sim$1.5 μM were achieved after a 400-mg once daily oral dose of STI-571 (6), with a terminal half-life of 16.2 $\pm$ 4.4 h (7, 8). Ninety-eight percent of interferon-refractory chronic phase CML patients who were treated with $\geq$300 mg/day achieved hematologic response; complete cytogenetic responses were seen in 13% and major cytogenetic responses in 31%. Fifty-five percent of patients with

Figure 1 Schematic of signal transduction inhibitor action. Growth factors or matrix components stimulate the action of protein kinase receptors or guanine nucleotide binding protein–coupled receptors, respectively. Activation of ras-related signaling pathways occurs through guanine-nucleotide exchange factor regulators such as Sos, or after calcium release and phospholipase activation. Either one can activate Raf, which stimulates MAP kinase pathways through MEK. Ultimately, transcription of factors leads to entry into the cell cycle and activation of cyclin dependent kinases. Drugs discussed in this review indicate where in this sequence of events there is evidence of their action.


Receptor and Nonreceptor protein tyrosine kinases
Herceptin, C225
STI571, ZD1839, OSI-774, SU101, SU5416, 17AAG
Shc-Grb2-Sos
Grb2-Sos
PLC-γ
PIP2
DAG/IP3
Miltefosine Perifosine
PLC-β
IP3
AMP
cAMP
G-protein coupled receptors
ras-GTP
ras-GDP
Raf
PKC
Ca++
UCN-01
Bryostatin
CAMK
PKA
PTK
R115777, SCH66336, L778,123, BMS214662
MEKK
MEK1
MEK2
PD184352
MAPK1,2,3
cytosolic
nuclear
membrane
cPLA2
EGF-R
tau
p90rsk
caldesmon
c-Jun
c-Myc
NF-IL6
TAL1
ATF-2
Elk-1
cyclin phosphorylation and degradation
Flavopiridol
G2/M
CDK1 activated
CDK1 inactivated
cdc25 phosphatase
mlk1/wee1
cyclin B
Flavopiridol
CDK7/cyclin H (CAK/MO15)
G1/S
CDK2/cyclin A
CDK2/cyclin E
Flavopiridol
G0/G1
CDK4/cyclin D
CDK8/cyclin D

myeloid blast crisis responded to STI-571 treatment, and although 70% of patients with lymphoid phenotype, Philadelphia chromosome-positive ALL, or lymphoid blast crisis responded initially, the majority have relapsed since then (8). Similarly remarkable responses were noted in phase II studies of single agent STI-571 in resistant or refractory chronic phase CML (9), accelerated phase CML (10), and patients in myeloid crisis (11).

The effect of STI-571 on c-kit (CD117) positive tumors has generated much excitement owing to the paucity of interventions for these chemoresistant tumors. A significant response of heavily pretreated patients with rapidly progressive gastrointestinal stromal tumors (GIST) to 400 mg of STI-571 (12) and other GIST and soft-tissue sarcomas (13) has led to large phase II studies in c-kit positive GIST (14) and other tumors, with resulting FDA approval as safe and effective treatment for GIST as well as CML.

ZD 1839 (Iressa)

Inhibition of the epidermal growth factor receptor (EGFR) has been of interest owing to the autocrine activation of EGFR and several downstream pathways, such as ras/MAP kinase and STAT-3 transcription factors, in many tumors. The EGFR pathway induces entry into the cell cycle, inhibition of apoptosis, and activation of angiogenesis and motility. EGFR is overexpressed in an extensive range of human cancers including non-small cell lung (NSCLC), colorectal, head and neck, bladder, brain, pancreas, breast, ovary, prostate, and gastric cancers (15, 16). Overexpression of EGFR has been associated with invasiveness, resistance to treatment, and poor outcome in several tumor types (17, 18).

ZD1839 [4-(3-chloro-4-fluoroanilino)-7-methoxy-6-(3-morpholinopropoxy) quinazoline] is a synthetic anilinoquinazoline compound selected as a specific potent inhibitor of EGFR. ZD1839 inhibits EGFR through competitive binding to the ATP-binding site. ZD1839 inhibits EGFR autophosphorylation with an IC_{50} of 0.023–0.079 μM (19). ZD1839 produced supra-additive and enhanced antitumor effects of cisplatin, carboplatin, oxaliplatin, paclitaxel, docetaxel, etoposide, topotecan, ralitrexed, and doxorubicin in several tumor types, which resulted in complete regression in some xenograft tumors (20, 21). Similar responses are seen in combination with radiation (22). An important finding in several studies is the apparent effectiveness of ZD1839 regardless of the levels of EGFR protein (21) or gene expression (23). This raised the possibility of other EGFR-related receptors or additional targets for the drug.

Several phase I and II studies have been completed in humans (24–26). Daily oral administration with doses ranging from 50 mg to 700 mg on 14 of 28 consecutive days or continuous administration for 28 days was examined. With intermittent repeated daily dosing, a median half-life was estimated at 46–49 h allowing for once daily dosing (24, 25), with a mean C_{max} of 0.1–2.2 ng/ml (24). In combination with paclitaxel and carboplatin (27), 500 mg per day of ZD1839 was found to be safe with no change in the pharmacokinetics of either. ZD1839 resulted in partial responses in NSCLC and prostate cancer, and stable disease ($\geq$4 months) in several patients with various tumor types (24–26). Five of 23 Japanese patients

achieved partial response (25). Side effects have been relatively mild and have included secretory diarrhea and rash.

OSI-774 (Erlotinib, Tarceva™)

OSI-774 [6,7-bis(2-methoxy-ethoxy)-quinazolin-4-yl]-(3-ethynylphenyl)amine hydrochloride is another orally active and potent quinazoline derivative inhibitor of EGFR with an IC_{50} of 2 nM (purified EGFR inhibition in biochemical assays) or IC_{50} of 20 nM (EGFR autophosphorylation in intact cells). It reversibly inhibits EGFR-TK through competitive binding to the ATP site. Inhibition of EGFR and its downstream P13/MAPK signal transduction pathways by OSI-774 results in accumulation of $p27^{KIP1}$, cell-cycle arrest at G_1 phase and induction of apoptosis (28); EGFR is more than 1000-fold more sensitive to OSI-774 compared with other tyrosine kinases. Evaluation of pre- and posttreatment biopsy specimens from mice bearing xenograft models treated with OSI-774 revealed marked reduction of phosphorylated EGFR. Combination of OSI-774 with cisplatin produced augmented effects (29).

Hidalgo et al. (30) tested the safety and feasibility of protracted administration of OSI-774 in a phase I study. At the maximum tolerated dose (MTD) for continuous administration (150 mg/day), $C_{max} = \sim 1.2\ \mu$g/ml, without evidence of drug accumulation over 28 days and an elimination half-life of 24.4 ± 14.6 h. Diarrhea was dose-limiting in this study, observed in 86% of patients receiving OSI-774 at 150 mg/day. Other notable toxicities included an acneiform eruption. This study demonstrated clinical benefits, with partial responses in patients with renal cell carcinoma, colorectal cancer, and $\geq$5 months stabilization in colon, prostate, cervical, NSCLC, and head and neck cancers. In a phase II study of OSI-774 in patients with EGFR-positive, platinum-refractory NSCLC, 11% of patients achieved partial response and 34% had stable disease (31). These responses did not correlate with higher percent or intense EGFR staining.

SU101

SU101 (N-[4-(trifluoromethyl)phenyl] 5-methylisoxazole-4-carboxamide) (32, 33) was identified as a potent inhibitor of PDGFR. Activity against a rat C6 glioma model engineered to overexpress PDGFR showed diminished p185 phosphotyrosine content ($IC_{50} = 50$–60 uM) upon PDGF stimulation. Inhibition of PDGF-stimulated events such as ligand-induced DNA synthesis and G1/S cell-cycle progression was observed. More potent growth inhibition was observed in cell lines with detectable PDGFRβ relative to cells not expressing PDGFR (IC_{50} 0.2 to 40 uM versus $>$100 uM, respectively).

Eckhardt et al. reported the initial SU101 clinical experience in 26 patients with advanced solid malignancies and variable PDGF expression (34). Treatment cycles consisted of weekly 24-h infusions for four consecutive weeks followed by a two-week rest. Toxicities were mainly grade 1 and 2 nausea/vomiting, fever, and phlebitis, but two patients with prior exposure to myelosuppresive therapies developed grade 3 neutropenia. A maximum-feasible dose of 443 mg/m^2, and

not a MTD, was defined. This endpoint was chosen because this dose required a 2.6 liter infusion volume, and the next 33% higher dose would have required a 3.3 liter volume infusion. SU101 is metabolized to its primary metabolite SU0020 (A77 1726; N-[4-(trifluoromethyl)phenyl] 2-cyano-3-hydroxyl-2-butenamide) rapidly in human plasma at 37C° via an intramolecular rearrangement. This open-ringed metabolite is antiproliferative through inhibition of dihydro-orotate dehydrogenase (IC_{50} 80 to 200 nM), which is a mitochondrial enzyme crucial to pyrimidine biosynthesis. The relative contribution of the parent and the SU0020 metabolite and the actual mechanism of cell growth inhibition vary with the cell system studied. In the initial phase I trial, the half-lives of SU101 (1.8 h) and SU0020 (19 days), as well as the slow clearance rate of SU0020 (0.42 L/day/m^2), suggested that a minimal fraction of the circulating drug was in the SU101 form. In addition, the preponderance of the SU0020 form confounded identification of PDGF pathway inhibition-specific effects. These pharmaceutic challenges have rendered further development of this drug problematic, but interestingly a partial response was reported in a patient with anaplastic astrocytoma stabilized for $>$9 months.

SU5416

This ATP site antagonist of the vascular endothelial growth factor (VEGF) (Flk1/KDR) receptor was designed following crystallographic studies of the indolin-2-one pharmacophore and the fibroblast growth factor (FGF) receptor tyrosine kinase domain. Lineweaver-Burk analysis revealed SU5416 to be a competitive inhibitor with ATP for the Flk1/KDR and PDGF receptors (Ki 0.16 μM and 0.32 μM, respectively) (35, 36).

The first SU5416 clinical trial enrolled 63 patients and administered the drug intravenously on a twice-weekly schedule (37). At the higher dose levels, nausea, projectile vomiting, headache, and increased liver enzymes were prominent toxicities. Stable disease of greater than 6 months duration, but no objective responses, was seen in patients with a variety of advanced diseases (colorectal, lung, renal, and Kaposi's sarcoma). Pharmacokinetic studies revealed that SU5416 had a large volume of distribution (Vd 22 L/M^2), dose-independent clearance (52 L/h), and a half-life of 50 minutes. Peak plasma levels of 17 μM 1 h postinfusion and detectable levels above 5 μM 2 h postinfusion were reported. In an effort to identify surrogate markers of drug levels and antitumor effects, dynamic contrast MRI was performed to assess vascular permeability, and levels of VEGF, tumor necrosis factor alpha (TNF-α), tissue plasminogen activator (tPA), and coagulation parameters were measured (38, 39). A reduction was observed in vascular permeability of up to 37% after repeated doses in stable disease patients, while patients with disease progression had detectable increases in vascularity. Alternative schedules are being explored, although the occurrence of vascular complications, including thrombotic events, raises the possibility that broad application of this drug may be problematic (40).

17-Allyl-amino-17-Demethoxygeldanamycin (17AAG)

The preceding compounds inhibit tyrosine kinase action by competing with ATP. An alternative strategy to block tyrosine kinase signaling is to decrease the expression of relevant kinases. The benzoquinoid ansamycins herbimycin and geldanamycin were found in the 1980s to reverse the transformed phenotype of cells driven by a number of *src*-related tyrosine kinases (41). Detailed studies revealed that herbimycin and geldanamycin did not directly inhibit *src* family kinases but actually increased the rate of degradation of *src*, *lck*, *erbB*1, and *erbB*2 (42–45). An insightful series of experiments by Whitesell & Neckers elucidated a unifying mechanism (46) by showing that derivatized geldanamycin analogs could bind to the ubiquitously expressed cellular chaperone molecule *hsp*90 (heat shock protein 90).

*Hsp*90 acts to catalyze the proper folding and maturation of many proteins including tyrosine kinases (47). Improperly folded kinases are ubiquitinated and then degraded by the proteosome. Herbimycin and geldanamycin, by binding to *hsp*90, cause the displacement and degradation of the client proteins (48, 49). Structural studies (50) have confirmed that the benzoquinoid ansamycins bind to the amino-terminal domain of *hsp*90, and these studies provided a basis for the observation that derivatives in the 17 position, including 17-allyl-amino-17-demethoxy-geldanamycin (17AAG) retained the ability to modulate *hsp*90 client proteins, while fortunately possessing an improved toxicology profile in comparison to geldanamycin. As numerous proteins in addition to tyrosine kinases are affected, 17AAG might be considered a first generation or prototypic compound. Nonetheless, Rosen and colleagues have demonstrated enhanced susceptibility of cell types expressing *erbB*2 (51), and Vande Woude and colleagues have emphasized the susceptibility of *met*-related signals important for metastasis and invasion to geldanamycin congeners (52). These findings raise the possibility that tumors uniquely driven by some *hsp*90 client proteins, including, interestingly, many tyrosine kinase-driven cell types, would actually be selectively sensitive to the agent. Initial phase I trials on a variety of schedules are ongoing.

Herceptin

Yet another distinct strategy to affect tyrosine kinase signaling emerged through identification of antibodies that can bind to the extracellular domain of signaling molecules and alter their function. The HER2/*neu* gene is a Type I receptor tyrosine kinase encoding a 185-kD surface membrane receptor protein. Activation of HER2/*neu* results in an increase in its kinase activity, thus initiating signal transduction leading to proliferation and/or differentiation. Somatic gene amplification of HER2/*neu* occurs in 25%–30% of human breast cancers, to as many as 50 to 100 gene copies per cell (53, 54). Amplification leads to increased transcription and protein levels. HER2 overexpression is an independent prognostic factor predicting poor clinical outcome in breast cancer (55).

The murine monoclonal antibody Ab4D5 directed against human epidermal growth factor receptor 2 $p185^{HER2}$ specifically inhibited proliferation of human

tumor cells overexpressing $p185^{HER2}$, while having no effect on cells expressing physiologic levels of HER2 (56). Development of human antimurine antibodies (HAMA) that neutralize murine antibodies limited the efficacy of the murine monoclonal antibody. A "humanized" antibody, humAb4D5-1, was constructed containing only the antigen binding loops from Ab4D5, with human variable region framework residues plus a human IgG1 constant domain. Trastuzumab, the engineered recombinant, humanized monoclonal antibody directed against HER2 is now known as Herceptin® (57).

Phase I trials revealed that the dose of trastuzumab (i.v. 10–500 mg single dose or once weekly) could be increased without toxicity and that pharmacokinetics were dose dependent (58). A phase II, single agent trial was conducted in 46 HER2-positive [defined as >25% of cells showing membrane staining on immunohistochemistry (IHC)] metastatic breast cancer patients who had failed prior cytotoxic chemotherapy (59). Objective responses were seen in 5 of 43 assessable patients, including 1 complete remission and 4 partial remissions (overall response rate, 11.6%; 95% confidence interval, 4.36 to 25.9). Duration of response ranged from 1 to >60 months. A second phase II trial (60) combined trastuzumab with cisplatin in 39 HER2-positive (defined as light to strong complete membrane staining on IHC using antibody 4D5) metastatic breast cancer patients who had failed prior cytotoxic chemotherapy. Patients received trastuzumab i.v. on day 1 as a 250-mg initial dose followed by weekly doses of 100 mg for 9 weeks. Cisplatin was administered i.v. at a dose of 75 mg/m^2 on days 1, 29, and 57. Of 37 patients assessable for response, 9 achieved a partial response and 9 had a minor response or stable disease. The median duration of response was 5.3 months. The toxicity profile reflected that expected from cisplatin alone. Mean pharmacokinetic parameters of trastuzumab were unaltered by coadministration of cisplatin. This was a key study that clearly suggested a major role of the antibody in augmenting response to chemotherapy agents.

A randomized, placebo-controlled phase III trial was performed to determine efficacy and safety of adding trastuzumab to chemotherapy in breast cancer. Patients received doxorubicin (60 mg/m^2) or epirubicin (75 mg/m^2) plus cyclophosphamide (600 mg/m^2) as first-line therapy, or if they had received prior adjuvant anthracycline therapy, paclitaxel (175 mg/m^2) (61). The dose of trastuzumab added was initially 4 mg/kg followed by 2 mg/kg weekly. Twenty-eight percent of patients treated with chemotherapy and trastuzumab were free of disease progression at 12 months, compared with 9% of the patients treated with chemotherapy alone. The addition of trastuzumab to chemotherapy was associated with a longer time to disease progression (median 7.4 versus 4.6 months), a higher rate of objective response (50% versus 32%), a longer duration of response (median 9.1 versus 6.1 months), and a longer survival (median survival 25.1 versus 20.3 months). The most significant adverse event observed in studies of trastuzumab was cardiac dysfunction. Thirty-eight out of 143 patients receiving anthracycline plus trastuzumab and 11 out of 91 patients that received paclitaxel and trastuzumab had cardiac dysfunction, whereas 1 out of 95 patients that received paclitaxel alone and 10 out 135 patients that received anthracycline alone had cardiac dysfunction.

These clinical trials led to the approval by the FDA of trastuzumab for use in women with metastatic breast cancer with HER2-positive tumors. The treatment is indicated as a single agent for patients having failed earlier therapy and as first-line treatment for metastatic disease when used in combination with paclitaxel. A key issue in considering how to apply the strategy utilized for approval of Herceptin is understanding whether the effect of the antibody in antibody plus chemotherapy regimens actually derives from signaling, as compared to immunological mechanisms. To date this is an open question.

Cetuximab (C225)

Another antibody-based approach to interdicting tyrosine kinase signaling is represented by cetuximab, a humanized monoclonal antibody directed against the EGFR. MAb225, a murine monoclonal antibody that specifically binds to EGFR, specifically competes with signal transduction initiated by TGF-α (62). Cetuximab (also known as C225) is the human–mouse chimeric version of Mab225, which specifically binds to the EGFR with high affinity, preventing the ligand from interacting with the receptor. Preclinical studies have shown that cetuximab results in cell-cycle arrest as well as apoptosis in different contexts (63, 64). A synergistic effect of cetuximab with cytotoxic chemotherapy has been seen with cisplatin, doxorubicin (65), gemcitabine (66), docetaxel (67), and paclitaxel (68).

Early phase I trials demonstrated that cetuximab displays nonlinear, dose-dependent pharmacokinetics that are not altered by coadministration of cisplatin (69). There were three initial, multi-institutional clinical studies with cetuximab; the first was a single-dose trial, and the two subsequent studies administered the antibody on a weekly basis, either alone or in combination with cisplatin. These studies were conducted in patients with tumors overexpressing EGFR. The single dose trial had 13 patients receiving cetuximab in dose range from 5 to 100 mg/m^2. The weekly dose trials had 17 patients in the single agent arm and 22 patients in the arm with cisplatin. There were only 5 episodes of severe C225-related toxicities among the 52 patients. The most frequent adverse events were fever and chills, asthenia, transaminase elevation, nausea, and skin toxicities. Acneiform rash was seen in 6 patients at doses $\geq$100 mg/m^2. The study was completed through the planned dose levels without reaching a MTD. Two patients with head and neck tumors who received cetuximab at doses of 200 mg/m^2 and 400 mg/m^2 with cisplatin exhibited a partial response. In light of these results, the clinical development of cetuximab is continuing with a number of phase II and III studies.

SERINE/THREONINE KINASE ANTAGONISTS

Flavopiridol

Flavopiridol (L86-8275 or HMR 1275) is derived from rohitukine, an alkaloid isolated from *Dysoxylum binectariferum* (a plant indigenous to India). Flavopiridol

has potent antiproliferative activity and modulates several cell signaling pathways in vitro. In the National Cancer Institute (NCI) 60 cell line anticancer drug screen, flavopiridol exhibits significant in vitro activity against all 60 human tumor cell lines (average $IC_{50} = 66$ nM) (70).

Flavopiridol causes cell-cycle arrest at G_1/S phase transition and G_2/M phase transitions and also slows the progression of the cell cycle through the S phase (71). These findings prompted an evaluation of cyclin-dependent kinases, recognized as responsible for governing the orderly transition from G_2 to M phase (CDK1) and G_1 to S phase (CDK4 or 6 with CDK2). Indeed, flavopiridol inhibits all CDKs known so far ($IC_{50} \sim 100$ nM), inhibiting CDK1, CDK2, and CDK4 with a similar potency (72). Studies with purified starfish CDK1 revealed that flavopiridol competitively inhibits ATP with respect to CDK1 (*K*i 41 nM) and noncompetitively with respect to the substrate peptide (73). Cocrystallization studies confirmed binding of deschloroflavopiridol to the ATP binding pocket of CDK2 with its benzopyran ring occupying the same region as the purine ring of ATP (74). Flavopiridol causes loss of regulatory tyrosine and threonine phosphorylation of CDKs (75), and the loss of threonine 161 phosphate inactivates the kinase.

A broadening of the roles for CDKs in cellular regulation recently emerged from the elucidation that certain CDK family members (including CDKs 7, 8, and 9) also have roles in regulating normal transcription. For example, CDK7 is a member of the transcription factor TFIIH complex, and CDK9 corresponds with cyclin T to the transcription factor pTEFb. Flavopiridol can inhibit CDK9 activity potently and in a mechanistically unique way (76, 77), consistent with its formation of a "tightly bound" drug-enzyme complex. This may be the basis for the observation that at certain concentrations, flavopiridol can potently affect the levels of rapidly turning over mRNAs (78) and can serve as a basis for magnifying its capacity for cell-cycle arrest by direct transcription repression of cyclin D1 mRNA (79). This possibility is noteworthy because in mantle cell lymphoma, which overexpresses cyclin D1 in 95% of cases, flavopiridol was able to produce significant delay of disease progression in 84% of patients (80).

Preclinical studies of flavopiridol revealed wide differences in growth inhibition between cell types depending on the duration of exposure and concentration of the drug. Significant cytostasis is observed when flavopiridol is administered in protracted fashion to colorectal (colo 205) and prostate (LnCap/DU145) carcinoma xenograft models (70, 81). Shorter "bolus" administration of flavopiridol to a lymphoma/leukemia (HL60) cell line had a higher degree of apoptosis and cytotoxicity (82).

Two phase I clinical trials have tested the regimen of 72-h continuous infusion every two weeks in humans (83, 84). Seventy-six patients were treated in the NCI phase I trial. The dose-limiting toxicity (DLT) was secretory diarrhea with a maximally tolerated dose (MTD) of 50 mg/m^2/day for 3 days. Flavopiridol was later found to induce chloride ion secretion in intestinal epithelial cells (85) and to have an enterohepatic circulation that may play a role in potentiating this toxicity (86). With antidiarrheal prophylaxis a higher MTD is reachable at 78 mg/m^2/day for 3 days with orthostatic hypotension as the DLT. At the MTD in this study,

the mean steady state concentration (C_{ss}) was 271 nmol/L, well within the range needed to inhibit CDKs and cell proliferation.

Bolus schedules of flavopiridol administration over 1 h were employed to reach higher peak concentrations (87). This study reached an MTD of 37.5 mg/m^2/day for 5 days every three weeks. Mean C_{max} achieved was $\sim$2 μM (1.31–4.2 μM). Although diarrhea and proinflammatory syndrome remain common, bone marrow suppression and neutropenia emerged as a DLT. In some studies other toxicities included thrombosis. Although some cases of partial responses and stable disease have been reported in various phase I studies, several phase II studies revealed few conventionally defined responses in several tumor types, with the possible exception of mantle cell lymphoma (80, 88–90). A more promising role for flavopiridol is envisioned in combination with other agents, including taxol (91), irinotecan (92), and gemcitabine (93), as well as other signal transduction modulators (94–96).

UCN-01 (7-OH Staurosporine)

Staurosporine, a natural product isolated from *Streptomyces staurosporeus*, is a relatively broad, nonspecific protein kinase antagonist, originally isolated in an effort to define inhibitors of protein kinase C (PKC). 7-OH staurosporine (UCN-01) was defined as a more selective, but not specific, PKC antagonist. UCN-01 inhibits "classic" lipid and calcium-dependent PKCs α, β, and γ ($IC_{50} =$ 4–30 nM), Ca^{2+}-independent PKCs * and less potently (IC 50 approximately 500 nM), and is without effect on the atypical PKCs, e.g., PKC ζ (97).

Two prominent effects of UCN-01 have emerged in preclinical studies in vitro: induction of cell-cycle arrest, and abrogation of the checkpoint to cell-cycle progression induced by DNA damaging agents. UCN-01 inhibited cell growth in several in vitro and in vivo human tumor preclinical models (98); however, antiproliferative activity on the part of UCN-01 cannot be explained solely by inhibition of PKC. First, in cell-cycle analyses UCN-01 inhibits Rb^+ cells at G1/S phase of the cell cycle (99). In addition, cells treated with various concentrations of UCN-01 showed decreased pRb phosphorylation in a dose-dependent manner (100). These results suggest that CDK2- or CDK4-regulated steps are targets for UCN-01-induced cell-cycle arrest. As immunoprecipitated CDK1 and CDK2 activity are only moderately susceptible to inhibition by UCN-01 ($IC_{50} =$ 300–600 nM) (101), UCN-01 potentially acts on targets "upstream" of CDKs 1 and 2 rather than directly on the CDKs.

UCN-01 abrogates the DNA damage-induced checkpoints to cell-cycle progression in G2 (102, 103) and in S phase (104). It is noteworthy that these effects were apparent at drug concentrations that appeared to have little direct effect on cell proliferation or that caused enhanced cytotoxicity by clonogenic or proliferation assays. In addition, they provided a mechanistic framework for prior observations that DNA-damaging agents such as mitomycin (105) could greatly potentiate UCN-01 action. Subsequent studies documented that numerous DNA-damaging agents including radiation (103), 5-fluorouracil (5-FU) (106), camptothecin congeners

(104), and temozolamide (107) appeared to act supra-additively with UCN-01 in effecting cytotoxicity. Notably, microtubule-directed agents did not appear to have toxicity enhanced by UCN-01 (108).

A biochemical basis for G2 checkpoint abrogation emerged from the early observation that UCN-01-mediated loss of G2 checkpoint function was accompanied by its ability to decrease CDK1-associated Tyr phosphorylation (101). This phosphorylation is governed by the *wee*1 and *mik*1 kinases, in conjunction with cdc25C phosphatase. Yu et al. (109) demonstrated that UCN-01 mediated G2 checkpoint abrogation requires functional CDK1 and identified that UCN-01 action results both in inhibition of *wee*1 kinase and activation of cdc25C phosphatase. Graves et al. (110) extended these studies by providing evidence to support the idea that cdc25C phosphatase activation resulted from direct, potent inhibition of *chk*1, whose physiologic function is to regulate cdc25C Serine 216 phosphorylation and consequent suitability for 14-3-3 sequestration in the cytoplasm. At the same time, Sarkaria et al. (111) and Busby et al. (111a) also demonstrated potent inhibition of *chk*1 by UCN-01.

Following completion of safety testing in animals, and with demonstration of antitumor activity in its own right reviewed elsewhere (112), UCN-01 entered into initial human phase I clinical trials, administered as a 72-h continuous intravenous infusion every two weeks (113, 114). Surprisingly, and in contrast to studies in animals, UCN-01 displayed avid binding to human plasma proteins, apparently to the α1-acid glycoprotein (AAG). This resulted in a very long half-life and required adjustment of the administration schedule so that the drug was administered once per month, with second and subsequent courses of therapy consisting of 50% of the dose given during the first course. On this schedule, a maximal tolerated and recommended phase II dose of 42.5 mg/m^2/day for 3 days was elucidated. DLTs observed at higher doses included hyperglycemia, acidosis, and pulmonary adverse events, without neutropenia or thrombocytopenia. One partial response occurred in a patient with melanoma, and a protracted (>4 year) period of stabilization of minimal residual disease was observed in a patient with *alk*(+) anaplastic large cell lymphoma. Significantly, despite protein binding, salivary levels of drug (saliva contains low concentrations of AAG) consistent with ability to modulate *chk*1 were achieved.

BRYOSTATIN

The bryostatins represent a large family of secondary metabolites produced in extremely small amounts by the marine invertebrate, *Bugula neritina* of the phylum Ectoprocta (115). The various bryostatins are distinguished by varying side chains off the macrocyclic lactone ring structure. Despite this close structural relationship, these nontumor-promoting PKC activators have different biologic activities and spectrum of toxicity (116, 117). Bryostatin 1 (Bryo 1) is the prototype of this 17-member family and the most extensively studied in humans. Initial isolation of Bryo 1 was based on its antineoplastic activity against the murine P388 lymphocytic

leukemia. Bryo 1 is a potent and rapid activator of PKC; however, unlike other PKC activators, including phorbol myristate acetate (PMA), Bryo 1 lacks tumor-promoting capabilities.

The first two published phase I trials evaluated Bryo 1 administered as a 1 h intravenous infusion (118, 119). Bryo 1 was given every 2 weeks for a maximum of 3 cycles; doses were escalated in steps from 5 to 65 μg/m^2 in successive groups of patients. The DLT was myalgia, occurring approximately 48 h after treatment and lasting up to several weeks at the highest dose levels (65 μg/m^2/dose). The MTD was 50 μg/m^2, and the recommended dose for phase II trials was 35 to 50 μg/m^2 every two weeks. The other trial (119) evaluated three different regimens of Bryo 1 and also concluded that the DLT was myalgia, and the recommended dose for phase II is 25 μg/m^2 weekly for 3 weeks, repeated every 4 weeks. In this phase I study, partial responses were observed in two patients with malignant melanoma, which lasted 6 months and 10 months. Plasma levels of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) increased 2 h and 24 h after treatment, respectively, and were dose related.

RAPAMYCIN CONGENERS

Rapamycin (Sirolimus, Rapamune) is a macrolide fungicide that binds intracellularly to the immunophilin FKBP12, and the resultant complex inhibits the activity of a 290-kDa kinase known as mammalian target of rapamycin (mTOR). Rapamycin is isolated from the bacteria *Streptomyces hygroscopicus* and is found to have potent antimicrobial and immunosuppressive properties (120). Sirolimus was approved by the FDA for prevention of allograft rejection after organ transplantation (121). Further studies with rapamycin revealed significant antitumor activity (122). This is understandable given the importance of mTOR in mitogenic cell signaling. mTOR is a kinase member of PI3K-related kinase family that is activated in response to growth signaling through the PI3K/Akt pathway. Activation of mTOR results in increased translation of several critical cell-cycle regulatory mRNAs through two downstream effector kinases, p70S6K and 4E-BP1/PHAS (123, 124). Rapamycin causes G_1 cell-cycle arrest by increasing the turnover of cyclin D1 (125), preventing upregulation of cyclins D3 and E (126), upregulating p27^{KIP1}, and inhibiting cyclin A-dependent kinase activity (127). Blockage of mTOR function results in inhibition of PI3K/Akt-mediated proliferative signals and cell-cycle arrest. Mutation of PTEN has also been implicated in chemoresistance of these tumors. Rapamycin treatment of PTEN-deficient PC-3 prostate cancer cells reverses this resistance to doxorubicin (128). Several analogs of rapamycin have been selected for further development as anticancer agents.

CCI-779, an ester of rapamycin, has significant antiproliferative effect and favorable toxicology profile and is being studied in several phase I clinical trials in human cancer (129, 130). In these phase I studies, CCI-779 was administered on a weekly or daily schedule for 5 days every two weeks. Toxicities observed

with CCI-779 treatment included hypocalcemia, neutropenia, thrombocytopenia, mucositis, hypertriglyceridemia, rash, reversible decrease in testosterone levels in men, and allergic reactions. A median half-life of 17.3 h was documented (130). Several partial responses have been documented in renal cell carcinoma, NSCLC, neuroendocrine tumors, and breast cancer, in addition to minor responses or stable disease in several tumor types (129, 130). RAD001, an orally bioavailable hydroxyethyl ether derivative of rapamycin, also has potent activity against various animal xenograft models of human tumors; an antiangiogenic effect may account in part for its antiproliferative properties (131).

MEK Inhibitor PD 184352

The stimulation of Ras-mediated signal pathways results in a cascade of downstream kinase activation including Raf, which phosphorylates two distinct serine residues on the dual-specificity kinase MEK (MAP kinase kinase) (132). MEK, in turn, activates and exclusively phosphorylates two subsequent kinases, ERK1 and ERK2 (MAPK), on specific tyrosine and threonine residues within each kinase. These kinases phosphorylate a variety of substrates including transcription factors critical to cell proliferation and tumor invasion (e.g., 133). Constitutive activation of MEK leads to cellular transformation and may be involved in apoptosis, differentiation, and angiogenesis. The substrate selectivity exhibited by MEK distinguishes the kinase as a potentially important pharmacologic target.

Screening for small molecule MEK inhibitors was implemented with an in vitro assay in which MEK activation of MAPK resulted in a quantifiable phosphorylation of myelin basic protein (134). This assay identified PD184352 as a MEK inhibitor with an IC_{50} of 17 nM. Biochemical analyses supported inhibition by an allosteric mechanism without interference with the ATP binding site or the MAPK site on MEK. A dose-dependent G_1 cell-cycle arrest and reversal of transformed morphology (from rounded to flattened appearances) were subsequently demonstrated with PD184352 exposure (135). Interestingly, in cytotoxicity studies, correlation between sensitivity to PD184352 and increased activated MAPK levels was observed in some cells—in particular, colon cancer cells. Higher levels of MAPK activation were observed in colon tumor tissue versus normal mucosa as this event occurs late in colon carcinogenesis (135). In mice with colon 26 xenograft model treated with PD184352, excision and assay of tumor cells revealed diminished phospho-MAPK levels. After drug withdrawal, a return to baseline levels was observed reflecting the cytostatic nature of the inhibition. The pharmacodynamic measurement of activated MAPK in tumor tissue may be used as a biological marker of drug activity as antibodies specific for phosphorylated MAPK are available.

Miltefosine and Perifosine

Certain alkylphospholipids (ALP), for example, Rac-1-O-octadecyl-2-O-methyl-glycero-3-phosphocholine (ET-18-OCH_3, edelfosine) when given to mice prior

to transplantation of Ehrlich ascites carcinoma cells, effectively prevent growth of this tumor (136). Enhancement of immune defense against tumor cells was initially considered a plausible mechanism and has been demonstrated on multiple occasions by a number of ALP analogs. For example, maturation and activation of macrophages have been seen in in vitro and in vivo experiments (137).

Edelfosine is also able to induce apoptosis in HL60 leukemic cells, even in low concentrations and after short incubation times. In U937 leukemic cells, the compound induced apoptosis rapidly, whereas in epithelial HeLa tumor cells this induction required prolonged times of treatment (138). Octadecyl-(1,1-dimethyl-4-piperidino-4-yl) phosphate, perifosine, induced intranucleosomal DNA fragmentation in human squamous cell carcinoma KB cells and induced activation of caspase 3–like protease; pretreatment with the caspase inhibitor Z-Asp-CH2-DCB inhibited perifosine-induced apoptosis and activation of caspase 3–like protease. However, the mechanism of activation of caspase 3–like protease is unclear; it is speculated to occur through the interference with molecules working in intracellular signal transduction pathways to activate caspases and cause apoptosis.

Initial experiments suggested that protein kinase C (PKC) was the target for ALP analogs (139). However, some ALP analogs that are equally potent antiproliferative agents to miltifosine do not affect PKC in cell-free extracts. Thus, a direct inhibition of PKC does not appear as a prerequisite for antitumor activity of these agents. However, all ALP analogs studied so far cause an indirect inhibition of PKC, most likely as a result of the reduced formation of diacylglycerol through inhibition of phospholipase C (139, 140). Additional antiproliferative mechanisms could involve altered growth factor receptor function, as well as recent evidence of p21 induction by an as yet undefined pathway (142) irrespective of p53 function.

Several phase I and phase II studies were initiated using oral formulations of miltefosine, but gastrointestinal intolerance was observed. Because of the hemolytic tendency of alkylphosphocholine analogs, intravenous application of miltefosine was not possible. In view of the high in vitro cytotoxic activity of miltefosine and its low toxicity against normal cells, development as a cutaneous preparation was pursued. Eight phase I–II studies, consisting of 443 patients using topically applied miltefosine 2%–8% for skin metastases in patients with breast cancer, showed a median response rate of 38% (range 12%–50%) (143–145). The response rate was found to be higher in patients with multiple small nodular or purely superficial infiltrations (rate: 41%) than in patients with predominantly large tumorous nodes that infiltrate the skin (rate: 15%). Evidence from the trials led to the approval of miltefosine, licensed as Miltex©, in Germany for the treatment of cutaneous breast cancer and cutaneous lymphomas.

The heterocyclic alkylphosphocholine derivative octadecyl-(1,1-dimethyl-piperidino-4-yl) phosphate (D-21266; perifosine) was developed and selected for improved gastrointestinal tolerability in animal experiments. A number of phase I studies are presently ongoing in Europe and the United States; early evidence

points to better tolerability and less gastrointestinal toxicity when given as a loading dose followed by a low maintenance dose (146).

Proteosome Inhibitor PS-341

The proteasome, a multicatalytic protease responsible for degradation of most proteins with the cell, has emerged as a new target for anticancer drug development. The 20S proteasome is involved in the degradation of several cell-cycle regulatory proteins such as cyclins (A, B, D, E), cyclin-dependent kinase inhibitors ($p21^{WAF1/CIP1}$ and p27), oncogenes (c-fos/c-jun, c-myc, N-myc), and p53 and regulatory proteins (IκB, p130) (147). Inhibition of the 20S proteasome pathway, therefore, aims at altering the cell cycle to promote apoptosis (148). Although the proteasome is present in all cells, transformed and dividing cells are most sensitive to its inhibition (149).

PS-341 is the first proteasome inhibitor to enter human trials. It is a boronic acid dipeptide that specifically inhibits the 20S proteasome presumably through the stability of a boron-threonine bond that forms at the active site of the proteasome. It was found to have substantial cytotoxicity against a wide range of human tumor cells in the NCI 60 cell line anticancer drug screen (150). The antitumor activity of a series of PS-341 analogs positively correlates with their respective ability to cause proteasome inhibition. PS-341 causes accumulation of cyclin A, cyclin B, $p21^{WAF1/CIP1}$, and wild-type p53 and arrests the cells at the S and G_2/M phases followed by nuclear fragmentation and apoptosis. PS-341 significantly inhibited NF-κB DNA binding and functional reporter activity (151).

More than 90% of PS-341 is rapidly removed from the vascular compartment within 15 min of IV administration (152), and therefore the degree of 20S proteasome inhibition in whole blood was adopted as a surrogate marker for the drug activity (153). Animal studies demonstrated significant toxicities when the proteasome is greater than 80% inhibited. Proteasome activity returned to baseline level within 48 to 72 h of treatment.

Several phase I studies evaluated various schedules of PS-341 administration. At the MTD recommended for phase II studies (1.25 mg/m^2–1.3 mg/m^2), a 65%–72% inhibition of 20S proteasome was achieved (154, 155). An average 54% inhibition of proteasome was achieved in patients' tumors (156). In these phase I studies several patients achieved partial responses and disease stabilization including a bronchoalveolar NSCLC, melanoma, sarcoma, lung adenocarcinoma, and malignant fibrous histiocytoma. The major toxicities observed with PS-341 treatments were painful neuropathy, diarrhea, fatigue, orthostatic hypotension, nausea, vomiting, fever, and thrombocytopenia. Patients usually had more toxicity with the second cycle of treatment. Currently several phase II clinical trials are evaluating PS-341 as a single agent in hematologic malignancies, neuroendocrine, renal cell, melanoma, breast, brain, pediatric tumors, and several other solid tumors. Significant antitumor effects were documented in a phase II study of PS-341 in refractory multiple myeloma (157).

FARNESYL TRANSFERASE INHIBITORS

Ras genes are mutated in 30% of all human cancers with K-Ras being the most common. This family of genes encodes GTP binding proteins important in malignant transformation, cell growth, and intracellular signal transduction. Normal ras binds GTP and in the GTP-bound state interacts with numerous effectors including the *raf* proto-oncogene kinase and phosphatidyl-inositol 3-kinase. Its intrinsic GTPase activity terminates the signal. Three isoforms, Harvey(Ha), Kirsten(K), and N-isoforms have been described, with mutation of the GTPase of the K isoform resulting in a persisting signaling capacity in approximately 20% of human epithelial tumors. N-ras is mutated in a smaller proportion of malignancies, predominantly leukemias. Ras function requires lipophilic anchorage to the cell membrane by lipid prenylation. This requires posttranslational modification or covalent thioether bond formation between a farnesyl group (C15) and a cysteine residue at the ras carboxy terminus. The cysteine lies within a four-amino-acid consensus sequence or CAAX motif (where C = cysteine, A = any aliphatic amino acid, X = serine or methionine), and this prenylation reaction is mediated by the enzyme farnesyl protein transferase (FT). Another important enzyme in protein prenylation is geranylgeranyl transferase (GTT), which can catalyze addition of geranylgeranyl (C20) group to the CAAX motif. The two enzymes exhibit substrate selectivity with FT preferring CAAX-containing proteins ending with Ser, Met, or Gln, whereas GTT targets proteins ending with Leu. The lack of substrate specificity is evident as compensatory geranylgeranyl prenylation of K-Ras and N-Ras by GTT occurs after FT inhibition. This "GTT shunt pathway" maintains K-Ras in an active prenylated, membrane-bound form and explains in part the requirements for higher farnasyl transferase inhibitor (FTI) dose or cotreatment with a GTT inhibitor for significant growth inhibition in K-Ras models (158). Several classes of FTIs have been developed in an initial effort to define inhibitors of Ras function and, in general, compete with the enzyme substrates, the CAAX tetrapeptide, and farnesyl pyrophosphate (FFP). The CAAX competitors are generally peptidomimetic agents that mimic the carboxy terminal portion of the Ras protein. "Bisubstrate" inhibitors have also been evaluated (159).

L-778,123

L-778,123 is a competitive peptidomimetic CAAX analog with a Ki of 1.6 nM for H-ras and 0.3 nM for N-Ras. As with other FTIs, it is capable of reversing anchorage-independent growth of ras-transformed Rat1 fibroblasts. Activity against various cell lines has been reported including K-Ras harboring cells with an IC_{50} of 2 to 5 μM. In available preclinical studies, toxicities included myelosuppression and an increase in the QT interval (QTc).

The initial experience in humans was reported in a study of 25 patients. Patients with history of significant cardiac dysrhythmias or retinal disease were excluded. Concomitant use of medications capable of causing dysrhythmias or CYP3A

induction was not allowed. The drug was administered as a 24-h continuous infusion daily for 7 days every 3 weeks. DLT was observed at 1120 mg/m^2/day with one patient experiencing a 30% QTc prolongation whereas another had grade 4 thrombocytopenia. Grade 3 fatigue was noted in two patients treated at this level. Eight patients treated at 560 mg/m^2/day experienced no DLTs, and this dose level was defined as the phase II recommended dose. Pharmacokinetic studies revealed that at this dose a biologically relevant steady state concentration of 8 μM was attained and exceeded the IC_{50} values for the human cell lines evaluated in cell culture studies. No objective antitumor effects were observed, but evidence of an effect on the prenylation status of a marker protein (HDJ2) was obtained. At the recommended phase II 520 mg/m^2/day dose, the percentage of unprenylated HDJ2 increased from 1% to 30% by day 4 and remained at that level through day 8. One week after the infusion was stopped, pretreatment levels of unprenylated HDJ2 were restored (160, 161).

BMS-214662

BMS-214662 is an imidazole-containing tetrahydrobenzodiazepine, which lacks a thiol moiety or a peptide backbone (162). It is a competitive FT inhibitor and was identified by in vitro screening assay using purified recombinant FT. The degree of enzyme inhibition depended on whether H-Ras or K-Ras was used as the farnesyl substrate as fivefold-less potent inhibition was observed with H-Ras versus K-Ras (IC_{50} 1.3 nM and 8.4 nM, respectively) (163). In contrast, inhibition of GTT required 1000-fold-higher concentrations (IC_{50} H-Ras 1.3 uM and K-Ras 2.3 μM). BMS-214662 also blocks activated ras-mediated events in cell-based studies leading to morphologic reversion to flat monolayer cells and inhibition of anchorage-independent growth. Despite inhibiting soft agarose growth of both H-Ras and K-Ras transformed cells (IC_{50} 0.0025 uM versus 0.3 μM), the compound did not induce the reversion of K-Ras-dependent cells. In a panel of murine and human cell lines from a variety of tumor types, BMS-214662 exhibited extensive activity with a mean IC_{50} of 0.20 μM. Importantly, a lack of correlation between Ras mutational status and cytotoxicity was noted with two of four sensitive cell lines (A431 and OVCAR-3) not expressing Ras mutations. BMS-214662 exhibits nonspecific cytotoxicity at doses higher than 2 μM (164).

The initial clinical reports with BMS-214662 have used various schedules of administration. The drug was initially given intravenously every three weeks and as a single oral dose during cycle 2 (165). Thirty-eight patients were evaluated, and a 225 mg/m^2 intravenous dose and 168 mg/m^2 oral dose were reached. Elevation of transaminases, nausea, vomiting, and diarrhea were observed requiring expansion of the 225 mg/m^2 dose level. Linear pharmacokinetics with rapid systemic clearance, a half-life of 2 to 4 h and 36% oral bioavailability, were reported (166). FT inhibition in peripheral blood mononuclear cells (PBMCs) was measured 1 and 6 h post treatment and returned to baseline by 24 h. At the 225 mg/m^2 dose level, greater than 90% FT inhibition was observed. The only objective response was in a

NSCLC patient with a 40% reduction in undefined measurable disease. A weekly schedule has also been evaluated and an MTD of 245 mg/m^2 defined (167). Thirty patients were treated for a median of 6 weeks, and grade 3 DLTs included vomiting, diarrhea, dehydration, and transient elevations in creatinine and transaminases. As with the previous schedule, 80% FT inhibition in PBMCs was noted at the higher dose levels (168). In addition, FT inhibition by a mean of 80% was observed in 14 posttreatment tumor samples at 2 h and persisted at 30% inhibition at 24 h. In some tumor samples, assessment of apoptosis by a DNA break labeling assay revealed induction of apoptosis after drug exposure, for example, a refractory breast cancer patient with a 5-month minor response. Two other schedules (169, 170), weekly for 4 weeks followed by a 2-week rest and oral twice a day for 14 days every 21 days, have been evaluated with no objective responses reported.

SCH66336

SCH66336 is a novel oral agent derived from a class of nonpeptide, nonthiol-containing, CAAX mimetic FTIs (171). The pyridobenzocycloheptene class of competitive inhibitors contains a common tricyclic nucleus, and SCH66336 is an 11-piperidinyl trihalogenated compound. The drug inhibits in vitro FT activity with an IC_{50} of 1.9 nM for H-ras, 2.8 nM for N-ras, and 5.2 nM for K-ras. Inhibition of cells with activated ras and anchorage-independent growth was noted with IC_{50} 75 nM in H-ras versus 400 nM with K-ras-driven cells (172). The compound also exhibited an IC_{50} of less than 500 nM against a panel of human ras-activated tumor cell lines. The observed growth inhibition of tumor cells in soft agar and in xenografts was independent of ras mutational status because even wild-type ras cells were sensitive (173).

The phase I experience with SCH66336 involved 20 patients using a twice a day schedule over 7 days every 21 days. A recommended phase II dose of 350 mg was determined, with DLT consisting of severe fatigue. Eight patients had stable disease, and treatment for up to 10 cycles was possible in a few patients. Antitumor activity was reported in one patient with advanced NSCLC who had a greater than 50% reduction in an adrenal metastasis and received treatment for 14 months (174).

R115777

R115777 is a substituted quinolone and competitive inhibitor of the CAAX peptide binding site of FT (175). The compound inhibits in vitro K-Ras farnesylation (IC_{50} 7.9 nM) and exerts antiproliferative effects in cell lines such as H-Ras-transformed fibroblasts (IC_{50} 1.7 nM) and K-Ras-driven colon and pancreatic cells lines (at roughly IC_{50} 20 nM) (176). Activity is also reported in cell lines with wild-type ras. In mouse xenograft studies with these sets of cells, growth inhibition is also observed, which further confirms the antitumor activity of R115777. The initial clinical experience with R115777 in 27 patients was reported by Zujewski et al.

(177) using a twice a day schedule for 5 days by oral administration. Patients were retreated after at least a 7-day rest period, intrapatient dose escalation was allowed in this trial, and a DLT (grade 3 peripheral neuropathy in a patient with a history of taxol-induced neuropathy) was observed in 1 of 6 patients at the level of 1300 mg twice a day. A true MTD was not determined, and the recommended phase II dose was 500 mg twice a day for 5 days every 2 weeks. Other observed toxicities included clinically significant fatigue and transient serum creatinine elevations reflecting acute tubular injury. Minimal ophthalmologic and hematologic toxicity was observed, although antiemetic therapy was necessary in 75% of patients. Of the 27 patients treated, 8 had stable disease after 3 treatment cycles, and 4 patients continued treatment with the longest reaching 5 months. A patient with metastatic colon cancer had symptomatic improvement and a 50% reduction in carcinoembryonic antigen (CEA) levels.

Pharmacokinetic analysis revealed R115777 to be rapidly absorbed with peak plasma concentrations being reached by 3 to 4 h and steady state levels after 3 days. The degree of bioavailability and the steady state levels attained were sufficient pharmacologically for antitumor effects as predicted by preclinical experiments. The drug exhibits biphasic elimination with half-lives of 4 h and 16 h.

In another phase I study with R115777 given orally twice a day for 21 consecutive days every 28 days, an estimated MTD of 240 mg/m^2 was established (178). DLTs included grade 3/4 neutropenia and thrombocytopenia in addition to grade 3 fatigue, confusion, and bilirubin elevation. Two patients with stable disease exceeding 6 months were reported. Another chronic dosing trial recommended a dose of 300 mg twice a day with similar toxicities as reported above. A partial response in a NSCLC patient lasting 4 months was reported (179). Finally, 3 advanced breast cancer patients treated continuously at 300 mg twice a day attained confirmed partial responses whereas another 9 patients had stable disease of at least 3 months duration (180).

A most interesting outcome was obtained in patients with myelodysplastic syndrome or relapsed or poor prognosis leukemias, where a phase I dose escalation study revealed DLT at 1200 mg twice per day, consisting of neurotoxicity, with non-DLTs including renal insufficiency and myelosuppression. There was clear evidence of downmodulation of erk kinase activity, along with the farnesylation status of lamin A and HDJ-2. Clinical responses occurred in 29% of 34 evaluable patients, including 2 complete responses (181). Though there were no mutations in N-Ras detected in this patient population, this study did suggest that in addition to clinical activity there was some evidence of downmodulation of signaling as well as farnesylation-directed activities.

SUMMARY AND CONCLUSION

The interim results presented in this review convey both the promise and the difficulties encountered in developing signal transduction inhibitors for cancer treatment. These molecules represent a marked departure from prior therapeutic

approaches based on cytotoxic activity in tumor models without reference to underlying mechanism. The fact that responses have been seen at all, on one hand, reaffirms the relevance of tumor cell biology in charting the further course of cancer developmental therapeutics. On the other hand, the initial experiences raise a number of issues that should be considered as the field moves forward.

First, with a number of agents the actual magnitude of conventionally described responses, though real, is lower than would usually be associated with clinical value. The implication of this finding is that more accurate means of diagnosing the dependence of a tumor on a particular signaling pathway or target must be defined. Microarray and proteomic approaches offer this promise, but these must be integrated into the clinical trials process. Alternatively, intelligent ways of efficiently combining inhibitors of multiple pathways must be discerned and implemented. For example, a logical evolution dictated by the biology of known pathways would consider combinations of signal transduction inhibitors in blast phase CML, which in some forms is only transiently responsive to STI-571. Logical combinations might, for example, include a *bcr-abl*-directed therapy plus PI-3 kinase/*akt*-pathway-directed inhibitors.

A second issue concerns the place of target assessment, or biomarkers, in early clinical trials. Some agents have entered initial clinical trials with extensive efforts to document target-based effects in conjunction with pharmacology and clinical toxicity evaluations; others have not, and in those latter instances one is left at the end of the phase I with little sense that, lacking clear evidence of clinical response, one can confidently move forward to later phase development. Likewise, intelligent design of combinations with standard cytotoxic agents also remains a challenge. Preclinical models of synergistic effect with signaling agents often proceed from empiricism without understanding a mechanistic basis that would guide clinical implementation. These circumstances call for renewed efforts to define robust assays of target effect in the preclinical phase of a drug's development that can be translated to the clinical arena. An additional issue is how one might integrate evidence of effect on the target into dose definition or regimen design. In this regard, studies with signaling molecules might profitably seek to define the biologically effective dose rather than a maximum tolerated dose.

A final point of concern is that many of the agents in both model systems and in initial clinical observations in patients might be associated with protracted periods of cytostasis or disease stability, rather than overt cytotoxic effects that might be attributed to the initiation of an apoptotic response. Though such stable disease can be readily observed in animal tumor models, it is uncertain whether it can be meaningfully and efficiently captured in clinical populations of patients with advanced malignancy. It is important to develop decision-making steps that would aid in the use of the drugs in patients with earlier stage or indeed adjuvant or prevention strategies, and to develop clinical study algorithms that address this biologically relevant possibility in a way that does not compromise safety.

Despite these issues, it is clear that this generation of molecules has marked a turning point in cancer therapeutics and defined a path for future progress. We look forward to the day when cytotoxic strategies will be employed circumspectly and in

coordination with rationally based signaling strategies that address the molecular disorder of cancer as a basis for benefit to patients.

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REGULATORY MECHANISMS CONTROLLING GENE EXPRESSION MEDIATED BY THE ANTIOXIDANT RESPONSE ELEMENT

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■ **Abstract** The expression of genes encoding antioxidative and Phase II detoxification enzymes is induced in cells exposed to electrophilic compounds and phenolic antioxidants. Induction of these enzymes is regulated at the transcriptional level and is mediated by a specific enhancer, the antioxidant response element or ARE, found in the promoter of the enzyme's gene. The transcription factor Nrf2 has been implicated as the central protein that interacts with the ARE to activate gene transcription constitutively or in response to an oxidative stress signal. This review focuses on the molecular mechanisms whereby the trancriptional activation mediated by the interaction between the ARE and NF-E2-related factor 2 (Nrf2) is regulated. Recent studies suggest that the sequence context of the ARE, the nature of the chemical inducers, and the cell type are important for determining the activity of the enhancer in a particular gene.

INTRODUCTION

The antioxidant response element (ARE) is a *cis*-acting enhancer sequence that mediates transcriptional activation of genes in cells exposed to oxidative stress (1). The term oxidative stress encompasses a broad spectrum of circumstances that cause a change in the cellular redox status such as an increased production of free radical species within the cell or by pro-oxidant xenobiotics that are thiol reactive and mimic an oxidative insult. In keeping with this, genes that are regulated by the ARE encode proteins that help control the cellular redox status and defend the cell against oxidative damage (2). Proteins that are encoded by the ARE gene battery include enzymes associated with glutathione biosynthesis (3, 4), redox proteins with active sulfhydryl moieties (5, 6), and drug-metabolizing enzymes (7–10).

The first evidence for the existence of the ARE pathway for gene regulation came from the observation that certain xenobiotics could modulate the regulation of Phase I and Phase II drug-metabolizing enzymes in different ways (11). The Phase I drug-metabolizing enzyme cytochrome P450 (CYP) 1A1 is induced in cells following exposure to endogenous and xenobiotic compounds that are ligands for the aryl-hydrocarbon receptor (AhR) (12). This well-characterized pathway involves the AhR-ligand complex dimerizing with the AhR-nuclear translocator protein (ARNT) and then binding to a DNA enhancer region known as the xenobiotic response element (XRE) (13, 14). Certain AhR ligands are also capable of inducing Phase II drug-metabolizing enzymes such as the glutathione *S*-transferases (GST) (15) and NAD(P)H:quinone oxidoreductase (NQO1) (8). Compounds that can induce both Phase I and Phase II drug-metabolizing enzymes were designated bifunctional inducers. In addition, a number of compounds were identified that could only regulate the expression of Phase II enzymes; these chemicals were referred to as monofunctional inducers (16).

Further experiments using cell lines with a low level of AhR expression and mice that expressed high- or low-affinity AhR showed that the induction of Phase II drug-metabolizing enzymes following exposure to bifunctional inducers was impaired. However, the compounds that were classed as monofunctional inducers were still capable of increasing the expression of Phase II enzymes. For the bifunctional inducers to increase Phase II detoxification enzyme expression, the inducer has to be metabolized by the monooxygenase system to compounds that are chemically similar to the monofunctional inducers. This is facilitated by their ability to induce *CYP 1A1*, thus increasing their own metabolism (7). This work demonstrated the existence of a distinct pathway for Phase II enzyme induction that was independent of the AhR.

Following these studies, a novel *cis*-acting element was discovered within the 5′ flanking region of the rat *GSTA2* subunit gene that was responsive to β-naphthoflavone (β-NF), a bifunctional inducer, in the presence of a functional AhR (17). Promoter analysis showed that the DNA sequence of this 41-bp regulatory region was distinct from the XRE sequence (15). This unique enhancer region was later designated the ARE, as it was not only responsive to β-NF but also to phenolic antioxidants (7). Thus, the ARE can mediate a transcriptional response to a broad spectrum of structurally diverse chemicals (Figure 1).

The core DNA sequence essential for the response to these chemicals was determined through deletion and mutational analysis. The ARE consensus sequence is defined as 5′-TGACnnnGC-3′ (1). Subsequent to these studies, other genes have been identified that are induced by monofunctional and bifunctional agents possessing this *cis*-acting enhancer, including the mouse *Gsta1* subunit gene (18) and the rat and human *NQO1* genes (8, 19–21).

This review intends to provide an account of the current knowledge on the ARE gene battery and focuses on the most recent topics of research to determine the transcription factors and signaling events that are important in regulating this enhancer.

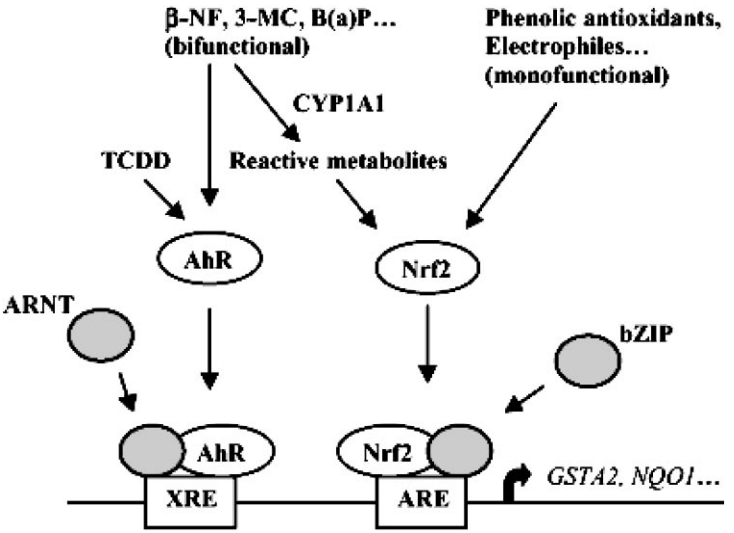


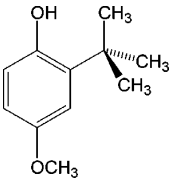
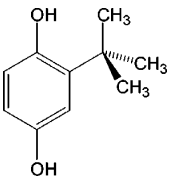
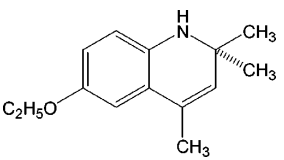
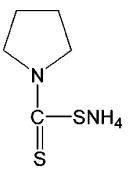
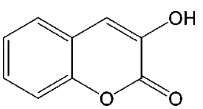
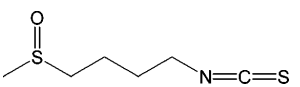
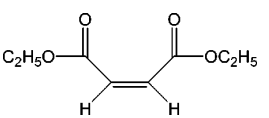
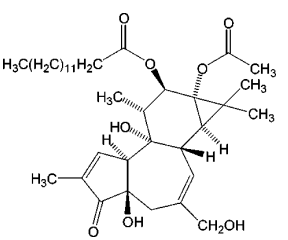
Figure 1 Transcriptional regulation of the rat *GSTA2* and *NQO1* genes by bifunctional and monofunctional inducers. The bifunctional inducers and the dioxin TCDD bind to and activate the AhR, which then translocates into the nucleus and associates with ARNT to activate transcription through the XRE. The bifunctional inducers can also activate transcription through the ARE via a separate pathway following their biotransformation into reactive metabolites that have characteristics of the monofunctional inducers. The monofunctional inducers can only act through the ARE-mediated pathway. 3-MC, 3-methylcholanthrene; B(a)P, benzo(a)pyrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

ENZYME INDUCTION BY XENOBIOTICS MEDIATED BY THE ARE

A wide range of structurally diverse compounds can activate the ARE. Classes of xenobiotics that can stimulate ARE-driven transcription include large planar compounds such as flavonoids and phenolic antioxidants (1), thiol-containing compounds such as isothiocyanates (22, 23) and 1,2-dithiole-3-thiones (24), heavy metals (25), and heme complexes (26, 27). Table 1 shows classes and examples of xenobiotics that are known to stimulate ARE-driven transcription.

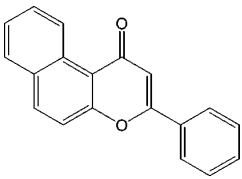
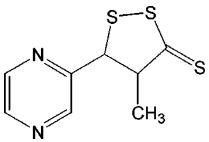
Monofunctional inducers share certain common chemical properties. They are electrophilic compounds that are capable of reacting with sulfhydryl groups; in addition, certain compounds can also undergo oxidation-reduction reactions (28). The bifunctional inducers acquire these properties following oxidative metabolism. Compounds such as the isothiocyanates and diethyl maleate do not require metabolism and can directly react with sulfhydryl groups, oxidizing cysteine

TABLE 1 Types of compounds that can stimulate ARE-driven transcription

Compound	Structure	Class	Nature of compound
Butylated hydroxyanisole		Synthetic phenolic antioxidant	A compound used as a food preservative and antioxidant in dermatological creams
tert-butyl-hydroquinone		Synthetic phenolic antioxidant	Metabolite of BHA used as a model inducing agent for ARE-driven transcription
Ethoxyquin		Synthetic antioxidant	A compound used as a pesticide and insecticide. Currently used as a pet food preservative
Pyrrolidin-edithiocarbamate		Synthetic antioxidant	A molecule that blocks activation of NF-κB and induction of iNOS
3-hydroxycoumarin		Coumarin	A naturally occurring chemopreventive agent found in leguminous vegetables
Sulforaphane		Isothiocyanate	A naturally occurring chemopreventive agent found in cruciferous vegetables
Diethyl maleate		GSH-depleting agent	A synthetic compound used as a model inducer of drug-metabolizing enzymes
Phorbol 12-myristate 13-acetate		Phorbol Ester	A potent tumor-promoting agent in mouse skin and has numerous biochemical effects such as activating PKC isoenzymes and stimulating expression of iNOS and COX-2

(*Continued*)

TABLE 1 (*Continued*)

Compound	Structure	Class	Nature of compound
β-naphthoflavone		Flavonoid	Synthetic compound used as a model inducer of drug-metabolizing enzymes
Oltipraz		1,2-dithiole-3-thione	Antischistosomal drug and cancer chemopreventive agent

residues and depleting reduced cellular glutathione (GSH); in this way, they mimic an oxidative insult. Compounds such as the phenolic antioxidant butylated hydroxyanisole (BHA) are metabolized to *tert*-butylhydroquinone (*t*BHQ), which following a dealkylation step can undergo oxidation-reduction reactions within cells. In this case, BHA is acting as a pro-oxidant. A model bifunctional inducer is β-NF. This synthetic flavonoid is metabolized by CYP 1A1 to a quinone intermediate that can undergo redox cycling (7).

In correlation with the nature of compounds that can induce ARE-driven transcription, many of the proteins whose expression is mediated by the ARE have an endogenous role in regulating cellular redox status and protecting the cell from oxidative damage. Enzymes such as GST, NQO1, and HO-1 function to detoxify harmful by-products of oxidative stress, including lipid and DNA base hydroperoxides (29, 30), quinones (31), and heme-containing molecules (32). The induction of enzymes involved in GSH biosynthesis leads to an increase in cellular GSH levels that provides a buffer against oxidative insult (2).

Apart from protecting the cell against endogenous toxic compounds, proteins associated with Phase II of drug metabolism are important for detoxifying genotoxic compounds that may be produced as a consequence of Phase I xenobiotic metabolism (33). For this reason, the use of monofunctional inducers to increase the capacity to detoxify chemical carcinogens during Phase II of drug metabolism has some therapeutic potential in cancer chemoprevention (34).

A number of studies have shown that synthetic and naturally occurring compounds, when included in the diet of rodents, can induce genes under the control of an ARE that provides protection against chemical carcinogens. For example, the inclusion of ethoxyquin or coumarin in the diet of rats can protect against the hepatocarcinogen aflatoxin B1 (35, 36). This compound is converted into the genotoxic intermediate aflatoxin B1 8,9-*exo*-epoxide by the action of CYP isoenzymes. The highly inducible GST A5-5 and aflatoxin-aldehyde reductase (AFAR) enzymes in rat liver that are regulated by the ARE detoxify this intermediate. Another

chemopreventive agent, oltipraz, which is a member of the 1,2-dithiole-3-thione class of compounds, is currently being used in a clinical trial in a region of China where aflatoxin B1-induced hepatocarcinogenesis is a real problem (37). There is also a good correlation between consuming a diet high in fruits and vegetables and a decreased risk in developing cancer (38). Many fruits and vegetables are natural sources of compounds such as isothiocyanates and coumarins that possess chemopreventive properties (39, 40).

THE ANTIOXIDANT RESPONSE ELEMENT

The existence of mono- and bifunctional inducers as proposed by Talalay et al. (11, 16) suggests that expression of many Phase II drug-metabolizing enzymes may be regulated by an additional pathway that is distinct from that mediated by the AhR via the XRE. Following the isolation and characterization of the structural gene encoding the rat GSTA2 subunit (17), a reporter construct was generated in which the 5′ regulatory sequence of this gene was fused to that encoding the bacterial enzyme chloramphenicol acetyl transferase (CAT). Analysis of this construct in transient transfection experiments showed that HepG2 cells containing this plasmid exhibited elevated CAT activity when exposed to β-NF (17). These observations provided evidence that expression of rat *GSTA2* is regulated at the transcriptional level in response to xenobiotics. A series of deletion mutants were then generated from the original reporter construct and tested in transient transfection assays in order to identify *cis*-acting sequences responsible for the responsiveness to β-NF. Results obtained from these experiments eventually revealed the presence of a novel *cis*-acting element that is distinct from and in addition to the XRE (15). When a reporter construct under the control of this *cis*-acting element was transfected into cells, induction of CAT activity was observed not only in response to β-NF but also to the phenolic antioxidant *t*BHQ, a compound unable to elicit a response via the XRE (1, 7). Because of its responsiveness to *t*BHQ, this enhancer was therefore referred to as the ARE. At the same time, an identical enhancer element was also discovered in the mouse *Gsta1* subunit gene, which is the orthologue of the rat *GSTA2* subunit gene, and was referred to as the electrophile response element or EpRE (18). Another feature of the ARE that distinguishes it from the XRE is its ability to mediate a response to *t*BHQ in mutant cell lines lacking either a functional AhR or CYP 1A1 (7) (Figure 1). Thus, these data strongly confirm the proposal that induction of Phase II enzymes may also be mediated by an AhR-independent mechanism (11, 16). Further characterization of the ARE in the rat *GSTA2* gene by point mutation analysis led to the identification of a core sequence 5′-TGACnnnGC-3′, which was shown to be essential for both basal and/or inducible activity (1).

In addition to the genes that encode the rat GSTA2 and mouse GSTA1 polypeptides, genes encoding the rat and human NQO1 proteins (8, 9), γ-glutamylcysteine catalytic (γ-GCS_h) and regulatory (γ-GCS_l) subunits (3, 4, 41), and HO-1 (27) were also found to be transcriptionally regulated via the ARE. Through computer

searches of various databases, a number of other genes, including those encoding human phenolic sulfotransferases, were found to contain ARE-like sequences (42, 43), though they are yet to be functionally characterized.

As discussed in the previous section, the ARE mediates gene expression in response to a wide variety of compounds. The observations that these compounds affect the cellular redox status along with the similarity in sequence identified between the ARE and the TPA-response element (TRE) raised the possibility that the ARE may be regulated by members of the AP-1 protein family. These transcription factors are known to activate gene transcription in response to numerous signals, including phorbol esters, growth factors, UV irradiation, and oxidative stress (44). However, despite their close similarity in sequence, the ARE has unique features that distinguish it from the TRE. The main difference between these two enhancers is the presence of the -GC- box at the 3′ end of the ARE core sequence, which has been shown to be critical for its inducibility (1, 19, 45). Thus, cells transfected with an ARE/TRE-CAT construct in which the ARE sequence had been mutated at noncritical nucleotides so as to also contain a consensus TRE motif (5′-TGACAAAGC-3′ to 5′-TGACTCAGC-3′) were found to be responsive to both *t*BHQ and 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Mutation of the -GC- box in this construct resulted in a failure to respond to *t*BHQ; however, the reporter construct was still responsive to phorbol ester treatment (45). Furthermore, induction of CAT activity was only observed in HepG2 cells transfected with an ARE-CAT reporter construct, but not with a TRE-CAT reporter construct, in response to *t*BHQ treatment (45). These data correlate with those from other studies that suggest that activation of gene expression via the ARE and TRE are mediated by different signaling pathways (25, 46).

It should be noted, however, that the ARE found in some of the genes contains an embedded TRE sequence (see Table 2), and it is possible that these genes are

TABLE 2 Sequences of ARE found in different genes

GSTA2 (rat)	5′-AAATGGCATTGCTAATGG **TGACAAAGC** AACT-3′
Gsta1 (mouse)	5′-AAATGACATTGCTAATGG **TGACAAAGC** AACT-3′
GSTP (rat)	5′-AGTAGTCAGTCACTA **TGATTCAGC** AACA-3′
NQO1 (rat)	5′-AGTCTAGAGTCACAG **TGACTTGGC** AAAA-3′
NQO1 (human)	5′-AAATCGCAGTCACAG **TGACTCAGC** AGAA-3′
GCS_h (human)	5′-AGC **GCTGAGTCA** CGGGG-. . . .-CCCGCGCAGTCAC-3′
GCS_l (human)	5′-GGATGAGTAACGG-. . . .-TTCT **GCTTAGTCA** TTG-3′
Ho-1 (mouse)	5′-TCT **GCTGAGTCA** AGGTCCG-3′
NF-E2 (consensus)	5′-TGT **GCTGAGTCA** CTG-3′
TRE (consensus)	5′-GCA **TGAGTCA** GAC-3′

The core ARE sequence is indicated by the nucleotides in boldface type. Underlined nucleotides represent the AP-1-like sequence required for full basal expression for certain genes.

under the control of both enhancers. In fact, in vitro DNA binding studies using antibody supershift assays have detected a number of transcription factors that can bind to the ARE, notably Nrf1 and Nrf2 (NF-E2-related factor), members of the AP-1 family, and small Maf proteins (discussed below). This is not surprising as many of these basic region-leucine zipper (bZIP) proteins can potentially dimerize with each other to generate a diverse array of functional protein complexes that bind to DNA with unique and/or overlapping specificity.

TRANSCRIPTION FACTORS ACTING ON THE ARE

The ARE contains the 5′-TGAC-3′ tetranucleotide within its core sequence that resembles the half-site recognized by members of the AP-1 (consensus: 5′-TGACTCA-3′) and the ATF/CREB (consensus: 5′-TGACGTCA-3′) families of transcription factors. All members of these two families of proteins belong to a class of transcription factors collectively known as bZIP proteins, which can bind to DNA as part of a homodimer and/or heterodimer complex. These observations suggest that bZIP proteins may be involved in regulating the ARE. In earlier studies, members of the AP-1 protein family were believed to act on the ARE on the basis that these proteins are known to be activated by various signals that cause cellular stress (20, 21, 47). In addition, the presence of a consensus TRE sequence within the ARE of the human *NQO1* gene further strengthened this belief (20, 21). However, a number of studies have failed to confirm that AP-1 proteins play a major role in mediating the ARE response, largely based on data showing that *t*BHQ, a model inducing agent for the ARE, could not elicit a response through the TRE (25, 45, 46). Furthermore, in vitro DNA binding assays have shown that the AP-1 proteins cannot bind to the ARE with high affinity and specificity (19, 45). In more recent studies, compelling evidence has been obtained from in vitro and in vivo studies suggesting that the bZIP transcription factor Nrf2 is intimately involved in mediating the ARE-driven response to oxidative stress and xenobiotics (10, 48–52).

The Nrf2 Protein

The Nrf2 protein was first isolated by an expression cloning procedure using an oligonucleotide containing the NF-E2 DNA binding motif as probe to screen for closely related proteins (53). NF-E2 is a dimeric protein originally identified as involved in the regulation of globin gene expression in hematopoietic cells (54, 55). The NF-E2 protein activates gene transcription following binding to its consensus DNA binding motif 5′-TGCTGAGTCAC-3′ as a heterodimer consisting of a 45-Kda and an 18-Kda subunit (56). The p45 subunit is a bZIP protein consisting of a transactivation domain within the N-terminal region and a basic DNA binding region/leucine zipper structure in the C-terminal region of the protein (54, 55). The p18 subunit was subsequently identified as a member of the small Maf proteins containing the basic region/leucine zipper but lacking any apparent transactivation

domain (56). Whereas expression of the p45 NF-E2 subunit is restricted to hematopoietic tissues (54, 55), two other related bZIP family members, Nrf1 and Nrf2, are ubiquitously expressed in a wide range of tissues and cell types (10, 53, 57). The tissue distribution profile of these proteins combined with their DNA binding motif being similar in comparison to that of the ARE led to the suggestion that ARE-responsive genes may be regulated by Nrf1 and/or Nrf2 (48). Their involvement was confirmed in transfection experiments where ectopic expression of either Nrf1 or Nrf2 in HepG2 cells was found to increase CAT activity from an ARE-CAT reporter construct derived from the human *NQO1* gene but was unable to induce CAT activity from a mutant ARE-CAT construct (48). Nrf2 was subsequently demonstrated to be involved in the transcriptional activation of other ARE-responsive genes including those encoding the human γ-GCS_h and γ-GCS_l subunits (49), mouse HO-1 (50), and rat NQO1 and GSTA2 subunits (51). More significantly, data from in vivo studies using gene knockout mice clearly implicated Nrf2 as a critical protein in regulating the expression of the *Gsta1* and *Nqo1* genes (52). Immunoblot analysis demonstrated that the expression level of these enzymes was reduced in Nrf2 (−/−) mice as compared to heterozygous animals. Of particular importance was the finding that induction of these enzymes by BHA, an inducer of ARE activity, was greatly attenuated in the null mice (52). These data suggest that Nrf2 mediates both the basal and inducible activity of the ARE. Further detailed characterization of these knockout mice demonstrated that the loss of Nrf2 resulted in a profound reduction in the expression and enzyme activities of NQO1, certain GST isoenzymes, and the γ-GCS_h subunit (10, 58).

Of particular interest from these studies were observations demonstrating the different effects Nrf2 may have on basal as well as inducible expression of these enzymes. For instance, the basal expression of NQO1 and certain GST subunits was significantly lower in Nrf2 (−/−) mice than in the wild-type animals. Whereas the inducible expression of NQO1 was mostly abolished in knockout mice, that of GSTs, particularly the GSTA1 subunit, was unaffected in the same animals in response to known ARE inducers such as BHA, ethoxyquin, and coumarin. By contrast, in the same studies the basal expression of the γ-GCS_h subunit was found to be similar in Nrf2 (−/−) and wild-type mice; however, its induction by the ARE inducers was abolished with the loss of Nrf2 (10). These data provide evidence suggesting the differential mode of regulation by Nrf2 for different genes. The biochemical basis for the differences observed in the involvement of Nrf2 in basal and/or inducible expression of these enzymes is currently not known.

The differential regulation of these genes may be explained at least in part by the sequence context of their ARE. Data from in vitro DNA footprinting analysis of the rat *GSTA2* and *NQO1* gene promoters showed that the DNase I–protection patterns produced by nuclear extracts isolated from human HepG2 or rat H4IIE cells were different for the two genes (51). Furthermore, in vitro synthesized Nrf2/MafK was found to exhibit a DNA binding activity with a higher affinity for the rat *NQO1*

ARE than the rat *GSTA2* subunit gene ARE by both DNA footprinting analysis and gel mobility shift assays (51). These results suggest that the context of the ARE, outside its core sequence, of each particular gene contributes an important role in its regulation. This was also confirmed by previous transfection experiments, which showed that mutating the rat *GSTA2* subunit ARE to contain a perfect TRE (i.e., ARE/TRE, see above) sequence significantly increased its basal activity and induction in response to β-NF, *t*BHQ, and TPA (45). The increased responsiveness is possibly due to the enhancer representing a higher-affinity binding site for Nrf2 as well as a site for AP-1 proteins. The ARE of the human *NQO1* gene, which contains an embedded TRE consensus sequence, may represent this type of dual enhancer. It has been shown by cotransfection experiments that Nrf1/Nrf2 and Jun proteins can synergize to potentiate the reporter gene expression under control of this ARE sequence as well as that of the rat *GSTA2* gene (59). Although DNA binding assays using nuclear extracts from mouse hepa1c1c7 cells and transcription factor antibodies showed that Nrf1, c-Fos, and JunD interacted with the *NQO1* ARE (48), it is not clear from these experiments whether the Nrf and Jun proteins acted independently or interacted together to enhance the ARE-mediated response because overexpression of these proteins individually also led to an increase of expression (59). Unexpectedly, overexpression of c-Jun and c-Fos proteins together failed to activate CAT gene transcription and caused a decrease in CAT activity in transfected cells, suggesting that AP-1 complexes may have a negative effect upon the ARE (48). Interestingly, an independent study demonstrated that phenolic antioxidants can inhibit AP-1 activity (46). The physiological role of members of the AP-1 protein family in regulating the expression of the human *NQO1* gene therefore remains to be established.

In the case of genes encoding the human γ-GCS_h and γ-GCS_l subunits, full basal and inducible expression has been shown to be controlled by two separate enhancers in close proximity to each other. One enhancer contains an AP-1 binding site, whereas the second is an ARE with an embedded AP-1 site (see Table 2). For the gene encoding the γ-GCS_h subunit, the presence of both enhancers is required for full basal activity, but only the ARE/AP-1 enhancer was important for β-NF induction (41). The promoter region responsible for the induction of the γ-*GCS*$_l$ subunit gene by β-NF also contains two enhancers that appear to act in a cooperative manner to produce a full inducible response (3). In addition to Nrf2, overexpression of JunD and small Maf was found to have an effect on the expression of these subunits (49). These data suggest that interactions may occur between other signaling pathways and the ARE to regulate the expression of certain genes. This may explain in vivo data that indicates that full basal expression of the γ-GCS_h subunit was unaffected in Nrf2 ($-/-$) mice, whereas its upregulation by known ARE inducers was essentially abolished (discussed above; 10).

Current data from in vitro DNA binding assays and transfection experiments point to Nrf2 as the most important protein involved in stimulating ARE-driven transcription. Using the chromatin immunoprecipitation assay (ChIP) studies from

this laboratory, we have observed that Nrf2 does interact directly with the rat *GSTA2* ARE in the nuclear compartment of rat H4IIE cells (unpublished observation). These data support the notion provided by in vivo studies in Nrf2 (−/−) mice that Nrf2 is important in basal as well as inducible gene expression. The role of Nrf2 has been demonstrated to be critical in recent studies that report that the reduced expression of Phase II drug-metabolizing enzymes in Nrf2 (−/−) mice confers a sensitive phenotype to the toxic effects of carcinogens and inflammatory drugs compared to wild-type animals (60–63).

Small Maf Proteins

As mentioned above, the bZIP proteins function as dimers in order to bind to DNA. Presently, there is no conclusive evidence to indicate whether Nrf2 may activate gene expression as a homodimer or whether it is obligated to form heterodimeric complexes. Because p45 NF-E2, the first family member of these proteins, was found to dimerize with MafK in order to bind DNA and activate transcription (56), it was hypothesized that small Maf proteins may represent a partner for Nrf2 in effecting transcriptional activation (52).

There are three known small Maf proteins, designated MafF, G, and K, each consisting of a basic DNA binding region and a leucine zipper; however, they lack a transactivation domain (64). These proteins also possess a cap 'and' collar (CNC) homology domain that has been shown to be important for recognition of an extended AP1-like binding motif (65). Small Mafs are present in the nucleus where they can form heterodimers with all members of the CNC protein family including the p45 NF-E2 and related factors (56, 66–68), the BACH proteins (69), and some of the AP-1 transcription factors (70).

The physiological role of small Maf proteins in terms of ARE regulation remains unresolved. Results from in vitro DNA binding experiments clearly demonstrated that Nrf2 only binds to DNA with a high affinity and specificity in the presence of small Maf proteins (51, 68). Nrf2 has not been shown to bind to DNA independently. This is presumably because it cannot either form a homodimer (53) or bind to DNA with a high affinity as a homodimer. Although small Maf proteins facilitate the ability of Nrf2 to bind to its target DNA sequence with a high affinity, this does not appear to effect an increase in transcriptional activity. In transfection experiments, the converse appears to be the case; MafK acts as a repressor of ARE-driven transcription. An increase in the amount of a plasmid that expresses the rat MafK inherently leads to decreasing levels of rat *GSTA2* and *NQO1* ARE-CAT activity in the presence of a constant amount of Nrf2 (51). Independent studies have also reported similar repressive effects associated with small Maf proteins on the human *NQO1* gene (71) and the human γ-GCS_h subunit gene (49). Interestingly, experiments employing a reporter gene construct containing the promoter region of the human γ-GCS_l subunit gene when transfected into HepG2 cells with a plasmid expressing Nrf2 and approximately a 10-fold lower molar amount of a plasmid expressing MafG showed a 1.4-fold increase in reporter gene activity

compared to cells overexpressing Nrf2 alone. However, increasing the amount of MafG plasmid in the transfection caused a decrease in the reporter gene activity (49). In the same studies, overexpression of a dominant-negative form of MafK (MafK DN), which cannot bind to DNA, also led to negative effects on the expression of both the catalytic and regulatory subunit genes. It is interesting that similar effects were observed for both wild-type MafG and MafK DN; therefore, further studies are required to determine the role of small Maf in the regulation of the γ-*GCS*$_l$ subunit gene expression.

In an independent study, in vitro gel mobility shift assays showed that MafG was present in a nuclear protein complex from CHO cells that interacted with an ARE-like enhancer in the mouse *Ho-1* gene, designated the StRE. In transfection experiments, overexpression of MafK DN was shown to block activity of this enhancer in response to cobalt (72). Although overexpression of MafK DN may have sequestered Nrf2 away from the StRE, these data alone were not sufficient to demonstrate a direct role for small Maf in transcriptional activation of *Ho-1* gene. The only evidence of small Maf proteins participating in transcriptional activation is when they act in concert with p45 NF-E2 (56, 66, 67, 73).

Unlike p45 NF-E2 and related factors, small Maf proteins are capable of binding DNA either as homodimers or heterodimers formed among other small Maf family members. It has therefore been postulated that small Maf dimers may bind to DNA and act as transcriptional repressors because they lack any transactivation domain (64). This would suggest that there is competition within the nucleus between small Mafs and transcriptional activators for the same binding site that determines a balance between repression and active (or derepressed) gene transcription. This was demonstrated in transfection experiments in Cos-1 cells, where at a low concentration MafK activated reporter gene transcription in concert with p45 NF-E2, whereas at higher concentrations its repression activity overcame the transactivation potential of p45 NF-E2 (74). In similar experiments employing the rat *GSTA2* and *NQO1* ARE, as described earlier, at no time did MafK elicit a positive effect on reporter gene expression even when cotransfected with Nrf2.

To assess whether endogenous levels of small Maf proteins are affected by known ARE inducers, various cell lines were treated with *t*BHQ, pyrrolidinedithiocarbamate (PDTC), and phenylethyl isothiocyanate (PEITC) (75). In these studies, all three small Maf proteins were constitutively expressed at various levels in different cell lines. Interestingly, the expression of the three proteins increased following exposure of the cells to PDTC and PEITC. However, treatment with *t*BHQ did not appear to induce expression levels of these proteins, suggesting that their induction may not be essential for ARE-mediated gene expression (75). These findings show some of the subtleties involved in regulation of the ARE and infer a possible feedback mechanism involving small Maf proteins in responses to certain stimuli.

All of the in vitro DNA binding studies to date have shown that small Maf binds to DNA more readily as a heterodimer with either p45 NF-E2, Nrf1, Nrf2,

or Nrf3 than as a homodimer or heterodimer formed between themselves. Thus, for small Maf dimers to compete with p45-small Maf complexes for the same binding site, the level of small Maf proteins would likely have to be greater than that of p45, assuming that the dimerization kinetics are similar among these proteins. Therefore, these observations raise the question of how a slight increase in the abundance of small Maf may tip the balance from enhanced to repressed gene expression, and this was shown in transfection studies (74) as well as by in vivo studies (76) that examined the transcriptional activation of genes regulated by NF-E2. A potential mechanism that may account for this effect is the association of small Maf with other bZIP proteins that may result in the formation of a repression complex that can effectively compete with NF-E2 for their binding site. MafK can dimerize with c-Fos to form a high-affinity DNA binding complex that acts as transcriptional repressor of NF-E2-mediated gene expression (70). However, this mechanism of repression does not explain the lack of any activation potential of small Maf proteins together with Nrf2 on ARE-mediated gene transcription.

Currently, the precise role of small Maf proteins in the regulation of ARE-driven transcription is unresolved. Further experiments using cell-based studies combined with in vivo gene knockout studies similar to those examining the basal and inducible expression of ARE-responsive genes in Nrf2 (−/−) mice (10, 52) involving individual and multiple Maf proteins would provide a better insight on this question. For instance, is BHA induction of *Gsta1* and *Nqo1* lost in small Maf (−/−) mice or mutant cell lines?

Other Proteins

Nrf2 is established as having a central role in the regulation of ARE-mediated gene expression; however, questions remain as to whether other related bZIP proteins may also be involved. There is some evidence indicating that Nrf1 may contribute to the expression of ARE-responsive genes. Ectopic expression of Nrf1 activates the human *NQO1* ARE reporter gene in HepG2 cells (48). Nrf1 also activates a reporter gene linked to the promoter of the γ-*GCS*$_h$ subunit gene and increases the intracellular level of GSH (77). A role for Nrf1 has been demonstrated further in studies employing cells containing a targeted disruption of the *Nrf1* locus. Although the loss of Nrf1 is lethal in early embryonic development (78), an analysis of mouse fibroblasts derived from Nrf1 (−/−) mouse embryonic tissue showed reduced levels of GSH and of γ-GCS$_l$ subunit expression compared to wild-type cells. Interestingly, induction of this enzyme was not affected by the loss of Nrf1, as treatment of the Nrf1-deficient fibroblasts with paraquat resulted in an increased level of its mRNA (79). These data and those obtained from studies involving Nrf2 (−/−) mice suggest that the expression of γ-*GCS* genes is mediated by both Nrf1 and Nrf2, with Nrf1 being involved in basal expression and Nrf2 in the inducible expression. It is not known whether Nrf1 plays a similar role, if any, on the ARE of other genes.

The p45 NF-E2 is unlikely to have any role, as its expression is restricted to hematopoietic cell lineages. Ectopic expression of p45 NF-E2, unlike Nrf2, did not increase reporter gene activity linked to the rat *GSTA2* and *NQO1* ARE in HepG2 cells (our unpublished observation), presumably because HepG2 cells as well as other nonhematopoietic cells lack specific cofactors required for full NF-E2 transcriptional activity. These observations are supported by a recent study that demonstrated in transient transfection experiments that overexpression of p45 NF-E2 could activate a reporter gene linked to an NF-E2 binding site in the erythroid cell line K562 but not in Cos-1 cells (73). The role of another member of the CNC protein family, Nrf3, in the regulation of ARE-responsive genes is not known, although overexpression of this protein activates a reporter gene linked to an enhancer containing the NF-E2 binding site (80).

In other recent studies, yeast two-hybrid screening experiments were employed to identify potential proteins that interact with Nrf2. In one such study, the bZIP protein ATF4 was identified and presumed to associate with Nrf2 by dimerization via its leucine zipper. The ATF4 protein bound and activated the mouse *Ho-1* ARE-reporter gene only in the presence of Nrf2 (81). At present, it is not known whether ATF4 has any function in the expression of other ARE-responsive genes. Using the same screening procedure, the polyamine-modulated factor-1 protein (PMF-1) has also been reported to be able to interact with Nrf2 and may contribute to the transcriptional regulation of the *spermidine/spermine N^1-acetyltransferase* gene via the polyamine response element (PRE, an ARE-like enhancer). Because overexpression of Nrf2 did not transcriptionally activate a reporter gene linked to the PRE, the significance of the interaction between Nrf2 and PMF-1 on ARE-responsive genes is not apparent (82, 83).

CELL SIGNALING PATHWAYS ASSOCIATED WITH THE ARE

Another major question is how does the cell sense and respond to the appropriate signals to initiate the cascade of signaling events leading to the activation of the ARE? Recent studies have implicated protein phosphorylation as being of major importance in the response to xenobiotics and oxidative stress that stimulates ARE-driven transcription. In addition to the action of kinases, a protein designated Kelch-like-ECH-associated protein 1 (Keap1) has been shown to repress Nrf2 activity within cells. It has been postulated that sulfhydryl group chemistry may also play an important part in the regulation of cell signaling pathways and in protein-protein interaction such as between Keap1 and Nrf2.

Signaling Pathways

To date, three major signal transduction pathways have been implicated in regulation of the ARE, which include those mediated by the MAPK cascades, PI3K, and PKC (Figure 2).

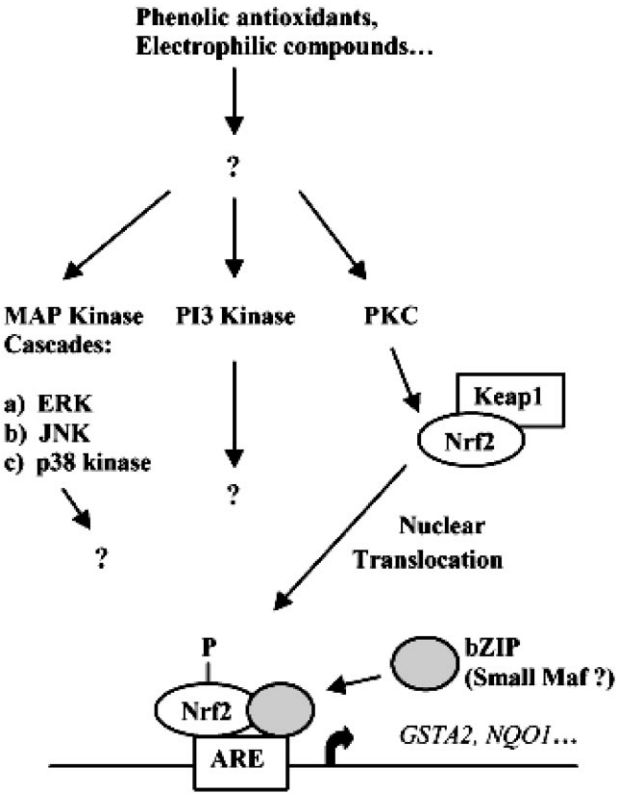


Figure 2 Signaling events involved in the transcriptional regulation of gene expression mediated by the ARE. Three major signaling pathways have been implicated in the regulation of the ARE-mediated transcriptional response to chemical stress. In vitro data suggest that direct phosphorylation by PKC may promote Nrf2 nuclear translocation as a mechanism leading to transcriptional activation of the ARE. Nrf2 has been proposed to be retained in the cytoplasm through an interaction with Keap1 and it is possible that phosphorylation of Nrf2 may also cause the disruption of this interaction. As a bZIP protein, Nrf2 binds to the ARE as a dimer. Although small Maf proteins have been proposed to represent the dimerizing partners for Nrf2 in the activation complex, this has not been conclusively demonstrated. The molecular mechanisms controlling the ARE-mediated transcription by the MAP kinase and PI3 kinase pathways remain to be determined (see text for detailed discussion).

MAPK SIGNALING PATHWAYS The first signal transduction pathways to be investigated in relation to the ARE were the mitogen activated protein kinase (MAPK) cascades. The first studies investigating their involvement reported that the classical extracellular regulated kinase (ERK) pathway made a positive contribution to ARE-driven gene expression (84). Experiments showed that ARE reporter gene

activity stimulated by the monofunctional inducers *t*BHQ and the isothiocyanate sulforophane (Sul) was mediated at least in part by ERK2 in human and mouse hepatoma cell lines. These compounds were found to cause an increase in the phosphorylation of ERK2 in hepatoma cells, and the MEK1 inhibitor PD 98059 could attenuate this activation. This was supported by data from reporter gene transfection experiments where the overexpression of a dominant-negative form of ERK2 also attenuated induction by *t*BHQ and Sul. In the mouse hepatoma cell line hepa1c1c7 cells, the induction of NQO1 activity by *t*BHQ and Sul could also be impaired by PD 98059. Interestingly, through the use of dominant-negative mutants of kinases upstream of ERK2, it was demonstrated that activation required raf-1 and occurred in a ras-independent manner.

In a parallel study, *t*BHQ and the bifunctional inducer β-NF, but not Sul, were shown to activate the p38 MAPK pathway in the same cell lines (85). The involvement of this pathway was identified by the use of SB 203580, an inhibitor of p38 kinase activity. Contrary to findings with the ERK pathway, the activation of p38 was found to be correlated with a down-regulation of basal and inducible ARE-reporter gene activity by this MAPK pathway. This was supported by transfection experiments showing that overexpression of dominant-negative mutants of other protein kinases that are components of the p38 pathway also effected a decrease in basal and inducible reporter gene activity in transfected cells. These data led to the suggestion that p38 exerts a general repressive effect upon the ARE and thus acts in opposition to the ERK pathway in response to *t*BHQ and β-NF in hepatoma cells.

A follow-up study investigating the third well characterized MAPK pathway showed that the c-jun N-terminal kinase (JNK) pathway may be activated in response to sodium arsenite and mercury chloride (86). This activation had a positive effect on the ARE and was dependent upon Nrf2, but not the c-jun transcription factor, a down-stream target of JNK. It was noted that JNK activity had no bearing on the activation of the ARE by *t*BHQ. Similar to ERK, the actions of JNK were also antagonized by the p38 pathway. These studies surmise that contribution of the MAPK signaling pathways in regulating the ARE depends upon the nature of the inducing agent.

A study to deduce the upstream signaling events leading to induction of the γ-GCS_h and γ-GCS_l subunits by PDTC reported slightly different findings (87). The treatment of human hepatoma cells with PDTC activated both the ERK and p38 MAPK pathways. It was discovered that these two pathways were acting synergistically to increase the transcription of the γ-*GCS* subunit genes. These conclusions were based upon the observation that a complete inhibition of PDTC induction could only be achieved following cotreatment with both PD 98059 and SB 202190. Although in partial agreement with previous studies as to the role of ERK, the role of p38 in the response to PDTC is different from that observed for *t*BHQ or β-NF induction.

So what factors may explain the differences observed for the contribution of the p38 pathway? The two studies do employ chemically distinct xenobiotics to

stimulate the ARE, and it may be that cell signaling pathways are tailored to specific stimuli. The genes of interest were also different between the two bodies of work. The endogenous gene of choice for the first set of data was the mouse *Nqo1* gene for which the promoter has not been characterized; the orthologous rat *NQO1* gene contains a putative ARE sequence, whereas the orthologous human *NQO1* gene contains an ARE with an embedded TRE (see Table 2). It is likely that the mouse *Nqo1* gene is regulated by an ARE, but it is not known whether AP-1 factors may also be important. These workers also used a reporter gene construct that contained the mouse *Gsta1* ARE sequence, which does not contain a TRE. The second study focused on the human γ-*GCS* subunit genes. The ARE sequence within theses genes contains an embedded AP1 site, like the human *NQO1* gene ARE, and also a farther AP1 site in close proximity to this ARE (Table 2). In addition, the ARE used in the respective reporter gene construct was from the human γ-GCS_l subunit gene that contains these ARE and AP-1 enhancers. It has been shown that the p38 MAPK cascade can elicit a positive effect upon the TRE, such as in the case of the MMP-9 collagenase gene (88). Thus, the differences in the role of p38 may be accounted for by the context of the ARE and how AP-1 transcription factors may interact with Nrf2.

In addition, there were differences in the finer details of the experimental conditions employed. The implication of p38 as a negative effector on the regulation of the ARE was demonstrated employing SB 203580 at a concentration of 5 μM, which is sufficient to inhibit p38 activity in cell lines. These data were supported by the use of dominant-negative mutant kinases. The implication of p38 as a positive regulator of the ARE was demonstrated using the pharmacological inhibitor SB 202190, which has a very similar specificity to SB 203580 but is more potent (89), at a concentration of 25 μM. The latter study did not employ the use of dominant-negative kinase mutants to support the findings from the inhibitor data. It has been noted that SB 203580 may have inhibitory effects on ARE-driven transcription at higher doses above 5 μM (our unpublished data), thus the differences observed could also be accounted for by the dose of compound used. Whether or not the inhibitory effects of high dose SB 202190 on γ-*GCS* induction are directly associated with the p38 pathway are therefore uncertain. There were also differences in cell culture conditions; the initial studies used serum-starved culture conditions, which is thought to be important in reducing basal MAPK activity within cells, particularly the ERK pathway. The second body of work carried out experiments in media containing high serum (10% v/v). The effects of serum upon the ARE have not been rigorously tested.

From other work, it appears possible that cell-specific effects could impact on the importance of certain transduction pathways that are associated with the ARE. Two independent studies that looked at the MAPK pathways involved in the regulation of human *HO-1* induction in response to cadmium reported different findings. In MCF-7 human mammary epithelial cells, cadmium activated the ERK, JNK, and p38 MAPK pathways (90). These experiments employed pharmacological inhibitors and dominant-negative mutants to deduce that basal and

cadmium- inducible HO-1 activity was dependent upon the p38 pathway and not the ERK or JNK pathways. However, in an alternative study, induction of the *HO-1* gene by cadmium was found to utilize the ERK pathway as opposed to the p38 pathway in HeLa cells (91). These conclusions were made employing the kinase inhibitors PD 98059 and SB 203580. It was not determined whether the p38 pathway had any repressive effects in either of the studies on *HO-1* regulation.

It appears that the many factors leading to the signaling context for a particular ARE may determine which of the MAPK signaling pathways are important in a given circumstance.

THE PI3K SIGNALING PATHWAY Separate from studies on the association between the ARE and the MAPK pathways, other studies have suggested that phosphatidylinositol 3-kinase (PI3K) is important in transducing a response to phenolic antioxidants and oxidative stress through to the ARE. This kinase is an integral component of the insulin signaling pathway. Experiments have shown that pretreatment of the H4IIE rat hepatoma cell line with the PI3K inhibitors, Wortmannin, and LY 294002 abolishes the induction of rat *GSTA2* mRNA by *t*BHQ and sulfur amino acid deprivation (SAAD) (92). In addition, it was shown that *t*BHQ treatment caused an increase in phosphorylation of Akt/PKB, a kinase downstream of PI3K in the insulin signaling pathway (93). No direct evidence was provided to implicate Akt/PKB as having an effect on the ARE. This report also suggested that ERK activity antagonizes ARE-mediated transcriptional activation from experimental data using PD 98059. The protein kinases, p38 and JNK1, did not affect induction of rat *GSTA2* by *t*BHQ in these experiments. It should be noted that the rat *GSTA2* subunit gene also contains other regulatory elements within its 5′ flanking region that includes an XRE (15). Whereas *t*BHQ does not affect the XRE (23), the pharmacological inhibitor PD 98059, which is a flavonoid, may do so. This compound has been shown to be a ligand for the AhR (94) so the clarity of findings with this compound on the role of ERK in H4IIE cells is uncertain. Flavonoids may be metabolized to inducing agents that activate the ARE; such is the case for β-NF. This emphasizes the importance of using more than one chemically distinct kinase inhibitor where possible to support findings using such compounds.

In a second body of work, there is further support for the notion that PI3K is important for enhancement of transcription at the ARE. These workers utilized the IMR-32 neuroblastoma cell line and demonstrated that pharmacological inhibitors of PI3K attenuated ARE-driven transcription in response to *t*BHQ (95). In experiments where the expression of a reporter gene is controlled by the human *NQO1* ARE, induction by *t*BHQ was attenuated by the PI3K pharmacological inhibitor LY 294002. In addition to chemical inhibitors, these studies employed a constitutively active PI3K, which increased the activity of a reporter gene construct containing the human *NQO1* ARE. However, contrary to the studies discussed above, there appeared to be no activation of PKB downstream of PI3K in cells exposed to *t*BHQ. In addition, these workers also provided evidence that activation of ERK is not important for *t*BHQ induction of *NQO1* in these cells based upon experiments

employing PD 98059. It is not known whether insulin can stimulate ARE-driven transcription.

In a follow-up study, a microarray approach was applied to identify *t*BHQ-inducible genes in the neuroblastoma cell line (96). IMR-32 cells were also cotreated with LY 294002 to determine which of these genes were regulated in a PI3K-dependent manner. The results highlighted 63 genes that were induced by *t*BHQ; the PI3K inhibitor attenuated the induction of 43 of these genes. Among the genes induced by *t*BHQ were those encoding NQO1, HO-1, and the γ-GCS subunits. Of these, *NQO1* and *HO-1* were induced in a PI3K-dependent manner, but the *γ-GCS* subunit genes were not. Interestingly, the gene for the human homologue of the Keap1 protein (discussed later) was also induced by *t*BHQ in a PI3K-dependent manner; it is not known what regulatory elements may be involved in controlling the expression of this gene. These data make a strong case for the importance of PI3K in the neuroblastoma cell line, although its importance in other cell lines is not clear.

THE PKC SIGNALING PATHWAY Of the investigations into regulation of the ARE by signal transduction pathways discussed so far, none have shown that phosphorylation of the transcription factor Nrf2 itself correlates with an increase in ARE activity. The first evidence for a direct effect of a kinase upon the transcription factor Nrf2 came from studies into the role of the PKC signaling pathways (97). Early reports characterizing the ARE demonstrated that phorbol esters as well as redox cycling compounds could stimulate transcription (45). From this fact, it was hypothesized that protein kinase C (PKC) isoenzymes may be involved in regulating the ARE.

The involvement of PKC in the regulation of the ARE was studied in the human hepatoma cell line HepG2 and the rat hepatoma cell line H4IIE (97). In transfection experiments, ARE reporter gene constructs were employed that contained either the rat *GSTA2* or the rat *NQO1* ARE. The use of staurosporine and RO-32-0432, which are broad-spectrum PKC inhibitors, impaired ARE reporter gene activity stimulated by TPA, *t*BHQ, and β-NF. In vivo labeling experiments were used to demonstrate that the Nrf2 protein became phosphorylated in response to *t*BHQ. In addition, it was shown that the translocation of endogenous Nrf2 from the cytoplasm to the nucleus and phosphorylation of the transcription factor were inhibited by pharmacological inhibitors of PKC. These events were not inhibited by the MEK1 and p38 kinase inhibitors U0126 or SB 203580, respectively. Importantly, it was subsequently shown that the PKC-catalytic subunit and PKC immunoprecipitated from cells could directly phosphorylate Nrf2 in vitro. The activity of immunoprecipitated PKC toward Nrf2 was higher in cells that had been stimulated with TPA, *t*BHQ, or β-NF. These experiments provide the first evidence that a kinase may have a direct effect on activation of the ARE in an Nrf2-dependent mechanism.

There is certain evidence that protein phosphorylation is important for stimulating and also possibly repressing ARE-driven transcription. Factors that appear

to determine which of the signal transduction pathways are important include the nature of the inducing agent, the cell type, and the context of the ARE in the gene of study. A heavy reliance has been placed upon information obtained from the use of pharmacological inhibitors of signaling pathways. Due to the frequency of contrasting data, in particular surrounding the MAPK cascades, more rigorous experiments are required using multiple approaches when deducing the signaling events leading to a specific ARE response. It is possible, however, that all of the signaling pathways discussed may play some role in regulating the ARE because there is the capacity for cross-talk to occur between these kinases. For example, it has been demonstrated that PI3K can activate PKC isoenzymes (98), and in turn that PKC isoenszymes can activate the MAPK cascades (99, 100). There is likely to be many complexities surrounding the association of protein kinases with the ARE.

The Importance of Other Cell Signaling Mechanisms and ARE Gene Expression

Apart from the phosphorylation events that occur within cells in response to xenobiotics, it has been suggested that sulfhydryl group chemistry is also an important factor in regulation of the ARE. There are a number of instances where the redox status of cysteine residues plays a crucial role in the activity of signal transduction pathways and transcription factors. As mentioned previously, the common factor linking compounds that induce ARE-driven transcription is that they can either directly or following metabolism give rise to thiol-reactive compounds. It has been hypothesized that activation of the ARE involves an intracellular redox sensor that possesses reactive cysteine residues that are sensitive to modification. One candidate for this redox sensor comes in the form of the Keap1 protein (101). Experiments have shown that Keap1 functions as a cytoplasmic effector for Nrf2 and thus inhibits its ability to transactivate the ARE (101, 102) (Figure 2). The two proteins associate through the double glycine-rich domains of Keap1 and a hydrophilic region in the Neh2 domain of Nrf2. The Keap1 protein contains a number of potential reactive cysteine residues. It has been postulated therefore that oxidative stress leads to modification of these cysteine residues, which results in the release of Nrf2, allowing it to translocate to the nucleus and transactivate the ARE. Electrophilic compounds such as diethyl maleate and catechol that are thiol reactive have been shown to promote the dissociation of Nrf2 from Keap1. It is also possible, however, that Keap1 may be a target for phosphorylation; computer-aided analysis shows that the protein does contain potential phosphorylation sites. It is not currently known whether Keap1 is phosphorylated. What is certain is that Keap1 along with Nrf2 is expressed in most tissues and cell lines studied so far and that it is a pivotal component of the ARE pathway.

There is also the potential for some feedback mechanism in the signaling pathways that involve proteins that are products of ARE-containing genes. Examples of these include thioredoxin, which is an endogenous inhibitor of ASK1, an

upstream kinase of both the JNK and p38 MAPK pathways (103). It has been shown that GSTs may also directly attenuate ASK1 (104) or JNK activity within cells (105). When cells become chemically stressed, thioredoxin and GST dissociate from ASK1, and JNK in the case for GST, to leave the kinases susceptible to activation. The inhibition of these kinases by thioredoxin and GST is suggested to be dependent upon interaction of these proteins through reactive cysteine residues.

There is likely to be a highly ordered network of signaling mechanisms that act to regulate the ARE in an appropriate manner. Many studies are required to determine the moment where each component of the signaling network, whether it be a kinase, Keap1, or other associated factors, impact to either promote or repress ARE-driven transcription. There appears likely to be a high degree of communication and cross-talk between signaling pathways associated with such an important response mechanism that we are beginning to elucidate.

CONCLUDING REMARKS

In this review, we have provided an overview of the link between detoxification enzyme monofunctional inducers and the discovery and initial characterization of the ARE. This has created an area of research that has given insights into cellular function and potential for the development of therapeutics that will benefit human health. Apart from the expanding field of cancer chemoprevention, enzymes that are regulated by the ARE may also have a protective role in other diseases, such as Alzheimer's disease and arthritis, where oxidative stress has been linked to their pathology. The number of genes that constitute the ARE gene battery is likely to increase with the use of modern genomic technology such as microarray analysis. This will provide further information as to the importance of this gene regulatory pathway within cells.

The focus of this review has centered upon the most recent studies into the regulation of the ARE at the molecular level. Currently, researchers are working to characterize the specific transcription factors and cell signaling pathways that are responsible for eliciting a transcriptional response. Where it is certain that proteins such as Nrf2 and Keap1 are critical in this response, the role of small Maf proteins, which to date are the only potential partners for Nrf2 in binding to the ARE, and other transcription factors requires further clarification. It is also certain that protein phosphorylation is important in regulating the ARE. The participation of protein kinase signaling pathways is complicated and appears to vary according to the nature of the inducing agent, cell type, and sequence context of the ARE. How the MAPK, PI3K, and PKC function and interact within cells to regulate the ARE requires careful dissection. What is not known is how the ARE response is initiated. It would be most valuable to identify the sensing mechanism within cells that leads to oxidative stress activating the ARE pathway. It is hoped that these studies will lead to the design of safe therapeutic agents that may help to prevent the progression of cancer as well as inflammatory and neurodegenerative disease.

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MONOAMINE TRANSPORTERS: From Genes to Behavior

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■ **Abstract** Modulation of fast neurotransmission by monoamines is critically involved in numerous physiological functions and pathological conditions. Plasma membrane monoamine transporters provide one of the most efficient mechanisms controlling functional extracellular monoamine concentrations. These transporters for dopamine (DAT), serotonin (SERT), and norepinephrine (NET), which are expressed selectively on the corresponding neurons, are established targets of many psychostimulants, antidepressants, and neurotoxins. Recently, genetic animal models with targeted disruption of these transporters have become available. These mice have provided opportunities to investigate the functional importance of transporters in homeostatic control of monoaminergic transmission and to evaluate, in an in vivo model system, their roles in physiology and pathology. The use of these mice as test subjects has been helpful in resolving several important issues on specificity and mechanisms of action of certain pharmacological agents. In the present review, we summarize recent advances in understanding the physiology and pharmacology of monoamine transporters gained in mice with targeted genetic deletion of DAT, SERT, and NET.

INTRODUCTION

Monoaminergic transmission is involved in a variety of physiological, behavioral, and endocrine functions (1, 2). These systems are believed to be critically important in both the pathophysiology and the pharmacotherapeutics of a number of brain disorders, including Parkinson's disease, depression, drug abuse, schizophrenia, attention-deficit hyperactivity disorder (ADHD), and Tourette syndrome (1–3). A complex balance between the amount of neurotransmitter synthesized, stored, released, metabolized, and recaptured determines the intensity of monoaminergic signaling (3). Monoamines released into the extracellular space can undergo enzymatic degradation and dilution by diffusion; however, the major mechanism controlling extracellular monoamine dynamics has proven to be reuptake by presynaptic neurons via plasma membrane monoamine transporters (4–9). Monoamine transporters, such as that for dopamine (DAT), serotonin (SERT),

and norepinephrine (NET), that most likely localized perisynaptically (10–12) in corresponding neurons, remove neurotransmitters from outside cells and recycle it back into the releasing and/or neighboring terminals (4–9). In most cases, this uptake is neurotransmitter specific; however, under certain conditions or in distinct brain regions, monoamines can be cleared from extracellular space heterologously by multiple monoamine transporters (13–16).

The DAT, NET, and SERT are members of the family of Na^+, Cl^--dependent substrate-specific neuronal membrane transporters, which includes transporters for GABA, glycine, taurine, proline, betaine, and creatine (4–8). The putative structure of these transporters consists of 12 transmembrane domains with both the N- and C-terminal domains located within the cytoplasm. The mechanism of the transporter-mediated uptake of monoamines is believed to involve an electrogenic transport of monoamines by sequential binding and cotransport of Na^+ and Cl^- ions (4–8).

These transporters represent established targets of many psychostimulants and antidepressants, which exert their potent psychotropic action via interference with transporter function, resulting in a rise in extracellular levels of monoamines (4, 8). Some neuron-specific toxins can enter the cells through plasma membrane monoamine transporters, thereby revealing the additional functional role of transporters as a molecular gateway for neurotoxins (6, 9).

Relatively little attention has been given to another important aspect of transporter function. Several lines of evidence have suggested that drugs affecting monoamine transporters can significantly modulate presynaptic neuronal homeostasis (17–22). However, until genetic animal models with targeted disruption of these transporters became available (23–25), this homeostatic role of plasma membrane transporters was not fully appreciated.

One of the attractive applications of mice having genetic deletions of certain proteins is their use as test subjects to evaluate the role of these molecules in therapeutic or pathological actions of psychotropic drugs. Several important observations in this context were made in transporter mutant mice (26).

Another important implication of genetically altered animals is to model, in vivo, the pathological conditions underlying human diseases. Profound physiological and behavioral changes detected in these mice have provided some intriguing insights on the potential role of these proteins in human diseases (27).

In this essay, recent findings on DAT (23), SERT (24), and NET (25) knockout (KO) mice on the homeostatic function of monoamine transporters, their involvement in the psychotropic or neurotoxic actions of various drugs, and their role in aberrant behaviors are discussed.

DOPAMINE TRANSPORTER-DEFICIENT MICE

The unique role of dopamine (DA) as a mediator of such critical functions as movement, emotion, and affect determines the involvement of this neurotransmitter in a variety of pathological conditions and disorders (1–3). It is not surprising,

therefore, that mice lacking the DAT have attracted continued interest, and a considerable amount of data on transporter function and pharmacology have been gained using this model. The DAT knockout (DAT-KO) mice, generated through genetic deletion of the DAT by homologous recombination (23, 28), display a distinct behavioral phenotype. The DAT-KO mice are hyperactive (23, 28–30), dwarf (31), display cognitive (29) and sensorimotor gating (32) deficits, and sleep dysregulation (33). The mutant mice demonstrate normal social interaction (30), but females lacking the DAT show an impaired capability to care for their offspring (23), most likely due to anterior pituitary hypoplasia-related hormonal dysregulation (31). Abnormalities in skeletal structure (34) and altered regulation of gastrointestinal tract motility (35) have been also described in DAT-KO mice.

Neurochemistry

EXTRACELLULAR DA DYNAMICS Hyperactivity of central dopaminergic transmission in DAT-KO mice was first demonstrated in cyclic voltammetry experiments in mouse striatal slices, which showed a 300-fold increase in extracellular lifetime of DA released by single pulse stimulation (23, 36). As might be expected, cocaine and amphetamine did not affect DA clearance in the striatum of DAT-KO mice (23, 36, 37). Furthermore, neither serotonin and norepinephrine transporter inhibitors nor inhibitors of the DA degradative enzymes monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT) were able to modulate the rate of DA clearance in the striatum of these mice (36). Thus, it has been demonstrated that within the time frame of single pulse stimulation, only diffusion may be involved in removing extracellular DA in the striatum of DAT-KO mice (36). Similar conclusions were reached when a complementary technique to study evoked DA release and the kinetics of DA elimination, carbon fiber amperometry, was used (38). In these in vivo experiments in anesthetized mice, striatal DA release was evoked by electrical stimulation of the medial forebrain bundle. Extracellular DA half-life was estimated to be at least two orders of magnitude higher in DAT-KO mice. Again, the inhibition of COMT did not significantly affect DA clearance. However, inhibition of MAO by pargyline modestly slowed down DA elimination in DAT-KO mice, suggesting that metabolism of DA by MAO may play some role, in addition to diffusion, in the clearance of DA released over the course of multiple stimulations. It has also been demonstrated that in mice lacking the DAT, low-frequency firing resulted in consistently high extracellular DA levels that could not be distinguished from DA levels achieved by high-frequency firing. These observations were interpreted to suggest that in DAT-KO mice the burst firing activity cannot be specifically translated into phasic changes in extracellular DA as happens in normal animals (38).

Overall, both voltammetric and amperometric studies convincingly demonstrated remarkably prolonged lifetime of extracellular DA in DAT-KO mice (36, 38). However, both investigations also revealed a significantly decreased (by 75% in voltammetric experiments and by 93% in amperometric studies) amount of

DA molecules released in response to stimulation (36, 38). To directly prove that the absence of DAT can induce elevation in extracellular DA concentrations, an alternative approach to assess basal extracellular DA level, a quantitative "no net flux" microdialysis technique was used (36, 39). These studies documented that, despite decreased levels of releasable DA, the greatly prolonged extracellular lifetime of DA in DAT-KO mice results in significantly elevated basal extracellular DA levels (fivefold). Nevertheless, these abnormal extracellular concentrations of DA were substantially lower than might be inferred from alterations in the clearance rate observed in DAT-KO mice (36, 38). It is important to underscore that extracellular DA levels in DAT-KO mice were still reflective of depolarization-dependent vesicular exocytosis. In microdialysis experiments, infusion of tetrodotoxin or Ca^{2+}-free artificial media gradually reduced levels of extracellular DA to undetectable levels (40). Altogether, these neurochemical investigations firmly establish the DAT-KO mice as a genetic model of persistent hyperdopaminergia (40).

PRESYNAPTIC HOMEOSTASIS Although profound alterations in extracellular DA dynamics in mice lacking the DAT were predictable from previous pharmacological experience, dramatic changes in the regulation of DA transmission at the level of presynaptic terminals were unexpected. Most notably, total tissue DA levels in the striatum, which generally reflect the intraneuronal vesicular storage pool of DA, were found drastically (20-fold) reduced in DAT-KO mice (36). Furthermore, these low levels of DA in the striatum of DAT-KO mice were extremely sensitive to the inhibition of tyrosine hydroxylase (TH)-mediated synthesis of DA, suggesting that they represent a newly synthesized pool of DA (36).

The striatal levels of TH protein were also markedly decreased in DAT-KO mice (36, 41). However, these reductions in DA and TH levels cannot be explained as a consequence of abnormal development or degeneration of DA neurons, due to several reasons. First, TH-positive neurons in the substantia nigra (SN), where cell bodies of nigrostriatal DA neurons are located, were only marginally decreased with no modification in the ratio of TH mRNA levels per neuron. Second, the striatal levels of the other enzyme involved in DA synthesis, DOPA decarboxylase, were not modified, and, finally, the vesicular transporter VMAT2 levels were only marginally decreased (39, 41). Furthermore, functional activity of TH in the striatum as measured by L-DOPA accumulation was in fact increased in mutant mice (36). Although the exact mechanisms responsible for such dramatic alterations in TH regulation are not clear, it is likely that the decrease in intracellular DA itself, rather than loss of dopaminergic projections, is responsible, at least in part, for this effect.

It is important to note that depleted striatal DA storage in DAT-KO mice cannot be explained by a deficit in VMAT2-mediated vesicular uptake mechanisms. Neither VMAT2 mRNA in the SN or tetrabenazine binding, VMAT2 protein levels, nor functional vesicular uptake of DA in the striatum was significantly altered in these mice (39). Thus, the depletion of the DA storage pools and the decreased

DA release in DAT-KO mice could be direct consequences of the lack of inward transport of DA through the DAT. Consequently, in the normal situation, a dependence of DA storage on recycled, rather than newly synthesized, DA must exist (Figure 1) (36, 39).

In fact, depletion of DA stores in DAT-KO mice occurred even though the DA synthesis rate was elevated (twofold) (36). Accordingly, the tissue levels of DA metabolites were either unaltered (DOPAC) or elevated (HVA) in DAT-KO mice (36). Thus, both DA synthesis and turnover are extremely high in the mutant animals, despite the low levels of striatal TH protein (36). This apparently paradoxical data may be explained by the disinhibition of TH, which is a known subject of tonic feedback inhibition by both intraneuronal and extraneuronal DA (3, 42). In the DAT-KO mice, intraneuronal DA is reduced; this could account for the disinhibition of TH. Alternatively, activation of TH in DAT-KO mice may be explained by dysregulation of autoreceptor control due to pronounced extracellular DA concentrations (3). Indeed, D2 DA receptor mRNA and binding of selective ligands were decreased by 50% in dopaminergic cell body regions in DAT-KO mice (23, 43). Neurochemical and electrophysiological studies also revealed a marked desensitization in major autoreceptor functions (43). The firing rate of DA neurons in the ventral midbrain was elevated and only slightly sensitive to DA agonist application. Striatal nerve terminal release-regulating autoreceptors were also essentially ineffective. The D2/D3 DA receptor agonist quinpirole elicited only a slight decrease in striatal DA release in DAT-KO mice, as measured by both cyclic voltammetry and in vivo microdialysis. Similarly, terminal DA autoreceptors controlling DA synthesis were also found to be nearly nonfunctional (43). This down-regulation of autoreceptor function, which is likely a consequence of persistently elevated extracellular DA, once more underscores an important homeostatic control that DAT exerts over presynaptic neuronal function.

POSTSYNAPTIC RECEPTOR RESPONSIVENESS Another important consequence of the marked alterations in extracellular DA dynamics is dysregulation in postsynaptic DA receptor responsiveness. As might be expected in the striatum of DAT-KO mice, mRNA and protein levels of the two major postsynaptic DA receptors, D1 and D2, were down-regulated by approximately 50% (23). However, some populations of postsynaptic DA receptors appear to be up-regulated. In quantitative in situ hybridization studies, decreased mRNA levels for both D1 and D2 receptors but increased levels of the D3 receptor mRNA (+40%–110%) were found in DAT-KO mice (44). Similarly, an increased density of preproenkephalin A-negative neurons that express the D3 receptor mRNA was described (44). In addition, investigations of the firing rate of DA-responsive neurons in the nucleus accumbens of DAT-KO mice have shown unaltered responsiveness of postsynaptic receptors to a microiontophoretically applied D1 receptor agonist, despite the apparent decrease in receptor numbers (45). A similar decrease in the number of D2 DA receptors that result in a reduced efficiency of coupling to G proteins (L.M. Bohn & M.G. Caron, unpublished data) is surprisingly associated with a conversion from

the normal inhibitory electrophysiological response to quinpirole to an excitatory effect (45). Another paradoxical observation in this regard was gained using an in vivo approach (40). In DA-depleted DAT-KO mice, the D1/D2 DA receptor agonist apomorphine induced more pronounced locomotor activation in comparison to wild-type controls. Thus, it appears that postsynaptic receptors are not uniformly adapted to the inactivation of DAT, with some populations becoming down-regulated but others being supersensitive.

GENE-DOSE EFFECT OF THE LEVEL OF DOPAMINE TRANSPORTER EXPRESSION Interestingly, essentially all of the neurochemical alterations listed above display a clear gene-dose effect (36, 43, 46). Thus, mice heterozygous for DAT deletion displayed intermediate neurochemical profiles between mutant and wild-type mice. Particularly, in heterozygous mice, the striatal tissue level of DA is decreased by 30%, DA synthesis is modestly elevated, TH inhibition results in faster depletion of DA storage, autoreceptor regulation is partially diminished, DA clearance is prolonged by approximately twofold, and extracellular DA is twofold higher in comparison to wild-type mice (36, 43). Similar observations have been gained in mice expressing less than 10% of DAT (46). Importantly, however, the magnitude of these changes was not directly proportional to the level of DAT expression. For example, the alterations in DA homeostasis observed in mice expressing 10% of the DAT (46) were generally more pronounced than in DAT heterozygous mice, but substantially less than the full magnitude of effect observed in DAT-KO mice (36, 43).

DOPAMINE TRANSPORTER AS DETERMINANT OF THE MODE OF DA TRANSMISSION Importantly, the alterations described in the striatum of DAT-KO mice are less evident in other brain areas of these mice. For example, less pronounced depletions in tissue DA were observed in the hypothalamus and pituitary of DAT-KO mice (31), and only minimal alterations in DA and metabolite concentrations were found in the frontal cortex (R.R. Gainetdinov & M.G. Caron, unpublished data). In fact, in DAT-KO mice, the mode of striatal DA transmission closely resembles that described in the frontal cortex of normal animals, where relatively low DAT expression (47) and DA uptake rates are found (48). Several characteristics of mesocortical DA neurons are markedly different from nigrostriatal neurons (3, 49). The firing rate of mesocortical neurons is elevated, possibly indicating less activity of impulse flow-regulating autoreceptors at the level of cell bodies (49). There are few DA synthesis-modulating autoreceptors in the frontal cortex (50). Tissue DA content is disproportionally low in comparison to both the basal extracellular DA level (51) and stimulation-evoked DA release (48). Moreover, DA storage in the frontal cortex is tightly dependent on ongoing synthesis, as evidenced by an increased sensitivity to TH inhibition (49). Similar characteristics of nigrostriatal neuron homeostasis observed in mice lacking the DAT strongly suggest that a low level of DAT expression is a primary determinant of these features of mesocortical neurons in normal animals.

The DAT, as a critical mechanism terminating extracellular DA signals, can play an important role in determining the mode of extracellular transmission from more synaptically limited to "volume"-like or "nonsynaptic" (36, 52) transmission. Profound alterations in presynaptic DA neurochemistry found in DAT-KO mice highlight an additional physiological role of DAT as a key controller in the presynaptic DA homeostasis (36, 39). Different dopaminergic groups of cells expressing various levels of DAT may have markedly different profiles of transmission. In addition, factors affecting DAT expression and regulation, such as development (53, 54), aging (55), and exposure to pharmacological or environmental agents (9, 56), may induce substantial shifts between these modalities. Particularly, because DAT is subject to substantial structural and functional maturation postnatally (53, 54), a specific developmentally determined mode of DA transmission may occur at earlier ages.

Pharmacology

ESTABLISHING THE MAJOR DRUG TARGET AND UNMASKING SECONDARY TARGETS

It is well known that DAT is the major drug target for psychostimulants like cocaine, methylphenidate, and amphetamine (4, 8). Accordingly, in the striatum of DAT-KO mice, these psychostimulants are unable to affect DA clearance or extracellular levels (23, 29, 37, 57). Whereas cocaine and methylphenidate are classical inhibitors of the DAT (3, 5, 8) and their ineffectiveness could be simply explained by the absence of their primary target, a more complex picture emerges with amphetamine (58, 59). Amphetamine enters the DA terminal not only through the DAT but also by diffusion (58). Then, via VMAT2 and/or diffusion, the drug enters vesicles and disrupts the vesicular pH gradient (59). As a result, amphetamine produces a redistribution of stored monoamine from vesicles into the cytoplasm, from which it is transported into the extracellular space by reverse DAT-mediated transport (59). In addition, while inside the cell, amphetamine decreases intraneuronal metabolism of DA via direct inhibition of MAO (58). As a result of all of these actions, amphetamine markedly elevates extracellular DA concentrations in the brain. Although most of the actions of amphetamine are attributed to its effect on the DA system, it is important to underscore that it similarly affects norepinephrine (NE) and serotonin (5-hydroxytryptamine, 5-HT) transmission (58).

Experiments in DAT-KO mice (37) clearly illustrated that although the interaction of amphetamine with the DAT is pivotal in its action, an involvement of other critical processes is important as well. Particularly, the role of vesicular storage of DA in amphetamine action has been highlighted in these mice (37). For example, while amphetamine-induced elevation of striatal DA did not occur in mice lacking DAT, the vesicle-depleting action of amphetamine was still observed (37). Furthermore, it has been suggested that amphetamine-triggered reverse transport of DA from cytoplasm to extracellular space does not occur simply due to an elevated cytoplasmic concentration of DA but requires direct action of amphetamine on the DAT (37).

High doses or chronic treatment with amphetamine and related drugs are known to induce dopaminergic and serotonergic neurotoxicity in several brain regions (60). For example, in normal animals, a neurotoxic regimen of methamphetamine administration produces massive DA outflow, free radical formation, and neuronal damage as evidenced by reactive astrogliosis and depletion of DA levels in striatal tissue. However, the same treatment failed to modify any of these parameters in the striatum of DAT-KO mice, whereas modest decreases in striatal and hippocampal 5-HT levels were observed (61). Thus, a critical role of DAT as a mediator of methamphetamine-induced dopaminergic but not serotonergic neurotoxicity has been demonstrated (61).

Similarly, a crucial role of DAT in the neurotoxic action of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been supported in mice lacking DAT (62, 63). In numerous previous studies it had been demonstrated that MPTP neurotoxicity involves selective uptake of its active metabolite, 1-methyl-4-phenylpyridinium (MPP^+) into the DA neuron via the DAT (6, 9). In DAT-KO mice treated with MPTP, markers of striatal neurotoxicity, such as depletion in DA levels and reactive astrogliosis, were virtually absent, whereas in DAT heterozygotes a partial sensitivity was observed (62). In another investigation, no apparent DA cell body loss was found in the substantia nigra of MPTP-treated DAT-KO mice (63). Thus, an absolute requirement of DAT for development of MPTP toxicity in vivo has been firmly established (9, 62, 63).

The wake-promoting action of psychostimulants as well as the antinarcoleptic compound modafinil was also probed in DAT-KO mice (33). Despite the widespread use of this antinarcoleptic drug in the clinic, the mechanism of its action had not been established. In DAT-KO mice, the wake-promoting action of modafinil, methamphetamine, and the DAT inhibitor GBR12909 was virtually absent. Thus, a role of DAT and DA in the antinarcoleptic action of psychostimulants and modafinil has been postulated (33).

One of the most surprizing findings made in DAT-KO mice is that the rewarding properties of cocaine are preserved in DAT-KO mice (28, 57) despite the demonstrated lack of effect of cocaine on striatal extracellular DA levels (23, 57). The ability of cocaine to block DAT and elevate extracellular DA is widely considered to be the primary determinant of its reinforcing and addictive properties (64, 65). However, DAT-KO mice were still able to self-administer cocaine (57) and display cocaine-conditioned place preference (28). These observations demonstrate that the DAT is not the sole mediator of the rewarding properties of cocaine and that other actions of cocaine should be also considered. It is well known that cocaine blocks not only DAT but can potently inhibit other monoamine transporters, such as SERT and NET (4, 8, 57). Furthermore, it has been demonstrated that DA can be captured by the NET in brain regions with low DAT expression, such as the frontal cortex (13, 16). The nucleus accumbens is the brain region that is primarily associated with reward-related behaviors (64, 65). It is known to have high densities of both DAT and NET, unlike the striatum where only DAT is present (66). The possibility has therefore been raised that the ability of cocaine to block NET

may produce the necessary rise in DA levels in the nucleus accumbens to induce cocaine reward in the DAT-KO mice (66). In fact, it has been observed that despite the lack of effect of these psychostimulants in the striatum of DAT-KO mice, both cocaine and amphetamine are able to elevate DA levels in the nucleus accumbens (66). At the same time, inhibition of the NET by reboxetine resulted in elevation of DA levels in the nucleus accumbens in microdialysis experiments (66), whereas another NET inhibitor nisoxetine was somewhat effective in blocking residual DA uptake in the nucleus accumbens synaptosomes from DAT-KO mice (16). These data have been tentatively interpreted to indicate that the ability of cocaine to block NET in the nucleus accumbens of DAT-KO mice is responsible for the elevation of DA levels and for inducing reward (66). However, in recent experiments using fast-scan cyclic voltammetry in brain slices, no such effect of locally applied cocaine or NET inhibitor desipramine on electrically evoked DA release and clearance in the shell of nucleus accumbens of DAT-KO mice was found (67). Similarly, no inhibition of residual DA uptake in nucleus accumbens synaptosomes from DAT-KO mice was observed after cocaine (16). Thus, the mechanism of this elevation of DA in the nucleus accumbens of DAT-KO mice by cocaine and amphetamine remains to be clarified. One possibility may involve an indirect modulation of DA neurons via NE (or 5-HT) inputs at the level of DA cell bodies in the ventral tegmental area leading to elevated DA release in the nucleus accumbens. It should be cautioned, however, that it is unclear whether any further elevation in DA levels could be rewarding in DAT-KO mice because extracellular levels of DA are already elevated fivefold both in the striatum and nucleus accumbens (36, 66).

There are additional reasons to believe that the 5-HT system could be primarily responsible for the unmasked mechanism of cocaine reward provided by elimination of DAT-mediated effects of the drug in DAT-KO mice. Numerous pharmacological investigations have suggested the potential involvement of 5-HT in cocaine responses (68). In DAT-KO mice, the role of 5-HT mechanisms has been suggested by analysis of cocaine analogue binding and mapping of cocaine-induced neuronal activation sites (57). Most crucial evidence for this hypothesis has been gained recently in double-mutant mice lacking both DAT and SERT (69). Unlike single mutants of DAT and SERT, these double-mutant mice do not display conditioned place preference for cocaine, strongly suggesting that, at least in this paradigm, the interaction of cocaine with the SERT is required for cocaine reward in DAT-KO mice. Future investigations of cocaine-induced behaviors in double-knockout mice lacking DAT and NET, recently developed in our laboratory, would be helpful to resolve this issue.

HYPERDOPAMINERGIA AS A MODEL TO TEST DRUG ACTIONS Neurochemical investigations have established the DAT-KO mice as a genetic model of persistent hyperdopaminergia (40). This hyperdopaminergia is revealed functionally as pronounced locomotor hyperactivity, thereby providing a simple model in which the effects of pharmacological agents in modulating DA-regulated behaviors can be assessed. Hyperactivity of these mice is extremely sensitive to treatments

diminishing DA transmission, such as inhibition of DA synthesis (29) or blockade of DA receptors with haloperidol (29, 30), clozapine (30), raclopride (32), and SCH23390 (32). Surprisingly, however, some drugs can modulate this hyperactivity without a direct effect on striatal DA function. Particularly, psychostimulants, like amphetamine, cocaine, and methylphenidate, paradoxically inhibited hyperactivity of DAT-KO mice (29, 70). A similar hypolocomotor effect of amphetamine was observed in modestly hyperactive mice with reduced expression (<10%) of DAT (46) and even in normally active DAT heterozygous mice (70). Although the lack of stimulatory action of psychostimulants in DAT-KO mice could be explained by the lack of striatal DA elevation (29, 37, 57, 66), the inhibitory effect reflects the action of these drugs on molecular targets other than the DAT. In fact, this inhibitory action of psychostimulants was found to be related to the other well-known but largely ignored target of these drugs—the SERT (29, 70). Both direct and indirect 5-HT agonists, including the selective serotonin transporter inhibitor (SSRI) fluoxetine, 5-HT agonist quipazine, and 5-HT precursors tryptophan and 5-hydroxytryptophan, but not the NET inhibitor nisoxetine, potently inhibited the hyperactivity of DAT-KO mice (29). Notably, the inhibitory role of 5-HT on DA-mediated activation and the role of this 5-HT/DA interaction in psychostimulant-induced behaviors were suggested more than 20 years ago (71).

Importantly, this paradoxical inhibitory effect of psychostimulants on hyperactivity occurs without direct involvement of striatal DA transmission (29). Striatal control of motor behaviors involves a reciprocal functional interaction of nigrostriatal dopaminergic and frontostriatal glutamatergic pathways (2, 72). These systems are known to converge at the level of the striatal medium spiny GABA neurons (2, 72). Because a complex modulatory action of 5-HT on the glutamatergic neurons is well-known (72), a role for glutamatergic mechanisms as an intermediate in the inhibitory action of psychostimulants has been suspected (73). In fact, it has been observed that the hyperactivity of DAT-KO mice can be markedly further enhanced when N-methyl-D-aspartate (NMDA) receptor-mediated glutamatergic transmission is blocked. Both DAT-KO and heterozygous mice were more sensitive to this effect of the NMDA antagonist (+)-MK-801 (73). Conversely, drugs that enhance glutamatergic transmission through positive modulation of AMPA glutamate receptors, such as AMPAkines (73), or increasing glycine concentration, such as glycine transporter (Glyt1) inhibitors (74), inhibited hyperactivity in DAT-KO mice. Furthermore, blockade of NMDA receptors by (+)-MK-801 effectively prevented the inhibitory effects of both psychostimulant and serotonergic drugs on hyperactivity, suggesting that intact glutamate transmission is required for the inhibitory effect of 5-HT on hyperactivity (73). These initial investigations suggested a dependence of DA-related locomotor responses on the intensity of frontostriatal glutamatergic signaling, which, in turn, can be potently modulated via serotonergic input. Therefore, because modulation by serotonin of glutamatergic transmission can counteract dopaminergic hyperactivity (29, 73), the potential therapeutic value of serotonergic and glutamatergic drugs in conditions associated with dopamine hyperfunction, such as schizophrenia and ADHD, might be contemplated.

Besides hyperactivity, the DAT-KO mice exhibit deficient sensorimotor gating as measured by prepulse inhibition (PPI) of the startle response (32), perseverative patterns of locomotion (32), and learning and memory deficits (29). To date, only few drugs have been tested in these paradigms. As might be expected, deficits in PPI were corrected by the D2 DA receptor antagonist raclopride, but not by the D1 DA receptor antagonist SCH23390 (32). In contrast, antagonism of D1 but not D2 DA receptors significantly attenuated the perseverative patterns of locomotion (32).

Another important question to be addressed in these mice is how the drugs that have a dopaminergic component as a part of their action could manifest their effects under conditions of a persistently elevated DA tone. For example, morphine was able to further elevate extracellular DA levels in the nucleus accumbens and induce enhanced reward in CPP test, but failed to increase locomotor activity in DAT-KO mice (75). In addition, whereas morphine-induced analgesia was unaffected in mutant mice, the behavioral manifestations of naloxone-induced withdrawal were blunted (75).

Dopamine Transporter-Deficient Mice as a Potential Animal Model of ADHD

Attention-deficit hyperactivity disorder (ADHD) is a common developmental disorder that manifests mostly in school-aged populations as impulsivity, hyperactivity, and inattention (76). The causes and pathophysiology of ADHD are unknown, but compelling evidence suggests an involvement of genetic factors. DA is believed to play a major role in ADHD, but a role for NE and 5-HT systems has also been suspected (76). The most commonly used pharmacotherapy for ADHD is based on the paradoxical ability of psychostimulants to produce an ameliorating (calming) effect; however, some antidepressants are also effective (77). Several studies have reported an association between a polymorphism in the noncoding regions of DAT gene and ADHD (78–80). These molecular genetic studies provide provocative evidence to anticipate that alterations in DAT-mediated processes could significantly contribute to the pathogenesis of this disorder. The functional consequence of this association is still unclear.

Several lines of evidence suggest that the observations gained in DAT-KO mice may be relevant for this disorder (29, 81, 82). As noted above, in DAT-KO mice, a fivefold increase in extracellular DA in the brain is associated with remarkable hyperactivity (36). This hyperactivity is triggered by exposure of mice to a novel environment. No corresponding rise in extracellular DA accompanies this novelty-driven hyperactivity, suggesting that these behavioral changes are regulated through more than just the DA system (29). These mice also showed impairments in tests assessing cognitive function, most likely related to poor behavioral inhibition as evidenced by both perseverative errors in eight-arm maze test and perseverative pattern of locomotion (29, 32). Most intriguingly, these mice responded to psychostimulants in the same way as individuals with ADHD. Administration of psychostimulants amphetamine, methylphenidate, or even

cocaine to the hyperactive DAT-KO mice potently inhibited their activity. Conversely, normal mice become hyperactive when given these psychostimulants (29). As described above, these mice provided a unique model to investigate a neuronal circuitry involved in the hypolocomotor action of psychostimulants (29, 73). Particularly, it has been demontrated that psychostimulants would decrease activity in DAT-KO mice by enhancing serotonin's inhibitory effects over dopaminergic hyperactivity, rather than by acting directly on the DA system (29). It should be mentioned, however, that 5-HT exerts an extremely complex set of actions on locomotor behaviors (72), and some of the 14 subtypes of 5-HT receptors known to date can induce opposite actions on locomotion. To determine which subtype(s) of 5-HT receptors are primarily involved in the hypolocomotor effect of psychostimulants represents a major challenge for future research.

Overall, the DAT-KO mice display several key characteristics of ADHD, including hyperactivity, impairments in cognitive tests, and paradoxical inhibitory responses to psychostimulants (29, 81, 82). Similarly, mice with reduced (<10%) expression of DAT demonstrate a modest hyperactivity, impaired response habituation, and paradoxical hypolocomotor reactions to amphetamine (46). In striking contrast, transgenic mice with modestly increased DAT expression (+20%–30%) show hypoactivity, which is particularly evident in a new environment (83).

One intriguing feature of ADHD is that in this developmental disorder, a significant reduction in the number of affected individuals occurs with age (76). These observations indicate that during the course of the disorder susceptibility to pathological manifestations and therapeutic responses to psychostimulants may vary. It is worthwhile mentioning that the DAT and SERT follow divergent patterns of expression in various brain areas through postnatal development (53, 54, 84), and altered responses to psychostimulants in different age groups, even in normal subjects, might occur (85).

It should be noted, however, that there are obvious caveats about this model (29, 81, 82). It is unlikely that complete functional absence of DAT occurs in ADHD patients, and as such DAT-KO mice represent an extreme case of a potential DAT dysfunction. Furthermore, multiple genes most likely contribute to this disorder and DAT-KO mice illustrate only one potential cause of these manifestations. It should be emphasized that dopaminergic dysregulation, as that observed with the DAT-KO mice, might be produced by defects in components of the system other than DAT. Moreover, dysregulation of the DA system is unlikely to be the exclusive mechanism responsible for the development of ADHD.

SEROTONIN TRANSPORTER-DEFICIENT MICE

Behavior and Pharmacology

Mice lacking the serotonin transporter (SERT-KO) were developed by homologous recombination (24). Despite evidence that an excess of 5-HT during

development may disrupt several critical processes of embryogenesis, only minor developmental abnormalities (24), such as abnormal gastrointestinal motility (86), were observed in the mutants. The role of SERT in locomotor and rewarding effects of several psychostimulants has been probed in SERT-KO mice. It has been reported that the ability of SERT-specific amphetamine derivative (+)-3,4-methylenedioxymethamphetamine (MDMA) (87) to induce locomotor activation was disrupted in mutant mice but *d*-amphetamine at high doses induced hyperactivity similarly in both SERT-KO and normal mice (24). Rewarding properties of cocaine were preserved in these mice as reflected by pronounced, and even increased, conditioned place preference to cocaine, in comparison to wild-type mice (28). Most intriguingly, in double-knockout mice lacking both DAT and SERT, no place preference for cocaine was observed, highlighting the contribution of SERT-mediated effects of cocaine to the reward process (69). Thus, these results suggest that in the absence of DAT the action of cocaine at the SERT may be sufficient to induce cocaine reward. However, it should be underscored that elimination of DAT creates a situation when extracellular levels of DA are abnormally elevated fivefold (36), which is in fact higher than what maximal doses of cocaine can produce in wild-type mice. This genetic unmasking indicates that SERT-mediated effects may play an important role in the effects of psychostimulants, and certainly these serotonergic mechanisms deserve closer scrutiny as potential ways to modulate reward mechanisms. In contrast, in double-knockout mice lacking both SERT and NET, rewarding properties of cocaine in CPP test were enhanced, suggesting that the actions of cocaine on the NET system may result, in fact, in aversive effects (88).

Extracellular 5-HT Dynamics, Presynaptic Homeostasis, and 5-HT Receptor Responsiveness

Neurochemical studies on 5-HT neuron homeostasis performed in SERT-KO mice in general recapitulate the observations gained in DAT-KO mice with respect to DA transmission (36, 39). In initial studies, a lack of high-affinity [^{3}H]5-HT uptake in brain synaptosomes from SERT-KO mice was found (24), but in primary neuronal cultures from embryonic SERT-KO mice, [^{3}H]5-HT uptake, although very weak, was observed (15). In vivo microdialysis studies have shown that disrupted uptake of 5-HT in SERT-KO mice results in a substantial increase in extracellular levels of 5-HT (five- to sixfold) (89, 90). A marked reduction (60%–80%) in 5-HT tissue levels was found in several brain regions of SERT-KO mice (24, 89, 90), suggesting deficient intraneuronal storage of 5-HT. Furthermore, 5-HT synthesis was disinhibited in SERT-KO mice (89).

The consequences of altered neurotransmission in SERT-KO mice on the responsiveness of 5-HT receptors have been extensively documented. In electrophysiological studies (91), a marked desensitization of both pre- and postsynaptic 5-HT1A receptors was found in SERT-KO mice, whereas only presynaptic receptors were affected in the heterozygous mice. Accordingly, 5-HT1A binding

sites, mRNA, and protein levels were significantly decreased in certain, but not all, serotonergic brain areas (92). Altered hypothermic and neuroendocrine responses to the 5-HT1A agonist 8-OH-DPAT were also noted in SERT-KO mice (92). Similar alterations were also found with respect to 5-HT1B receptors (90). Quantification of [^{35}S]GTP-γ-S binding evoked by potent 5-HT1A and 5-HT1B receptor agonists revealed a decrease in receptor coupling in the dorsal raphe nucleus and the substantia nigra, but not in other brain areas in SERT-KO mice (90). Accordingly, a decrease in brain 5-HT turnover rate after administration of the 5-HT1A agonist ipsapirone and an increased 5-HT release in the substantia nigra by 5-HT1B/1D antagonist GR 127935 were disrupted in SERT-KO mice (90). In addition, the density of postsynaptic 5-HT2A receptors in SERT mutants was decreased in some brain areas (93). These data convincingly demonstrate that autoreceptor function is remarkably desensitized or down-regulated in SERT-KO mice, whereas postsynaptic receptor regulation may be more complex, depending on the brain area and the receptor subtype.

NOREPINEPHRINE TRANSPORTER-DEFICIENT MICE

Behavior and Pharmacology

Using homologous recombination, mice lacking the NET (NET-KO) have recently become available (25, 94). The homozygous NET-KO mice display both lower body weight and locomotor activity in a new environment. Similar to wild-type mice treated with antidepressants, untreated NET-KO mice exhibit prolonged escape attempts in either tail suspension test or the forced swim test, two commonly used tests for behavioral screening of antidepressants (25). No additional effect of NET-specific antidepressant desipramine in the tail suspension test was found in mutant mice, but, interestingly, antidepressants affecting primarily SERT and DAT (paroxetine and bupropione, respectively) were also ineffective (25). In the warm water tail immersion pain assay, a greater degree of morphine analgesia was found in NET-KO mice; the effect apparently mediated by enhanced NE stimulation of α2-adrenoreceptors (95). Interestingly, desipramine did not affect basal level of analgesia in the NET-KO mice, but was still able to produce significant inhibitory action on the locomotor activity of these mutants, suggesting that not all the effects of this drug are mediated exclusively via interaction with the NET (95). Both cocaine and amphetamine were more effective in stimulating locomotion of NET-KO mice (25). Moreover, chronic cocaine treatment produced no significant enhancement of cocaine responses in NET-KO mice, while producing a robust sensitized effect in wild-type controls. In addition, somewhat increased rewarding properties of cocaine were observed in NET-KO mice (25), and an even more pronounced CPP to cocaine was observed in SERT/NET double-knockout mutant mice (88). These enhanced responses to psychostimulants have been accompanied with suppression of striatal dopaminergic function and

postsynaptic D2/D3 DA receptor supersensitivity (25). Intriguingly, D2/D3 DA receptor supersensitivity has been previously found in experimental animals treated chronically with either noradrenergic or serotonergic antidepressants, and this supersensitivity has been hypothesized to be a common final pathway of antidepressant action (96).

Extracellular NE Dynamics, Presynaptic Homeostasis, and Adrenergic Receptor Responsiveness

Like DAT-KO and SERT-KO mice, the NET-KO mice demonstrated profound alterations in extracellular monoamine dynamics, presynaptic neuron homeostasis, and postsynaptic receptor regulation (25). In the fast-scan cyclic voltammetry experiments performed in the bed nucleus of the stria terminalis, pars ventralis, the release of NE in response to electrical stimulation was reduced by approximately twofold and the rates of clearance following stimulation were at least sixfold slower in the NET-KO mice (25). The extracellular NE levels in the cerebellum were elevated twofold in mutant mice as evidenced by quantitative microdialysis (25). In NE-enriched brain regions such as prefrontal cortex, hippocampus, cerebellum, and spinal cord tissue, concentrations of NE, reflective of intraneuronal NE storage, were approximately 55%–70% lower in mutant mice (25, 95). The synthesis rate of NE, assessed in the hippocampus, was augmented approximately 1.7-fold over that of wild-type littermates (25). As a result of elevated extracellular levels of NE, a significant decrease (30%) in postsynaptic α1-adrenergic receptor binding in hippocampus of NET-KO mice was observed (25). However, in the binding assessment of α2-adrenergic receptor density in the spinal cord, no significant alterations were found in these mutants (95).

NET-KO mice were used to demonstrate heterologous uptake of catecholamines in certain brain areas. Particularly, in the frontal cortex synaptosomes from NET-KO mice, no inhibitory effect of cocaine and nisoxetine on uptake of DA was found, whereas effectiveness of cocaine in the nucleus accumbens was somewhat reduced (16). Thus, it has been demonstrated that DA uptake in brain regions with low levels of the DAT may occur via NET (16). Similar conclusions were reached recently when voltammetric analyses of catecholamine clearance in the frontal cortex (97) and bed nucleus of the stria terminalis (98) were performed.

Taken together, these results support and extend the observations made in the DAT-KO and SERT-KO mice, where disruption of transporters is found to exert similar changes in homeostasis of corresponding neurons.

CONCLUSIONS

Neurochemical investigations performed in transporter mutant mice clearly illustrate that elimination of the active transport process results in a fundamental shift in the mode of neuronal transmission (23–25, 36, 39, 89) (Table 1). Absence

TABLE 1 Alterations in monoamine homeostasis in DAT-KO, SERT-KO, and NET-KO mice

Neurochemical parameters of respective monoamines	DAT-KO mice	SERT-KO mice	NET-KO mice
Extracellular clearance rate	Prolonged (300–fold) (23, 36, 38)	Not tested	Prolonged (sixfold) (25)
Amplitude of stimulated release	Decreased by 75%–90% (23, 36, 38)	Not tested	Decreased by 55% (25)
Basal extracellular levels	Elevated fivefold (36)	Elevated five- to sixfold (89, 90)	Elevated twofold (25)
Tissue content (storage)	Decreased by 95% (36)	Decreased by 65%–80% (24, 89, 90)	Decreased by 55%–75% (25, 95)
Synthesis rate	Elevated twofold (36)	Elevated (89)	Elevated by 70% (25)
Autoreceptor function	Disrupted (43)	Disrupted (90–92)	Not tested
Postsynaptic receptors	Down-regulated, but some population is supersensitive (23, 40, 44)	Down-regulated, but not uniformly (90–93)	Down-regulated (25)

of transporter function results in expected disruption of extracellular monoamine clearance and prolonged extracellular lifetime of monoamines. The most remarkable (300-fold) protraction of extracellular lifetime was found for DA in the striatum of DAT-KO mice (36). Currently, data on 5-HT clearance in the SERT-KO mice are not available to make a direct comparison. In the NET-KO mice (25), the clearance rate of NE was prolonged by only sixfold. The difference in the magnitude of alterations in clearance of monoamines observed in DAT-KO and NET-KO mice is essentially due to differences in the intrinsic clearance rates of NE and DA in normal animals because the residual clearance of NE and DA in the two respective mutants is essentially identical (25, 36). Furthermore, the assessment of DA clearance was performed in the striatum of DAT-KO mice where the highest levels of DAT are normally expressed, whereas measurements of NE dynamics were performed in the bed nucleus stria terminalis, one of the many areas expressing modest levels of NET (10, 12, 98). Thus, regional differences in transporter expression per neuron may contribute to these differences.

As a result of the protracted clearance, extracellular levels of the respective monoamines are elevated in all three mutant strains (25, 36, 89, 90), but the degree of elevation is quite variable. For example, a fivefold increase in extracellular DA was observed in DAT-KO mice (36), five- to sixfold elevation of 5-HT levels in SERT-KO mice (89, 90), but only a twofold elevation in extracellular

NE levels was found in NET-KO mice (25). Importantly, these extracellular levels of monoamines are substantially lower than could be predicted from alterations in clearance of monoamines. It is worth mentioning in this regard, that the actual amount of monoamine released per pulse was decreased for both DA and NE in mutant mice (25, 36). In addition, metabolic enzymes and potentially, additional transporter systems could contribute to the clearance of monoamines, and this contribution could vary for each neurotransmitter in each particular region.

In general, a concept of a diffusion-mediated extrasynaptic mode of neurotransmission, termed volume, paracrine, or nonsynaptic transmission (11, 52, 99), may be a suitable framework to describe the extracellular fate of monoamines in mice lacking plasma membrane monoamine transporters (36, 39). Volume transmission has been postulated for neuropeptides (52, 99) and also for classical neurotransmitters, including DA in the frontal cortex (11, 47, 48, 97) and 5-HT in many regions of the brain (11). Levels of transporter expression per neuron, alongside with the synaptic organization of the anatomical area and the proximity of release sites and receptors, could be an important contributor to the mode of transmission for a given neurotransmitter in a given brain area (36, 52).

One of the most striking observations made in mice lacking monoamine transporters is the depletion of intraneuronal storage of monoamines. These observations indicate a tight dependence of the intraneuronal storage on the monoamine uptake system (36, 39). Furthermore, monoamine synthesis in mutant mice is not decreased, but in fact seems to be elevated, strongly indicating that the contribution of newly synthesized monoamines to maintenance of storage is negligible. It is tempting to speculate that depleted monoamine storage in these mice may change several critical intracellular processes and, for example, may account for the altered regulation of monoamine synthesis (36) in the mutant mice.

There are several reports describing depletions in monoamine content after chronic administration of drugs interacting with monoamine transporters (17–22, 87). However, the depletions induced by drugs like amphetamines or cocaine were, as a rule, interpreted as a consequence of neurotoxicity induced be these drugs (60). Nevertheless, several observations have shown that these depletions do not necessarily reflect damage or loss of DA neurons (17, 18). For example, it has been reported that in chronic methamphetamine abusers, reductions in striatal DA, TH, and DAT levels, but not VMAT2 or DOPA decarboxylase levels, occur, demonstrating that DA depletion following methamphetamine in these subjects does not necessarily reflect the destruction of DA neurons, but might be a consequence of a chronically diminished DA reuptake process (18). Modest decreases in 5-HT content are found in brain tissues following chronic treatment with drugs interacting with the SERT (19, 20, 87). Similar decreases in brain NE levels are observed following chronic treatment with NET inhibitors (21, 22). These observations, along with the findings from transporter mutant mice, suggest that caution should be taken in the interpretation of monoamine depletion produced by the

drugs that interfere with monoamine transporters. Direct depletion of monoamine storage due to diminished monoamine transporter function may also account for these effects. The relative inefficiency of most current monoamine transporter inhibitors to significantly affect monoamine storage may be due to the relatively low potency or short-term duration of action of these drugs. It might be proposed that depletion of monoamines by monoamine transporter inhibitors may take place only following long-term effective blockade of reuptake. The studies employing new generations of extremely potent monoamine transporter inhibitors (100, 101) could potentially resolve this issue. It would be of interest to explore how these homeostatic changes can contribute to adverse or beneficial effects of treatments with monoamine transporter inhibitors.

Furthermore, because pharmacological effects or adverse actions of many drugs interacting with monoamine transporters involve chronic treatment, an important lesson learned from these mice relates to the remarkable plasticity in the regulation of receptor function in response to persistently elevated monoaminergic tone. The persistently elevated monoamine levels result in down-regulation and functional desensitization of presynaptic autoreceptors in both DAT-KO (43) and SERT-KO (90–92) mice. With regard to postsynaptic receptors, a more complex picture seems to emerge. In general, studies in all three mutant mice depict a down-regulation of major postsynaptic receptors (23, 25, 44, 90–93). However, this down-regulation was not uniform, and some populations of receptors were found to be up-regulated (44, 90) or not affected (90). Although this nonuniform pattern in postsynaptic receptor regulation is not well understood, it is possible that the relative localization of postsynaptic receptors (synaptic versus extrasynaptic) may determine these differences.

An interesting aspect of CNS function revealed by the studies of monoamine transporter mutant mice is the subtle interplay between neurotransmitter systems. Genetic manipulation of one system can create a situation that results in the unmasking of subtle, but nonetheless important, contributions of another neurotransmitter system. For example, the contribution of 5-HT and/or NE systems to the rewarding properties of psychostimulants had been suspected but never demonstrated in such a direct way (28, 57, 69, 88).

In summary, protracted clearance of monoamine from synaptic cleft, elevated monoamine extracellular levels, depletion of intraneuronal stores of transmitter, and disinhibition of neuronal amine synthesis can be considered as hallmarks of neuronal systems without active monoamine reuptake. Thus, the expression level of transporters in monoaminergic cell groups may determine both the profile of presynaptic monoaminergic homeostasis and the mode of extracellular monoamine transmission. The factors that affect transporter function such as development, aging, and pharmacological or environmental interventions may produce substantial shifts between these modalities. It would be of considerable interest to determine whether the regulatory control that the monoamine transporters exert over monoaminergic signaling could extend to all neurotransmitter systems with transporters.

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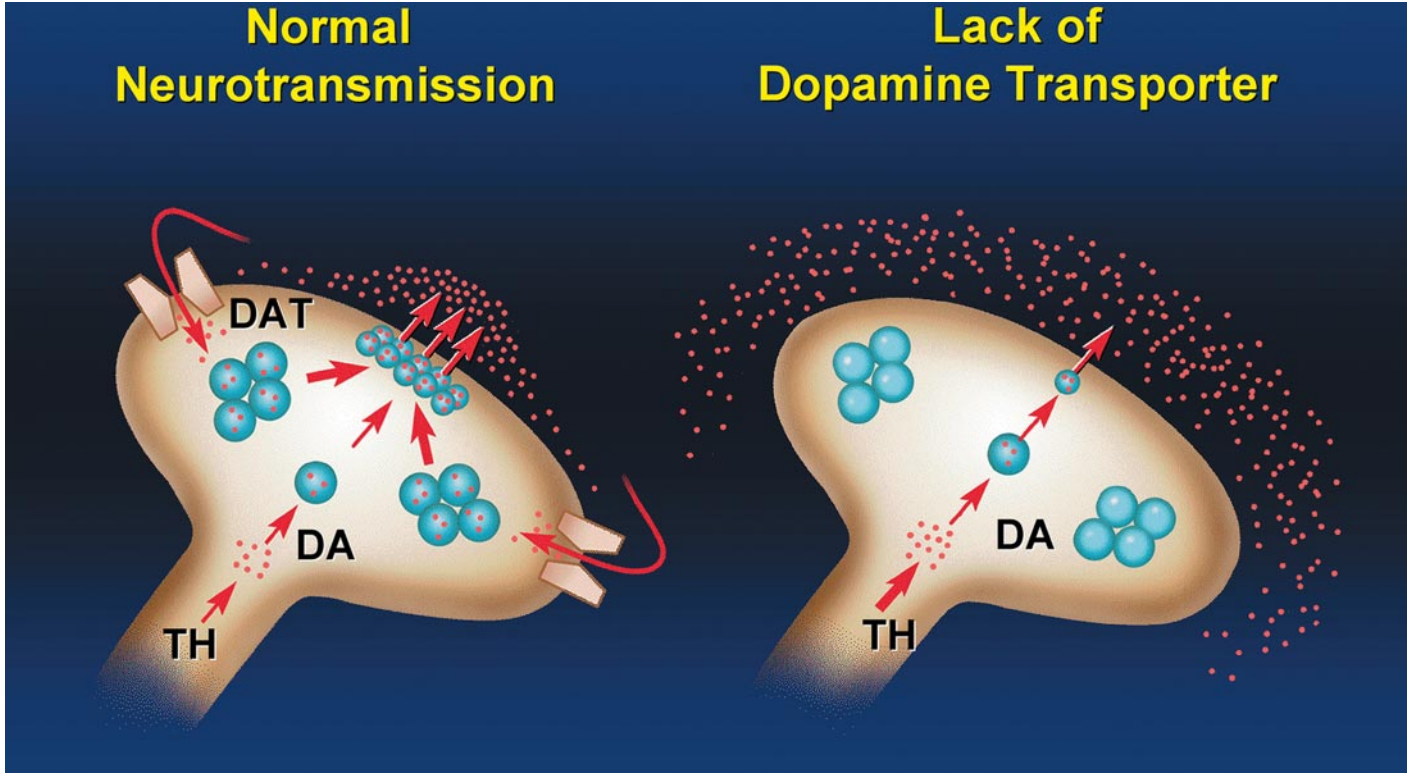

Figure 1 Hypothetical model of striatal dopaminergic terminal in normal and DAT-deficient mice. Elimination of the DAT results in fivefold elevated extracellular levels of DA. Lack of inward transport in DAT-KO mice also results in 20-fold decreased intracellular DA stores. These low intracellular levels of DA are extremely sensitive to inhibition of DA synthesis, suggesting that they represent the newly synthesized DA pool. Note that synthesis rate is increased in DAT-KO mice, but cannot restore the deficiency in DA storage in DAT-KO mice (36, 39). Whether in DAT-KO mice only a few vesicles contain substantial amount of DA to account for the remaining (5%) levels of stored DA or whether all vesicles contain 5% of the normal content has not been formally tested and requires further investigations.

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GENETIC POLYMORPHISMS OF THE HUMAN *MDR1* DRUG TRANSPORTER*

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■ **Abstract** P-glycoprotein is an ATP-dependent efflux pump that contributes to the protection of the body from environmental toxins. It transports a huge variety of structurally diverse compounds. P-glycoprotein is involved in limiting absorption of xenobiotics from the gut lumen, in protection of sensitive tissues (brain, fetus, testis), and in biliary and urinary excretion of its substrates. P-glycoprotein can be inhibited or induced by xenobiotics, thereby contributing to variable drug disposition and drug interactions. Recently, several SNPs have been identified in the *MDR1* gene, some of which can affect P-glycoprotein expression and function. Potential implications of *MDR1* polymorphisms for drug disposition, drug effects, and disease risk are discussed.

OVERVIEW

ABC Transporters

Translocation of endogenous compounds and xenobiotics across biological membranes not only occurs via passive diffusion, but also by carrier-mediated processes. In recent years, multiple transporter genes and proteins have been identified. Knowledge about cellular and tissue-specific transporter expression, as well as characterization of substrates of individual transporters, leads to better understanding of the role of these proteins for physiological processes and elimination of xenobiotics. The ATP-binding (ABC) superfamily is a large family of

*Abbreviations used in text: ALL, acute lymphoblastic leukemia; CD, Crohn's disease; MDR, multidrug resistance; PBMC, peripheral blood mononuclear cells; SNP, single nucleotide polymorphism; UC, ulcerative colitits.

different transporters expressed in a broad variety of organisms (1). In humans, 48 ABC transporters have been identified (2). ABC transporters are located in the plasma membrane or in intracellular membranes. Conserved ABC motifs (Walker A and Walker B) are involved in ATP binding and hydrolysis (3). In general, ABC superfamily members transport a wide range of substances including ions, sugars, amino acids, glycans, peptides, proteins, phospholipids, toxins, and drugs (1). Human ABC transporters are divided into seven subfamilies, each containing several individual members. These subfamilies are ABC1 (12 members), MDR/TAP (11 members), MRP/CFTR (12 members), ALD (4 members), OABP (1 member), GCN20 (3 members), and White [5 members; (1); http://nutrigene.4t.com/humanabc.htm]. Proteins of the MDR/TAP and MRP/CFTR families appear to be most relevant for disposition of xenobiotics in humans.

THE *MDR1* GENE AND ITS PRODUCT P-GLYCOPROTEIN

The *MDR1* gene (ABCB1) product P-glycoprotein is probably one of the most important ABC transporters for drug disposition in humans. It is assumed to be a protective mechanism against potentially toxic xenobiotics that are ingested with the diet. It was first described in tumor cells where it contributed to the phenomenon of MDR against anticancer agents (4). The *MDR1* gene is located on chromosomal region 7q21 and consists of 28 exons, encoding a 1280–amino acid transporter (~170 kDa). It has two homologous halves, each containing six transmembrane domains and an ATP binding site (5, 6). It is currently assumed that P-glycoprotein acts as a "flippase" or a "hydrophobic vacuum cleaner" that removes its substrates from the membrane lipid bilayer into the extracellular space (6, 7). Mutational analysis was intensively used to investigate P-glycoprotein structure-function relationships. In particular, multiple mutations in *MDR1* regions encoding for transmembrane domains 5, 6, 11, and 12 alter P-glycoprotein substrate specificity (1, 6). However, mutations in *MDR1* regions encoding for other domains such as intra- or extracellular loops or ATP binding sites also alter the transporter's substrate specificity (1, 6).

Substrates of P-glycoprotein

P-glycoprotein transports a wide range of substances with diverse chemical structures, among them anticancer agents, cardiac drugs (e.g., digoxin, quinidine), HIV protease inhibitors, immunosuppressants (e.g., cyclosporine), and ß-adrenoceptor antagonists. A summary of important drugs transported by P-glycoprotein is given in Table 1. Attempts to predict P-glycoprotein-mediated transport of a substance from its chemical structure have not been particularly successful. However, P-glycoprotein substrates in general appear to be lipophilic and amphiphatic (6). Interestingly, most substrates of P-glycoprotein are also metabolized by the major drug metabolizing enzyme CYP3A4 (8). This is of particular importance because

TABLE 1 List of clinically important drugs that are substrates of human P-glycoprotein

Drug	Reference
actinomycin D	(82)
amprenavir	(63)
atorvastatin	(83)
bunitrolol	(84)
celiprolol	(85)
colchicine	(86)
cyclosporine A	(87)
daunorubicin	(88)
dexamethasone	(15)
digitoxin	(89)
digoxin	(15)
diltiazem	(90)
docetaxel	(91)
domperidon	(52)
doxorubicin	(88)
erythromycin	(92)
etoposide	(52)
fexofenadine	(59)
indinavir	(60)
irinotecan	(93)
levofloxacin	(94)
loperamide	(52)
losartan	(95)
lovastatin	(13)
mitomycin C	(96)
mitoxantrone	(96)
morphine	(15)
nelfinavir	(60)
ondansetron	(52)
paclitaxel	(97)
phenytoin	(52)
quinidine	(16)
rifampin	(98)

(*Continued*)

TABLE 1 (*Continued*)

Drug	Reference
ritonavir	(61)
saquinavir	(60)
sparfloxacin	(99)
tacrolimus	(100)
talinolol	(101)
teniposide	(96)
topotecan	(96)
verapamil	(102)
vinblastine	(103)
vincristine	(104)

P-glycoprotein and CYP3A4 are colocalized in tissues with major function for drug disposition, such as small intestine and liver. CYP3A4 and P-glycoprotein work in these tissues in a coordinate fashion in order to prevent entry of orally ingested xenobiotics into the body (9–12). However, it should be noted that the overlap in substrate specificity between P-glycoprotein and CYP3A4 is not complete. For example, nifedipine and midazolam, well-known substrates of CYP3A4, are not transported by human P-glycoprotein (13).

Drug Interactions Involving P-glycoprotein

Traditionally, most drug-drug interactions have been attributed to inhibition or induction of drug metabolizing enzymes. It is now well established that modification of P-glycoprotein function by concomitantly administered drugs is another important mechanism of drug-drug interactions. For example, in the late 1970s, it was shown that administration of the antiarrhythmic quinidine significantly increases digoxin serum concentrations in humans (14). Because digoxin is not metabolized to a major extent in humans, the mechanism underlying this interaction remained unclear. However, recent studies identified digoxin as a P-glycoprotein substrate and quinidine as a potent inhibitor of P-glycoprotein function (15, 16). On the other hand, induction of P-glycoprotein by rifampin or St. John's wort results in decreased plasma concentrations of its substrates [e.g., digoxin, talinolol; (17–19)]. Because most drugs are substrates of both CYP3A4 and P-glycoprotein and because inhibitors or inducers of P-glycoprotein in most cases also affect CYP3A4 function, the majority of drug-drug interactions are due to alteration of both CYP3A4 and P-glycoprotein function. Whereas factors determining basal promoter activity of *MDR1* are poorly characterized (1, 20), it

is now established that rifampin-mediated induction of P-glycoprotein occurs by a DR4 motif in the upstream enhancer at approximately −8 kb pairs, to which the nuclear receptor PXR binds (21).

GENETIC VARIABILITY

The first evidence on structural variability of the *MDR1* locus was obtained by screening a large number of samples including normal tissues, unselected, and drug-selected cell lines by RNase protection and subsequent sequencing analysis (22). Two SNPs in exon 21 (G2677T) and exon 24 (G2995A) were identified, of which the single base pair mismatch at position 2677 results in an amino acid change from Ala to Ser (23, 24). A first systematic screen of the *MDR1* gene for the presence of additional SNPs was performed by Hoffmeyer et al. (25). All 28 exons, including the core promotor region and exon-intron boundaries, were sequenced using specific oligonucleotide primers for amplification by PCR derived from the original *MDR1* sequence (Gene Bank accession #AC002457 and #AC005068), which was defined as wild-type sequence. Of the 15 identified SNPs, three polymorphisms resulted in protein alterations, one in exon 2 (Asn21Asp), in exon 5 (Phe103Leu), and in exon 11 (Ser400Asn). Seven synonymous mutations are located in introns 4, 6, 12, 16, and 17, close to exon-intron boundaries, and three SNPs at wobble positions with no amino acid changes [one in exon 12 (C1236T) and two in exon 26 (C3435T, C3396T)]. The SNPs at positions A61G (Asn21Asp), C1236T, and C3435T had been reported previously (23, 26). Subsequently, an ethnic screen of 461 German Caucasians for allele and genotype distribution revealed two further rare mutations [2677A (893Thr), 3320C (1107Pro); (27)]. Further SNPs of the *MDR1* gene were identified in Asians, an A to G transversion 41 bases upstream from the initial position of exon 1a (A-41aG) and a C to G transversion at −145 in exon 1a (C-145G) (28), as well as three nonsynonymous mutations A548G (Asn183Ser), C1474T (Arg492Cys), and T3421A (Ser1141Thr) in different ethnic populations (29, 30). Table 2 summarizes all mutations identified until now. Different genotyping methods, conventional PCR-based (RFLP, SSCP) (25, 27–29) and highly automated screening techniques (DHPLC, Light Cycler^{TM}) (31, 32), were established and recently reported.

Detailed linkage disequilibrium analysis of the different SNPs has not yet been described, but haplotype assignment can be performed in part when subjects who are homozygous at a single polymorphic site or multiple sites are identified. Thus, a cosegregation of the silent mutation 3435T in 62% with the T allele of the nonsynonymous exon 21 SNP 2677T and the T allele of the synonymous exon 12 polymorphism T1236C was reported for European Americans (29). Additionally, in a Japanese population ($n = 65$) a strong association, 93.8% ($n = 61$), was reported using placental cDNA between the 3435T allele and the 2677A and T alleles (33). In a Northern Italian population, the extent of linkage disequilibrium

TABLE 2 Summary of *MDR1* genetic variants in different ethnic groups

Location	Position	Allele	Effect	Reference
promotor	5′flanking/−41a	A G		(28)
exon 1a	exon 1a/−145	C G		(28)
exon 1b	exon 1b/−129	T C		(25, 33)
intron 1	exon 2/−4	C T		(29)
intron 1	exon 2/−1	G A	initiation of translation	(25, 27, 29)
exon 2	exon 2/61	A G	Asn21Asp	(25–27, 29)
intron 4	exon 5/−35	G C		(25)
intron 4	exon 5/−25	G T		(25)
exon 5	exon 5/307	T C	Phe103Leu	(25)
intron 6	exon 6/+139	C T		(25, 27)
intron 6	exon 6/+145	C T		(25)
exon 7	exon 7/548	A G	Asn183Ser	(29)
exon 11	exon 11/1199	G A	Ser400Asn	(25, 27, 29)
exon 12	exon 12/1236	C T	wobble (Gly412Gly)	(23, 25, 27, 29)
intron 12	exon 12/+44	C T		(25, 27)
exon 13	exon 13/1474	C T	Arg492Cys	(29)
intron 16	exon 17/−76	T A		(25, 27)
intron 17	exon 17/137	A G		(25)
exon 21	exon 21/2650	C T	wobble (Leu884Leu)	(29)

(*Continued*)

TABLE 2 (*Continued*)

Location	Position	Allele	Effect	Reference
exon 21	exon 21/2677	G		(22, 23, 27, 29)
		T	Ala893Ser	
		A	Ala893Thr	
exon 24	exon 24/2956	*A*	Met986Val	(33)
		G		
exon 24	exon 24/2995	*G*	Ala999Thr	(22)
		A		
exon 26	exon 26/3320	A	Gln1107Pro	(27)
		C		
exon 26	exon 26/3396	C	wobble	(25)
		T		
exon 26	exon 26/3421	T	Ser1141Thr	(29, 30)
		A		
exon 26	exon 26/3435	C	wobble	(23, 25, 29)
		T	(Ile1145Ile)	
exon 28	exon 28/4030	G		(33)
		C		
exon 28	exon 28/4036	A		(23, 33)
		G		

The positions of the polymorphisms correspond to positions of *MDR1* cDNA with the first base of the ATG start codon set to 1 (GenBank accession # M14758). Mutations located in introns are given as position downstream (−) or upstream (+) of the respective exon according to the genomic organization of *MDR1* as described by Chen et al. (105).

between 3435TT subjects and 2677TT carriers appeared to be somewhat less tight (73.3%) (34a). Taken together, the knowledge of the haplotype structure across the entire *MDR1* gene in different populations is of major interest and could shed light on the mechanisms to identify associations between polymorphism represented by each haplotype and expression and function of P-glycoprotein. To take this issue into account, the first *MDR1* nomenclature was proposed by Kim et al. (29). Very recently, a first haplotype profiling using computational algorithms (Arlequin software) has been reported for three ethnic Asian populations (Chinese, Malays, Indians) with regard to four polymorphic sites (34a). These data indicate a strong linkage disequilibrium between the silent 3435 SNP and an unobserved causal SNP, which underlies the observed association between the 3435 polymorphism and functional consequences.

Potential functional consequences of *MDR1* polymorphisms can be deduced from their location within the *MDR1* gene in relation to the domain structure of P-glycoprotein. The A61G mutation (Asn21Asp) results in a net charge change (basic to acidic) close to the N-terminus of P-glycoprotein, which appears to be of minor functional importance if recombinant mutational analyses of P-glycoprotein

are considered (6). The protein alteration Phe103Leu in exon 5 is located next to the second transmembrane domain on the extracellular side of P-glycoprotein and is in close vinicity to glycosylation sites of P-glycoprotein. The change from a large aromatic to a large lipophilic residue may contribute to a structural alteration of protein by disturbing the side chain packing. The nonsynonymous G1199A SNP in exon 11 (Ser400Asn) results in a significant size change dependent on pH and isoelectric environment of the residue, leading possibly to a charge change in the protein. This SNP is located on the cytoplasmatic side just preceding the first ATP-binding domain. Both polymorphisms, G2677T/A and G2995A, resulting in amino acid exchanges in exon 21 (Ala893Ser/Thr) and exon 24 (Ala999Thr), respectively, are located in the second transmembrane domain, the exon G2995A polymorphism closer to the ABC domain. For Ser893, it can be supposed that certain serine residues in P-glycoprotein are subject to phosphorylation by protein kinase C, resulting in altered protein function (35). In fact, a different multidrug resistance pattern in AdrR MCF-7 cells with Ser893 substitution was described (24). Finally, the C3435T SNP at a wobble position in exon 26 does not alter its encoded Ile amino acid (Ile1145Ile) and is therefore of apparent silent nature. Nevertheless, this polymorphism was associated with altered P-glycoprotein expression and function (see below). The molecular basis of this observation is still poorly understood, but the following mechanisms are conceivable (34a). First, a linkage of the C3435T SNP to other, so far unidentified, mutations elsewhere within the *MDR1* gene, e.g., in the promoter/enhancer or intronic regions, or in another nearby gene can be assumed. Second, silent mutations may yet alter downstream mRNA splicing by allele-specific differences in RNA folding (36) resulting in disrupted exon skipping, a mechanism that has so far probably been vastly underestimated (37). Third, further mechanisms, such as alteration of RNA processing (38) or of translational control, as well as regulatory processes have been described to influence protein expression/function (39, 40a). Finally, a reduced translation efficiency has been discussed to explain how a silent mutation may have functional consequences. In the case of the 3435T allele, an Ile codon is created that is infrequently utilized in the human genome (http://iubio.bio.indiana.edu/soft/molbio/codon/hum.cod or http://www.kazusa.or.jp/codon/).

The only data for retroviral expression of genetic variants of MDR1 are available for the G2677T polymorphism using NIH3T3 GP+E86 cells stably transduced with MDR1-Ser893 or MDR1-Ala893 compared to untransduced cells (29). Whereas P-glycoprotein expression was found to be similar, after incubation with digoxin intracellular concentration was 47% lower for the Ser893 variant than for Ala893, which implies an enhanced efflux transporting ability of the MDR1 Ser893. However, a recent publication characterized the functional consequences of five coding SNPs (Asn21Asp, Phe103Leu, Ser400Asn, Ala893Ser, Ala999Thr) using a vaccinia virus–based transient expression system (40a). Interestingly, cell surface expression and function was not altered even in some common double polymorphisms. The reasons for the contradictory findings between these two publications regarding the G2677T polymorphisms are unclear at the moment (29, 40a).

INTERETHNIC VARIABILITY

Geographic, ethnic, and racial differences in the frequency of variant alleles provide a mechanistic basis for at least some of the observed differences in pharmacokinetics and/or drug effect or toxicity between populations.

Significant ethnic differences exist in the frequency of allele and genotype distribution of the C3435T polymorphism of *MDR1*. Whereas in European and American Caucasians the frequency of individuals homozygous for the C and T allele, respectively, is approximately 25% for each genotype, in Africans the TT genotype has only a frequency of up to 6%. Table 3 summarizes interethnic differences observed for the C3435T SNP in various populations. Data on the allele

TABLE 3 Ethnic distribution of allele and genotype frequencies of the *MDR1* exon 26 SNP C3435T

		Allele frequency		Genotype frequency			
Population	**n**	**C**	**T**	**CC**	**CT**	**TT**	**Reference**
CAUCASIANS							
German Caucasian	188	0.52	0.48	0.28	0.48	0.24	(25)
German Caucasian	461	0.46	0.54	0.21	0.51	0.29	(27)
German Caucasian	537	0.50	0.50	0.26	0.48	0.26	(106)
Caucasian, UK	190	0.48	0.52	0.24	0.48	0.28	(107)
European American	37	0.46	0.54	n.a.	n.a.	n.a.	(29)
Portuguese	100	0.43	0.57	0.22	0.42	0.36	(107)
Northern Italian	106	0.54	0.46	0.26	0.55	0.19	(34)
EAST ASIAN							
Japanese	50	0.57	0.43	0.34	0.46	0.20	(106)
Japanese	100	0.58	0.42	0.35	0.46	0.19	(33)
Japanese	114	0.61	0.39	0.35	0.53	0.12	(51)
Chinese	132	0.53	0.47	0.32	0.42	0.26	(107)
Filipino	60	0.59	0.41	0.38	0.42	0.20	(107)
Saudi	96	0.55	0.45	0.37	0.38	0.26	(107)
SOUTH ASIAN							
Southwest Asians	89	0.34	0.66	0.15	0.38	0.47	(107)
AFRICANS							
Ghanaian	206	0.83	0.17	0.67	0.34	0.00	(107)
Ghanaian	172	0.90	0.10	0.83	0.16	0.02	(106)
Kenyan	80	0.83	0.17	0.70	0.26	0.04	(107)
Sudanese	51	0.73	0.27	0.52	0.43	0.06	(107)
African American	88	0.84	0.16	0.68	0.31	0.01	(107)
African American	41	0.78	0.22	0.61	0.34	0.05	(106)
African American	23	0.74	0.26	n.a.	n.a.	n.a.	(29)

n.a.: data not available.

frequency for other SNPs are limited. In a German population, the 2677T allele was observed in 42% (27) and in Japanese 41.7% (33), whereas in African Americans, the frequency is only 13% (29). Additionally, the frequency of Caucasians homozygous for the wobble mutation at position 1236 (1236TT) is about one-third of the value in Japanese (13.3% versus 37.5%) (25, 28). Moreover, as described above, several mutations are only identified in single ethnic populations. For example, the nonsynonymous SNP at position 3421 was only found in Ghanaians (1.2%) and African Americans (2.4% and 4.3%, respectively), but not in Caucasians (29, 30).

Whether these differences are of any functional relevance remains to be determined. In the case of the C3435T polymorphism in which the T allele is associated with reduced P-glycoprotein expression [see below; (25)], a selective advantage of the CC genotype can be supposed because P-glycoprotein plays an important role in defense against several toxins including bacterial and viral particles. Overdominance of a genotype as a consequence of natural selection by infectious diseases has been demonstrated for other polymorphisms, such as the glucose-6-phosphate dehydrogenase gene (41). In the case of P-glycoprotein, it can be assumed that the much higher frequency of the P-glycoprotein high expression 3435CC genotype in Africans compared to Caucasians or Japanese may be protective for developing gastrointestinal-tract infections, which are endemic in tropical countries.

These interethnic variabilities could have an impact on drug disposition. For example, drug disposition of cyclosporine and tacrolimus is mainly affected by CYP3A4 and P-glycoprotein. Moreover, intestinal P-glycoprotein has been shown to determine oral clearance of cyclosporine (42). Comparison of pharmacokinetics of oral cyclosporine in Black and White renal transplant recipients showed a significantly lower bioavailability of cyclosporine in Blacks (mean 30.9%) than in Whites or Hispanics [mean 39.6% ($p = 0.0009$) and 42.1% ($p = 0.0003$), respectively] (43). Furthermore, the mean dosage requirement of the immunosuppressant tacrolimus was 96% higher in Black recipients compared to Whites or Asians ($p < 0.001$) (44). Racial differences in the clearance of cyclosporine as well as tacrolimus were also found among healthy African Americans and White volunteers (45, 46). In this context, it is interesting to note that several studies indicate a poorer outcome for Africans after renal transplantation than for White patients, although the immunosuppressive therapy including cyclosporine or tacrolimus is similar. Hence, the high frequency of the high expression *MDR1* genotype CC in Africans/African Americans versus Caucasians may be of clinical relevance.

MDR1 POLYMORPHISMS AND P-GLYCOPROTEIN EXPRESSION IN HUMANS

P-glycoprotein Expression

The only polymorphism identified so far that affects P-glycoprotein expression in different human tissues is the silent mutation at position 3435 in exon 26 (C3435).

P-glycoprotein expression levels in upper small intestine of healthy volunteers and patients were determined by quantitative immunohistochemistry and Western blot analysis (17, 25). Carriers homozygous for the T-allele had on average more than twofold lower intestinal MDR1 expression levels compared to the CC genotype. Additionally, the subject with the lowest and highest intestinal P-glycoprotein level had the TT and CC genotype, respectively. A similar relationship between P-glycoprotein expression in human kidney and the C3435T polymorphism was found using quantitative immunohistochemistry. Subjects with the TT genotype had on average a significantly (1.5-fold) lower P-glycoprotein expression compared to the CC genotype group ($p = 0.0065$) (47). The specific expression of P-glycoprotein in placenta of women from Japan was significantly correlated to the T-129C mutation (allele frequency: 8.3%) in exon 1b with twofold lower expression levels in heterozygous samples than in homozygous TT subjects ($p = 0.002$) (33). A trend was observed for the polymorphism at position 2677, with lowest P-glycoprotein expression levels in homozygous mutant individuals, intermediate expression in heterozygous subjects, and highest in the wild-type group. With respect to the C3435T SNP, a similar (nonsignificant) trend was reported (CC > CT > TT), however, with a large standard deviation for each genotype group.

MDR1 mRNA Expression

On the level of total cellular MDR1 mRNA expression obtained from PBMC, two independent studies confirmed an association toward lower values in 3435TT subjects as compared to higher levels in individuals with the CT and CC genotype. Whereas these results were not statistically significant in healthy volunteers [$p = 0.33$; (31)], patients with HIV-1 infection before commencing antiretroviral therapy, who were carriers of the 3435TT genotype, showed significantly lower mRNA levels compared to heterozygotes and subjects homozygous for CC ($p = 0.02$) (48). These findings were confirmed by fluorescence-activated cell-sorter analysis of P-glycoprotein expression in PBMCs. In contrast to these studies, mRNA quantification in duodenal enterocytes from 13 healthy Japanese individuals showed higher mean expression levels of MDR1 mRNA in homozygous carriers for 3435T as compared to subjects with a CT or CC genotype (49). Assuming a linkage disequilibrium between the C3435T polymorphism and the genetic variant G2677T, these findings corroborate the in vitro results of retroviral expression of the Ser893 variant (2677T) by Kim et al. (29) leading to an enhanced activity of P-glycoprotein. Consequently, racial differences in the relation between C3435T and C2677T should be considered as a possible explanation for seemingly contradictory findings. Furthermore, tissue specific expression of MDR1 mRNA cannot be completely ruled out as an additional mechanism for different results in PBMCs and intestinal mucosa.

MDR1 POLYMORPHISMS AND P-GLYCOPROTEIN FUNCTION IN HUMANS

Several studies addressed the association of *MDR1* genotypes with disposition of P-glycoprotein substrates in humans. These investigations were based on the initial observation that *MDR1* genotype 3435TT is associated with a lower intestinal P-glycoprotein expression in humans in comparison to individuals with the 3435CT or CC genotypes (25). Thus, in individuals with lower intestinal P-glycoprotein concentration the extent of drug absorption from the gastrointestinal (GI) tract should be higher and result in increased plasma levels in comparison to the remainder of the population. Accordingly, Hoffmeyer et al. (25) detected significantly higher maximum digoxin plasma concentrations in seven healthy volunteers with the 3435TT genotype (+38%), compared to seven individuals with the 3435CC genotype during steady-state conditions (0.25 mg/day). Similarly, a reduced digoxin oral clearance (−26.6%) was reported for Korean patients with the one or two T alleles at position 3435 using a population pharmacokinetic approach (50). However, administration of a single oral dose of digoxin to healthy Japanese subjects resulted in a lower AUC_{0-4h} (−20.4%) in individuals with the 3435TT genotype compared to the CT and CC groups (51). The antiepileptic drug phenytoin is primarily metabolized by polymorphic CYP2C9. Moreover, it is transported to some extent by P-glycoprotein (52). Accordingly, Kerb et al. (53) identified the *CYP2C9* genotype as a major determinant of phenytoin disposition in humans. In addition, the *MDR1* 3435 CC genotype was more common in volunteers with low phenytoin plasma concentrations ($p \leq 0.01$).

So far, no clear relationship was observed between the *MDR1* genotype and the disposition of other P-glycoprotein substrates [e.g., the antihistaminic drug fexofenadine; (29, 54, 55)]. Moreover, *MDR1* 3435 polymorphism was not associated with altered disposition of cyclosporine, talinolol, and loperamide (56–58). An interesting finding was also reported for nelfinavir plasma concentrations in relation to the *MDR1* genotype (48). In patients on antiretroviral therapy, nelfinavir plasma concentrations (but also plasma concentrations of the non-P-glycoprotein substrate efavirenz) were highest with the CC genotype compared to the CT and TT groups. In the latter case, it was speculated that an indirect effect of the *MDR1* genotype on nelfinavir disposition could explain these findings (48). For example, low intestinal P-glycoprotein expression increases concentrations of nelfinavir in enterocytes, subsequently leading to induction of other intestinal transporters and/or drug metabolizing enzymes (CYP3A4).

Taken together, in spite of several studies relating the 3435T polymorphism with low tissue expression of P-glycoprotein, there is not a clear trend toward higher plasma concentrations of all P-glycoprotein substrates investigated so far. This could be due to the following confounding factors. (*a*) The effect of the *MDR1* 3435 polymorphism on P-glycoprotein tissue levels is rather modest (approximately twofold). (*b*) Disposition of most P-glycoprotein substrates is also determined by other factors, such as metabolism (e.g., nelfinavir or cyclosporine

metabolism via CYP3A4) or active transport [e.g., fexofenadine uptake via OATP-A; (59)]. (*c*) In addition, modification of drug disposition may occur by exogenous factors (e.g., diet, drugs), which could explain different results, even if the same drug is investigated. (*d*) Due to the presence of multiple SNPs in the *MDR1* gene and pronounced interethnic differences in frequencies of some of these polymorphisms (see above), a detailed haplotype analysis should be performed, rather than determination of one SNP. (*e*) Not all pharmacokinetic parameters are likely to be determined to a major extent by modulation of intestinal P-glycoprotein expression [AUC for the first few hours after oral drug administration or C_{max} are preferable over trough concentrations; (42, 56)].

In accordance with the above-mentioned lower MDR1 mRNA and P-glycoprotein expression in lymphocytes (31, 48), ex vivo investigations using $CD56^+$natural killer cells revealed a significantly lower P-glycoprotein function in cells obtained from healthy volunteers with the 3435TT genotype in comparison to the CT and CC groups ($p = 0.0015$) (31). The potential relevance of these findings is discussed in the following paragraph.

MDR1 POLYMORPHISMS AND CLINICAL OUTCOME

HIV

All currently marketed HIV protease inhibitors are P-glycoprotein substrates (60–63). Studies with P-glycoprotein knockout mice revealed that intestinal P-glycoprotein has a major impact on bioavailability of HIV protease inhibitors (60). P-glycoprotein expressed in the blood-brain or blood-testis barriers limited accumulation of these drugs in the CNS and testis, respectively (60, 64). The presence of a substantial barrier to the drugs' distribution into these tissues suggests that the ability to achieve therapeutic brain or testis concentrations is limited, creating a potential sanctuary for virus replication. A similar scenario is possible for $CD4^+$ cells, which are a major target of the HIV virus. In lymphocytes, P-glycoprotein expression is highly variable, with approximately 25% of $CD4^+$ cells displaying considerable activity (65). Interestingly, penetration and antiviral activity of indinavir, saquinavir, and ritonavir are diminished in HIV-1 infected cells with high P-glycoprotein expression (62). Moreover, *MDR1* genotype–related differences in P-glycoprotein function have been described in $CD56^+$ natural killer cells using rhodamine efflux. The 3435 TT genotype was associated with a significantly reduced P-glycoprotein function in $CD56^+$ natural killer cells as well as reduced P-glycoprotein expression in PBMCs (31, 48). Because P-glycoprotein is expressed in $CD4^+$ subpopulations, intracellular concentrations of HIV protease inhibitors and antiretroviral efficacy are affected by variable P-glycoprotein expression. Indeed, in a recent study it was shown for the first time that *MDR1* genotype was significantly related to response to antiretroviral treatment. The 3435 TT genotype was associated with a significantly greater increase in CD4-cell count and a trend toward a more pronounced decrease in viral load compared to patients

with the 3435 CT or CC genotypes six months after antiretroviral therapy was started (48). It is important to determine whether this effect persists for a longer period than six months in order to investigate particular antiretroviral regimens containing different HIV protease inhibitors, which are affected by *MDR1* genotype, and to prove that genotyping of the patients before initiation of treatment will help to further improve the outcome in HIV infected patients (e.g., by additional treatment with selective P-glycoprotein inhibitors).

Childhood ALL

P-glycoprotein is an essential part of the blood-brain barrier. Several P-glycoprotein substrates (doxorubicin, vincristin, etoposide) are used in treatment protocols for childhood ALL. In spite of considerable progress in treatment of childhood ALL, central nervous system relapses still occur in approximately 3%–5% of the patients. The *MDR1* genotype could contribute to a patient's risk for the development of CNS relapse due to a potentially lower P-glycoprotein expression in the blood-brain barrier of individuals with the 3435 TT genotype. Therefore, a better CNS penetration of some anticancer agents may occur in spite of similar plasma concentrations (66). In accordance with this hypothesis, patients (at intermediate or high risk for treatment failure) with the 3435 CT or TT genotype had a significantly lower rate of CNS relapse compared to the CC group (66).

Nortriptyline-Induced Postural Hypotension

Data obtained with P-glycoprotein knockout mice indicate that nortriptyline may be a P-glycoprotein substrate (67). Accordingly, Roberts et al. (68) tested the hypothesis whether side effects of the antidepressant nortriptyline might be associated with the *MDR1* 3435 polymorphism. Indeed, the presence of two T alleles at position 3435 of the *MDR1* gene was associated with a higher risk for the occurrence of postural hypotension in nortriptyline-treated patients in comparison to patients with the 3435 CC or CT genotype (68).

MDR1 POLYMORPHISMS AND DISEASE SUSCEPTIBILITY

Although the physiological role of P-glycoprotein is not fully understood, it is conceivable that transporter proteins, such as P-glycoprotein, prevent intracellular accumulation of potentially toxic substances and metabolites (69). Because the exposure of epithelial cells depends on the entry and efflux of xenobiotics via transporter proteins, it is plausible to hypothesize that genotype-dependent P-glycoprotein expression may contribute to a certain disease susceptibility. This may either indicate that *MDR1* as marker polymorphism causes disease or that this marker is closely linked to a disease locus.

Renal Epithelial Tumor

Except for hereditary diseases (e.g., von Hippel-Lindau syndrome), genetic factors contributing to the development of renal epithelial tumors remain elusive. Because P-glycoprotein mediates active secretion of its substrates into urine and may be involved in the clearance of carcinogens or water-soluble metabolites via the brush border of the proximal tubular lumen, P-glycoprotein may have a protective role as a renal biological barrier protein. In a first case-control study including patients with clear cell renal cell carcinoma (CCRCC; $n = 179$) and non-CCRCC ($n = 33$), a significant association between the 3435T allele frequency and the occurrence of tumors was found [$p = 0.007$; (47)]. Additionally, a second case-control study established the T-allele as a risk factor, especially for non-CCRCC (OR 2.3, $p = 0.0005$), with the highest risk for homozygote TT carriers (OR 21.7, $p < 0.001$). These data indicate that different expression levels of P-glycoprotein may be associated with the susceptibility to develop rare renal epithelial tumors by virtue of the C3435T polymorphism.

Inflammatory Bowel Disease

To date, the exact etiology of IBD, CD, and UC is still unknown. There is evidence that microbial and genetic factors as well as the immune system play a major role in pathogenesis of IBD (70, 71). Genome-wide screening and candidate-gene analysis have been used to search for IBD-susceptibility genes. Although UC and CD share some of these genes, separate genes appear to be associated with disease severity, extent steroid response, and steroid requirements in patients with UC (70).

Evidence for the role of microbial factors in the pathogenesis of UC comes from experimental animal models of intestinal inflammation (72), but the majority of IBD mouse models are very susceptible to immunological dysregulation due to cytokine imbalance or T cell defect (72). Recent findings with *mdr1a*-P-glycoprotein deficient mice (mdr1a−/−), which are immunologically normal, indicate that an intestinal epithelial barrier defect may contribute to spontaneously severe colitis, which resembles human UC (73, 74). The development of spontaneous colitis in *mdr1a*−/− mice could be prevented when treated with oral antibiotics. Based on the assumption that interindividual variability of intestinal P-glycoprotein expression is linked to the *MDR1* C3435T SNP in exon 26 and the TT-genotype is associated with significantly lower intestinal P-glycoprotein expression, the hypothesis of whether this genetic variant may predispose to UC was tested (75). Compared to sex-matched controls, the 3435T allele frequency was significantly higher in UC patients ($n = 149$; 56.7% versus 48.3%; $p = 0.049$; OR 1.4; 95% CI, 1.02–1.94). Interestingly, no differences were found between CD patients and controls ($n = 126$; $p = 0.66$; OR, 0.9; 95% CI, 0.6–1.3). An overrepresentation of patients homozygous for TT (30.9% versus 22.8%, $p = 0.045$) was likewise found only for UC patients, with a twofold increased overall risk (OR 2.03; 95% CI, 1.04–3.95). The higher frequency of both the 3435T allele and TT genotype in patients with UC corroborates the experimental findings from the *mdr1a*-knockout

mouse model. The results support the notion that P-glycoprotein plays an important role in the defense against intestinal bacteria and suggest that the *MDR1* gene is associated with susceptibility to develop UC.

Parkinson's Disease

Epidemiological studies suggest that both genetic and environmental factors (e.g., neurotoxic xenobiotics) play a role in the development of Parkinson's disease (PD) (76). Therefore, host factors that contribute to variability in uptake and distribution of xenobiotics in the brain can be expected to modulate individual risk, especially because P-glycoprotein has been characterized as a neuroprotective barrier protein in endothelial cells of brain capillaries (77). In a pilot case-control study investigating *MDR1* polymorphisms in relation to the risk for development of PD, the frequency of the 3435 TT genotype was highest in the early-onset PD group (36.0%), second highest in the late-onset PD group (22.9%), and lowest in controls [18.9%, $p = 0.08$; (34a)]. These data provide some evidence that the *MDR1* polymorphism, via altered P-glycoprotein expression in the blood-brain barrier would affect the intracellular concentrations of potentially neurotoxic substances leading to an increased susceptibility factor for PD and/or lead to earlier onset of disease symptoms.

SUMMARY AND FUTURE PERSPECTIVES

P-glycoprotein plays a major role in drug disposition and drug efficacy by providing a barrier for the entry of orally ingested compounds into the body as well as controlling their rate of transfer into different tissues. So far, several SNPs have been identified in the *MDR1* gene that might alter P-glycoprotein expression and function in humans. In contrast to the extensive knowledge of molecular mechanisms of polymorphically expressed drug metabolizing enzymes (e.g., cytochrome P450 2D6), only limited data are available to explain contradictory findings of the observed functional differences when *MDR1* mutations were correlated with P-glycoprotein expression and/or function. Although the data indicate that intestinal P-glycoprotein expression is affected on average twofold by a certain *MDR1* genotype [C3435T; (25)], pronounced intersubject variability exists (up to 10-fold). Moreover, there is an apparent discrepancy in approximately 5%–10% of individuals who are homozygous for the putative P-glycoprotein high (or low) expression genotype 3435CC (or TT) but show a decreased (or increased) P-glycoprotein expression/function (25, 31). The reasons for these observations are unknown; however, it can be speculated that a strong linkage disequilibrium of the wobble mutation C3435T exists with a so far unidentified SNP. Additionally, confounding factors, such as environmental alterations (e.g., dietary salt exposure) (78, 79) as well as comedications [e.g., St John's wort, rifampin; (17, 19)], can modify P-glycoprotein expression, especially if surgical specimens obtained from patients were used for phenotype-genotype correlation studies. Interindividual differences

in transcriptional control of human *MDR1* (20, 21), including genetic variants of transcription factors (e.g., PXR) (80, 81), may also contribute to variability of P-glycoprotein expression.

Future studies need to elucidate the molecular mechanisms of the association of certain *MDR1* genotypes with altered P-glycoprotein expression and function. Moreover, more detailed studies relating *MDR1* haplotypes with P-glycoprotein expression and function will help to improve our understanding of variable P-glycoprotein function. Finally, a better knowledge of the major impact of environmental factors as well as interethnic differences in P-glycoprotein function is necessary for investigations on an association of *MDR1* genotype with P-glycoprotein expression, drug disposition, and risk for certain diseases.

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ACTIVATION OF THE ARYL HYDROCARBON RECEPTOR BY STRUCTURALLY DIVERSE EXOGENOUS AND ENDOGENOUS CHEMICALS*

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Key Words Ah receptor, 2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD, Ah receptor ligands, CYP1A1

■ **Abstract** The induction of expression of genes for xenobiotic metabolizing enzymes in response to chemical insult is an adaptive response found in most organisms. In vertebrates, the AhR is one of several chemical/ligand-dependent intracellular receptors that can stimulate gene transcription in response to xenobiotics. The ability of the AhR to bind and be activated by a range of structurally divergent chemicals suggests that the AhR contains a rather promiscuous ligand binding site. In addition to synthetic and environmental chemicals, numerous naturally occurring dietary and endogenous AhR ligands have also been identified. In this review, we describe evidence for the structural promiscuity of AhR ligand binding and discuss the current state of knowledge with regards to the activation of the AhR signaling pathway by naturally occurring exogenous and endogenous ligands.

Ah RECEPTOR SIGNAL TRANSDUCTION

The AhR is a ligand-dependent transcription factor that regulates the expression of a battery of genes in a wide range of species and tissues (1–5). Environmental contaminants, such as the HAHs and nonhalogenated PAHs, represent the most extensively characterized classes of AhR ligands (6–9), although naturally occurring ligands do exist. Exposure to TCDD (dioxin), the prototypical and most potent HAH, and related compounds produces a diverse array of species- and tissue-specific toxic and biological effects, the majority of which are AhR dependent

*Abbreviations used in text: AA, arachidonic acid; AhR, aryl hydrocarbon receptor; Arnt, AhR nuclear translocator; BR, bilirubin; BV, biliverdin; CYP1A1, cytochrome P4501A1; DRE, dioxin responsive element; FICZ, 6-formylindolo(3,2b)carbazole; HAH, halogenated aromatic hydrocarbon; I3C, indole 3-carbinol; ICZ, indolo-(3,2,-b)-carbazole; PAH, polycyclic aromatic hydrocarbon; RAR, retinoic acid receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; Trp, tryptophan; UGT*01, UDP-glucuronosyl transferase *01

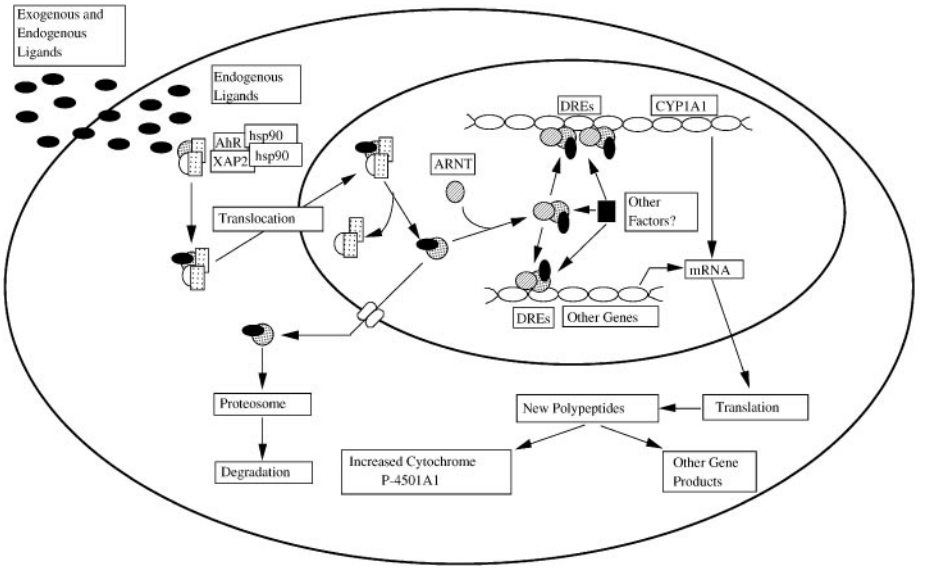

Figure 1 The molecular mechanism of activation of gene expression by the AhR. See text for more details.

(8–11). In fact, knockout of the AhR results in loss of responsiveness to TCDD and related chemicals (12–14). Although numerous genes are regulated by the AhR (6), the best studied are those encoding xenobiotic metabolizing enzymes, such as CYP1A1. The induction of CYP1A1 is one AhR-dependent response that has been consistently observed in most species, and it has been used as the model system to define the mechanism by which the AhR regulates gene expression. The current model of AhR action is presented in Figure 1. The inducing chemical enters the responsive cell and binds with high affinity to the cytosolic AhR, which exists as a multiprotein complex, containing two molecules of the chaperone protein hsp90 (a heat shock protein of 90 kDa), the X-associated protein 2 [XAP2 (15)], and a recently identified 23-kDa co-chaperone protein referred to as p23 (16). Following ligand binding, the AhR is presumed to undergo a conformation change that exposes a nuclear localization sequence(s), resulting in translocation of the complex into the nucleus (17, 18). Release of the ligand:AhR from this complex and its subsequent dimerization with a related nuclear protein called Arnt converts the AhR into its high affinity DNA binding form (1, 19). A nuclear export sequence present in the AhR is responsible for the cytoplasmic shuttling of nuclear AhR complexes that fail to dimerize with Arnt and/or bind to DNA and leads to its ubiquitination and degradation (20). Binding of the heteromeric ligand:AhR:Arnt complex to its specific DNA recognition site, the DRE, upstream of the CYP1A1 and other AhR-responsive genes stimulate transcription of these genes (3, 5, 21). The presence of the AhR and AhR signal transduction pathways in a diverse range

of species, tissues, and cell types (22–24), combined with its ability to act as a ligand-dependent transcription factor, suggests that many of the toxic and biological effects of AhR ligands result from differential alteration of gene expression in susceptible cells. Because many of the adverse effects of TCDD/HAHs are not observed until days to weeks following chemical exposure (8, 11), the adverse effects of these chemicals likely result from the continuous and inappropriate expression of specific genes in responsive cells. This hypothesis is consistent with the lack of TCDD-like toxic effects produced by PAHs and other relatively weak ligands, which produce only transient activation of the AhR signaling pathway. Although the role of AhR in the toxic and biological effects produced by AhR ligands is well documented, the exact biochemical events and responsible gene products responsible for the adverse effects of these chemicals still remain to be elucidated.

The physiological role of the AhR remains a key question, and to date no high affinity endogenous ligand has been identified. Detailed analysis of AhR ligand binding has predominantly focused on the structurally related HAHs and PAHs; however, recent studies have demonstrated the ability of a structurally diverse range of chemicals to bind to and/or activate AhR-dependent gene expression [reviewed in (6) and (7)]. These results suggest that the AhR has a promiscuous ligand binding site. In addition, the identification and characterization of a variety of naturally occurring AhR ligands has begun to redefine our ideas as to the structural specificity of AhR ligand binding. In this review, we describe recent developments in our understanding of the structural diversity of AhR ligands with an emphasis on those ligands and inducers that are naturally occurring exogenous and endogenous ligands. More details on the AhR and AhR signal transduction can be found in other excellent reviews (1–5, 7, 11).

AhR LIGANDS AND INDUCERS

In this review, AhR ligands have been separated into two major categories, those that are synthetic in nature (i.e., formed as a result of anthropogenic or nonbiological activity) and those that occur naturally (i.e., formed in biological systems as a result of natural processes). The majority of the high affinity AhR ligands that have been identified and characterized to date are members of the first category and include planar, hydrophobic HAHs (such as the polyhalogenated dibenzo-p-dioxins, dibenzofurans, and biphenyls) and PAHs (such as 3-methylcholanthrene, benzo(a)pyrene, benzanthracenes, and benzoflavones), and related compounds (6, 8, 9, 25, 26). The metabolically more stable HAHs represent the most potent class of AhR ligands, with binding affinities in the pM to nM range, whereas the metabolically more labile PAHs bind with relatively lower affinity (nM to μM range). Structure activity relationship analysis using a large number of HAHs and PAHs has suggested that the AhR ligand binding pocket can bind planar ligands with maximal dimensions of 14 Å $\times$ 12 Å $\times$ 5 Å and that high affinity ligand binding appears to be dependent upon key electronic and thermodynamic

characteristics of the ligand (26–31). There are many excellent reviews on the physiochemical characteristics and biological/toxicology potency of these "synthetic" HAH/PAH AhR ligands (26, 31) and they are not discussed here. An interesting recent development is the identification of a relatively large number of AhR ligands whose structure and physiochemical characteristics are dramatically different than that of the "classical" HAH and PAH ligands [reviewed in (6) and (7)]. The structures of some "classical" and "nonclassical" synthetic AhR ligands are shown in Figure 2. Interestingly, high-throughput screening analysis of a combinatorial

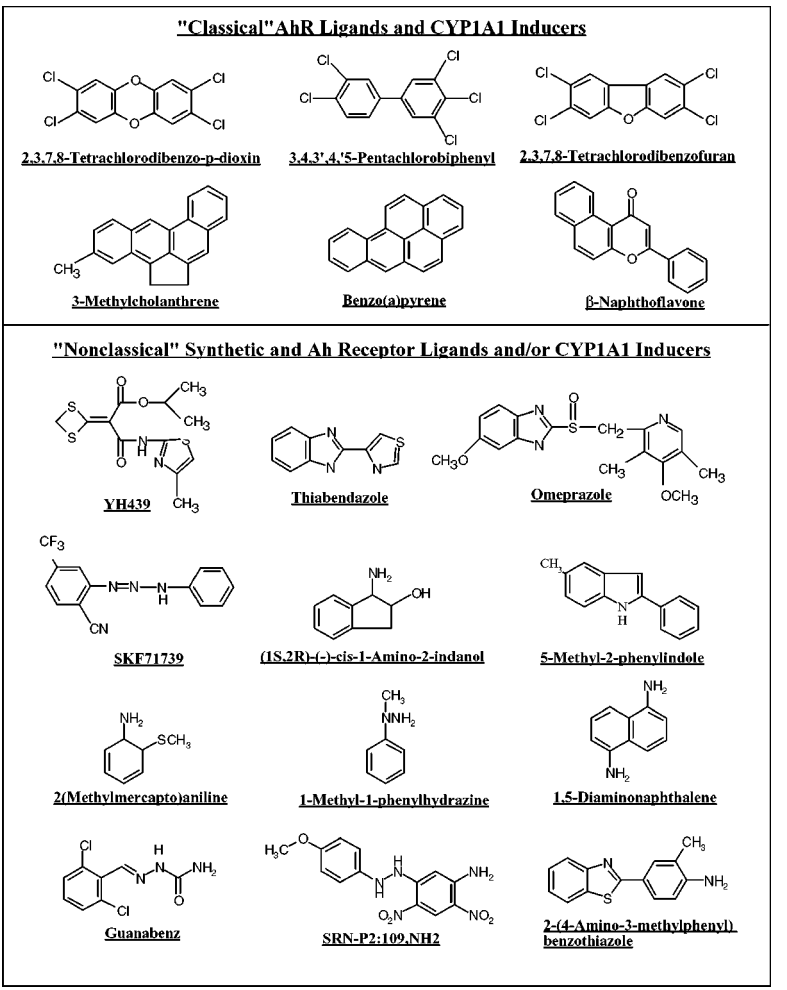

Figure 2 Structures of selected classical and nonclassical AhR ligands and inducers of AhR-dependent gene expression. See text and Reference 6 for more details.

chemical library using an AhR-responsive reporter gene system (32) has allowed identification of numerous novel AhR agonists, including several activators whose structures contain only a single unsaturated ring (33). Although the majority of the currently identified nonclassical AhR ligands/agonists are relatively weak inducers of CYP1A1 and/or low affinity AhR ligands (when compared with TCDD), the identification of this striking structural diversity of AhR ligands is important because it indicates that the spectrum of synthetic AhR ligands is likely to be much broader than was originally thought. Thus, attempts to identify endogenous and natural ligands should not be restricted by previous views of the structural requirements for AhR ligands.

NATURALLY OCCURRING DIETARY AhR LIGANDS AND INDUCERS

The greatest source of exposure of animals and humans to AhR ligands (synthetic and natural) comes from the diet. Numerous studies have described and characterized a variety of naturally occurring dietary chemicals that can directly activate and/or inhibit the AhR signaling pathway. The structures of some of these chemicals are shown in Figures 3 and 4. The earliest reports of natural Ah inducers came from observations that extracts of vegetables or vegetable-derived materials could induce CYP1A1 activity (34, 35). Subsequently, the ability of several dietary plant compounds, such as I3C (25, 35), 7,8-dihydrorutacarpine (36), dibenzoylmethanes (37), curcumin (38), and carotinoids [e.g., canthaxanthin, astaxanthin, and the apo-carotinoid, β-apo-8′carotenal (39, 40)], to competitively bind to the AhR and/or induce AhR-dependent gene expression was reported. Conversion of dietary indoles (including I3C and Trp) in the mammalian digestive tract to significantly more potent AhR ligands/agonists (Figure 3) was also demonstrated (35, 41). In fact, ICZ, an acidic condensation product formed from I3C (itself a weak AhR ligand), has perhaps the highest affinity of any "natural" AhR ligand identified to date ($\sim$0.2–3.6 nM), and it is a potent inducer of AhR-dependent gene expression in cells in culture (25, 35). 3,3′-Diindolylmethane, another acidic condensation product of I3C, is also an established AhR agonist (42). The formation of relatively potent AhR ligands from precursors that have little or no AhR agonist activity is significant, especially considering that most dietary ligands are themselves relatively weak AhR ligands/agonists. Flavonoids, including flavones, flavanols, flavanones, and isoflavones, represent the largest group of naturally occurring dietary AhR ligands. Although the majority of these natural plant products are AhR antagonists (43–47), numerous agonists, such as quercetin (48), diosmin (49), tangeritin (50), and tamarixetin (43), have also been identified. In addition to interacting with the AhR, many of these flavonoids are also substrates for CYP1A1 (51). These chemicals are widely distributed in dietary vegetables, fruits, and teas (52–55), and flavonoid levels in human blood have been reported to be in the low μM range (56–58), concentrations sufficient to inhibit/activate the AhR. Thus, it is not surprising that crude extracts of a large number of different

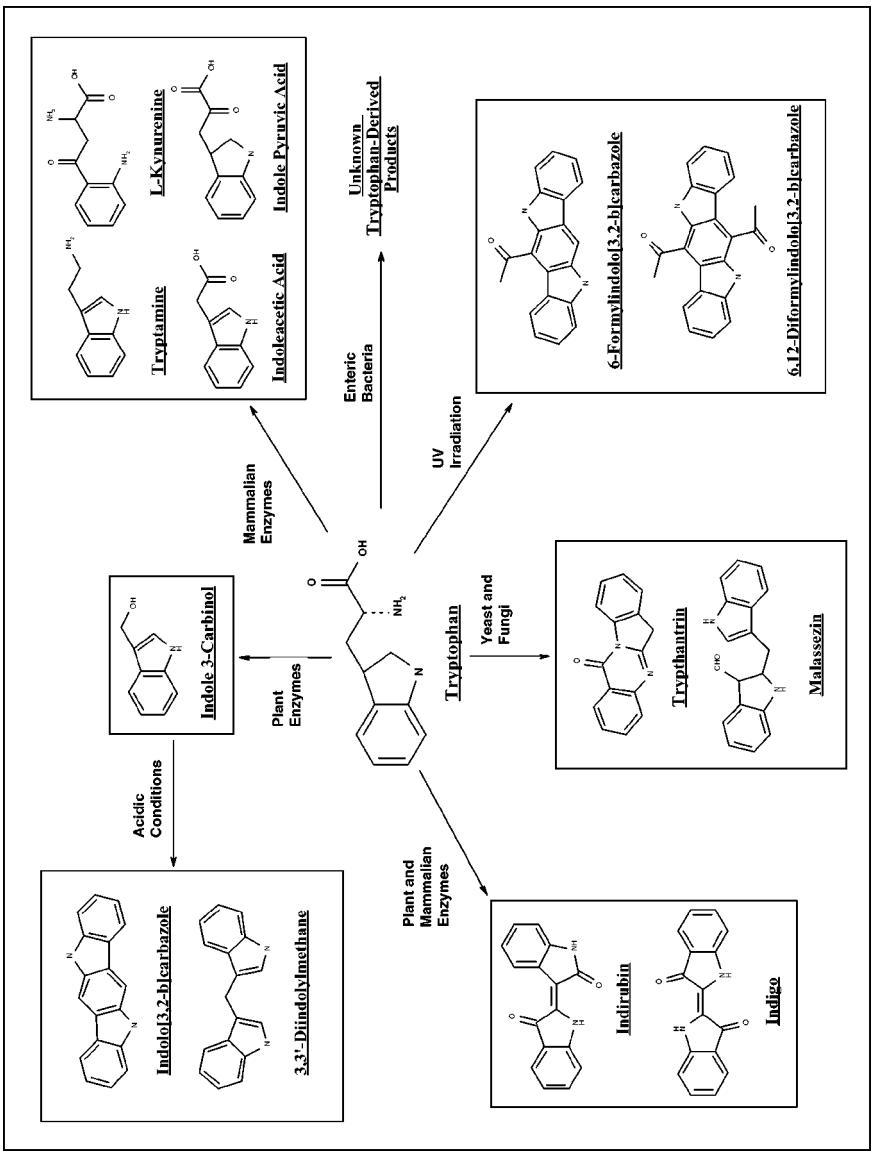

Figure 3 Tryptophan can be converted by a variety of mechanisms into AhR ligands and inducers of AhR-dependent gene expression. See text for details.

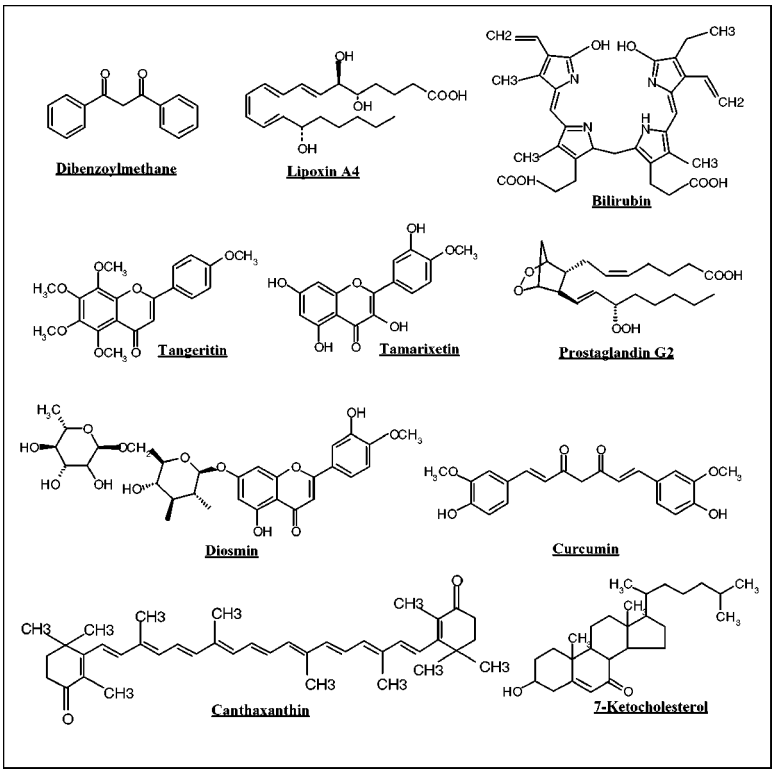

Figure 4 Structures of selected naturally occurring AhR ligands and inducers of AhR-dependent gene expression. See text for details.

vegetables, teas, fruits, and natural herbal products have AhR agonist and/or antagonist activity (59, 60). Thus, plant-derived materials appear to commonly contain AhR ligands or products that can readily be converted into AhR ligands, and as such, they are perhaps the largest class of natural AhR ligands to which humans and animals are exposed.

EVIDENCE FOR ENDOGENOUS LIGANDS FOR THE AhR

The existence of endogenous physiological AhR ligands has been suggested by numerous studies in which the AhR signaling pathway is active in the absence of exogenous ligands. The identification of nuclear AhR complexes in unexposed cells in culture and tissue slices (61–63), combined with the demonstration that disruption of AhR expression using antisense resulted in decreased development of mouse blastocysts (64) and alternations in normal cell cycle progression (65, 66), supports the existence of endogenous AhR ligands. The ability of hydrodynamic shear stress conditions (67) as well as methylcellulose suspension (68, 69) to

induce CYP1A1 in cells in culture and of hyperoxia to induce CYP1A1 in rat lungs and liver in vivo (70, 71) are consistent with the formation of an endogenous AhR ligand in these conditions. The best evidence for a role of the AhR in normal development and physiological/biochemical processes derives from the occurrence of numerous physiological changes and developmental abnormalities in AhR knockout animals (12, 72, 73). These changes are presumed to result from loss of AhR activation by an endogenous ligand, although the identity of the responsible chemical(s) remains to be determined. Recently, however, a variety of endogenous chemicals have been identified that can bind to the AhR and/or active AhR-dependent gene expression. Although the majority of these chemicals are relatively weak when compared to TCDD, these studies confirm that such ligands do exist. Not surprisingly, these endogenous activators represent several structurally distinct classes of chemicals. For this review, we have grouped these "endogenous" ligands into several categories, including indoles, tetrapyroles, AA metabolites, and other ligands. Although the role of these chemicals in AhR signaling in vivo remains to be confirmed, their ability to activate the AhR in vitro and in cells in culture suggests that they may also play a role in regulating AhR function in vivo.

Indoles

Numerous laboratories have reported activation of the AhR by indole-containing chemicals whether they are present in the diet or are endogenous substances. The majority of these AhR ligands are formed from Trp as a result of various biological and physiochemical processes (Figure 3). One of the earliest studies reported that UV illumination of tissue culture media induced aryl hydrocarbon hydroxylase, an enzymatic activity generally associated with CYP1A1, and although this effect appeared to result predominantly from histidine oxidation products, Trp was required for this response (74, 75). Subsequent studies demonstrated the ability of UV irradiation to induce CYP1A1 in the skin and liver of rats and mice (76, 77), suggesting that a diffusible AhR ligand was generated in the skin. The results of Paine and coworkers (74, 75), combined with the fact that Trp is a strong near-UV absorbing amino acid, led several groups to examine the ability of Trp photoproducts to induce CYP1A1. These studies led to the identification of several Trp photooxidation products that competitively bind to the AhR with high affinity and can activate the AhR and AhR-dependent gene expression (78–81). Rannug and coworkers (82) determined the structure of two of the photoproducts, the most active being that of FICZ, a chemical with significant structural similarity to the potent exogenous indole ligand ICZ (35). Although formation of FICZ and other photooxidation products in vivo remains to be confirmed, it was hypothesized that Trp photoproducts formed in the skin of UV irradiated animals might be responsible for the CYP1A1 induction response. The relationship between light exposure and Trp and/or Trp-metabolites is well documented. Indole acetic acid, a plant growth regulator, and its precursor, indole-3-acetaldehyde, are formed from Trp in response to light. Serotonin and melatonin, two other Trp-derived biomolecules,

are important neuroendocrine modulators; the latter of which is important in normal circadian rhythms in mammalian species. As a result of this information, it was recently proposed that FICZ and other photooxidation products of Trp may actually be novel chemical messengers of light (80). This hypothesis takes on more potential significance when one considers that other members of the PAS domain superfamily (83–85) include a variety of light-activated photoreceptors and phytochromes (photoactive yellow protein, phototrophins, phytochrome A, and others) and numerous genes involved in circadian rhythm (Clock, Per, White Collar, and others). Further work in this area may provide interesting insights into the role of these Trp photoproducts in normal endogenous signaling pathways.

The ability of other endogenous indoles and indole metabolites to bind to the AhR has also been reported by Miller and coworkers (86) and our laboratory (87). These studies demonstrated that Trp and naturally occurring Trp metabolites (tryptamine and indole acetic acid) can bind to and activate the AhR and AhR-dependent gene expression in both yeast and mammalian cells in culture. Tryptamine was also shown to be a relatively potent competitive inhibitor of CYP1A1-dependent enzymatic activity, suggesting that it may be a substrate for this enzyme (87). More recently, we have also observed that several kyneurinines, additional metabolic breakdown products of Trp, can activate the AhR signaling pathway (88). Because these chemicals are relatively weak ligands and only found at low concentration in cells, they are likely not endogenous activators in normal physiological conditions. However, if cellular concentrations of some Trp metabolites (i.e., tryptamine) are significantly elevated, as can occur during some abnormal conditions (i.e., when monoamine oxidase activity is inhibited), concentrations have been reported to reach levels that are sufficient to activate the AhR [i.e., $\sim$700 nM (89)].

Indigo and indirubin (Figure 3), two Trp metabolites isolated from human urine, were recently reported to activate the AhR in an AhR-Arnt-containing yeast cell bioassay system (90). These chemicals were reported to be extremely potent AhR agonists, with indigo and indirubin being equipotent or 50-fold more potent than TCDD, respectively. However, in mammalian cells these compounds are 50,000- to 100,000-fold less potent than TCDD as activators of the AhR signaling pathway (E. Fairbairn & M.S. Denison, manuscript in preparation). This significant difference in biological potency likely results from a combination of both their predicted metabolic lability in mammalian cells and a technical limitation of the yeast bioassay in accurately measuring the relative activity of TCDD (91). The EC_{50} for induction of AhR-dependent gene expression by TCDD is $\sim$9 nM in the yeast bioassay (90) compared to that of 6–10 pM in mammalian cells (32). In contrast, β-naphthoflavone, a PAH ligand for the AhR is only 3-fold less potent than TCDD in yeast cells (91), whereas it is 5000-fold less potent in mammalian cells (D.H. Han & M.S. Denison, unpublished results). The principal differences in the relative potency of TCDD between yeast and mammalian cells most likely results from the extreme hydrophobic character of TCDD. When TCDD is added to highly aqueous solutions, like that of yeast culture media, there would likely be a significant loss of

TCDD from the media directly onto the wall of the assay microplate. Although the concentration of indirubin and indigo in human serum remains to be established, fetal bovine serum contains ~0.07 nM indirubin, a concentration that is sufficient to activate the AhR in the yeast cell bioassay (90). Finally, it might be questioned as to whether indigo and indirubin should be considered endogenous ligands because they are predominantly derived from plants. However, because these products can also be formed from cytochrome P450- (CYP2A6, 2C19, and 2E1) dependent-metabolism of indole (92, 93) and they have been identified in the urine of patients with pathological conditions such as leukemia and porphyria cutanea tarda (94, 95), it is clear that they can be produced in human in vivo.

Tetrapyroles

The relationship between the AhR and heme biosynthetic and degradation pathways has been previously established. TCDD is known to disrupt heme biosynthesis, resulting in uroporphyria and hepatocellular damage (7, 11, 96, 97). Although the exact mechanism has not yet been defined, this effect has been proposed to result from a combination of AhR-dependent induction of CYP1A2 and the inhibition of uroporphyrin decarboxylase activity (98). In addition, TCDD treatment has also been observed to enhance the degradation of BR, the primary heme degradation product, presumably by its ability to induce AhR- and DRE-dependent expression of CYP1A1, CYP1A2, and UGT*01, enzymes that can metabolize BR. Interestingly, persistent expression of CYP1A1 in congenitally jaundiced Gunn rats that lack functional UDPGT*01 suggested the presence of endogenous AhR ligands in the blood of these animals (99). Subsequent studies demonstrated that BR (Figure 4), present in high levels in the blood of Gunn rats, can induce expression of CYP1A1 and a DRE-dependent reporter gene in a dose- and AhR-dependent manner in cultured cells (100, 101). This induction was observed using physiologically relevant concentrations of BR. In addition, BV, the metabolic precursor of BR, also induced DRE-dependent gene transcription in several species, although it is likely that it does so indirectly by serving as a metabolic precursor of BR. Subsequent experiments have demonstrated not only that BR can directly stimulate AhR transformation and DNA binding in vitro and in cells in culture, but that it is a competitive ligand for the AhR, albeit a relatively weak one (99). Thus, available evidence demonstrates that the heme degradation products BR and possibly BV are ligands and agonists of the AhR signal transduction pathway. The physiological relevance of these results is likely related to BR's ability to simulate its own metabolism. Because congenitally jaundiced Gunn rats or human infants with Crigler-Naijar syndrome type-I lack functional UGT*01, the primary BR detoxification enzyme, the ability of BR to induce expression of CYP1A1/1A2 [both of which can metabolize BR (102, 103)] provides an AhR-dependent feedback mechanism to reduce circulating levels of BR. Support for this hypothesis comes from studies in which oral administration of I3C, a naturally occurring plant product that is converted in acidic conditions in the stomach into the potent AhR

agonist ICZ, to jaundiced Gunn rats or Crigler-Naijar infants resulted in a significant reduction in plasma BR levels in both situations (104). In addition, TCDD pretreatment has been shown to enhance oxidative metabolism of BR as well as biliary excretion of BR-glutathione conjugates in Gunn rats (105). Interestingly, these and other studies (106) demonstrate the utility of AhR agonists as potential therapeutic agents.

Arachidonic Acid Metabolites

A relationship between TCDD, the AhR, and AA metabolites has also been demonstrated. TCDD can increase the release of AA from membranes as a result of its ability to stimulate membrane lipid oxidation and phospholipase A activity (107–111). In addition, it can induce AA-metabolizing cytochrome P450s [such as CYP1A1 (112–114)] as well as prostaglandin synthase H2 [PGSH2 (115, 116)], which converts AA to prostaglandins. TCDD and other AhR ligands are also reported to increase cardiac release of prostaglandins in vitro (117). These results demonstrate an effect of TCDD and the AhR pathway on AA metabolism, but the effect of AA or AA metabolites on the AhR signaling pathway has not been examined. Recently, it was proposed that AA metabolites may play a role in the hydrodynamic shear-stress induction of CYP1A1 in cells in culture (67). Furthermore, suspension of cells in culture in methylcellulose results in CYP1A1 induction (68, 69), an effect proposed to result from the production or release of an endogenous ligand, possibly from the cell membrane in response to changes in membrane conformation/structure. Given the structural diversity and general hydrophobic nature of AhR ligands, it seems reasonable to suggest that some biological lipids and/or steroids may be endogenous AhR ligands. The ability of lipoxin A4 (Figure 4), a lipoxygenase product of AA, and several prostaglandins [most notably prostaglandin G_2 (Figure 4)] to both bind to the AhR and activate AhR-dependent gene expression supports this hypothesis (118, 119). Lipoxin A4 reportedly induces a transient expression of CYP1A1 and that of a DRE-dependent reporter gene at concentrations near physiological in some situations (120) and it is a competitive substrate for CYP1A1 (118). In contrast, the prostaglandins are relatively weak AhR agonists in cells in culture (119), transiently inducing AhR-dependent gene expression only at concentrations >1 μM, much greater than their normal physiological levels (121). However, when one considers that prostaglandins are charged at physiological pH and thus have limited ability to diffuse through biological membranes (122, 123), the actual potency of these chemicals may be significantly greater than that determined in the cell culture experiments (119). It has been reported that prostaglandin concentrations may actually reach 5–10 μM in the proximity of hepatocytes due to nonparenchymal liver cells secreting these "local hormones" into the narrow space of Disse (124). Thus, select prostaglandins, or a combination of prostaglandins, may be able to activate the AhR in vivo. Perhaps the most intriguing aspect of these studies is that although the most active prostaglandins stimulate AhR transformation and DNA binding to a maximum

of 40%–60% of that obtained using TCDD, several prostaglandins induced AhR-dependent reporter gene expression up to a level three- to fivefold greater than that produced by a maximal inducing dose of TCDD (119). It was proposed that this synergistic response resulted from both a direct action on the AhR and activation of a secondary signal transduction system that augments the AhR-dependent gene expression response. Given previous studies demonstrating a synergistic increase in AhR-dependent gene expression following concomitant activation of both the AhR and protein kinase C (125, 126), it is possible that this type of signaling cross-talk mechanism may also occur with the prostaglandins. The specific cellular signaling pathway(s) affected by the prostaglandins responsible for this dramatic increase in AhR-dependent gene expression and whether other AA metabolites or related biological lipids also activate the AhR signaling pathway is currently unknown.

Other Ligands

The ability of several carotinoids [canthxanthin (Figure 4), astaxanthin, and β-apo-8′-carotinal], but not others (vitamin A, β-carotene, lypene, or lutein), to induce CYP1A1 and other members of the Ah gene battery in rats and mice has been described (39, 40, 127, 128). Although the induction of CYP1A1 in mice by carotinoids was demonstrated to be AhR dependent, neither canthaxanthin nor β-apo-8′-carotinal were observed to competitively bind to the AhR (127). Whether these chemicals are simply weak AhR ligands and unable to compete effectively with the high affinity ligand TCDD or are converted in vivo into more potent ligands/inducers remains to be determined. Dietary carotinoids are cleaved in vivo into retinol and converted into other vitamin A metabolites (retinoids), and previous studies have demonstrated a link between retinoids and the AhR signaling pathway. Not only are various retinoids known to be substrates for CYP1A1 (114, 129–131), but one of the hallmark effects of TCDD is an AhR-dependent alteration in retinoid homeostasis and metabolism, resulting in enhanced retinoid mobilization and decreased hepatic vitamin A levels (7, 11, 132). Also consistent with a role for the AhR in normal retinoid homeostasis is the observation of retinoid accumulation and decreased vitamin A metabolism in the livers of AhR knockout animals (133). This link between retinoids and the AhR, combined with the ability of retinoids to alter gene expression through the retinoid dependent RARs and retinoid X receptor, led several groups to examine the ability of retinoids to directly activate the AhR. Although the initial observation of the ability of retinoic acid to induce CYP1A1 gene expression in human but not rodent cells in culture (112, 134–136) suggested that retinoic acid may be an endogenous ligand, it was subsequently demonstrated that this induction was regulated by RARs and a functional retinoic acid responsive element present in the upstream region of the human, but not rodent, CYP1A1 gene (135). More recently, however, the ability of several synthetic retinoids to directly activate the AhR and AhR-dependent gene expression was observed (136, 137). Although the most AhR-active retinoids in

these studies had the weakest relative affinity for the RARs (136), these studies provide intriguing evidence for the possibility of retinoid or retinoid-like AhR ligands.

The suggested role of the AhR in cardiovascular disease (138–141) led Savouret and coworkers (142) to examine the ability of a series of oxysterols normally present in blood to bind to the AhR. Their analysis revealed that 7-ketocholesterol (Figure 4) could competitively bind to the AhR and function as an AhR antagonist. Ligand binding experiments indicate that the affinity of 7-ketocholesterol for the AhR is $\sim 10^5$ lower than that for TCDD; however, concentrations of 7-ketocholesterol found in blood in vivo are sufficient to inhibit TCDD-induced gene expression in cells in culture (142). Although 7-ketocholesterol is not an AhR agonist, it is possible that it may represent the first identified member of a novel group of AhR ligands in which an endogenous activator does exist.

Ligand-Dependent Versus Ligand-Independent Activation of the AhR

The AhR can be activated by a structurally diverse range of chemicals, and although the ability of many of these chemicals to directly bind to and activate the AhR remains to be confirmed, their ability to induce CYP1A1 and/or activate the AhR and AhR-regulated gene expression in animals or cells in culture indirectly supports their interaction with the AhR. Interestingly, some chemicals have been identified that can induce AhR-dependent gene expression, yet they reportedly fail to competitively bind to the AhR (Table 1). It has been proposed that these chemicals are not AhR ligands themselves, but that they can activate AhR-dependent gene expression indirectly, either via metabolic conversion into a ligand or by their ability to affect some cellular pathway that results in AhR activation. These conclusions are difficult to reconcile, especially given what is known about the AhR-dependent mechanism of gene activation. Although these weak inducers have not been observed to competitively bind to the AhR, they may still be AhR ligands, albeit ligands that bind with relatively low affinity. Demonstration of the ability of weak ligands (Kd in the μM range) to competitively bind to the AhR in standard binding assays is technically challenging, especially given the extremely high AhR binding affinity of TCDD [Kd in the pM range (143)]. Recent modifications of the AhR ligand binding assay that favor competitive binding by weak AhR ligands (i.e., reduction of [^{3}H]TCDD and increased competitor concentration) have been used to demonstrate that carbaryl, previously reported to not bind to the AhR (144, 145), is actually a weak AhR ligand (146). The 300,000-fold lower potency of carbaryl, as compared to TCDD (146), likely explains its inability to competitively displace [^{3}H]TCDD from the AhR ligand binding domain using the ligand binding assay conditions described by those investigators. In addition, the competitive binding of Trp metabolites (namely tryptamine and indole acetic acid) and several benzimidazoles (omeprazole, thiabendazole, albendazole, and

TABLE 1 Chemicals that are reported to activate the AhR and induce AhR-dependent gene expression, yet do not competitively bind to the AhR

Chemical	Reference
Omeprazole	(148, 150)
Thiabendazole	(162)
Oxfendazole	(163)
Myristicin	(164)
Methylenedioxyphenyls (isosafrole, PBO)	(157, 165)
Carbaryl	(144, 145)
Mevinolin	(166)
Canathaxanthin	(128)
β-Apo-8′-carotinal	(128)
11-Ethoxy-cyclopenta(a)phenanthrene-17-one	(167)
Primaquine	(168)
Caffeine	(169)
Cypermethrin	(170)
Diflubenzuron	(170)
Tetrachlorvinphos	(170)
Lanosperole	(171)
Nicotine	(172)
Pyridines	(173)

fenbendazole) has also been observed (87, 147), even though these chemicals were previously reported to induce AhR-dependent gene expression in a ligand independent manner (148–151). Not only are these binding results consistent with what is known about other CYP1A1 inducers and AhR activators, but it is likely that many (if not all) of the other chemicals that reportedly induce in an AhR-independent manner (Table 1) are actually weak AhR ligands. The absolute requirement for the AhR in the chemical-inducible response, combined with the demonstration that some "ligand independent" inducers actually bind to the AhR, raises questions as to the existence of these proposed alternative induction pathways. Unlike other ligand-dependent receptors (i.e., steroid hormone receptors) that can be activated in a ligand-independent manner by processes such as phosphorylation (152, 153), ligand-independent activation for the AhR remains to be confirmed. Accordingly, in our view, the available data are still consistent with the established mechanism in which the ability of a chemical(s) to activate AhR-dependent gene expression is dependent upon its interaction with the AhR ligand binding site.

CONCLUDING COMMENTS

In most biological systems, ligand binding to receptors is generally of high affinity and high chemical specificity. However, the structural diversity of AhR ligands is somewhat similar to that reported for the peroxisome proliferator activated receptor and pregnane X receptor, orphan receptors of the steroid hormone receptor superfamily that also respond to xenobiotics (154–157). Although the rather "sloppy" or promiscuous ligand binding specificity of these xenobiotic receptors may at first seem to be incompatible with their role as selective ligand-dependent receptors, a case can be made that this characteristic may actually confer some adaptive advantage to the organism. These receptors are known to induce expression of cytochrome P450s as well as that of other xenobiotic metabolizing enzymes (at least in the case of the AhR). Because activation of these receptor systems enhances expression of numerous detoxification enzymes, each of which exhibits broad substrate specificity, the promiscuous ligand binding activity of these receptors would provide the organism with a greater dynamic range of "chemical detection" and metabolism. In addition to xenobiotic-mediated induction, the promiscuous nature of AhR ligand binding could also increase the spectrum of endogenous chemicals that could activate AhR. One can imagine that distinct endogenous ligands present in different cell types could activate AhR and thus induce expression of gene products important for a desired biological activity in a cell-specific manner. Although it is possible that there is a high affinity endogenous AhR ligand, one has yet to be identified. We envision the existence of numerous endogenous physiological AhR ligands that have relatively weak affinity compared to TCDD and are rapidly degraded by the coordinately induced detoxification enzymes. Accordingly, these endogenous ligands would act as transient inducers of AhR-dependent gene expression, a situation similar to that produced by the prostaglandins (119). In fact, many AhR ligands are known substrates for CYP1A1 and/or other members of the Ah gene battery. The observation of elevated levels of AhR-dependent gene expression, as well as reduced cytosolic AhR concentrations in cells lacking CYP1A1 activity, and loss of this constitutive AhR-dependent response when CYP1A1 activity was restored are also consistent with these enzymes metabolizing an endogenous AhR ligand(s) (63, 66). Purification and characterization of the endogenous AhR inducer from CYP1A1 deficient cells and other tissues containing constitutively active AhR complexes is an interesting direction for future research.

The structural diversity of CYP1A1 inducers was first reported by Owens & Nebert (158), who demonstrated the ability of numerous hydrophobic compounds to induce CYP1A1-associated enzyme activity. Although the list of inducers has expanded greatly since that time, the actual spectrum of chemicals that can bind to and activate the AhR is still an area of ongoing research. Elucidation of the spectrum of physiochemical and structural characteristics of AhR ligands may provide insights into the identity of other exogenous and endogenous ligands for the AhR. The recent development of several very sensitive, rapid, and high throughput AhR-based screening bioassay systems (32, 159, 160) now provides avenues in

which to identify, isolate, and characterize AhR ligands from a variety of matrices and biological samples. Although tissue fractionation approaches had failed to identify endogenous ligands in the past, the increased sensitivity of these new screening bioassays should improve chances of identifying these ligands. Similar cell bioassay approaches have been successfully utilized to identify endogenous ligands for orphan nuclear receptors (161). In addition, the recently published homology model of the murine AhR ligand binding site (162) will now allow detailed modeling studies of the binding and specificity of these diverse ligands to the AhR. These are exciting areas for future studies. Overall, identification and characterization of the spectrum of endogenous and exogenous ligands of the AhR will provide insights into the biochemical and molecular mechanisms by which ligands can activate the AhR signaling pathway, but they will also facilitate studies into the endogenous role of this novel receptor system.

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TRAFFICKING OF NMDA RECEPTORS[1]

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■ **Abstract** The NMDA receptor (NMDAR) plays a central role in the function of excitatory synapses. Recent studies have provided interesting insights into several aspects of the trafficking of this receptor in neurons. The NMDAR is not a static resident of the synapse. Rather, the number and composition of synaptic NMDARs can be modulated by several factors. The interaction of PDZ proteins, generally thought to occur at the synapse, appears to occur early in the secretory pathway; this interaction may play a role in the assembly of the receptor complex and its exit from the endoplasmic reticulum. This review addresses recent advances in our understanding of NMDAR trafficking and its synaptic delivery and maintenance.

INTRODUCTION

Since its identification in the late 1970s, the NMDA receptor (NMDAR) has been the object of an intense and diverse research effort, which has implicated this receptor in multiple neuronal functions ranging from synapse formation to ischemic damage to learning and memory. The demonstration in 1983 that antagonists of the NMDAR could block long-term potentiation (LTP) in the hippocampus elevated the NMDAR to the role of a key player in synaptic plasticity (1). This role was supported by studies showing the voltage dependency of the magnesium block and calcium permeability of the channel (2, 3). With the cloning of the NMDAR subunits, first NR1 in 1991 (4), the NR2 subunits in 1992 (5, 6), and the NR3 subunit in 1995 (7), the molecular bases for these functional properties could be defined. However, our understanding of the cell biology of the NMDAR has lagged, and questions concerning the assembly of the receptor complex, its trafficking in the neuron, and the mechanisms controlling its addition to and removal from the synapse have only recently been addressed. Its companion glutamate receptor, the AMPA receptor, has been shown to be added to and removed from the synapse

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based on activity, providing a possible molecular mechanism for LTP and long-term depression (LTD), which are cellular models of learning and memory (8). Although the NMDAR has been viewed as a more stable component of the synapse, recent data indicate that the number of receptors can be changed rapidly by internalization (9–12). NMDARs are present at synapses early in development, and the addition of AMPA receptors is dependent on the activity of NMDARs, forming the basis of the "silent synapse hypothesis" (13). These studies point to the central role of trafficking in the regulation of functional NMDARs and provide a clear incentive to understand the mechanisms underlying these events.

STRUCTURE, SUBUNIT COMPOSITION, AND FUNCTIONAL PROPERTIES OF NMDA RECEPTORS

Subunit Assembly

To form a functional receptor complex, the NR1 subunit must assemble with one of the four NR2 subunits (NR2A, NR2B, NR2C, NR2D) into what is generally thought to be a tetrameric complex. The NMDA receptor is unique among ligand-gated ion channels in its requirement for two agonists, glutamate and glycine. The NR1 and NR2 subunits contain the glycine and glutamate binding sites, respectively. There are three sites of alternative splicing in the NR1 subunit, one in the N terminus and two in the C terminus, resulting in a total of eight possible splice variants. The C-terminal splice variant compositions affect the early trafficking of this subunit and are discussed in detail later. In addition, the NR3 subunit assembles with NR1 and NR2, resulting in a receptor with diminished activity (14), and NR3 subunits can assemble with NR1 alone to create a functional glycine receptor (15). However, it appears that most NMDARs are made up of an NR1 subunit and one or more NR2 subunits. The NR2A and 2B subunits are the major and most widespread NR2 subunits, with NR2C largely restricted to the cerebellum and NR2D most heavily expressed early in development. The NR2B subunit predominates early in development and then gradually decreases, whereas expression of NR2A is low shortly after birth but continues to increase. Therefore, NR2B is the major subunit during the early period of a neuron's life, whereas NR2A is predominant in the later stages, suggesting that the NR2B to 2A switch is responsible for the transition of a synapse from a more plastic to a less plastic state. The importance of having the correct number and composition of NMDAR subunits is demonstrated by genetic studies. Targeted disruption of the NR2A subunit produces mice with a reduction in LTP and deficiencies in some learning tests (16). Animals with enhanced production of NR2B show superior ability in learning and memory in behavioral tasks (17), whereas a dramatic decrease in the amount of NR1 produces animals with symptoms characteristic of schizophrenia (18).

Neurons have a large excess of the NR1 subunit relative to the NR2 subunits; this pool of NR1 is unassembled with NR2, exists primarily as a monomer, does not reach the cell surface, and is rapidly degraded if it does not assemble (19, 20).

This suggests that the availability of the NR2 subunits is the limiting factor in production of functional receptors. This seemingly inefficient mechanism, in which most of the NR1 is never used to produce a functional receptor, stresses the critical importance of mechanisms that regulate rapid changes in availability of NR2 subunits. In cortical neurons of four-week-old rats, three types of NMDARs were identified: those made up of NR1/NR2A, NR1/NR2B, and NR1/NR2A/NR2B (21). Because these receptor complexes have different functional characteristics, their localization and ontogeny could dramatically affect the neuron's response to synaptic stimulation. It is not known if the assembly process is simply based on the availability of subunits or regulated in some fashion, but based on similar data on AMPA receptors, it is suggested that the assembly process is regulated and may vary with cell type and stage of development (22).

Proteins that Interact with NMDA Receptors

The targeting of the receptor to the synapse and its localization at the synapse depend on a series of interactions with other proteins. Many of these interactions may involve the receptor's carboxy terminal domain, which is located in the cytoplasm (Figure 1), and are likely candidates to influence the trafficking of the receptor. The carboxy terminal domains are large compared to those of other ionotropic glutamate receptors. For example, the NR2A subunit has 627 amino acids and the NR1-1 subunit has 105 amino acids. The first yeast two-hybrid screen with the NR2 C-terminal tail identified PSD-95, a highly abundant protein in the postsynaptic density (PSD), as an interacting protein that associates with the last four amino acids of the NR2 subunit (23). Additional members of the PSD-95 family were identified and include SAP102, SAP97, and PSD-93 [see (24) for review]. Like PSD-95, they all have three PDZ domains, one SH3 domain, and one guanylate kinase (GK) domain. Proteins in this family are referred to as membrane-associated guanylate kinases (MAGUKs) and are proposed to anchor NMDARs at the synapse. The NR2 subunits have been shown to bind to the first two PDZ domains of PSD-95, PSD-93, and SAP102 (24). The NR1 splice variant, NR1-4, contains a consensus PDZ interacting domain that interacts with all these MAGUKs (25).

Although the PDZ interacting domain of NR2 subunits has received most of the attention, a number of other proteins have been shown to interact with both the NR1 and NR2 subunits (Figure 1), and this number will certainly grow. For example, recently two sites on the NR2B tail, one near TM4 and the other near the C terminus, have been shown to be involved in clathrin-mediated internalization of the receptor (9, 10). These are consensus sequences for binding to AP-2 adaptor proteins that initiate internalization of membrane proteins. In addition, a number of phosphorylation sites that affect the function or trafficking of the receptor have been identified on the C terminus. The roles of these sites are discussed in more detail later. In addition, although the C terminus is critical for mediating many protein-protein interactions, co-assembly of subunits into a complex has been

shown to involve interactions of the N terminus of NR1 and NR2 subunits (26), and interactions of NMDARs with the EphB receptor appear to be mediated by the N terminus of NR1 (27).

PROCESSING OF NMDA RECEPTORS IN INTRACELLULAR COMPARTMENTS

Because both NR1 and NR2 subunits assemble together to form a functional receptor complex, a mechanism must exist to ensure that only complexes that contain the proper combination of both subunits are allowed to reach the cell surface. For other complex proteins, this is often achieved by retaining the unassembled subunits in the endoplasmic reticulum (ER); a similar mechanism appears to play a role in NMDAR assembly.

Subunit Assembly and Quality Control in the Endoplasmic Reticulum

Newly synthesized proteins undergo essential folding, maturation, and oligomerization in the ER. The ER uses a rigorous quality control process to ensure that both lumenal and transmembrane proteins conform to their proper states before exiting. Thus, unfolded, misfolded, and unassembled proteins can be retained in the ER by molecular chaperones [see (28) for review]. Although some multimeric proteins require only chaperone-based quality control for proper oligomerization, many transmembrane proteins contain a short amino sequence on their cytoplasmic or ER lumenal domains that acts as an ER retention signal. ER retention signals were first defined in single transmembrane domain Type-I transmembrane proteins (N terminus in the ER lumen and C terminus in the cytoplasm) that form heteromultimers and Type-II transmembrane proteins (C terminus in the ER lumen and N terminus in the cytoplasm) [see (29) for review]. Different motifs are distinguished as functional in either Type I (KKXX-COOH; di-lysine motif), or Type II (NH_2-XXRR; di-arginine, and KDEL/HDEL-COOH) proteins. Detailed analyses of several proteins that contain retention signals and form complexes that are destined for the plasma membrane have shown that ER exit is achieved by steric masking of retention signals. Assembly of the subunits, therefore, results in masking of the retention signals and exit from the ER.

Recently, another ER retention signal, RXR, has been defined in multitransmembrane domain proteins. In this case, X is less well defined, but can be any basic and some neutral amino acids depending on the protein. RXR retention motifs have been characterized in a number of different proteins, the first being the ATP-sensitive potassium channel (K_{ATP}) (30). Fully assembled channels contain eight subunits; four core α-subunits (Kir 6.1/6.2) that form the pore and four β-auxiliary subunits (SUR1). All of the subunits contain the RXR ER retention motif, and through analysis of mutants and surface expression in *Xenopus* oocytes, Zerangue et al. (30) were able to demonstrate an even more complex interplay between

subunits and steric blockade of ER retention. ER retention signals on core α-subunits require the β-auxiliary subunits for steric masking. α-Subunits do not reciprocate by blocking the β-subunit's signal. Rather, individual β-subunits occlude the α-subunit and contribute to masking of the neighboring β-subunit. This sets up exquisite criteria for ER exit; only fully assembled octamers are able to pass quality control by ER retention. If α- and β-subunits were capable of mutual retention blockade, one might imagine that individual α- and β-subunits could pass quality control by this mechanism. Zerangue et al. (30) discovered that by mutating all of the retention signals in every subunit, functional channels were still formed. The mutant channels showed higher basal responses that were insensitive to modulation by ATP. However, the channels were expressed in greater abundance on the surface. Considering the investment that cells put into regulating trafficking via ER retention, these signals might have the additional role of reducing cell-surface expression and creating a larger intracellular pool that could act as a buffer to meet a wide range of demands for cell-surface expression. Thus, the presence of ER retention motifs may not only reduce surface expression, but also may allow fine regulation of the number of proteins delivered to the cell surface.

$GABA_B$ receptors possess distinctly different subunits, GB1 and GB2, that are required for functional expression of the $GABA_B$ complex. The GB1 subunit has a retention signal similar to the RXR (RXR[R]) (31). The GB2 has no ER retention signal and is expressed on the surface in heterologous cells, but it does not seem to form a functional receptor in the absence of GB1. Classical steric masking through a proximal coiled-coil domain in each cytoplasmic tail has been identified as the mechanism for blocking the retention signal on GB1 (31). GB2 does not appear to have a function independent of GB1, as no GB2 homodimers can be found on the neurons (32). Supposing that GB2's synthesis is rate-limiting to expression of GB1, GB1 retention acts as a "checkpoint" along the secretory pathway for normal assembly (31). Essentially, GB1 proteins could remain in ER-residence and become associated with any newly synthesized GB2.

ER Retention of the NR1 Subunit

NR2 subunits do not form functional receptors when expressed alone in heterologous cells (5), and those that have been studied (NR2A and NR2B) do not reach the cell surface (33). NR1 subunits also do not form functional receptors alone, but their cell surface expression depends on the splice variant. As mentioned above, there are eight splice variants of NR1, and four of these are generated by alternative splicing of the C terminus. The NR1 C terminus (Figure 1) is made up of four cassettes. C0 is present in all splice variants. C1 is optional and is present in four of the eight splice variants. Alternative splicing also determines if a C2 or C2′ cassette is present, and each is present in four of the eight variants. The most abundant variant contains C0, C1, and C2. This variant, NR1-1, does not reach the cell surface when expressed without NR2 in heterologous cells and is retained in the ER. NR1 with the other three combinations of C termini will all reach the

cell surface. The ER retention of NR1 is dependent on an RXR motif in one of the alternatively expressed cassettes, C1 (25, 34, 35).

Although the ER retention of NR1-1 (C1C2) and the cell surface expression of NR1-2 (C2) and NR1-4 (C2′) are clearly explained by the presence of the ER retention motif in C1, it does not explain the surface expression of NR1-3 (C1C2′). It suggests that another signal, present in C2′, can negate the RXR retention signal in C1. This signal was shown to be the PDZ-binding domain at the distal end of C2′ (STVV). This could result from masking of the RXR motif by the PDZ proteins that interact with the C2′ cassette, or the PDZ protein could be acting as an export protein that simply overrides the ER retention. The last six amino acids of C2′ are sufficient to suppress ER retention, as shown by deleting the rest of C2′, but experiments to lengthen the distance between the RXR motif and the PDZ binding domain remain to be done. Such experiments may help determine the mechanism by which the retention is negated. Soluble fusion proteins containing the PDZ interacting domain of C2′ can effectively block the surface expression of NR1-4, showing that saturation of the trafficking pathway leads to an intracellular buildup of NR1-4 (36). Subunits that lack the C2′ cassette are not affected by this treatment. In addition to providing an interesting mechanism to overcome ER retention, these results show that the PDZ protein can interact early in the secretory pathway in addition to serving as an anchor at the synapse.

The functional significance of the multiple C-terminal configurations of NR1 and their various behaviors in the ER remain unclear. Homomeric NR1 has not been detected on the surface of neurons. It is interesting that NR1 can assemble with NR3 to produce functional glycine receptors; this assembly is also sufficient to relieve the ER retention of NR1 (37).

Assembly Releases ER Retention of NR1 and NR2 Subunits

The NR2 subunit is retained in the ER, and like NR1, its retention appears to depend on a signal in the C terminus. The mechanism by which assembly overcomes ER retention of both subunits is not clear. Cytoplasmic tails of each subunit type have been truncated in a number of experiments in different preparations; the sum of the observations do not support simple masking of the ER retention signal via complementary cytoplasmic tails (10, 38–40). With the entire NR1 C-terminal domain truncated, functional heteromeric receptors are expressed on the cell surface (10, 39, 40). Similar results are obtained when NR2 C-terminal domains are truncated. For example, mice lacking nearly all of the C-terminal cytoplasmic domain of NR2A still have functional NMDARs containing this subunit, although they fail to be appropriately clustered at the synapse (41). If complementary masking is not involved, a number of other possibilities must be considered. First, like subunits may mask each other. The argument against this is that dimerization of NR1 subunits is believed to be an early step in assembly; in heterologous cells, NR1-1 can form dimers in the absence of NR2, but they are still retained in the ER (26). Thus, masking of their retention signals does not occur. However co-expression of

NR1-1 and NR1-4 resulted in a limited surface expression of NR1-1, showing that the subunits interacted and in some way negated the ER retention of NR1-1 (42). Second, NMDAR C termini may undergo sustained conformational changes upon proper quaternary folding, acting as a "folding sensor" that occludes the motifs. Finally, an unknown protein may interact with one or both cytoplasmic tails and thereby suppress ER retention, as PDZ proteins do for the NR1-3 variant. This may or may not involve masking of the retention signals.

Trafficking of NMDA Receptors from the ER to the Plasma Membrane

After they are released from the ER, membrane proteins such as the NMDAR are further processed in the Golgi apparatus and then distributed to the *trans* Golgi network (TGN) and endosomes. Because functional ER and Golgi complexes are present in many dendrites and even dendritic spines, it is possible that some of the processing related to these compartments actually occurs near the synapse. This would provide a mechanism for a rapid and local response. Most receptors, however, are likely processed in the cell body and then transported to the synapse. In determining the function of a novel kinesin (KIF17), Setou et al. (43) found KIF17 to be indirectly associated with NMDARs. Their data suggest that a complex of mLin-7, mLin-2, and mLin-10 associates with a cargo vesicle containing NMDARs and with KIF17, which transports it along microtubules in dendrites to the synapse. Little is known about the nature or formation of these vesicles that contain the newly processed receptors. Because it would require creating a large number of discrete vesicles, it is unlikely that each type of receptor is parceled into its own vesicle population, but whether or not the packaging follows any particular organization remains to be determined.

Several findings suggest that the NMDAR interacts with PDZ proteins before it reaches the synapses and that these interactions may be involved in the synaptic delivery (25, 43). A yeast two-hybrid analysis showed that one of the PDZ partners of the NR2 subunit, SAP102, interacts through its PDZ domains with sec8, a protein of the exocyst or sec6/8 complex (44). The exocyst is a complex of eight proteins first identified in yeast and later in mammals that was shown to be involved in targeting of secretory vesicles to the plasma membrane (45). The initial site of interaction is unclear, with some studies indicating the ER and others the Golgi or TGN (45–47); the site of interaction may vary with the cell type or particular protein. The fact that SAP102 interacts with sec8 suggests that the NMDAR may indirectly associate with the exocyst complex through SAP102. Several studies, including functional analyses, confirm that the NMDAR, SAP102, and sec8 form a complex in the brain and suggest that formation of this complex is involved in the synaptic delivery of NMDARs. Receptors without the PDZ interacting domain bypass the exocyst interaction and reach the cell surface. This raises the possibility that there are two mechanisms for delivery of NMDARs to the cell surface, one which involves the PDZ interacting domain and one that does not.

REGULATION OF NMDA RECEPTORS AT THE SYNAPSE

Many factors could conceivably influence the number and composition of NMDA receptors at the synapse. These include the availability of receptors, either in an intracellular pool or a nonsynaptic surface pool, the stability of receptors at the synapse, and the removal of receptors from the synapse either through endocytosis or diffusion in the plasma membrane.

NMDA Receptors and Organization of the Synapse

NMDARs are found at both synaptic and extrasynaptic sites, but are present at a much higher density at the synapse. This clustering at the synapse is believed to involve an interaction of the receptor with proteins that are part of the PSD. Identification of the interacting proteins and how these interactions are regulated is central to understanding the trafficking of NMDARs at the synapse.

The PSD is an electron-dense organelle that is localized along the postsynaptic membrane of excitatory synapses. A major component of the PSD is the PSD-95 family of MAGUKs (PSD-95, PSD-93, SAP97, and SAP102) (24) (Figure 2). Through their PDZ domains, these proteins can bind directly to NMDARs (at least two other NMDAR-associated proteins, S-SCAM and CIPP, have been reported) (48). From this basic scaffold, other proteins can bind in chains that link the NMDAR to the other glutamate receptors and to ion channels in the postsynaptic reticulum. For example, GKAP can bind to the GK-like domain that is found in MAGUKs and S-SCAM (49); GKAP then can bind to Shank, which can bind to dimers of Homer that bind to type 1 metabotropic glutamate receptors in the postsynaptic or adjacent perisynaptic membrane. Shank also can bind to Homer dimers that bind to inositol 1,4,5-triphosphate (IP3) receptors in extensions of the reticulum that lie subjacent to the PSD (24, 50). Another chain of proteins can be formed between NMDARs and AMPA receptors via a combination of calcium/calmodulin-dependent protein kinase II (CaMKII) and an AMPA receptor channel anchoring an assembly of proteins, which consists of actinin, actin, 4.1N protein, and SAP97/GluR1 [see (51) for review]. An additional method of linking NMDA and AMPA receptors is through PSD-95 and other MAGUKs via Stargazin, which binds to the PDZ domains of the MAGUKs and mediates synaptic targeting of AMPA receptors (52). Finally, the postsynaptic complex is linked to actin filaments, which control the overall structure of the postsynaptic spine and may form pathways for transport of proteins to and from the postsynaptic membrane. At least three such kinds of connections exist in addition to the one associated with GluR1: actinin-actin-NMDARs, GKAP-Shank-cortactin-actin, and PSD-95-SPAR-actin [see (53) for review]. SPAR is a Rap-specific GTPase-activating protein; the latter proteins are implicated in regulation of MAP kinase cascades, cell adhesion, and activation of integrins. Like GKAP, SPAR can bind to the GK domain of PSD-95 (54). It regulates spine morphology, both through its direct interaction with F-actin and also probably via Rap

signaling. Nevertheless, whereas actin-protein associations play significant roles in synaptic structure and function, anchoring of NMDAR/PSD-95 complexes at synapses appears to be independent of actin associations (55).

Many other proteins associate with the NMDAR complex. For example, at least two proteins that may regulate the MAP kinase pathway can bind to the PDZ domains of the major scaffolding proteins: SynGAP (synaptic Ras-GTPase activating protein) binds to PSD-95 (56, 57) and nRap GEP (neural GDP/GTP exchange protein for Rap1 small G-protein) binds to S-SCAM (58). MAGUIN, which binds to both PSD-95 and S-SCAM, also may regulate the MAP kinase pathway (59). SynGAP is believed to maintain a low steady-state level of active Ras near the synapse by catalyzing rapid hydrolysis of Ras-GTP to Ras-GDP. Calcium entry through NMDARs activates CaMKII, which can inactivate SynGAP via phosphorylation. Without the influence of SynGAP, Ras-GTP accumulates and increases the activation of the MAP kinase cascade, which is associated with long-term potentiation of synaptic function. Another PSD-95-associated protein, Citron, is limited to certain populations of neurons in the brain, and is a target of Rho, which is a small GTPase that regulates actin cytoskeleton organization (60, 61). Citron may mediate forms of NMDAR-dependent synaptic plasticity that are limited to certain specialized groups of neurons.

Finally, pre- and postsynaptic components of glutamatergic synapses can be linked by proteins, including neuroligin/neurexin complexes, L1, and cadherins (48). Neuroligin/neurexin complexes cross the synaptic cleft and connect to other PDZ domain-containing proteins in the presynaptic terminal. Cadherins dimerize in the synaptic cleft and link via catenins to the actin cytoskeleton. Both cadherins and catenins are part of the NMDA receptor complex (62). This dimerization of cadherin is linked directly to NMDAR activation, so that the stability of the synaptic contact may be regulated by NMDARs (63). Interestingly, NMDARs plus PSD-95 also are found in cadherin-based attachment plaques in cerebellar glomeruli, suggesting that the overall stability of the glomerulus is controlled similarly, in this case, via glutamate spillover from adjacent synapses in the glomerulus (64).

Kinase/Phosphatase Interactions with NMDA Receptors

Phosphorylation of NMDARs produces a wide variety of effects under various experimental conditions [see (65) for review], suggesting that this may be a major mechanism for regulating receptor trafficking at the synapse. In some cases, the effect may be directly on the ion channel. In this section, we limit the discussion to effects that alter the distribution of the NMDAR or its interaction with other proteins.

CaMKII can associate with NR1, NR2A, and NR2B. Binding of CaMKII to NR2A and NR2B does not involve the direct binding of CaMKII to its major phosphorylation site on the NMDAR subunit—serine 1289 for NR2A (66) or serine 1303 for NR2B (67). Association of CaMKII with NMDARs is believed to occur following autophosphorylation of CaMKII due to calcium entry from activated

NMDARs and induction of LTP. It is believed that this association between CaMKII and NMDARs brings CaMKII into close proximity with AMPA receptors. Subsequent phosphorylation of AMPA receptors results in potentiation of the synapse by inducing synaptic insertion and increasing single-channel conductance of AMPA receptors (67, 68). However, autophosphorylation of CAMKII (via T286) is not necessary for binding to NR2B. In fact, following stimulation by calcium and calmodulin, CaMKII can bind to NR2B (68). NR2B then regulates the function of the bound CaMKII, putting it in an autonomous, calmodulin-trapping state that cannot be reversed by phosphatases, and suppresses inhibitory autophosphorylation of T305/306, thus preventing dissociation of the CaMKII from the synapse. This association may also induce autophosphorylation of neighboring CaMKII molecules. CaMKII forms stable complexes with NR1 and NR2B but not with NR2A; stimulation of NMDARs increases this association (67). Another study shows that CaMKII and PSD-95 compete for binding to NR2A in a hippocampal PSD preparation (66). They also show that in LTP-potentiated hippocampal slices, both CaMKII-dependent activity and CaMKII association with NR2A/B (ratio of CaMKII/PSD-95 after NR2A/B precipitation) increases, with a concomitant decrease in association between PSD-95 and NR2A/B. Clearly, there are complex relationships of CaMKII with NMDARs and these vary even with different NR2 subunits.

Studies on the effects of protein kinase C (PKC) on NMDARs have yielded conflicting results, probably because PKC has multiple effects depending on cell type, sites of action, and variable associations of NMDARs with other proteins. Liao et al. (69) found that PSD-95 has a profound influence on insulin/PKC and Src potentiation of currents of NMDARs expressed in *Xenopus* oocytes. Potentiation of NR1/NR2A currents by Src requires co-expression of PSD-95. In contrast, PSD-95 co-expression eliminates insulin—(probably via PKC) or phorbol ester (PE; activates PKC)—mediated potentiation of NR1/NR2A currents. Furthermore, both of these responses differ from those involving NR1/NR2B currents, which are potentiated by insulin or PE with or without co-expression of PSD-95 (Src failed to potentiate these currents in either case). The effect of PKC on NR1/NR2A receptors in *Xenopus* oocytes involves an increase in NMDAR channel opening rate and delivery of new NMDAR channels to the surface through regulated exocytosis (70). PKC activation of NMDARs also influences the association of CaMKII with NMDARs. PKC-dependent phosphorylation [due to stimulation with PE or the metabotropic glutamate receptor (mGluR)-specific agonist, t-ACPD] of NR2A at serine 1416 inhibits CaMKII binding, thus promoting the dissociation of the CaMKII-NR2A complex (71). In addition, Fong et al. (72) found that PE activation of PKC induces translocation of CaMKII to synapses in cultured hippocampal neurons; perhaps related to this, immunogold studies indicate that CaMKII in the PSD increases fivefold following depolarization (73). Fong et al. (72) also found that the PKC activation induces rapid dispersal of NMDARs from the synapse to the extrasynaptic membrane; this may be a way that phosphorylation can downregulate synaptic NMDARs. Overall, the last four studies (in addition to studies

described in the previous paragraph) suggest that synapse potentiation involves an initial recruitment of CaMKII to the synapse. CaMKII would first associate with NMDARs; this association would have different consequences for binding to NR2A versus NR2B. Ultimately, this would translate into an optimum effect on the potentiation of the synapse by inducing synaptic insertion and increasing single-channel conductance of AMPA receptors, as noted above.

Other important kinases include the Src protein tyrosine kinase (PTK) family, cyclin-dependent kinase-5 (Cdk5), and cyclic AMP-dependent protein kinases (PKAs). PTKs include five members in the CNS—Src, Fyn, Lyn, Lck, and Yes [see (74) for review]. Tyrosine phosphorylation of NR2A by Fyn is promoted by PSD-95, possibly because PSD-95 acts as a physical intermediate to bring Fyn close to NR2A (75). Other PTKs including Src, Yes, and Lyn associate with PSD-95. Indeed, Liao et al. (69) showed that PSD-95 is required for the Src-mediated potentiation of NR1/NR2A receptor current in *Xenopus* oocytes. Also, Fyn and other Src PTKs may be important in mediating the action of ephrins and their receptors, the Eph tyrosine kinases, which are involved in the establishment of axon-dendrite connections during development (76). Cdk5 phosphorylates NR2A at serine 1232 and appears to be necessary for LTP (77). PKAs, which are known to increase the activity of NMDARs, can phosphorylate NR1, NR2A, and NR2B (78) and can induce synaptic targeting of NMDARs (79). PKA can overcome constitutive type 1 protein phosphatase (PP1) activity, resulting in rapid enhancement of NMDAR currents (80). This is due to a selective anchoring of PKA to NMDARs via yotiao, a protein that binds to the C1 exon cassette, found in the C-terminal of some NR1 splice variants.

Compared to kinases and phosphorylation of NMDARs, less is known about the role of phosphatases and dephosphorylation of NMDARs, but overall evidence suggests that phosphatases downregulate the function of NMDARs (80).

Subunit Composition of Synaptic and Extrasynaptic Receptors

Functional and immunocytochemical studies have shown that NMDARs are present at both synaptic and extrasynaptic sites. It is possible that the extrasynaptic population simply represents receptors that have been delivered to the plasma membrane and are awaiting incorporation into the synapse. This idea is supported by recent work that shows that NMDARs can rapidly move between synaptic and extrasynaptic sites, possibly by lateral diffusion of the receptors (81). However, the possibility remains that there is a distinct population of extrasynaptic receptors with a specific function. Extrasynaptic receptors respond differently to excitotoxic drugs (82) and may be activated under physiological conditions (83). There is also evidence of a selective coupling of NMDA channels at extrasynaptic sites to inhibitory currents, which may limit excitation during periods of intense activity (84).

Synaptic and extrasynaptic receptors may also differ in their subunit compositions. The kinetics of NMDA excitatory postsynaptic currents (EPSCs) become faster during development in the CNS and correlate with an increase in expression

of the NR2A subunit (85) and a decrease in the sensitivity to NR2B-selective antagonists (86, 87). At ages when EPSCs are relatively insensitive to NR2B-selective antagonists, however, extrasynaptic receptors still show considerable block by these agents (87–89). In addition, the decay kinetics of the EPSC are significantly faster than the decay kinetics of direct glutamate application to extrasynaptic receptors (87), providing evidence for NR2B expression in the extrasynaptic pool. These results are consistent with synaptic and extrasynaptic receptor pools having different subunit compositions and forming distinct populations of receptors. Although NR2A and NR2B are thought to be the major subunits comprising synaptic receptors, the reduced Mg^{++} sensitivity and slower deactivation kinetics of NMDA-EPSCs at the mature mossy fiber synapse implies that NR2C-containing receptors are present at this synapse (90). A recent study (90b) showed that NR2A- and NR2B-containing receptors are added to the synapse using different mechanisms. Ligand binding to synaptic NMDARs leads to the delivery of NR2A-containing receptors to the synapse, but not of NR2B-containing receptors. Furthermore, NR2A-containing receptors can replace synaptic NR2B-containing receptors, but not vice versa. These results are consistent with the changes in subunit composition seen in synaptic NMDARs during development and begin to reveal the mechanism by which this occurs.

Recent work has indicated that subunit composition at the synapse can regulate synapse maturation through communication of the pre- and postsynaptic neuron by integrins (91). This study found that blocking activity in hippocampal neurons in culture kept the neurons in an immature state with high expression of the NR2B subunit. Expression of the NR2B subunit was correlated with a high release probability that decreased as the neurons matured; NR2B expression and high release probability could be maintained in the culture by blocking integrin signaling. Although this concept of cross-talk between the pre- and postsynaptic neurons via integrins is only beginning to be studied, it appears to be a critical means by which subunit composition at the postsynaptic density can control NMDA-EPSC properties.

Sensory experience can strongly regulate NMDAR subunit expression and incorporation at the synapse. Ocular dominance plasticity is a well-described phenomenon (92), and dark-reared rats have significantly lower expression of the NR2A subunit (93). Visual experience causes a decrease in NR2B-antagonist sensitivity of EPSCs along with a shortening of EPSC duration; deprivation of visual input has the opposite effect (94). Although the deprivation-induced change to NR2B-like characteristics occurred over a time course of days, visual experience could cause increased expression of the NR2A protein and a change to NR2A-like characteristics in synaptic transmission within hours, implying distinct mechanisms for these changes (93, 94). Interestingly, it has been shown that in NR2A knockout mice, the critical period of plasticity at thalamocortical synapses closes even when the NMDA channels retain NR2B-like characteristics (95). Therefore, changes in synaptic NMDAR subunit composition may be influenced by sensory experience, but the role of these changes in controlling plasticity is not fully clear.

Activity-Dependent Changes in NMDA Receptor Subunit Localization

Blockade of NMDAR activity can cause a dramatic increase in expression of NR2 subunits and an increase in surface expression of the NR1 subunit (79, 96, 97). Blockade of synaptic activity or treatment with NMDAR antagonists has been shown to increase NMDAR colocalization with PSD-95 (a marker of excitatory synapses) without an apparent increase in the number of synapses (97). However, those authors have also shown that significant synaptic rearrangement can occur in the absence of protein synthesis, implicating a role for changes in subunit trafficking (79). The data indicate that PKA may control this activity-dependent change in receptor localization downstream of NMDAR activation.

Functional studies have also tested changes in NMDAR properties in response to receptor blockade. Blockade of NMDARs during development in culture leads to a large increase in the frequency of NMDAR-mediated miniature excitatory postsynaptic currents (NMDA-mEPSCs) with no change in amplitude (98). In contrast, blockade of synaptic transmission with tetrodotoxin (TTX) leads to an increase in the amplitude of NMDA-mEPSCs with no change in frequency (98, 99). With changes in synaptic activity, there were parallel changes in AMPA and NMDAR currents, implying that there is activity-dependent scaling of the number of AMPA and NMDARs at the synapse to maintain a constant ratio between these two receptor types (99). Excitatory synaptic innervation is critical for clustering of AMPA receptors, but PSD-95 and NMDARs can cluster in the absence of this input (100). Therefore, these data indicate that both synaptic activity and NMDAR activation are critical regulators of excitatory synapses, but that the initial formation of the postsynaptic density may be independent of this activity.

Synaptic Targeting of NMDA Receptors

Studies with transgenic mice have been used extensively to study NMDAR trafficking [see (101) for review]. Knockout mice lacking NR1 and NR2B die shortly after birth, but in both cases, synaptic morphology appears normal. If embryonic cultures are made from NR2B knockout mice, NMDAR responses are seen that are significantly faster than normal, indicating that the NR2A subunit can be delivered normally to the synapse in the absence of the NR2B subunit (102). Knockout mice lacking NR2A have NMDARs with slow kinetics and show reduced LTP (16, 103), and mice lacking the NR2C subunit show increased amplitude of NMDAR-EPSCs with faster kinetics (104). However, motor discoordination in mice is seen only with loss of both NR2A and NR2C, with knockouts of either individual subunit showing normal motor coordination (103). NR2D knockout mice develop normally, but have deficits in certain locomotor activities and monoamine metabolism (105, 106). The ability of mice lacking different NR2 subunits to form functional synapses implies that subunit trafficking to the synapse can occur effectively even in the absence of the normal complement of NR2 subunits.

Transgenic mice expressing NR2A with a deletion of its C terminus showed an absence of NMDARs from the synapse, with receptor expression limited to extrasynaptic sites (41). Mice expressing truncated NR2B had a perinatally lethal phenotype, but in cultures made from these animals, there was a decrease in localization of NR2B at synapses (38, 107). In both of these cases, because the transgenic mice had a loss of a large region of the NR2 C terminus, the actual region(s) responsible for synaptic delivery cannot be determined. The PDZ-binding domain on the NR2 subunits would be a likely candidate for controlling synaptic delivery because it mediates interaction with several MAGUK proteins that are localized to the synapse (108). Several studies in which these proteins were deleted or overexpressed, however, were inconclusive in establishing their role in the delivery or stabilization of NMDARs at the synapses (109–111). This may be due to compensatory mechanisms for regulating other MAGUKs when one is increased or lost (112).

Recent work has studied the effect of NMDAR subunit overexpression by transfection on NMDAR properties in cultured cerebellar granule cells. Overexpression of the NR1 subunit does not alter the total number of functional channels in neurons, but overexpression of either NR2A or NR2B causes an increase in receptor number, implying that synthesis of NR2 subunits controls the number of functional channels expressed by neurons (113). In contrast, the amplitude of NMDAR-EPSCs was not increased by overexpression of NR2 subunits, indicating that subunit availability is not the major factor in determining the number of synaptic NMDARs. Transfected NR2 subunits are targeted to the synapse because the deactivation kinetics of NMDA-EPSCs were controlled by the NR2 subunit overexpressed. NR2 subunits lacking the *PDZ-binding* domain did not alter synaptic kinetics, however, indicating that the PDZ domain is necessary for entry of receptors into the functional pool at the synapse. These findings would be consistent with the interpretation that the receptor without its PDZ interacting domain cannot be clustered at the synapse. However, these results also raise the possibility that there are two distinct mechanisms for delivery of synaptic and extrasynaptic NMDARs, requiring and not requiring the PDZ-binding domain, respectively. The latter interpretation would support recent work that defines a role for MAGUKs in the early trafficking of NMDAR subunits from the ER/Golgi via association with the exocyst complex through the PDZ-binding domain of the sec8 protein (44).

Subunit overexpression has also been studied in transgenic mice. Overexpression of the NR2B subunit in the forebrain produced the "smart mouse," which showed improved performance on memory tasks and larger NMDA-mediated currents in the hippocampus (17). Interestingly, studies have also shown that this mouse had an increased sensitivity to pain (114), emphasizing the complex systems controlled by NMDA-mediated transmission. Recent studies have shown in this same strain of mice that there is no change in NMDA-EPSC properties in the visual cortex (115). Thus, the ability of NR2 subunit overexpression to modulate synaptic NMDA responses may be dependent on the brain region studied. In contrast, overexpression of the NR2D subunit in mice causes a marked impairment

of LTP generation with NMDA-evoked currents that were slower and had smaller amplitude (116). Therefore, maintenance of the subunit composition may be a critical means of determining NMDA receptor responses at synapses.

Internalization of NMDA Receptors

NMDARs are relatively stable components of the postsynaptic membrane. For example, the basal rate of endocytosis of surface AMPA receptors in cultured cortical neurons is nearly threefold that of NMDARs (20, 117). Thus, it is not surprising that synaptic plasticity is linked more to changes in numbers and distribution of surface AMPA receptors than it is to NMDARs; consequently, studies on glutamate receptor internalization have focused more on AMPA receptors than on NMDARs.

What regulates NMDAR internalization and where does it occur? Mechanisms of NMDAR internalization involve at least three different regions of the receptor molecule, including a PDZ-binding motif and two different tyrosine motifs. First, NMDAR internalization is regulated by its association with PSD-95 and other PDZ proteins. These proteins form the backbone of the NMDAR complex at the postsynaptic membrane. NMDARs that are bound to these proteins are stabilized and are less likely to be internalized, whereas NMDARs that are not bound to these proteins may be internalized readily. This would be a useful mechanism for removing surface NMDARs that are not bound at the synapse. This phenomenon has been demonstrated recently: When a chimera of surface integral membrane protein Tac and the distal C terminus of NR2B is transfected into cultured hippocampal neurons, only 15% of the cells show internalized constructs after a 15-minute incubation at 37°C. In contrast, when the neurons are transfected with modified constructs that lack the last seven amino acid residues [TacNR2BΔ7], two thirds of the cells show distinct internalization. The latter constructs lack the PDZ-binding domain and thus cannot be stabilized by association with the MAGUKs (9). Furthermore, when the same constructs are transfected into HeLa cells with/without cotransfection of PSD-95, the latter protein significantly inhibits internalization of TacNR2B and stabilizes it at the cell surface. The control of internalization of NMDARs via binding to PSD-95 is likely a dynamic event. First, Roche et al. (9) suggest that internalization of the receptor/PSD-95 complex may occur under conditions leading to the dissociation of PSD-95 from other components of the postsynaptic complex. Second, binding of NMDARs to PSD-95 appears to be affected by phosphorylation of the receptor, as discussed in the previous section. Interestingly, the main site of tyrosine phosphorylation of NR2B is Y1472, which is very close to the PDZ-binding domain on the C-terminal (118). Increased phosphorylation of this site, as occurs following tetanic stimulation of Schaffer collaterals in the CA1 region of the hippocampus, might interfere with PSD-95 binding, and perhaps makes the receptor more available for internalization.

Indeed, the latter tyrosine is part of a motif, YEKL, that signals clathrin-mediated endocytosis by binding to AP-2 adaptor complexes. Roche et al. (9)

transfected TacNR2B, TacNR2BΔ7 (which lacks the last 7 amino acids) and TacNR2BΔ11 (lacking the last 11 amino acids) into HeLa cells; the last construct, which lacks YEKL, conferred a 50% inhibition of internalization. The authors also tried a TacNR2BΔ11 with a mutated dileucine motif, which can signal clathrin-mediated endocytosis in some systems, but this mutation did not produce any additional inhibition of internalization. Thus, the remaining 50% internalization must be regulated by some other motif. Internalization of NMDARs also involves a ring of tyrosines on the C termini of NMDAR subunits, just distal to the last transmembrane domain. These include tyrosine 837 of NR1 and 842 of NR2A (10). Dephosphorylation of these tyrosines may allow AP-2 binding, leading to clathrin-mediated endocytosis of the NMDAR. Interestingly, agonist binding to the NMDAR mediates this dephosphorylation independent of ion flux. This suggests that, in this way, an NMDAR could trigger intracellular signal cascades, independent of its function as an ion channel, and thus would behave, in some ways, like metabotropic glutamate receptors (mGluRs). In fact, group 1 mGluRs also can induce NMDAR internalization, perhaps by indirectly affecting the binding of NMDARs to PSD-95 (11).

Identification of the site of glutamate receptor exo- and endocytosis has always been problematic. There is at least circumstantial evidence that glutamate receptors move to and from synapses from either the sides of the spine or from the edge of the active zone (50, 119–120). Passaforo et al. (121) used a thrombin cleavage assay in cultured hippocampal neurons to provide evidence that GluR2/3 AMPA receptors are inserted at the synapse. They also suggest that endocytosis of these receptors would occur at an extrasynaptic site; this method would avoid co-internalization of the latter receptors with synaptic NMDARs and other membrane proteins. Nevertheless, endocytosis of at least some kinds of glutamate receptors directly from the synaptic active zone could be possible. Dynamin 2, which is involved in endocytosis, can specifically interact with Shank proteins and is prevalent in the postsynaptic density (122). In addition, we see some evidence of clathrin, adaptin, and dynamin immunogold labeling at and just below the postsynaptic density, and show some associations there with AMPA and NMDA receptors (123).

CONCLUSION

As is apparent from this review, there is considerable information available on the trafficking of NMDARs in neurons. But most of it is fragmentary and fails to address in detail any one step in the assembly, processing, and synaptic delivery of the receptor. For one of the best-characterized NMDAR interacting proteins, PSD-95, it is still unknown where and when this interaction occurs and what regulates its association with the NMDAR. More than 70 proteins have been shown to interact either directly or indirectly with the NMDAR (62). Our challenge is to determine how each of these proteins, in addition to many others yet to be identified, participates in the trafficking of the NMDAR.

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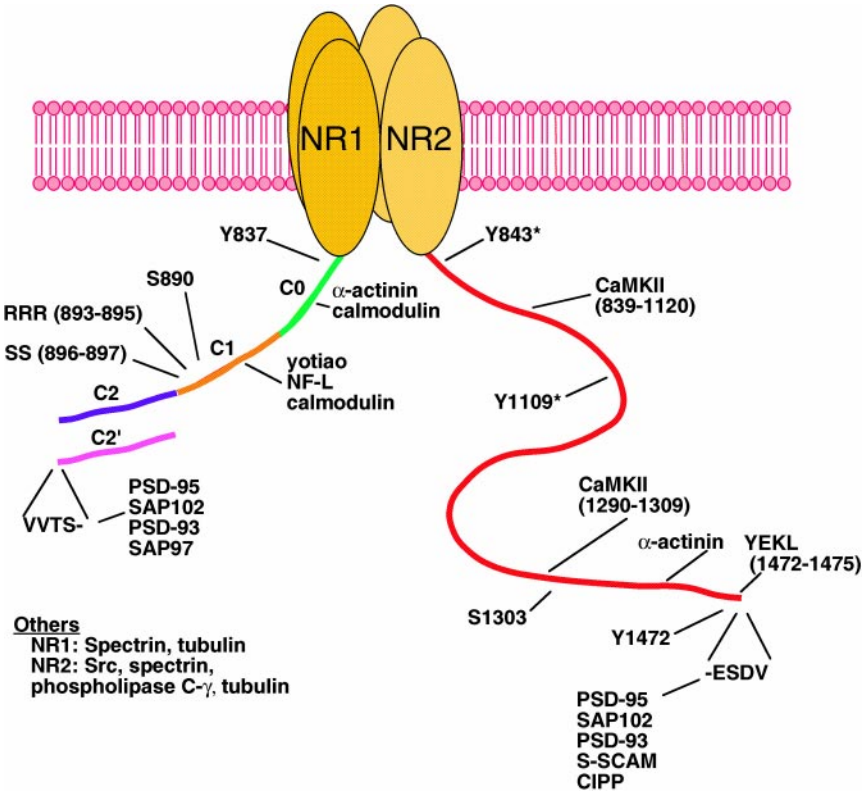

Figure 1 Important motifs and proteins that interact with the C-terminus domains of NR1 and NR2B (residues 839–1482). The splice variant NR1-1 (residues 834–938) is illustrated along with an alternatively spliced cassette, C2′. In NR1, Y837 has been implicated in endocytosis of the receptor (10). S890 and S896 are PKC phosphorylation sites and S897 is a PKA phosphorylation site (124). RRR is an ER retention signal in the C1 cassette of NR1. In NR2, Y843 has been implicated in endocytosis and may be phosphorylated (10). Y1472 is the major phosphorylation site of Fyn (118). Y1109 and Y1281 are phosphorylated by Src (125) and S1303 by CaMKII (126). Some phosphorylation sites were demonstrated on the homologous subunit, NR2A, and corresponding sites (*asterisks*) are present on NR2B. A cdk5 site (S1232) found on the NR2A subunit is not present on NR2B. YEKL is a consensus AP-2 adaptor binding site and plays a role in the endocytosis of the NMDAR (9). Proteins that interact with NR1 include α-actinin (alpha-actinin) (127), calmodulin (128), yotiao (129), neurofilament-L (130), spectrin (131), tubulin (132), and the MAGUKs (PSD-95, SAP102, PSD-93, and SAP97) (25). Proteins that interact with NR2 include CaMKII (133), α-actinin (127), PSD-95 (24), SAP102 (24), PSD-93 (24), S-SCAM (49), CIPP (134), Src (135), spectrin (131), phospholipase C-γ (136) and tubulin (132).

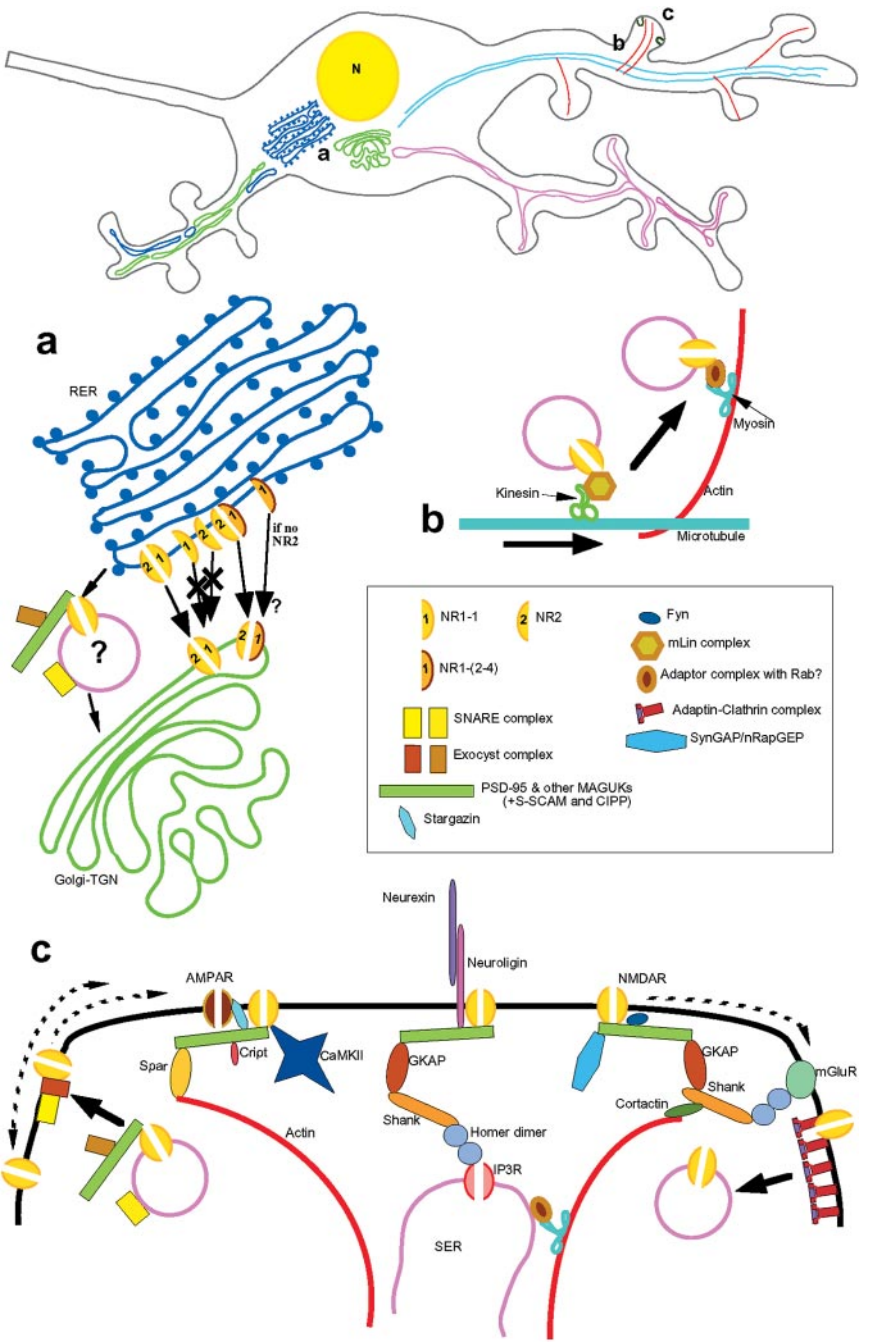

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Figure 2 (See figure on previous page) Diagram representing the major steps in the trafficking of NMDARs. The lower magnification drawing represents a typical neuron with an axon and three dendrites. As is typical of cells, there are major concentrations of RER and Golgi/TGN adjacent to the nucleus (*N*). Dendrites contain cytoskeletal elements (*upper dendrite*) and several systems of tubulovesicular organelles (*lower dendrites*) including ER, Golgi, TGN, and endosomes. For clarity, these elements are divided amongst the three dendrites in the drawing; major areas are indicated and enlarged in drawings (*a*)–(*c*). (*a*) Exit of NMDARs from the ER and subsequent processing and delivery to the cell membrane depends on subunit composition; NR2 subunits must combine (heteromeric complexes) with one or more NR1 variants (NR1-1 to NR1-4) for exit. In addition, NR1-2 to NR1-4 (presumably in homomeric complexes) are expressed on the surface in heterologous cells lacking NR2, but it is not clear whether this occurs in vivo. NMDARs leaving the ER probably traffic with the assistance of SNARE and exocyst complexes. In addition, PDZ-containing proteins, including MAGUKs, may link the NMDAR to components of the exocyst complex during this trafficking. (*b*) Passage of NMDARs along dendrites requires kinesin motors, which are linked to the receptors via an mLin complex. The receptors would travel on the surface of a vesicle or tubulovesicular organelle. Presumably, the receptor switches to myosin motors traveling along actin for passage into the spine and to the spine synapse. The proteins that connect NMDARs to myosin motors are not known but perhaps involve Rab proteins, as described for melanosome trafficking (137). The SNARE and exocyst complex components and MAGUKs, which probably also are associated with the receptors in these stages, have been excluded from the drawing for clarity. (*c*) NMDARs can reach the synapse via exocytosis onto the side of the synaptic spine and perhaps close to or within the active zone itself. At the synapse, NMDARs are linked to many other proteins and are endocytosed, probably from the sides of the spines, typically via adaptin/clathrin complexes. NMDARs move between the synapse, extrasynaptic sites, and sites of exocytosis and endocytosis (*dotted arrows*).

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TELOMERE INHIBITION AND TELOMERE DISRUPTION AS PROCESSES FOR DRUG TARGETING

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■ **Abstract** The components and cofactors of the holoenzyme telomerase and its substrate telomeric DNA are attractive targets for anticancer agents that act by inhibiting the activity of telomerase. This review outlines recent advances in telomerase inhibition that have been achieved using antisense oligonucleotides and ribozymes that target the telomerase mRNA or its hTR RNA template. Although these are potent catalytic inhibitors of telomerase, they are challenging to implement in the clinic due to their delayed effectiveness. Drugs that directly bind to the telomeres, the complex structures that are associated at the telomeric ends, and stabilize secondary DNA structures such as G-quadruplexes are also potent inhibitors of telomerase. Special focus is given here to the telomeres, the biological machinery that works in tandem with telomerase to elongate telomeres, the causes of telomere disruption or dysfunction, and the consequences of disruption/dysfunction on the activity and design of anticancer agents.

INTRODUCTION

Human telomerase is a structurally complex ribonucleoprotein that is responsible for the maintenance of telomeric DNA at the ends of chromosomes. Telomerase acts to synthesize and add a simple six-base motif (of TTAGGG in the human case) to the ends of the chromosomes, resulting in stable telomere length that would otherwise be gradually eroded after each cell replication. Active telomerase has been detected in a majority of human cancer, embryonic, and germline cells but not in normal somatic cells, with the exception of some stem cells, such as those involved in tissue renewal.

The telomerase holoenzyme core consists of a catalytic subunit, the reverse transcriptase protein hTERT (1–3), and an RNA template subunit, hTR (4), which are essential for telomerase activity (5). Other proteins (6, 7) and kinases (8–11) are

important transient components of the holoenzyme and play a role in the activation, stabilization, and regulation of telomerase. Telomerase enzyme processivity is also dependent on the availability of the substrate telomeric DNA, dNTPs, and primer (12, 13).

This review explores the various approaches to and recent advances in the inhibition of telomerase, an enzyme whose activation and on-going activity are generally accepted to be paramount to the survival and proliferation of the majority of cancer cells. The processes and mechanisms of telomerase component assembly, activation, and activity have enabled the development of distinct targets and strategies for the inhibition of telomerase. These targets fall into three major categories that allow for the introduction of various agents to bring about the inhibition of telomerase activity. First, the processes of production and assembly of the elements necessary for telomerase activity yield potential targets, such as the genes (and therefore their messenger RNA) that express for the telomerase components, and the chaperone proteins and kinases that are required in the assembly process. Second, the active telomerase enzyme can be switched off by agents that target its components, hTERT and hTR, or other cofactors necessary for ongoing activity. Finally, agents can be introduced to bind to telomeric DNA, the substrate of telomerase, thus inhibiting telomerase activity by making it unavailable to the enzyme. Accordingly, this review deals with each of these important areas in turn; however, particular emphasis is given to the structure and role of the telomeres and the indicators of telomere disruption or dysfunction with a view to constructively examine the causal relationship among telomeric DNA length, cell senescence, and cell death.

THE TELOMERES

Structure and Function of Telomeres

Telomeres are specialized DNA protein structures that cap the ends of linear chromosomes. Mammalian telomeres consist of tandem repeats of the six nucleotides TTAGGG, which are repeated for 5–25 kilobase pairs in length 5′ to 3′ toward the chromosome end (14–17). Several recent studies have suggested that the structure of the ends of telomeres may be more complex than originally thought. Griffith and colleagues have found that telomeres do not end in a linear manner (18). Instead, the end of the telomere forms a loop structure with the 3′ G-rich strand (referred to as the T-loop), invading the duplex telomeric repeats and forming a displacement loop (D-loop) (Figure 1). Telomere-associated proteins may facilitate the formation and maintenance of both the T- and D-loops, suggesting the presence of a large DNA-protein structure at the end of each chromosome. In addition, most human telomeres appear to terminate in a single-stranded 3′ GT-rich overhang, which is thought to play an important role in telomere structure and function (19–21). These fundamental findings of the structure of telomeres have enormous implications for the design of compounds that can target and disrupt the telomere structure.

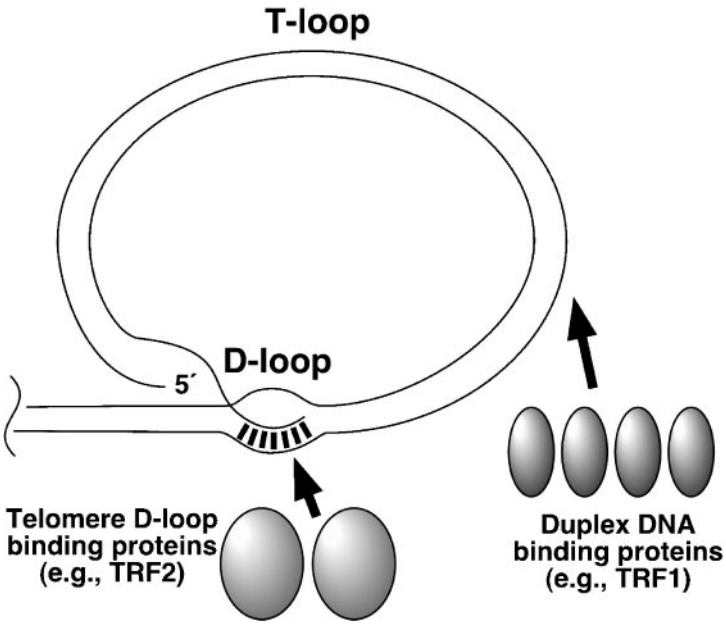


Figure 1 Cartoon showing the T-loop and D-loop and associated telomere binding proteins (18).

Telomeres are highly conserved in organisms ranging from unicellular eukaryotes to mammals, indicating a strong preservation of their protective mechanisms for preventing chromosomal ends from undergoing degradation and ligation with other chromosomes. Without telomeric caps, human chromosomes undergo end-to-end fusions and form dicentric and multicentric chromosomes (22–25). These abnormal chromosomes would break during mitosis, resulting in severe damage to the genome and the activation of DNA damage checkpoints. This leads to cell senescence or the initiation of apoptosis cell death pathways (26).

In addition to protecting chromosomes from end-to-end fusion, telomeres are also thought to protect against the loss of DNA at the end of each chromosome upon the completion of DNA replication. Dividing cells have been shown to undergo a progressive loss of 25–200 DNA base pairs following each cell division (22, 27, 28). This loss of telomeric DNA is largely due to the "end replication problem," which refers to the inability of the DNA replication machinery to copy the final few base pairs of the lagging strand during DNA synthesis (29, 30). Another possible cause for loss of telomeric sequence is by a 5′ to 3′ exonuclease activity that recesses the telomeric CA-rich strand (19, 31). Because the telomere consists of a repetitive DNA sequence, its loss is thought to be less important to the cell than the loss of critical gene encoding sequences that may be near the end of a

chromosome; therefore, telomeric sequences protect from the loss of more critical gene encoding sequences.

Crucial Characteristics Necessary for Senescence at the Telomeres

Telomerase has been thought of as an attractive anticancer drug target because of the compelling correlation between telomerase reactivation and cellular immortalization. The difficulty in designing therapeutic strategies with telomerase inhibitors is that to achieve telomere shortening, one would have to continuously treat the patient for multiple tumor cell population doublings. Most solid tumors have population doubling times of several days to several weeks, suggesting that anti-telomerase therapies could take months to produce any effect in a patient's cancer (32). These difficulties have slowed the development of telomerase inhibitors for the clinic. A key development in the approach to targeting telomeres is the discovery that alterations in telomere function, not the loss of telomere sequence, initiate cell crisis events such as replicative senescence (33). This understanding comes from the results of a study showing that inhibition of the function of telomere binding proteins leads to senescence in the absence of telomere sequence shortening (33), thereby implying that destabilization of the telomere may lead to the same effects that have been associated with critically short telomeres, namely, the induction of cell senescence and cell death.

The idea that telomere capping, not length, determines whether or not a telomere is functional has been proposed (34, 35). Telomere ends are capped by the binding of a number of telomeric proteins, including TRF-1 and TRF-2 (36). These proteins bind in a sequence-specific manner to telomeric DNA and protect chromosome ends from end-to-end fusion (25). The protection of the telomere ends is important to cell survival because loss of normal telomere capping leads to cell death by apoptosis (37). Response to the destabilization of telomeres appears to be mediated through an ataxia-telangiectasia mutated–dependent pathway, suggesting that unprotected telomeres can be recognized as DNA damage (37). Further evidence linking the telomere with DNA damage response pathways is the observation that components of DNA damage response pathways are localized at telomeres and are required for normal telomere maintenance. For example, the Ku protein involved in nonhomologous end-joining is localized to telomeres in budding yeast and human cells. Ku is involved in the localization of telomeres at nuclear pores, and loss of Ku function leads to end-to-end chromosome fusion (38, 39). Taken together, these results suggest that loss or uncapping of telomeric sequences can be sensed as a DNA break. It has been proposed that the DNA damage repair apparatus can allow for cell cycle checkpoint control so that cell cycle progression will not proceed until telomere replication is complete (40). The discovery of this monitoring system of telomere integrity within the cell leads to possible strategies to target telomere integrity for the treatment of cancer.

STRATEGIES FOR THE INHIBITION OF TELOMERASE

Control of the Production and Assembly of the Elements Necessary for Telomerase Activity

The assembly of the active telomerase enzyme complex in cells requires the catalytic hTERT protein and the hTR template RNA, components of the enzyme complex, as well as several accessory proteins, including Hsp90, p23, and TEP-1. Therefore, inhibition of any one of these proteins can lead to a decrease in telomerase activity, making these accessory factors potential molecular targets. Various strategies employing antisense oligonucleotides, ribozymes, or small molecules have been used to target telomerase by regulating the expression and activity of the proteins necessary for its assembly.

The Hsp90 and p23 proteins are molecular chaperones, which probably form a "foldosome" that facilitates and mediates the assembly of a biologically active telomerase complex (41). If the interaction of either Hsp90 or p23 with the catalytic subunit of telomerase is blocked, the assembly of active telomerase is blocked in vitro (41). Inhibition of Hsp90 function in cells with geldanamycin (an Hsp90 inhibitor) also blocks the assembly of active telomerase, suggesting that the targeting of the assembly of telomerase may be a very attractive way of inhibiting telomerase (41).

The hTERT messenger RNA has also been targeted with antisense oligonucleotides that are designed to hybridize with complementary sequences of hTERT mRNA. The recent clinical success of the first antisense drug provides the impetus for further development of these strategies for selective disruption of telomerase expression (42).

In addition to antisense targeting, hammerhead ribozymes have also been used to inhibit the expression of hTERT messenger RNA. These ribozymes are small catalytic RNA molecules that consist of a catalytic core flanked by antisense sequences that function in the recognition of the target sequence. These RNAs possess endoribonuclease activity that allows for the degradation of target transcripts. A hammerhead ribozyme has recently been used to cleave the hTERT mRNA in breast epithelial cells that inhibited telomerase activity and resulted in shortened telomeres, decreased net growth, and apoptosis (43).

Regulation of hTERT expression appears to be largely through transcriptional control (2, 3, 10, 44–46). Several transcription factors have been identified as having a role in the regulation of hTERT gene expression (47). Of note is the observation that c-*MYC* can induce telomerase activity by increasing the transcription of hTERT mRNA (48). There are c-*MYC/MAX* E-box binding sequences within the hTERT gene promoter, and several studies have addressed the importance of c-*MYC* in regulating the expression of telomerase in cancer cells (47, 49). Therefore, inhibition of c-*MYC* expression and interference with c-*MYC* regulation of gene expression are additional mechanisms for preventing telomerase activity (50, 50a). To explore this targeting strategy, we have recently identified compounds that can

down-regulate c-*MYC* expression, leading to repression of telomerase activity. These compounds recognize a specific G-quadruplex structure that forms in the nuclease hypersensitive element within the c-*MYC* promoter. The stabilization of this G-quadruplex leads to inhibition of c-*MYC* expression (50, 50a). Recently, a new DNA-binding small molecule, WP631 (Figure 2), has also been found to inhibit the transcription of c-*MYC* and *p53* genes that induce cell arrest at the G_2 checkpoint in the cell cycle and limited apoptosis in Jurkat T lymphocytes (51). These results are consistent with altered c-*MYC* expression by WP631 being linked to cell pathways leading to growth arrest in Jurkat T lymphocytes (51) that most likely involve inhibition of telomerase activity.

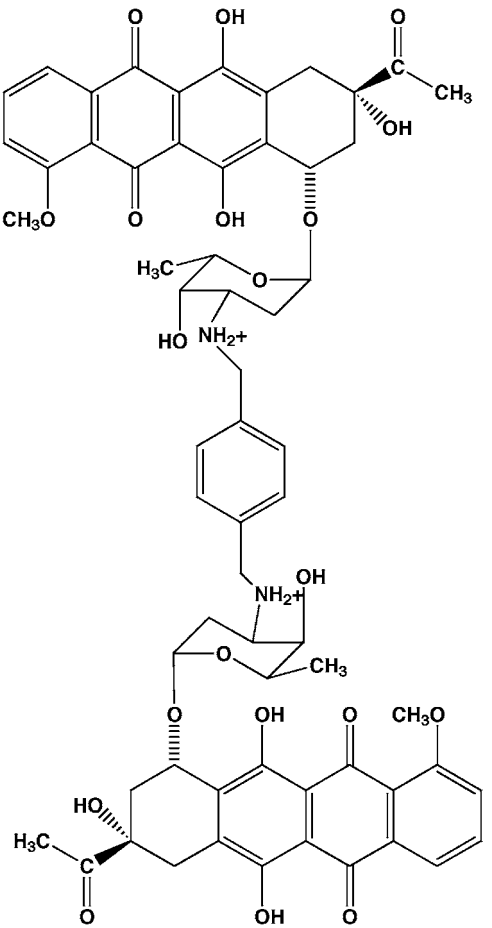


Figure 2 Structure of WP631.

Targeting of the Telomerase Components Necessary For and During Telomerase Activity

The intracellular introduction of synthetic molecules that bind to a specific component (or components) of the holoenzyme telomerase can serve to literally switch off the activity of telomerase once it is already in full-swing (as is the case in most cancer cells). To date, the agents employed to successfully down-regulate or inhibit telomerase activity by directly binding to one of its components have mainly targeted hTERT and hTR, the core subunits of telomerase. These inhibition agents are shown in Figures 3*A* and 3*B*.

Modified short DNA and RNA molecules with novel bond linkages between the bases have been designed with the aim to use the antisense approach by binding to the RNA template in the hTR subunit(s) to prevent or halt transcription and thereby act as competitive inhibitors of telomerase activity. Hence, the hTR RNA template is unavailable to hTERT for reverse transcription (52). The various types of sugar phosphodiester backbone modifications in these molecules are intended to confer certain desirable characteristics or properties, such as intracellular penetration, superior binding affinity, and therefore specificity, to the hTR RNA template and in order to enable intact delivery to their target.

In recent years, many researchers have extensively tested the efficacy of telomerase inhibition by peptide nucleic acids (PNAs) that contain *N*-(2-aminoethyl) glycine linkages between bases (52, 53), DNA-oligomers with phosphorothioate (PS) linkages (52), DNA-oligomers with phosphoramidite (PN) linkages (54, 55), RNA oligomers with methyl-substituted (56, 57) or methoxyethyl-substituted (58) ribose sugar rings (2-OMe RNA and 2-MOE RNA, respectively), locked nucleic acid RNA oligomers that have constraining ribose ring methylene bridges (54), various substituted RNA oligomers with PS linkages (56–58), and hybrid RNA–DNA molecules (termed as chimera molecules) consisting of a variety of combinations of the aforementioned oligomer types (54, 56–58). These agents are stable against intracellular degradation, such as nuclease digestion (54), and effect telomerase inhibition at the pico- to micromolar levels, depending on the cell line assayed (58, 59). Repeated transfection of cells with cationic lipids is required for delivery to cells and to maintain their effectiveness against telomerase activity (60). The impact of sustained treatment on telomere length, antiproliferation of cells, and even apoptosis (due to inhibition of telomerase activity) are all critically dependent on the initial telomere length and cell type (59). For example, in some cases treatment with agents such as substituted RNA oligomers prevented spontaneous immortalization of epithelial cells, even though cell apoptosis was not achieved (57).

The best of these agents are PNAs, which were shown to target specific regions in the hTR RNA template, with activities at pico- to nanomolar concentrations, an inhibition level 10 to 50 times more efficient than for PS-DNA oligomers (52). However, PNAs were found to have poor pharmacokinetic properties and higher toxicities relative to PS-containing oligomers (59). Also, PN-DNA oligomer derivatives containing complementary sequences to those at specific sites in the

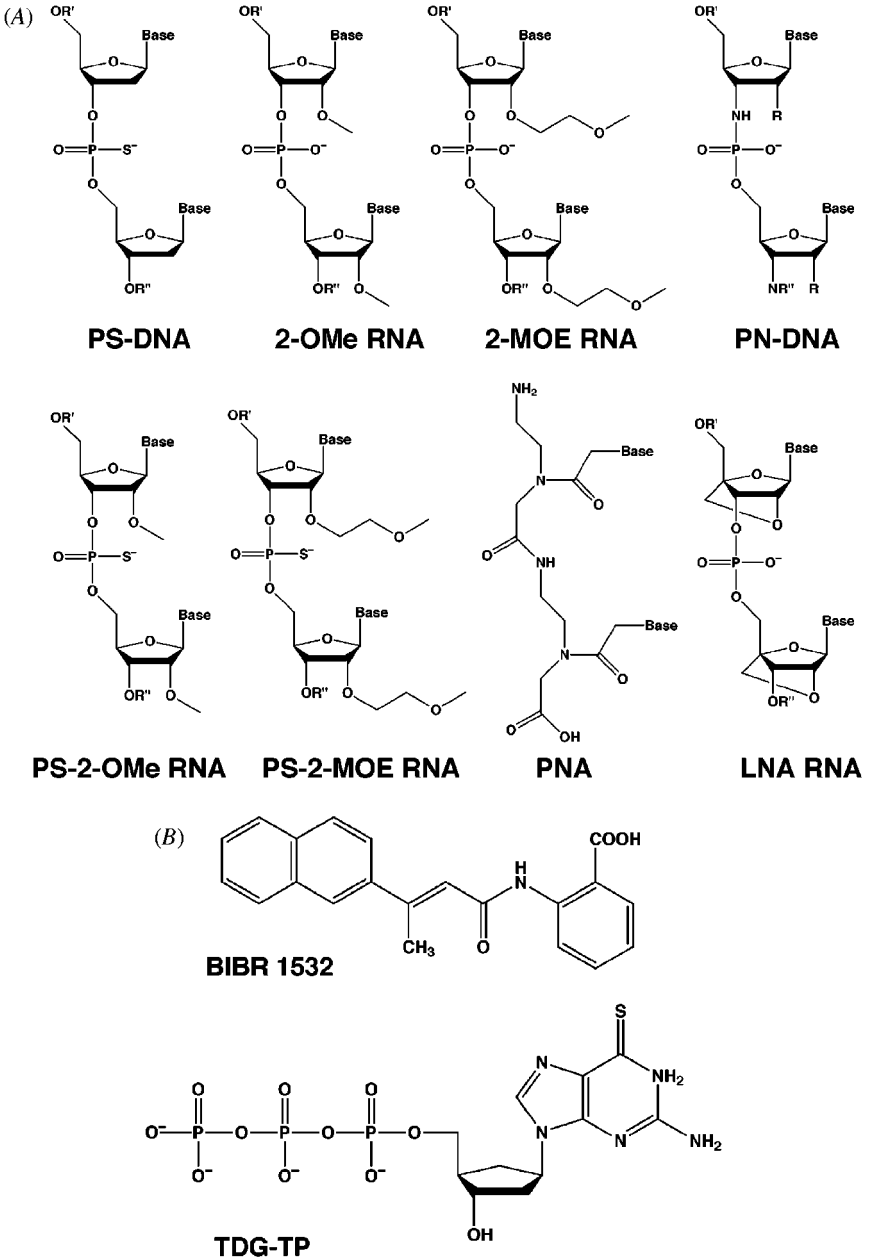


Figure 3 (*A*) The different types of antisense oligonucleotides that inhibit telomerase by targeting the hTR RNA template. (*B*) Two of the inhibitors of telomerase that target hTERT.

target hTR RNA template were efficient against telomerase activity at pico- to nanomolar concentrations (55). Interestingly, the PS-DNA oligomers have an enhanced nonspecific type of binding to proteins in general (59) and probably inhibit telomerase activity by interacting with the hTERT protein subunit of telomerase rather than with the hTR RNA template (59, 61). The 2-OMe and 2-MOE RNA oligomers and their DNA hybrids possess increased binding affinity at the target hTR RNA template and inhibit telomerase activity at the nanomolar level (56–58). The addition of PS linkages to these substituted RNA oligomers and hybrids confers better pharmacokinetic properties to these agents while still maintaining similar telomerase inhibition levels (56–58), giving them promising clinical properties (59).

Another promising approach employs a chimera molecule constituting an antisense DNA oligomer with an attached 2′,5′-oligoadenylate (2-5A) to induce massive apoptosis of ovarian cancer cells after sustained treatment over one to two weeks, but with no effect on normal ovarian cells (62). It is postulated that this chimera's antisense DNA oligomer enables direct binding to the hTR RNA template, while the 2′,5′-oligoadenylate recruits and activates an endoribonuclease (RNase L), which then cleaves the proximal hTR RNA template, thus inhibiting telomerase activity (62). Similarly, in several other studies the 2-5A antisense chimeras were used against prostate and bladder cancers and malignant gliomas in vitro and in vivo in nude mice with encouraging results (63–65).

Hammerhead ribozymes that cleave the hTR RNA template have been shown to inhibit telomerase activity in human (66) and endometrial carcinomas (67, 68) and in human melanoma cell extracts (69). Also, telomerase activity was downregulated in vitro in endometrial carcinoma and human melanoma cells (68, 69); however, no telomere shortening was observed in the human melanoma cells (69), and this approach to inhibition of telomerase activity has not been further investigated.

Telomerase activity can also be inhibited by the direct binding of small nonnucleosidic synthetic compounds to the hTERT reverse transcriptase component of telomerase. Schnapp and coworkers have recently reported the first mixed-type noncompetitive (70) catalytic telomerase inhibitor, (2-((E)-3-naphtalen-2-yl-but-2-enoylamino)-benzoic acid) (BIBR1532), which causes telomere shortening and senescence characteristics in various types of cancer cells in vitro and in vivo in mouse xenograft models at nanomolar concentrations (71).

Last, the hTERT reverse transcriptase inhibitors do not effect their activity by specifically and persistently binding to hTERT; rather, they act as competitors for the substrate deoxyribonucleotides used by reverse transcriptases, such as hTERT, to construct DNA chains (or more specifically for hTERT, to construct telomeric DNA extensions). Small nucleoside analogues can act as reverse transcriptase inhibitors, although only some of these compounds, such as 6-thio-2′-deoxyguanosine 5′-triphosphate (TDG-TP), are selective against the hTERT reverse transcriptase (72). TDG-TP is effective at low micromolar concentrations (72) and stops telomeric DNA extension after incorporation into the DNA (73).

Targeting of Telomeres

Inhibition of telomerase can be achieved by sequestration of the primer (the single-stranded telomeric end) required for the reverse transcriptase activity of this enzyme. This was first demonstrated by showing that K^+ inhibited telomere activity, presumably by facilitation of folding of the single-stranded telomeric DNA into a G-quadruplex structure (74). G-quadruplexes are composed of two or more G-tetrads (Figure 4*A*) assembled into either intermolecular (Figures 4*B,C*) or intramolecular (Figure 4*D*) structures. Human telomeric DNA can form an intramolecular G-quadruplex structure, characterized as a basket, having three G-tetrads, each stabilized by Hoogsteen base pairing (Figure 4*A*) (75). Based on the observations that K^+ facilitates and stabilizes G-quadruplex structures, small molecules that mimic the K^+ effect were also found to inhibit telomerase activity

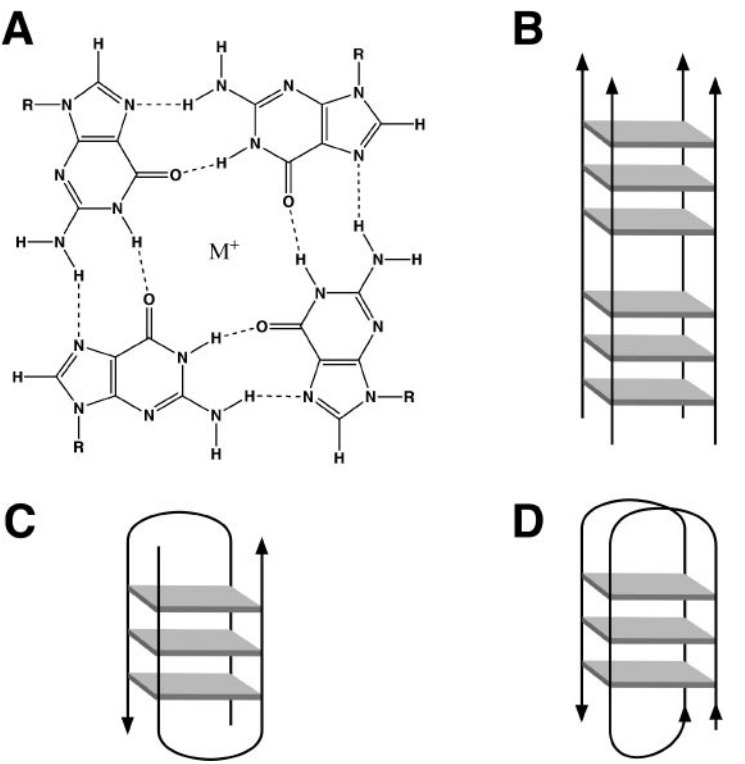


Figure 4 G-tetrad and G-quadruplexes. (*A*) Four guanine residues forming a planar structure G-tetrad through Hoogsteen hydrogen bonding. (*B*) A parallel G-quadruplex model. (*C*) An intermolecular antiparallel G-quadruplex model. (*D*) An intramolecular basket G-quadruplex model. Each parallelogram in (*B*), (*C*), and (*D*) represents a G-tetrad.

(76). The first proof of this principle was that telomerase inhibition did not take place until sufficient telomeric repeats were assembled by telomerase extension of the DNA primer to form a G-quadruplex structure (76). Consequently, the direct telomerase assay (77) (Figure 5), alongside the polymerase stop assay (78) (Figure 6), provides important cell-free signatures of G-quadruplex-interactive compounds. In addition to cell-free assays, in vitro and in vivo assays have also been used to characterize the effects of G-quadruplex-interactive compounds. In in vitro systems, inhibition of telomerase (79), telomere shortening (80), cell senescence, and delayed growth inhibition (80) have been demonstrated with G-quadruplex-interactive compounds. Unlike telomerase inhibitors, G-quadruplex-interactive compounds would be expected to affect cells that maintain telomeres by telomerase-dependent as well as telomerase-independent mechanisms because the latter involve recombination mechanisms quite possibly involving G-quadruplex structures (81). The formation of G-quadruplex structures in regions other than telomeres, for example, in the promoter region of c-myc, may also lead to effects on telomerase because c-myc controls hTERT. Indeed, the G-quadruplex-interactive compound TMPyP4, but not its isomer TMPyP2, which does not interact with G-quadruplex, is able to down-regulate c-myc and hTERT (50, 50a).

As described before, an altered telomere state may be a more important consequence than critical telomere shortening (33). Thus, disruption of telesomes by either depletion of proteins involved in telomere binding (e.g., telomerase) or sequestration of telomere ends by stabilization of G-quadruplex structures, or both, may lead to chromosome end-to-end fusion in presenescence cells. In fact, TMPyP4 and the fluoroquinophenoxazines have both been demonstrated to produce anaphase bridges, a hallmark of chromosome end fusions, in relatively short periods of time (78).

It has been frequently pointed out that G-quadruplex-interactive compounds lack the cancer cell selectivity imparted by the fairly selective occurrence of telomerase in cancer cells. However, uncapping and recapping of telomerase-dependent cells may provide some opportunities for selectivity of G-quadruplex-interactive compounds. In cells switching between these two states, this most likely occurs in S or G2/M phases in the cell cycle. In cancer cells, where it is necessary to add telomeric sequences via a telomerase-dependent or telomerase-independent mechanism, the single-stranded DNA template becomes exposed in the uncapped state, and if either telomerase is deficient or G-quadruplex-interactive compounds are present, which facilitate folding of the G-quadruplex (e.g., TMPyP4, telomestatin), irreversible uncapped telomeric ends and chromosome end-to-end fusion may result (78). In contrast, normal cells that do not need to elongate their ends may remain stable through multiple cell divisions (35).

The natural occurrence of G-quadruplex in human telomeric sequences remains unproven, although a recent report provides convincing evidence of their occurrence in *Stylonychia lemnae* telomeres (80). Nevertheless, the existence of

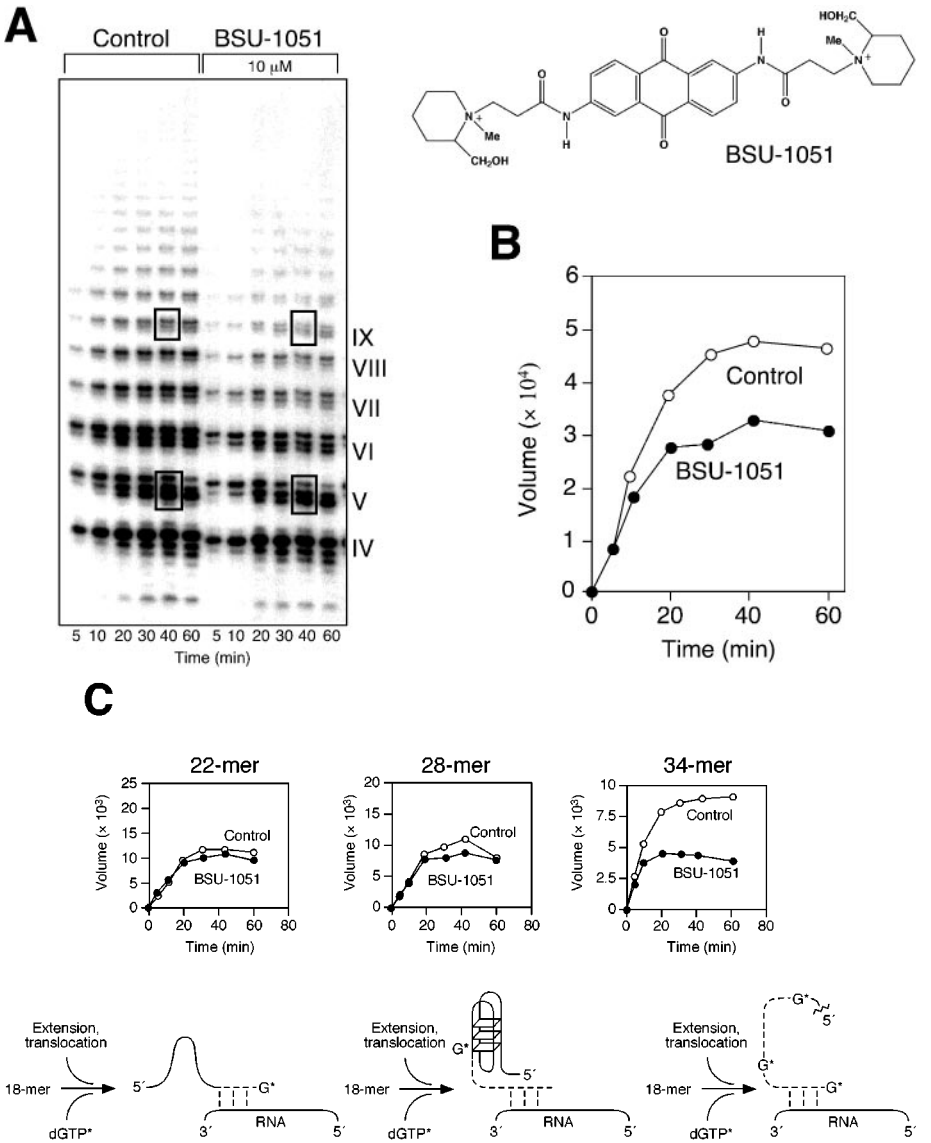


Figure 5 Effect of BSU-1051 on the time-course of telomerase activity using (*A*) the 18-mer telomeric primer d[TTAGGG]$_3$ (1 μM) without (*left-hand lanes*) or with (*right-hand lanes*) BSU-1051 added at 10 μM (76). The boxes identify the 40-min samples, which show altered multiple band patterns due to 3′-exonuclease activity. (*B*) Time-course of total amount of [α-^{32}P]-dGTP incorporated into the extension products of the d[TTAGGG]$_3$ primer in the presence and absence of BSU-1051. (*C*) Time-course incorporation of [α-^{32}P]-dGTP into the 22-mer, 28-mer, and 34-mer and comparison of patterns of sets of multimers for the 22-mer and 28-mer in the presence and absence of BSU-1051. The diagrams between the two sets of results show the proposed structures of the species formed at each step.

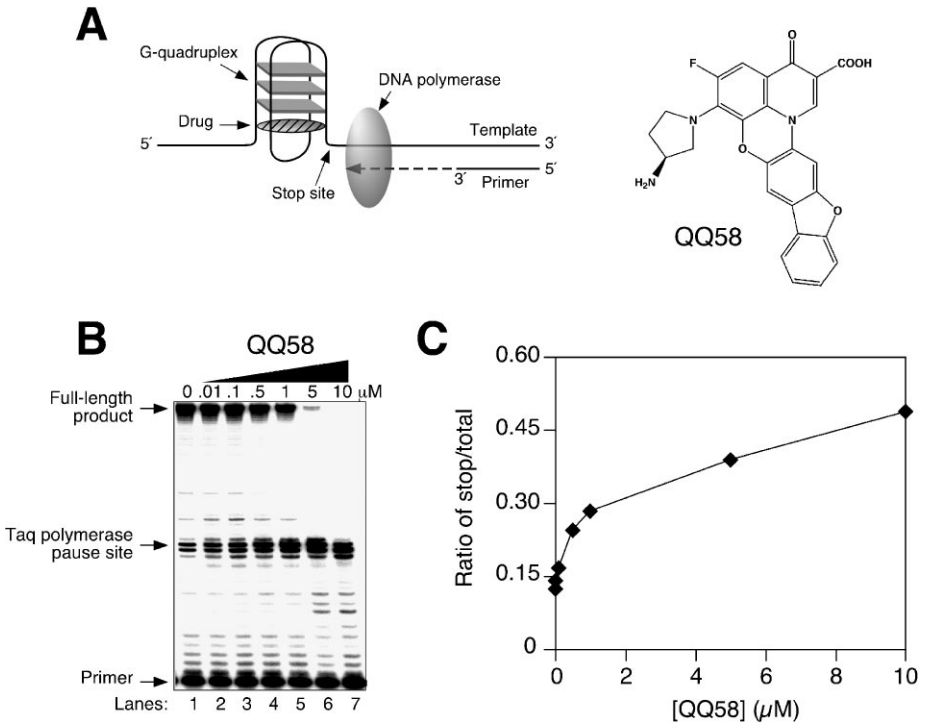


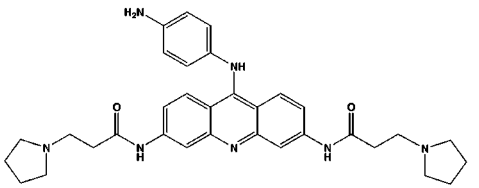
Figure 6 Inhibition of Taq polymerase with increasing concentrations of the fluoroquinophenoxazine QQ58. (*A*) Cartoon of the assay and structure of QQ58. (*B*) Autoradiogram of the sequencing gel showing enhanced DNA synthesis pausing at the G-quadruplex site with increasing concentrations of QQ58 (*lanes 1–7*). The free primer, the pause site, and the full-length product are indicated. (*C*) Graphical representation of the quantification of the sequencing gel shown in (*B*), showing the concentration of QQ58 to the ratio of intensity of the bands obtained for the pausing site/total intensity per lane.

helicases such as Sgs1 and Cdc13p in yeast and Werner's and Bloom's helicases in human cells, which resolve G-quadruplex structures, suggests that such structures may exist at least transiently (82–86). Whether or not these structures form naturally, it is likely that their formation and subsequent resolution need to be controlled so that free single-stranded telomeric primer can be available for telomerase reverse transcriptase. Therefore, if G-quadruplex-interactive compounds interfere with the dynamics of interconversion between G-quadruplex or single-stranded telomeric sequences, they will likely have effects on telomeric states of DNA. It was first demonstrated with a perylene (PIPER) that G-quadruplex-interactive molecules could facilitate the formation and also inhibit the Sgs1 helicase unwinding of G-quadruplex structures (87, 88). Subsequently, a number of other compounds, including the cationic porphyrins, telomestatin, and 9-anilino-proflavins, have been

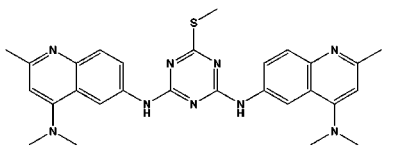
shown to facilitate or inhibit G-quadruplex conversion to single-stranded DNA (89–91).

The first G-quadruplex-interactive compounds were relatively nonpotent (IC_{50} in the micromolar range) (92) and nonselective for G-quadruplex versus other forms of DNA. More recently, more potent and selective molecules have been designed or discovered (78, 80, 90, 93–95). Of particular interest are 9-anilino proflavine, the triazines, the fluoroquinophenoxazines, telomestatin, and the pentacyclic acridines (Figure 7).

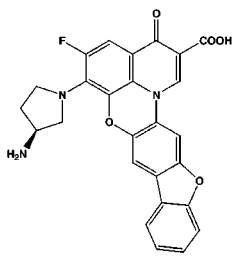
The 9-anilino proflavine derivative was designed to optimize the interaction with the intramolecular G-quadruplex from human telomere and minimize that with duplex DNA. These compounds have 60 to 100 nM potency in a modified TRAP assay and corresponding low cytotoxicity (93). The triazines have been demonstrated to produce telomere shortening, which is associated with delayed growth arrest and cell senescence (80). The fluoroquinophenoxazines are redesigned topoisomerase II poisons that now interact more specifically with G-quadruplex structures, and this activity is correlated with production of anaphase bridges (78), a property also shared by the cationic porphyrin TMPyP4 (96) and



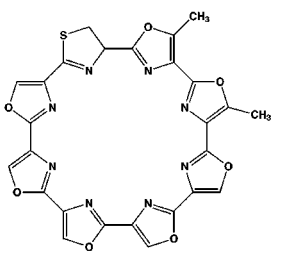
9-anilino proflavine



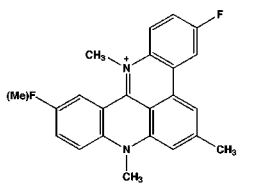
triazine (115405)



fluoroquinophenoxazine (QQ58)



telomestatin



pentacyclic acridine (RHPS4)

Figure 7 Chemical formulae of some of the inhibitors of telomerase that target its substrate (telomeres).

the triazines (80), both of which are also G-quadruplex-interactive compounds. Telomestatin, a natural product, is the most potent of the telomerase inhibitors (97) and also a G-quadruplex-interactive compound (90). This compound shows remarkable specificity for intramolecular versus intermolecular G-quadruplex structures. The pentacyclic acridine RHPS4, like the other G-quadruplex-interactive compounds reported above, produces cellular effects within a two- to three-week period at noncytotoxic concentrations. A number of reviews on drug targeting of G-quadruplex have appeared (60, 98–100).

In addition to the exciting developments described previously, leading to more potent and G-quadruplex-specific agents, underlying concepts for how G-quadruplex-interactive compounds may affect biological processes have been advanced. It is likely that, in most cases, the occurrence of G-quadruplex in genomic DNA is undesirable unless the facile conversion between duplex or single-stranded DNA and G-quadruplex DNA can be achieved (101). In the latter case, their involvement as switch signals in transcriptional control might be a useful and even primordial mechanism for regulation of gene expression. Therefore, agents such as the perylene and cationic porphyrins, which both facilitate the formation of G-quadruplex (91) and inhibit their resolution by helicases such as Sgs1 (87, 91), are likely to affect the natural equilibrium between duplex and single-stranded DNA and G-quadruplex structures, and thus have effects on biological processes mediated either by duplex or single-stranded DNA or by G-quadruplex DNA forms (101).

Last, Brad Chaires has developed an extremely useful method to determine the DNA structural selectivity for binding of ligands to different forms of DNA (102). Thus it is possible to determine if there is a correlation between binding affinities to G-quadruplex and biological activities for a range of analogues. However, for this to be predictive, the biologically relevant G-quadruplex must first be known.

CONCLUSIONS

Telomerase has alternately waxed and waned as a potential cancer-specific target. The first expectations following the discovery of telomerase were overly optimistic in accordance with the lack of appreciation of the processes that maintain telomere length. It is more than a decade since these halcyon days, but telomerase is now enjoying a revival as a more validated molecular target. The concept of altered telomere states that prompt chromosomal end-to-end fusion in presenescence cells, thus negating the need for critical telomere shortening, will affect the way we think about treating cancer cells with telomerase inhibitors and telomere-interactive compounds. For us to take full advantage of this new concept, we need more complete information on the uncapping and recapping of telomeres. Compounds such as the G-quadruplex-interactive drugs, which interfere with telomere structures and presumably produce altered telomere states, assume new

significance under this new insight. Moreover, compounds that lower c-myc and hTERT and interfere with telomere integrity assume even more significance. From the discovery of small organic compounds to larger synthetically modified oligonucleotides that target defined macromolecular structures, the field has moved to measuring effects on telomerase, telomeres, and associated biological processes. Methodologically driven, the telomere/telomerase research area is moving toward clinical trials of the best chemical agents. One hopes that the first clinical trials will be conducted in a manner such that if the results are negative, there will be a sufficient level of science so that a poor study design will not lead to the premature demise of this exciting area.

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PHARMACOLOGY AND PHYSIOLOGY OF HUMAN ADRENERGIC RECEPTOR POLYMORPHISMS

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■ **Abstract** Adrenergic receptors are expressed on virtually every cell type in the body and are the receptors for epinephrine and norepinephrine within the sympathetic nervous system. They serve critical roles in maintaining homeostasis in normal physiologic settings as well as pathologic states. These receptors are also targets for therapeutically administered agonists and antagonists. Recent studies have shown that at least seven adrenergic receptor subtypes display variation in amino acid sequence in the human population due to common genetic polymorphisms. Variations in potential regulatory domains in noncoding sequence are also present. Here, we review the consequences of these polymorphisms in terms of signaling, human physiology and disease, and response to therapy.

INTRODUCTION

Adrenergic receptors are the targets for epinephrine and norepinephrine and thus represent a critical component in the sympathetic nervous system for maintenance of homeostasis and response to disease. Adrenergic receptors are members of the superfamily of cell surface receptors that carry out signaling via coupling to guanine nucleotide binding proteins (G-proteins). G-protein coupled receptors (GPCRs), including adrenergic receptors, are targets for many therapeutic agonists and antagonists in current use. The family of human adrenergic receptors consists of nine subtypes: α_{1A}-, α_{1B}-, α_{1D}AR; α_{2A}-, α_{2B}-, α_{2C}AR; and β_1-, β_2-, and β_3AR. Although several of these receptors can couple to more than one G-protein, the classic coupling pathways for α_1AR, α_2AR, and βAR are via G_q (stimulation of phopholipase C), G_i (inhibition of adenylyl cyclase), and G_s (stimulation of adenylyl cyclase), respectively. As such, assays measuring inositol phosphate stimulation, inhibition or stimulation of adenylyl cyclase activities, or cAMP levels are often utilized to ascertain receptor function. However, other signals may be physiologically relevant in various cell types. For example, the α_2AR also couples to stimulation of adenylyl cyclase, inhibition of inwardly rectifying potassium channels, stimulation of voltage gated calcium channels, increases in intracellular

calcium release, and stimulation of mitogen activated protein (MAP) kinase. The signaling pathways of adrenergic receptors and their organ and cell-type distribution have been extensively described (1, 2).

For many years it has been recognized that physiologic responses, expression, and function of adrenergic receptors, as well as the response to adrenergic receptor agonists and antagonists, display marked interindividual variation within the human population. Although environmental factors and the heterogeneity of diseases, such as asthma and hypertension, for which these drugs are utilized are undoubtedly important, recent efforts have been under way to discern the impact of genetic variations of the receptor genes as a basis for some of this interindividual variation.

A variability in DNA sequence that occurs with an allele frequency of $>1\%$ in the population is termed a polymorphism. This is in contrast to mutations, which are rare variants and may be the single basis of an inherited disease (i.e., cystic fibrosis, nephrogenic diabetes insipidus). In such cases, these mutations are necessary and sufficient for the disease. On the other hand, polymorphisms may have no effect, have effects that are clinically silent but can be delineated with physiologic testing, have an increased prevalence in certain diseases and thus act as low-level risk factors, can act to modify diseases, or can alter the response to therapy. Whereas nucleotide deletions and insertions are observed in the human genome, by far the most common polymorphisms are single nucleotide substitutions (single nucleotide polymorphisms, frequently abbreviated as SNPs). Within coding regions of genes, polymorphisms may encode different amino acids (denoted as nonsynonymous polymorphisms) or because of redundancy in the genetic code, may have no effect on the encoded amino acid (synonymous polymorphisms). Polymorphisms occur in 5′ UTR, promoter, 3′ UTR, and introns as well, and in general, these are more common than coding polymorphisms.

Seven of the nine adrenergic genes have been found to have nonsynonymous or noncoding polymorphisms (Figure 1). In this paper, the genetics, signaling consequences, and clinical relevance (when known) of these polymorphisms are reviewed. Particular attention is devoted to the effects of receptor polymorphisms on cellular signaling, which provides a molecular mechanism for the physiologic and pharmacologic phenotypes observed in the limited number of human studies and provides a basis for additional clinical studies. The issues regarding initial discovery and subsequent rapid detection of adrenergic receptor polymorphisms (3), "in silico" methods (4), sample size considerations (4), and transfected cell-based characterization methods (5) are described elsewhere.

β_1-ADRENERGIC RECEPTOR POLYMORPHISMS

Localization and Characterization

There are two nonsynonymous coding polymorphisms of the β_1AR found in the human population (Figure 1, Table 1). At nucleotide 145, variation is present that results in either Ser or Gly at amino acid position 49. This position is within

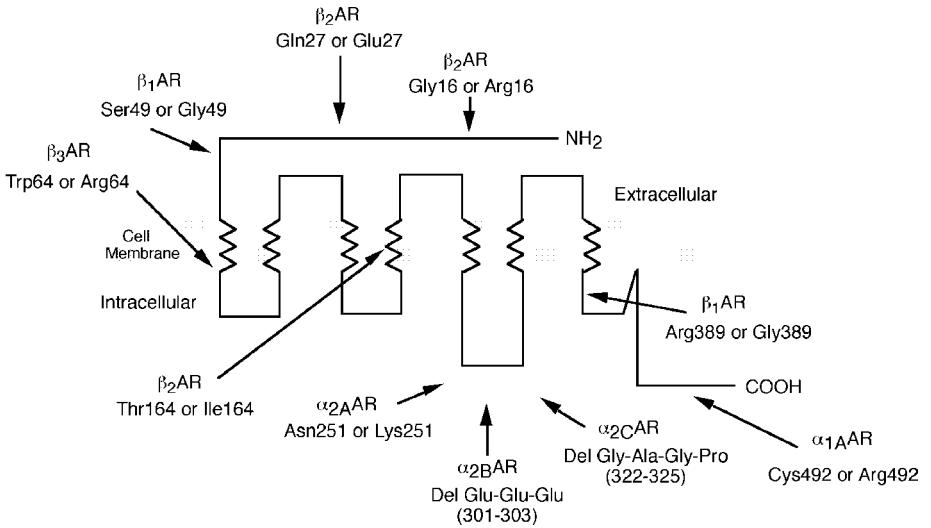

Figure 1 Localization of nonsynonymous polymorphisms of human adrenergic receptors. Shown is a prototypic adrenergic receptor and the approximate location of amino acid substitutions or deletions.

the extracellular amino terminus, ~9 amino acids from the cell membrane. The minor allele is Gly, which has a frequency of ~15% (Table 1). We have found no differences in the frequency of this β_1AR polymorphism between Caucasians and African Americans (6). Polymorphic variation of the β_1AR also occurs at nucleotide 1165, resulting in either Arg or Gly being encoded at amino acid 389. This locus of variability is in the proximal portion of the carboxy terminus. This region, between the seventh transmembrane spanning domain and the palmitoylated cysteine(s), is predicted to be an α-helix. The first human β_1AR to be cloned actually represents the minor allele (Gly), and has been referred to as the wild-type receptor. Indeed, virtually all recombinant structure/function studies have been performed with the Gly389 β_1AR. The allele frequency of Gly389 differs between Caucasians and African Americans, with the latter ethnic group having a higher frequency (~42% versus ~27%, Table 1). In a preliminary study, several very uncommon variations have been reported by one group (7) at amino acids 399, 402, 404, and 418. Apparently, these have been detected as heterozygotes in one or two subjects in their cohorts. Verification and pharmacologic studies have not been reported to date.

The localization of the common amino acid substitutions at positions 49 and 389 (Figure 1) suggested potential phenotypes of the polymorphic receptors. For the position 49 variation, it seemed unlikely that agonist binding or G-protein coupling would be affected. However, expression or trafficking of GPCRs can be affected by mutations imposed in the amino terminus. In this regard, we were hampered by the

TABLE 1 Adrenergic receptor polymorphisms

Receptor	Position		Alleles		Minor allele frequency (%)	
	Nucleotide	Amino acid	Major	Minor	Caucasians	African Americans
α_{1A}AR	1441	492	Cys[a]	Arg	46	70
α_{2A}AR	753	251	Asn	Lys	0.4	5
α_{2B}AR	901–909	301–303	No deletion	Delete Glu-Glu-Glu	31	12
α_{2C}AR	964–975	322–325	No deletion	Delete Gly-Ala-Gly-Pro	4	38
β_1AR						
	145	49	Ser	Gly	15	13
	1165	389	Arg	Gly	27	42
β_2AR						
	46	16	Gly	Arg	39	50
	79	27	Gln	Glu	43	27
	491	164	Thr	Ile	2–5	2–5
β_3AR	190	64	Trp	Arg	10	?

[a]In African-Americans, Arg is the major allele.

well-recognized observation in recombinantly expressed cells, that β_1ARs display very little agonist-promoted internalization or agonist-promoted down-regulation via protein degradation (8). On the other hand, in many endogenous expressing cells, such as the cardiomyocyte, agonist-promoted down-regulation of the β_1AR clearly occurs, which is thought to be due primarily to decreases in mRNA. Taken together, it is clear that the recombinant approach, with cells transiently or stably expressing the β_1AR, represents only a model system. Nevertheless, because the cDNAs used for transfections are in identical expression vectors and differ only at nucleotide 145, the system can be useful to differentiate potential phenotypes between the Ser49 and Gly49 receptors.

HEK293 cells stably expressing the two β_1ARs have been utilized for the majority of studies with the position 49 polymorphism (9). Agonist and antagonist-binding affinities, as well as coupling to adenylyl cyclase, were not different between Ser49 and Gly49 β_1AR. Agonist-promoted internalization was minimal ($\sim$28% after 30 min of exposure to 10 μM isoproterenol) and not different between the two receptors. However, with 24 h of exposure to isoproterenol, a clear difference in receptor down-regulation was observed (9). The Ser49 receptor showed no down-regulation; indeed, receptor expression actually increased with this receptor (Figure 2*A*). In contrast, the Gly49 receptor underwent a 24 $\pm$ 3% decrease in expression in paired studies. These findings were confirmed in multiple cell lines and were independent of the initial levels of receptor expression. The basal

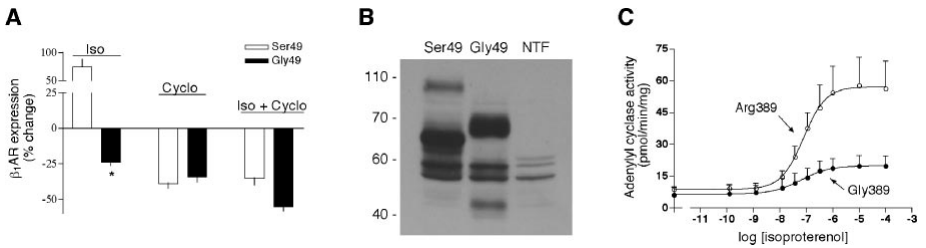

Figure 2 Characteristics of β_1AR polymorphisms in transfected cells. (*A*) Agonist-promoted down-regulation phenotypes of the Ser49 and Gly49 β_1ARs. (*B*) Western analysis of the Ser49 and Gly49 β_1ARs. (*C*) Adenylyl cyclase activities of the Arg389 and Gly389 β_1ARs. ISO = isoproterenol, Cyclo = cycloheximide.

(non-agonist promoted) turnover rates were assessed by treating cells with the protein synthesis inhibitor cycloheximide. Under these conditions, both receptors displayed a similar decrease in expression after 18 h of exposure, consistent with basal degradation rates being equivalent. To approximate the scenario in myocytes, where receptor synthesis is markedly reduced due to a decrease in transcripts, cells were treated with both cycloheximide and isoproterenol. Here again, the Gly49 receptor displayed greater down-regulation than Ser49 (55 $\pm$ 3% versus 36 $\pm$ 5%, Figure 2*A*).

Western blots have shown differences in the immunoreactive bands between the two receptors (Figure 2*B*) (9). Additional studies have indicated that a high molecular weight band ($\sim$105 kDa) was observed only with the Ser49 receptor and represents a high degree of glycosylation (rather than nonglycosylated receptor dimers). The major glycosylated forms also differed between the Ser49 ($\sim$63 kDa) and Gly49 ($\sim$69 kDa) receptors. Both were sensitive to tunicamycin and N-glycosidases. Although the loss of Ser in the amino terminus might represent a loss of an O-glycosylation site, this was not the case, as the immunoreactive bands were not affected by O-glycosidase. Of note, the site for N-linked glycosylation of the β_1AR is amino acid position 15. Thus, it is not clear how a polymorphism at position 49 can sufficiently alter the conformation of the amino terminus so as to alter glycosylation at Asn15. Although there are limitations to this model system, the data indicate that the Gly49 receptor displays enhanced agonist-promoted down-regulation compared to the Ser49 receptor. This effect appears to occur after the internalization process and may be related to altered intracellular trafficking to degradation pathways, perhaps due to differences in receptor glycosylation status.

The Arg389 and Gly389 receptors were stably expressed in Chinese hamster fibroblasts (CHW cells) (10). In the presence of guanine nucleotide, agonist competition studies in cell membranes revealed no differences in affinity binding constants. However, functional studies revealed a marked difference in coupling of the two receptors to adenylyl cyclase (Figure 2*C*). As is shown, maximal agonist stimulation of adenylyl cyclase of Gly389 is only $\sim$1/3 that of the Arg389

receptor. In agonist competition studies carried out with membranes in the absence of GTP, high- and low-affinity binding sites could only be detected with the Arg389 receptor. This indicates impaired formation of the agonist-receptor-G_s complex with the Gly389 receptor. In addition, agonist stimulation of [^{35}S]GTPγS was less with Gly389 compared to the Arg389 receptor (10). Thus, the decreased adenylyl cyclase stimulation is due to a less favorable receptor conformation for agonist stabilization and coupling to $G_{\alpha s}$. Residue 389 is within a G_s coupling domain of the β_1AR, and Gly is considered likely to disrupt the predicted α-helix in this region. Thus, depressed agonist-promoted stimulation of adenylyl cyclase is consistent with the location of the polymorphism and the nature of the substitution. Because the Gly389 receptor was originally denoted as the wild-type, the Arg389 receptor was characterized as a gain-of-function variant. Because Gly is the minor allele, we have now denoted it as the polymorphism, which has a phenotype of depressed functional coupling.

Three SNPs in the 5′ promoter region of the human β_1AR have been reported at positions −93, −210, and −2146 (11). However, they are rare (allele frequencies 1%–10%) and have not been studied as to functional significance. The −2146 allele is in strong linkage disequilibrium with the position 145 locus in the coding block (11).

Human Studies

There have been several clinical association studies addressing whether β_1AR polymorphisms are risk factors for disease, modifiers of disease, or modifiers of the response to βAR agonists or antagonists. Most of these have centered on cardiovascular phenotypes. In regard to heart failure, the basis of these inquiries include studies with various animal models, showing that prolonged stimulation of cardiac β_1AR via drug infusions, enhanced presynaptic norepinephrine release, or transgenic expression of β_1AR results in severe cardiomyopathy (12–15). Patients with heart failure show improvements (albeit to variable extents) in contractility, morbidity, and mortality when treated with β-blockers (16). In case-control studies, no difference in allele frequencies of the position 49 or 389 genotypes was noted in one study (17), but another study by Podlowski et al. (7) showed an increase in the frequency of the Gly49 allele in idiopathic dilated cardiomyopathy. In this latter study, no individuals with Gly49 were found (i.e., allele frequency of 0%) in the control (normal) population. This is in marked contrast to data from a number of other studies that show an allele frequency of ∼10%–15% for the Gly49 polymorphism in normals (6, 11, 17–19) and in patients with various forms of heart failure (11, 17, 20). Given that the Podlowski study had only 37 patients and 40 controls, their finding of no controls with Gly49, combined with the results of these other studies, make this proposed association seem erroneous.

In considering β_1AR polymorphisms as risk factors for heart failure, it would seem that the combination of increased norepinephrine release from cardiac presynaptic nerves and a hyperactive β_1AR would be a condition whereby the probability

of catecholamine-evoked cardiomyopathy might be the greatest. Indeed, African Americans with the combination of a dysfunctional α_{2C}AR polymorphism (α_{2C}AR Del322-325, see below), which is localized to cardiac presynaptic nerves and is responsible for basal norepinephrine release, and the Arg389 polymorphism, which has enhanced coupling compared to the Gly389 polymorphism, have a marked risk for heart failure (odds ratio = 10.11, 95% CI = 2.11−48.53, p = 0.004) (21). The effect is synergistic, rather than additive. In fact, the odds ratio for heart failure was 0.55 (p = 0.23) for the β_1AR Arg389 polymorphism alone. It thus appears that Arg389 is a risk factor for heart failure, but only in conjunction with α_{2C} Del322-325. The Gly49 allele has been shown to be associated with a small (5 beats/min) but statistically significant decrease in resting heart rate (19). Interestingly, in this study the position 389 polymorphism was not associated with heart rate or hypertension. In another study, however, Arg389 was found to be associated with increased diastolic blood pressure in discordant sibling-pairs and hypertension (defined as elevated systolic or diastolic pressure) in a case-control analysis (22). In addition, Arg389 has been shown to be associated with increased systolic blood pressure (∼12 mmHg) in patients with heart failure (20).

Concerning disease modification and drug response, the β_1AR Gly49 allele has been associated with improved five-year survival (risk = 2.34, 95% CI = 1.3−4.20, p = 0.003) (17). This finding is consistent with the phenotype delineated in transfected cells (9) because down-regulation of the β_1AR may serve as a chronic protective effect in the failing heart (23). Another study explored potential relationships between β_1AR polymorphisms and patients with heart failure and exercise performance (20). The primary outcome variable was oxygen consumption (VO_2), which is considered a relevant measure of the capacity of the heart to increase cardiac output as well as overall cardiovascular status. The results revealed a highly clinically significant decreased VO_2 in patients homozygous for Gly389 compared to those homozygous for Arg389 ($VO_2 = 14.5 \pm 0.6$ versus 17.7 ± 0.4 ml/kg/min, respectively; p = 0.006). Heterozygous individuals had intermediate levels of VO_2. The position 49 polymorphisms were also associated with a difference in exercise capacity but only when heterozygous and homozygous Gly49 individuals were considered as one group. However, a robust relationship between haplotypes consisting of the combination of polymorphisms at positions 49 and 389 and VO_2 was found. It was concluded that the major determinant of exercise capacity, in regards to β_1AR polymorphisms, is at the 389 locus based on allele frequency and a greater absolute effect, and that haplotypes may provide an even greater predictive value. In another study, exercise-induced increases in heart rates, and systolic time intervals have been reported to be the same between groups of normal individuals with Arg389 or Gly389 (24), but VO_2 was not measured. This may suggest that these β_1AR variants play a more prominent role when β_1ARs are desensitized, as occurs in heart failure. No studies to date have assessed the potential for β_1AR polymorphisms to effect responsiveness to β-blockers in heart failure. However, one study showed no association between blood pressure or heart rate responses to β-blockers in the treatment of hypertension (25).

β_2-ADRENERGIC RECEPTOR POLYMORPHISMS

Localization and Characterization

In the coding region of the human β_2AR, nine polymorphisms have been identified, three of which are nonsynonymous (Table 1) (26). A nonsynonymous variation at codon 34 has been reported (26), but the allele frequency is <1%. As shown in Figure 1, the common nonsynonymous polymorphisms occur at nucleotides 47 (amino acid 16) and 79 (amino acid 27). Both display differences in allele frequencies between Caucasians and African-Americans (Table 1) (27). Of note, the Arg16 receptor was first cloned and has been referred to as wild-type, but is in fact the minor allelic variant.

Prior to a full appreciation of the linkage disequilibrium between the position 16 and 27 polymorphisms, all combinations of the two (Arg16/Gln27, Gly16/Gln27, Arg16/Glu27, and Gly16/Glu27) were studied in recombinant CHW cells (28). Given their location in the amino terminus of the receptor, it was not unexpected to find no differences in agonist binding or agonist-stimulated adenylyl cyclase activities. In addition, receptor synthesis rates and agonist-promoted internalization were not different between the receptors. However, the extent of agonist-promoted down-regulation was affected by these substitutions (Figure 3*A*) (28). Cells were exposed to 10 μM isoproterenol or carrier for up to 24 h in culture, washed, membranes prepared, and [^{125}I]CYP radioligand binding utilized to quantitate receptor density. The Arg16/Gln27 receptor underwent 26 $\pm$ 3% down-regulation. In contrast, the Gly16/Gln27 receptor displayed 41 $\pm$ 3% down-regulation. Furthermore, the rare Arg16/Glu27 receptor failed to downregulate. The Gly16/Glu27 receptor displayed a similar level of down-regulation (39 $\pm$ 4%) compared to the Gly16/Gln27 receptor. Taken together, the data suggest that the major polymorphic

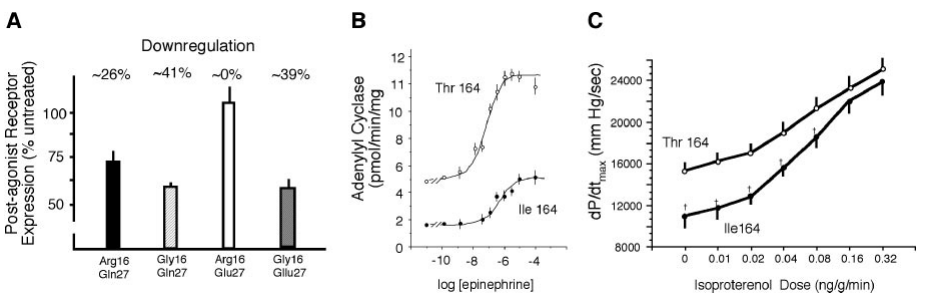

Figure 3 Characteristics of the β_2AR polymorphisms in transfected cells or transgenic mice. (*A*) Agonist-promoted down-regulation phenotypes of the position 16 and 27 polymorphisms in various combinations. (*B*) Adenylyl cyclase activities of the Thr164 and Ile164 β_2ARs. (*C*) Contractility of hearts from transgenic mice with targeted expression of the Thr164 and Ile164 β_2ARs.

locus that affects agonist-promoted down-regulation is at position 16. That is, whenever Gly16 is present, down-regulation is enhanced compared to Arg16. The molecular basis of these phenotypes is not clear, but it appears to occur after the internalization process, prior to or during passage through the degradation pathway. Efforts to further explore this mechanism are somewhat hampered in that little is known about how β_2ARs are targeted to degradative pathways.

In a study of cultured human airway smooth muscle cells natively expressing several of the β_2AR genotypic combinations (29), down-regulation (decrease in receptor density) promoted by 24 h of agonist exposure followed the same pattern as that observed in the transfected cell studies (28). However, another study using human airway smooth muscle cells showed a somewhat different phenotype (30). In this study, changes in receptor expression were not determined, but rather changes in function (cell stiffness and cAMP accumulation) after agonist exposure were delineated. In these studies, the presence of any Glu27 allele was associated with enhanced desensitization of these functions. This finding was observed with both 24-h exposures as well as 1-h exposures to agonist in culture. Because agonist-promoted down-regulation of β_2AR density requires many hours (minimal of $\sim$6 h for earliest detection regardless of cell-type), one must conclude that these protocols serve to study event(s) other than down-regulation alone. Based on the location of residue 27 and the known time courses of the short- and long-term desensitization processes, it is difficult to assign a common mechanistic basis for these observations. The effect of the position 16 genotype could not be fully assessed due to the distribution of genotypes. In another study with natively expressing polymorphic β_2AR, human lung mast cell function (agonist-promoted inhibition of histamine release) was examined (31). Desensitization of this response after 24 h of agonist exposure showed results that were the opposite of that predicted by the study where receptor expression was quantitated (29). Even though the above three studies utilized different protocols and outcome measures, one must also consider that polymorphisms in other genes whose products are involved in the various pathways being investigated may account for some of these apparent discrepancies.

The Ile164 polymorphism is localized to the fourth transmembrane spanning domain of the receptor (32). The Ile164 allele is uncommon in all populations studied to date. The heterozygous frequency is $\sim$2%–5% (Table 1). We have never found an individual homozygous for Ile164. When expressed in CHW cells, a two- to threefold decrease in affinities for agonists, and some antagonists, was observed in membrane competition studies in the presence of guanine nucleotide. Further agonist competition studies in the absence of GTP revealed very little accumulation of high-affinity binding with Ile164, such that the curves were essentially monophasic. In contrast, the Thr164 receptor displayed biphasic curves with readily resolved high- and low-affinity binding constants. Thus, the Ile164 receptor has depressed formation of the agonist-receptor-G_s complex as a result of this substitution in the ligand-binding pocket of the receptor. This was manifested as a decrease in basal and agonist-stimulated adenylyl cyclase activities

(Figure 3*B*) (32). The lower basal activity is consistent with the current concept concerning spontaneous activation of GPCRs in the absence of agonist. With high expression levels, the basal phenotype of an uncoupled receptor would thus be more readily observed. Indeed, expression levels for the Thr164 and Ile164 receptors were ~1000 fmol/mg. In contrast, the studies described above with the β_1AR Arg389 and Gly389 receptor were at ~250 fmol/mg, where an increase in agonist-stimulated, but not basal, levels was observed, due to the lower expression levels.

The Thr164 and Ile164 β_2AR have also been studied in targeted transgenic mice (33). For these studies, the α–myosin heavy chain promoter was utilized to direct expression to the heart. Expression levels were ~1000 fmol/mg for both receptors. Adenylyl cyclase studies in cardiac membranes revealed the same signaling phenotype as was observed using the recombinant CHW cells. The endogenous βAR in the heart were still present, but the levels of adenylyl cyclase were below those of the transgenic mice so that the phenotypic differences between the two receptors could be delineated. Both sets of transgenic mice displayed normal development, and at one year showed no evidence of cardiac pathology. Invasive studies (cardiac catheterization) revealed lower heart rates and contractility ($^{+}$dP/dt) in the Ile164 mice compared to the Thr164 mice (Figure 3*C*). It is interesting to note that the physiologic responses of the Ile164 mice were found to be essentially the same as those of nontransgenic mice despite ~40-fold overexpression (33). This suggests that physiologically, Ile164 may be even more impaired than that indicated by the adenylyl cyclase studies. We conclude that the phenotype of the Ile164 β_2AR receptor is that of depressed basal and agonist-promoted function, which likely has physiologic relevance in target tissues.

The degree of variability of the β_2AR gene has been further explored by interrogating ~1100 bp of the 5′ upstream region of this intronless gene (34). This region includes a short open reading frame, denoted the 5′-leader cistron. The encoded peptide from the cistron (β_2AR upstream protein or BUP) alters translation of the β_2AR and a SNP that alters the most 3′ amino acid (Cys to Arg) was found. In addition, the promoter and 5′ UTR regions contain multiple potential *cis*-acting elements. For this study, sequencing did not extend into the 3′ UTR and in fact terminated after the synonymous SNP at nucleotide 523. The results are shown in Table 2. Altogether, 13 SNPs were identified. Of the 2^{13} (8192) possible haplotypic combinations, only 12 were found. As can be seen, a number of these haplotypes are uncommon, with four major haplotypes identified. For some haplotypes, there were marked differences (>20-fold) in frequency between various ethnic groups. To ascertain the relevance of these haplotypes to receptor function, the two most common Caucasian homozygous haplotypes (2/2 and 4/4) were studied in a transient expression system. The constructs used for transfection lacked the typical eukaryotic promoters, but instead used the two β_2AR 5′ upstream sequences as the promoter in the exact context found in the native gene. As shown in Figure 4, the levels of β_2AR mRNA and protein expression were indeed different between haplotypes 2 and 4. As is discussed below, these haplotypes were associated with a

TABLE 2 Haplotypes of the human beta-2 adrenergic receptor

														Frequency (%)			
Nucleotide:	-1023	-709	-654	-468	-406	-367	-47	-20	46	79	252	491	523	Ca	A-A	As	H-L
Alleles:	G/A	C/A	G/A	C/G	C/T	T/C	T/C	T/C	G/A	C/G	G/A	C/T	C/A				
Haplotype																	
1	A	C	G	C	C	T	T	T	A	C	G	C	C	0.7	25.0	12.5	10.0
2	A	C	G	G	C	C	C	C	G	G	G	C	C	48.3	6.3	10.0	26.7
3	G	A	A	C	C	T	T	T	A	C	G	C	C	0.7	0.0	0.0	0.0
4	G	C	A	C	C	T	T	T	A	C	G	C	C	33.0	29.7	45.0	40.0
5	G	C	A	C	C	T	T	T	G	C	G	C	C	1.4	0.0	0.0	0.0
6	G	C	G	C	C	T	T	T	G	C	A	C	A	13.2	31.3	30.0	13.3
7	G	C	G	C	C	T	T	T	G	C	A	T	A	1.0	1.6	0.0	3.3
8	G	C	A	C	C	T	T	T	A	C	A	C	A	0.7	0.0	0.0	0.0
9	A	C	G	C	T	T	T	T	A	C	G	C	C	0.0	4.7	0.0	0.0
10	G	C	G	C	C	T	T	T	G	C	A	C	C	0.7	0.0	0.0	3.3
11	G	C	G	C	C	T	T	T	G	C	G	C	C	0.3	0.0	2.5	0.0
12	A	C	G	G	C	T	T	T	A	C	G	C	C	0.0	1.6	0.0	3.3
Location:	5'	5'	5'	5'	5'	5'	AA19 BUP Cys/Arg	5'	AA16 Gly/Arg	AA27 Gln/Glu	syn	AA164 Thr/Ile	syn				

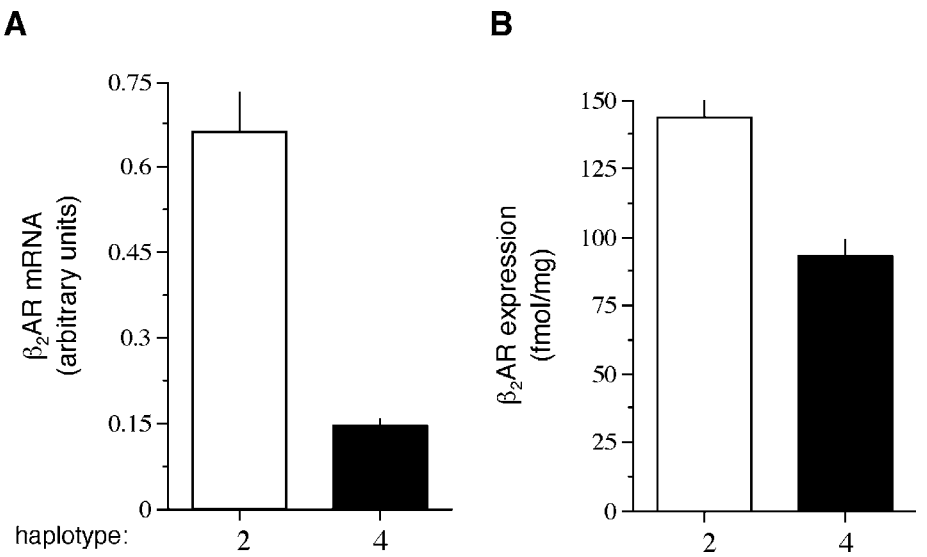

Figure 4 β_2-adrenergic receptor haplotypes affect receptor expression. See Table 2 and text for localization of the polymorphisms that encompass haplotypes 2 and 4.

differential response to albuterol in asthmatics of the same direction and magnitude as found in these in vitro studies (34). Thus the β_2AR gene is highly polymorphic, with all the nonsynonymous SNPs resulting in receptors that have distinct characteristics that include ligand binding, functional coupling, and agonist-promoted regulation. In addition, 5′ upstream SNPs, likely in combination with each other and the coding SNPs, may act to direct expression of the receptor. Additional studies, however, are necessary to delineate the nature of these effects and interactions and to discover which SNPs are primarily responsible for the expression phenotype.

Human Studies

Many clinical association studies have been carried out with β_2AR SNPs or haplotypes, examining if they are risk factors or modifiers of disease or determinants of the response to β-agonists. The majority have been within the context of asthma (26, 27, 34–56) because β_2ARs expressed on airway smooth muscle regulate bronchomotor tone and β-agonists are utilized for treating asthma since they serve to bronchodilate. In assessing the body of studies collectively, it is clear that most, but not all, have shown some association between β_2AR genotypes and various asthma phenotypes. These have included severity and other clinical subsets, hyperresponsiveness, bronchodilator response to β-agonist, tachyphylaxis to β-agonist, "asthma control," and others. A few of these asthma studies are discussed here in some detail in order to highlight important points relevant to genotype-phenotype

association studies in asthma and similar complex syndromes. In a study by Israel et al., relationships between position 16 and 27 polymorphisms and tachyphylaxis to the β-agonist albuterol were sought in a cohort of mild-moderate asthmatics (49). Patients were treated as needed with albuterol (i.e., only when symptoms occurred) or on a regular schedule (four times/day) with a standard dose of inhaled albuterol. The outcome measure was the change in morning and evening peak expiratory flow rates, with worsening rates over the 16-week treatment plus the 4-week washout period being considered evidence for tachyphylaxis. In the analysis, only homozygous patients were considered. Morning and evening peak expiratory flow rates declined only in patients receiving regular albuterol who had the homozygous Arg16 genotype. Gly16 patients showed no decline, nor did Arg16 patients who received intermittent β-agonist.

The above results from this clinical study appeared to be in contrast to what would be predicted from the recombinant cell culture studies (28). In the latter studies, the Gly16 β_2AR underwent the greatest degree of down-regulation after prolonged exposure to agonist in the culture medium. However, we have proposed (57) that the results of the Israel et al. study are consistent with the recombinant studies if one considers a dynamic, rather than static, model of receptor regulation. The concept is relevant to a variety of regulatory processes and is depicted in a generic manner in Figure 5. In the static model, the expression/function of β_2AR is not modulated by endogenous agonist (epinephrine). As such, when challenged by chronic β-agonist administration, the Gly16 receptor ("SNP B" in the model) would display the greatest degree of desensitization, clinically manifested

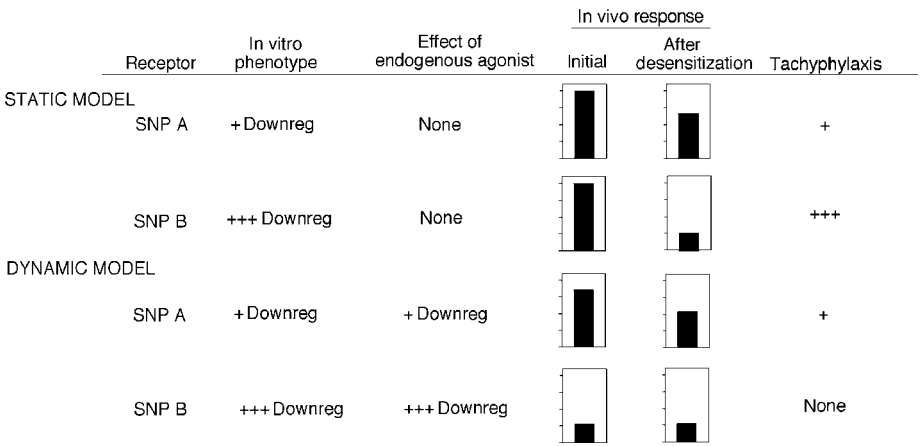

Figure 5 Comparison of tachyphylaxis phenotypes in the static versus dynamic models of receptor regulation. The models differ as to whether endogenous agonist "pre-regulates" receptor function prior to exogenous agonist exposure. The predicted clinical effect (tachyphylaxis) differs depending on the model. For β_2AR, SNP A is the Arg16 receptor and SNP B is Gly16. The bars represent arbitrary physiologic responses, such as FEV_1.

as tachyphylaxis. However, ample evidence points to the fact that GPCRs, including the β_2AR, are under constant regulation by their cognate endogenous agonists, thereby providing a means to integrate the multitude of incoming signals and to ultimately modulate complex physiological functions. Under such circumstances, one can consider that the Gly16 β_2AR, which is the most sensitive to agonist-promoted down-regulation, may be substantially, or maximally, downregulated by endogenous epinephrine before exposure to exogenous agonist such as albuterol. As such, patients with this genotype would show no tachyphylaxis because further down-regulation by albuterol does not occur. In contrast, the Arg16 receptor ("SNP A" in the model) undergoes relatively less down-regulation by endogenous epinephrine, but with exposure to chronic exogenous albuterol, undergoes down-regulation. The difference between the pre- and post-albuterol state is thus measurable, and so patients with Arg16 in fact display tachyphylaxis (Figure 5). A recent study of desensitization of vascular blood flow to isoproterenol supports the dynamic model in the context of β_2AR polymorphisms as well (58). The above concepts emphasize the need to carry out both in vitro and human studies to fully appreciate the effects and mechanisms of polymorphisms on differential responses to therapeutics (59, 60).

In addition to considering the impact of polymorphisms within the context of the intact individual, recent studies have also shown the importance of taking into account the context of polymorphisms within a functional unit (i.e., the gene) rather than in isolation. In the most straightforward illustration, one could have a scenario whereby a SNP at one position could negate, or accentuate, the effect of a SNP at another position. Thus, knowing the SNP at only one position may not provide ample predictive power. To address this, we examined the haplotypes of the β_2AR gene (34) as described above (see also Table 2). The common Caucasian haplotype pairs were 2/2, 4/4, 2/4, 2/6, and 4/6. In a cohort of 131 Caucasian asthmatics, the forced expiratory volume in one second (FEV_1) was determined before and 30 min after inhalation of a standard dose of albuterol. Initially, analysis was conducted to assess whether any one SNP was associated with the bronchodilator response (considered as a continuous variable). No associations were found. However, haplotype pair was found to be significantly associated with the FEV_1 response ($p = 0.007$ by ANCOVA). The greatest differences were between those with haplotype pair 4/6 and 4/4 (19.1 versus 8.5 change in % predicted FEV_1). We had no patients who were homozygous 6/6. Those with haplotypes 2/2 and 2/6 had intermediate responses between those with 4/4 and those with 4/6. The data with the 4/4 and 2/2 individuals were consistent with the transfection studies (see above and Figure 4) that showed greater expression of β_2AR mRNA and protein expression with the haplotype 2 construct. This study emphasizes, particularly in somewhat heterogenous groups, that β_2AR haplotypes provide greater predictive power of the bronchodilator response to β-agonists than individual SNPs. A similar finding was made by Weir et al. (27) when asthma severity was examined. Here, only haplotypes composed of chromosomally phased SNPs at positions 16 and 27 revealed an association. In another study, a three-SNP haplotype, but not individual

β_2AR SNPs, was strongly predictive of protection against bronchial hyperreactivity (47). Taken together, these studies indicate, at least with some phenotypic traits, the need for more extensive information about β_2AR genetic variation than individual SNPs or unphased genotypic combinations of a few SNPs to further enhance predictive power.

Some (61), but not all (62–67), studies have shown relationships between β_2AR polymorphisms and hypertension, presumably because of the expression of these receptors on vascular smooth muscle that serve to vasodilate. Indeed, several studies have revealed a relationship between genotype and some measure of vascular relaxation in response to agonist infusion (62, 68, 69). Perhaps the most definitive study, by Boerwinkle and colleagues (65), which utilized sib-pairs from 55 pedigrees and ~2500 individuals from 589 families, revealed that the β_2AR polymorphisms are susceptibility loci for essential hypertension. The risk was greater for Gly16 and Glu27 alleles, the latter having an odds ratio for occurrence of hypertension of 1.80 (95% CI = 1.08−3.0, p = 0.023). Given the heterogeneity of hypertension, it is not surprising to find that β_2AR polymorphisms are responsible for a relatively small effect. However, in combination with other genetic variants and substantial environmental interactions, β_2AR polymorphisms appear to represent one component in this complex syndrome.

Several studies have been carried out examining β_2AR polymorphisms and heart failure. The basis of these studies is the growing body of evidence that myocardial β_2AR signals somewhat differently than β_1AR, with the former having anti-apoptotic effects (70). In addition, β_2ARs have positive inotropic and chronotropic effects in the human heart, and on vascular smooth muscle they contribute to vascular tone, particularly during exercise. An initial analysis of cases and controls showed no association between β_2AR genotypes at positions 16, 27, and 164 and heart failure. However, longitudinal follow-up for three years revealed that individuals with the Ile164 allele had a rapid progression to either death or transplantation (adjusted relative risk = 4.81, 95% CI = 2.0−11.5, $p < 0.001$) (71). Indeed, one year survival was 42% for patients with Ile164 compared to 70% for Thr164 patients. There was no significant confounding effect of age, sex, etiology of failure, medication use, or initial left ventricular ejection fraction. A subsequent study (72) examined patients early in the course of the disease, matched for clinical and demographic parameters, but either homozygous for Thr164 or heterozygous for Ile164. Exercise capacity was measured using a graded treadmill protocol, with VO_2 as the primary outcome measure. Those with Ile164 had substantially depressed VO_2 (15.0 $\pm$ 0.9 versus 17.9 $\pm$ 0.9 ml/kg/min). The odds ratio of having $VO_2 \leq 14$ ml/kg/min was 8.0 ($p = 0.009$). Importantly, these patients could not be differentiated by standard clinical tests. Of note, a $VO_2 \leq 14$ is one of the criteria for placement on the cardiac transplantation list. So, even at early stages, with more intense investigation a pathophysiologic effect of Ile164 is observed. A study by Brodde et al. (73) with normal volunteers showed a decreased responsiveness (heart rate and systolic time interval) to infusions of the β_2AR agonist terbutaline in those with the Ile164 allele compared to Thr164 homozygotes. This

indicates that this polymorphism has physiologic effects even in the absence of a disease such as heart failure.

There have been several studies assessing potential associations between β_2AR polymorphisms and obesity (74). These were based on the fact that β_2ARs are expressed on white adipose tissue where activation results in lipolysis. In obese women, homozygosity for Glu27 was associated with ~20 kg higher fat mass and ~50% larger fat cells in the obese compared to nonobese women (75). However, in isolated adipocytes from such individuals, Glu27 was not associated with increased sensitivity or maximal glycerol release from terbutaline exposure ex vivo. Instead, the sensitivity was related to the position 16 polymorphisms. In another study, obesity in males was shown to be positively associated with the Gln27 polymorphisms (or negatively associated with Glu27) (76). It was concluded that gender may play a role in the influence of β_2AR genotype on obesity. Interestingly, the effect of position 27 polymorphisms may be modified by exercise (77) or, stated another way, may identify patients likely to achieve weight loss with exercise (78). Other studies, however, have failed to observe any relationship, or only a small risk, for obesity (79–82). Studies have also suggested associations with dyslipoproteinemia (79, 83) and type II diabetes (79). At this juncture, then, it is difficult to ascertain the role of β_2AR polymorphisms as predisposing factors for obesity. This is most likely due to the extreme clinical heterogeneity of the syndrome, gender effects, interaction with other genes, influences of other related disease such as diabetes, and environmental influences.

β_3-ADRENERGIC RECEPTOR POLYMORPHISMS

Localization and Characterization

One nonsynonymous polymorphism of the β_3AR gene has been reported (84) that results in a substitution of Arg for Trp (the major human allele) at amino acid position 64. This residue is localized either to the most distal residue within the first transmembrane spanning domain or the most proximal residue of the first intracellular loop (Figure 1) of the receptor. It is interesting to note that in virtually all β_3AR genes cloned from various species, Arg is found at position 64. In most cases that we are aware of, the human major allele is the one found in the other species, rather than the apparent situation with the β_3AR. This suggests very strong, human-specific, evolutionary pressure for dominance of the Trp residue. Indeed, the pharmacologic properties between rodent and human β_3AR are quite different (85), suggesting that the receptor subserves somewhat different functions in the two species. The Arg64 β_3AR polymorphism occurs with an allele frequency of ~8%–10% in Caucasian populations; to our knowledge the frequency in African or African-American populations has not been reported. However, Japanese and Alaskan Eskimos have higher allele frequencies (74).

Two studies with discrepant results have been published on the pharmacologic effect of the Arg64 substitution in the β_3AR using recombinant expression. In CHO(dhfr-) cells, Strader and colleagues (86) found no differences in agonist

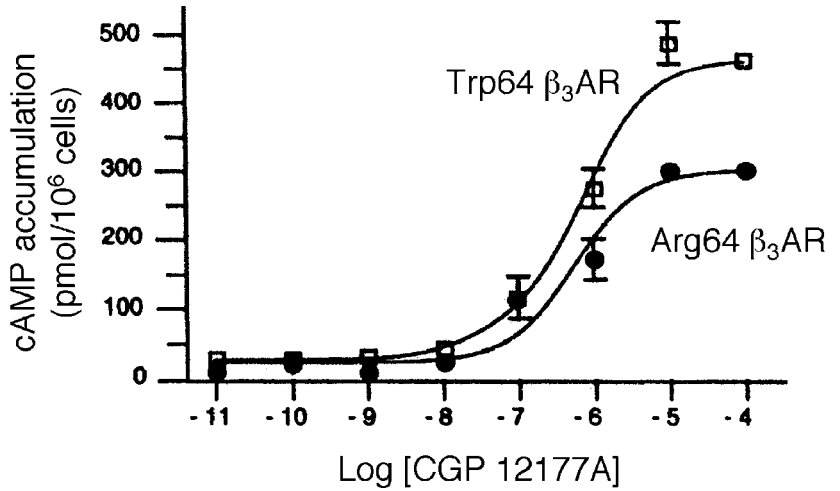

Figure 6 Adenylyl cyclase activities of the β_3AR polymorphism in transfected cells.

binding parameters or agonist stimulation of cellular cAMP accumulation between the Trp64 and Arg64 receptors. In contrast, another group has reported a decrease in the maximal stimulation of cAMP accumulation in CHO-K1 and HEK293 cells with the Arg64 β_3AR compared to its allelic counterpart (Figure 6). The CHO-K1 cells expressing the Arg64 β_3AR displayed lower agonist-promoted cAMP accumulation compared to the Trp64 cells. However, forskolin-stimulated accumulation was also depressed. In HEK-293 cells, the forskolin-stimulated cAMP levels were similar between the cell lines, with agonist-stimulated cAMP levels being modestly depressed with the Arg64 β_3AR (Figure 6). The reduction was observed with all agonists tested, albeit to different extents. Agonist affinity was not tested. The reasons for the discrepancy between these two studies are not clear. In human fat cells, an ~10-fold increase in EC_{50} (i.e., a decrease in sensitivity) to a β_3AR agonist in stimulation of lypolysis ex vivo has been reported (87). No differences in β_1AR- or β_2AR-mediated lipolysis were noted. β_3AR expression levels were not measured, so it is not clear whether the polymorphism may alter expression of the receptor in adipocytes. Taken together, it appears that there may be a difference in coupling between Arg64 and Trp64 β_3ARs, but additional studies may help to clarify the pharmacologic characteristics and mechanistic basis for the phenotype.

Human Studies

Quite a few studies have been published examining potential relationships between indices of obesity or type II diabetes and the Arg64 β_3AR polymorphism (74). The basis for such studies is the expression of the β_3AR in brown adipose tissue, where receptor activation increases thermogenesis, and in white adipose tissue,

where activation results in lipolysis. The β_3AR is also expressed in several other tissues, such as heart and gall bladder, but its relevance at these sites is not well established. Well over 30 studies have been published in regard to indices of obesity or type II diabetes, with ∼one half reporting positive associations and the remainder failing to note associations. Many of these studies had large numbers of subjects and had similar study designs yet reached very different conclusions. The reasons for such extensive discrepancies in this field are not entirely clear. Again, the heterogeneity of obesity, gender, and racial factors, and the presence of comorbid conditions have made interpretation of these studies as a group difficult. An interaction with other obesity-related genes is highly likely. To our knowledge, effects of a β_3AR agonist administered for weight loss in humans, stratified by the Arg64 and Trp64 genotypes, have not been published.

α_1-ADRENERGIC RECEPTOR POLYMORPHISMS

Localization and Characterization

There was some initial confusion as to the pharmacologic classification of the cloned α_1AR subtypes. The current classification is as follows: α_1A (originally designated as the $\alpha_{1c}$when cloned), α_{1B} (same as cloned α_{1b}), and α_{1d} (cloned α_{1d} but also designated by some as the $\alpha_{1A/D}$ or $\alpha_{1a/d}$). In this review, the current classification as indicated above is utilized.To date, only one polymorphism in an α_1AR subtype gene that alters the amino acid sequence has been described (Table 1). This polymorphism, initially identified as a Pst I restriction fragment length polymorphism (RFLP), is located within the α_{1A}AR gene and corresponds to either a C or T at nucleotide 1441 encoding Arg or Cys at amino acid 492 (88). This residue is located in the carboxy terminus of the receptor (Figure 1). The frequency of the Cys492 allele was found to be more common in Caucasians than in African Americans, with frequencies of 54% and 30%, respectively (89). For the α_{1B}AR gene, sequence analysis of exonic regions from 51 individuals revealed only two synonymous polymorphisms (90). Studies designed to identify polymorphisms of the α_{1D}AR have not been described.

Comparison of the Arg492 and Cys492 α_{1A}AR function has been investigated using transfected CHO cells stably expressing each receptor (91). Radioligand binding studies using [^{125}I]-HEAT showed no differences in agonist or antagonist binding. In addition, receptor-mediated calcium signaling, as well as the extent of receptor desensitization following agonist exposure, were also found to be similar for both receptors. Given the location of this SNP and the nature of the substitution, these findings were not altogether unexpected.

Human Studies

Although functional differences between the Arg492 and Cys492 receptors have yet to be identified, the potential relevance of α_{1A}AR genetic variation to multiple pathophysiological conditions has been examined. In these studies, the prevalence

of each polymorphic allele was ascertained in control individuals and patients with hypertension, benign prostatic hypertrophy, schizophrenia, or clozapine-induced urinary incontinence. Despite compelling evidence to support a role for α_{1A}AR in these conditions, no association of either polymorphic allele was found (89, 91–93). A trend showing an increase in the frequency of the Arg492 allele in patients with Alzheimer's disease has been reported, but the significance of this observation remains to be explored (94).

In summary, the Arg492 and Cys492 α_{1A}ARs have been shown to have similar pharmacological and functional characteristics, results consistent with a lack of association of either polymorphic allele with multiple pathophysiological conditions involving α_{1A}AR function. At this time, a complete interrogation of sequence spanning all coding and noncoding regions of each α_1AR subtype, performed using an appropriately powered repository of ethnically diverse DNA samples, has not been described. Thus, relevant α_1AR polymorphisms may remain unidentified.

α_{2A}-ADRENERGIC RECEPTOR POLYMORPHISMS

Localization and Characterization

Direct sequence analysis of overlapping PCR products identified a SNP within the coding region of the α_{2A}AR gene (95). This polymorphism consists of a C to G transversion at nucleotide 753 that results in an Asn to Lys change at amino acid 251, a highly conserved residue within the third intracellular loop of the receptor (Figure 1). The Lys251 α_{2A}AR allele was found to be relatively rare, with frequencies of 0.4% and 5% in Caucasians and African-Americans, respectively (Table 1).

The consequences of this polymorphism on ligand binding and receptor coupling were assessed in CHO cells stably expressing either the Asn251 or Lys251 α_{2A}AR (95). Ligand binding was not altered by the presence of the polymorphism as shown by virtually identical dissociation binding constants for the α_2AR antagonist [^{3}H]yohimbine and no differences in binding to the agonist epinephrine. Activation of the polymorphic Lys251 receptor with the agonist epinephrine, however, resulted in ~40% increase in [^{35}S]GTPγS binding. This enhanced agonist-promoted G-protein coupling was also evident in multiple signaling pathways, with the Lys251 receptor showing increased agonist-promoted inhibition of forskolin-stimulated adenylyl cyclase activity and activation of MAP kinase (Figure 7). In each case, basal receptor function was equivalent for each receptor, whereas enhanced function was observed with several full and partial agonists in selected assays. It is interesting to note that the gain of function was much more for MAP kinase stimulation (~280%) compared to inhibition of adenylyl cyclase (~30%), which highlights the need to assess multiple pathways when determining phenotype. In addition to the Lys251 polymorphism within the coding region of the α_{2A}AR, several polymorphisms have also been identified that are located within noncoding regions of the gene (96–99).

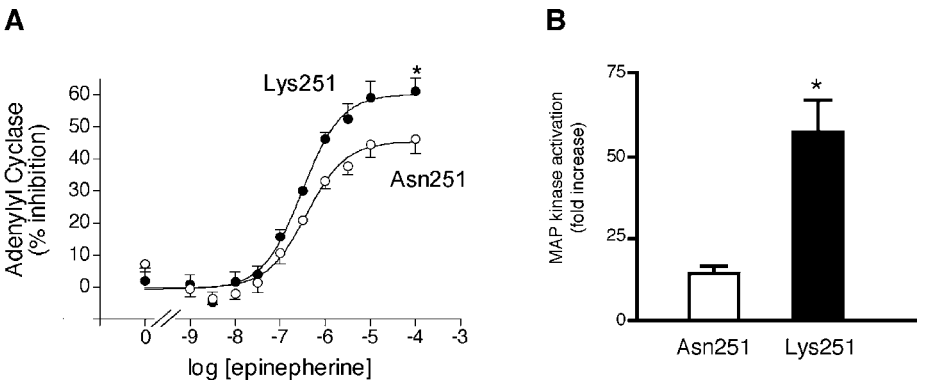

Figure 7 Enhanced coupling of the Lys251 α_{2A}AR polymorphism in transfected cells.

Human Studies

To date, no associations have been established for the Lys251 α_{2A}AR polymorphism and clinical phenotypes in which α_{2A}ARs are thought to play a role. One case-control study has been performed to ascertain the frequency of this polymorphism in patients with essential hypertension (95); however, considering the gain-of-function phenotype of the polymorphic receptor and the known centrally mediated hypotensive functions of the α_{2A}AR (100), results showing a lack of association with this polymorphism and essential hypertension are not surprising.

Although the functional consequences of the noncoding α_{2A}AR polymorphisms remain unknown, several clinical studies have been performed to examine potential associations of these polymorphisms with various disease states. For most of the noncoding polymorphisms, no associations have been found with multiple pathological conditions linked to α_{2A}AR function, including hypertension, panic and mood disorders, schizophrenia, and obesity (92, 99, 101–105). In contrast, several clinical phenotypes, including hypertension, body fat distribution, and glucose metabolism, have been shown to be associated with a Dra I restriction fragment length polymorphism within the 3′UTR (106–109). However, because the functional relevance of this polymorphism has not been determined, the significance of these results remains unclear.

α_{2B}-ADRENERGIC RECEPTOR POLYMORPHISMS

Localization and Characterization

One polymorphic form of the α_{2B}AR, consisting of a three–amino acid deletion (denoted Del301-303) located within the third intracellular loop of the receptor, has been described (110). This polymorphism consists of an in-frame nine-bp deletion beginning at nucleotide 901 that results in the loss of three Glu residues at positions

301–303 (Figure 1). PCR amplification of the region spanning this polymorphism followed by agarose gel electrophoresis to distinguish the insertion/deletion alleles based on size was performed to determine the frequency of each allele in various populations. In doing so, the deletion allele was found to occur at a frequency of 31% in Caucasians and 12% in African-Americans (Table 1).

As with the polymorphisms of the other α_2AR subtypes, the effects of the α_{2B}AR deletion polymorphism on ligand binding and receptor function were assessed in CHO cells stably expressing the wild-type and the polymorphic Del301-303 receptors (110). Saturation binding with the antagonists [^{3}H]yohimbine and [^{125}I]aminoclonidine, as well as competition binding with the agonists epinephrine, revealed that deletion of amino acids 301–303 had little effect on ligand binding. In addition, receptor coupling to G_i, as determined by agonist-promoted inhibition of forskolin-stimulated adenylyl cyclase activity, showed only a modest decrease in Del301-303 receptor function as compared to the wild-type α_{2B}AR, manifested as an ~18 % decrease in the maximal inhibition of adenylyl cyclase activity and a ~twofold increase in the EC_{50} for this response. Because these deleted Glu amino acids are localized to an acidic region of the third loop that had been found to be essential for GRK-mediated phosphorylation and agonist-promoted desensitization (111), studies were carried out to investigate these functions. COS-7 cells were transiently transfected to express GRK2 and the two α_{2B}AR receptors and whole-cell agonist stimulated phosphorylation studies carried out (110). In such experiments, the Del301-303 receptor displayed ~56% of wild-type agonist-promoted phosphorylation (Figure 8*A*). For the wild-type α_{2B}AR receptor, agonist-promoted phosphorylation results in receptor desensitization, which is manifested as an increase in the EC_{50} for agonist-mediated inhibition of adenylyl cyclase activity. At a concentration of agonist equal to the EC_{50}, the differences in inhibition of adenylyl cyclase between control and agonist pre-exposed cells represents 54% desensitization with the wild-type α_{2B}AR (Figure 8*B*). In contrast to the

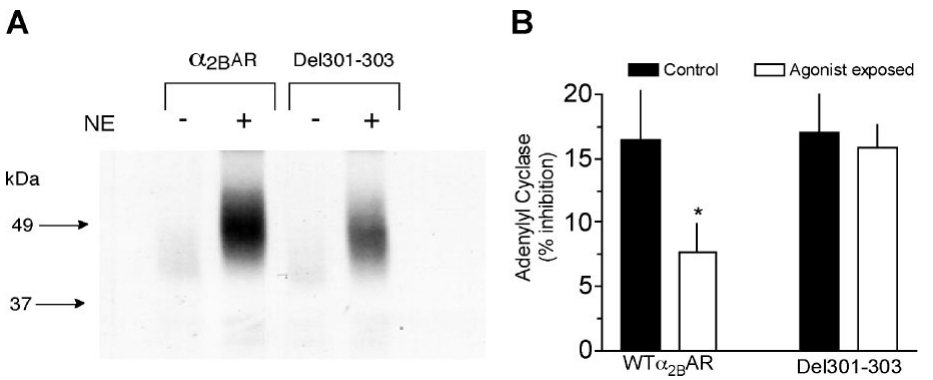

Figure 8 Altered agonist-promoted phosphorylation and desensitization of the Del301-303 α_{2B}-adrenergic receptor polymorphism in transfected cells.

desensitization observed with the wild-type receptor, the α_{2B}Del301-303 displayed a complete absence of desensitization (Figure 8*B*). This total loss of desensitization, despite only a partial loss of phosphorylation, is consistent with other studies of GRK-mediated phosphorylation of the α_{2A}AR and α_{2B}AR, which indicate that "full" phosphorylation (i.e., all sites are phosphorylated) is necessary to evoke desensitization (111, 112).

Human Studies

Several clinical studies have been performed to assess the role of the α_{2B}AR deletion polymorphism as a risk factor for disease. In terms of cardiovascular disease, it has been hypothesized that the presence of the deletion polymorphism may lead to increased vasoconstriction in humans (113, 114). This is based on the fact that α_{2B}AR have been shown to mediate the hypertensive effects of α_2AR stimulation (115) and that the polymorphic loss of three glutamic acid residues from the third intracellular loop of the receptor results in a complete loss of receptor desensitization (110). In a recent prospective study of Finnish men, individuals homozygous for the deletion polymorphism had 2.2 times the risk (95% CI = 1.1−4.4, p = 0.02) for experiencing acute coronary events, defined as prolonged chest pain or acute myocardial infarction. Thus, the presence of the deletion polymorphism may be a risk factor for the occurrence of acute coronary events (114). In contrast, no significant associations for this polymorphism have been observed for patients with essential hypertension (113, 114). In addition, because α_2ARs are also known to influence energy metabolism through inhibition of insulin secretion and lipolysis, several studies have been performed to investigate the potential roles of the α_{2B}AR deletion polymorphism in clinical parameters related to obesity. Heinonen et al. have shown that the deletion polymorphism is associated with reduced basal metabolic rates in obese patients (116), whereas Sivenius et al. found an increase in body weight in nondiabetic individuals homozygous for this polymorphism (117). In another study, a lack of association for either the α_{2B}AR deletion polymorphism or the β_3AR Arg64 polymorphism alone with various clinical parameters of obesity was noted, but a significant interaction of these two variants on fat mass and percentage of fat in Caucasian women was found (118).

α_{2C}-ADRENERGIC RECEPTOR POLYMORPHISMS

Localization and Characterization

One polymorphic form of the α_{2C}AR (Figure 1), consisting of a four–amino acid deletion (denoted Del322-325), has been identified within the third intracellular loop of this receptor (119). This polymorphism consists of a 12-bp in-frame deletion beginning at nucleotide 964 that results in the deletion of amino acids 322–325 (Gly-Ala-Gly-Pro). The Del322-325 allele was found to be common in African-Americans, with an allele frequency of ∼40%. In contrast, this polymorphism

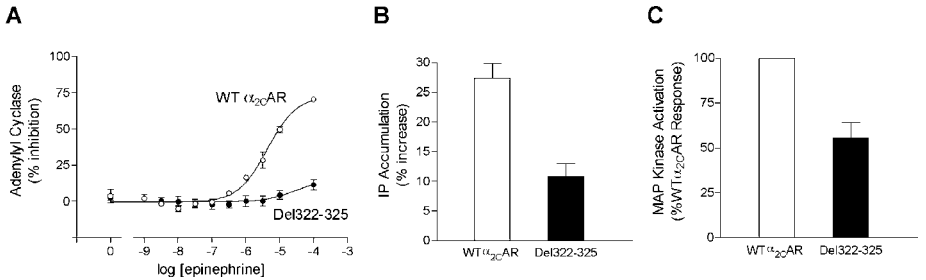

Figure 9 Decreased function of the Del322-325 α_{2C}AR polymorphism in transfected cells. IP = inositol phosphates.

was found to be relatively rare in Caucasians, with an allele frequency of ∼4% (Table 1).

The consequences of the Del322-325 α_{2C}AR polymorphism have been studied in CHO cells permanently expressing equivalent levels of wild-type (i.e., no deletion) and the α_{2C}Del322-325 receptor (119). In competition binding studies with the agonist epinephrine, carried out in the absence of GTP, the deletion receptor showed reduced high-affinity agonist binding, indicating an impaired ability of this receptor to form the high-affinity agonist-receptor-Gi/Go complex. Indeed, additional analyses showed impaired coupling of the polymorphic receptor to multiple signaling pathways. In cell lines expressing high levels of receptor (∼1200 fmol/mg), a ∼50% reduction in agonist-promoted inhibition of adenylyl cyclase was observed for the Del322-325 compared to the wild-type receptor. The impaired function of the deletion receptor was even more striking (∼86% reduction) in cell lines with low levels of receptor expression (∼500 fmol/mg) (Figure 9*A*). In addition, agonist-promoted coupling to activation of MAP kinase as well as accumulation of inositol phosphates via activation of phospholipase C were impaired 71% and ∼60%, respectively (Figure 9*B*,*C*).

Human Studies

In a recent study, the prevalence of the Del322-325 α_{2C}AR polymorphism in control and heart failure patients was ascertained. The study was based on the location and function of this subtype in cardiac presynaptic nerve terminals. Presynaptic autoinhibition of norepinephrine release is regulated by both the α_{2A}- and α_{2C}AR subtypes. The α_{2A}AR regulates release due to high-frequency stimulation, whereas the α_{2C}AR regulates release from low-frequency (basal) stimulation (13). In various animal models, prolonged stimulation of cardiac β_1AR via drug infusions, ablation of α_{2A}- and α_{2C}AR, or transgenic expression of β_1AR results in severe cardiomyopathy (12–15). We then considered that a defective α_{2C}AR, which would lead to chronic increased norepinephrine release, may predispose individuals to heart failure. The major findings in this case-control study were in

African-Americans, where the prevalence of the α_{2C}Del322-325 polymorphism is common. The odds ratio for heart failure in individuals with Del322-325 was found to be 5.65 (95% CI = 2.67−11.95, $p<0.0001$). Indeed, 53% of African-Americans with heart failure were homozygous for α_{2C}Del322-325, compared to 18% of controls. The association held for both idiopathic dilated and ischemic cardiomyopathies. As introduced earlier, a significant gene-gene interaction was observed with the α_{2C}Del322-325 and the β_1AR Arg389 alleles. The latter receptor has ∼threefold enhanced stimulation of adenylyl cyclase compared to the β_1AR Gly389. Thus, from a biological standpoint, the enhanced risk (odds ratio ∼10, see above) resulting from the synergistic actions of these receptor variants makes sense. Additional studies with β_1AR, α_2AR, and polymorphisms of other biologically linked genes need to be carried out in order to fully appreciate the genetic component in these complex heart failure and other cardiovascular syndromes.

CONCLUSIONS

Adrenergic receptors display a substantial degree of polymorphic variation in multiple different structural domains of the encoded proteins. Although not as extensively investigated, 5′ and 3′ noncoding regions also are variable. Both recombinant expression systems and human studies have indicated that many of the polymorphisms alter some aspect of receptor signaling. Clinical studies have further revealed that these polymorphisms may be risk factors for disease, modify disease, or alter the response to therapy. In most cases, the diseases studied are complex, with multiple phenotypes and environmental influences. Investigations utilizing haplotypes of these receptors and polymorphisms of other genes within common signal transduction pathways may lead to a further refinement in our understanding of their physiologic, pathologic, and pharmacologic importance.

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GENE THERAPY WITH VIRAL VECTORS

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■ **Abstract** A key factor in the success of gene therapy is the development of gene delivery systems that are capable of efficient gene transfer in a broad variety of tissues, without causing any pathogenic effect. Currently, viral vectors based on many different viruses have been developed, and their performance and pathogenicity has been evaluated in animal models. The results of these studies form the basis for the first clinical trials for correcting genetic disorders using retroviral, adenoviral, and adeno-associated viral vectors. Even though the results of these trials are encouraging, vector development is still required to improve and refine future treatment of hereditary disorders.

INTRODUCTION

The major goal of gene therapy is to introduce a functional gene into a target cell and restore protein production that is absent or deficient due to a genetic disorder. Although the basic principle of gene therapy is quite simple, successes rely considerably on the development of the gene transfer vectors.

Over the years, a number of gene transfer vehicles have been developed that can roughly be divided into two categories: synthetic and virus-based gene delivery systems. Synthetic gene delivery systems depend on direct delivery of genetic information into a target cell and include direct injection of naked DNA and encapsulation of DNA with cationic lipids (liposomes). Although these delivery systems exhibit low toxicity, gene transfer in general is inefficient and often transient.

Viral delivery systems are based on replicating viruses that have the ability to deliver genetic information into the host cell. In general, genomes of replicating viruses contain coding regions and *cis*-acting regulatory elements. The coding sequences enclose the genetic information of the viral structural and regulatory proteins and are required for propagation of infectious viruses, whereas *cis*-acting sequences are essential for packaging of viral genomes and integration into the host cell. To generate a replication-defective viral vector, the coding regions of the virus are replaced by the genetic information of a therapeutic gene, leaving the *cis*-acting sequences intact. When the viral vector is introduced

into producer cells providing the structural viral proteins in *trans*, production of nonreplicating virus particles containing the genetic information of a therapeutic gene is established (Figures 1, 3, and 4). The ability to generate replication-defective viral vectors is the backbone of developing virus-based gene delivery vehicles.

Viral vectors currently available for gene therapy are based on different viruses and can roughly be categorized into integrating and nonintegrating vectors. Vectors based on adeno-associated virus and retroviruses (including lentivirus and foamy virus) have the ability to integrate their viral genome into the chromosomal DNA of the host cell, which will possibly achieve lifelong gene expression. Vectors based on adenovirus (Ad) and herpes simplex virus type 1 (HSV-1) represent the nonintegrating vectors. These vectors deliver their genomes into the nucleus of the target cell, where they remain episomal.

In this review, we give an overview of the development of vectors derived from viruses, discussing their specific properties and problems. Furthermore, we give a brief overview of clinical studies using viral vectors.

ADENO-ASSOCIATED VIRAL (AAV) VECTORS

AAV Structure and Replication

AAV is a member of the dependoviruses, a subfamily of the parvoviridae. The virus is nonpathogenic and by itself nonreplicating. As the name suggests, virus replication can be propagated only upon coinfection with a helper virus, which explains why AAV has frequently been found as a contaminant in Ad and herpes virus isolates.

AAV virions are small nonenveloped particles (20–25 nm) that carry a linear single-stranded DNA (ssDNA) genome, which is 4.7 kb in size. Two open reading frames (ORFs), *rep* and *cap*, have been identified in the viral genome and are flanked by T-shaped inverted terminal repeats (ITRs). The *cap* ORF encodes for the structural proteins that form the capsid, whereas the regulatory proteins are produced from the *rep* ORF (Figure 1*A*). [For more details see (1).]

After binding to its receptor, the virus enters the cell through the endocytic pathway and is subsequently transported to the nucleus. Before the viral genome can integrate into the host cell genome, the ssDNA has to be duplicated. This occurs either by annealing with a complementary DNA strand from a second AAV virus or by the host cell machinery. Subsequently, the double-stranded DNA (dsDNA) genome is directed to a specific site in chromosome 19 by *rep* proteins, where it integrates by nonhomologous recombination. After integration, the virus remains silent and persists for the lifetime of the cell.

Subsequent steps in AAV replication depend completely on gene products of unrelated helper viruses, and therefore virus production is propagated only upon coinfection. Ad or herpes virus can serve as a helper virus, and upon infection their viral proteins regulate AAV replication from initiation of transcription to

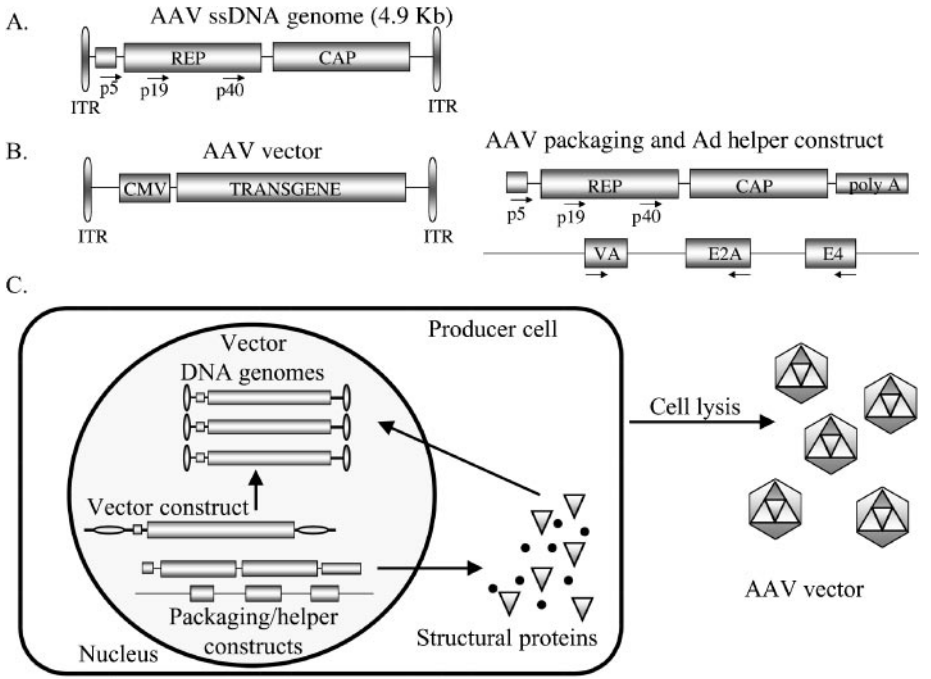

Figure 1 AAV vectors. (*A*) The AAV viral genome containing *rep* and *cap* genes flanked by ITRs. (*B*) In the AAV vector, the *rep* and *cap* genes are replaced by promotor and transgene sequences. The *rep* and *cap* genes are provided from a packaging construct in which expression is regulated by the endogenous promoters p5, p40, and p19. The Ad helper construct provides expression of E2, VA, and E4, which are essential for AAV vector production, whereas Ad E1 genes are provided by the 293 producer cells. (*C*) Nonreplicating AAV vector is produced by simultaneous expression of the viral vector, packaging, and helper constructs in producer cells. Vector ssDNA genomes are packaged by the structural proteins in the nucleus and are released from the cell by lysis.

DNA replication. New virus particles assemble in the nucleus, packaging either plus or minus ssDNA viral genomes, and are released from the cell together with the helper virus. [For more details see (1).]

AAV Vector Development and Production

The AAV viral vector system was initially developed by replacing the viral genes *cap* and *rep* with the transgene sequences. During vector production, *cap* and *rep* sequences are provided from a helper plasmid, and an infectious vector is easily rescued by coinfection with Ad (2). However, the viral vector still contains over 400 bp of the viral genome, allowing recombination between homologous sequences in the helper construct and the viral vector, resulting in the emergence of wild-type AAV.

The next generation of AAV vectors contain only the ITRs and 45 adjacent bp that display *cis*-acting functions essential for virus production and integration (3). In essence, these vectors consist of only a promotor and a transgene flanked by ITRs, preventing the formation of replication competent AAV during vector production (Figure 1). *Rep* and *cap* proteins are produced in *trans* in the packaging cells, whereas coinfection with Ad provides the necessary Ad proteins for initiation of vector replication. Although high-titer AAV vectors can be produced using this system, the coproduced wild-type Ad contaminates these vector preparations.

Recently, new vector production systems have been developed that are free of replicating Ad (4). In this system, the Ad proteins E2A, VA, and E4 are expressed from a second helper construct in 293 cells, which provides E1A and E1B gene products (Figure 1) (5). Furthermore, the reduction of *rep* production from the helper construct prevents the cytotoxicity in the packaging cells, which subsequently improves vector production (6).

AAV Vector Tropism and Transduction

AAV serotype 2 (AAV-2) is commonly used for gene therapy studies and shares the natural tropism of the wild-type virus. The primary receptor for AAV-2 is heparan sulfate proteoglycans, whereas fibroblast growth factor receptor 1 and integrin $\alpha_v\beta_5$ serve as coreceptors and facilitate internalization by endocytosis (7–9). Although AAV-2 displays a broad host range, it has been reported that certain cell types are resistant to AAV-2 infection, probably due to the lack of appropriate receptors. Including AAV-2, five serotypes of AAV have been identified. Sequence analysis of the *cap* proteins reveals considerable diversity, indicating that different receptors and coreceptors can be used among the serotypes. AAV-1, AAV-2, and AAV-3 share homology across the *cap* proteins and are all able to bind to heparan sulfate (7), whereas AAV-4 and -5 show more diversity in the *cap* proteins (10, 11). Indeed, differences in cellular tropism have been observed (12–15). Of all serotypes, AAV-1 shows the highest transduction efficiency in muscle and liver, whereas AAV-5 displays high tropism for retina (12). Furthermore, AAV-5 is able to transduce airway epithelia cells (13), and AAV-3 shows tropism for hematopoietic stem cells (14), which were resistant to transduction by AAV-2. These studies suggest that the use of different AAV serotypes may allow targeting of the vector for tissue-specific transduction. Altering the tropism of AAV vectors has also been explored by chemical cross-linking of bispecific antibodies to the viral capsid (16) and by the insertion of receptor specific epitopes in the *cap* proteins (17, 18).

To establish efficient transgene expression, the ssDNA genome of the vectors has to be converted into a dsDNA, and this appears to be the rate-limiting step in AAV transduction (19). Unless the ssDNA genome is converted into a stable dsDNA genome, it is lost rapidly after transduction. Because transduction occurs in the absence of helper virus and *rep* proteins, AAV relies solely on cellular conditions supporting this event. When the ssDNA is converted into dsDNA by the host cell machinery, transgene expression is increased concomitantly (20, 21).

AAV transduction can occur in the absence of cell cycle; however, transduction efficiency is markedly improved in cells in S-phase (22). Furthermore, activation of the cellular DNA repair machinery also supports second strand synthesis, thus improving AAV transduction (23, 24). The latter suggests that transduction of terminally differentiated postmitotic cells may be hampered to some extent due to insufficient second strand synthesis.

AAV vector dsDNA genomes can persist in transduced cells for long periods of time and are able to form concatamers by head to tail recombination of the ITRs. Integration of single and concatameric genomes occurs randomly in the host cell genome at low frequency because vectors are deprived of all *rep* sequences (22, 25). This indicates that the transgene is predominantly expressed from episomal forms, and expression may decline over time due to loss of the episomal genome by degradation.

The major limitation in the use of AAV as a gene delivery vehicle is the small packaging capacity. In order to deliver a large gene, the unique ability of AAV to form concatamers by head to tail recombination of the ITRs has been explored, increasing delivery size up to 10 kb. In this approach, promotor and transgene sequences are split over two AAV vectors (26, 27). When these two vectors were used to transduce cells, expression of a functional gene was obtained after head to tail recombination of the two viral genomes. Although successful transgene expression was demonstrated using this strategy, transduction efficiency was indeed reduced compared with single vector transduction.

Another hurdle in the use of AAV vectors for gene therapy is the presence of circulating neutralizing antibodies against AAV in the majority of the population as a result of natural infection (28). Furthermore, one single injection of AAV vector elicits a strong humoral immune response against the viral capsid, which interferes with re-administration of the vector (29). The use of AAV vectors containing *cap* proteins from different serotypes may overcome the problems of neutralizing antibodies (11).

RETROVIRAL VECTORS

General Features of Retroviruses (Structure and Replication)

Retroviruses are a large family of enveloped RNA viruses found in all vertebrates and can be classified into oncoretroviruses, lentiviruses, and spumaviruses. The enveloped virus particle contains two copies of the viral RNA genome, which are surrounded by a cone-shaped core (30). The viral RNA contains three essential genes, *gag*, *pol*, and *env*, and is flanked by long terminal repeats (LTR). The *gag* gene encodes for the core proteins capsid, matrix, and nucleocapsid, which are generated by proteolytic cleavage of the *gag* precursor protein. The *pol* gene encodes for the viral enzymes protease, reverse transcriptase, and integrase, and is usually derived from the *gag-pol* precursor. The *env* gene encodes for the envelope glycoproteins, which mediate virus entry.

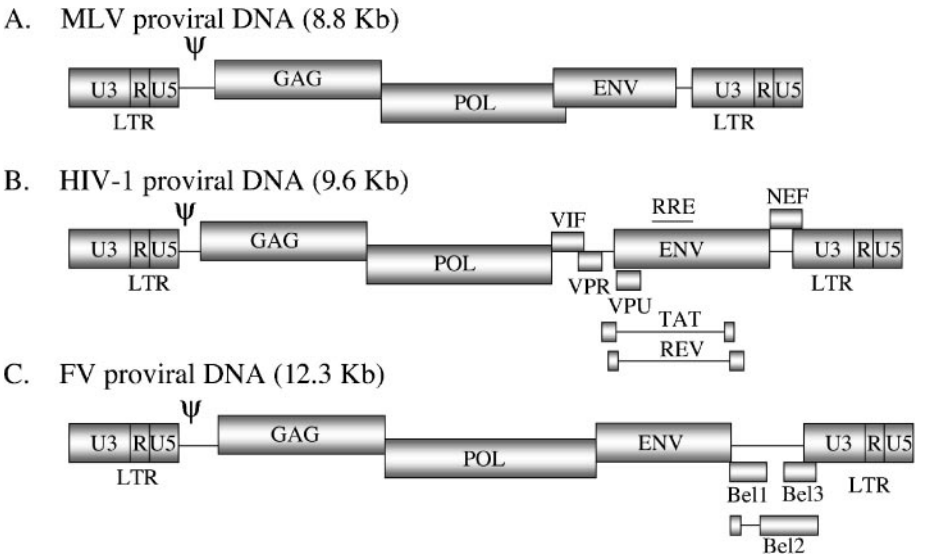

Figure 2 Schematic presentation of the proviral DNA organization of retroviruses. (*A*) The MLV provirus contains *gag*, *pol*, and *env* coding regions flanked by LTRs. The LTR is comprised of three regions, U3, R, and U5, which are essential for reverse transcription, proviral integration, and transcriptional activation. Ψ indicates the packaging signal. (*B*) The HIV-1 proviral DNA encodes for six additional proteins *vif*, *vpr*, *vpu*, *tat*, *rev*, and *nef*, and contains the *cis*-acting element RRE. (*C*) The FV provirus contains three additional ORFs: *bel*1, *bel*2, and *bel*3.

Oncoretroviruses are simple viruses encoding only the structural genes *gag*, *pol*, and *env*, whereas lentiviruses and spumaviruses have a more complex organization and encode for additional viral proteins (Figure 2). Lentiviruses encode three to six additional viral proteins that are essential for virus replication and persistence of infection. Two of the accessory proteins, tat and rev, are present in all lentiviruses and mediate transactivation of viral transcription (31, 32) and nuclear export of unspliced viral RNA, respectively (33). Spumaviruses, also called foamy viruses (FV), contain, in addition to the structural proteins, three ORFs (*tas*/*bel*1, *bel*-2, and *bel*-3), of which *tas*/*bel*1 has been identified as a coactivator of viral transcription (34).

After binding to its receptor, the viral capsid containing the RNA genome enters the cell through membrane fusion. The viral RNA genome is subsequently converted into a double-stranded proviral DNA by the viral enzyme reverse transcriptase. The proviral DNA is heavily associated with viral proteins like nucleocapsid, reverse transcriptase, and integrase (preintegration complex), and translocates to the nucleus where the viral enzyme integrase mediates integration of the provirus into the host cell genome.

Host cell transcription factors initiate transcription from the LTR, and new viral particles are formed at the plasma membrane. *Gag-pol* and *gag* precursors assemble together with two copies of viral RNA, and during the budding process *env* glycoproteins are incorporated into the viral membrane. In the newly formed virion, *gag* and *gag-pol* precursors are subjected to processing by the viral enzyme protease, which results in maturation of the virion. [For more details see (30).]

Retroviral Vector Development and Production

ONCORETROVIRAL VECTORS Viral vectors have been derived from different oncoretroviruses like murine leukemia virus (MLV), spleen necrosis virus, Rous sarcoma virus, and avian leukosis virus, but MLV-based vectors are most frequently used.

Replication-defective MLV vectors are generated by replacing all viral protein encoding sequences with the exogenous promotor-driven transgene of interest (35). Besides the packaging signal, the viral LTRs and adjacent sequences, which are essential for reverse transcription and integration, remain in the vector (36). In this system, vector RNA production is driven by the U3 region of the LTR and results only in low titers of the vector due to the low transcriptional activity of the LTR. Therefore, the U3 of the 5′ LTR is replaced by a CMV promotor resulting in a CMV/LTR hybrid with high transcriptional activity (37). The 3′ U3 region of the LTR remained intact and is copied over to the 5′ LTR during reverse transcription, allowing efficient integration and LTR-driven transgene expression in the transduced cell.

Major concerns in the use of retroviral vectors are the possibility of vector mobilization and recombination with defective (endogenous) retroviruses in the target cell. This led to the development of self-inactivating vectors (SIN) (38). In these vectors, the viral promotor and enhancer regions in the 3′ U3 are deleted, thus preventing LTR-driven transcription in the transduced cells. Furthermore, transgene expression in these vectors is exclusively driven by an internal promotor, which improves the use of regulatory and tissue-specific promoters.

For the packaging of retroviral vectors, the structural proteins are provided in *trans* in packaging cells. The first packaging cell lines expressed *gag*, *pol*, and *env* from a complete proviral DNA lacking only the packaging signal (35). However, sequence homology between the vector and packaging constructs facilitated recombination, resulting in the generation of replication-competent virus. To prevent homologous recombination, packaging cells have been developed expressing *gag*/*pol* and *env* from separate constructs. Furthermore, expression from the packaging constructs is no longer driven by the viral LTR, but by constitutive promoters, thus allowing a high level of virus production (35, 39).

LENTIVIRAL VECTORS Although lentiviral vectors based on primate as well as nonprimate lentiviruses have been developed, lentiviral vector development has mainly focused on human immunodeficiency virus type 1 (HIV-1) because this virus has been studied extensively. Besides the structural proteins, HIV-1 encodes for six

additional accessory proteins (*tat*, *rev*, *vif*, *vpr*, *nef*, and *vpu*) that play an important role in the virus replication and persistence of infection (Figure 2*B*). For example, *tat* transactivates viral transcription (31, 32) and *rev* facilitates nuclear export of unspliced viral RNA (33).

The development of lentiviral vectors closely resembles the retroviral vector design. Similar to retroviral vectors, the HIV-1-based lentiviral vector is deprived of all viral sequences apart from the *cis*-acting sequences like LTRs and the packaging signal. In addition, the *rev* responsive element (RRE) is included in the vector. This region in the viral RNA binds the *rev* protein, which is provided in *trans*, ensuring efficient nuclear export of the full-length viral RNA genomes (40).

Vector RNA expression in this system is driven by the endogenous LTR and relies on transactivation by the *tat* protein for its transcriptional activity. Subsequent construction of a CMV/LTR hybrid markedly increased vector production and, more importantly, made vector production independent of *tat* expression (41).

Furthermore, the biosafety of the vectors is markedly improved by the development of SIN vectors. These vectors contain large deletions in the transcriptional activation unit in the 3′ U3 region of the LTR (42, 43), which results in inactivation of LTR and reduces the risk of recombination with wild-type virus. Recently, another *cis*-acting sequence has been added to the vector. This sequence, the central polypurine tract (cPPT) from the *pol* ORF, has been demonstrated to improve nuclear import of the proviral DNA and subsequently accelerates transduction (Figure 3) (44, 45).

During lentiviral vector production, all of the essential viral gene products are provided in *trans* from a packaging construct. The first versions of the packaging construct consist of the full-length proviral DNA lacking a packaging signal and contain a frame shift in the *env* and *vpu* ORF leaving the RRE intact, thus promoting nuclear export of the mRNAs in the presence of *rev*. Furthermore, the LTRs are removed from the construct and transcription is driven by a CMV promotor, which allows a high level of viral protein expression and reduces the sequence homology between the vector and packaging construct (40).

To improve the biosafety of the lentiviral vectors, all nonessential viral sequences are removed from the packaging constructs. The HIV-1 accessory genes *vif*, *vpr*, *nef*, and *vpu* are dispensable for lentiviral vector production and transduction and were deleted from the packaging construct (41). Subsequent modifications in the transfer vector constructs made vector production independent of *tat* expression, resulting in the third generation packaging system containing only the coding region for *gag*/*pol* and the *cis*-acting RRE, whereas *rev* is expressed from a separate construct (Figure 3) (46).

Given the restricted host range of the HIV-1 *env* glycoprotein, lentiviral vectors are pseudotyped with the vesicular stomatitis virus glycoproteins (VSV-G). The VSV-G *env* displays a broad host range and is expressed from a distinct construct during vector production (Figure 3) (40, 41, 46).

Recently, stable packaging cell lines have been developed. These stable producer cells express the structural proteins from minimal packaging constructs and

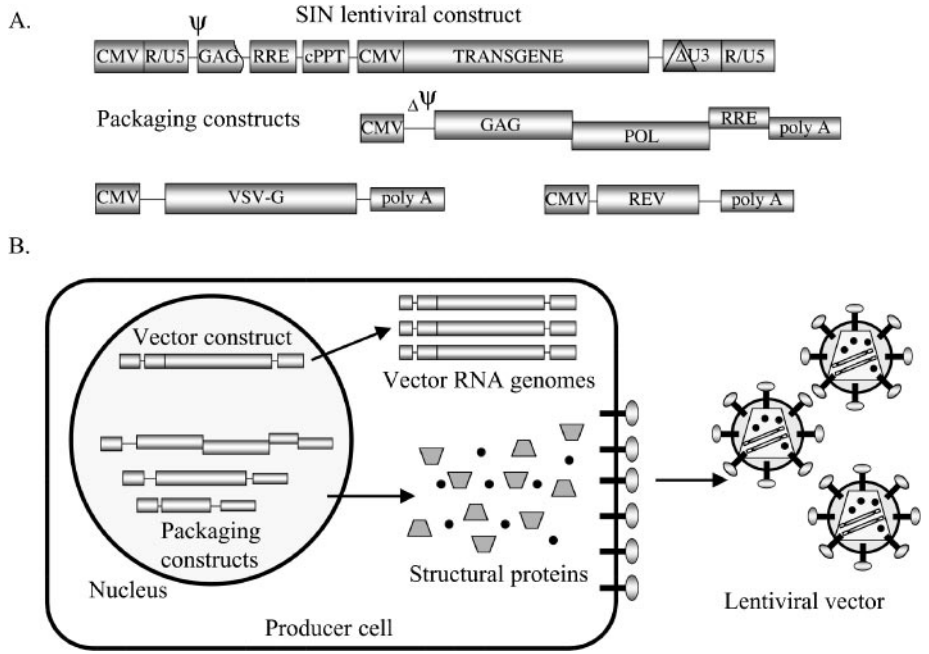

Figure 3 Lentiviral vectors. (*A*) In the HIV-1-based lentiviral vector, the viral genes *gag*, *pol*, and *env* have been replaced by promotor and transgene sequences and are flanked by the viral LTRs. Packaging of the viral RNA genome is ensured by the presence of the packaging signal (Ψ) comprised of the 5′ untranslated region and the 5′ sequence of the *gag* ORF. In addition, the vector contains two additional *cis*-acting sequences, the RRE, which is essential for nuclear export of unspliced viral RNA in the presence of *rev*, and the cPPT, which supports nuclear import of the proviral DNA in the transduced cell. The 3′ LTR contains a large deletion in the U3 region (depicted as ΔU3) to prevent transcription from the LTR. The lentiviral packaging system consists of three constructs encoding for *gag*/*pol*, VSV-G, and *rev*. The *gag*/*pol* construct contains the *cis*-acting RRE and requires expression of *rev* for efficient nuclear export similar to the vector construct. (*B*) Lentiviral vectors are produced by transient transfection of the vector construct together with the packaging constructs in producer cells. Vector RNA genomes are packaged by *gag* and *gag*/*pol* precursor proteins at the cellular membrane. Subsequently, the vector particles bud through the cellular membrane, obtaining the viral envelope that contains the VSV-G glycoproteins.

expression is driven by an inducible promotor to minimize the toxicity of the VSV-G envelope protein (47–49).

FOAMY VIRAL VECTORS FV vectors have only recently been developed and are quite similar to retroviral and lentiviral vectors. In addition to the packaging signal that consists of the 5′ untranslated region and the 5′ portion of the *gag* ORF present

in all retroviral vectors, FV vectors contain the 3′ region of the *pol* ORF, which is critical for efficient packaging of these vectors (50, 51). Similar to other retroviral vectors, the 5′ U3 region of the LTR in the vectors plasmid has been replaced by a CMV promotor, which increased vector expression and made vector production independent of *tas*/*bel1*.

FV vectors are produced by transient transfection of the vector construct and the packaging constructs encoding for the structural proteins *gag*, *pol*, and *env* in 293T cells. Because the FV envelope has a broad cellular host range, it is used by the vector and therefore the *env* sequences are included in the packaging construct or expressed from a separate construct (50, 51). The FV *env* contains, in contrast to other retroviruses, an ER sorting signal, which allows FV particles to bud from intracellular membranes, and, therefore, the majority of the infectious virions are cell associated (52). Consequently, the infectious particles have to be released from the packaging cells by freeze-thawing.

Retroviral Cellular Tropism and Transduction

The limited cellular tropism of the natural envelope of wild-type viruses is one of the barriers for retroviral transduction. However, retroviruses have the ability to incorporate *env* glycoproteins from related as well as unrelated viruses, thus allowing pseudotyping with alternative glycoproteins. Retroviral vectors are usually pseudotyped with glycoproteins from the VSV-G, a member of the rhabdovirus family, or the amphotropic MLV envelope in order to broaden their host range (53–59). Moreover, pseudotyping allows transfer of specific tropisms to the vector. Neurotropism and retrograde axonal transport were accomplished by the vector by pseudotyping with the G protein of Mokola lyssaviruses (60, 61), and the filovirus (Ebola Zaire) envelope supported transduction of airway epithelia, whereas only minimal transduction was observed with VSV-G (62). Additionally, glycoproteins obtained from other retroviruses (MLV 10A1, gibbon ape leukemia virus, RD114/endogenous feline virus) have been used for pseudotyping, which proved to be useful for transduction of hematopoietic progenitor cells (63, 64).

Interestingly, the entry pathway of the retroviral vector has evidently no effect on the transduction efficiency. Retroviral glycoproteins mediate virus entry by membrane fusion, whereas VSV-G pseudotyped vectors enter the cell through the endocytic pathway. Apparently, the virus is able to escape from the endosome by membrane fusion, induced by VSV-G at low pH, without being degraded.

Reverse transcription and nuclear translocation of the preintegration complex are thought to be limiting steps in retroviral transduction, especially in terminally differentiated postmitotic cells. Proviral DNA synthesis of all retroviruses depends strongly on cellular conditions, and low nucleoside pools or absence of cellular cofactors may explain the incomplete reverse transcription in quiescent or stationary cells (56, 65–70).

In contrast to other retroviral vectors, FV vector particles can contain fully reverse transcribed viral DNA-activation through reverse transcription before

virus assembly (71, 72). This suggests that FV vector gene transfer may be more efficient in certain postmitotic cells in which reverse transcription is limited.

A major limitation in the use of oncoretroviral vectors is their inability to transduce nondividing cells. Oncoretroviruses are unable to transport their preintegration complex containing the proviral DNA across the nuclear membrane in the absence of cell division. During mitosis, the nuclear membrane breaks down and only then is the large preintegration complex able to enter the nucleus (73, 74).

Lentiviral vectors on the other hand are able to transduce nondividing cells. The HIV-1 preintegration complex has karyophilic properties due to the presence of nuclear localization signals (NLS) in the viral proteins matrix and integrase. These unique features allow the preintegration complex to cross the nuclear membrane using the cellular nuclear import machinery in the absence of mitosis (75–78).

FV, however, is unable to replicate in nondividing cells despite efficient nuclear localization of the preintegration complex due to a NLS in *gag* (79, 80). Nevertheless, efficient transduction of postmitotic cells has been demonstrated using FV vectors in which transgene expression is driven by an internal CMV promotor (81), indicating that virus replication in nondividing cells is probably blocked at the transcriptional level.

Because HIV-1 is a human pathogen, there is some concern about the use of HIV-1-based lentiviral vectors. The current HIV-1 lentiviral vector system is deprived of all accessory proteins (except *rev*) and viral sequences in the vectors have been minimized; therefore, replication of these vectors is highly disabled and the possibility of homologous recombination is minimized. In addition, codon-optimization of the packaging construct further decreases the risk of homologous recombination. Furthermore, changes in the codons makes the production of the structural proteins independent of *rev*, and therefore additional viral sequences (RRE) can be eliminated from the packaging construct (82).

The use of vectors based on other primate lentiviruses (83, 84) may also eliminate some of the concerns; however, HIV-2 and simian immunodeficiency virus (SIV) are closely related to HIV-1. Therefore, vectors based on nonprimate lentiviruses like feline immunodeficiency virus (FIV), equine infectious anemia virus, and visna may be more acceptable (85–87). These viruses do not cause infection in humans due to restrictions in the envelope tropism. However, the risk associated with the introduction of nonhuman lentiviral vectors in human tissues is unknown, and the actual safety of these lentiviral vectors remains to be evaluated.

Because many steps in lentiviral infection (reverse transcription, nuclear transport, and integration) depend on cellular cofactors (65, 66, 68–70), there may be serious limitations in the use of nonhuman lentiviral vectors in primary human tissues. Replication of lentiviruses is highly adapted to their natural host, indicating that cross species variability of cellular factors essential for virus replication, and thus vector transduction, may impair the transduction efficiency of nonhuman lentiviral vectors in human cells. These restrictions may be overcome by the use of chimeric lentiviral vectors. Indeed, cross-packaging of FIV RNA by HIV-1 and SIV packaging systems has been demonstrated, and viral proteins were able to

support transduction, indicating that recognition of *cis*-acting sequences is highly promiscuous among lentiviruses (88). Furthermore, cross-packaging of nonhuman viral RNA by HIV-1-based virions may further eliminate any sequence homology between the vector and packaging constructs. However, for biosafety reasons, the introduction of chimeric lentiviral vectors for gene therapy is less desirable.

ADENOVIRAL VECTORS

Adenovirus Structure and Replication

Ad are icosahedral particles existing of a viral capsid that surrounds the viral core containing the large DNA genome of 36 kb. The viral linear dsDNA genome coding region is flanked by ITRs, and contains five early transcription regions (E1A, E1B, E2, E3, E4) and one late transcription region from which five families of late mRNAs (L1-5) are generated (Figure 4*A*). The Ad genome is intimately associated with viral proteins (core) and is packaged in the viral capsid, which consists primarily of three proteins: hexon, penton base, and knobbed fibers (89).

The Ad replication cycle can be divided in two phases, early and late. During the early phase of the replication cycle, the viral DNA is transported to the nucleus and transcription of early viral genes is initiated. Early gene products interfere with antiviral host cell defense mechanisms and direct the host cell to enter the cell cycle, supporting transcription and DNA replication. As soon as DNA replication is initiated by the E2 gene product, late events in the viral replication start. During this phase, gene expression of mRNA regulated by the late promotor increases, which results in high production of structural proteins that assemble together with viral genomes in the nucleus. The newly synthesized virions are released from the cell by the induction of cell lysis. [For more details see (89).]

Adenoviral Vector Development and Production

The first recombinant Ad vectors have been generated by deleting the E1 and/or E3 gene regions in the viral genome, allowing the introduction of promotor and transgene sequences up to 6.5–8.3 kb (90, 91). However, removal of the E1 gene from the vector, markedly hampers transcription of E2 genes and, consequently, DNA replication and production of structural viral proteins. E1-deleted Ad vectors can be efficiently propagated in 293 cells, complementing E1 gene products (5). Moreover, deletions in the E2 and/or E4 regions were made that increased the packaging capacity of the vector but also required the development of suitable complementing cells additionally expressing E2 and/or E4 (92, 93).

One major concern in the use of Ad recombinant vectors is the emergence of replication competent Ad virus as a result of recombination events between the viral sequences. Ad protein expression by replication-competent Ad virus, but also from the vector itself, results in the in vivo induction of a potent immune response.

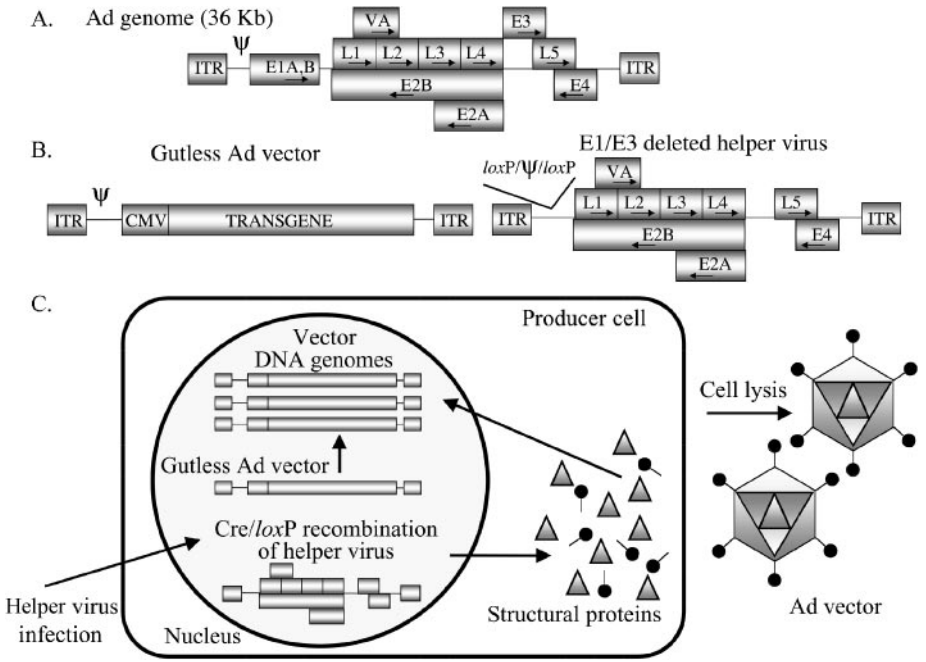

Figure 4 Gutless Ad vectors. (*A*) Schematic presentation of the organization of the Ad viral genome. (*B*) Gutless Ad vectors contain, besides promotor and transgene sequences, the *cis*-acting ITRs and the packaging signal (Ψ). Structural and regulatory Ad genes essential for vector production are provided from an E1/E3 deleted helper virus in which the packaging signal is flanked by *lox*P sites. (*C*) The gutless Ad vector is produced in 293 cells, which complement for the Ad E1 and express Cre recombinase. The vector construct is transiently transfected into the producer cells and vector production is propagated by infection with the E1/E3 deleted helper virus. Packaging of the helper virus is prevented by excision of the Ψ element by Cre/*lox*P recombination.

The immune system eliminates transduced cells that express viral proteins as well as the transgene, and therefore only transient transgene expression is observed.

More recently, Ad vectors have been developed that are deprived of nearly all viral genes. These so-called gutless Ad vectors retain only the viral ITRs and the packaging signal and require a helper virus for their replication (94). The helper virus, in general, is an E1/E3 deleted recombinant Ad vector that provides the necessary viral proteins in *trans*. However, the helper virus is also packaged in this system, resulting in the generation of replication competent Ad. To prevent packaging of the helper virus genome, the packaging signal is removed by Cre/*lox*P recombination in the producer cells expressing Cre recombinase (Figure 4). This helper virus maintains the ability to facilitate efficient virus replication but minimizes the generation of replication competent virus.

Because the majority of the viral coding sequences have been deleted in the gutless Ad vector, the addition of stuffer DNA is essential to maintain the optimum packaging size of the vector (95). Moreover, removal of viral genes from the vector prevents the induction of an immune response, and long-term gene expression can be obtained using the gutless Ad vector.

Adenoviral Vector Tropism and Transduction

Ad serotype 5 is commonly used for gene therapy studies, and consequently the Ad vector host range is similar to that of the wild-type virus. Recently, the viral receptor and coreceptor of this serotype have been identified. The virus binds to the cellular receptor CAR (coxsackievirus and adenovirus receptor) (96), a member of the immunoglobulin superfamily, through the knob of the fiber, and virus entry occurs through clathrin-mediated endocytosis after binding of the penton to integrins ($\alpha V\beta 3$ and $\alpha V\beta 5$ integrin) (97). Although the receptors for Ad are ubiquitously expressed, inefficient transduction due to low level expression of the receptors has been observed in some tissues like airway epithelia (98). To permit virus entry in cells lacking the CAR receptor or to target specific cell types, retargeting of Ad vectors was essential, and this has been explored extensively. Bispecific conjugates or retargeting complexes that cross-link the virus with alternate receptors have been developed. Retargeting complexes have been designed using either Ad neutralizing antibodies (fiber or penton base specific) chemically linked to ligands or antibodies specific for cellular receptors (99–101) or fusion proteins of the ectodomain of the Ad receptor CAR and ligands (102). Using this method, retargeting of Ad vector via epidermal growth factor (99, 102) and E-selectin (101) has been demonstrated. Alternatively, the Ad vector host range can be altered by incorporation of binding motifs in the C terminus of the fiber protein and by modifications in the RGD motif of the penton base (103–106).

Differences in the host-range of Ad serotypes indicate that besides CAR, other cellular receptors are involved, and consequently, the cellular tropism of Ad vectors can also be altered by the use of alternative serotypes. For instance, Ad17 has enhanced tropism for airway epithelia (107), Ad35 is able to infect hematopoietic progenitor cells (108), and Ad subgroup D shows higher infectivity in the central nervous system (109).

Ad vectors efficiently transduce dividing as well as nondividing cells, and high levels of transgene expression have been observed from the episomal dsDNA genome. However, the synthesis of viral proteins from the vector elicits a strong cellular and humoral immune response (110). This results in clearance of the transduced cells by cytotoxic T cells, whereas the humoral immune response precludes re-administration of the vector.

The development of the gutless Ad vector, which is deprived of nearly all viral sequences, prevents elimination of transduced cells by the immune system, thus allowing long term transgene expression. However, the vector DNA genome exists episomally and is nonreplicating. Therefore, transgene expression may be lost over

time due to dilution in replicating cells, whereas the episomal genome is subjected to degradation in nondividing cells. Recently, Ad/AAV and Ad/retrovirus hybrid vectors have been developed (111, 112). These vectors are devoid of all viral genes, and the transgene sequence is flanked by AAV ITRs or retroviral LTRs. These *cis*-acting elements from unrelated viruses allow integration of the transgene by host cell enzymes in the absence of viral proteins (AAV *rep* and retroviral integrase) supporting these events. Successful transduction and integration has been observed using these hybrid vectors, albeit at low efficiency.

One of the major concerns in the use of Ad vectors for gene therapy purposes is the induction of an innate immune response. Administration of an Ad vector causes inflammation of the infected tissue, especially in the liver (110, 113–115). The induction of chemokine production at the infected site attracts neutrophils, leading to necrosis and apoptosis in the liver. The development of gutless Ad vectors markedly reduce inflammatory responses and cellular infiltration (116). However, inflammation induced by the viral capsid proteins itself cannot be prevented (110).

HERPES SIMPLEX VIRUS TYPE 1 (HSV-1) VECTORS

HSV-1 Structure and Replication

The HSV-1 virion is approximately 20 nm in diameter and consists of four components: envelope, tegument, capsid, and viral genome. The envelope is derived from the cellular membrane and contains approximately 12 viral glycoproteins essential for viral entry. The tegument is the protein layer between the capsid and the envelope and contains at least 10 viral proteins, including VP16 (essential for transactivation and virion envelopment), VP22 (membrane translocation domain), and virion host shut off (*vhs*) protein. The capsid consists of 7 viral proteins and contains the linear dsDNA genome, which is 152 kb in size.

The viral genome can be divided in unique long (U_L) and unique short (U_S) regions flanked by terminal repeats (TR), and encodes for at least 80 viral proteins. However, the function of a large number of these viral proteins is still unknown.

The fate of HSV-1 infection can be either lytic or latent. During the lytic replication cycle, which occurs in mucosal or epithelial cells, the host cell protein synthesis is shut off by the tegument *vhs* protein immediately after infection. Subsequently, the viral capsid releases the viral DNA into the nucleus where it will circularize.

Within several hours after infection, protein expression from the circular genome is initiated. Protein expression occurs in a highly regulated fashion and can be divided into three groups of sequentially expressed proteins: α- or immediate early genes, β- or early genes, and γ- or late genes. Once β-gene products are present, DNA replication and γ-gene production is initiated and progeny virus is produced. In the nucleus, capsid proteins assemble together with the viral DNA genomes and the newly formed viral capsids bud through the nuclear membrane. On their way

to the Golgi apparatus, the virion obtains the tegument and the viral envelope and, subsequently, the virus is released from the cell through secretory vesicles.

In neurons, HSV-1 infection can result in a latent infection. Upon entry, the virion is transported to the nucleus by retrograde transport along the axon. It is currently unknown which viral genes are involved in the establishment of a latent infection, but de novo viral protein synthesis is not required. However, latency is related to the expression of latency associated transcripts (LAT), which are expressed from a promotor that is highly active in neurons. LATs prevent the lytic replication cycle by down-regulation of genes associated with lytic infection. Reactivation of the latent virus can be induced by different stimuli like stress and UV irradiation. [For more details see (117).]

HSV-1 Vector Development and Production

The development of vectors based on HSV-1 has produced two different viral vector systems: recombinant HSV-1 vectors and HSV-1 amplicons.

Recombinant HSV-1 vectors contain a number of deletions in the α-genes and the *vhs* and can harbor large transgenes up to 30 kb in size (118, 119). These vectors are nonreplicating and can be propagated in complementing cell lines providing the essential α-genes in *trans*. Nevertheless, recombinant HSV-1 vectors still retain large proportions of the HSV-1 genome and can express viral genes that induce cytotoxicity and immune responses. Moreover, transgene expression by recombinant HSV-1 vectors is usually transient.

The HSV-1 amplicon vector system is based on the ability of HSV-1 to package defective genomes containing the *cis*-acting sequences *ori* (origin of viral DNA replication) and *pac* (packaging and cleavage signal). HSV-1 amplicon vectors contain, besides these *cis*-acting elements, no viral genes (120–122). However, packaging of the amplicon vectors requires a replicating helper virus, resulting in high level contamination with replication competent virus. This problem has been overcome by the development of a helper-free packaging system in which viral genes are provided in *trans* from five cosmids spanning the HSV-1 genome but lacking the *pac* signal (123). This packaging system markedly decreases the generation of replication competent virus and cytotoxicity; however, only low titers of amplicon vectors are generated. Recently, the entire HSV-1 genome lacking the *pac* signal has been cloned as a bacterial artificial chromosome (*bac*) that simplifies amplicon packaging and results in increased vector titers (124, 125).

HSV-1 Vector Tropism and Transduction

HSV-1 virus entry is mediated by multiple glycoproteins present in the envelope and is a rather complex process. Initial adhesion to the cellular membrane is mediated by the interaction of gC and gB with glycosaminoglycan heparan sulfate. Subsequently, gD binds to a specific cellular receptor, either a member of the TNF receptor family [herpes virus entry mediator A (HveA)], immunoglobulin superfamily (HveB, HveC), or 3-O-sulfated heparan sulfate. Then, the virus enters the cell through membrane fusion promoted by the gH/gL complex and gB (117).

Although the HSV-1 envelope has a broad host range, efforts to target and alter the cellular tropism of the vectors have been made. The HSV-1 vector host range can be altered by the incorporation of the VSV-G envelope, which circumvents the receptor-specific binding of gD and supports attachment and entry of gD-deficient HSV-1 vectors, albeit at low levels (126). Furthermore, construction of a chimeric gC-containing ligands for cellular membrane receptors allows targeting of HSV-1 vector deficient for gC and lacking the receptor-binding domain in gB. However, the vector enters the cell by endocytosis instead of membrane fusion, which results in degradation of the vector in the endosome (127).

The major problem of recombinant HSV-1 vectors is their cytopathic effect and the induction of an immune response by viral gene expression. The development of amplicon vectors and a helper virus–free packaging system has overcome this problem to a great extent (123–125). However, additional deletion of nonessential genes from the *bac* packaging system may be necessary to further prevent the cytotoxicity of this vector system.

The large packaging capacity of HSV-1 amplicons (up to 152 kb in theory) may be very useful for gene therapy purposes to deliver complex genes and regulatory sequences or to deliver multiple copies of the transgene. However, long-term gene expression using HSV-1 amplicons has not been demonstrated. Because the amplicon DNA exists extra-chromosomal in a circular form and does not integrate, it is subjected to loss by cell division and degradation. The incorporation of elements from the Epstein-Barr virus (*oriP* and EBNA-1) has been shown to maintain the viral DNA and subsequently prolongs transgene expression (123).

VIRAL VECTORS IN CLINICAL TRIALS

In recent years, many clinical trials have been conducted and the first successes have been reported. Most prominent is the treatment of two young children suffering from a fatal form of severe combined immunodeficiency-X1 (SCID-X1). This disease is an X-linked hereditary disorder characterized by an early block in the development of T and natural killer (NK) cells due to mutations in the γc cytokine receptor subunit. Hematopoietic stem cells from the patients were stimulated and transduced ex vivo with an MLV-based retroviral vector expressing the γc cytokine receptor subunit and were re-infused into the patients (128). During a 10-month follow-up, γc-expressing T and NK cells could be detected, and cell counts and function were comparable to aged-matched controls. The selective advantage of the γc-expressing lymphocyte progenitors, enabling the development of mature T and NK cells, contributed considerably to the success of this study. Two additional patients have since been treated and the outcome is very promising. However, earlier attempts to treat SCID patients suffering from adenosine deaminase (ADA) deficiency using retroviral vectors failed to show a long-term beneficial effect even though long-term reconstitution from transduced progenitor cells was observed at low levels. Patients in these studies received, in addition to the transduced cells, ADA enzyme preparations, which

may have prevented the selective outgrowth of the transduced progenitor cells (129–133). Indeed, discontinuation of ADA replacement therapy showed a selective increase in the number of peripheral blood mononuclear cells containing the transgene. Although T cell counts and function remained within normal limits, a loss of B and NK cells was observed in these patients. Moreover, the accumulation of toxic adenosine metabolites in erythrocytes required reinstatement of the therapy (133, 134) and indicated that ADA production was insufficient due to limited numbers of transduced cells or low ADA expression levels.

Although successful transduction of hematopoietic stem cells has been demonstrated using MLV-based retroviral vectors, the extensive manipulation of the hematopoietic stem cells to achieve efficient transduction is undesirable and may induce differentiation of the early progenitors. The development of lentiviral vectors, which are able to transduce hematopoietic progenitor in the absence of cytokines, may further improve stem cell gene therapy. Although these vectors have not yet been approved for use in clinical trials, some remarkable results have been obtained in animal models. Lentiviral vectors were successfully used to introduce a functional β-globin gene into hematopoietic stem cells and corrected β-thalassaemia and sickle cell disease in mice models (135, 136). Furthermore, lentiviral vectors hold great promise in the treatment of neurological diseases as demonstrated in a rhesus monkey model for Parkinson's disease (137) and a mouse model for metachromatic leukodystrophy, a lysosomal storage disease affecting the central nervous system (138).

In another clinical study, patients suffering from hemophilia B, which is a bleeding disorder caused by a deficiency of coagulation factor IX, were treated with AAV vectors expressing human factor IX (139). These patients participated in a Phase I trial and received intramuscular injections of AAV vectors. Although only very low levels of secreted factor IX could be detected in the plasma of one patient, the treated patients showed some clinical benefits and a reduced intake of factor IX infusions. Moreover, no vector-related toxicity and germ line transmission was observed.

The treatment of genetic diseases using Ad vectors has recently been tempered. As a result of in vivo studies, it became clear that administration of Ad vector induced a potent immune response and inflammation of the transduced tissue (113, 114). The significance of this problem became even more evident after the tragic death of a participant in a Phase I trial receiving Ad vector therapy (115), emphasizing the need to understand and control the host responses.

CLOSING REMARKS

This review focuses on viral vectors commonly used in gene therapy studies. Their specific properties are summarized in Table 1. Although remarkable progress has been made in the development of viral vectors, the ideal vector remains elusive. The wide variety of diseases that may benefit from gene therapy will mandate specific

TABLE 1 Properties of viral vector systems

		Retroviral			Adenoviral		HSV-1	
	AAV	**Onco**	**Lenti**	**Foamy**	**Recomb**	**Gutless**	**Recomb**	**Amplicon**
Viral genome	ssDNA	RNA	RNA	RNA dsDNA	dsDNA	dsDNA	dsDNA	dsDNA
Packaging size[a] (transgene)	4.9	8.8	9.6	12.3	36 (8.3)	36	152 (30)	152
Integration	Y/N[b]	Y	Y	Y	N	N	N	N
Nondividing cells	Y	N	Y	Y	Y	Y	Y	Y
Duration of expression	L	L	L	L	S	S/L	S	S
Induction of CTL response	N	N	N	N	Y	Y	Y	Y
Pre-existing immunity	Y	N	N[c]	N	Y	Y	Y	Y
Safety concerns	Insertional mutagenesis		Insertional mutagenesis		Inflammation cytotoxicity		Inflammation cytotoxicity	

[a]Size of the viral genome packaged by the virion (Kb).

[b]Integration is inefficient in the absence of *rep* protein.

[c]With the exception of HIV-1 patients.

Y: yes; N: no; L: long; S: short.

requirements of viral vectors, such as tissue-specific transduction and regulated gene expression. Therefore, it is unlikely that one single vector system will suffice for all gene therapy purposes. Nevertheless, gene therapy approaches have great promise to influence human health in the future.

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K^+ Channel Structure-Activity Relationships and Mechanisms of Drug-Induced QT Prolongation

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■ **Abstract** Pharmacological intervention, often for the purpose of treating syndromes unrelated to cardiac disease, can increase the vulnerability of some patients to life-threatening rhythm disturbances. This may be due to an underlying propensity stemming from genetic defects or polymorphisms, or structural abnormalities that provide a substrate allowing for the initiation of arrhythmic triggers. A number of pharmacological agents that have proven useful in the treatment of allergic reactions, gastrointestinal disorders, and psychotic disorders, among others, have been shown to reduce repolarizing K^+ currents and prolong the QT interval on the electrocardiogram. Understanding the structural determinants of K^+ channel blockade may provide new insights into the mechanism and rate-dependent effects of drugs on cellular physiology. Drug-induced disruption of cellular repolarization underlies electrocardiographic abnormalities that are diagnostic indicators of arrhythmia susceptibility.

INTRODUCTION

The delicate synchronization of cardiac ion channels underlies the spread of cardiac electrical excitation that is coupled to continuous rhythmic contraction of the heart. The disruption of the precise ionic balance resulting from administration of pharmacological agents or congenital defects may undermine the cardiac electrical syncytium, which can undermine coordinated contraction and lead to insufficient pressure for blood circulation.

Cardiac excitation originates in the sino-atrial node and propagates through the atria into the atrial-ventricular node. The impulse then enters the Purkinje conduction system, which delivers the excitatory wave to the ventricles. Ventricular excitation spreads from the endocardium to the epicardium and is coupled to the

contraction of the ventricles that generates systolic blood pressure. The wave of excitation that spreads over the heart reflects membrane depolarization of cardiac myocytes, due primarily to activation of fast voltage-dependent Na^+ channels that underlie the action potential upstroke. Activation is followed by a long depolarized plateau phase that permits Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum, binding of Ca^{2+} to contractile proteins on the sarcomeres, and coordinated contraction (systole). Repolarization follows due to the activation of time- and voltage-dependent activation of repolarizing potassium currents. Relaxation of contraction is coupled to the electrical repolarization phase, which allows filling of the ventricles (diastole) prior to the next excitation. Each of these electrical processes can be detected on the body surface electrocardiogram (ECG) as a signal average of the temporal and spatial gradients (∇Vm) generated during each phase (1–3) (ECG, Figure 1*A*). Electrical excitation gradients in the atria (atrial depolarization) manifest on the ECG as P waves, whereas gradients of ventricular depolarization are seen as the QRS complex. Gradients in ventricular repolarization are reflected in the T wave (Figure 1).

Electrocardiographic abnormalities are related to changes in cellular action potential morphologies, which may be due to altered cell-to-cell coupling, congenital ion channel abnormalities, drug intervention, or electrolyte imbalance (1–3). Conduction abnormalities can be detected as changes in the QRS complex. Widening of the QRS reflects reduced conduction velocity, which typically

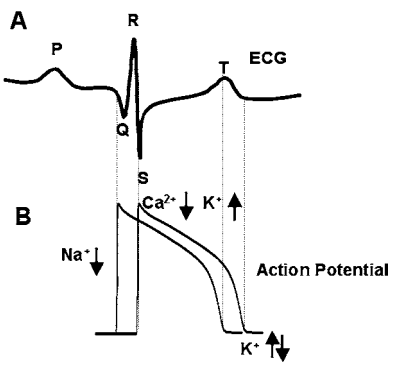

Figure 1 Electrical gradients in the myocardium can be detected on the body surface ECG. (*A*) An illustrative example of a single cardiac cycle detected as spatial and temporal electrical gradients on the ECG. The P wave is generated by the spread of excitation through the atria. The QRS complex represents ventricular activation and is followed by the T wave reflecting ventricular repolarization gradients. (*B*) Schematic representation of cellular electrical activity underlying the ECG [see text and (1) for details]. Arrows indicate the direction of ion flow during each phase of the action potential.

stems from altered Na^+ channel function (4). ST segment elevation reflects transmural voltage gradients during the action potential plateau, a hallmark of congenital forms or drug-provoked Brugada syndrome (5–7). Prolongation of the action potential duration (APD) (delayed repolarization) results in long QT intervals and may result in morphological changes in the T wave that can provide insight as to the underlying cellular mechanism of APD prolongation (1, 8–10).

Individuals displaying ECG abnormalities may be at higher risk of lethal arrhythmias associated with syncope and sudden death. Many such arrhythmic events are rate dependent and may be linked to sudden changes in heart rate due to exercise or auditory stimulation that may trigger life-threatening arrhythmias (10–14).

Pharmacological intervention, often for the purpose of treating syndromes unrelated to cardiac disease, can increase the susceptibility of some patients to life-threatening rhythm disturbances (15–17). This may be due to underlying vulnerability stemming from genetic defects or polymorphisms, gender, or structural abnormalities that provide a substrate allowing for the initiation of arrhythmic triggers (16–19). Indeed, a number of histamine-blocking drugs, including antihistamines astemizole and terfenadine and more recently loratadine, have been shown to block HERG (human ether-a-go-go-related gene) channels as an adverse side effect and prolong the QT interval of the ECG (20). Cisapride (Propulsid®) is a gastrointestinal agent used to treat nocturnal heartburn as well as a variety of other gastrointestinal disorders, which also as a side effect blocks HERG K^+ channels and is associated with acquired long QT syndrome and ventricular arrhythmias, such as torsades de pointes (21, 22). In a study of patients with paranoid psychosis, changes in the morphology of the ECG T wave were observed in more than 85% of traces when the plasma concentration of the antipsychotic drug thioridazine was greater than 1 μM (23) due to blockade of I_{Kr} (IC_{50}, 1.25 μM) and I_{Ks} (IC_{50}, 14 μM). Unfortunately, examples of inadvertent side effects of pharmacological agents on cardiac K^+ channels are plentiful (Table 1). As a result, screening affinity of new therapeutic agents for cardiac K^+ channels is becoming commonplace in the drug development process.

Supraventricular tachyarrhythmias are generally treated with pharmacological intervention. K^+ channel blockers are one such therapy that acts to increase action potential duration and the effective refractory period to prevent premature re-excitation. Although these interventions can be useful in targeting tachyarrhythmias, they may predispose some patients to the development of other types of arrhythmias (24). However, the proarrhythmic effects of some drugs may actually prove useful as diagnostic indicators of underlying vulnerability to arrhythmia. HERG K^+ channels and to a lesser extent I_{Ks} are of considerable pharmaceutical interest as potential therapeutic targets for antiarrhythmic agents and as the molecular targets responsible for the cardiac toxicity of a wide range of pharmaceutical agents (25, 26).

TABLE 1 Classes of drugs with QT-prolonging potential (16, 17, 123)

Drug class	Drug (trade name)
Class IA antiarrhythmic agents	qunidine procainamide (Procan, Procanbid) disopyramide (Norpace)
Class III antiarrhythimic agents	sotalol (Betapace) dofetilide (Tikosyn) ibutilide (Covert)
Antianginal agents	bepridil (Vascor)
Hypocholesterolemic agents	pubucol (Lorelco)
Catecholaminergic agents	epinephrine (Adrenaline)
Antihistamines	terfenadine (Seldane) astemizole (Hismanol) loratadine (Claritin) diphenhydramine (Benadryl)
Antibiotics	erythromycin (E-Mycin, EES, EryPeds, PCE) pentamidine (Pentam) grepafloxacin (Raxar) sparfloxacin (Zagam) moxifloxacin (Avelox)
Antimalarials	halofantrine mefloquine (Lariam) chloroquine
Antifungal agents	ketoconazole (Nizoral) fluconazole (Diflucan) itraconazole (Sporanox)
Antidepressants	desipramine (Norpramin) nortriptyline amitriptyline (Elavil)
Antipsychotics	chlorpromazine (Thorazine) haloperidol (Haldol) thioridazine (Mellaril) droperidol (Inapsine) pimozide (Orap) risperidone (Risperdal) sertindole (Serdolect)
Gastrointestinal	cisapride (Propulsid)
Diuretics	indapamide (Lozol)
Impotence	sildenafil (Viagra)
Miscellaneous	ketanserin, tacrolimus (FK506) (Prograf) tamoxifen (Nolvacex) indapamide (Lozol) terodiline, potassium loss

I_{Kr}, THE RAPIDLY ACTIVATING COMPONENT OF THE DELAYED RECTIFIER K^+ CURRENT

The Physiological Role of I_{Kr} in the Heart

Two components of I_K (delayed rectifier K^+ current) have been separated on the basis of the activation kinetics: a rapidly activating component (I_{Kr}, encoded by HERG) and slowly activating component (I_{Ks}) (27). I_{Kr} can also be revealed pharmacologically by its sensitivity to block by class III antiarrhythmic drugs, such as E-4031 (28, 29) and dofetilide (30–32). HERG K^+ channels exhibit strong inward rectification (33) due to rapid voltage-dependent C-type inactivation (34, 35). Unlike C-type inactivation in many other channels, HERG inactivation appears to be unique in that it possesses intrinsic voltage dependence (34, 35).

HERG channels activate from closed to open states ($C \rightarrow O$) upon depolarization, but pass very little outward current because they rapidly inactivate ($O \rightarrow I$). HERG channels can also inactivate from closed states ($C \rightarrow I$) (36). Inactivation from both pathways results in the accumulation of HERG channels in inactivated states during depolarization. Channels then reopen, or open for the first time, during repolarization as they recover from inactivation through the open state ($I \rightarrow O$) (Figure 2*A*). HERG has unique channel properties that give rise to I_{Kr} current during repolarization of the cardiac action potential (Figure 2*B*) (36–38).

The Molecular Basis of HERG and I_{Kr} Current

The α-subunit of I_{Kr} is encoded by HERG (33, 39, 40). The topology of HERG channels is similar to many voltage-gated channels in that they are homo-tetramers of identical six *trans*-membrane spanning domains (S1–S6). A cluster of positive charges is localized in the S4 domain and acts as the putative activation voltage sensor (41).

There are, however, marked differences between native I_{Kr} current and HERG-induced currents in heterologous expression systems in terms of gating (42, 43), regulation by external K^+ (44–46), and sensitivity to antiarrhythmics (33). These data suggest the presence of a modulating subunit that co-assembles with HERG in order to reconstitute native I_{Kr} currents. A likely candidate is the minK-related protein 1 (MiRP1 = KCNE2), which when co-expressed with HERG, results in currents similar to native I_{Kr} (42). Coexpression with MiRP1 causes a $+5 \rightarrow 10$ mV depolarizing shift in steady-state activation, accelerates the rate of deactivation, and causes a decrease in single channel conductance from 13 to 8 pS (42). However, a specific and selective interaction of HERG and MiRP1 in the myocardium has not yet been demonstrated (47, 48), and other factors may contribute to the functional differences between native I_{Kr} current and HERG-induced currents in heterologous expression systems. Several alternatively spliced ERG1 variants have been demonstrated in the heart (49–51) and there is evidence for posttranslational modification of HERG proteins (52, 53).

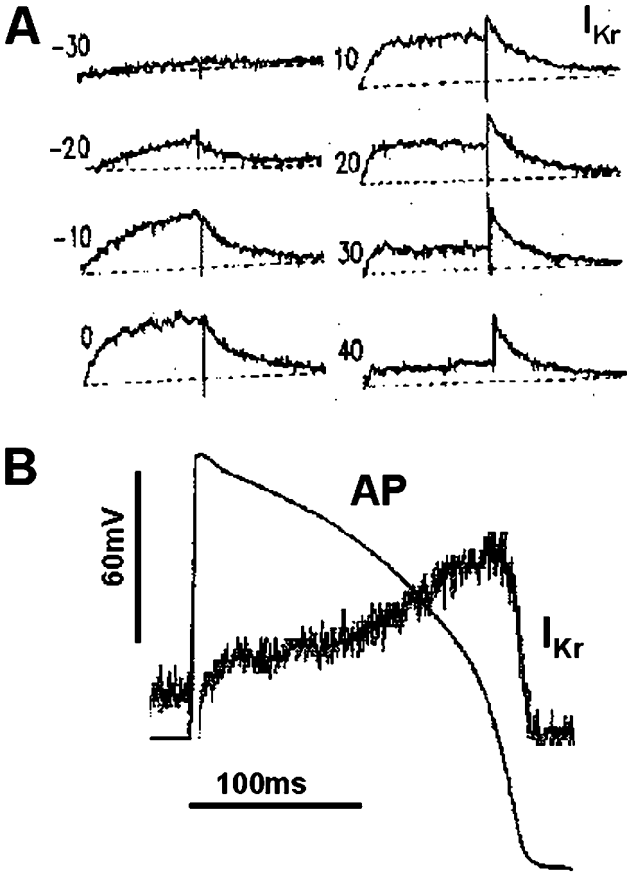

Figure 2 I_{Kr}, the fast component of the delayed rectifier current. (*A*) Time course of I_{Kr} at physiological temperature and ion concentrations (27). The cell is depolarized to the indicated test potential for 250 ms from a holding potential of −40 mV and then repolarized to −40 mV. The hook in the tail current reflects recovery from inactivation through the open state. (*B*) I_{Kr} recorded during an action potential clamp (121). Because recovery from inactivation (I → O) is very fast compared to deactivation (O → C), a large outward current appears during repolarization as channels slowly return to the closed resting states.

Structural Basis of I_{Kr}/HERG Blockade

Recent studies have revealed the molecular basis of the promiscuity of HERG K^+ channel drug binding and have provided further insight into the structure and function of HERG K^+ channels. I_{Kr} is the primary target of methanesulfonanilides (dofetilide, E-4031, ibutilide, and MK-499), a group of potent and specific class III antiarrhythmic drugs that prolong APD (27, 54). HERG channels can also be

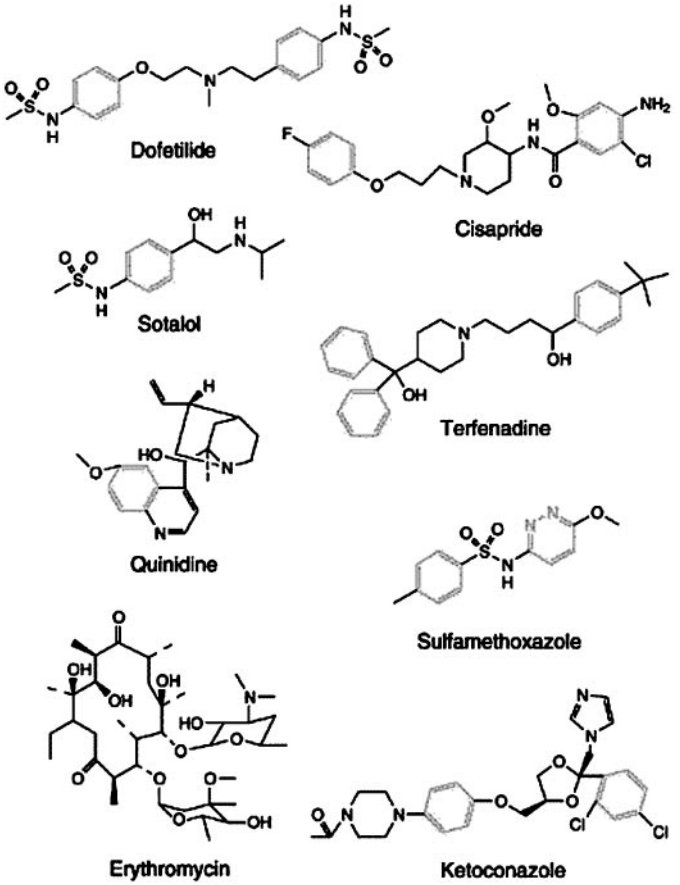

Figure 3 Promiscuous HERG channels are blocked by structurally diverse molecules [from (122)]. In addition to compounds developed for K^+ channel blockade (Dofetilide and Sotalol), a wide range of compounds, from antihistamines (Terfenadine) to antibiotics (Erythromycin), block HERG as an adverse side effect.

blocked by an array of other pharmacological agents with diverse chemical structures (55) (Figure 3). Recent studies have suggested the involvement of aromatic residues in the S6 domain (Y652 and F656) unique to eag/erg K^+ channels that may underlie the structural mechanism of preferential block of HERG by a number of commonly prescribed drugs (56) (Figure 4).

Initial investigation of the HERG antagonist binding site was carried out via site-directed mutagenesis techniques. One study (32) revealed that a single residue, Ser620 in the H5 domain of the S5-S6 linker of HERG, altered the sensitivity of the channel to dofetilide. The altered residue was believed to affect drug binding via an allosteric effect related to loss of inactivation. A more recent study (57) reported

that Phe656 in S6 was necessary, although not sufficient, for high-affinity binding of dofetilide and quinidine, but did not affect binding of tetraethyl ammonium (TEA) and did not disrupt inactivation.

Homology modeling based on crystallographic structure of the bacterial K^+ channel KcsA (58) predicts that Phe656 falls within the HERG pore region. This result was confirmed and extended in an elegant study by Mitcheson et al. (56) who identified four residues in addition to Phe656 that were crucial for high-affinity binding by methanesulfonanilides, namely Tyr652, Gly648, Val625, and Thr623. Using similar homology modeling of HERG channels, the authors showed that the aromatic rings of methanesulfonanilides are likely to interact with the aromatic rings of Tyr652 and Phe656 [Figure 4; from (56)] The crucial role of Tyr652 and Phe656 was confirmed by studies using cisapride and terfenadine, whereas Gly648, Val625, and Thr623 were found to be more specific for methanesulfonanilides (56).

The importance of residues Tyr652 and Phe656 was also demonstrated for the low affinity ligand choloroquine, an antimalarial agent that appears to preferentially block open HERG channels. Blockade of HERG by chloroquine requires channel opening followed by interactions of the drug with the aromatic residues in the S6 domain that face the central cavity of the HERG channel pore (59).

State-Specific Block of I_{Kr}

The biophysical properties of HERG blockade are consistent with a discrete state-dependent blocking mechanism (60, 61). Initial HERG channel studies demonstrated that methanesulfanonilides require channel opening for access to a presumptive intracellular binding site (54). Mutations that result in loss of inactivation act to reduce affinity for methanesulfonanilides, suggesting that inactivation may be required for drug binding. However, methanesulfanonilides are less effective at inhibiting HERG K^+ channels during strong depolarization (e.g., +60 mV), which promotes inactivation (60–62) and would therefore be expected to favor drug binding if the drugs bind to the inactivated state. A possible explanation for this apparent discrepancy may be that channel opening is required for a drug to contact its binding site, which becomes accessible as channels inactivate. At positive voltages, extremely rapid inactivation may reduce the channel open time sufficiently to prevent the drug from accessing the binding site. This idea has recently been proposed as a mechanism of flecainide binding to Na^+ channels, where channel opening is required for flecainide to bind to inactivated channels (63).

Recovery from HERG blockade by methanesulfonanilides is extremely slow, even at negative holding potentials when most channels are in closed states. Using a mutant HERG (D540K) channel that has the unusual property of opening in response to hyperpolarization (64), it was shown that methanesulfonanilides are trapped in the inner vestibule by closure of the activation gate. Opening of the channel in response to hyperpolarization allowed release of the drug from its receptor.

HERG trapping of MK-499, despite its large size, suggests that the vestibule of the HERG channel is larger than the well-studied Shaker K^+ channel. Indeed,

homology modeling based on the KcsA structure revealed two unusual features of the HERG inner vestibule (the site of drug block) that are unique among potassium channels (56). Other voltage-gated K^+ channels contain a Pro-X-Pro sequence in the S6 domain that has been predicted to "kink" the S6 segment and therefore limit the size of the inner vestibule (65). In addition, HERG K^+ channels have two aromatic residues predicted to face the inner pore, whereas other K^+ channels lack these residues, or in the case of KCNQ1, contain only one. As shown in the molecular model of Mitcheson et al. (56), these residues (Y652 and F656) are crucial for electrostatic interactions between aromatic rings of Y652/F652 and the drug molecules.

Cellular Consequences of I_{Kr} Blockade

The HERG channel subunit was originally identified by genetic studies on patients with the congenital long QT syndrome. Incorporation of mutated HERG subunits in the channel tetramer generally cause a reduction of I_{Kr} current, which leads to prolongation of the ventricular action potential (Figure 5). A delay in ventricular repolarization predisposes the heart to arrhythmogenic early afterdepolarizations (38, 66, 67).

Both the cellular effects of these congenital defects and the resulting electrocardiographic abnormalities are analogous to those seen with inhibition of HERG channels by a variety of compounds. Reductions in I_{Kr} result in prolongation of APD and dispersion of repolarization across the wall of the ventricle, which manifests on the ECG as prolongation of the QT interval and widening of the T wave, respectively (1). The ECG alterations have been associated with an increased risk of arrhythmias and sudden cardiac death. Certain factors can increase the disruption of the repolarization balance (e.g., hypokalemia due to diuretics and sudden changes in pacing rate), and can exacerbate the arrhythmogenic effect of HERG-blocking drugs. These additional interventions may result in the appearance of notched T waves on the ECG (1, 2). The recognition of the fundamental role played by the K^+ channels encoded by HERG in cardiac pathophysiology has the potential to improve the understanding of mechanisms of arrhythmogenesis.

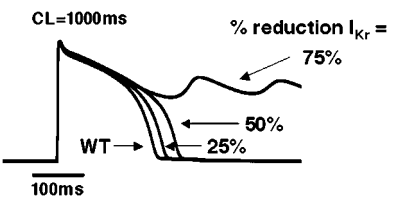

Figure 5 Reduction of I_{Kr} prolongs action potential duration. Here, the prolongation effects of 25%, 50%, and 75% reduction of I_{Kr} on APD are shown in a virtual cell model after 1000 paced beats at a cycle length (CL) = 1000 ms. A 75% current reduction results in the development of arrhythmogenic early afterdepolarizations (EADs). [Adapted from (38).]

I_{Ks}, THE SLOWLY ACTIVATING COMPONENT OF THE DELAYED RECTIFIER K^+ CURRENT

I_{Ks} in Cardiac Repolarization

I_{Ks}, the slowly activating component of I_K, is a major contributor to repolarization of the cardiac action potential (AP) (68). Moreover, I_{Ks} is a dominant determinant of the physiological heart rate–dependent shortening of APD (69). At fast rates, I_{Ks} underlies the rate-dependent adaptation of the APD (70). Fast pacing results in short diastolic (recovery) intervals that prevent complete deactivation ($O \rightarrow C$) of I_{Ks}, resulting in the build-up of instantaneous I_{Ks} repolarizing current at the AP onset [Figure 6, from (70)]. At slower rates, less repolarizing current exists during each action potential due to sufficient time between beats to allow for complete deactivation of I_{Ks} (30, 70, 71). In some species, I_{Ks} deactivation results in a reduction of outward current in pacemaker cells (sino-atrial node), which allows for the slow diastolic depolarization preceding the action potential upstroke (72, 73).

The Molecular Basis of I_{Ks}

I_{Ks} results from co-assembly of two subunits, KCNQ1 (KvLQT1) and KCNE1 (minK) (74, 75). KCNQ1 was identified by positional cloning and mapped by linkage analysis to chromosome 11 (76). KCNQ1, the α-subunit of I_{Ks}, shares topological homology with other voltage-gated K^+ channels in that its 676 amino

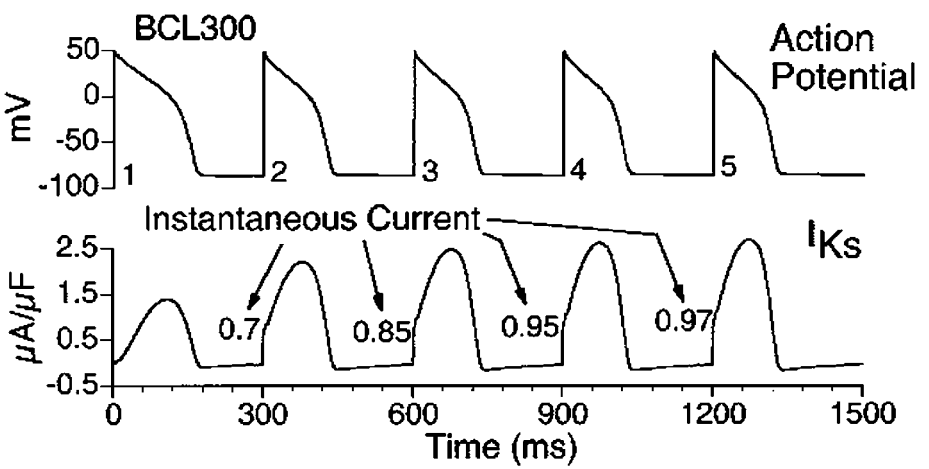

Figure 6 The role of I_{Ks} in APD shortening during rapid pacing (70). A simulated cell is paced from rest at a rate of 300 ms. Five action potentials and corresponding I_{Ks} currents are shown at the onset of rapid pacing. Rapid shortening of APD occurs due to an increase in the instantaneous I_{Ks} (*indicated by the arrows*) caused by incomplete deactivation of I_{Ks} between beats.

acids consist of six transmembrane domains and a pore-forming region. KCNE1, the β-subunit of I_{Ks}, was cloned from human cardiac tissue and encodes a protein containing 129–130 amino acids consisting of a single transmembrane spanning domain (77–80).

Autonomic Regulation of I_{Ks}

The contribution of I_{Ks} to regulation of APD is augmented by the sympathetic branch of the autonomic nervous system, which increases I_{Ks} through primary and secondary effects on channel gating kinetics (27, 81). β-adrenergic receptor (β-AR) stimulation acts to increase the heart rate, which results in rate-dependent shortening of the APD, resulting from the slow deactivation of I_{Ks} (as described above). I_{Ks} amplitude is also directly mediated by β-AR stimulation through PKA phosphorylation (81, 82) (Figure 7). PKA phosphorylation of I_{Ks} considerably increases current amplitude by increasing the rate of channel activation (C $\rightarrow$ O transition) and reducing the rate of channel deactivation (O $\rightarrow$ C transition) (81). Each of these outcomes acts to increase the channel open probability, leading to increased current amplitude and faster cardiac repolarization. β-AR stimulation also hastens

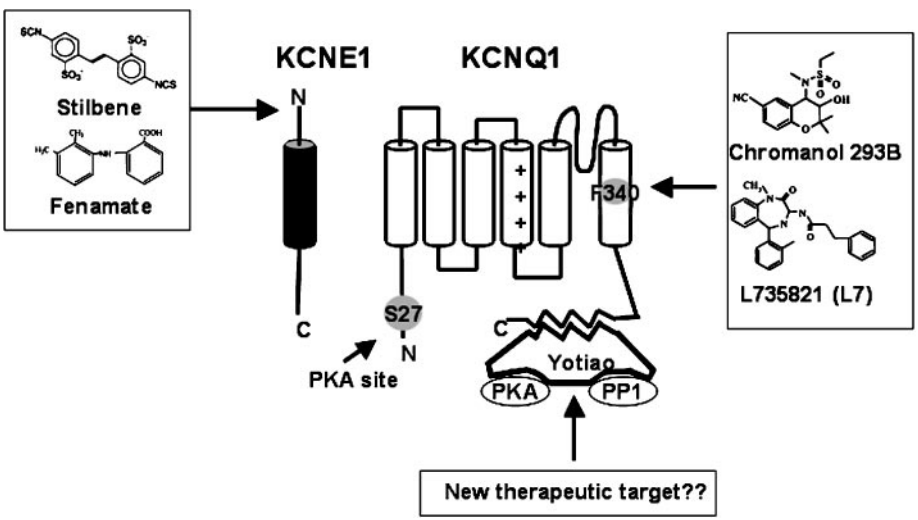

Figure 7 Sympathetic regulation of I_{Ks} requires a macromolecular signaling complex (82). KCNQ1 and KCNE1 co-assemble to form I_{Ks}. Sympathetic stimulation results in the activation of PKA, which is recruited to the channel C-terminus in conjunction with protein phosphatase 1 (PP1) by Yotiao (an AKAP scaffolding protein). PKA phosphorylation of serine 27 (*arrow*) ensues and I_{Ks} is upregulated, allowing for rate-dependent adaptation of the APD. Both KCNQ1 and KCNE1 are targets for pharmacological agents. Stilbene and Fenamate bind to the extracellular domain of KCNE1 and increase I_{Ks} (91). Chromanol 293B and L7 interact with the S6 domain of KCNQ1 and reduce I_{Ks} (87).

diastolic depolarization in the sinus node mainly by enhancing the L-type Ca^{2+} and I_f channels (83). Hypothetically, the simultaneous reduction in the rate of I_{Ks} deactivation during β-AR stimulation may act to regulate the slow diastolic depolarization, thereby adding another layer of regulation to the modulation of heart rate.

Additionally, endothelin-1, a myocardial and endothelial peptide hormone, inhibits I_{Ks} currents presumably through inhibition of adenylate cyclase via a PTX-sensitive G protein (84), and results in APD prolongation. Because both β-AR signaling and ET_A receptor signaling result in PKA phosphorylation, the molecular mechanisms of phosphorylation and dephosphorylation of I_{Ks} are of major interest as potential therapeutic targets.

Recently, the requirement of a macromolecular signaling complex for PKA phosphorylation of I_{Ks} has been shown (82). A leucine zipper motif in the C-terminus of KCNQ1 coordinates the binding of a targeting protein yotiao (85, 86), which in turn binds to and recruits PKA and protein phosphatase 1 (PP1) to the channel. The complex then regulates the phosphorylation of Ser^{27} in the N-terminus of KCNQ1 (Figure 7).

Structural Basis of I_{Ks} Blockade

Investigation into the structural determinants of I_{Ks} blockade has only recently begun. Not unlike HERG drug interaction sites, preliminary studies revealed a common site for binding of I_{Ks} blockers, including chromanol 293B and L735821 (L7) in the S6-domain (F340) of the KCNQ1 subunit (Figure 7). Other putative interaction sites in the S6-domain (T312 and A344) and the pore-helix (I337) may lend specificity to pharmacological interactions (87). Interestingly, these binding sites are located near an aqueous crevice in KCNQ1 that is thought to be important for interactions with KCNE1 that allosterically affect pore geometry (88–90). Drug interaction sites for channel agonists stilbene and fenamate have also been elucidated on extracellular domains in KCNE1 (Figure 7) (91).

Cellular Consequences of I_{Ks} Channel Blockade

Although the efficacy of I_{Kr} blockade is reduced at fast pacing rates due to reverse use dependence (i.e., APD-prolonging effects are least pronounced at fast stimulation rates), I_{Ks} blockade may be expected to be more useful in prolonging APD at fast rates, whereas I_{Ks} accumulates due to slow deactivation (30, 92).

Some pure class III compounds block both native and heterologously expressed I_{Ks} currents. Chromanol 293B and the benzodiazepine L7, which are distinct in their chemical structures (Figure 7), as well as the diuretic agent indapamide were some of the first compounds discovered to selectively block I_{Ks} (93–96). The application of chromanol 293B revealed that I_{Ks} inhibition appears to have rate-independent effects on human and guinea pig myocytes (94). Chromanol 293B exhibits slow binding kinetics to open channels and blocks I_{Ks} in a voltage-dependent manner, favoring positive potentials (97). It is possible that this type of voltage and time dependence of drug-induced I_{Ks} blockade may have less proarrhythmic potency compared to other compounds. Azimilide, a class III compound that blocks both

I_{Kr} and I_{Ks}, also appears to have rate-independent effects that are maintained under ischemic or hypoxic conditions, properties of potential clinical significance (98).

Some evidence suggests that I_{Ks} blockers can prolong QT intervals in a dose-dependent manner, an effect that is exacerbated when administered in combination with isoproterenol (99). These studies in canine preparations may even underestimate the proarrhythmia potential of I_{Ks} blockade because canine repolarization appears to be less dependent upon I_{Ks} than other species (100), and chromanol 293B was shown to markedly prolong human and guinea pig APD (94).

The sensitivity of I_{Ks} to blockade by chromanol 293B (101) and XE991 (102) is modulated by the presence of KCNE1. KCNE1 is itself a distinct receptor for the I_{Ks} agonists stilbene and fenamate (101), which bind to an extracellular domain on KCNE1. Stilbene and fenamate have been shown to be useful in reversing dominant negative effects of some LQT5 C-terminal mutations and restoring I_{Ks} channel function (91). On the other hand, a 1,4-benzodiazepine compound, L364,373, was an effective agonistic on KCNQ1 currents only in the absence of KCNE1 (103). These types of studies illustrate the importance of accessory subunits in determining the pharmacological properties of I_{Ks}. Variable subunit expression may determine tissue selectivity or electrical heterogeneity of pharmacological action that could exacerbate dispersion of repolarization (71, 104).

Blockade of I_{Ks} and β-Adrenergic Stimulation

In a model of acquired LQTS (I_{Ks} blockade by chromanol 293B), the addition of the β-adrenergic agonist isopreterenol induced the development of torsades de pointes (99). These results are consistent with the clinical findings that cardiac events are more likely to be associated with sympathetic nervous system stimulation in LQT1 patients than in either LQT2 or LQT3 patients (11, 105, 106). Moreover, β-blockers were reported to reduce cardiac events dramatically in LQT1 patients (106–108). Indeed, clinical data indicate that sudden cardiac death is significantly associated with sympathetic nervous system stimulation (106).

Gene Defects in KCNQ1 or KCNE1 Can Disrupt Cellular Repolarization

Mutations in either KCNQ1 or KCNE1 can reduce I_{Ks} amplitude, resulting in abnormal cardiac phenotypes and the development of lethal arrhythmias. Reduction of I_{Ks} during the delicate plateau phase of the action potential disrupts the balance of inward and outward current, leading to delayed repolarization. Prolongation of APD manifests clinically as forms of LQTS that are characterized by extended QT intervals on the ECG. Gene defects in KCNQ1 and KCNE1 are associated with distinct disease forms, LQT1 and LQT5, respectively (109–114).

In general, mutations in KCNQ1 or KCNE1 act to reduce I_{Ks} through dominant negative effects (76, 109, 113, 115–118), reduced responsiveness to β-AR signaling (82, 106), or alterations in channel gating (112, 119, 120). The latter effects typically manifest as either reduction in the rate of channel activation, such as R539W KCNQ1 (109), R555C KCNQ1 (118), or an increased rate of channel

deactivation, including S74L (119), V47F, W87R (112), KCNE1, and W248R KCNQ1 (120). An LQTS-associated KCNQ1 C-terminal mutation, G589D, disrupts the leucine zipper motif and prevents cAMP-dependent regulation of I_{Ks} (82). The reduction of sensitivity to sympathetic activity likely prevents appropriate shortening of the action potential duration in response to increases in heart rate.

Despite their distinct origins, congenital and drug induced forms of ECG abnormalities related to alterations in I_{Ks} are remarkably similar. In either case, reduction in I_{Ks} results in prolongation of the QT interval on the ECG without an accompanying broadening of the T wave, as observed in other forms of LQTs (1). Reduced I_{Ks} leads to loss of rate-dependent adaptation in APD, which is consistent with the clinical manifestation of arrhythmias associated with LQT1 and LQT5, which tend to occur due to sudden increases in heart rate. This strongly suggests that investigation of congenital forms of electrical abnormalities may act as a paradigm for drug-induced forms of clinical syndromes that is simplified by the absence of accompanying structural heart disease.

SUMMARY

In general, the broad diversity in response to pharmacologic intervention in the presence or absence of gene defects among individuals prone to arrhythmia is likely influenced by other genetic traits separate from the primary disease locus. Clinical presentation is determined by complex interactions between pharmacology, causal genes, genetic background (modifier genes), and environmental factors. Although individual modifier genes for lethal arrhythmias remain largely unknown, potential modifiers of cardiac arrhythmias include, but are not limited to, gender, febrile states, adrenergic stimulation, signaling molecules, channel-associated protein kinases, channel-associated protein phosphatases, and individual electrophysiological and morphological substrates. Identification of modifier genes will complement the current studies that have identified and characterized causative genes, which may improve upon genetically based diagnosis, risk stratification, and implementation of preventive and therapeutic interventions in patients with drug-induced arrhythmias.

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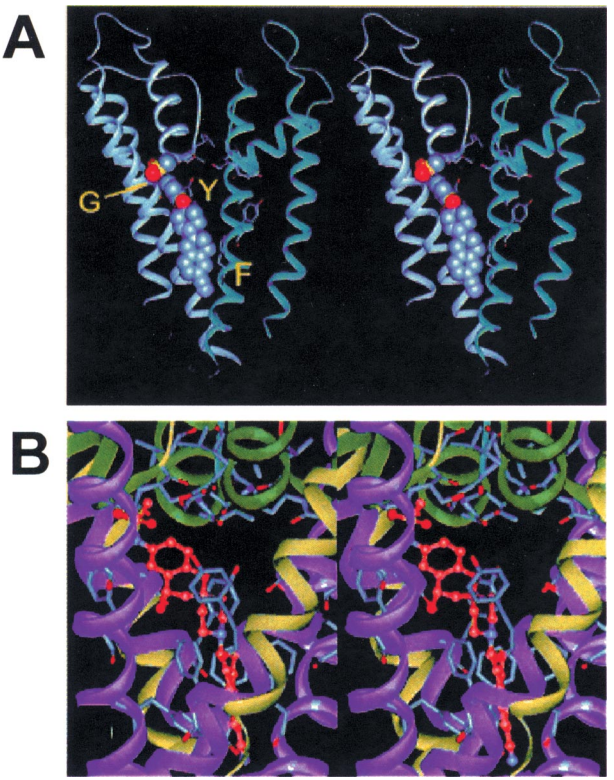

Figure 4 Drug docking in a homology model of the HERG K^+ channel based on the KcsA (58) channel structure. (*A*) Stereo view of the S5-S6 domains of two HERG subunits with docked molecule of MK-499 (shown as a space-filling model). T623, S624, V625, G648, Y652, and F656 are shown as sticks. (*B*) Close-up stereo view of MK-499 in a four-subunit model of the channel. T623, S624, and V625 of the pore helix (green), and G648, Y652, and F656 of the S6 domain (magenta) are shown as sticks; MK-499 is shown as a ball and stick model. Only two of the four S5 domains (yellow) are shown (from Reference 56).

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COMPLEMENTARY AND ALTERNATIVE THERAPEUTICS: Rigorous Research is Needed to Support Claims[1]

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■ **Abstract** The establishment of the National Center for Complementary and Alternative Medicine (NCCAM) in 1998 as a part of the National Institutes of Health was catalyzed by the increasing interest and use of complementary and alternative medicine (CAM) modalities by the public. This article presents an overview of CAM, summarizes similarities and differences between the regulatory requirements for drugs and CAM/botanical products, identifies several challenges and opportunities for conducting research to demonstrate the safety and efficacy of CAM therapeutics, and highlights the role of NCCAM in supporting and stimulating research in this area.

WHAT IS CAM? AN OVERVIEW OF CAM AS THERAPEUTICS

Complementary and alternative medicine (CAM) practices can be described as those not currently considered to be an integral part of conventional medicine. As CAM practices are proven safe and effective, they may become integrated into mainstream medicine. The majority of patients use CAM approaches to complement conventional health care, rather than as an alternative to it (1). CAM practices can be grouped into five major domains (Figure 1).

MARKET FACTORS

Advances in biomedical science over the past century, coupled with improved sanitation measures and public health practices (2), have led to remarkable gains in the health of the American people and an increase in the life expectancy from

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47 years in 1900 to 77 years today (3). This impressive achievement is due in large part to the development and use of vaccines and antibiotics, which have significantly reduced the number of deaths from acute infectious diseases, such as tuberculosis, influenza, and pneumonia. Nonetheless, new and re-emerging diseases, such as the resurgence of cholera in the Americas after over a century, the emergence of hantavirus in the continental United States, and the westward spread of West Nile fever from the East Coast, remain a serious threat. In addition, the world now faces the menace of bioterrorism.

The predominant challenges for medicine today, however, come increasingly from chronic diseases that are prevalent among the growing numbers of aging Americans. Chronic diseases often resist cure and may coexist with unrelieved pain. As the members of the "baby boomer" generation age, they have hopes of continuing to live free of disease and disability. They are usually well informed and want to participate actively in decisions about their own health care. These factors contribute to an increased interest in CAM. Looking at long-term trends of CAM use in the United States, Kessler et al. (4) found that 68% of adults had used at least one CAM therapy in their lifetime and that lifetime use increased steadily with age.

The elderly are not the only group to be attracted to the use of CAM. Frustrated by the inability of mainstream medicine to treat, prevent, or cure all illnesses, many people have turned to CAM approaches. The expenditures for alternative medicine professional services in the United States are estimated at $21.2 billion, with the preponderance of costs paid out of pocket. Out-of-pocket expenditures for CAM therapies were estimated at $27 billion, comparable to out-of-pocket costs for all U.S. physician services (5).

In the United States, the increase in use of CAM since the 1950s (4, 5) has been accelerated in the past few years by a number of factors. With the explosive growth of the Internet, consumers have greater access to an increasing body of information, including advertising and marketing of CAM practices. Consumers, armed with more (though not necessarily more reliable) information, are asking for products and services that they believe to be safe and efficacious. In addition, a number of CAM practices serve as either the first line of medical care or augment conventional medical treatment in both developed and developing countries around the world. The world is becoming a global community, due in part to the enormous volume of international travel, and individuals are exposed to the health practices of peoples who live far away. Finally, racial/ethnic minorities who have immigrated recently to the United States may continue to use the health practices of their countries of origin.

INTEGRATION: FROM CAM TO CONVENTIONAL

In some cases, the existence of a widely used natural product has informed the development of a conventional drug product and its movement into medical practice. The development of aspirin is but one example (Table 1). Therapeutic benefits of the leaves and bark of the willow tree were known by Hippocrates and used by many indigenous peoples, such as Native Americans, to relieve pain and fever. In

TABLE 1 Aspirin: integration of a botanical product into medicinal practice

400 BC
The Greek physician Hippocrates prescribes the bark and leaves of the willow tree to prevent pain fever.
Middle Ages
Reportedly, demand for wicker furniture takes precedence for use of willow bark and Europeans stop using willow bark remedies.
1500s–1700s
A number of ethnic groups, including Native Americans, use various parts of the willow tree for its analgesic properties.
1763
In England, Edward Stone used willow tree bark to reduce the fever caused by malaria.
1832
A German chemist isolates salicin and synthesizes improved analogs, including acetyl salicylic acid (aspirin).
1899
Aspirin becomes the top drug worldwide.
1915
Aspirin is moved to over-the-counter status.
1915–1999
Various formulations of aspirin (low strength, children's chewable, enteric coated, extra strength, timed release) are manufactured for targeted markets.
Early 1970s
Aspirin is determined to act by inhibiting the production of prostaglandins.

1897, a German chemist with the Bayer Company isolated a chemical compound, salicin, from willow bark. Several analogs were successively synthesized to reduce irritating side effects and improve stability. By 1899, one of the synthesized entities, acetyl salicylic acid, commonly known as aspirin, had become the number one drug worldwide. The mechanism of action of aspirin was not known, however, until the 1970s, when scientists discovered its ability to inhibit the production of prostaglandins that are involved in inflammation. Even a century after its development, new uses are still being identified for aspirin. The U.S. Food and Drug Administration (FDA) has approved the use of aspirin to reduce the risk of recurrent myocardial infarction or heart attack, to prevent a first myocardial infarction in patients with unstable angina, to reduce the risk of death during a suspected heart attack, and to prevent a recurrent stroke. Research continues to explore the use of aspirin to prevent selected cancers, such as colon and esophageal. Willow bark–containing products are still in use, although the concentrations of salicylates vary greatly among species of Salix (6).

Similarly, for many centuries, native people in many parts of the world have used extracts of the foxglove (*Digitalis purpurea*) plant as a diuretic, heart tonic,

emetic, and rat poison. In the eighteenth century, British surgeon William Withering conducted experiments to demonstrate the uses and side effects of foxglove and determined that the dried powdered leaf of the plant was more effective than the fresh leaf. Crude extracts of the plant were hard to prepare in a reproducible manner. With the discovery of the active component of foxglove, an analog, digoxin, was synthesized, which has a short half-life in the body. Digoxin has been used as a prescription drug for decades in patients with heart failure and to this day remains a first line therapy for congestive heart failure. Digitalis is no longer recommended for self-medication (7).

THE REGULATORY ENVIRONMENT

Not all natural products have served as the basis for classical drug discovery and development. Most continue to be used as they have for eons, as complex mixtures that today are regulated in the United States differently from drugs. An overview of the legal framework under which conventional drugs and diverse natural products are regulated in the United States (Table 2) shows the difference between the development of CAM products and conventional drugs, either prescription or over the counter (OTC).

The emphasis in early federal regulatory statutes was on safety. Until the early twentieth century, the Bureau of Chemistry in the Department of Agriculture was responsible for assuring the safety of both foods and medicines. The Biologics Control Act of 1902 (8) ensured the purity and safety of vaccines and the Federal Food and Drugs Act of 1906 (9) prohibited interstate commerce of misbranded or adulterated foods and drugs. The poisoning of more than 100 people, mostly children, with a sulfanilamide elixir containing a component of antifreeze, ethylene glycol, led to the enactment of the Federal Food, Drug, and Cosmetic Act in 1938 (10). This act created the present day FDA in 1940 and required medicines to be demonstrated safe prior to marketing.

Two important amendments to the FDC Act followed. The Durham-Humphrey Amendments in 1951 (11) required certain drugs to be labeled as prescription only; a drug that could be labeled for use without professional supervision was to be available OTC, without a prescription. The thalidomide crisis in 1962 led Congress to enact the Kefauver-Harris Drug Amendments (12), which required manufacturers to prove both product effectiveness and safety in well-controlled studies and also applied certain requirements such as informed consent to clinical studies. In addition, regulations require manufacturers of both prescription and OTC products to follow current Good Manufacturing Practice (cGMP) to assure quality and standardization of their drugs and to list their facilities and products with the FDA.

Later legislation for drug products focused on providing financial incentives to the pharmaceutical industry. The Orphan Drug Act of 1983 (13) stimulated investment in the development of drugs to treat rare diseases or conditions affecting fewer that 200,000 persons in the United States. Manufacturers of orphan drug products enjoy seven years of market exclusivity if the products are not patentable

TABLE 2 Selected legislation concerning the regulation of drugs and dietary supplements

Year	Legislation	Main provisions
1902	Biologics Control Act	Ensured the purity and safety of vaccines
1906	Pure Food and Drugs Act	Prohibited interstate commerce of misbranded and adulterated foods, drinks, and drugs
1938	Federal Food, Drug, and Cosmetic Act	Required demonstrated safety for medicines (no clear-cut distinction between prescription and OTC drugs)
1951	Durham-Humphrey Amendments	Established specific standards for classification of prescription and nonprescription drugs[a]
1962	Kefauver-Harris Drug Amendments	Required manufacturers to prove both effectiveness and safety for prescription drugs
1983	Orphan Drug Act	Provided financial incentives for investment in therapeutics for rare diseases[b]
1984	Drug Price Competition and Patent Term Restoration Act (Waxman-Hatch Act)	Allowed FDA to accept abbreviated new drug applications for generic products after patent expiration
1995	Dietary Supplement Health and Education Act	Amended the Federal Food, Drug, and Cosmetic act for dietary supplements

[a]In 1972, the FDA began a comprehensive review of the active ingredients in OTC drug products; some were removed from the market because they were found to be unsafe and/or ineffective.

[b]Rare diseases are defined as affecting fewer than 200,000 individuals in the United States.

and a 50% tax credit for research and development expenses. Sponsors may also apply for a clinical research grant from the FDA. The Drug Price and Patent Term Restoration Act of 1984 (14), commonly called the Waxman-Hatch Act after the congressional sponsors of the bill, authorized the FDA to accept abbreviated new drug applications (ANDAs) from manufacturers of a generic product once the patent on the innovator's product has expired; innovator companies also gained restoration of up to five years of a product's patent life that had been lost during the process of gaining approval from the FDA to market the product.

The regulation of herbal products has been complex. Prior to 1994, these products were marketed either as foods or drugs, depending on their intended use and whether any health claims were made. All of this changed in 1994 when Congress passed the Dietary Supplement Health and Education Act (DSHEA) (15) to protect access by consumers to safe dietary supplements. This law defined a new category of food for regulatory purposes, the dietary supplement (16), which includes herbs, other botanicals, vitamins, and minerals.

DSHEA requires manufacturers to clearly state on the product label that it is not intended to diagnose, treat, cure, or prevent any disease, but rather will be used to supplement the diet. Statements can be made claiming benefits related to a classical nutrient deficiency disease, describing the role of the supplement intended to affect the structure or function, characterizing the documented mechanism by which the supplement maintains structure or function, or describing the general well-being from consuming the supplement. These are generally referred to as structure/function claims. For example, improving cardiovascular health may be such a claim, rather than preventing a heart attack, which would be a specific disease claim that a dietary supplement could not legally make.

Drugs and dietary supplements are subject to different requirements concerning their manufacture and their standards for safety and efficacy (Table 3).

- Manufacturing. Drug products must meet cGMP requirements. Although the law permits the FDA to develop specific GMPs for dietary supplements, to date such GMPs have not been issued. Currently, companies must follow existing manufacturing requirements for foods.
- Product characterization. Sponsors of prescription products must document information about the chemical purity of the active ingredient as well as product formulation and stability. An OTC drug product must meet the OTC monograph standards. DSHEA does not require any chemical characterization or standardization.
- Safety. Drug products must be approved by the FDA as safe prior to marketing (prescription) or be contained in one of the OTC monographs. Manufacturers of dietary supplements are responsible for ensuring that their products are safe, but the FDA bears the burden of proof to show that a product is adulterated. For example, in February 2002, the FDA issued a safety alert warning consumers to stop taking the dietary supplement/herbal product PC SPES because the marketed product was found to contain undeclared prescription drug ingredients (17). Although the FDA monitors adverse effects after either drug or dietary supplement products are on the market, newly marketed dietary supplements are not subjected to premarket approval or a specific postmarket surveillance period. In the face of a rapidly expanding market, some observers have expressed concern that the FDA has inadequate resources to monitor the safety of dietary supplements (18).
- Efficacy. Although DSHEA requires companies to substantiate claims of benefit for dietary supplements, citation of existing literature is sufficient. In contrast, extensive premarket testing is required for prescription drug products; OTC drug products must meet OTC monograph standards.
- Claims. For dietary supplements and for OTC drugs, the FDA has primary responsibility for claims on product labeling, whereas the Federal Trade Commission has primary responsibility for claims in advertising. The FDA has responsibility in both areas for prescription drugs.

TABLE 3 A comparison of requirements for drugs and dietary supplements[a]

	Prescription drug product	Over-the-counter (OTC) drug product	Dietary supplement
Label claim	Intended to treat or prevent disease	Intended to treat or prevent disease	Intended to affect general well-being or to counteract nutritient deficiency
Labeling oversight	Food and Drug Administration (FDA)	FDA	FDA
Product characterization	Must include information about ■ Chemical purity of active component ■ Manufacturing method and facilities ■ Product formulation and stability	Manufacturer must meet OTC monograph standards	None needed
Product review	Final formulation	Active ingredient	None
Data review	Private	Public	None
IND/NDA	Required for new chemical entity or for new indication for existing product	■ NDA may be submitted to market a current prescription drug in a new dosage or formulation (e.g., ibuprofen) ■ NDA is required for new drug delivery systems or formulations (e.g., sustained release formulation) ■ Grants exclusive marketing rights for a number of years	Not required for commercial marketing

(*Continued*)

TABLE 3 (*Continued*)

	Prescription drug product	Over-the-counter (OTC) drug product	Dietary supplement
Manufacturing requirements	current Good Manufacturing Practices (cGMPs)	cGMPs	cGMPs modeled on food practices
Demonstration of safety	Premarket approval required ■ Testing in animals ■ Phase I/II/III clinical trials ■ Post marketing surveillance system	Manufacturer must meet OTC monograph standards[b]	Premarketing approval or postmarketing surveillance not required Citation of existing literature
Demonstration of efficacy	Premarket approval required ■ Testing in animals ■ Phase I/II/III clinical trials	Manufacturer must meet OTC monograph standards	Citation of existing literature
Regulation of advertising claims	FDA	Federal Trade Commission (FTC)	FTC
Financial incentives	■ Process patent ■ Use patent (e.g., new formulation) ■ Market exclusivity (e.g., seven years for rare disease indication)	Marketing rights granted for drug product if NDA submitted	None

[a]A dietary supplement is defined by the Dietary Supplement Health and Education Act of 1994 as a product intended to supplement the diet that contains one or more of the following: a vitamin, a mineral, an herb, a botanical, an amino acid, or a dietary substance to supplement the diet.

[b]http://www.fda.gov/cder/otc/index.htm

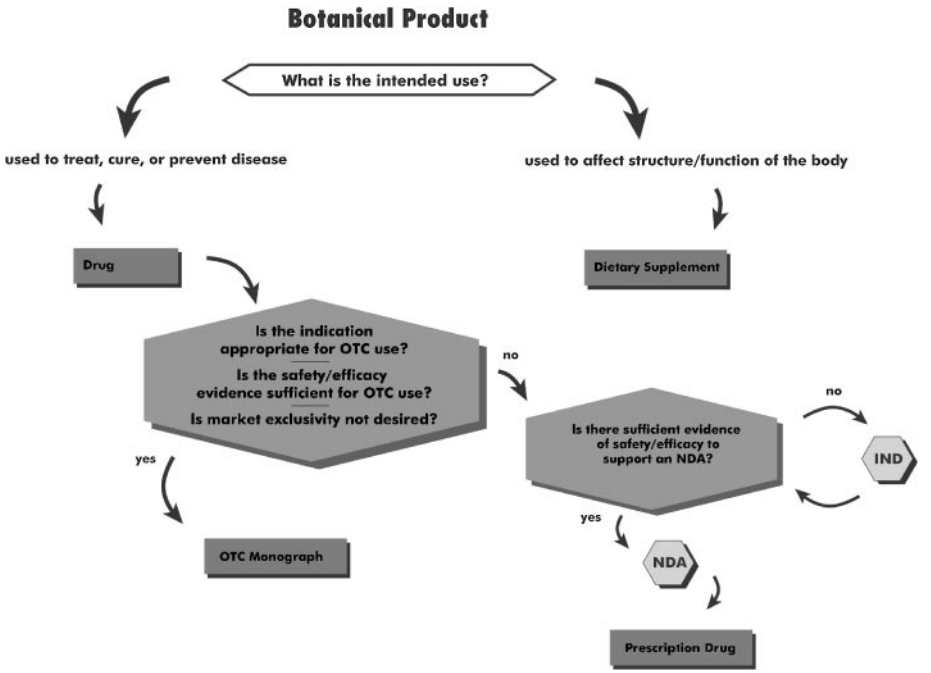

Figure 2 Dietary supplements proceed through the approval process depending on the claims that are made for the product (a more detailed process flow chart can be found at http://www.fda.gov/cder/guidance/1221dft.pdf, attachments A and B, pp. 39–40).

Again, when sold as dietary supplements, CAM products cannot claim to treat or prevent a disease. CAM therapies that are intended to treat or prevent disease would have to proceed through the drug approval pathway (Figure 2). These therapeutic products are subject to the same requirements as are other drugs, such as purity, stability, safety, and efficacy, and must adhere to stringent manufacturing requirements. For example, edetate, or EDTA, was approved by the FDA half a century ago to treat heavy metal poisoning through its action of forming chelates with divalent and trivalent metals. More recently, chelation therapy has been used for off-label purposes to ameliorate angina pectoris and to reduce the symptoms of peripheral vascular disease, coronary artery disease, and cerebrovascular disease (19). There are little data from controlled clinical trials regarding the efficacy of chelation for these indications. For manufacturers to make claims regarding EDTA-containing solutions for these indications, but more importantly, for public health purposes, rigorous studies are required. To this end, the National Center for Complementary and Alternative Medicine (NCCAM), in collaboration with the National Heart, Lung, and Blood Institute, has launched a $30 M, five-year, multisite, randomized, double-blinded, placebo-controlled trial to investigate the

safety and efficacy of EDTA chelation therapy in individuals who are suffering from coronary artery diseases (the Request for Applications announcement can be found at http://www.nccam.nih.gov).

A drug product that contains the same active constituent as a botanical product would be regulated according to different paths, as long as they are marketed with different intent. The combination of caffeine with any other stimulant, such as ephedrine alkaloids[2], may not be sold as an OTC drug product (20). However, dietary supplement products that contain ma huang (a source of ephedrine) and natural product stimulants such as kola nut (50% caffeine) are permitted on the market under DSHEA.

FEWER FINANCIAL INCENTIVES FOR BOTANICAL PRODUCTS

Manufacturers and producers of CAM products intended to treat or prevent disease and that will be marketed directly to consumers might not enjoy the same financial incentives, such as market exclusivity and patent protection as do drugs. For example, a drug that is approved by the FDA for prescription use is eligible for any protection that remains under an existing patent. A pharmaceutical manufacturer may extend product exclusivity by obtaining a new patent for a revised formulation, such as a sustained release product that alters the bioavailability profile of the active component, or for a new indication.

Market exclusivity is awarded for certain uses for a drug product, such as to treat or prevent orphan diseases. In this case, the most powerful incentive for a company to develop a product with a small target population is the Orphan Drug Act's market exclusivity clause.

Under certain circumstances, manufacturers may submit a new drug application (NDA) for an OTC drug product, which grants market exclusivity for a certain number of years. An NDA may be submitted for a current prescription drug to be marketed in a new formulation or dosage. A manufacturer of a product for which an OTC monograph exists is required to submit an NDA for a new drug delivery formulation, such as a sustained release product.

THE RESEARCH ENTERPRISE

Prioritizing Research Needs and Opportunities

Given the widespread use of CAM practices and products and the lack of private sector investment to prove their safety and efficacy, the responsibility to do so fell to the National Institutes of Health (NIH). In 1998, the NCCAM was created by Congress as a component of the NIH to conduct basic and applied research

[2]Ephedrine alkaloids include ephedrine, pseudoephedrine, and norephedrine (phenylpropanolamine, PPA).

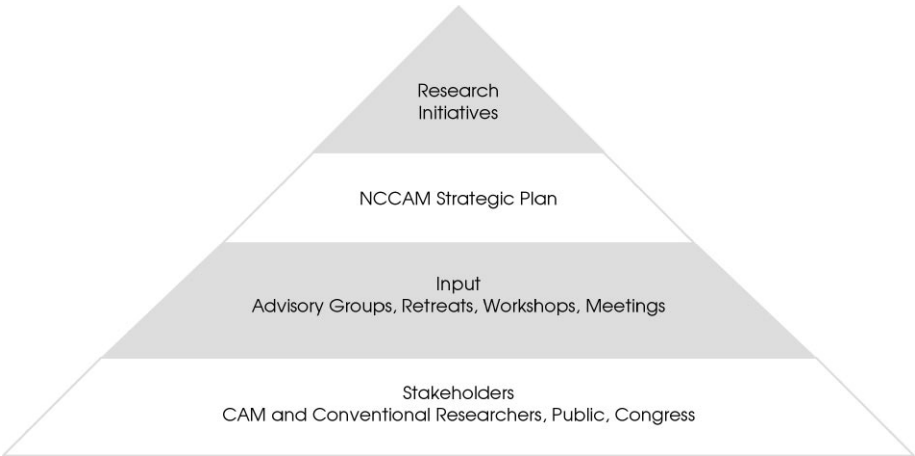

Figure 3 NCCAM's planning process solicits input from a broad base of constituents to develop research plans and initiatives.

(intramural and extramural) and research training, disseminate health information, and other programs with respect to identifying, investigating, and validating CAM treatments, diagnostic and prevention modalities, disciplines, and systems. With the funds appropriated by Congress to the Center each year, NCCAM provides support for research that is initiated by research investigators as well as for research in targeted areas. These areas of need and opportunity are identified through NCCAM's planning process (Figure 3). NCCAM relies on input from its advisory groups (e.g., the National Advisory Council for Complementary and Alternative Medicine), researchers in the CAM and conventional communities, the public, and Congress to develop and select initiatives that solicit research applications in specific areas. Factors that are considered to determine the highest priority initiatives include the extent of credible preliminary data, use by the U.S. public, significance from a public health point of view, availability of appropriate scientific expertise to conduct the research, and, for clinical trials, the availability of a patient population to study. Semiannual planning retreats provide a forum for NCCAM staff to identify and discuss gaps in the research portfolio, propose approaches/initiatives to respond to the identified opportunities and needs, prioritize initiatives within the budgetary framework, and make decisions about activities to be initiated in future years.

In 2000, NCCAM developed a strategic plan, *Expanding Horizons of Healthcare* (http://nccam.nih.gov/strategic), that delineates four key areas as part of the Center's mission: investing in high quality research, training CAM investigators, expanding outreach activities, and facilitating integration of CAM and conventional medicine. The strategic plan, developed with input from a broad range of stakeholders, outlines the Center's research agenda and will guide the development of initiatives and activities for the coming five years.

Research on CAM Therapeutics

With the support that has been provided since its creation, NCCAM has invested in a broad array of scientific research projects that span the spectrum from basic research, such as interactions between botanical products and conventional drugs, to the conduct of clinical trials to study the safety and efficacy of CAM modalities. The NCCAM portfolio also includes support for research training and education grants. In addition, the Center disseminates information through the NCCAM Clearinghouse (info@nccam.nih.gov) and the NCCAM website (http://www.nccam.nih.gov).

Because CAM interventions are already being used, clinical research is NCCAM's highest research priority and serves as one entry point for a product into the research pipeline. Thus, the study of CAM products differs from the model of conventional drug development (Figure 4), where basic research serves as the foundation for drug development. Scientific advances help to identify potential strategies for further testing in animal model systems and clinical trials in humans. In an idealized drug development model, single, defined chemical entities are synthesized or extracted, purified and characterized, and preclinical studies are conducted to determine activity in in vitro test systems. Promising compounds progress to studies in animals to study physiological and pharmacological effects, bioactivity, mechanisms of action, adverse effects, and toxicology. The most promising entities with respect to safety and efficacy move forward to small clinical

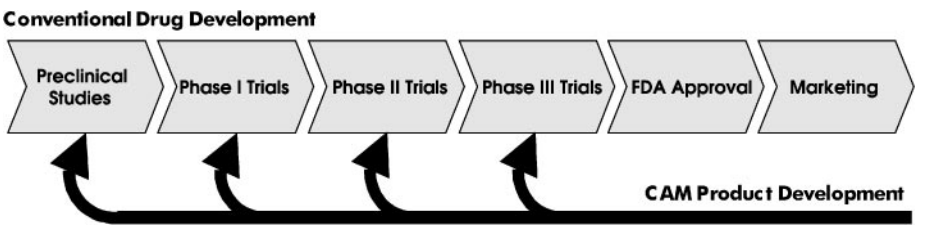

Figure 4 Stages in development of conventional drugs and CAM products. The conventional model of drug development proceeds in a sequential fashion. The process begins with the conduct of preclinical studies in which single, defined molecular entities are synthesized, purified, and characterized, and animal studies are conducted to determine bioactivity, mechanisms of action, and adverse effects. Promising compounds are then evaluated in Phase I human trials for safety, in Phase II trials for clinical activity and dose ranging, and in larger Phase III trials to determine efficacy. Marketing approval is granted by the FDA on the basis of successful demonstration of safety and efficacy. The NCCAM model for the development of CAM products operates in reverse. Phase I, II, and even Phase III trials of natural products are undertaken based on their history of presumed safe use, the extent of public use, the public health opportunities, and the existing level of evidence about them. Investigators then "back-fill" the knowledge base by conducting preclinical studies. (Reprinted with permission from *Nature Reviews Drug Discovery* "Stages in the Development of Conventional Drugs and CAM Products." Copyright 2002 Macmillan Magazines Ltd.)

trials in humans to evaluate tolerability (Phase I), controlled trials to assess clinical activity and determine dose range for study (Phase II), and larger controlled trials to establish clinical efficacy (Phase III). Thousands of compounds may be screened before a successful new drug is identified. Postmarketing surveillance studies and reporting of adverse events to the FDA provide an umbrella to identify those products that may show higher-than-anticipated problems when introduced into the larger population.

Because many CAM therapeutics are already being used by the public, research is conducted on products that are already on the market. Phase II and even Phase III trials of CAM products are undertaken based on their history of presumed safe use, the extent of public use, the public health opportunities, and the existing level of evidence about them. Investigators then add to the knowledge base by further evaluating dose response, obtaining physiological and pharmacological data, and determining bioactivity, mechanisms of action, and adverse effects (21).

NCCAM also relies on reviews of the current literature to help identify areas for clinical investigations. For CAM products, the information ranges from anecdotes and case studies to uncontrolled trials and small randomized controlled trials. In striving to elevate CAM research to a higher standard, NCCAM views randomized, double-blind controlled trials as the "gold standard," and designs of clinical trials supported by NCCAM are the most rigorous possible. Of course, not all CAM approaches, like many conventional practices such as surgical interventions, can be well blinded or placebo controlled, but one must still depend on designs that incorporate adequate sample sizes and validated endpoints.

RESEARCH ON BOTANICAL PRODUCTS: CHALLENGES AND OPPORTUNITIES

The lack of consistent and reliable botanical products represents a formidable challenge to conducting clinical trials, as well as basic research. Although many botanicals are widely used, most have not been sufficiently characterized or standardized for the conduct of clinical trials capable of adequately demonstrating safety or efficacy, or predicting that similarly prepared products would also be safe and effective in wider public use. Consequently, obtaining sufficient quantities of well-characterized products for evaluation in clinical trials would be advantageous. Several issues regarding the choice of the clinical trial material require special attention, for example, (*a*) use of different parts of the plants (e.g., roots, seeds, aerial parts, whole plant), (*b*) use of different cultivars and species, (*c*) optimal growing and harvesting conditions, (*d*) use of the whole extract or a specific fraction, (*e*) the method of extraction (e.g., alcoholic, tea, pressed juice), (*f*) chemical standardization of the product, (*g*) bioavailability of the formulation (e.g., extract, tablet, capsule), and (*h*) the dose and length of administration.

Unlike conventional drugs, herbal products are not regulated for purity and potency. Some of the adverse effects and drug interactions reported for herbal

products could be caused by impurities, unnamed adulterants, or batch-to-batch variability. The chemical characterization (or fingerprint analysis) and standardization of botanical products would facilitate their evaluation in basic research and clinical studies. A study (22) supported by NCCAM compared the labeled amount and type of ginseng in 25 commercial products with the actual content. The study showed that, although each product was appropriately labeled for the type of ginseng contained within, the concentrations of ginseng, as determined by analysis of marker compounds, differed widely from that stated on the label.

Preparation of Standardized Products

Given the popularity of botanical products, NCCAM has taken several steps to ensure the development of well-defined products to conduct conclusive clinical trials. With the great need for standardized botanical products, industry involvement is critical. NCCAM is working with industrial and academic partners through several mechanisms; echinacea is illustrative of these interactions (Table 4). One investigator is attempting to identify the best source of crude echinacea (*Echinacea angustifolia*), an herb used for treatment of common respiratory infections. The investigator is biochemically profiling "marker" compounds in the plant to determine the optimal conditions for cultivation relative to the yield of potentially medicinal components. Later work will focus on the isolation and characterization of the most biologically active components. Another investigator is examining the correlation between composition and bioactivity of echinacea and is using an in vitro model to determine the circumstances under which the liver activates specific echinacea components possessing the kinds of immunostimulating effects that are thought to help speed the resolution of infections.

In addition, NCCAM plans to support a contract for the development and production of research grade cranberry (*Vaccinium macrocarpon*) products and placebos for use in clinical studies. There is evidence from small clinical trials suggesting that cranberry may relieve symptoms of urinary tract infection (UTI) and may reduce the need for antibiotics in treating such infections (23). The products developed under the contract will be evaluated in basic and clinical research studies on the role of cranberry in the prevention and treatment of UTIs and other conditions for which there is credible evidence of efficacy.

A critical step in ensuring that NCCAM can secure sufficient standardized botanical products is to build collaborative relationships with industry. In May 2001, NCCAM and the NIH Office of Dietary Supplements (ODS) convened a colloquium to begin a dialogue regarding how NCCAM, ODS, and industry can work together to definitively evaluate CAM therapeutic products for composition, safety, and efficacy (24). The meeting involved two key groups: industrial stakeholders that supply raw materials and manufacture and market CAM therapeutics (e.g., dietary supplements), and organizations that develop and apply standards to determine the identity, quality, and safety of these products. Industry representatives

TABLE 4 NCCAM-supported research on echinacea[a]

Stage of study	NIH grant mechanism[b]	Principal investigator/ institution	Study summary
Standardization of echinacea	R44	Xiping Wang/Gaia Herbs, Inc.	■ Prepare raw material and collect data on plant identity ■ Prepare marker compounds and testing methods ■ Test marker compounds to identify promising end products using different extraction methods ■ Evaluate optimum delivery presentation and product stability
In vitro studies	K01	Cynthia Wenner/ Bastyr University	■ Investigate the correlation between formulation composition and bioactivity of echinacea ■ Study herb/drug interactions by examining echinacea's effects on drug-induced inhibition of Cytochrome P450 isoenzymes involved in drug metabolism
Phase II trials	P50	Fayez Ghishan/ University of Arizona	■ Determine the efficacy of herbal therapy and craniosacral manipulation to prevent acute otitis media in children with recurrent otitis media infection
Phase III trials	R01	Ronald Turner/ University of Virginia	■ Evaluate the effect of different echinacea constituents on rhinovirus infection and rhinovirus-induced illness (experimental common cold model)
Phase III trials	R01	James Taylor/ University of Washington	■ Study echinacea for the treatment of upper respiratory infection
Phase III trials	K23	Bruce Barrett/ University of Wisconsin	■ Test the efficacy of echinacea as early treatment for upper respiratory infection: explore dose dependency, compare specific preparations, investigate mechanisms of action

[a]Echinacea is a widely used herbal remedy for the common cold. NCCAM supports a broad array of studies on echinacea. Investigators supported by NCCAM are using crude preparations of echinacea in clinical trials in order to determine if the herb is effective in preventing and/or treating upper respiratory and middle ear infections. The studies differ in the type of echinacea preparation used, product form, duration of administration, patient population type and size, and outcome measurements. Together, however, these studies may demonstrate some applicability to these conditions, providing information about the best opportunities for conducting conclusive clinical trials employing a highly characterized and standardized product.

[b]R44: Small Business Innovation Business Research (SBIR) Grant; K01: Mentored Scientist Award; P50: Center Grant; R01: Research Project Grant; K23: Mentored Patient-Oriented Research Career Development Award.

are increasingly involved in self-regulation with respect to product standardization and quality assurance. Because there are few financial incentives for industry, NIH plays an important role in supporting basic research on mechanism of action and modeling studies and clinical trials to determine safety and efficacy. Based on the understanding gained of the common interests and the complementary roles each group can play, a foundation has been laid for pursuing opportunities to develop future collaborations.

Bioavailability/Bioequivalence Issues

Manufacturers of both prescription and OTC drug products must demonstrate not only that the product contains the amount of drug that is stated on the label, but that the active component is bioavailable. For prescription drug products, manufacturers measure the blood level concentrations of the drug over a time interval. A generic product is shown to be comparable to an innovator drug product in dosage form, strength, route of administration, quality, performance characteristics, and intended use. Generic applicants must demonstrate that their product is bioequivalent to the innovator drug by measuring the concentrations of both products in blood or plasma in 24 to 36 healthy volunteers (25). OTC drug products must meet specifications identified in the drug monograph, such as dissolution and disintegration test standards. Various forms of a botanical product are available on the market—dried forms, which may be milled to different particle sizes, tinctures, extracts, capsules, and tablets—yet the bioavailability and comparability of these different products has not been well studied. Likewise, the bioequivalence of products from different manufacturers has not been demonstrated. Such studies will prove increasingly important to advance the CAM research field.

CAM-Drug Interactions

Each year a number of deaths occur when patients take a drug that interacts with other medications in their regimen or incites reactions that might not have been predicted from the studies that permitted the drug's licensure. A small number of drugs are withdrawn each year as a result of harmful or fatal adverse drug reactions and interactions. Even though the safety of new drugs is determined through extensive premarket clinical testing, certain patient populations not adequately represented in the clinical trial stage may react more strongly. In addition, the younger, healthier patient who participates in many clinical trials is not always representative of the individuals who will be taking the drug. It may take months or years of actual use before problems are discovered.

The causes and significance of drug interactions are multifaceted and can affect the processes by which pharmacologically active entities are absorbed, distributed, metabolized, and excreted (26). An enhanced or diminished modulation of the pharmacologic properties of a drug by another entitity may be harmful (administration of tetracycline with an antacid that contains divalent or trivalent

metallic ions results in formation of poorly soluble chelates and reduction in antibiotic absorption) or beneficial (because probenicid competes with penicillin for renal excretion, administration of the two drugs together increases serum levels of penicillin and prolongs its half life).

Because of widespread use, often for centuries, and because the products are "natural," many people assume complementary and alternative medicines to be inert or at least innocuous. Yet interactions between these products and drugs do occur and may have profound clinical consequences. For example, the active ingredients in Ginkgo biloba extract, a leading herbal supplement in the United States (27), are reported to have antioxidant properties and to inhibit platelet aggregation (28). This herbal supplement is promoted for use in improving cognitive function and blood flow (29). Several reports have been published on the increased bleeding associated with the use of Ginkgo biloba alone or in combination with other drugs that have anticoagulant or antiplatelet effects, such as warfarin and aspirin. Of note is information from a national survey showing that fewer than 40% of patients disclose the use of CAM therapies to their physician (30).

St. John's wort (*Hypericum perforatum*) is another leading herbal supplement sold in the United States (31), taken most often to treat various forms of depression. Reported cases of interactions with drugs together with pharmacokinetic data strongly suggest that St. John's wort is an inducer of a broad range of enzymes that metabolize drugs (32); a recent article suggests applying knowledge about the role of two hormone receptors that may regulate cytochrome P450 expression to the development of toxicological screens in the drug discovery process (33). In this regard, St. John's wort has been shown to interact with a number of drugs that serve as substrates for the cytochrome P450 CYP 3A enzymes responsible for metabolism of approximately 60% of current pharmaceutical agents. Substrates for the CYP 3A subfamily include cyclosporin, oral contraceptives, indinavir, sertraline, estrogen, and progesterone (34). A study conducted at the NIH (35) found that St. John's wort, when taken together with the HIV protease-inhibitor drug, indinavir, significantly reduced the plasma concentrations and approximately halved the area under the curve for orally administered indinavir. Inadequate plasma concentrations of protease inhibitors are a cause of antiretroviral resistance and treatment failure. Preliminary evidence indicates that St. John's wort also lowers the therapeutic activity of some types of oral contraceptives (36). Heart transplant rejection was reported as soon as three weeks after St. John's wort was added to the drug regimen of heart transplant patients on cyclosporin therapy (37) to prevent transplant rejection. Finally, the results of recent study suggest that St. John's wort may compromise the effects of certain cancer treatments, such as irinotecan (38). The FDA has issued a public health advisory warning physicians of potential adverse interactions with St. John's wort and advising them to alert their patients (39).

NCCAM supports a number of studies investigating the mechanism of action of CAM therapies and interactions with other drugs. In 2001, NCCAM issued a

TABLE 5 Selected NCCAM-funded studies on mechanism of action of CAM therapies and interactions with other drugs

Principal investigator/ institution	Study aim	Type of study
B. Timmermann/U. of Arizona	Curcuma longa rhizome (turmeric), Zingiber Officinale Rhizome (ginger), Boswellia serrata (boswellin)	
	■ Determine the active components and their ability to regulate inflammation	In vitro
	■ Characterize the disposition, gastrointestinal absorption kinetics, and bioavailability	Animals
	■ Assess the pharmacokinetic and pharmacodynamic characteristics	Phase I trial
G. Henderson/U. of California	Investigate the pharmacologic interactions between herbal products and asthma medications	In vitro
A. Hurwitz/U. of Kansas Medical Center	Investigate interactions of ginseng and ginkgo with various drugs	In vitro
S. Liao/U. of Chicago	Determine if green tea extracts, a complex herbal known as PCSPES, and extracts of individual PCSPES components affect the chemotherapeutic effect of growth of a drug used for prostate cancer	In vitro
D. Shen/Fred Hutchinson Cancer Research Center	Investigate whether significant interactions occur between two widely used opioid analgesics, oxycodone and fentanyl, and St. John's wort	Phase I
J. Markowitz/Medical University of South Carolina	Evaluate 10 commonly used herbs for inhibition/induction of enzymes that metabolize drugs (e.g., CYP3A4, 2136)	In vitro
P. Murphy/Columbia University	Evaluate the effects of St. John's wort on oral contraceptives	Phase I/II

request for applications to further stimulate research in this area. A list of selected studies is shown in Table 5.

Safety and Efficacy

Although participants at the NIH-Industry Colloquium mentioned earlier agreed that studies on the safety and efficacy of biologically based products are important, they disagreed on the level of evidence that is needed. Some advocates of herbal medicines are satisfied with the existing evidence that these products are safe and

effective. Because there are currently no regulatory requirements or guidelines for what constitutes adequate studies, the private and public sectors must continue to work together to provide the public and health professionals with reliable research data.

Compelling data are now beginning to emerge from NCCAM-funded research studies. A report in *The British Medical Journal* (40) showed that St. John's wort is more effective than placebo in treatment of depression, and perhaps as effective as an older generation antidepressant drug, imipramine. Because of the intense interest in the use of St. John's wort, NCCAM and its research partners are collaborating on several studies of the safety and effectiveness of St. John's wort in treating depression. One study compared St. John's wort with placebo and sertraline, currently one of the most commonly used antidepressants. The results of the study show that St. John's wort is no more effective for treating major depression of moderate severity than placebo (41). Other studies on St. John's wort are under way.

NCCAM's research interests are broad, encompassing virtually all branches of medicine and health conditions across the lifespan. Data from basic and early clinical research studies are used as the basis for launching major clinical trials. In addition to the study of St. John's wort, NCCAM is conducting the largest and most definitive Phase III clinical trials ever underaken for a range of CAM therapies, and thousands of research subjects have been enrolled. In collaboration with research partners at NIH, NCCAM is sponsoring several large clinical trials to address a variety of popular CAM modalities for important public health issues such as depression, arthritis, heart disease, and dementia (Table 6).

TABLE 6 NCCAM is conducting the largest and most definitive multicenter, Phase III clinical trials ever undertaken for a range of CAM therapies (as of September 1, 2002)

Condition	Therapy	NIH Cosponsor[a]
Dementia	Ginkgo biloba	NIA, NHLBI, NINDS
Osteoarthritis	Accupuncture	NIAMS
Osteoarthritis	Glucosamine/chondroitin	NIAMS
Lung cancer	Shark cartilage	NCI
Prostate cancer	Vitamin E/selenium	NCI
Minor depression	St. John's wort	NIMH, ODS
Coronary artery disease	EDTA chelation therapy	NHLBI
Benign prostatic hypertrophy	Saw palmetto/African plum	NIDDK, ODS

[a]NIA: National Institute on Aging; NHLBI: National Heart, Lung, and Blood Institute; NINDS: National Institute of Neurological Disorders and Stroke; NIAMS: National Institute of Arthritis and Musculoskeletal and Skin Diseases; NCI: National Cancer Institute; NIMH: National Institute of Mental Health; ODS: Office of Dietary Supplements; NIDDK: National Institute of Diabetes and Digestive and Kidney Diseases.

CONCLUSIONS

The examples cited illustrate both the promise and challenges presented by CAM therapies. Through rigorous research, we will be able to determine not only to what extent each therapy is safe or effective, but also under what circumstances an effective CAM modality may be contraindicated. As the recent studies with St. John's wort show, a widely used botanical drug may impart unexpected adverse consequences when taken together with drugs that are part of the contemporary pharmaceutical arsenal. It is critical that untested but widely used CAM treatments be rigorously evaluated both for safety and efficacy. In the current regulatory climate, maintaining a strong research effort is critical and the NIH has stepped into the void left by the private sector. Studies on the underlying mechanisms of action, safety, efficacy, and purity of products will provide answers regarding which are suitable to be incorporated into medical practice. Only when providers and consumers have reliable information can they make well-informed decisions about which products and practices are appropriate and which should be rejected.

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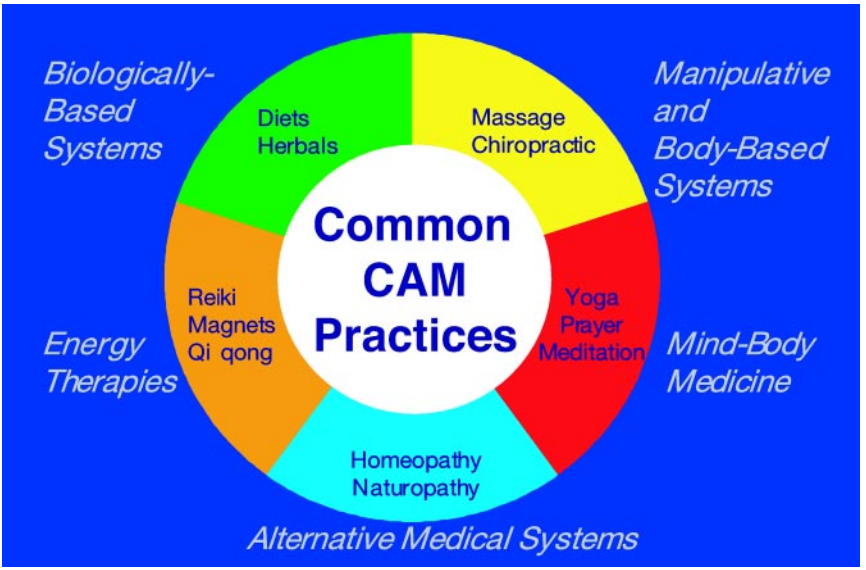

Figure 1 Five major CAM domains. NCCAM defines complementary and alternative medicine (CAM) practices as those not presently considered an integral part of conventional medicine. As CAM modalities are proven safe and effective, they may become adopted into mainstream medical practice. The five areas shown in the schematic represent but one way to array CAM approaches. Examples in each area are provided below (see NCCAM strategic plan at http://www.nccam.nih.gov for more details):

- Alternative medical systems—traditional oriental medicine, homeopathy, Ayurveda.
- Mind-body interventions—meditation, prayer, biofeedback.
- Biologically-based therapies—botanicals, herbs, special diet therapies.
- Manipulative and body-based methods—chiropractic, massage.
- Energy therapies—Qi gong, Reiki, magnets.

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CHALLENGING DOGMA: Thresholds for Genotoxic Carcinogens? The Case of Vinyl Acetate

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Key Words physiological modeling, acetaldehyde, acetic acid, DNA damage, olfactory tissue

■ **Abstract** Although many questions remain unanswered, the general principle of the sequence of events leading to cancer after exposure to genotoxic carcinogens has become increasingly clear. This helps to understand the parameters that influence the shape of the dose-effect curve for carcinogenesis, including metabolic activation and inactivation of carcinogens, DNA repair, cell cycle control, apoptosis, and control by the immune system. A linear dose-response relationship with no observable threshold seems to be a conservative but adequate description for the carcinogenic activity of many genotoxic carcinogens, such as aflatoxin B1, the tobacco-specific nitrosoketone NNK, and probably N,N-diethylnitrosamine. However, extrapolation models connecting the high-level risk to the zero intercept have clearly resulted in overestimations of risk. Vinyl acetate is an example that is discussed extensively in this review. At extremely high and toxic doses, vinyl acetate is carcinogenic in rats and mice and causes chromosomal aberrations. In tissues of contact, vinyl acetate is converted to acetic acid and acetaldehyde. Only when threshold levels are achieved do critical steps in the mechanism ultimately leading to cancer become active, namely pH reduction in exposed cells of more than 0.15 units leading to cytotoxicity, damage to DNA, and regenerative proliferation. Consistent with the known exposure to endogenous acetic acid and acetaldehyde, tissues sustain a certain level of exposure without adverse effects. Physiological modeling shows that the conditions necessary for carcinogenesis are in place only when threshold levels of vinyl acetate are exceeded. The example of vinyl acetate underlines the importance of toxicological research that unequivocally identifies genotoxic carcinogens acting through a threshold process.

INTRODUCTION

A conservative strategy of carcinogenic risk evaluation is to connect the high-level risk to the zero intercept and define the slope of the line as a risk coefficient for carcinogens per unit of dose (1). However, in some cases this type of calculation may result in unreasonable conclusions. One of the most provocative examples is presented by Goldman (1): Assume that every human being on Earth adds a 3-cm lift to his shoes for 20 years. The resulting increase in cosmic ray dose, which doubles for every 2000 m in altitude, is extremely small, but multiplied by the population of Earth results in a dose that causes cancer in approximately 30,000 individuals in 50 years. Although the mathematics in this example are correct, it would rightfully never be accepted as a scientific basis to ban high shoes.

The (unacceptable) linear extrapolation model of radiation risk represents only one of several examples showing that the old dogma based on the assumption of "no threshold" for genotoxic carcinogens does not hold true in all cases. Such considerations stimulated opposition against the no threshold assumption inherent in extrapolating risk linearly to the zero dose intercept. Several examples have been published in *Science*:

- "The current mode of extrapolating high-dose to low-dose effects is erroneous for both chemicals and radiation. Safe levels of exposure exist. The public has been needlessly frightened and deceived, and hundreds of billion of dollars wasted" (2).
- ". . . it is time to seriously consider the utility of implementing a concept of an effective or practical threshold for risk, that is, negligible risk" (1).
- "Toxic effects of high risk doses often do not occur at low doses . . . the last thing we want to do . . . is to put our limited resources into protecting people from things that are harmless" (3).

Without any doubt, these and other provocative statements have initiated a fruitful discussion about risk assessments of low-level exposures. However, it is dangerous to generalize that safe levels of exposure exist. For instance, there is no convincing evidence showing that aflatoxin B1 (AFB1) acts by a threshold mechanism. This leads to the question of whether modern toxicology should differentiate between the two types of genotoxic carcinogens, those acting by a practical threshold and those acting by a nonthreshold mechanism (or at least by a mechanism for which a threshold has not yet been shown). In this article, the molecular mechanisms are reviewed, explaining the difference between both types of genotoxic carcinogens. Special attention is given to the case of vinyl acetate. Vinyl acetate serves as an example for a number of high-volume industrial chemicals, including trichloroethylene (4), acrylonitrile (5), and 1,3-butadiene (6), for which threshold or practical threshold mechanisms of carcinogenesis are currently being debated.

THRESHOLD MECHANISMS

Although many questions remain unanswered, the general principle of the sequence of events leading to cancer after exposure to genotoxic carcinogens has become increasingly clear (Figure 1). This helps to understand the parameters that influence the shape of the dose-effect curve for carcinogenesis. Lack of metabolic activation of a precarcinogen in a certain animal species (Figure 1) would result in a mechanism that precludes its carcinogenic action in that species. This is obvious because, per definition, a substance is not a genotoxic carcinogen if it cannot be activated to damage DNA in that species. Nevertheless, the capacity for metabolic

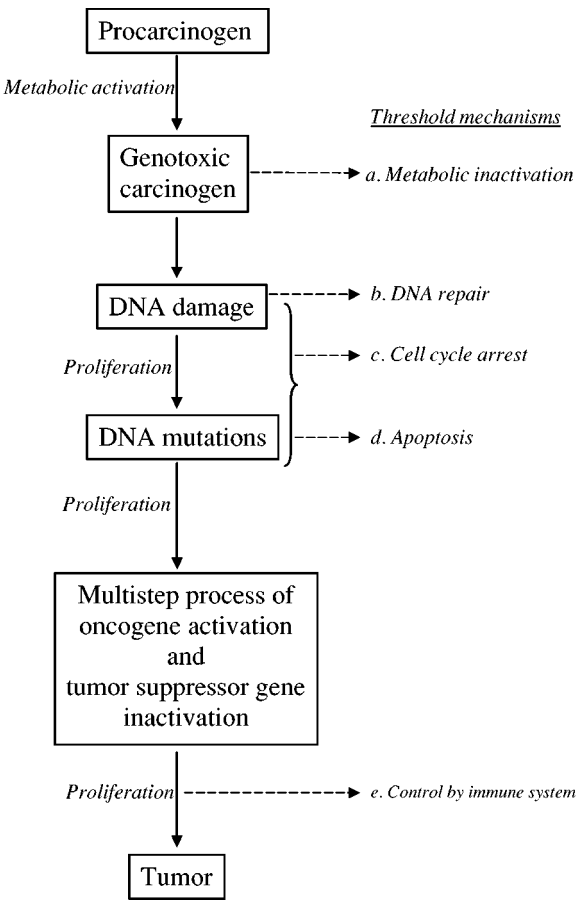

Figure 1 Possible threshold mechanisms of genotoxic carcinogens.

activation influences the shape of the dose-effect curve. For instance, the slope of the dose-effect curve of N,N-diethylnitrosamine (DEN)- or the tobacco-specific nitrosoketone NNK-induced liver tumor incidence in rats decreases at high doses due to saturation of the metabolic activation by CYP2E1 (Figures 6 and 9).

The next step on the path to cancer that influences the shape of the dose-effect curve is the metabolic inactivation of genotoxic carcinogens (Figure 1*a*). As a typical example, we present the influence of microsomal epoxide hydrolase (mEH), which influences the dose-effect curve in a way characteristic for the influence of metabolic inactivation. mEH inactivates styrene oxide by hydrolysis. Styrene oxide binds to DNA, predominantly to the N7-positon of guanine, which finally leads to DNA strand breaks. Human mEH was transfected into a Chinese hamster cell line (V79 cells) that, before transfection, expressed only very low activities of this enzyme [Figure 2*A*,*B*; (7)]. In V79 cells that express only very low mEH, styrene oxide leads to the formation of DNA strand breaks in a dose-dependent manner with no observable threshold [Figure 2*C*; (7–9)]. Chinese hamster cells genetically engineered to express human mEH at levels giving rise to similar activities as observed in human liver are protected from measurable genotoxic effects of styrene oxide up to 100 μM. Due to the detection limit of the assay, it is not possible to differentiate whether transfection of mEH introduced a "practical threshold," characterized by a very low level of DNA damage (not measurably exceeding the background in absence of styrene oxide), or a "real threshold," characterized by a dose-effect curve crossing the abscissa at 100-μM styrene oxide, i.e., representing truly zero effect up to a concentration of 100-μM styrene oxide. Although a conclusion cannot be obtained experimentally, we favor the model of a practical threshold because it seems improbable that a detoxifying enzyme can completely exclude small numbers of genotoxic molecules from reaching the DNA. If the assumption that detoxification does not guarantee completeness is accepted, it is possible to conclude that metabolic inactivation results in practical, albeit not perfect, thresholds. Whether a detoxifying enzyme qualifies as a basis for a practical threshold depends on the speed and capacity of removal of the reactive species from the system compared with the speed of the translocation of the reactive species from the site of its generation to the nucleus and reaction with the DNA. The recently discovered two-step mechanism of the xenobiotic metabolizing mEH leads to the observed practical threshold (Figure 2*C*). This mechanism consists of a very fast first step of removal of the epoxide by covalent interaction with the enzyme followed by the much slower hydrolysis of the complex. The high capacity of this mechanism is provided by an unusually high amount of enzyme. Thus, mEH soaks up the genotoxic epoxide like a sponge up to a concentration of epoxide that has titrated out the high amount of enzyme (7, 8). Such mechanisms are also being discussed for trichloroethylene (4).

DNA repair is known to protect cells from fixation of DNA damage in the newly synthesized DNA strand as heritable mutations, thereby generating the situation of a race between repair and proliferation-dependent DNA synthesis (Figure 1*b*). It is not a matter of debate that DNA repair can effectively reduce tumor incidence.

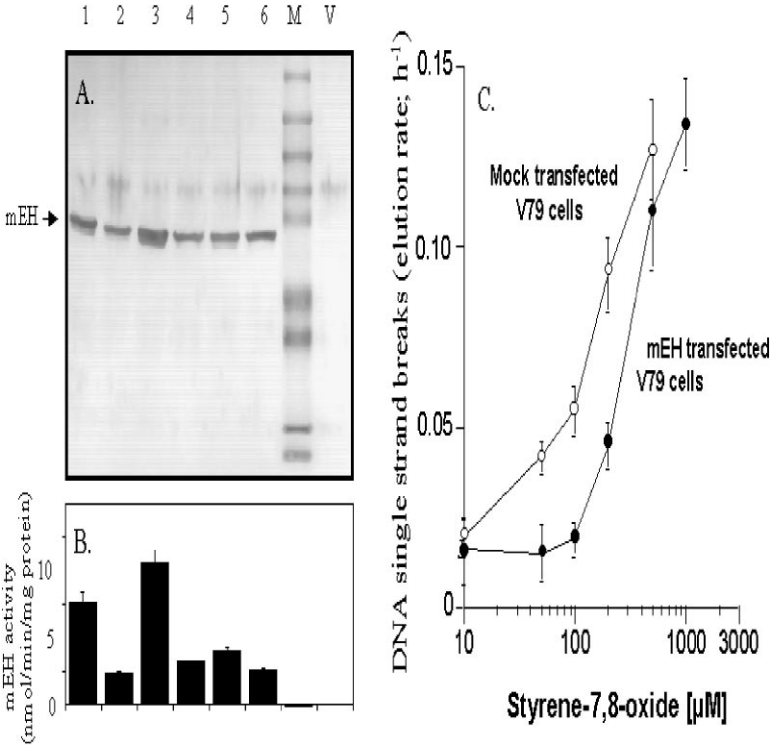

Figure 2 Recombinant expression of human mEH protects V79 Chinese hamster cells (V79 cells) from styrene-7,8-oxide-induced DNA single-strand breaks. (*A*) Western blot analysis of mEH transfected (*lanes 1–6*) and mock transfected (*lane V*) V79 cells. S9 fractions of the different clones were analyzed with a polyclonal antibody against purified human mEH raised in rabbits. Lane M: prestained molecular weight markers (195, 112, 84, 63, 52, 35, and 32 kd). (*B*) mEH activity of the same clones using styrene-7,8-oxide as a substrate. Values are expressed as nanomoles of styrene glycol formed per minute and milligrams of protein. (*C*) Effect of styrene-7,8-oxide on DNA single-strand breaks using mEH transfected clone No. 3 [from (*A*) and (*B*)] and mock transfected V79 cells (7).

However, it seems unlikely that DNA repair mechanisms are perfect. If an extremely low dose of a carcinogen induces only a single DNA adduct in a cell, this adduct has a probability to persist unrepaired until fixation as a mutation occurs. Although this probability may be low, it is unlikely zero. If this assumption is accepted, the influence of DNA repair may be regarded analogously to the discussions above concerning metabolic inactivation: Practical, but not perfect, thresholds may be introduced. A carcinogen may induce DNA repair enzymes. For instance, the DNA repair protein O6-methylguanine-DNA-methyltransferase (MGMT) that is

responsible for direct reversal of the miscoding DNA lesion O6-methylguanine has been reported to be inducible by diethylnitrosamine and ethylnitrosourea in mammalian tissues (9a). One might expect that the increased capacity for repair may reduce levels of DNA damage below baseline levels (i.e., below the levels of "spontaneous" DNA lesions), leading to a tumor incidence lower than in the control group. Although treatment with low doses of some carcinogens has led to a lower yield of some tumors than in the control group, to our knowledge, this theoretical possibility with respect to induction of DNA repair enzymes has not yet been supported by convincing overall tumor incidence data.

Cell cycle arrest can be induced as a consequence of DNA damage or interference with signal transduction in target cells (Figure 1*c*). Low levels of a carcinogen may even decrease cell cycle progression below baseline rates (10, 11). Because under specific circumstances the protective influence of decreased cell division can be stronger than the deleterious influence of increased DNA damage, the combination of both effects may result in a decreased tumor incidence. Higher levels of the same substance increase cell cycle progression due to cytotoxicity and regenerative cell proliferation, resulting in an increased tumor incidence. As a consequence, a J-shaped dose-effect curve results. This mechanism has been observed for nongenotoxic carcinogens, such as TCDD (12) or caffeic acid [(13), reviewed in (10)], but has also been postulated for genotoxic carcinogens, such as 2-acetylaminofluorene and ionizing radiation. The benefit of the first, decreasing part of a J-shaped dose-effect curve must be interpreted with caution because a decrease in cell proliferation below baseline may interfere with normal tissue regeneration. In addition, the protective influence may be tissue or cell-type specific. Cell cycle delay may be induced in one cell type, whereas other, more sensitive cell types may respond with regenerative proliferation. Nevertheless, cell cycle progression and regenerative proliferation probably represent the most relevant key parameters concerning threshold mechanisms. Due to the lack of fixation of DNA damage as a stable mutation in a newly synthesized daughter strand of DNA, a genotoxic substance will not be able to induce tumors in tissues that do not proliferate. For instance, the extremely low proliferative capacity of cardiomyocytes protects this cell type from carcinogenesis even if genotoxic substances induce DNA damage in cardiomyocytes. The latter has been shown for heterocyclic amines as assessed by ^{32}P-postlabelling analysis (13a), suggesting that cardiomyocytes are not more resistant to primary DNA damage than cells of organs that are susceptible to carcinogenesis. On the other hand, a genotoxic substance causes tumors with an extremely high probability when both DNA damage and cell proliferation are induced in target tissues. The situation becomes complex when a given dose of a substance induces DNA damage but not cytotoxicity and proliferative cell regeneration. In this circumstance, the result may depend on baseline proliferation of the relevant tissue. Rapidly proliferating cells in bone marrow or the crypt cells of the colon are at high risk for neoplastic transformation. On the other hand, for cells with a relatively low baseline proliferation, such as the olfactory epithelium, the latency period for carcinogenesis may exceed life expectancy (14, 15). Of high interest

are genotoxic substances for which both induction of regenerative proliferation and genotoxicity act through a threshold process. For instance, paracetamol and vinyl acetate belong to this class of substances (see below).

Apoptosis (Figure 1*d*) and the control of neoplastically transformed cells by the immune system (Figure 1*e*) are additional mechanisms influencing the shape of the dose-effect curve. Cells may undergo apoptosis as a consequence of DNA damage induced by relatively high doses. There is no doubt that this process can reduce tumor rates. However, little is known about the efficiency of apoptotic mechanisms at low doses and whether such mechanisms can lead to thresholds for carcinogenesis.

Considering the complexity of mechanisms that may introduce practical or perfect thresholds, it becomes clear that evaluation of the risk of genotoxic substances is not easy, but also not impossible. Selected examples of genotoxic carcinogens with and without practical threshold mechanisms are reviewed in the next section.

EXAMPLES OF CARCINOGENS WITH AND WITHOUT PRACTICAL THRESHOLDS

Aflatoxin B1

Aflatoxin B1 (AFB1) is one of the most potent human hepatocarcinogens known. It represents a liver cell type–specific toxin because it induces the formation of tumors developing from parenchymal and bile duct epithelial liver cells, but not from other cell types present in the liver, such as Kupffer or endothelial cells (16). AFB1 is a typical representative of the class of carcinogens showing a linear dose-response relationship in the low dose range. For instance, a 24-month study with male Fischer rats exposed to five doses between 1 and 50 ppb in the drinking water resulted in a linear dose-response curve, with the liver tumor incidence being 80% at the highest dose [Figure 3; (17), reviewed in (18)]. The linear increase in liver tumors corresponds well to the also linear induction of the main DNA adduct (dG-N7-AFB1) formed by AFB1 [Figure 3; (19)]. DNA adducts were measured after eight weeks of continuous administration of AFB1 in the drinking water. After this period, a steady state is achieved between adduct formation and removal. A linear low dose-response to AFB1 has also been shown in other species. For instance, tumor incidence in rainbow trout exposed to 50–250-ppb AFB1 as embryos increased without an obvious threshold [Figure 4; (20)]. Large species differences in AFB1 susceptiblilty are known (21). Rats represent a very sensitive species, whereas mice are much more resistant. It is important to use the rat model when extrapolating to human carcinogenicity because both rats and man—compared to mice—are relatively poor conjugators of activated AFB1. The molecular mechanisms of these interspecies differences have been reviewed (21).

AFB1 requires metabolic conversion to AFB1 exo-8,9-epoxide in order to cause DNA damage (22–24). In humans, AFB1 is activated primarily by CYP3A4 and 1A2. The AFB1 epoxide reacts with guanine, resulting in 8,9-dihydro-8-(N^7guanyl)-9-hydroxyaflatoxin B1 (AFB1-N7-Gua) as a main DNA adduct. The

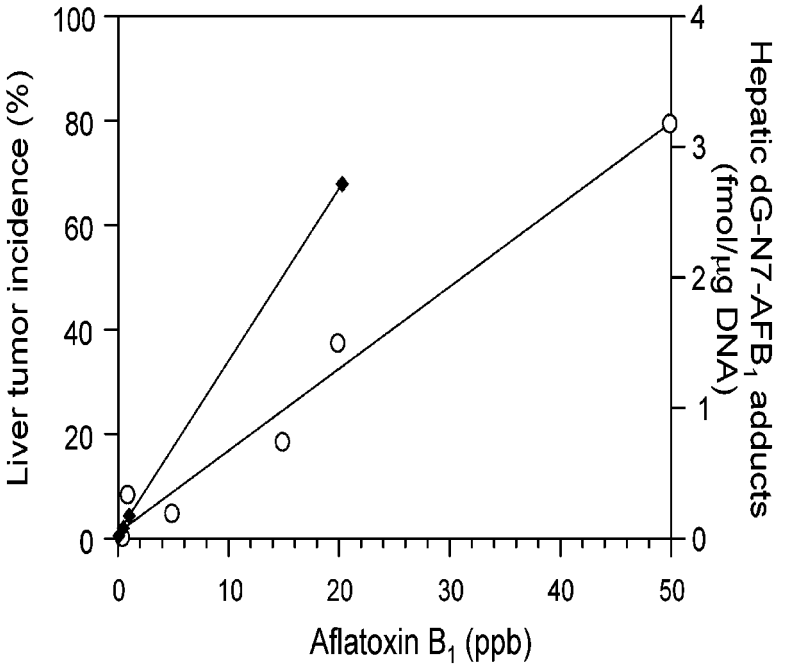

Figure 3 Liver tumors (○) and DNA adducts (♦) induced by AFB1. Male Fischer rats were exposed to five doses of AFB1 between 1 and 50 ppb in the drinking water for 24 months for analysis of tumor incidence. DNA adducts were measured after eight weeks of continuous administration of AFB1 in the drinking water (17–19).

positively charged imidazole ring of the resulting molecule facilitates depurination, leading to an apurinic site. Alternatively, the imidazole ring of AFB1-N7-Gua opens to form the stable AFB1 formamidopyrimidine (AFB1-FAPY). The initial AFB1-N7-Gua, AFB1-FAPY, and the apurinic site represent likely precursors to the mutagenic effects of AFB1. Bailey et al. presented strong evidence that the initial AFB1-N7-Gua adduct is extremely efficient in inducing mutations (25). Two main mutations are induced (Figure 5): (*a*) G→T transversions are targeted to the original site of the adduct (Figure 5, *upper panel*). Such G→T transversions have been identified in the p53 tumor suppressor gene in the third position of codon 249 (AGG) in approximately half of all examined hepatocellular carcinomas of humans exposed to AFB1 (26, 27). (*b*) The C→T transition occurs on the 3′ face of the modified guanine (Figure 5, *lower panel*). The AFB1 moiety of the AFB1-N7-Gua adduct intercalates on the 3′ face of guanine. As a consequence, the base 3′ of the adduct (a cytosine in Figure 5) may rotate out of the helix, leading to the insertion of adenine across from the AFB1 adduct, finally resulting in a C→T transition (25). Such C→T transitions have been identified in codon 12 of the c-ki-ras oncogene of rat hepatocellular carcinomas (28, 29). It can be assumed that the linear relationship between AFB1 exposure and tumor incidence at low doses as well as

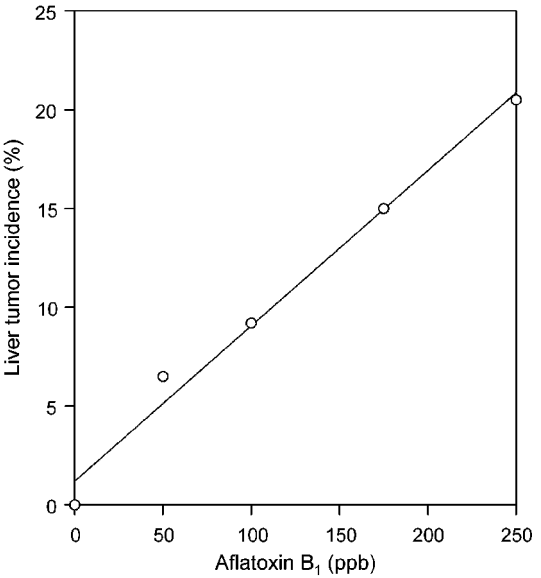

Figure 4 AFB1 liver carcinogenicity in rainbow trout. Rainbow trout embryos were exposed to AFB1 for 30 min. At 11 months of age, trouts were sacrificed and analyzed for liver tumors. The number of fish were 200, 370, 249, 400, and 400 in the 0, 50, 100, 175, and 250 ppb exposure groups, respectively (20).

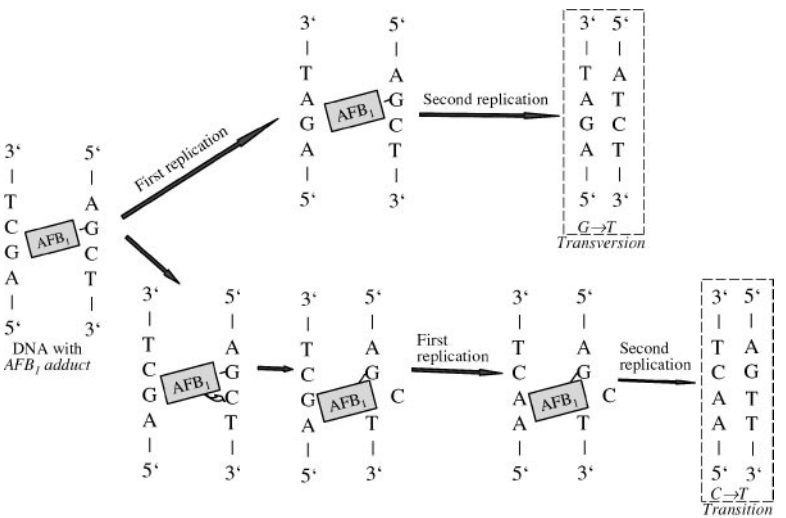

Figure 5 Two types of mutations (G→T transversion and C→T transition) resulting from the AFB1-N^7-guanine adduct [8,9-dihydro-8-(N^7 guanyl)-9-hydroxyaflatoxin] B1. Newly synthesized DNA strand: dashed boxes.

the direct relationship between AFB1-DNA adducts and tumor incidence require several preconditions. In the case of AFB1, none of the possible mechanisms (*a–e* in Figure 1) seem to be effective enough to introduce a measurable threshold. The possibility that a threshold could be observed for doses lower than those used in the experiments shown in Figures 3 and 4 cannot be excluded. Such experiments would be difficult because extremely large numbers of animals would be required. However, unless data are available, the most conservative model, which is a linear dose-response extrapolation with no assumed threshold, should be used for AFB1 risk evaluation.

N,N-Diethylnitrosamine

Similar to AFB1, N,N-diethylnitrosamine (DEN) is a rodent liver carcinogen when administered continuously in low doses. In a large lifetime tumorigenesis study involving 1140 male Wistar rats, 15 different doses ranging between 0.033 and 16.9 ppm were given in the drinking water (30). No threshold was observed in the low dose range, although the dose-response curve was not linear but approached a plateau at higher doses (Figure 6). O^4-ethylthymidine (O^4-Et-dT) was considered

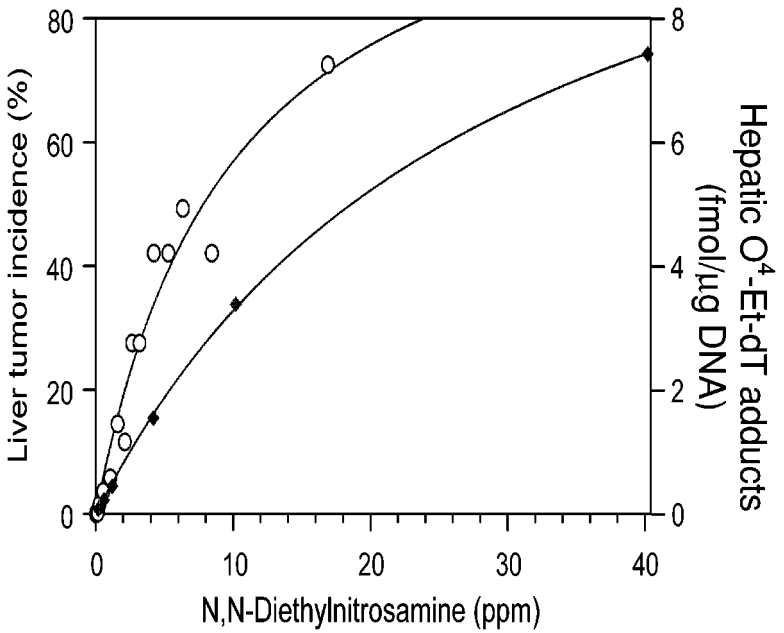

Figure 6 Liver tumor incidence (○) in a large lifetime study involving 1140 male Wistar rats. Fifteen different doses of DEN ranging between 0.033 and 16.9 ppm were given in the drinking water (18, 30). Data for O^4-ethylthymidine (♦), the major promutagenic DNA adduct responsible for induction of liver tumors, were obtained from another study with male Fischer rats (31) because adduct data from Wistar rats are not available.

to represent the major promutagenic DNA adduct responsible for induction of liver tumors (31). When DNA adducts were analyzed in another rat strain (male Fischer rats), a similar shape of the dose-effect curve was obtained as in liver tumor incidence (Figure 6). Thus, DEN represents a carcinogen showing a nonlinear dose-response without a threshold and with a very good correlation between DNA adduct induction and tumor incidence when administered continuously in the drinking water. However, different results were obtained in studies using short-term or intermittent instead of chronic lifetime exposures. Seven hundred fifty 14-day post-hatch medaka were divided into 10 groups of 75 fish each, and replicate groups were exposed to 0, 10, 25, 50, and 100 ppm DEN for 48 h (32). No increase in hepatic adenomas or carcinomas was observed for the two lowest doses of 10 and 25 ppm DEN, whereas an increase was reported for 50 and 100 ppm (Figure 7). The different dose-response curves for short- versus long-term administration may be due to interspecies differences. Williams et al. also observed nonlinearities in several studies with male Fischer 344 rats (33–36). The data shown in Figure 8 were obtained with five doses ranging from a cumulative total of 0.5 to 4 mmol DEN per kg body weight given intermittently as weekly i.p. injections for 10 weeks. No liver carcinomas were observed up to an exposure of 1 mmol/kg. Exposure to 2 mmol/kg and greater caused liver carcinomas in almost all of the 12 exposed rats [Figure 8; (35)]. Whereas these data were obtained by the i.p. administration route, the authors later obtained similar results by once weekly intragastric instillation

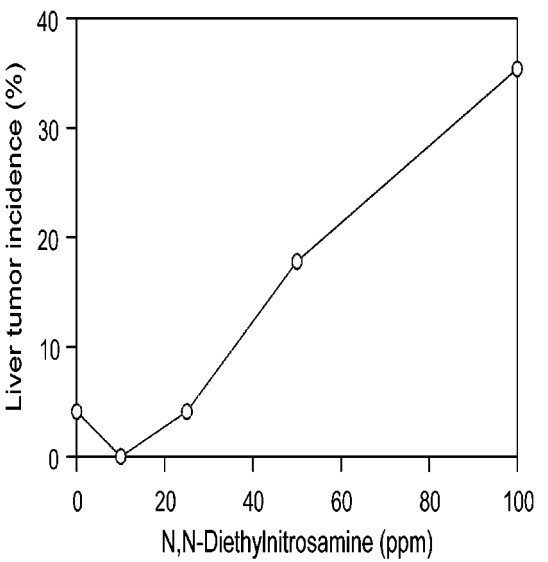

Figure 7 Liver tumor induction by DEN in fish. Japanese medaka were exposed to DEN for 48 h at 14 days post hatch. At 6 months of age, fish were sacrificed and analyzed for liver tumors. The number of fish were 49, 44, 49, 45, and 48 in the 0, 10, 25, 50, and 100 ppm exposure groups, respectively (32).

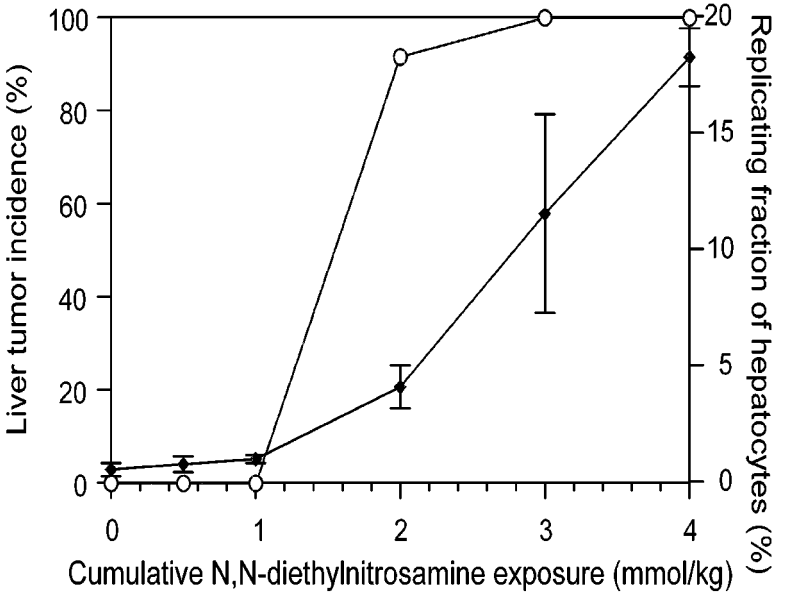

Figure 8 Incidence of liver carcinomas (○) in male Fischer 344 rats (35). Ten doses ranging from a cumulative total of 0.5 to 4 mmol DEN per kg body weight were given as weekly i.p. injections for 10 weeks. After a period of 38 weeks 12 rats were examined per dose group. For determination of the replicating fraction (♦) of hepatocytes, the same administration schedule was applied to male Fischer 344 rats. Values are means and standard deviations of five rats. The replicating fraction was determined by immunohistochemical analysis of bromodeoxyuridine incorporation.

(34). Thus, the type of exposure, continuous for lifetime versus intermittent or short-term exposure, appears to strongly influence the low dose–response curve. The cumulative exposure of 2 mmol/kg yielded a 92% liver cancer incidence (35). This effect can be best compared to that of the highest cumulative exposure achieved in the continuous lifetime study of Peto et al. (30) that was calculated to be approximately 10-mmol/kg cumulative exposure resulting in a 78% incidence of liver tumors. Thus, the cumulative exposure in the study of Williams et al. was even smaller compared to the study of Peto et al. But because DEN was administered in only ten (individual) doses, the single doses in the Williams et al. study were much higher. The mechanisms responsible for the observed nonlinearity were also examined by Williams et al. (34, 35). Interestingly, induction of DNA adducts does not explain the observed nonlinearity because the lowest exposures also produced a clear level of DNA ethylation in the liver, even when given as only a single dose (data not shown). However, cytotoxicity and cell proliferation in rat liver correlated well with tumor incidence. Whereas the nontumorigenic cumulative doses of 0.5 and 1 mmol/kg did not cause a significant increase in the replicating fraction

of hepatocytes, a strong increase was observed for higher doses (Figure 8). It is tempting to speculate that the differences between the studies of Peto et al. and Williams et al. are due to differences in toxicity in the low-dose range (with an expected linear toxicity dose response in the Peto study), but the relevant data are not presently available.

In conclusion, the dose-response relationship for DEN in the low-dose range appears to depend on the schedule of administration of the test substance, resulting in a linear relationship for continuous lifetime exposure but a nonlinear relationship with a practical threshold at 1-mmol DEN/kg for intermittent weekly administration. We believe that the difference between lifetime exposure to very low daily doses (30) and intermittent weekly administration of relatively high doses (35) can be explained by the difference in the period between administration of DEN and analysis of tumors, and in differences of cytotoxicity. Although the low daily doses of DEN in the study of Peto et al. (30) induced DNA adducts, they were so low that probably no significant toxicity was induced. Nevertheless, the period of more than two years in this lifetime study was long enough to allow formation of mutations and later carcinomas due to the low baseline proliferation of hepatocytes. Thus, in absence of a DEN-induced influence on hepatocyte proliferation, the linear induction of DNA adducts explains the also linear dose-response relationship of liver tumors. In contrast to the study of Peto et al. (30), a shorter period between administration of DEN and analysis of carcinomas was chosen in the study of Williams [only 38 weeks; (35)]. This time period was probably not long enough to allow formation of tumors at normal (low) baseline proliferation of hepatocytes. However, when proliferation was increased by administration of cytotoxic doses of DEN, carcinomas could be induced also during the relatively short latency period of 38 weeks. Thus, cytotoxicity-induced proliferation seems to be a necessary prerequisite to cause carcinomas after relatively short latency periods. Because cytotoxicity and the induced proliferation were not linear with dose in William's study (Figure 8), a nonlinear dose response for carcinomas is a plausible consequence. Based on the assumption that human exposure to environmental carcinogens is intermittent (35), it has been suggested that low-level exposures to DEN may represent no cancer risk to humans (34). However, due to the remaining uncertainties (such as the effect of intermittent low-dose DEN on human hepatocyte proliferation and the question of whether individuals may also be exposed rather continuously), the conservative linear model with no threshold assumption may be most appropriate for extrapolation of human cancer risk due to DEN until additional clarifying data become available.

NNK [4-(N-Methyl-N-nitrosoamino)-1-(3-pyridyl)-1-butanone]

In the previous sections, AFB1 and DEN are discussed as nonthreshold carcinogens of the liver. Similarly, the linear dose-response relationship with no observable threshold seems to be an adequate description for the carcinogenic activity of several genotoxic carcinogens in lung. One of the best documented examples

is the tabacco-specific nitrosoketone NNK, which is a nicotine derivative forming the promutagenic O^6-methylguanine and pyridyloxobutyl DNA adducts (37–39) that are responsible for lung cancer in rats. NNK is also relevant for nonsmokers because nonsmoking women exposed to environmental tobacco smoke have been reported to take up and metabolize NNK, which could increase their risk of lung cancer [(40); review in (18)]. Analogous to DEN, a good correlation between DNA adducts (O^6-methylguanine) and tumor incidence has been observed (Figure 9). Both tumor incidence and DNA adducts show a nonlinear dose-response curve without an obvious threshold.

2-Acetylaminofluorene (2-AAF)

Probably the largest tumorigenicity study ever conducted was with the polycyclic aromatic amide 2-AAF published in 1979 (41). Approximately 24,000 female

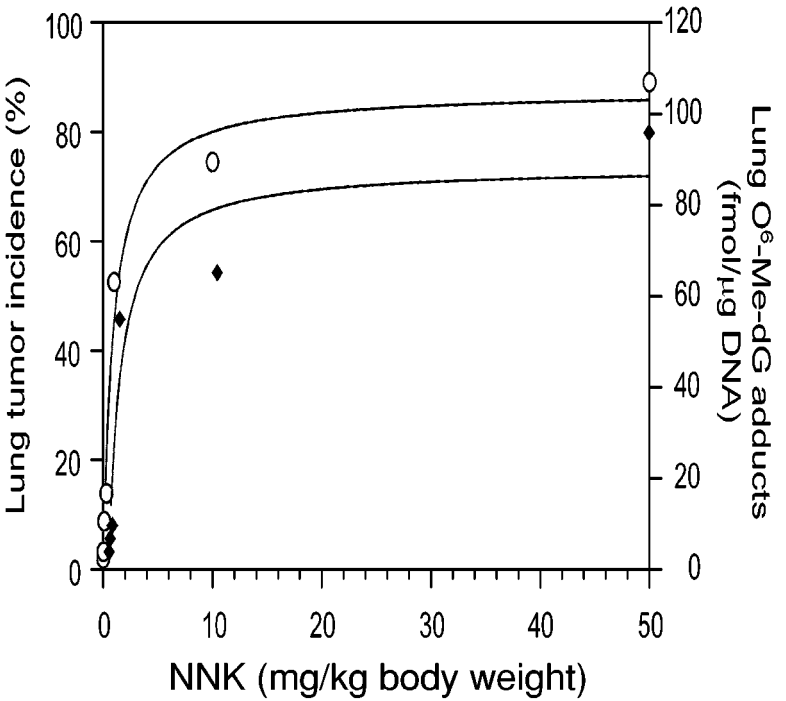

Figure 9 Lung tumor incidence (○) was determined in male Fischer rats (experimental data and modeling of dose dependence). The N-nitrosoketone was administered as subcutaneous injections three times a week for 20 weeks. Rats were sacrificed and analyzed for lung tumors after 31 months. O^6-Methyldeoxyguanosine (O^6-Me-dG) (♦) was measured in lung Clara cells at four weeks in male Fischer rats given subcutaneous injections three times a week (18, 38, 103).

BALB/c mice were given continuous administration of seven concentrations of 2-AAF in the diet, ranging from 30–150 ppm. Tumor incidence after 24 months is shown in Figure 10. In the bladder, tumor incidence was not increased for 30, 34, and 45 ppm, but increased weakly for 75 ppm, and a steep increase followed at doses of 100 and 150 ppm (Figure 10*A*). In contrast to bladder tumor incidence, the level of DNA adducts in bladder tissue increased linearly in the dose range between 15 and 150 ppm (Figure 10*A*). The tumorigenic effects of 2-AAF in mouse target organs have been reported to be associated with the formation of only one DNA adduct, N-(deoxyguanosine-8-yl)-2-aminofluorene (dG-C8-AF) (18, 42). However, a different scenario was observed in the liver (Figure 10*B*). Liver incidence and DNA adducts increased linearly with dose. Thus, the scenario observed with 2-AAF in liver is similar to that of AFB1. The example of 2-AAF shows that dose-effect relationships at low doses do not only depend on the nature of the substance tested, but may also be tissue specific.

4-Aminobiphenyl

Exposure of mice to 4-aminobiphenyl (4-ABP) has been shown to result primarily in the formation of one adduct, N-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-C8-ABP) (43). DNA adducts resulted in a linear dose-response relationship in liver and bladder tissue of male BALB/c mice 28 days after administration of six doses between 7 and 220 ppm in the drinking water [Figure 11*A*,*B*; (43, 44)]. In contrast, tumor incidence was not increased for 7, 14, and 28 ppm in the bladder, and no increase in liver tumors was observed in male BALB/c mice. The situation is complicated by a gender difference in the susceptibility to 4-ABP. In contrast to male BALB/c mice (Figure11*A*), a linear low-dose-effect relationship for hepatic DNA adducts and liver tumor incidence was observed for female mice (Figure 11*C*). In contrast to male mice, only relatively low bladder tumor incidences could be induced in female animals (Figure 11*D*). This example shows that in rodents, occurrence of thresholds can be sex and tissue dependent.

THE CASE OF VINYL ACETATE

The genotoxic carcinogens discussed thus far did not exhibit thresholds in all organs or under all treatment modes. Because the same seems to be the case for the majority of genotoxic carcinogens, it has been proposed that a nonthreshold dose-response relationship should always be assumed. However, this assumption is no longer acceptable. At least some genotoxic carcinogens induce tumors via mechanisms that have highly nonlinear dose-response curves in which practical, if not true, thresholds exist. A well-studied example to illustrate this point is vinyl acetate. Vinyl acetate is genotoxic because it induces chromosomal aberrations, DNA protein cross-links, and sister chromatid exchanges. Bioassay data show that vinyl acetate is carcinogenic in rats and mice by the oral route and in rats by the inhalation route. However, all carcinogenic responses are expressed at very high

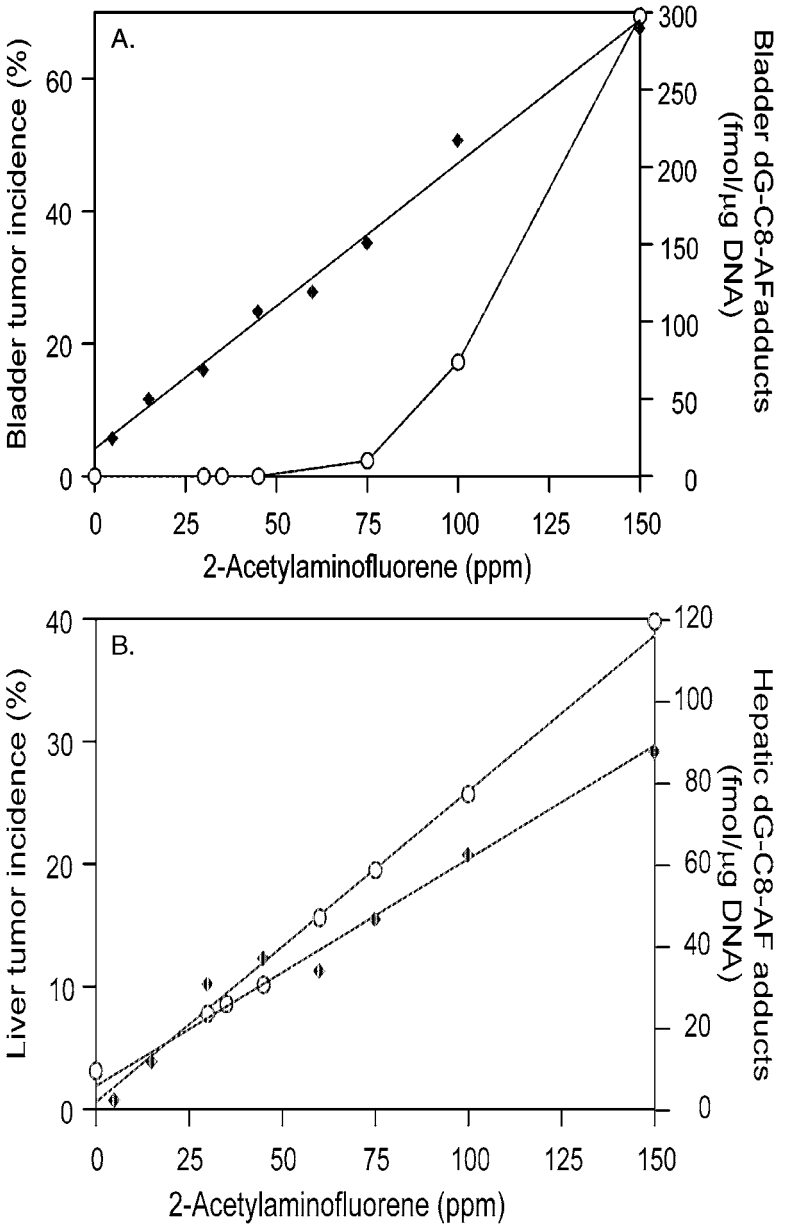

Figure 10 (*A*) Bladder tumor incidence (○) in female BALB/c mice after administration of 2-AAF in the diet (41). Mice were analyzed for tumors after 24 months. In the similarly conducted DNA adduct study, N-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF) (♦) was measured in bladder tissue of mice (18). (*B*) Liver tumor incidence (○) in female BALB/c mice after administration of 2-AAF in the diet (41). Mice were analyzed for tumors after 24 months. In the similarly conducted DNA adduct study, N-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF) (♦) was measured in liver tissue of mice (18).

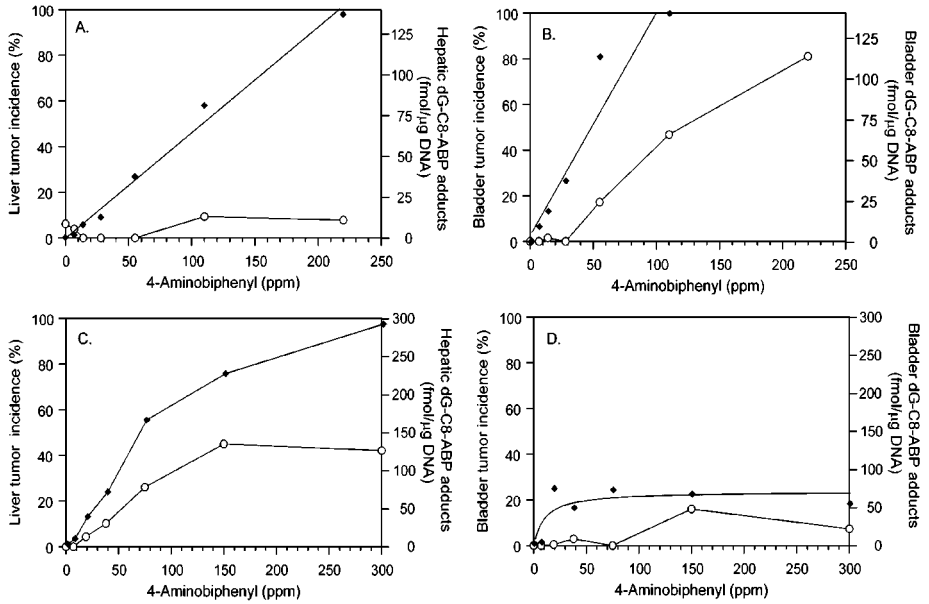

Figure 11 4-ABP induced liver (*A* and *C*) and bladder (*B* and *D*) tumors (○) in relation to the DNA adduct N-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-C8-ABP) in male (*A* and *B*) and female (*C* and *D*) BALB/c mice. ABP was administered chronically in the drinking water. Tumor incidence was examined after 24 months, whereas DNA adducts (♦) were measured after 28 days of exposure (18, 42–44).

dose levels that exceed standard definitions of maximum tolerated dose (MTD). In this section, we show that the dose-response curve of vinyl acetate has a practical, if not a perfect, threshold.

Carcinogenicity in Rats and Mice

ORAL EXPOSURE Vinyl acetate has been tested extensively for carcinogenic activity after oral exposure. Four studies have been conducted, all with administration via the drinking water.

1. Data from a study conducted by the Japanese Bioassay Research Center (JBRC) (45) are reviewed here. Data summary tables and summarized methodological details are available. Groups of 50 male and female rats and mice were administered 0, 400, 2000, or 10,000 ppm vinyl acetate for up to 104 weeks. The stock solution (98% pure vinyl acetate) contained impurities of hydroquinone (5 ppm), acetic acid, and water. Except for hydroquinone, the concentrations of the impurities were not specified. Vinyl acetate drinking water solutions were prepared twice a week. The solutions were kept in airtight containers and analyzed for vinyl acetate content before and after

administration. Body weight gain was significantly reduced in male and female rats and mice in the 10,000-ppm group. The only tumors seen in the JBRC study that were clearly attributable to vinyl acetate were of the upper digestive tract. In rats, oral cavity squamous cell carcinomas were the only tumors observed to be statistically significant. These were observed in the 10,000-ppm group. In mice, tumors of the oral cavity, esophagus, and stomach were observed. With the exception of one squamous cell papilloma, there were no tumors of the upper digestive tract observed in mice of the 0, 400, or 2000 ppm groups. The single squamous cell papilloma was seen in the esophagus of a 2000-ppm female mouse. Tumor incidence was higher in mice than rats. This difference is likely to be related to the higher mg/kg/day intake in mice relative to rats. The data suggest that the mice received approximately twice the dose received by the rats, on a mg/kg/day basis.

2. Maltoni et al. (46) exposed mice to concentrations of 0, 1000, or 5000 ppm vinyl acetate in drinking water. A number of contaminants in the stock test sample (benzene, 30–45 ppm; methyl and ethyl acetate, 50 ppm; crotonaldehyde, 6–16 ppm; acetaldehyde, 2–11 ppm; and acetone, 330–500 ppm) were identified. Although the presence of benzene in the sample is a concern, the levels of all of these contaminants are too low to be definitively confounding factors. This study appears to have taken appropriate precautions against vinyl acetate degradation. Drinking water solutions were prepared daily. Mice were exposed in utero, from the twelfth day of pregnancy. Both breeders and offspring were exposed throughout their lifespan, i.e., approximately 136 weeks. Group sizes of the male and female breeders were 13 and 37, respectively. Group sizes of the offspring ranged from 37 females in the 1000 ppm group to 49 males in the 5000 ppm group. A variety of tumors were observed in this study, many of which can be clearly attributed to vinyl acetate. The compound-related tumors are largely of the upper digestive tract. A number of nondigestive tract tumors (e.g., zymbal gland, lung, liver, uterine, and mammary gland) were observed, but these all occurred with high background incidence. Therefore, without adjusting for age, these tumor data cannot be evaluated with certainty. Squamous cell carcinoma of the oral cavity, tongue, esophagus, and forestomach were all treatment related at 5000 ppm. There were no tumors among mice administered 1000 ppm. Because drinking water consumption data were not collected, the concentrations used cannot be precisely converted to mg/kg/day dose rates. However, the dose received by the mice can be estimated from a recent 90-day drinking water study in mice conducted at 1000 and 5000 ppm (47). Male mice administered 1000 ppm or 5000 ppm consumed 254 mg/kg/day or 1185 mg/kg/day, respectively.
3. Bogdanffy et al. (48) administered vinyl acetate in drinking water at concentrations of 0, 200, 1000, or 5000 ppm for 10 weeks to male and female rats that were subsequently mated. The offspring were then culled into two

groups of 60 for the main study and 30 for satellite groups. Exposure to the drinking water then continued to 104 weeks. Drinking water solutions used in this study have been carefully controlled for both contaminants and spontaneous breakdown of vinyl acetate. A number of contaminants in the stock test sample (hydroquinone $\leq$1 ppm, acetic acid $\leq$11.5 ppm, acetaldehyde $\leq$71 ppm, and water $\leq$389 ppm) were identified but were too low to be of concern as confounding factors. The drinking water was formulated daily (and slightly overformulated to account for decomposition), and therefore exposure of rats to acetaldehyde or acetic acid was minimal. Other than decreased water and food consumption, there were no effects reported on standard parameters measuring systemic toxicity such as body weight gain or clinical parameters (hematology, chemistry, urinalysis). The dose rates for the 200, 1000, and 5000 ppm groups were approximately 13, 62, and 252 mg/kg/day, respectively. The no observed adverse effect level (NOAEL) of 200 ppm was based on the effects on food and water consumption. The authors conclude that there were no non-neoplastic or neoplastic lesions observed that were compound related. Two squamous carcinomas were observed in the oral cavity of males exposed to 5000 ppm. Because the incidence of these tumors was within historical control ranges, they cannot be considered compound related. The MTD was not exceeded, but effects on the animals (e.g., water and food consumption) clearly demonstrate adequate exposure. It must be concluded, therefore, that based on this study alone vinyl acetate would not be considered carcinogenic in rats via the oral route. Conversion of doses to mg/kg/day shows that the dose rate of 252 mg/kg/day (5000 ppm) in the rat study of Bogdanffy et al. (48) is approximately equivalent to the dose rate at the NOAEL of approximately 254 mg/kg/day (1000 ppm) in the mouse study of Maltoni et al. (46). From these calculations, it can be seen that, when adjusted for dose, the rat and the mouse studies appear to be similar. These estimations would also suggest the high dose of the Maltoni study exceeded the limit dose of 1000 mg/kg/day according to OECD (Organization for Economic Co-Operation and Development) (49, 50).

4. In the oldest published oral carcinogenicity study, Lijinksy & Reuber (51) administered vinyl acetate in the drinking water to male and female rats for two years at concentrations of 1000 or 2500 ppm. However, this study is deficient in several aspects, most notably that the drinking water solutions were prepared only once per week. The authors recognized a decomposition rate of approximately 8.5% per day. Therefore, by the end of the week the animals in the 2500 group, for example, were exposed to approximately 1300 ppm vinyl acetate and significant quantities of breakdown products, including acetaldehyde and acetic acid. The authors also did not purify the vinyl acetate prior to preparation of the drinking water solutions. Thus, the rats were also exposed to unspecified impurities. These problems have been discussed by the authors. In addition, only 20 rats were in each group, whereas the standard for such a study is generally approximately

50. Thus, the statistical power for detecting true positive responses and for discriminating against false positive and false negative outcomes is compromised. Therefore, we do not consider the data of this study in the present review.

Summary of carcinogenicity studies with oral exposure Vinyl acetate is clearly carcinogenic in mice and rats by oral ingestion. When the data are plotted collectively, there is a clear break in the dose-response curve (Figure 12). Responsiveness of rats and mice is similar when the dose is expressed in a mg/kg/day format. Because carcinogenicity only occurs at dose levels that exceed an MTD and are beyond what is expected of a limit test of 1000 mg/kg/day according to OECD guidelines (49, 50), the respective studies must be considered excessive. Regarding multiple sites of carcinogenicity, the only tumors clearly associated with vinyl acetate exposure are of the upper digestive tract. Tumors are located in the oral cavity, esophagus, and forestomach. All of these tissues are lined with squamous epithelium and do not display marked differences in histological makeup of the lining epithelium. Not surprisingly, all tumors are of the same histiogenic origin. Generally, multiple-site carcinogenesis is considered for agents, such as nitrosamines, that affect a variety of systemic organs and different cell types. Nitrosamines are carcinogenic to the esophagus and the brain, tissues of entirely

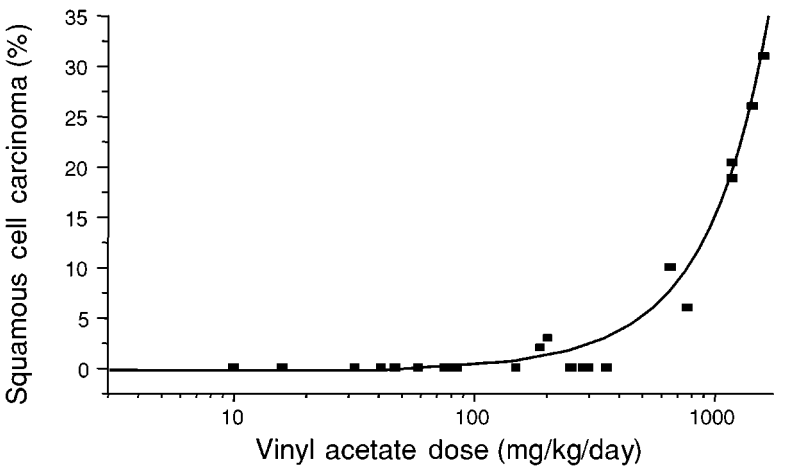

Figure 12 Composite dose-response data for rat and mouse drinking water bioassays. Vinyl acetate induced squamous cell carcinoma of the oral cavity, esophagus, and forestomach. The tumor incidence was greatest for the oral cavity. These data, which include male and female rats and mice, illustrate the sharp break in the dose-response curve with clear evidence for a practical threshold. Data are from Bogdanffy et al. (65), Maltoni et al. (46), and JBRC (45).

different histology (52). It is not appropriate to consider the oral cavity, esophagus, and forestomach as multiple organs when the tumors all appear to be derived from squamous epithelial lining and all of the tumors are histologically similar. This argument is consistent with published guidelines for combining neoplasms for evaluation in carcinogenesis studies (53). There is, therefore, no evidence for multiplicity of tumor sites. Furthermore, the tumor incidence decreases from the oral cavity to the forestomach. This is a characteristic that seems to be more appropriately associated with a site-of-contact carcinogen.

INHALATION EXPOSURE There are two studies that have tested the carcinogenic potential of vinyl acetate by the inhalation route.

1. Bogdanffy et al. (54) exposed rats and mice (60 per sex per group) to 0, 50, 200, or 600 ppm vinyl acetate for 104 weeks. The test material purity was characterized and found to be >99% pure. Impurities in the stock material included acetic acid (≤10 ppm), acetaldehyde (≤65 ppm), water (≤472 ppm), and hydroquinone (<1 ppm). None of these impurities are likely to have confounded the results. Body weight gain in rats and mice was reduced in the 600 ppm groups, and in mice in the 200 ppm groups. At the end of the study, body weight gain was 10% and 15% below controls for rats and mice, respectively. There was no negative effect on survival. Vinyl acetate induced nasal tumors in both male and female rats. No nasal tumors, or other tumors, were noted in mice. The nasal cavity tumors were all discovered at the terminal kill, with the exception of one benign tumor found in an animal that died two weeks before termination of the study. Of the 12 tumors diagnosed, 5 were benign and 7 were malignant. Most of the malignant tumors were squamous cell carcinomas; 1 tumor was a carcinoma in situ. The tumors were localized to regions lined with olfactory epithelium (n = 5), respiratory epithelium (n = 2), cuboidal epithelium (n = 2), and three were of unknown origin. Published historical control data for rat nasal tumors show that nasal tumors in unexposed controls are rare (55).
2. Maltoni et al. (56) performed an inhalation bioassay primarily to study vinyl chloride. Groups of rats were exposed to 0 ppm (68 male and female combined) or 2500 ppm (96 male and female combined) vinyl acetate for 4 hours per day, 5 days per week, for 52 weeks. After the exposure period, all animals were kept under observation until spontaneous death (135 weeks total). There was significant mortality among the rats. Fifty-eight and 49 rats (male and female combined) remained after the treatment period in the 0 ppm and 2500 ppm groups, respectively. The authors did not observe tumor induction in rats exposed to vinyl acetate. However, the authors conclude that exposure to vinyl acetate caused death of the animals within a period that did not allow a correct carcinogenicity test. However, it is worth noting that even at such high exposure concentrations, there were no tumors reported during the first 12 months of testing or during the exposure-free phase.

Summary of carcinogenicity studies with inhalation exposure Vinyl acetate is clearly carcinogenic by the inhalation route in rats, but not mice. Whereas the Maltoni et al. (56) study is inadequate for assessing carcinogenicity, the Bogdanffy et al. (54) study is sufficient. Although vinyl acetate was clearly carcinogenic in rats, carcinogenicity was only expressed at high exposure levels (600 ppm). The MTD (defined as 10% retardation in weight gain) was exceeded in rats exposed to 600 ppm. There was no evidence for systemic carcinogenicity.

Other In Vivo Studies in Experimental Animals

Induction of hepatic enzyme-altered foci (ATPase, GGTase) was investigated after administration of vinyl acetate (200 and 400 mg/kg/day, orally) to newborn rats for 3 weeks, with or without subsequent promotion by phenobarbital (57). No foci were observed in vinyl acetate–treated animals at the age of 14 weeks, whereas the structurally related compounds vinyl carbamate and vinyl chloride induced enzyme-altered foci under comparable experimental conditions. The negative result was not surprising considering the short exposure period and relatively low doses compared to the carcinogenicity studies discussed above.

Another in vivo study with a negative result was performed by Simon et al. (58). After administration of (^{14}C) vinyl acetate to male and female rats, either orally or by inhalation, no specific hepatic DNA adducts, known to occur after administration of labelled vinyl halides or vinyl carbamates, could be observed in liver.

Epidemiological Studies of Vinyl Acetate Carcinogenicity

A cohort study including 4806 individuals employed at a plant for the manufacture of synthetic chemicals in the United States was performed between 1942 and 1973 by Waxweiler et al. (59). The cohort had an excess risk for cancer of the respiratory system [resulting in a standardized mortality ratio of 1.5 (95 % confidence interval: 1.1–2.0)]. Thus, exposure of these cancer patients to 19 chemicals, including vinyl acetate, was examined. Exposure of the patients with cancer of the respiratory system to vinyl acetate was below the mean exposure expected for the members of the cohort with the same year of birth and age at commencement of work in the plant. A subgroup of employees with undifferentiated non-small-cell lung cancer had a slight, but statistically nonsignificant, cumulative exposure to vinyl acetate. Thus, this (59) does not provide evidence for a carcinogenic effect of vinyl acetate in humans.

A case-control study was performed in a cohort of 29,139 men employed in a chemical manufacturing environment (60). Nested case-control studies of non-Hodgkin's lymphoma, multiple myeloma, nonlymphocytic leukemia, and lymphocytic leukemia were conducted in men from two chemical facilities and a research center. Exposure odds ratios were examined in relation to 21 specific chemicals. The results are difficult to interpret because exposure to vinyl acetate was associated with a decreased risk for nonlymphocytic leukemia (odds ratio:

0.5), but slightly increased odds ratios for non-Hodgkin's lymphoma (odds ratio: 1.2) or multiple myeloma (odds ratio: 1.6).

In conclusion, evaluation of epidemiologic data on a possible carcinogenic effect of vinyl acetate is difficult because most individuals in the existing epidemiological studies were exposed to several chemicals. Nevertheless, the existing data do not support a carcinogenic effect of vinyl acetate in humans.

Metabolism and Genotoxicity of Vinyl Acetate

Exposure of tissues to vinyl acetate results in metabolic conversion to acetic acid and acetaldehyde at the site of contact. The histochemical localization of carboxylesterase and aldehyde dehydrogenase in nasal tissue have been described in detail (61–65). These enzymes rapidly and almost completely convert vinyl acetate to acetic acid and acetaldehyde in nasal tissue. At high concentrations, acetaldehyde induces DNA-protein cross-links that lead to chromosomal aberrations. Formation of DNA-protein cross-links is facilitated by low intracellular pH (pH_i). A low pH microenvironment is caused by acetic acid formation from both vinyl acetate hydrolysis and acetaldehyde oxidation to acetic acid and liberation of protons (66). Acetaldehyde, as discussed above, is a known clastogen but does not appear to induce point mutations. In fact, the profiles of genotoxic activity for acetaldehyde and vinyl acetate are almost identical and vinyl acetate is not active as a clastogen without a source of carboxylesterase added. Thus, the clastogenic activity of vinyl acetate must be attributed to metabolic formation of acetaldehyde. It has been reported that acetic acid, formed intracellularly from vinyl acetate hydrolysis, contributes to the genotoxic activity (67, 68) and tumor progression (69). It is well known that a low pH can have a confounding effect on genetic toxicity tests using mammalian cells (70). Low pH has also been shown to induce cellular transformation of Syrian hamster embryo cells (71, 72). In fact, acetic acid induces chromosomal aberrations in Chinese hamster ovary (CHO) cells. It is likely that the genotoxic activity of acetaldehyde is attributable, at least in part, to intracellular acidification because two protons are released when acetaldehyde is oxidized to acetic acid in the presence of aldehyde dehydrogenase and NAD^+. Intracellular acidification has been reported to facilitate acetaldehyde-induced genotoxicity (73). The authors used a model system for measuring DNA-protein cross-links (the initial event finally leading to chromosomal breaks) involving incubations of calf thymus histone protein with plasmid DNA and measurement of covalently bound DNA-histone protein complexes. Cross-links appeared to be between DNA and amino acid residues, guanosine and lysine, respectively. In their studies, it was first shown that DNA-protein complex formation requires carboxylesterase-dependent metabolism of vinyl acetate to acetaldehyde and acetic acid. Next, it was shown that the formation of acetaldehyde-induced DNA-protein cross-links is increased in the presence of increasing concentrations of acetic acid. Finally, they demonstrated that acetaldehyde-induced DNA-protein cross-links are increased with simple reduction in pH. The proposed mechanism for this increase is ionization to positively

charged amino acid groups of histone proteins, resulting in a higher affinity for the negatively charged DNA. The resulting tight association of histone protein with DNA may be a prerequisite for the formation of DNA-protein cross-links by acetaldehyde, which, in addition, is increased by the higher electrophilicity of the carbonyl carbon upon protonation of acetaldehyde. In conclusion, although further studies are required in this field, strong evidence has been presented showing that intracellular acidification is a prerequisite for the genotoxic activity of vinyl acetate and acetaldehyde. In addition, the acetaldehyde-DNA-protein cross-link was found to be very unstable, with a half-life of approximately six hours (74, 75).

Mechanistic Data Support a Practical Threshold for Vinyl Acetate

Tissues can accommodate exposure to acetic acid and acetaldehyde without adverse effects up to a certain level of exposure (76). This is consistent with the known exposure of tissues to endogenous acetic acid and acetaldehyde. It is known that acetaldehyde is a natural constituent in the body and a metabolic by-product of threonine metabolism (77, 78). Background levels of approximately 0.3-μg/mL acetaldehyde exist in blood. Therefore, it would seem reasonable that exposures to vinyl acetate that do not raise tissue acetaldehyde levels beyond the range of natural background levels in blood or tissues would also be below biological thresholds. Using a physiologically based pharmacokinetic (PBPK) model, it is possible to predict tissue exposure to acetaldehyde resulting from inhalation exposure to vinyl acetate. Figure 13 shows the predicted basal cell acetaldehyde levels in humans during exposure to 1-ppm vinyl acetate. When critical levels of vinyl acetate are achieved, thresholds are exceeded and five critical steps in the mechanism ultimately leading to cancer become active (Figure 14). The threshold for pH_i reduction in neuronal cells that does not induce cytotoxicity in vitro is 0.15 pH unit (79). The lowest concentration of acetaldehyde that has induced sister chromatid exchanges (SCEs) in vitro in CHO cells is 3.9 μg/mL (80). SCEs are not considered to be a valid marker of mutagenic damage and are generally overly sensitive. A more appropriate and more widely accepted benchmark genetic toxicity endpoint would be chromosomal aberrations. Chromosomal aberrations are also mechanistically consistent with the data, suggesting that acetaldehyde induces DNA-protein cross-links. However, because SCEs already occur at lower concentrations than chromosomal aberrations, they were chosen as an endpoint in order to overestimate, rather than underestimate, the risk due to vinyl acetate exposure.

When critical levels of exposure to the ensuing acetaldehyde and acetic acid are achieved, thresholds are exceeded and further critical steps in the mechanism ultimately leading to cancer become active. These steps are illustrated for olfactory epithelium in Figure 14. The PBPK model predicts that in rat nasal olfactory tissue, exposure to 50-ppm vinyl acetate causes a 0.08 unit reduction in pH_i and a basal cell acetaldehyde concentration of 1.7 μg/mL. Fifty-ppm vinyl acetate is a NOAEL,

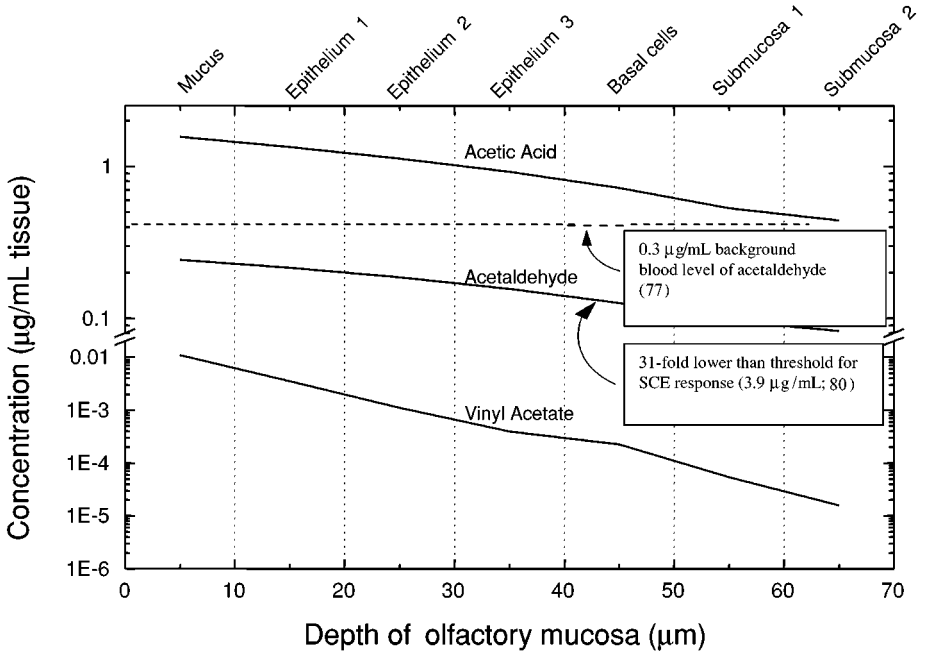

Figure 13 Dosimetry in human epithelium at an exposure to 1-ppm vinyl acetate (104). Predicted steady state concentrations of acetaldehyde, acetic acid, and vinyl acetate throughout the olfactory nasal mucosa of humans exposed continuously to 1 ppm vinyl acetate. Concentrations of acetaldehyde at the basal cell layer are critical for consideration because basal cells are the progenitor cells for the epithelium and are the target cell for carcinogenesis. The figure illustrates that at 1 ppm vinyl acetate, basal cell acetaldehyde concentrations are predicted to be approximately 3 times lower than background blood acetaldehyde levels and more than 31 times lower than the lowest concentration shown to induce SCE. SCE are sensitive markers of genetic damage and of questionable relevance. The margin of safety relative to the more appropriate endpoint of chromosomal aberrations is much greater. Obe et al. (80) reported the lowest level to induce SCE in normal human lymphocytes or lymphocytes from Fanconi's anemia patients to be 15.6 μg/mL or 7.8 μg/mL acetaldehyde, respectively. The margin of safety below the chromosomal aberrations endpoint is 124-fold.

and the pH_i reduction and basal cell acetaldehyde levels are below the thresholds of their effects. As the dose level increases to 200 ppm, pH_i is predicted to be reduced by 0.25-pH units, a value slightly above the threshold, and cytotoxicity, such as olfactory degeneration, occurs at an incidence between 8% and 10% (Figure 14, *step 3*). However, the cell proliferation response at 200 ppm is weak (*step 4*). Levels of acetaldehyde of 5.4 μg/mL are slightly in excess of the threshold for genotoxicity. Thus, at 200 ppm there is minimal exposure above threshold levels of acetaldehyde (*step 1*), minimal pH_i reduction above the threshold (*step 2*), enhanced olfactory

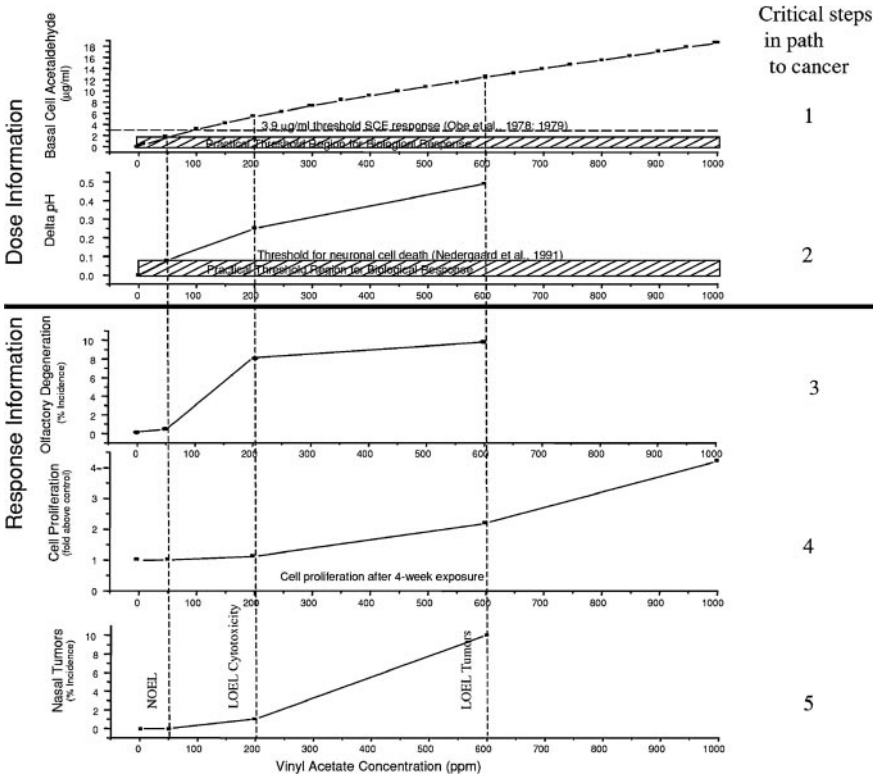

Figure 14 Composite presentation of dose and response data for the five critical steps on the pathway to carcinogenesis in nasal olfactory epithelium. Panel 1 shows the predicted steady state concentration of acetaldehyde at the basal cells, the progenitor cells of nasal cancer, in relation to in vitro doses that produce sister chromatide exchanges. Panel 2 shows predicted pH_i changes in olfactory epithelium of the rat in relation to changes in pH_i that are cytotoxic to neuronal cells in vitro. pH_i reduction is proposed to be the critical step leading to cytotoxicity. Panel 3 shows olfactory degeneration in rats as a cytotoxic endpoint. Basal cell proliferation and the incidence of nasal tumors in rats is presented in panels 4 and 5. Olfactory degeneration (cytotoxicity) is observed at 200 ppm. Because acetaldehyde levels are only slightly above thresholds, there is no significant tumor response. At 600 ppm, all thresholds are exceeded, cell proliferation is significantly enhanced, and a significant incidence of nasal tumors is observed. The mechanism of action for nasal respiratory and oral cavity tumors is similar, with the exception that the cause of the proliferative response in oral cavity may be only subtly related to cytotoxicity and is more likely the result of known mitogenic effects due to reduced pH_i.

degeneration (*step 3*), and slightly enhanced cell proliferation (*step 4*). At 200 ppm, one nasal tumor was observed that was not statistically significant (*step 5*). At 600 ppm, acetaldehyde levels are predicted to be markedly above threshold at 12.4 μg/mL (*step 1*), pH_i is predicted to be markedly reduced by 0.49-pH units (*step 2*), olfactory degeneration is strongly enhanced (*step 3*), and basal cell

proliferation is more than twofold above control (*step 4*). At 600 ppm, all of the critical steps in the mechanism of carcinogenesis are active and tumors now appear at a statistically significantly increased rate (*step 5*). This sequence of events and the physiological modeling suggest that only when critical exposure concentrations, threshold levels for pH_i reduction, and cellular proliferation (induced by cytotoxicity in olfactory epithelium) are achieved, all the conditions necessary for a complete carcinogenic mechanism in place.

There is another area of mechanistic work that deserves discussion regarding the generality of the mechanism discussed above for both olfactory and respiratory tissue as well as the upper digestive tract. The mechanism of action described above for olfactory epithelium suggests that cytotoxicity is the first adverse cellular response to vinyl acetate exposure of both respiratory and olfactory tissue. Events such as cell proliferation and tumor formation become significant only at higher concentrations. The data to support this mechanism are clear for olfactory tissue but require careful analysis for respiratory tissue. In respiratory tissue, there are several pieces of information that support cytotoxicity as the first step in carcinogenesis; for example, the observation of respiratory epithelial degeneration and cell proliferation in rats exposed for one or five days to 1000-ppm vinyl acetate (81) and the in vitro cytotoxicity studies that show acid phosphatase release from nasal turbinates in culture (74). The cell proliferation responses, which were also significant at 600 ppm, most likely represent subtle cytotoxic responses that are repaired or not evident microscopically. The lack of a more pronounced response in respiratory tissue has been recognized as an unresolved question (54, 82).

An alternative hypothesis for the cell proliferation stimulus in respiratory epithelium has recently emerged (66) that may also clarify the mode of action in the upper gastrointestinal tract. Literature reports suggest that reductions in pH_i can also induce mitogenesis. Alterations in pH_i are involved in stimulation of cell growth and transformation. For instance, Syrian hamster embryo cells cultured at pH 6.7 show a marked increase in life span compared to those cultured at pH 7.3, as measured by the number of population doublings that occur before cellular senescence (72). The higher proton burden of the intracellular environment has been shown to displace $Ca2^+$ from intracellular binding sites (83). $Ca2^+$ displaced from the growth and differentiation factor (GDF) protein blocks the intracellular signaling that leads to differentiation (84). Blockage of the differentiation pathway could promote sustained proliferation, expansion of the undifferentiated cell population, and clonal expansion of spontaneous or chemical-induced mutants. Although substantiation of the hypothesis that intracellular acidification is mitogenic in nasal or oral cavity mucosal cells suggests further experimentation, the proposal is supported by the literature and could provide a fundamental linkage to many tumor promotion mechanisms. Regardless of the mechanism for induction, it is clear that cell proliferation is induced in nasal respiratory and upper gastrointestinal tract epithelial tissues and that this step is critical to the complete expression of the carcinogenic potential of vinyl acetate (85).

It has been argued that respiratory and olfactory tissue are not likely to respond similarly to the same exposure to acetic acid or reduced pH_i and that respiratory and olfactory tissues may have different biochemical capacities for responding to alterations in pH_i. There is precedent in the literature to support this position (86–88). Similar to the nasal cavity, the oral cavity possesses carboxylesterase that has been localized to squamous epithelium (89, 90). The activity in oral mucosa of rats and mice was similar and was correlated to regions shown to be active by histochemistry (91). However, the carboxylesterase activity of the rat oral mucosa was approximately 100 times lower than that of nasal tissue. Recently, cell proliferation has been measured in the oral mucosa of rats and mice administered vinyl acetate in drinking water (92). The oral cavity is lined with squamous epithelium. Rats and mice were exposed to concentrations of up to 24,000 ppm for 92 days. Less than twofold, but significant, increases in oral mucosal basal cell proliferation were observed in rats evaluated on days 29 and 92. In mice, the responses were more pronounced, with approximately 2.4- and 3.4-fold increases being observed at day 92 in the 10,000 and 24,000 ppm groups, respectively. The greatest proliferative response observed in mice was in the lower jaw, which was also the region of greatest tumor formation observed in the JBRC study. In conclusion, although the support for the proposed mechanism of action of vinyl acetate on oral cavity mucosa is not as robust as for the nasal cavity, the research to date provides a parallel picture in which enhanced epithelial cell proliferation is induced when critical thresholds are exceeded. The data further suggest an even higher level of a practical threshold in these tissues for which the formation of some tumors have also been reported after exposure to very high doses of vinyl acetate. The implication of the discussed five-step mechanism is that practical thresholds of exposure exist below which there is no substance-related increased risk for cancer.

Values of Practical Thresholds in Relation to Human Vinyl Acetate Exposure

As shown above, there is clear evidence for a practical threshold for vinyl acetate–induced carcinogenesis; an exposure ranging between 50 and 200 ppm vinyl acetate was shown to cause cytotoxicity, but the proliferation response was only weak. In this concentration range, no significant increase in carcinogenicity was observed in experimental animals, but it began at 600 ppm. An even more conservative practical threshold is 50 ppm. Below 50 ppm, no cytotoxicity and no cell proliferation could be induced by vinyl acetate. Consequently, no carcinogenesis was observed in experimental animals in this dose range. In addition to these studies based on experimental animals, PBPK modeling predicts that exposure to 1-ppm vinyl acetate leads to a basal cell acetaldehyde level that is approximately three times lower than the endogenous concentration of acetaldehyde in vivo. Thus, a concentration of 1 ppm can be expected to be far below concentrations for which our organism would not have established compensatory mechanisms, such as pH-buffering of

the ensuing acetic acid to prevent significant damage. Based on limited data on the likely irritation threshold in humans, current occupational exposure limits have been set to 10 ppm in some countries (93, 94).

These practical thresholds can be compared to occupational vinyl acetate exposures. Concentrations of 0.07–0.57-ppm vinyl acetate were reported in ambient air in an area where several vinyl acetate manufacturers were located (95). An ambient air concentration of 0.14×10^{-3} ppm was detected near a chemical waste disposal site (96). Although most studies published in 1990 or later report relatively low vinyl acetate exposures, for instance <0.22 ppm for polyvinyl acetate painters (97) or <9.9 ppm in various Finnish industries (98), earlier studies report much higher occupational exposures. For instance, in 1969, a maximal exposure of 49 ppm was reported in vinyl acetate production and polymerization industries in the United States (99). As a consequence of the practical threshold for vinyl acetate, it seems to be very important to avoid extreme exposures because at concentrations of 600 ppm, albeit for a lifetime exposure, carcinogenesis was observed. On the other hand, to our knowledge, almost all occupational exposures reported in the past decade were much lower. Exposure limits for vinyl acetate are different in several countries. Examples for current national occupational exposure limits (time-weighted average) are 10 ppm in the United States and Germany, 8.5 ppm in France, and 2.8 ppm in Poland and the Russian Federation (93, 94, 100). Regarding ambient lifetime exposures, a limit of 0.4–1.0 ppm has been recommended (101). Lower concentrations are below a practical threshold, where the prerequisites for vinyl acetate carcinogenesis, namely cytotoxicity and regenerative cell proliferation, are not observed, whereas significant vinyl acetate–induced carcinogenesis itself is not observed below 600 ppm.

CONCLUSIONS

A linear low dose–response relationship with no observable threshold seems to be a conservative but adequate description for the carcinogenic activity of many potent genotoxic chemicals, such as AFB1 or NNK. However, for several genotoxic carcinogens, sufficient data provide an adequate base for the judgement that they operate by mechanisms that establish practical thresholds. For some, a nonlinear dose–response relationship at low doses has been observed for certain tissues and cell types, whereas other organs showed a linear dose–response relationship at low doses. 2-AAF and 4-ABP are examples of this class of carcinogens. On the other hand, vinyl acetate and formaldehyde belong to a class of carcinogens showing clear “practical” or possibly even “real” thresholds.

We finish with a question concerning an old but still controversially debated substance: Would you use a genotoxic carcinogen for alleviation of a harmless headache? Certainly the majority, including physicians and pharmacologists, would say no. However, reality is different. Although controversial, paracetamol (acetaminophen), one of the world’s most popular over-the-counter drugs may be considered as a genotoxic carcinogen because it causes liver and bladder tumors in

certain rat and mouse strains, covalently binds to DNA, and induces chromosomal aberrations (102). On the other hand, some negative carcinogenicity studies have been published. When carcinogenicity was observed, this occurred only at extremely high cytotoxic doses. The carcinogenicity studies and the experience with patients taking paracetamol taken together do not suggest a problem with carcinogenicity at therapeutic doses. Again, the reason for the lack of carcinogenicity seems to be the existence of a practical threshold concentration for carcinogenicity that is not reached at therapeutic doses. There are some solid biochemical findings inferring mechanistic reasons for the existence of a practical threshold dose of paracetamol, below which it may reasonably be expected that a significant increase in the cancer rate does not occur; paracetamol at therapeutic doses is almost completely conjugated to glucuronide and sulfate with very low amounts of electrophilic quinone imine formed, which is detoxified by conjugation with glutathione. The systems for conjugation to glucuronide and sulfate are overwhelmed, and consequently the formation of quinone imine increases only at higher doses. At much higher doses, glutathione is depleted, and then extensive covalent modification of cellular macromolecules starts. The example of paracetamol underlines the importance of toxicological research that unequivocally identifies genotoxic carcinogens acting through a practical threshold process.

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THE *CAENORHABDITIS ELEGANS* DOPAMINERGIC SYSTEM: Opportunities for Insights into Dopamine Transport and Neurodegeneration

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■ **Abstract** The neurotransmitter dopamine (DA) plays a central role in the coordination of movement, attention, and the recognition of reward. Loss of DA from the basal ganglia, as a consequence of degeneration of neurons in the substantia nigra, triggers postural instability and Parkinson's disease (PD). DA transporters (DATs) regulate synaptic DA availability and provide a conduit for the uptake of DA mimetic neurotoxins, which can be used to evoke neuronal death and Parkinson-like syndrome. Recently, we have explored the sensitivity of DA neurons in the nematode *Caenorhabditis elegans* to the Parkinsonian-inducing neurotoxin 6-hydroxydopamine (6-OHDA) and found striking similarities, including DAT dependence, to neurodegeneration observed in mammalian models. In this review, we present our findings in the context of molecular and behavioral dimensions of DA signaling in *C. elegans* with an eye toward opportunities for uncovering DAT mutants, DAT regulators, and components of toxin-mediated cell death.

INTRODUCTION

In the year 1817, the English neurologist James Parkinson published his "Essay on the Shaking Palsy," which described a devastating neurological disease that deteriorates into uncontrollable tremors, bradykinesia, and loss of motor skills. We now recognize this neurodegenerative disorder as Parkinson's disease (PD). It was not until almost a century and a half later that the biochemical basis for PD was clarified by Arvid Carlsson as a depletion of dopamine (DA) from the basal ganglia, studies for which he was jointly awarded the 2000 Nobel Prize in Physiology or Medicine. His investigations also led to the identification of the DA precursor L-DOPA, which thirty years later is still the principle medication used to alleviate the clinical manifestations of PD. Since these early studies, DA has been found to play a key role in a number of other physiological processes, including cognition, emotion, reward, memory, and endocrine function. Moreover, DA neuronal

dysfunction has been associated with several prevalent neurobehavioral disorders, including drug addiction, schizophrenia, and attention-deficit hyperactivity disorder (ADHD). But despite almost a half-century of intense investigations into DA neurotransmission and its clear role in neurological disorders, much remains to be understood regarding the regulation of DA signaling during normal physiological states as well as what confers the unique sensitivity of DA neurons to damage in PD. The complexity of the human brain, which contains over 100 billion neurons and tens of thousands of DA-containing cells (1), each capable of forming many thousands of synaptic connections, has greatly limited our ability to dissect the regulatory machinery involved in DA neurotransmission. Moreover, the relative inaccessability of DA neurons in vertebrates, and our inability to directly visualize DA neurons and their synaptic connections in vivo, significantly restricts the pace of progress in elucidating the molecular mechanisms involved in DA neurodegeneration. Recently, we and others have taken advantage of the high conservation that exists at a molecular level in DAergic signaling across phylogeny to propose new models for the study of DA neurotransmission in normal and pathogenic states (2–5). In the present review, we discuss how the opportunities presented by the nematode *Caenorhabditis elegans* can increase our understanding of DA signaling, dopamine transporter (DAT) regulation, and DA neuron pathogenisis. We also describe how this system can be utilized to identify new molecular components of DA signaling as well as evaluate pharmaceutics that may be protective in PD.

C. ELEGANS AS A MODEL SYSTEM: GENERAL FEATURES

C. elegans has been well recognized as a powerful model system for dissecting the components involved in neurotransmission and disease (2, 6, 7). Its genome, biosynthetic, and metabolic pathways are highly conserved with mammalian systems, yet their small size (adults are approximately 1-mm long), ease of maintenance in the laboratory (they grow on agar plates coated with bacteria), quick generation time (3 days), and large brood sizes (over 300 progeny per hermaphrodite) allow for the rapid growth of many animals for a variety of cellular, molecular, and genetic analysis (8–10). The transparency of the animal and the ease of making reporter gene fusions allow for examination of neuronal morphology and direct viewing of protein expression patterns within the living nervous system (11–14). Moreover, the availability of a complete three-dimensional map of the 302-cell nervous system allows for the identification of most synapses between neurons (15, 16). The self-fertilizing hermaphrodite permits quick and easy homozygosity of mutations, and males can be used for mating to generate lines with multiple mutations. The completed sequence of the genome, the ability to perform whole-animal PCR (“single-worm PCR”), and the existence of a high-density polymorphism map of a related strain of the wild-type (WT) *C. elegans* allows for quick and easy mapping of mutations within practically any gene (8, 17). Gene knockouts can also be generated (minimum of about one week),

and loss-of-function mutant phenotypes can be evaluated within most cell types with the use of RNA-mediated interference (RNAi) technology (18, 19). With the recent advances in primary *C. elegans* cultures, reliable knockout phenotypes can now be generated in vitro within the nervous system (20), and compounds that are impermeable to the whole animal due to the barrier of the cuticle or that could not be solubilized to sufficiently high concentrations to permit the drug to penetrate the cuticle can now be effectively applied to cultured cells. Also, electrophysiological methods have been developed to examine the electrical properties of a variety of neurons and other cell types both in vivo and in vitro (16, 20).

The *C. elegans* nervous system contains many of the known signaling components and neurotransmitter systems found in the mammalian nervous system. The worm contains acetylcholine, glutamate, γ-aminobutyric acid (GABA), serotonin (5-HT), DA, and neuropeptides (21), among other chemical messengers. Neurotransmitter-specific transporters (membrane and vesicular) and receptors (including G-protein-coupled) are also highly conserved with their mammalian counterparts (21). Most ligand-gated and voltage-gated ion channels are present within the worm, with the notable exception of the voltage-gated Na^+ channel (21); however, the voltage-gated Ca^+ channels are conserved and, as in mammals, could generate the necessary action potentials for neurotransmission (16). Finally, all the known synaptic components involved in synaptic vesicle plasma membrane interactions and exocytosis, including syntaxins, synaptotagmin, synaptobrevin, and SNAP-25, are highly conserved between the worm and mammals (21). This remarkable similarity between the mammalian and worm nervous systems has expedited the identification of novel proteins required for normal synaptic transmission in humans (22).

NEURONS AND GENES SUPPORTING DOPAMINE SIGNALING IN *C. ELEGANS*

The *C. elegans* hermaphrodite contains eight DA neurons, four symetrically arranged cephalic cells (CEPs) and two bilateral anterior deirids (ADEs) in the head, and two bilateral posterior deirids (PDEs), which contain processes that run through the main part of the body and tail (Figure 1) (23). These neurons are believed to be mechanosensory neurons because the microtubule-containing cilium at the end of the dendrites are embedded in the subcuticle and may detect movement or food (see below) (24–26). The CEPs (two dorsal and two ventral) contain a single long dendrite that extends from the cell body near the nerve ring, or "brain," through the length of the head where the ciliated endings enter the cuticle near the nose of the animal (15, 24). The ADE cell bodies are located behind the second bulb of the pharynx, and the dendrites contain ciliated endings that travel into the deirid sensilla (15). The axons of both the CEPs and ADEs are directed into the nerve ring, and the dorsal pair of CEPs may receive some synaptic input from the ADEs (15). The cell bodies of the PDEs are located posterior

to the vulva, and as with other DA neurons, the dendrites contain ciliated endings that enter the sensillum (15). The PDE axons enter the nerve chord, where the anterior process extends to near the nerve ring, and receive en passant synaptic connections from other neurons (15, 27). The *C. elegans* male contains another three pairs of DA neurons that are located within the tail, as well as four DA-containing male-specific spicule socket cells (28, 29).

The DA neurons were initially identified by Sulston and coworkers, who used the catecholamine-specific technique of formaldehyde-induced fluorescence (FIF) (23). DA cell bodies and processes were visualized using fluorescence microscopy, confirmed as DA by alumina absorption and thin-layer chromatography (TLC). The precursor L-DOPA was also identified but not the catecholamines norepinephrine and epinephrine. Based on FIF micrographs and worm DA content as well as the estimated volume of the cell bodies and processes, the concentration of DA in the nerve endings is predicted to be very similar to the concentration within mammalian varicosities (23). We and others [(6); R. Nass, unpublished data] have confirmed via high-perfomance liquid chromatography (HPLC) that DA and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) are present in the animal.

Sulston and coworkers isolated several mutant lines deficient in catecholamine fluorescence. Once cloned and characterized, these mutants reveal that the *C. elegans* DAergic system is at least fundamentally very similar to vertebrates (Figure 2). *cat-1* animals (catecholamine abnormal) have a dramatic reduction in DA content yet over a threefold increase in L-DOPA (23). FIF in this animal was also only seen in the cell bodies and not the processes. The animals were also similar to WT worms treated with the drug reserpine, which depletes vertebrate secretory vesicles of catecholamines (23). Taken together, these results suggest that these mutants are unable to load DA into synaptic vesicles (23). Duerr and coworkers confirmed these results when they identified the mutation in the *C. elegans* orthologue of mammalian vesicular monoamine transporters (VMATs) (30). And like the neuronal VMAT, VMAT2, *cat-1* is associated with synaptic vesicles that are localized to both DA and 5-HT neurons and has a high affinity for DA, serotonin, norepinephrine, and histamine. Furthermore, the *cat-1* behavioral phenotype (see below) can be rescued by the human VMAT2 transgene, which strongly supports the role of *cat-1* as a synaptic vesicle neurotransmitter transporter (30). The VMATs also transport octopamine. Octopamine is present within the worm (31) and is believed to be the norepinephrine analogue found in many invertebrate systems.

Sulston identified two other mutants that have altered DA production, and FIF patterns—*cat-2* and *cat-4*. *cat-2* mutants have a complete loss of DA, as detected by FIF and TLC (23). Lints & Emmons subsequently identified the mutant protein via BLAST and gene sequencing to encode a polypeptide with 50% amino acid identity to tyrosine hydroxylase (TH) (32). TH is the rate-limiting enzyme in DA biosynthesis and is expressed in mammalian DA neurons, and their GFP-reporter expression pattern using *cat-2* promotor fusions agreed with this functional

assignment, revealing expression limited to DA neurons. Based on reductions in 5-HT levels and cuticle defects in the *cat-4* mutants, it has been suggested that this gene encodes a pterin cofactor, GTP cyclohydrolase (GTPCH). The pterin cofactor is used in the biosynthetic pathway of both DA and 5-HT and is involved in worm cuticle formation (23, 33–35). Notably, dopa-responsive dystonia, a disease causing parkinsonian-like phenotypes, is due to mutations within the GTPCH gene (36).

Metabolic pathways for catecholamines also appear to be conserved from man to worm. Sulston identified aromatic amino acid decarboxylase (AAAD) activity in his *C. elegans* preparations (23). AAAD is an enzyme required for the conversion of L-DOPA to DA and for the conversion of 5-hydroxytryptophan (5-HTP) to 5-HT in mammals. Loer & Kenyon loaded 5-HTP in DA neurons and found that they became 5-HT immunoreactive, implying AAAD activity similar to what is found in mammalian systems (34). Mutants containing the *bas-1* (biogenic amine synthesis-defective) allele, which do not produce DA or 5-HT and cannot convert exogenous 5-HTP to 5-HT, maps to the putative AAAD gene (23, 34). *C. elegans* also contains homologues of mammalian DA metabolizing enzymes, including monoamine oxidase (MAO) and catechol-o-methyltransferase (COMT) (6, 37), enzymes involved in the pathways for the conversion of DA to DOPAC or to 3-methoxytyramine (3-MT) and HVA, and we and others have identified these metabolites in whole-animal worm preparations via HPLC [(6); R. Nass, unpublished data].

DA and mammalian DA receptor antagonists alter behaviors in *C. elegans* consistent with the presence of DA receptors in the worm [(38) and references therein]. Exposure of animals to DA results in a decrease of movement, grazing, and egg-laying; however, addition of the mammalian D2 antagonist haloperidol reverses this effect (38, 39). Chlorpromazine, another D2-antagonist, can also blunt the DA contribution to egg-laying (40). We have found that the DA transporter substrate amphetamine, which causes an increase in synaptic DA by eliciting efflux of DA from DAergic terminals, also causes a decrease in egg-laying and larval development (R. Nass, unpublished data) (41). This effect is also blunted in the ΔVMAT *cat-1* line, which cannot package DA for release and also exhibits lower whole-animal DA levels (R. Nass, unpublished data) (41). Finally, gain-of-function *egl-2* mutants contain a mutation in a voltage-gated eag-like K^+ channel that causes a defect in egg-laying and defecation; this phenotype can be suppressed in the presence of different D2 family DA antagonists, including chlorpromazine, haloperidol, butachlamol, droperido, and pimozide (38), suggesting that EGL-2 may participate in circuits modulated by DA. Recently, the first DA receptor was identified in *C. elegans*. CeDOP1 has high sequence similiarity (43%) to the human G-protein-coupled D1-like receptors (D1 and D5) (42). In vitro DA and lysergic acid diethylamide (LSD) binding assays suggest that DA is likely the natural ligand and that CeDOP1 is a DA receptor in vivo. At least nine other cDNAs encoding DA receptor homologs have been cloned from the worm, though the functional identity of these receptors has yet to be determined (6).

The behavior of *cat* mutants, as well as the results of DA neuron cell-ablation studies, support the role that DA is involved in mechanosensation (26). Worms normally move slowly on bacteria relative to when they are off their food source (26). Laser ablations of all the DA neurons [the DA neurons are not required for survival (an advantage in genetic screens, see below)] or mutations that affect DA synthesis or storage (*cat-1*, *cat-2*, *cat-4*, and *bas-1*), cause the animals to move at similar rates on or off bacteria; however, the addition of DA to *cat-2*, *cat-4*, and *bas-1* selectively restores the slower movement on the bacterial lawn, suggesting that DA plays a role in context-dependent locomotion (26). Furthermore, male mating is a complex behavior that requires the normal function of the DA neurons in the male tail; laser ablation of the tail DA ray neurons results in inefficient attempts to locate the vulva for copulation (43). Similarly, well-fed *cat-2* males, unlike WT males, often appear disinterested when encountering hermaphrodites and do not attempt to mate (34). Abnormal DA signaling in *cat-1*, *cat-4*, and *bas-1* males may also contribute to their disinterest in mating with the hermaphrodites (34). Finally, worms exposed to DA become paralyzed and do not lay eggs, but after a few hours are able to move normally (39). *unc-2* mutants do not adapt to DA. *unc-2* encodes for the α-1 subunit of a voltage-sensitive calcium-channel, and calcium-dependent transcription modulation has been implicated in DA reward pathways (39, 44). It will be interesting to determine if the ability of *C. elegans* to adapt to DA exposure shares features common to adaptations found in DA signaling pathways following chronic psychostimulant exposure (44).

MAMMALIAN AND *C. ELEGANS* DOPAMINE TRANSPORTERS

A unique and identifying characteristic of mammalian DA neurons is the presence of the DAT, which is responsible for the reuptake of DA in the presynaptic terminal following release into the synaptic cleft (45–47). DAT localizes to axons and dendrites of mesencephalic and hypothalamic DA neurons, innervating the striatum and frontal cortex, and directly or indirectly regulates locomotor activity, cognition, emotion, reward, and neuroendocrine function (48–50). The transporter is the target for many psychoactive drugs, including cocaine, amphetamine, and methylphenidate. These agents confer euphoria and can lead to addiction. DATs also provide the molecular gateway for the accumulation of neurotoxins that can evoke neuronal death (see below) (47, 51, 52).

Studies in knockout mice indicate that DAT significantly modulates many aspects of DA neuronal function and neurotransmission. DAT knockout mice display a 75% decrease in DA release into the synaptic cleft and a 300-fold decrease in extracellular DA clearance (clearance approximately correlates with normal diffusion) (53, 54). TH levels, as measured by immunohistochemistry, are also dramatically decreased to 90% of WT, although TH activity is increased 200-fold; this concomitant increase in TH activity is not sufficient to account for the reduction in

TH, and cellular DA levels are reduced 20-fold (53, 55). Furthermore, D1 and D2 DA receptors are reduced approximately twofold, and DA autoreceptor function is significantly reduced or completely lost (54, 56). Because of the importance of DAT in DA neurotransmission, it is likely that DAT is under strict regulatory control, but the mechanism of this regulation is poorly understood (51). DATs are now believed to be regulated by posttranslational modification and altered trafficking. Mammalian DATs contain multiple phosphorylation sites, including consensus sites for protein kinase A (PKA), protein kinase C (PKC), calcium calmodulin kinase, and cAMP-dependent protein kinase (57). PKC activation via phorbol esters causes DAT phosphorylation and leads to DAT redistribution from the plasma membrane to intracellular compartments, suggesting that phosphorylation may play a role in DAT endocytosis and localization (58–65). It is not clear though whether the direct phosphorylation of DAT is involved in the internalization or whether another protein mediating cell surface expression of DAT is responsible (66).

Evidence exists that DATs are regulated by multple cell surface receptors. D2 receptor (D_2R) antagonists decrease DA transport in vivo and in vitro, but not in all cell types (67–69). Recently, Mayfield & Zahniser showed that D_2R activation increases DAT expression on the cell surface in *Xenopus* oocytes, indicating that the presynaptic autoreceptor can play a significant role in DAT regulation. Muscarinic agonists also cause an increase in extracellular DA, which could be due to a reduction in DAT activity (70), and $\sigma 2$-receptors may regulate DAT activity via a PKC dependent pathway (71).

Recently, DAT has been shown to have direct interaction, both in vivo and in vitro, with two proteins that influence the density of DAT on the plasma membrane. PICK1, a PDZ domain-containing protein and a PKC binding partner, binds to the PDZ domain recognition sequence at the C-terminas of DAT (72). This association appears to enhance DA uptake by increasing the level of, and possibly stabilizing, DATs on the cell surface. Likewise, α-synuclein, a presynaptic protein that associates with synaptic vesicles and participates in excitation-secretion coupling, has been reported to form complexes with human DAT (hDAT) at the C-terminus that also increases DAT clustering at the cell surface and DA transport (73). Interestingly, the degree to which PICK1 and α-synuclein interact is unknown. Importantly, familial PD has been associated with two independent autosomal dominant missense mutations in α-synuclein (74, 75). Although α-synuclein is a major component of Lewey bodies, the pathological hallmark of PD, it is possible that certain facets of increased susceptibility to DA neurodegeneration in individuals carrying the mutant gene is due to altered DAT-α-synuclein interactions (see below).

We have previously cloned the *C. elegans* DAT, DAT-1 (76). It is a 615-residue polypeptide that is highly homologous to the mammalian DATs (45% amino acid identity), with 12 predicted transmembrane domains and multiple predicted N-glycosylation sites (77). We have expressed DAT-1 in mammalian cells, and similar to its mammalian ortholog, DAT-1 exhibits saturable and high-affinity, Na^+- and Cl^--dependent DA transport ($Km = 1.2\ \mu M$) (76, 77). Transport of DA by

transfected DAT-1 is also potently inhibited by the mammalian DAT antagonist cocaine, nomifensine, and GBR 12909, and the agonist amphetamine (76–78). DAT-1 also exhibits high affinity for tricyclic antidepressants such as imipramine and the more norepinephrine transporter (NET)-selective antagonists, such as nisoxetine. Like its mammalian counterpart, DAT-1 is also expressed exclusively in DA neurons (2, 3). We developed transgenic lines that contain 0.7 Kb of the sequence immediately upstream of DAT-1 fused to coding sequences of the green fluorescent protein (GFP) (2). This construct results in the specific expression of GFP in all eight DA neurons (Figure 1) in the hermaphrodite as well as the three pairs of the DA neurons in the male tail (3). The GFP expression level is intense, allowing for direct visualization of the neurons in live animals as they move underneath the objective of a fluorescent dissecting microscope. We have also generated a translational GFP fusion ($P_{dat\text{-}1}$::DAT-1::GFP) that results in a similar expression pattern, though the coincidence of this expression pattern with endogenous DAT-1 remains to be validated (3). Studies are currently underway to compare alternative GFP fusions and to identify native patterns of DAT localization using site-specific DAT-1 antibodies.

DOPAMINE, DOPAMINE TRANSPORTER, AND PARKINSON'S DISEASE

We have recently considered how the presence of DA neurons in *C. elegans* may be useful in modeling DA and DAT regulation and provide opportunities for PD research (2). PD results from the loss of greater than 80% of the DA neurons within the substantia nigra pars compacta (SNpc). It is the second most prevalent neurodegenerative disorder, affecting 1% of the population over 55 years (79). Although the specific etiology of PD is unknown, abundant pathological data suggests that oxidative stress and mitochondrial dysfunction play a role in the DA neuron degeneration (80, 81). DA itself may be an endogenous neurotoxin, clarifying the specificity of DA neuron vulnerablility seen in PD. Several studies have shown that the highly reactive DA molecule can cause striatal neuronal death both in vitro and in vivo in a variety of organisms (82, 83). DA can be oxidized by at least two independent pathways. It can be oxidized enzymatically by monoamine oxidase to produce the relatively inert metabolites HVA and DOPAC, and it can be nonenzymatically auto-oxidized to produce highly reactive quinones and the superoxide radical. Both pathways also produce hydrogen peroxide, which in the presence of transition metals, such as iron (which is found in higher abundance in PD substantial nigra, possibly due to abnormal ferritin metabolism), can further be oxidized to the highly reactive hydroxyl radical (82, 83). Several studies suggest these reactive oxygen species (ROS) and quinones could be major contributors to the DA neuron cell death because of their ability to cause protein denaturation, lipid peroxidation, and DNA damage (82, 83). DA has also been proposed to cause cellular death via calcium channel activation in a DA receptor-dependent

mechanism (82, 84). Finally, DA can confer toxicity to the DA neurons via catechol hydroxylation to form 6-OHDA (see below) (83).

Exposing the SNpc in rodents and mammals to neurotoxins that cause DA neurodegeneration is the most commonly utilized animal model for PD. Vertebrate exposure to the neurotoxins 6-OHDA, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1-methyl-4-phenylpyridinium ion [MPP+ (the active metabolite of MPTP)], or the insecticide rotenone causes SNpc DA neuronal death within several weeks, and the animals exhibit many of the same symptoms as PD (81, 85, 86). 6-OHDA lesioning of nigrostriatal neurons is a particularly intriguing model for PD, because 6-OHDA may be an endogenous neurotoxic metabolite of DA causing or contributing to the disease. The toxin has been identified in untreated rat brains, as well as in brains and urine of PD patients (87–90). It has been proposed that the in vivo production of 6-OHDA occurs via a nonenzymatic reaction between DA, hydrogen peroxide, and free iron at physiological concentrations and that this reaction may be more likely to occur in the highly oxidizing environment of DA neurons (90, 91). The substantia nigra, with its large stores of iron, may provide the catalyst for 6-OHDA production (83, 92). Furthermore, 6-OHDA could be generated in this environment in the presence of DA by either free nitrite ions or manganese (93, 94). 6-OHDA generates an increase in the production of hydrogen peroxide and free radicals, including the superoxide ion and hydroxyl radical (95, 96). These ROS are likely generated through the nonenzymatic breakdown of 6-OHDA and/or the direct inhibition of complex I and IV of the mitochondrial electron transport chain (87, 95, 97). The resulting ROS production leads to lipid peroxidation, protein denaturation, and a decrease in reduced glutathione, which are similar hallmarks found in postmortem PD patients (80, 81).

The specificity of 6-OHDA for the DA neurons lies in its affinity for DAT (54, 55, 87, 98–101). DAT antagonists can block cellular uptake of the neurotoxins both in vivo and in vitro (87, 99–101; R. Nass, unpublished data). Whether DAT expression is important for human PD is unknown; however, DA neurons of the substantia nigra that express the highest levels of DAT in vivo are most affected in PD, whereas the mesencephalic and hypothalamic DA systems that express lower levels of DAT are less severely compromised (52, 102). Because the *C. elegans* DAT-1 is both structurally and functionally similar to mammalian DATs, we asked whether DAergic neurotoxins could gain access to the worm DA neurons and effect their degeneration. Indeed, we found that brief (1 h) exposure of worms to 6-OHDA results in a time- and concentration-dependent loss of DA neuron GFP fluorescence (Figure 3) (R. Nass, unpublished data) (3). Moreover, coincubation of worms with either of two inhibitors of DAT-1, imipramine or D-amphetamine, completely blocked the 6-OHDA effects (3). Using a *dat-1* loss-of-function strain generated by J. Rand and J. Duerr [Oklahoma Medical Research Foundation (OMRF), Oklahoma City, Oklahoma], we found that DAT-1 is required for the 6-OHDA effects (Figure 3) (3). Furthermore, using other lines exhibiting GFP labeling in other neurons (i.e., lacking DAT), we found that the effects are selective for the DA neurons (3). The establishment

and initial characterization of our *C. elegans* model of DA neurodegeneration provides an opportunity to explore fundamental questions concerning the regulation of DAT-1. The 6-OHDA sensitivity of the DA neurons also provide an opportunity to examine the role various endogenous and exogenous compounds, as well as proteins involved in the biosynthetic pathways of DA neurotransmission, may play in normal DA neuron function and disease.

USING *C. ELEGANS* TO EXAMINE THE REGULATION OF DAT-1

The ability to observe live DA neurons in the living worm, to utilize a translational DAT-1::GFP (or CFP or YFP) fusion protein to follow DAT expression levels and localization, and the DAT-1 dependence of the 6-OHDA sensitivity, provide a unique opportunity to explore the structure, function, and regulation of the DAT. Trafficking-based regulation of DAT and the regulation of DA synaptic transmission are poorly understood, but they are under increasing scrutiny as modes by which to localize and control DA clearance (51). DATs also appear to reside, both at the plasma memebrane and intracellularly, in tubulovesicular structures that may represent intermediate trafficking compartments (50). Whether this compartment shares synaptic vesicle components is unknown. Transporters related to DATs appear to reside at steady-state, largely in synaptic vesicle-like structures (103, 104). All known proteins required for neurotransmission in *C. elegans* have homologs in mammalian systems, and well over two dozen mutant proteins that localize to the synapse have been identified (22). Fluorescently-tagged DAT-1-expressing animals (e.g., $P_{dat\text{-}1}$::DAT-1::GFP) can be mated with animals that contain mutations within specific synaptic-targeted proteins to determine in vivo whether these genes may play a role in DAT localization and function. For example, the DAT-1 translational fusion can be crossed into the *unc-104* or *unc-116* lines, which contain mutations within synaptic vesicle motor proteins (105, 106). These kinesin-like molecules are responsible for the transport of a number of synaptic vesicle–associated proteins to synapses, and mutations within these genes accumulate proteins within the neuronal somas (105, 107–109). KIF1A and KIF1B, the mammalian orthologues of UNC-104 and UNC-16, respectively, are also involved in transport of synaptic vesicles to neuronal processes (110, 111). DAT-1::GFP localization can be compared in WT and mutant backgrounds to determine if DAT-1 requires these proteins for proper localization. The sensitivity of these lines to 6-OHDA can also be utilized to determine if DAT-1 function is altered; decreased toxin sensitivity would suggest a failure of DAT-1 to reach or be retained at the plasma membrane. Furthermore, viable *C. elegans* lines exist with mutations in the soluble N-ethylmaleimide-sensitive attachment protein receptor complexes (v- and t-SNARE), *unc-18*, and *unc-64* syntaxin, which are all proteins that are highly conserved with mammals in synaptic vesicle fusion and plasma membrane protein regulation (22, 112, 113). Recently, syntaxin was also shown to directly

down-regulate the GABA transporter GAT1, a member of the DAT family of Na^+- and Cl^--dependent neurotransporters (114). Our translational DAT-1::GFP fusion strain can be crossed into these lines to explore DAT-1 plasma membrane and subcellular localization, regulation, and function.

Mammalian DATs are the target of multiple psychoactive drugs, including the DAT substrate amphetamine and antagonist cocaine. DA neuronal exposure to amphetamine has been reported to cause an internalization of DAT, whereas exposure to cocaine increases DAT on the cell surface (115, 116). The proteins involved in this trafficking modulation are not known; monitoring DAT-1 localization in various synaptic mutant backgrounds could yield insight into this phenomena. As noted, mammalian DATs have been shown to physically interact with two proteins, PICK-1 and α-synuclein, to effect DAT clustering on the plasma membrane and increase DA transport (see above) (72, 73). *C. elegans* contains a homolog to the mammalian PICK1 (Y57G11C.22, 48% AA identity); colocalization studies of PICK1 by fluorescently tagging the protein could help elucidate its regulatory role in vivo and whether sensitivity to 6-OHDA is influenced under normal and oxidizable (6-OHDA) conditions.

SCREEN FOR NOVEL GENES INVOLVED IN DAT-1 REGULATION AND CELL DEATH

Forward genetics provides an opportunity to search for novel genes involved in DAT regulation and DA neurodegeneration (2, 117). The power of a forward genetic screen is that no prior knowledge is necessary concerning the function of the gene in order to identify it as part of a particular regulatory pathway; the only requirement is that the gene is necessary for the phenotype that is assayed. Typically in this type of screen, older worm larvae (P_0) are exposed to a mutagen at a stage of development when the number of germ-line nuclei is at its highest concentration, which ensures the maximum number of mutagenized gametes (118). Following mutagenesis, the eggs from P_0 animals develop into F_1 adults that are allowed to self-fertilize to produce F_2 progeny. The F_2 progeny, in which recessive mutations can now be identified because of their homozygosity, are screened for disruption of behavioral, morphological, or biochemical phenotypes that reflect interference of normal physiological processes. The animals that contain mutant alleles can then be quickly mapped and identified as a protein necessary in the regulatory process.

We are now in the initial stages of a forward genetic screen using our DAT-1::GFP reporter line to identify molecules involved in DAT regulation and 6-OHDA-induced DA neuronal death. Second generation progeny (F_2) of mutagenized animals are being screened for their retention of GFP in the DA neurons following exposure to 6-OHDA. Animals with DA neurons that are insensitive to the neurotoxin could have mutations within DAT or DAT regulatory proteins (Figure 4), for example, which do not allow DAT to efficiently transport the toxin into the cell or localize DAT to the plasma membrane (2).

Alternatively, we envision that a mutation could exist within a cell death pathway that is involved in the toxin-induced neurodegeneration and that could inhibit the onset of neuronal death (see below) (2). Our pilot screen has yielded several dozen mutants that maintain varying degrees of DA neuronal resistance to 6-OHDA. Some of these lines, which are as much as 100% tolerant to the toxin, contain mutations within DAT. The identification of DAT mutants provides proof-of-concept that we should be able to isolate mutations within genes required for proper DAT regulation or genes involved in DA neurodegeneration. We also expect that these lines with mutations in DAT may be able to provide us with insight into critical regions and residues supporting DA transport. A recent study, such as those by Vasudevan et al. (119), with a nucleoside transporter demonstrates how forward genetic approaches can yield insights into facets of substrate translocation pathways (119). Proteins involved in DA production or breakdown may also be identified if cellular concentrations of DA play a role in DAT cell surface expression, regulation, or vulnerability to ROS-induced cell death (51, 82). To this end, DA and ROS have been reported to affect the regulation of DAT (51). In order to determine if DA may also play a role in 6-OHDA-induced DA neuron degenerations, we have crossed our DAT-1::GFP reporter line into mutant lines deficient in DA. Our preliminary results reveal that animals containing lower amounts of DA are more resistant to the toxin; whether this is directly due to a reduction in DAT expression or an overall reduction in ROS-mediated cellular damage due to the loss of DA is not yet clear. Nonetheless, these results are consistent with mammalian studies that suggest that DA itself may mediate the vulnerability of DA neurons to degeneration (82, 83).

Although this model provides significant possibilities for isolating regulators of DAT-1, the screen could miss identifying some proteins involved in proper DAT function. If the protein is required for viability, a loss-of-function mutation precludes isolation by classical means, although the existence of genetic balancers still allows for isolation of these mutations (120). Also, the loss of a regulator of DAT may be missed due to other existing orthologues or other proteins that could compensate for the functional loss of the gene in the worm. Fortunately *C. elegans* often contains only a single locus of a particular gene, allowing for the identification of many genes in biochemical pathways without the interfering aspect of genome redundancy (21).

CELL DEATH AND PARKINSON'S DISEASE

As stated above, our system should also allow for the identification of genes involved in 6-OHDA-induced DA neuron degeneration and cell death pathways reminiscent of PD. The two main types of cell death that can occur during normal development and disease are apoptosis and necrosis (121, 122). Both forms of cell death are morphologically distinct. Apoptosis results in cell shrinkage, nuclear fragmentation, and the formation of apoptotic bodies, whereas necrosis

typically features cell and mitochondrial swelling, cell rupture, and inflammation (122). There are at least two main types of apoptosis—caspase-dependent (classical apoptosis) and caspase-independent (see below). Caspase-dependent apoptosis is the most studied form of programmed cell death that has been identified in invertebrates and mammals (123). This apoptotic cell death pathway is strongly conserved between *C. elegans* and humans (124). Indeed, the molecular components in classical apoptosis were first identified in the worm (123). The BH3-domain protein EGL-1 triggers apoptosis by interacting with CED-9 (cell death abnormal), the mammalian proto-oncogene Bcl-2 homologue. CED-9 is released from the CED-3/CED-4 complex (homologous to the mammalian Caspase-9/Apaf-1 complex) at the mitochondria, and CED-4 translocates to the nuclear envelope where CED-3 is activated and initiates the breakdown of critical cellular components, resulting in cellular death (125, 126).

The mechanism of cell death in PD remains to be elucidated. Indeed, whether classical apoptosis or necrosis play a central role in DA neurodegeneration in PD remains controversial (83, 127, 128). Compared with controls, there are not significant differences in the number of apoptotic bodies, TUNEL-positive cell bodies, or the immunohistochemical patterns or expression levels of the late-stage apoptosis specific protein (ASP) c-Jun/AP-1, nor Bcl-2, or caspase-3 (127, 128). Furthermore, morphological characteristics of apotosis or necrosis, including chromatin condensation, nuclear fragmentation, or cell shrinkage or swelling are not readily apparent (127, 128). Confounding the problem in determining the mechanism of cell death in PD, however, is the apparent long, slow rate of degeneration that occurs within the nigra (at least before the idiopathic form of the disease manifests itself), the relatively quick rate of the apoptotic process (at least in experimental models), the normal background of neuronal cells dying via apoptosis, and the relative small number of cells that are dying at any given time (127–129).

In vivo and in vitro toxin-induced model studies of PD indicate either apoptosis or necrosis depending on the particular type of toxin to which the animal or culture system is exposed. Most in vivo and in vitro DA- or 6-OHDA-exposure experiments suggest an apoptotic form of cell death, whereas MPP^+/MPTP indicate necrotic cell death (130, 131). The pesticide rotenone has been implicated in both apoptotic and necrotic cell death (132–134).

Because classical apoptosis and necrosis are well-defined events in *C. elegans*, we asked whether either of these forms of cell death could be involved in the 6-OHDA-induced degeneration of DA neurons in the worm. Electron microscopy analysis of 6-OHDA-treated worms suggests that the DA neuron degeneration does not occur through a necrotic mechanism because the degenerating cells did not display any swollen organelles or swollen cell bodies or membranous whorls (a characteristic of necrotic cell death in *C. elegans*) (135). The only morphological changes we found relative to the control animals were small, dark, and rounded DA neuronal cell bodies and loss of dendritic endings (without apparent collateral cellular damage), which is most indicative of apoptosis (3, 135). We then crossed our DAT-1::GFP reporter into strains deficient for the cell death genes *ced-3* and

ced-4 and found the sensitivity to 6-OHDA to be indistinguishable from the lines that do not contain the *ced* mutations, which indicates that cell damage here does not involve the classical apoptotic pathway utilized for programmed cell death (3). These results are intriguing because they are consistent with the observations found in postmortem PD patients and could provide a model of DA neurodegeneration that occurs in vivo (127, 128).

If a *ced-3*/*ced-4* linked pathway is not involved in 6-OHDA induced neural degeneration, what molecular pathways may be involved in the 6-OHDA-induced cell death? *C. elegans* contains three other caspase-related (csp, caspase homolog) genes that could play a role in the cellular death. csp-1, csp-2, and csp-3 encode a total of seven transcripts in which a clear function in vivo has not been assigned (136). It could be that one or more of these caspases play a role in ROS-mediated cellular degeneration or, specifically, in DA neurodegeneration. *C. elegans* also contains a homologue to the mammalian caspase-independent death effector, apoptosis inducing factor (AIF) (GenBank accession number U50301) (137). Studies with mammalian AIFs show that following exposure to cell death stimuli, AIF translocates from the mitochondria to the nucleus where it initiates the breakdown of DNA, causing chromatin condensation and conferring cellular death (138, 139). The putative *C. elegans* orthologue is 56% similar (25% identical) to the human enzyme, contains a mitochondria localization domain, and like the mammalian gene, has significant homology with oxidoreductases (137). Considering that 6-OHDA can cause a dramatic increase in oxidative stress in the mitochondria and cytoplasm, the death effector could play a role in the 6-OHDA-mediated DA neuron degeneration (3).

FUTURE DIRECTIONS

C. elegans provides an opportunity to characterize potential human regulators of DAT and gain insight into the roles they may play in human disease (140). Mammalian proteins associated with diseases can compliment their endogenous function in invertebrate systems, such as *Drosophila melanogaster* or *C. elegans*, and recapitulate many aspects of their cellular function and pathology or behavior; examples include genes involved in polyglutamine disorders (Huntington's disease and Spinocerebellar Ataxias), Alzheimers, and prion disease (5, 141). Human WT and mutant α-synuclein, a major component of Lewey bodies, have also been expressed in DA neurons within the fly, and similar to its human counterpart, these animals display adult-onset DA neurodegeneration, filamentous intraneuronal inclusions, and progressive locomotor dysfunction (5, 142). Interestingly though, there does not appear to be a difference in the rate or severity of the degeneration found in the transgenic animal expressing the mutant form. Presently, it is not clear if α-synuclein inclusion contributes to or is a result of the degeneration. As α-synuclein is a proposed DAT regulator, expression studies of human WT and mutant α-synuclein in the worm could provide significant insight into the role

this protein plays both in DAT regulation and neurodegeneration (74, 75). It will be important to determine whether the mutant form of α-synuclein preferentially causes an increase in DA neurodegeneration relative to WT and whether an increase in sensitivity is due to altered interactions with DAT and/or inclusion body formation. Mutant α-synuclein has also been shown to cause an increase in DA toxicity in vitro (143); examination within DA deficient lines will assist in elucidating the role DA plays in the vulnerability of the neurons to degeneration in vivo. Moreover, it will be important to test whether mutant α-synuclein lines are more sensitive to 6-OHDA-induced DA neurodegeneration, further suggesting the role of α-synuclein in oxidative stress-induced cell death. To this end, in collaboration with G. Wong at the A.I. Virtanen Institute for Molecular Sciences in Kuopio, Finland, we are examining human α-synuclein-expressing worm lines for altered DAT expression, localization, and 6-OHDA sensitivity.

Expression of the human parkin gene, which is linked to an autosomal recessive juvenile form of PD, is also widely expressed in DA neurons, suggesting that facets of DA physiology intersect with mutant alleles to lead to DA neuron vulnerability (144, 145). Recent biochemical studies indicate that parkin may participate in the ubiquitin-proteosome pathway of protein degradation as a ubiquitin ligase (145). Interestingly, α-synuclein appears to be normally degraded by a proteosomal pathway, suggesting a link between the known causes of familial PD in the formation of α-synuclein aggregates and pathways that may be altered by oxidative stress. *C. elegans* also contains an orthologue to parkin (Wormpep, K08E3.7), with 53% similarity to the human gene (141); isolation of a worm parkin knockout line or expression of mutant human parkin genes in the worm could provide valuable insights into the role it may play in DA neurodegeneration.

As noted, the transcriptional and translational DAT-1::GFP reporter lines provide an opportunity to explore the role that drugs of abuse and environmental toxins may play in DAT regulation and vulnerability to DA neuron degeneration in vivo. Altered DAT trafficking and plasma membrane density has been proposed to play a role in cocaine and amphetamine toxicity and in neurological disorders such as PD and ADHD, although the precise mechanisms are not clear (51, 116, 146). DAT trafficking can be followed in real time following exposure to the drugs of abuse, and suppressor screens to identify proteins that alter DAT trafficking following drug exposure can be exploited to identify possible proteins involved in DA-mediated behaviors, such as reward and addiction. Furthermore, because environmental agents continue to lie at the forefront of explanations for idiopathic PD (147), exposure of WT or mutant worms with genetic backgrounds deficient in DA biosynthesis or metabolism or ROS protecting proteins (e.g., superoxide dismutase) to suspected pesticides, fungicides, or other xenobiotics will likely yield important clues to the role these compounds play in the vulnerability of DA neurons to degeneration.

Finally, we envision that the nematode model will allow the establishment of a high-throughput screen (HTS) for agents that can efficiently protect against

6-OHDA-induced neural degeneration. *C. elegans* can easily be grown in liquid medium in standard 96- and 384-well microtitre plates (148). These microcultures allow for rapid screening of animals with particular behavioral phenotypes, or optical properties. The high conservation of genes between mammals and worms suggest that the worm is a valid model for neuroprotective drug discovery (149). Indeed, the worm is already known to be sensitive to a wide range of human neuroreactive drugs, including acteylcholine receptor agonsist [e.g., levamisole and nicotine (150, 151)], anesthetics [e.g., halothane (152)], cholinesterase inhibitors [e.g., aldicarb (153)], caffeine (154), serotonin-related [e.g., imipramine and fluoxetine (38, 155)], GABA-related muscimol (156), and dopamine-related [cocaine and amphetamine (76)] compounds. Though inherently simple, the nematode offers a rich array of conserved molecular targets whose manipulation may prove of benefit to halt or perhaps prevent DA neuron degeneration.

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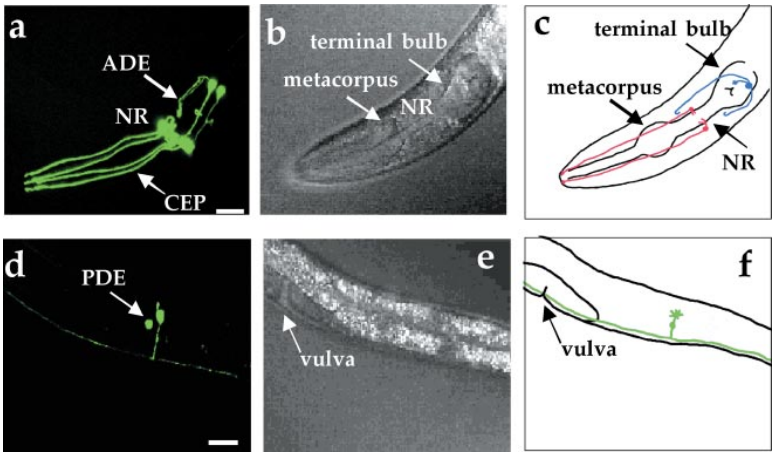

Figure 1 Visualization of all eight DA neurons in living, adult *C. elegans* hermaphrodites using DAT-1::GFP transcriptional fusions. (*a*) 3-D reconstruction of confocal epifluorescence from head DA neurons in a $P_{dat\text{-}1}$::GFP transgenic line. Arrows identify CEP and ADE processes. NR refers to the nerve ring. (*b*) DIC image of animal in panel (*a*). (*c*) Schematic drawing showing location of DA neurons in the head relative to the pharynx. In this top view, two pairs of CEP neurons (red) project dendritic endings to the tip of the nose and one pair of ADE neurons (blue) extend ciliated processes to amphids adjacent to the terminal bulb of the pharynx. (*d*) 3-D reconstructions of confocal epifluorescence of the PDE neurons. Both PDE cell bodies are apparent. (*e*) DIC image of animal in panel (*d*). (*f*) Schematic drawing showing left hand member of pair of PDE neurons (green) in lateral location posterior to vulva. All scale bars = 25 μm. Anterior is to the left. See Reference (3) for details. Reproduced with permission from PNAS (3).

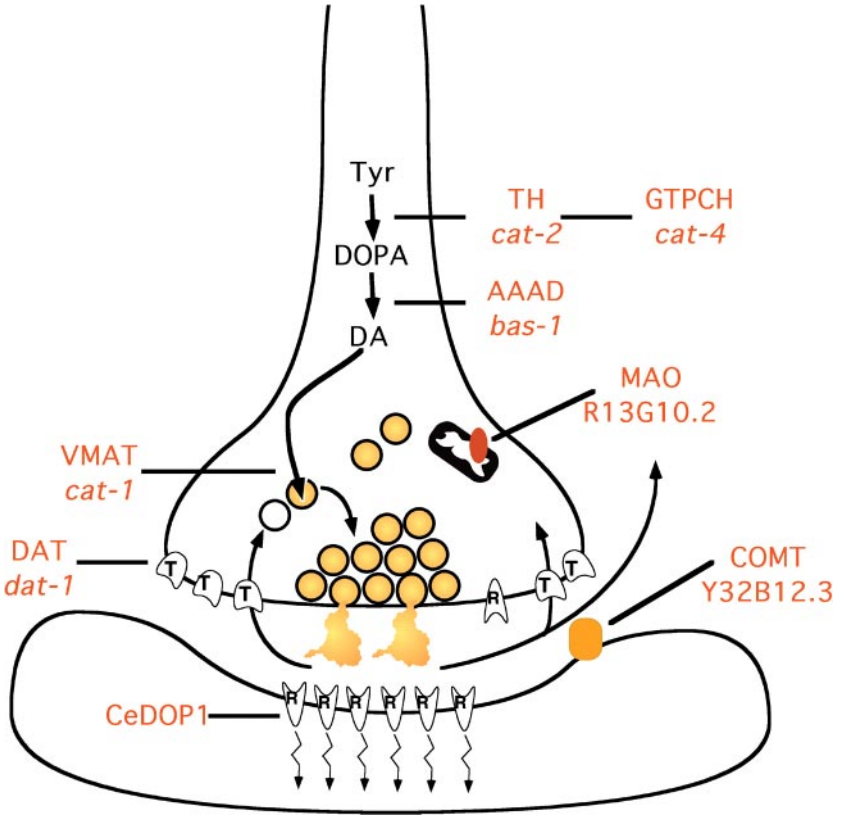

Figure 2 Schematic model of a *C. elegans* DA neuron containing known and predicted genes involved in DA biosynthesis and metabolism. Genetic mutants are indicated in italics. All protein locations are putative. TH, tyrosine hydroxylase; GTPCH, GTP cyclohydrolase; AAAD, aromatic L-amino acid decarboxylase; MAO, monoamine oxidase; VMAT, vesicular monoamine transporter; DAT-1, dopamine transporter; CeDOP1, D1-like DA receptor; COMT, cachol-O-methyltransferase.

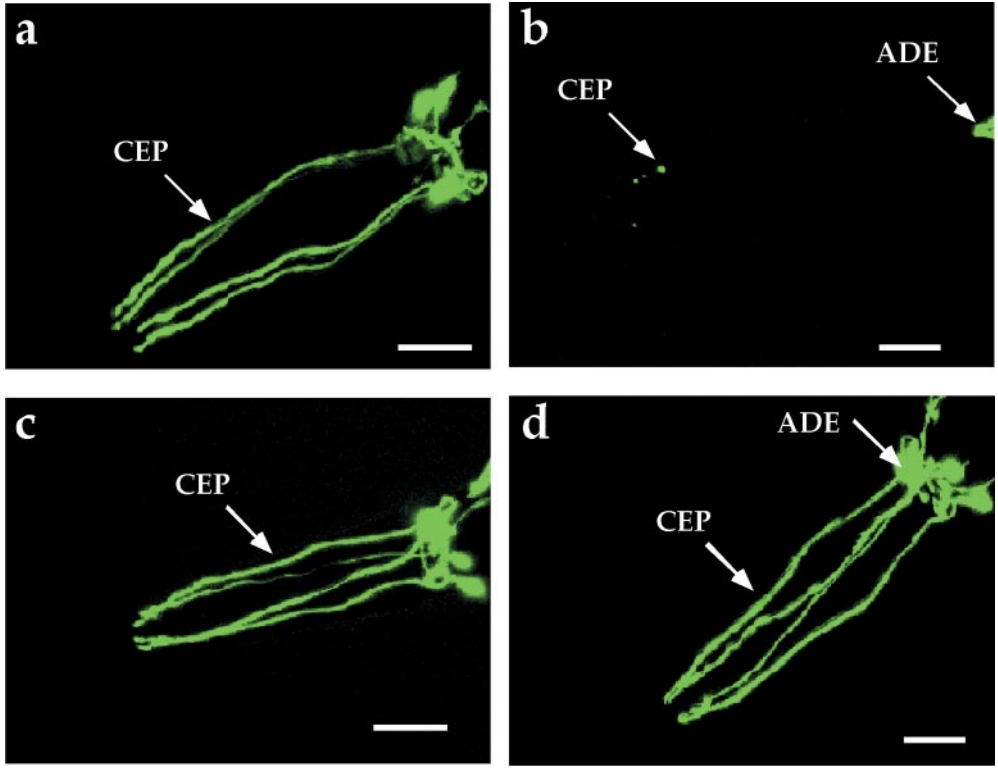

Figure 3 Suppression of 6-OHDA sensitivity of DA neurons. (*a*) $P_{dat\text{-}1}$::GFP animals exposed to vehicle; (*b*) $P_{dat\text{-}1}$::GFP worms exposed to 6-OHDA; (*c*) $P_{dat\text{-}1}$::GFP, *dat-1*(ΔDAT-1 worms) exposed to vehicle; (*d*) $P_{dat\text{-}1}$::GFP, *dat-1* worms exposed to 6-OHDA. Scale bars = 25 μm. See Reference (3) for details. Reproduced with permission from PNAS (3).

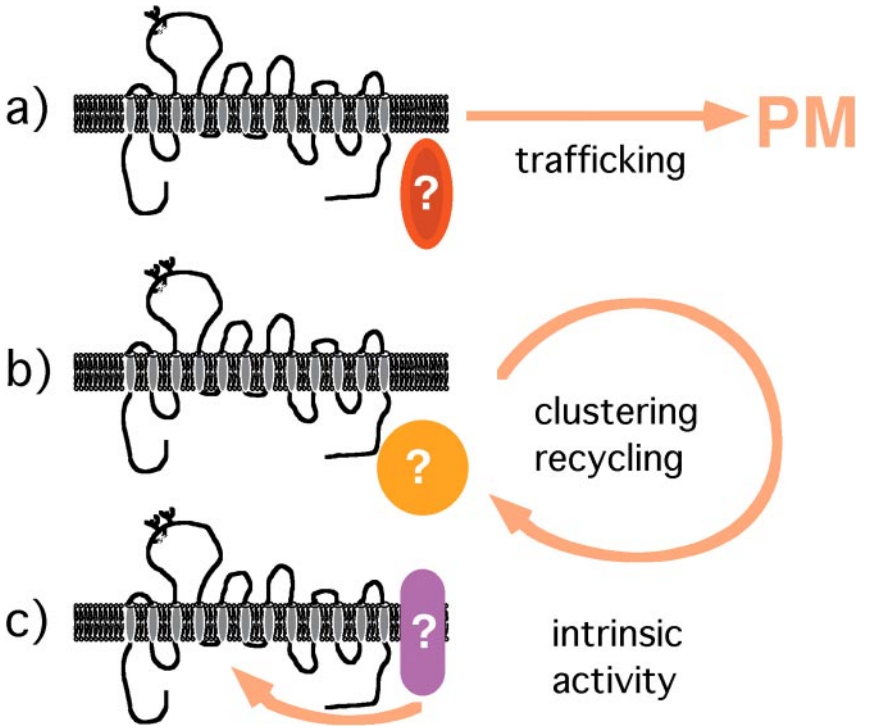

Figure 4 Predicted DAT-1 affected mutants generated from our 6-OHDA dependent DA neurodegeneration screen. The screen could yield mutants affecting DAT-1 trafficking (*a*), recycling from synaptic vesicles to the plasma membrane (*b*), or activity (*c*).

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ALZHEIMER'S DISEASE: Molecular Understanding Predicts Amyloid-Based Therapeutics

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■ **Abstract** Degenerative diseases of the brain were long considered among the most obscure and intractable of human maladies. However, recent advances in understanding their mechanisms have brought us to the verge of potential disease-modifying agents. This progress is perhaps best exemplified by the case of Alzheimer's disease. The application of molecular pathology and genetics has led to the recognition that the four genes implicated to date in familial Alzheimer's disease all chronically elevate cerebral levels of the amyloid β-protein (Aβ). Accordingly, small molecule inhibitors of the β- and γ-secretases, the proteases that generate Aβ from its precursor, are under active development, and some have shown in vivo efficacy in mouse models. An alternative approach, active or passive immunization against Aβ, has received extensive pre-clinical validation in mice, but an effective preparation free of significant side effects in humans is still awaited. Several other potential therapies are also reviewed here. If one or more of these varied approaches is ultimately proven to slow or prevent dementia, Alzheimer's disease will become a salient example of the successful application of reductionist biology to the most complex of organs, the human cerebral cortex.

INTRODUCTION

Few biomedical problems have captured the attention of the scientific and lay communities alike as has Alzheimer's disease (AD). This insidious and devastating brain degeneration that robs its victims of their most human qualities—memory, reasoning, abstraction, and language—is believed to afflict some 4 million Americans and perhaps 20–30 million people worldwide. Once classified as an obscure, "presenile" dementia that was relegated to a few short paragraphs in neurological textbooks, AD is now recognized as a major public health problem in developed nations, and knowledge of its causes and mechanisms has grown enormously in the past decade. In this review, we briefly summarize the current understanding and management of AD and then explore in detail several discrete therapeutic targets that have emerged from the ongoing elucidation of its molecular basis.

Defining Alzheimer's Disease

THE CLINICAL SYNDROME AD begins almost imperceptibly, most often with occasional, minor lapses in recalling recent events of daily life (episodic memory). Patients may fail to remember a conversation or activity, or may become confused about an item of information recently received. The syndrome referred to as mild cognitive impairment (MCI) is often a harbinger of AD and begins with pure amnestic symptoms, with little or no difficulty in other cognitive spheres (1, 2). Patients with MCI or early (mild) AD have fully preserved alertness, no significant language disturbance, and intact motor and sensory function.

During or after the first couple of years of amnestic symptoms, most patients develop additional minor problems with some aspects of general cognitive function, such as being oriented to time and place and executing complex tasks easily and correctly (executive function). As such deficits become increasingly noticeable, patients may experience disinterest in activities and hobbies, apathy, emotional lability, word-finding difficulty, spatial disorientation (e.g., getting lost), and trouble with mathematical concepts (e.g., keeping bank accounts). After a few years of progressive memory and cognitive decline, many patients begin to experience first mild and then more noticeable deficits in motor function, including stereotyped manual tasks (e.g., handiwork, writing, and drawing), balance, and walking. Over several years or even a decade or more, AD patients will gradually deteriorate into a marked dementia, with full disorientation, profound memory impairment, and global cognitive deficits. Many patients become immobile, confined to a chair or bed, and ultimately succumb to minor respiratory difficulties, such as aspiration or pneumonia.

THE NEUROPATHOLOGICAL PHENOTYPE Although a clinical diagnosis of AD based on the above signs and symptoms can be made with considerable certainty during life, confirmation still requires postmortem observation of the classical lesions in microscopic sections of hippocampus, amygdala, and the association cortices of the frontal, temporal, and parietal lobes. Although mild ($\sim$8%–15%) atrophy of the cerebral hemispheres may be observed on gross inspection of the brain, this is often hardly more than occurs with age in non-demented individuals. Light microscopy, however, reveals a myriad of neuritic ("senile") plaques and neurofibrillary tangles in the aforementioned brain areas of the AD patient. Neuritic plaques are roughly spherical, extracellular deposits of amyloid β-protein (Aβ) fibrils intimately surrounded by dystrophic axons and dendrites, activated microglia, and reactive astrocytes. Such fibrillar amyloid plaques are invariably accompanied by many "diffuse" (pre-amyloid) plaques in the same brain regions. These consist of amorphous extracellular deposits of Aβ-immunoreactive granular material that generally lack amyloid fibrils and are associated with very few or no dystrophic neurites or altered glia. In most AD cases, the number of diffuse plaques clearly exceeds that of the neuritic plaques. Diffuse plaques appear to represent the earliest light-microscopically detectable lesion in AD brains. They seem

to predate fibrillar neuritic plaques, as judged by their occurrence in cognitively normal, late middle-aged and elderly healthy individuals (3), as well as by their development in teenagers with Down's syndrome long before the latter individuals develop the neuritic plaques and neurofibrillary tangles typical of AD (4). It should be emphasized that diffuse and neuritic plaques actually exist in the cortex in a morphological continuum, rather than as two distinct types of lesions.

Aβ also accumulates in the small blood vessels of the meninges and cerebral cortex, mostly in the outer walls of arterioles and capillaries (5). The extent of this congophilic amyloid angiopathy (CAA) varies greatly among AD brains, even when parenchymal Aβ deposits are at roughly similar levels. The pathophysiological contribution of CAA to the dementing symptoms of AD, if any, remains unclear. CAA occurs not only in AD patients, but also in isolation in elderly humans lacking the clinical and neuropathological features of AD. If the amyloid deposition becomes severe enough to cause substantial hyaline necrosis of the microvessel wall, cerebral hemorrhage may ensue. However, this is a rare clinical complication in sporadic AD.

Besides the neuritic plaque, the other diagnostic lesion of AD is the neurofibrillary tangle. Tangles are non-membrane-bound masses of paired helical filaments, usually intermixed with straight filaments, found in the perinuclear cytoplasm of many limbic and cortical neuronal cell bodies. Smaller bundles of these abnormal filaments may occur in many, but not all, of the cortical dystrophic neurites found within and also separate from the neuritic plaques. Tangles are also observed in neurons of the subcortical nuclei (e.g., the cholinergic septal nuclei and nucleus basalis of Meynert) that project widely to limbic and association cortices rich in Aβ deposits.

A recurring concern in the study of AD is that Aβ plaques can be found at autopsy in individuals who had few or no cognitive symptoms during life. However, it is important to note that almost all of the plaques in aged normal brain tissue are of the diffuse type—that is, they lack associated neuritic and glial cytopatholgy—and they are accompanied by very few or no neocortical tangles. On this basis, it has been postulated that diffuse plaques are "pre-clinical" lesions not yet associated with microscopically visible injury to neurons and their processes. A rough analogy can be drawn to the fatty streaks of cholesterol in systemic blood vessels that occur in most older asymptomatic patients and are often a precursor to clinically important, mature atherosclerotic plaques.

THE NEUROTRANSMITTER PHENOTYPE The first transmitter abnormality to be documented in AD brain tissue was the loss of enzymes that synthesize and degrade acetylcholine (6–8). Accordingly, cholinergic neurons in the septum and basal forebrain were found to decline in both size and number in AD (9). However, these deficits were soon shown to be accompanied by losses of neurons using other neurotransmitters, including glutamate, GABA, somatostatin, corticotropin-releasing factor, serotonin, and several others (10–12). Thus, the neurotransmitter deficits of AD are multiple and provide no clear clue as to the process destroying

these diverse neuronal subtypes. To date, only the cholinergic deficiency has been seriously addressed therapeutically.

BASIC BIOLOGY OF AMYLOID β-PROTEIN PRECURSOR Aβ is a small hydrophobic peptide with N- and C-terminal heterogeneity that occurs in two principal lengths: Aβ40 and Aβ42. Aβ is proteolytically released from a large type 1 membrane glycoprotein of unknown function, the Aβ precursor (APP) (13), via sequential cleavages by two aspartyl proteases, referred to as the β- and γ-secretases (Figure 1) (14). The Aβ region of APP comprises the 28 residues just outside the single transmembrane domain (TMD), plus the first 12–14 residues of that buried domain. On this basis, Aβ was originally assumed to arise only under pathological circumstances, in that the second cleavage was thought to require some kind of prior membrane disruption to allow access of γ-secretase and a water molecule to

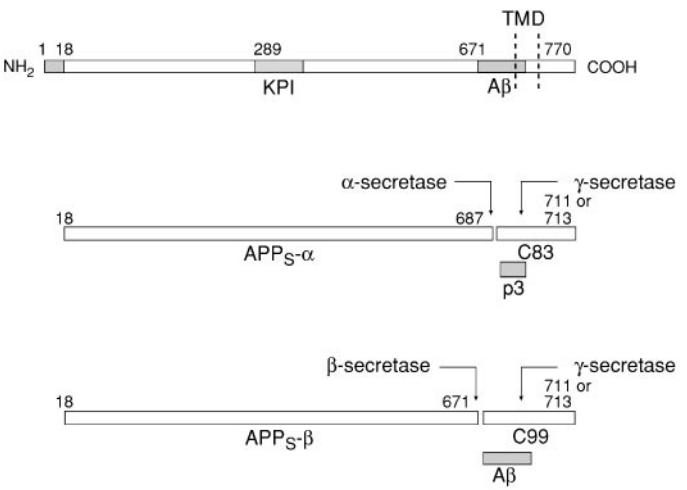

Figure 1 Schematic representation of APP processing. The top diagram represents the largest known splice variant of APP, which contains a signal peptide (residues 1–17), two alternatively spliced exons (at residue 289), and a single TMD (700-723). Constitutive α-secretase-mediated proteolytic cleavage of APP occurs after residue 687, yielding APP_s-α and the C83 fragment (*second line*) Alternatively, β-secretase-mediated cleavage occurs after residue 671, yielding APP_s-β and C99 (*third line*). γ-secretase cleavage at position 711 or 713 releases the p3 peptides from C83 and Aβ40 and Aβ42 from C99. Numbers represent amino acid positions; arrows indicate sites of secretase cleavages. Aβ: amyloid β-protein; APP: amyloid β-protein precursor; APP_s-α: soluble ectodomain of APP formed by α-secretase cleavage; APP_s-β: soluble ectodomain of APP formed by β-secretase cleavage; KPI: Kunitz type serine protease inhibitor motif; TMD: transmembrane domain. Reproduced with permission from Reference 14.

the otherwise intramembranous region. This concept was disproved in 1992, when Aβ was shown to be constitutively released from APP and secreted by mammalian cells throughout life and thus occur normally in plasma and cerebrospinal fluid (CSF) (15–17). This discovery enabled the dynamic study of Aβ production in cell culture and animal models, including examination of the effects of AD-causing genetic mutations. Moreover, high-throughput screening could now be conducted on cultured cells to identify Aβ-lowering compounds and determine their mechanism.

Most APP molecules that undergo secretory processing are cleaved by α-secretase, rather than β-secretase, near the middle of the Aβ region (18, 19). This releases the large, soluble ectodomain (APP_s-α) into the medium and allows the resultant 83-residue, membrane-retained, C-terminal fragment (C83) to be cleaved by γ-secretase, generating the small p3 peptide (Figure 1). α-secretase acts on APP molecules at the cell surface, although some processing also occurs in intracellular secretory compartments. The precise subcellular loci of the β- and γ-secretase cleavages are unclear, but likely include early, recycling endosomes (20, 21). The functional consequences of the proteolytic processing of APP remain ill-defined. The current leading hypothesis is that cleavage by α-secretase followed by γ-secretase enables the release of the APP intracellular domain (AICD) into the nucleus, where it may participate in transcriptional signaling (22–24). The APP_s-α derivative secreted as a result of this processing appears to have distinct extracellular functions. For example, those APP_s-α isoforms that contain an alternatively spliced Kunitz protease inhibitor domain function as serine protease inhibitors, including by inhibiting of Factor XIa in the coagulation cascade (25).

GENOTYPE TO PHENOTYPE CONVERSIONS IN FAMILIAL ALZHEIMER'S DISEASE As knowledge of the complex processing of APP unfolded, the question of its significance in the pathogenesis of AD came to the fore. This question was largely answered by the discovery of various genetic alterations that strongly predispose individuals to AD. The first familial AD gene to be identified was APP itself (26). Missense mutations located within and immediately flanking the Aβ sequence were found to cause autosomal dominant forms of early-onset AD and/or CAA (27). No AD-causing mutations in the large APP molecule have been found away from the Aβ region, strongly suggesting that the substitutions alter the proteolytic generation of Aβ and/or its aggregation into neurotoxic assemblies. This conclusion has been amply confirmed in cell culture assays, APP transgenic mice, and patients bearing these mutations.

The ε4 allele of the cholesterol transport protein, apolipoprotein E, was the second predisposing genetic factor to be discovered (28). Extensive genetic epidemiology has shown that inheritance of one apoε4 allele increases the likelihood of developing late-onset AD by $\sim$2–5-fold, and the inheritance of two alleles raises the risk by 4–10-fold or more (29). Interestingly, inheritance of the apoε2 allele appears to confer some protection from AD (30). The mechanism by which

apoE4 promotes the disease is unclear. Crossing mutant APP transgenic mice with mice lacking the endogenous apoE gene results in offspring with far fewer Aβ deposits (31). Expressing human apoE4 in such mice leads to heightened Aβ deposition, compared to expressing human apoE3 (32). In vitro studies also suggest that apoE4 is a less effective inhibitor of Aβ aggregation than is apoE3 (33, 34). Such experimental data fit well with neuropathological analyses in humans, which show that the inheritance of one or two apoE4 alleles significantly heightens cerebral Aβ burden, even in those who have not yet developed clinical AD (35, 36).

The two other genes clearly linked to AD are presenilins (PS) 1 and 2 (37, 38). Missense mutations in these homologous 8-TMD polypeptides cause the most aggressive form of AD known, with onset commonly occurring in the 40s and 50s and very rarely as early as the late teens. Many studies in cell culture, animal models, and patients demonstrate that presenilin missense mutations elevate the γ-secretase-mediated production of the strongly self-aggregating Aβ42 peptide, perhaps at the expense of the Aβ40 peptide (14). This mechanism is discussed in detail when we consider inhibition of γ-secretase as a therapeutic target.

The APP, PS1, and PS2 mutations cumulatively account for close to half of early-onset (<65 years) familial AD cases, with PS1 responsible for almost all of these. Inheritance of apoE4 is variously estimated to be the principal pathogenic factor in 10%–40% of all AD cases. It should be emphasized that apoε4 acts as a risk factor for AD, not a deterministic gene, so that octogenarians carrying one or even two ε4 alleles may have normal cognitive function. Several other chromosomal loci and candidate genes are under active study, but not unequivocally confirmed. When they are, the same kind of genotype-to-phenotype analysis should establish whether or not they operate by altering the economy of Aβ in the brain.

Progress in deciphering the genetic forms of AD has led to several compelling transgenic mouse models. The first models expressed human APP that contained missense mutations which cause autosomal dominant AD (39, 40). A number of distinct mouse lines ensued, carrying various mutant forms of human APP driven by different promoters and having subtly different neuropathological and biochemical phenotypes (41, 42). The cloning of PS1 led to crossed mouse lines expressing mutant human APP plus a missense mutation in human PS1, and these mice show accelerated Aβ deposition and generally more severe neuritic and glial cytopathology than APP single transgenic mice (43). Crossing mutant APP mice with those expressing human transforming growth factor-β produced a line with prominent microvascular amyloidosis (44). Particularly useful is the recent development of crossed mice expressing human mutant APP plus human mutant tau, as the progeny develop tangle-like neuronal cytopathology in addition to diffuse and neuritic plaques (45). In general, the various mouse models show progressive Aβ accumulation and deposition in the hippocampus and association cortices in anatomic patterns resembling those seen in AD and with the development of secondary neuritic, neuronal, and glial alterations.

Current Treatments are Largely Symptomatic

At the time of writing, no treatments have been clinically proven to modify the disease process in a way that significantly slows or prevents the progression of AD. However, there are several different pharmacological agents that may ameliorate or temporarily suppress certain debilitating symptoms. The most commonly used drugs that do so are reviewed here.

ACETYLCHOLINESTERASE INHIBITORS Four compounds in this class have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of AD. Of these, three are in active clinical use, whereas one [tetrahydroaminoacridine (Cognex)] has the potential to cause significant hepatotoxicity and is therefore rarely used. The other three [donepezil (Aricept), rivastigmine (Exelon), and galantamine (Reminyl)] have similar potential benefits and adverse events, including nausea, vomiting, diarrhea, and other cholinergic symptoms, and have generally been used interchangeably. In placebo-controlled studies of a few months in duration, these agents modestly improved certain cognitive test scores, although in clinical practice many patients show no objective evidence of improvement in memory or other cognitive tests. A minority of treated patients show modest improvement in certain mental status scores or no decline over a period of several months to a year. Patients who show no improvement or lose benefit over time can be switched to another inhibitor, but this does not usually result in a substantial difference in efficacy. Over time, many physicians and patients decide to discontinue acetylcholinesterase inhibitors due to lack of clear benefit. In Europe, the NMDA antagonist, memantine, is marketed for treatment of AD. This is the only non-cholinergic neurotransmitter agent currently in widespread use for symptomatic treatment.

PSYCHOTROPIC DRUGS THAT MODIFY BEHAVIORAL SYMPTOMS One of the most difficult aspects of AD for patients and caregivers is the occurrence of behavioral disturbances, including anxiety, apathy, agitation, aggressiveness, depression, serious delusions, and hallucinations (46). Although minor anxiolytics, such as benzodiazepines, can blunt some of these symptoms, their benefit is modest and often accompanied by unacceptable sedation or worsening of dementia. Non-phenothiazine antipsychotic agents in low-to-moderate doses are more widely used for this purpose. These include risperidone (Risperdal), olanzapine (Zyprexa), haloperidol (Haldol), and quetiapine (Seroquel). Occasional AD patients (~5%–10%) may display prominent symptoms of depression and therefore may benefit from low doses of selective serotonin re-uptake inhibitors, although these may also temporarily heighten the patient's cognitive deficits. Physicians sometimes prescribe stimulants, such as methylphenidate, for marked apathy, or anticonvulsants, such as devalproex sodium (Depakote) or carbamazepine (Tegretol), for agitation and anxiety. The administration of low-to-moderate doses of one, or sometimes more than one, psychotropic drug can significantly improve behavioral symptoms in AD patients. However, dose escalation should be slow,

and patients should later be gradually weaned off the agent to determine whether cognitive function is being compromised. Many AD patients experience significant behavioral aberration for relatively circumscribed periods of time; as their dementia worsens, they lose these symptoms and become more calm and mellow.

POTENTIAL NEUROPROTECTIVE STRATEGIES Although there are no available drugs clearly proven to modify the course of cognitive decline, several neuroprotective approaches have been suggested. Many clinicians recommend that patients take high doses (1000–2000 IU/day) of vitamin E. At least one placebo-controlled trial of vitamin E and selegiline (a monoamine oxidase inhibitor) has reported small but significant delays in time to both nursing home entry and death with each of these agents alone, but no additive effect of taking both. However, these effects may well represent benefits for cardiovascular and other systems, and there was no evidence of improvement in the cognitive symptoms of dementia. Although high doses of vitamin C have sometimes been recommended, no studies showing a definitive benefit in AD have appeared. Nonsteroidal anti-inflammatory drugs could potentially blunt the neurotoxic effects of peri-plaque microglial/astrocytic activation, cytokine release, and the acute phase protein response in the AD brain, but no prospective controlled trials have yet shown a benefit. Other neuroprotective strategies potentially relevant to AD include calcium channel modulators (47), free-radical scavengers (48), and metal ion chelators (49); published clinical trial results for the latter are emerging.

INHIBITION OF β-SECRETASE

Identification and Protease Biochemistry of β-Secretase

Early experiments established that a substantial portion of the APP normally secreted by human-mixed brain cultures, and some of that present in human CSF, is precisely cleaved at the amino terminus of Aβ (between residues 671 and 672) resulting in formation of the C99 fragment (Figure 1) (50). Well before the identification of the molecular structure of β-secretase, a considerable amount of information was obtained about its functional properties (Table 1) (51). This work culminated in the identification by five independent research groups in 1999 of a novel aspartic protease that exhibited the expected properties of β-secretase (52–56). Aspartyl proteases are a well-characterized class of proteases that include pepsin, renin, cathepsins D and E, and napsin A. Although each research group used a somewhat different detection approach [expression cloning (52), biochemical enzyme purification (53), and various genomic strategies (54–56)], the enzyme was identical (Figure 1), thus strengthening the conclusion that the structure identified was β-secretase.

β-secretase is a 501–amino acid type I transmembrane protein with a lumenal active site (Figure 2). It has an N-terminal signal peptide (residues 1–21), a

TABLE 1 Functional characteristics expected of β-secretase based on cell culture experiments

Present in neurons in the brain
Membrane-associated protease with lumenal active site
Present in Aβ-secreting cells
Co-localized with APP in Golgi vesicles
Overexpression increases APP_s-β, C99, and Aβ levels
Cleaves at Asp 1 of Aβ
Optimum pH ~4.5
Pepstatin-insensitive
Antisense inhibition reduces APP_s-β, C99, and Aβ expression
Cleavage preference at P1 (Leu $\gg$ Met $\gg$ Val)

Aβ: amyloid β-protein; APP: amyloid β-protein precursor; APP_s-β: soluble ectodomain of APP formed by β-secretase cleavage.

preprotein domain (residues 22–45), a catalytic domain (residues 45–459) with two conserved aspartyl protease active sites (DTGS at residues 93–96 and DSGT at residues 289–292), and a C-terminal extension with a TMD (residues 460–477) and a cytoplasmic tail (Figure 2) (51). The TMD distinguishes β-secretase from most other aspartic proteases and enables the enzyme to properly access the APP ectodomain at the β-cleavage site (57, 58). The lumenal part of the molecule includes four sites for N-glycosylation at 163, 172, 223, and 354 (52), and six cysteine residues (216, 278, 330, 380, 420, and 443) that can form up to three intramolecular disulfide bonds.

The newly identified β-secretase enzyme was initially designated variously as beta-site APP-cleaving enzyme, or BACE (52), novel aspartic protease 2 (Asp2) (54, 55), and membrane aspartic protease 2 (memapsin2) (56). However, with the identification of a close homolog of β-secretase (known as BACE2, Asp1, or memapsin1), which has a similar, but distinct, substrate profile (54, 59, 60), BACE is now more commonly referred to as BACE1.

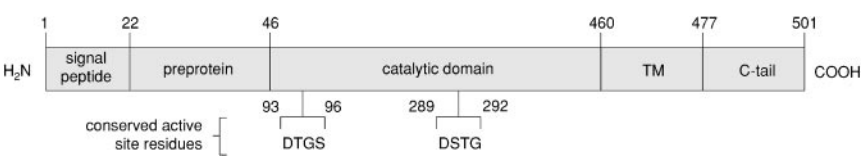

Figure 2 Schematic representation of the protein structure of human β-secretase, indicating the conserved active site residues. Numbers indicate amino acid positions. C-tail: cytoplasmic tail; TM: transmembrane domain.

Cell Biology of β-Secretase

β-secretase mRNA is widely expressed in both the peripheral tissues and brain, with the highest levels in the body found in the pancreas (52, 54, 61). In the brain, β-secretase mRNA is expressed at high levels in neurons of the hippocampus, cortex, and cerebellum (52, 61). Enzymatic activity is highest in neural tissue, and little activity occurs in peripheral tissues (52–54). Subcellular localization studies have revealed that β-secretase occurs mainly within the acidic subcellular compartments of the secretory pathway, the Golgi network and endosomes (52, 55, 58), with low amounts in the endoplasmic reticulum and lysosomes. Thus, β-secretase localizes to the same intracellular compartments at which β-cleavage of APP is believed to occur. The acidic pH of these compartments provides optimal conditions for the cleavage of APP by an aspartyl protease (52–54).

Initially expressed as an inactive pre-proprotein in the endoplasmic reticulum (57, 62), β-secretase subsequently undergoes intramolecular disulfide bond formation and N-linked glycosylation at three of its four potential N-glycosylation sites (62). On leaving the endoplasmic reticulum, the propeptide is cleaved by a furin-like endoprotease, followed by further glycosylation to produce the mature protease, which is rapidly and efficiently transported through the Golgi apparatus before being targeted to endosomes (57, 62). The cytoplasmic tail of β-secretase, and in particular its dileucine motif, appear to be important for the normal trafficking of the enzyme (62).

Substrate Specificity of β-Secretase

β-secretase cleaves APP at the N-terminus of Aβ by recognizing the VKM*DA sequence (residues 594–598). This sequence is labeled P3-P2-P1*P1′-P2′ in standard protease nomenclature, with the site of cleavage marked by *. Cleavage by β-secretase is highly sequence specific. APP containing the Swedish double mutation (KM to NL at positions 595 and 596) that is associated with early-onset AD seems to be a significantly better substrate for β-secretase than wild-type APP (63). In contrast, substitutions of other residues in the VKM*DA region, for example, valine at the P1 position, generally reduce Aβ production, sometimes markedly (64).

Sauder et al. (65) have used three-dimensional modeling to study the interactions between β-secretase and its APP substrate and to identify which residues confer specificity. This work established that the Arg296 residue of β-secretase makes a salt-bridge with the P1′ aspartic acid residue of the wild-type APP. Of additional importance are the interactions between the P2 lysine residue and Asp379 of β-secretase; the hydrophobic contacts between the P1 methionine residue and the enzyme at Leu91, Tyr132, and Ile179; and the P3 valine residue with Phe170 of β-secretase. The model also showed that the Swedish double mutation at P2-P1 interacts more favorably with Arg296 and the hydrophobic pocket of β-secretase than do residues of wild-type APP, thereby explaining the enhanced cleavage of this

mutated form of APP. Conversely, when the P1 residue is changed from methionine to valine, many of the stabilizing hydrophobic contacts between the substrate and enzyme are missing, whereas the catalytic Asp93 residue is blocked. The apparent importance of the Arg296 residue of β-secretase and the hydrophobic pocket of the active site in determining the substrate specificity of β-secretase have subsequently been confirmed by Hong et al. (66), who determined the crystal structure of the active domain of β-secretase complexed with a transition-state inhibitor.

More recently, the substrate specificity of β-secretase has been explored and compared with that of other aspartic proteases using a range of dodecameric substrates based mainly on the β-cleavage site of APP (67). The substrate recognition site of β-secretase extended over several amino acids, and β-secretase accepted a wide range of peptidic substrates. In common with other aspartic proteases, β-secretase prefers a leucine residue at position P1. However, unlike these enzymes, β-secretase accepts polar or even acidic residues at positions P1 and P2′, and prefers bulky hydrophobic residues, preferably valine, at position P3.

Strategies to Identify β-Secretase Inhibitors

β-secretase is an attractive target for a disease-modifying therapy for AD, as it initiates and is the rate-limiting step in the formation of Aβ. Now that the molecular sequence and crystal structure of the target protease have been identified, the development of specific β-secretase inhibitors is being actively pursued. The observation that mice deficient in β-secretase (BACE1 knockout mice) do not generate any Aβ (61, 68, 69) supports the validity of this target. Importantly, these mice appear to be healthy, with no obvious neurological or behavioral abnormalities, despite the complete lack of β-secretase activity in the brain (61, 68, 69).

Inhibitors of other aspartic proteases have been developed, but only those targeted against the retroviral human immunodeficiency virus protease are currently in therapeutic use (51, 70). Research groups attempting to develop β-secretase inhibitors are generally modifying peptidic molecules designed from the sequence of the APP cleavage site (53, 67, 71, 72). To date, only a few of the molecules that directly inhibit β-secretase activity at nanomolar concentrations have been described in published literature.

The first of these, StatV (Figure 3), a 14-residue transition-state analog that spans the P10 to P4′ region of Swedish mutant APP, was used as an affinity ligand by Sinha et al. when they purified and cloned β-secretase from human brain tissue (53). StatV has an IC_{50} of $\sim$30 nM. A second, smaller, and more potent compound that spans eight residues of Swedish mutant APP (P4 to P4′) is OM99-2, with an IC_{50} of $\sim$1.6 nM (Figure 3) (71). However, neither compound is entirely specific to β-secretase, as they both inhibit other aspartic proteases (67). Indeed, the IC_{50} of StatV against cathepsin D, cathepsin E, and pepsin is considerably lower than that for β-secretase (67). Furthermore, both molecules have a molecular weight that exceeds 1000 and are consequently too large for therapeutic use.

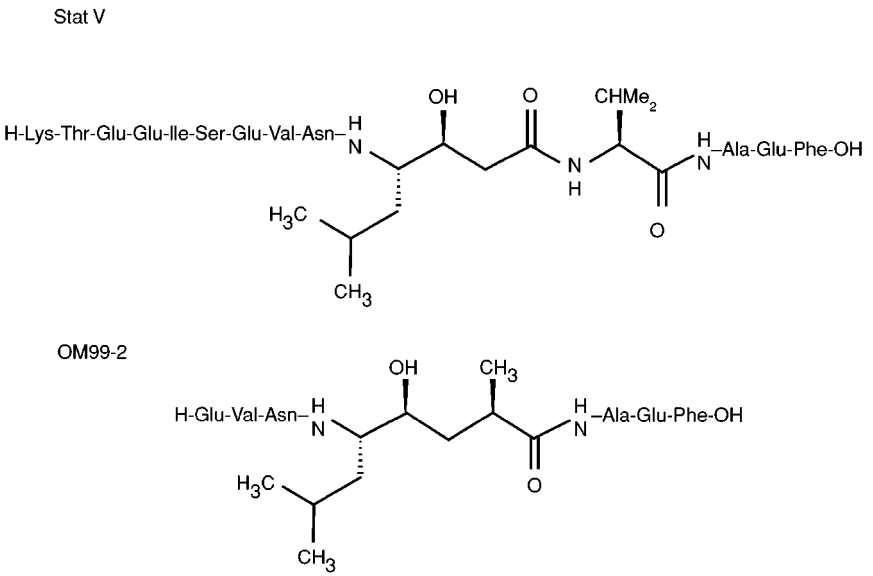

Figure 3 Structure of two direct inhibitors of β-secretase.

To facilitate the rational design of more specific, β-secretase inhibitors, Hong et al. (66) have co-crystallized OM99-2 with β-secretase with the aim of providing information on specific ligand-binding site interactions and the orientation of the inhibitor in the active domain. The group subsequently designed a series of peptidomimetic inhibitors of β-secretase using OM99-2 as a starting point. Some of these compounds were of similar potency to OM99-2, but had a much lower molecular weight (72).

INHIBITION OF γ-SECRETASE

The Concept of Intramembrane Proteolysis

When the precursor of Aβ was cloned in 1987 (13), it immediately became apparent that one of the two proteolytic scissions needed to release Aβ must occur within a hydrophobic, putatively membrane-spanning region of APP. As hydrolysis of peptide bonds requires water and as all known proteases had their active sites in aqueous compartments of the cell or extracellularly, it was assumed that at least part of the APP TMD must be transiently exposed outside of the membrane, presumably due to prior membrane injury. The only other possibility raised at the time was that the TMD of APP ended just before or at the γ-secretase cleavage site; however, this was incompatible with available information about the predicted

lengths of TMDs. The discovery of normal Aβ production in 1992 showed that neither of these explanations appeared to be correct. Rather, Aβ peptides ending at residues 40 and 42, which are clearly within the membrane-spanning region, were naturally secreted by healthy cells without evidence of membrane injury and were circulating peptides. With this discovery, it became apparent that some kind of proteolysis within the membrane could occur normally. In this sense, Aβ generation presaged the recognition of what is now appropriately referred to as regulated intramembrane proteolysis (73).

The first identification of a protease that appeared to cleave within the lipid bilayer emerged from the cloning of the site 2 protease (S2P), an unprecedented polytopic metalloprotease with its HEXXH catalytic motif interior to the membrane-cytosolic interface (74). S2P executes the second of the two cleavages of the sterol regulatory element-binding protein (SREBP), enabling the release of its N-terminal cytoplasmic domain to the nucleus, where it regulates the transcription of genes important for cholesterol homeostasis. Since this discovery, several novel and unrelated intramembrane proteases that process diverse membrane substrates have been found in organisms ranging from bacteria to man (73). A newly recognized example is the rhomboid family, responsible for releasing epidermal growth factor (75). Conserved intramembrane residues in rhomboid that are required for function (asparagine, histidine, and serine) are reminiscent of the catalytic triad typically found in serine proteases, and inhibitors of soluble serine proteases block rhomboid-mediated proteolysis. Many mechanistic details of how such molecules break amide bonds remain unknown, but progress in deciphering the identity and biochemistry of γ-secretase is helping define some of the principles of regulated intramembrane proteolysis.

Relationship of Presenilin to γ-Secretase

When presenilin was first identified (38), the mechanism by which mutations in this polytopic protein caused AD was unidentified, and early speculation about its function was wide ranging. However, it soon became clear that presenilin missense mutations alter the γ-secretase-mediated cleavage of APP in a way that elevates the ratio of Aβ42 to Aβ40 (76). Furthermore, the cloning of *sel-12*, a gene encoding the presenilin homologue in *Caenorhabditis elegans*, revealed presenilin to be critical for signaling by the Notch family of cell surface receptors (77). Deletion of the PS1 gene in mice resulted in an embryonic lethal phenotype that included severe developmental alterations in the Notch pathway (78, 79). Shortly thereafter, De Strooper et al. showed that such mice had a marked decrease in neuronal Aβ production due to a 60%–70% loss of γ-secretase activity (80), the remainder of which was later shown to be due to the residual function of PS2 (81, 82). This finding was interpreted to suggest that presenilins were critical cofactors of the unknown γ-secretase (80). An alternative explanation of these data soon emerged: that presenilin itself was γ-secretase (83). In addition to the

above findings, four principal lines of evidence led to this hypothesis. First, small amounts of APP and PS1 could be co-immunoprecipitated from the membranes of cells, and thus the two proteins could occur as complexes (84). This finding was later extended to show co-precipitation of presenilin with C83 and C99, the immediate substrates of γ-secretase (85). Second, presenilin and APP were localized to the same subcellular vesicle fractions, which mediated de novo generation of Aβ upon incubation at 37°C (86). Third, APP peptidomimetic inhibitors designed as transition-state analogs specific for an aspartyl protease blocked γ-secretase, strongly suggesting that γ-secretase was an aspartyl protease (87). Fourth, and most importantly, presenilins were observed to have two intramembrane aspartate residues predicted to lie in the middle of adjacent TMDs, flanking the hydrophobic region that undergoes endoproteolysis to create the biologically active presenilin heterodimer (83).

Based on this hypothesis, the aspartate residues in TMD 6 and TMD 7 of PS1 were independently mutated to alanine, resulting in an ~60% loss of γ-secretase-mediated Aβ generation and concomitant cellular accumulation of the C83 and C99 substrates (83). Intriguingly, mutation of either aspartate residue also abrogated the endoproteolysis of PS1. Engineering an aspartate mutation into a functional human variant of PS1 that does not undergo endoproteolysis (the ΔExon9 variant) showed that the mutation still blocked γ-secretase activity (i.e., Aβ generation), indicating that the TMD aspartates were independently required for both presenilin endoproteolysis and cleavage of C99 (83). Taken together, these findings were interpreted to suggest that presenilins were unprecedented intramembrane-cleaving aspartyl proteases activated by autoproteolysis (83, 88).

Subsequently, extensive data supporting the hypothesis that presenilins are the active site of γ-secretase has emerged. Antibodies directed against PS1 are able to precipitate in vitro γ-secretase activity from cell membranes solubilized in CHAPS and related detergents (89). Moreover, aspartyl protease transition-state analogue inhibitors of γ-secretase bind directly and specifically to both fragments of the biologically active presenilin heterodimer (90, 91), providing compelling evidence that presenilins contain the active site of γ-secretase. Deletion of both PS1 and PS2 from cells completely abrogates γ-secretase cleavage, both of APP and Notch (81, 82). Furthermore, co-expressing aspartate to alanine mutant forms of PS1 and PS2 in cells simultaneously reduces presenilin heterodimer levels to extremely low levels and essentially shuts down Aβ production, indicating that inactivation of just these residues is akin to deleting the entire protein (92). The presenilin intramembranous aspartate residues are part of signature motifs found in a bacterial family of polytopic aspartyl proteases called type-4 prepilin peptidases (93, 94). Most recently, a novel 7-TMD protein containing the two transmembrane aspartates and conserved flanking residues of presenilin has been shown to be the signal peptide peptidase (SPP), and expressing SPP alone in yeast (which have no endogenous SPP) reconstitutes proteolytic activity (95).

γ-Secretase is a Multi-Protein Complex

Despite the very substantial information implicating presenilin in γ-secretase, it has not been possible to increase substrate-cleaving activity by overexpressing PS1 and PS2 alone. This failure presumably relates to the observation that the endoproteolysis of presenilin and stabilization of the resultant heterodimers requires certain limiting cellular factor(s) (96). In other words, overexpressing presenilin by itself results in the replacement of endogenous presenilin heterodimers with their exogenous counterparts, so that little or no net increase in cleavage activity is obtained. Consequently, there has been an active search for protein cofactors that may help release this tight regulation of γ-secretase activity. The first protein found to potentially serve such a function was nicastrin (97). This large, single transmembrane glycoprotein co-precipitates with PS1 and APP, and mutations introduced into a conserved hydrophilic domain are reported to alter Aβ production (97). Presenilin heterodimers and nicastrin also co-localize in high molecular weight ($\sim$200 kDa) complexes during glycerol velocity gradient centrifugation of cellular microsomes (97, 99). Moreover, partial purification of human γ-secretase from HeLa cell microsomes yields PS1 and PS2 heterodimers and nicastrin in stoichiometric amounts, and the enriched complex cleaves the C100 fragment of APP and an equivalent Notch-based substrate in vitro (100). In accordance, conditions that prevent the co-precipitation of nicastrin and presenilin heterodimers (e.g., the presence of certain detergents) obviate γ-secretase activity (100). Although these data indicate that presenilin and nicastrin are obligatory members of the γ-secretase complex, their co-expression still does not overcome the tight cellular regulation of γ-secretase activity, and additional factors must exist in the active enzyme complex.

Evidence for two additional required components of γ-secretase has recently emerged from genetic analyses of Notch signaling in *C. elegans*. Mutational screens identified a putative 7-TMD protein, designated aph-1, mutations that result in a phenotype closely resembling loss of function in Notch (101) and pen-2, a 101-residue polypeptide with two TMDs (102). Both *aph-1* and *pen-2* showed strong genetic interaction with *sel-12*/PS and *aph-2*/nicastrin. Importantly, inactivation by RNA interference of *aph-1*, *pen-2*, and *nicastrin* in cultured *Drosophila* cells decreased γ-secretase cleavage of APP and Notch reporter substrates and reduced the levels of presenilin heterodimers (102). These results mean that all three proteins play a role in regulating presenilin, the likely catalytic component of the protease. Mutagenesis screens in nematodes have not revealed any other proteins absolutely required for Notch cleavage.

Thus, it currently appears that presenilin, nicastrin, aph-1, and pen-2 are the key components required for γ-secretase to cleave substrates. Accordingly, several experiments can now complete the story. RNA interference-mediated inactivation of each component in mammalian cells should confirm the necessity for each of them in substrate proteolysis in higher organisms. Similarly, transfection into mammalian cells, which have endogenous γ-secretase activity, could prove that

nicastrin, aph-1, and pen-2 together release the tight regulation of presenilin heterodimer levels, allowing more endoproteolysis of full-length presenilin and thus increased production of Aβ, AICD, and the Notch intracellular domain. Indeed, this has recently been accomplished (W.T. Kimberly, M. Wolfe, D.J. Selkoe, et al., unpublished data). Transfection of the four components individually and in various combinations into a cell that has no endogenous γ-secretase activity (e.g., yeast) could establish whether cleavage activity is only reconstituted when all four are present. As such experiments prove successful, the subunits of γ-secretase will have been unequivocally identified, and detailed study of the other biochemical requirements for efficient proteolysis can begin, including presenilin-associated proteins that may facilitate (or inhibit) the reaction, ionic and energy parameters, and the subcellular loci where the components assemble to yield mature enzyme.

How Does Scission by γ-Secretase Occur, and How do Presenilin Mutations Alter Cleavage Specificity?

Based on our current understanding of this very unusual protease, it appears that the two catalytic aspartates interact with a scissile bond in the TMD of a substrate within the lipid bilayer, and the protease initially engages the substrate in its α-helical conformation (103, 104). The tight α-helical conformation of the TMD presumably must be transiently relaxed to allow access of the presenilin aspartates and other residues of the active site to the scissile bond, but how this is accomplished is unclear. A water molecule is necessary for scission, and this may enter the protease via a pore-like structure formed by the eight TMDs of the presenilin heterodimer plus some or all of the TMDs of aph-1, pen-2, and/or nicastrin. The water molecules must somehow be sequestered from the substrate within the membrane until cleavage is actually required. It has been shown that deletion of TMDs 1 and 2 of PS1 abrogates its function, whereas deletion of much of the large cytoplasmic loop, which contains the binding site for the catenins, has no effect (105). Therefore, the protein-to-protein interactions needed for presenilin hydrolytic function are likely to principally involve the various TMDs and to occur within the membrane. In addition, nicastrin, the one required component of the complex with a large ectodomain, could potentially help anchor the ectodomains of the full-length substrates, prior to cleavage, and perhaps even "measure" their length in a way that allows correct cleavage by the proteases that shed the ectodomains of the substrates, such as members of the disintegrin and metalloprotease (ADAM) family. The latter speculation assumes that the α- and β-secretases transiently associate with the presenilin/γ-secretase complex to effect shedding of the substrate ectodomains, thus producing a conformational change in C83 or C99 that permits the secondary γ-secretase cleavage (106).

The two principal γ-secretase cleavage sites in APP, Aβ40 and Aβ42 are separated by two residues, i.e., they are about one half of a helical turn apart and thus on opposite faces of the substrate. It could be that the large majority of C99 and C83 substrate molecules have their Aβ40–41 bonds oriented towards the two

aspartates of particular presenilin heterodimers at the time of cleavage, whereas a small minority (~10%) have their 42–43 bonds oriented towards the aspartates of other presenilin heterodimers, thus yielding the ratio of Aß42 to Aß40 products (~1:10) documented in vivo. All of the known alterations in PS1 and 2 that cause AD are missense mutations, and most are located within the TMDs or adjacent to them. Presumably, these subtly alter the conformation of the active site and/or other critical portions of the TMDs in a way that decreases the efficiency of cleavage of C99 and C83 at the Aβ40–41 peptide bond and/or increases that at the Aβ42–43 bond. This would explain the increased ratio of Aβ42 to Aβ40 peptides that these mutations cause. Of course, even this model is a gross oversimplification because γ-secretase can cleave C99, and presumably C83 as well, at numerous different bonds between Aβ positions 35 and 43.

γ-Secretase Inhibitors Can Lower Aβ In Vitro and In Vivo and Decrease the Formation of Potentially Synaptotoxic Oligomers

Long before the identity of γ-secretase became apparent, the discovery of the normal cellular production of Aβ made the screening of compound libraries in whole cell assays possible, with the goal of detecting molecules that lowered Aβ secretion without inducing general cellular toxicity. Such screening should have uncovered both β- and γ-secretase inhibitors, but analyses of APP processing suggested that virtually all of the potential inhibitors identified acted at the level of γ-secretase. Despite the substantial difficulties of conducting screens in whole cells rather than in purified enzyme systems, the screening efforts of several companies yielded a substantial number of compounds of different structural classes that appear to inhibit the activity of γ-secretase. Extensive medicinal chemistry efforts on some of these molecules ensued. One of the resultant compounds (DAPT) has been reported to produce an acute, dose-dependent reduction of brain and plasma Aβ in vivo when administered orally to APP transgenic mice (107). A single dose of 30 mg/kg yielded an ~30% decrease in cortical Aβ40 and Aβ42 levels measured 3 h later (Figure 4). DAPT was shown to raise brain levels of C83 and C99 in vivo, confirming it as a bona fide γ-secretase inhibitor.

There is growing evidence that diffusible oligomeric assemblies of Aβ, rather than mature amyloid fibrils, may be the principal mediators of neurotoxicity in AD and models thereof (42, 108–111). In this context, certain APP-overexpressing cultured cells generate small amounts of stable Aβ oligomers that are detectable in the culture medium. Microinjection of such medium into the lateral ventricles of anesthetized rats resulted in a block of hippocampal long-term potentiation (LTP), an electrophysiological correlate of certain aspects of memory and learning (112). Moreover, pretreatment of the cultured cells with a γ-secretase inhibitor (a close analog of DAPT) partially lowered monomer production enough to markedly decrease oligomer formation, and microinjection of this medium into rat brain no longer interrupted LTP (112). These results suggest that soluble, potentially synaptotoxic oligomers of Aβ can be targeted therapeutically with γ-secretase inhibitors.

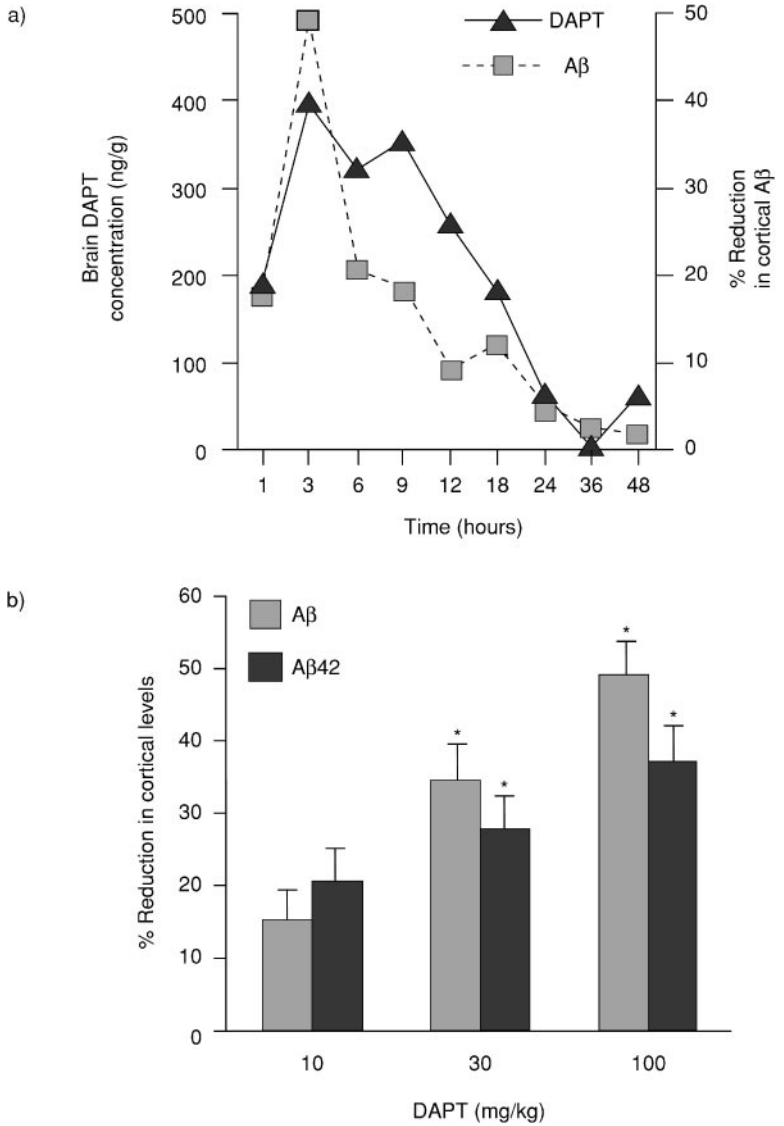

Figure 4 Effect of DAPT administration on Aβ in PDAPP mice. (*a*) Brain concentrations of DAPT and percentage reduction in cortical total Aβ levels after subcutaneous administration of DAPT (100 mg/kg). Aβ levels were significantly reduced compared with baseline from 1 to 18 h after administration, and a peak reduction of 40% occurred after 3 h. Aβ levels closely reflected DAPT concentrations. (*b*) Total cortical levels of Aβ and Aβ42 (expressed as a percentage of levels in vehicle-treated controls) 3 h after oral administration of DAPT (10, 30, or 100 mg/kg) to PDAPP mice. $^*p < 0.05$ versus vehicle-treated control. Reproduced with permission from Reference 107.

Concerns About Inhibiting the Cleavage of Notch and Other Substrates Limit the Potential Utility of Current γ-Secretase Inhibitors

The remarkably similar proteolytic processing of APP and the Notch receptors by α- and β-secretases has raised the question of whether it will be possible to reduce Aβ production by this approach without significantly interfering with the functions of Notch signaling in adults. These functions include a variety of cell fate decisions necessary for the proper differentiation of hematopoietic, immune, mucosal, and skin cells, among others. However, mutational and pharmacological studies examining Notch and presenilin suggest that presenilin-mediated cleavage can be inhibited rather substantially without significantly decreasing the amount of Notch-mediated signaling. For example, mice expressing just one allele of PS1 and no PS2 alleles show apparently normal development (113). As it is generally assumed that lowering the steady state levels of Aβ by just 30% may prove beneficial in slowing the progression of AD, this level of inhibition may leave a sufficient reserve of presenilin-mediated Notch signaling to avoid significant adverse events. However, increasing numbers of presenilin/γ-secretase substrates are emerging, and it will be necessary to determine whether chronic, partial interference with the processing of one or more of these produces adverse effects in vivo. If some of the components of the γ-secretase complex act to bind various presenilin substrates differentially and offer them to the active site, then inhibitors directed at one of these components and not the active site (the PS aspartates) may be less problematic. Of course, the use of a β-secretase inhibitor would not entail these particular risks. Resolution of the central issue of whether γ-secretase inhibitors can lower Aβ production without significantly impairing the normal functions of other substrates awaits further studies in animals and subsequent clinical trials.

An intriguing approach to the problem would be to inhibit production of only the highly amyloidogenic Aβ1–42 form of the peptide or to shift the cleavage specificity of γ-secretase away from position 42, without altering overall Aβ levels. A recent report suggests that certain members of an existing class of FDA-approved drugs, the non-steroidal anti-inflammatory drugs (NSAIDs), may do just that if administered at appropriate doses (114). The treatment of Aβ-secreting cells with the NSAIDs sulindac sulfate, ibuprofen, or indomethacin reduced Aβ42 and increased Aβ38 levels in the culture medium at doses that did not significantly decrease Aβ40 levels. This selective lowering of Aβ42 was also observed in the brains of APP transgenic mice in short-term in vivo experiments. Other NSAIDs (e.g., naproxen sodium) had no effect, indicating that the shift in Aβ production was unrelated to the inhibition of cyclo-oxygenase. This surprising result suggests that certain NSAIDs may have actions directly on γ-secretase that are independent of any anti-inflammatory effects. Chronic use in humans of some of the NSAIDs that lowered Aβ42 levels in this paradigm has been associated with a significantly lower risk of developing AD in epidemiological studies (115). Thus, this work raises

the specter of interfering selectively with Aβ42 production without otherwise perturbing γ-secretase-mediated processing of APP and other substrates. The doses of NSAIDs used to date are very high and considerable further work in animal models and prospective studies in humans is needed to determine whether chronic NSAID administration could have true therapeutic benefit.

CLEARING Aβ FROM THE BRAIN: THE IMMUNOLOGICAL APPROACH

Rationale

The immunological approach to the treatment of AD involves either stimulating the host immune system to recognize and attack Aβ or providing antibodies passively, thereby enhancing the clearance and/or preventing the deposition of Aβ plaques. The immune system can be stimulated in a number of ways, eliciting T cell–mediated and/or humoral responses. Several different types of immunotherapy are currently under investigation for AD: active immunization with synthetic intact Aβ42 or conjugated fragments thereof, and passive immunization with human anti-Aβ monoclonal antibodies (mAb). Immunization with intact Aβ42 has been evaluated in transgenic mouse models and has proceeded to clinical trials, whereas the other immunization strategies are approaching clinical development.

Active Immunization Strategies: Effects in Mouse Models

Aβ42 IMMUNIZATION The hypothesis that Aβ42 immunization could modify the disease process in AD was initially tested in young PDAPP transgenic mice carrying the V717F APP mutation (39), with treatment beginning at six weeks of age, prior to the development of any Aβ deposits (116). In the two active treatment groups, animals were injected with 100 μg of either synthetic Aβ42 or serum amyloid-P component (SAP, another protein associated with amyloid plaques) at monthly intervals for 11 months, combined with Freund's complete adjuvant for the first four doses. Control animals received adjuvant alone in phosphate-buffered saline (PBS) or no treatment. Quantitative image analysis of brains from 13-month-old mice indicated the almost complete absence of hippocampal Aβ42 plaque formation in the Aβ42 group (median plaque burden 0% of cross-sectional area versus 2.22% in adjuvant/PBS-treated controls and 2.65% in untreated mice; $p = 0.0005$). There were parallel reductions in dystrophic neurites (median 0% versus 0.28% in adjuvant-treated controls; $p = 0.0005$) and astrocytosis (median 1.55% versus >6% for all other groups; $p = 0.0017$). Histological examination of several organs, including the brain and kidneys, revealed no signs of immune-mediated complications in any treatment group. Serological analysis showed that the majority of mice immunized with Aβ42 (8 of 9) developed and maintained high serum antibody titers (>1:10,000) against Aβ42. SAP-treated mice mounted

an immune response to SAP (titers generally 1:1000–1:10,000), but exhibited an increase in plaque burden (median 5.74%), suggesting that immune responses against plaque components per se do not prevent or eliminate amyloid deposition. This particular experiment has potentially important implications regarding SAP, as it has been suggested that agents acting upon SAP may be useful in cleaving deposits in a broad range of amyloidoses (117). Subsequent experiments showed that Aβ production itself was not disrupted by the immunization. The paucity of neuritic and gliotic changes suggested that immunized mice did not develop the neurodegenerative cytopathology normally seen in the PDAPP model.

The next series of experiments were designed to establish whether the neuropathological outcome could be improved if Aβ42 immunization was initiated when there was already a substantial Aβ plaque burden (116). Immunization of PDAPP mice began at 11 months with monthly immunizations (100 μg) that continued for 4–7 months. Quantitative image analysis showed that the median cortical Aβ burden in 18-month-old immunized mice was 0.01%, compared with 4.87% for untreated age-matched controls and 0.28% for untreated 12-month-old mice. The reduction in Aβ burden was 96% after four months of treatment and $>$99% after seven months (Figure 5). Again, neuritic pathology and astrocytosis were reduced (by 55% and 34%, respectively, after seven months of immunization). The brains of 15- and 18-month-old immunized mice contained fewer diffuse and mature Aβ deposits than those of 12-month-old untreated mice, suggesting that immunization with Aβ42 had resulted in the clearance of pre-existing Aβ plaques. Analysis of different areas of the cortex indicated that immunization disrupted the normal progressive pattern of amyloidogenesis in PDAPP mice (which begins in the cingulate, frontal, and retrosplenial cortices and progresses in a lateral-ventral fashion). Immunization also prevented the usually heavy Aβ deposition in the outer molecular layer of the hippocampal dentate gyrus. Antibody titer responses to Aβ42 immunization in these experiments were similar to those described above for the younger (pre-plaque) animals.

Additional evidence supporting the concept of an immunotherapeutic approach for AD has been provided by assessments of the functional effects of Aβ42 immunization (118, 119). In experiments in a different transgenic model in which mice expressing APP (APP K670N/M671L and M146L) develop learning deficits as the amyloid burden accumulates, 5 months of immunizations with Aβ42 (100 μg/month), beginning at age 7.5 months, had no adverse effects on performance in a novel working-memory task (the radial-arm, water-maze test) (Figure 6*a*). Subsequently, at an age when the transgenic animals would be expected to exhibit cognitive deficits (15.5 months), Aβ42-immunized mice showed better cognitive performance than those who received a control immunization, and ultimately their performance became similar to that of non-transgenic mice (Figure 6*b*). Modest reductions in cortical and hippocampal Aβ40 and Aβ42 deposits were also observed (118). The observed difference in the magnitude of reductions in amyloid burden between this study and that of Schenk et al. (116) is

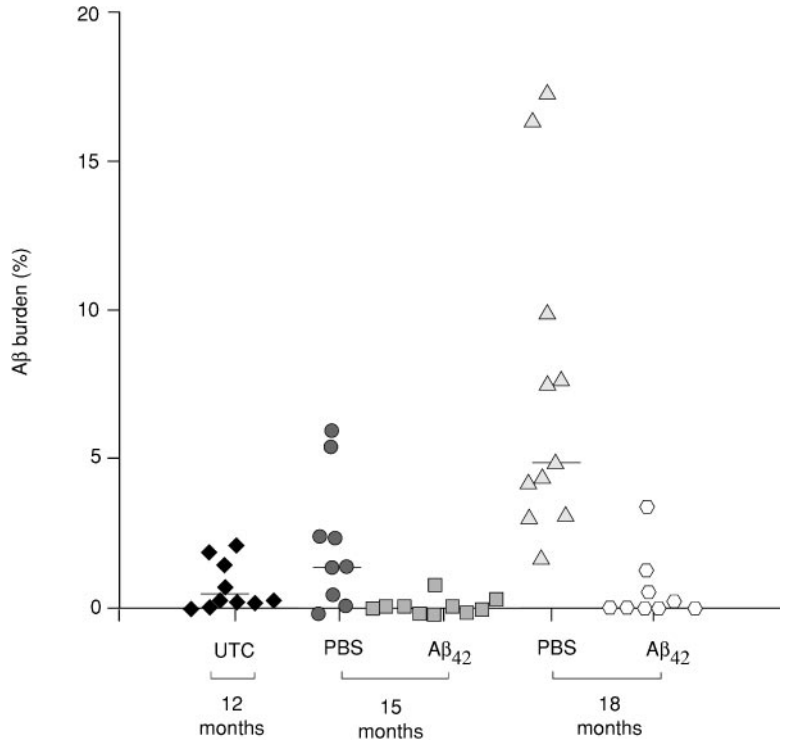

Figure 5 Quantitative image analysis of the cortical Aβ burden in older PDAPP mice (116). Treatment began at 11 months of age. Amyloid burden was significantly reduced in the Aβ group, compared with the PBS controls at both 15 and 18 months of age ($p = 0.003$ and 0.0002, respectively). The median value of the amyloid burden for each group is shown by the horizontal lines. UTC: untreated controls; PBS: phosphate-buffered saline controls; Aβ: amyloid β-protein. Reproduced with permission from Reference 116.

likely to be related to the mouse model used and the duration of the immunization period.

The behavioral consequences of Aβ42 immunization have also been explored in the TgCRND8 (APP K670N/M671L and V717F) transgenic murine model, in which the formation of Aβ plaques was accompanied by the development of spatial learning deficits by three months of age (119). Performance in the Morris water maze of 23-week-old mice immunized with Aβ42 protofibrillar assemblies in the β-pleated sheet form (100 μg/month) from 6 weeks of age was significantly better than that of control transgenic mice ($p < 0.05$), although inferior to that of non-transgenic littermates. Immunization resulted in the formation of antibodies that decorated extracellular dense-cored plaques and reduced Aβ plaque deposition,

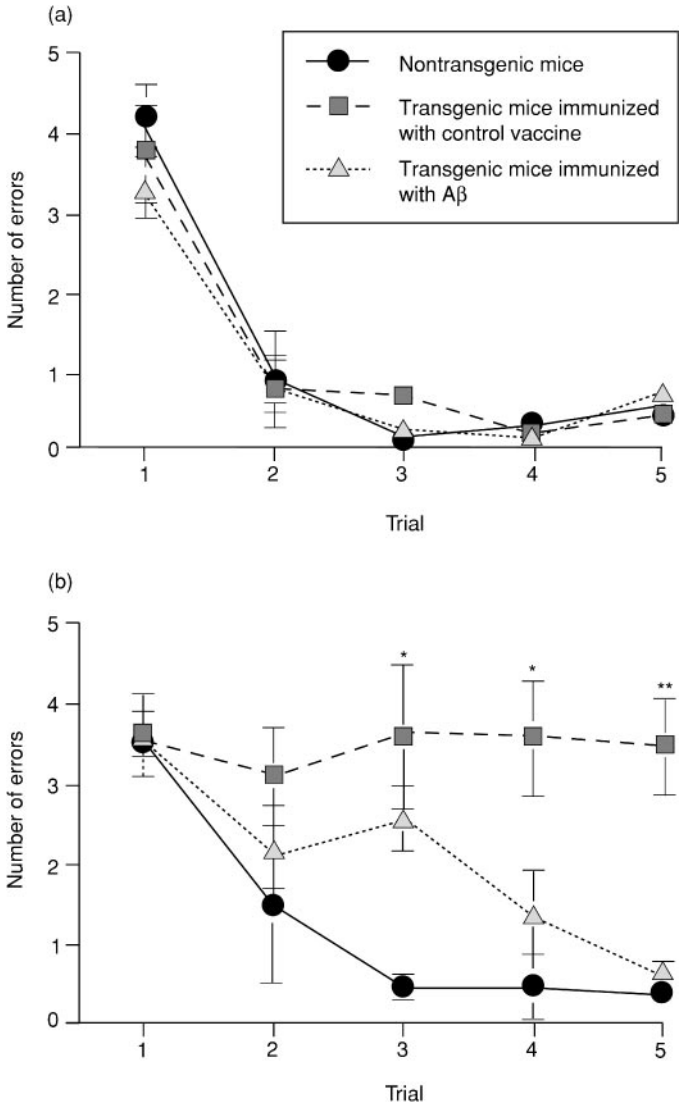

Figure 6 Effect of Aβ immunization on performance in the radial-arm water maze. (*a*) Nontransgenic mice, APP transgenic mice immunized with control vaccine, and transgenic mice immunized with Aβ were tested in the radial-arm water maze at 11.5 months of age (after 5 injections). All groups learned (*trial 4*) and remembered (*trial 5*) the platform location at this time point. (*b*) APP transgenic mice immunized with Aβ continued to show learning and memory of the platform location, whereas the transgenic mice who received the control treatment failed to show learning and memory for platform location on either trial 4 or 5 ($^{*}p < 0.05$, $^{**}p < 0.01$; control immunization group significantly different from the other two groups by LSD post-hoc analysis after MANOVA). Reproduced with permission from Reference 118.

although total brain levels of Aβ were unchanged. These results suggest that the induced antibodies were directed predominantly against Aβ in the β-sheet conformation, rather than in its more soluble form.

The nasal administration of Aβ in PDAPP mice has also been described (120). Weekly administration of Aβ40 (25 μg), beginning at age 5 months and continuing until age 12 months, was associated with a 60% reduction in plaque burden in the hippocampus, decreased neuritic dystrophy, and a reduced level of microglial and astrocytic activation. The presence of anti-Aβ antibodies was demonstrated in the serum of immunized mice, and a slight mononuclear cell infiltration in brain tissue was characterized by cytokine expression.

In summary, Aβ42 immunization results in the clearance of pre-existing Aβ deposits and also prevents the development of new Aβ plaques and associated neuropathology in transgenic murine models of AD. Furthermore, the reduction in Aβ burden is accompanied by improved cognitive performance. No adverse effects of Aβ42 immunization were observed in these models.

The underlying mechanism by which the beneficial effects of Aβ42 immunization are mediated has yet to be explained, although it is conceivable that more than one process is involved. One possibility is that the reduction in Aβ deposition is due to Fc-mediated microglial phagocytosis (116). This hypothesis is supported by the observation that a proportion of the plaques found in immunized mice were decorated with IgG and that microglia co-localized with Aβ within plaques. Furthermore, microglia/monocytes were invariably located near the remaining plaques. The effects of peripherally administered antibodies against Aβ are also consistent with this hypothesis (121).

THE IMMUNOCONJUGATE APPROACH A distinct approach to active immunization is the use of conjugated Aβ fragments from the region of the peptide that does not include the T cell epitopes (i.e., residues 16, 20, 25, and 30) and hence induces only a B cell response. The results of this immunization approach in mouse models have not yet been reported.

Passive Immunization Strategies: Effects in Mouse Models

Passive immunization using mAb raised against synthetic peptide fragments represents an alternative immunotherapeutic strategy. In addition, studies of this approach in transgenic mice have provided further insights into the possible mechanism(s) underlying the observed benefits of active immunization described above. These experiments showed that a humoral response alone, in the absence of a cellular response to Aβ, is sufficient to elicit a strongly efficacious.

In initial experiments, heterozygous PDAPP mice aged 8–10 months received weekly intravenous injections of mouse mAb, either 10D5 (Aβ1–16), 21F12 (Aβ33–42), or polyclonal immunoglobulins (Ig) to Aβ1–42. Stable serum antibody concentrations were maintained throughout the six-month study. At the study's end, mean cortical Aβ42 levels in the 10D5, 21F12, Ig, and control groups

were 6200, 13,580, 4890, and 13,800 ng/g tissue, respectively. Two further mAb, 3D6 (Aβ1−5) and 16C11 (to the tau protein), in addition to 10D5, were subsequently administered to slightly older mice (11.5–12 months). The reduction in cortical Aβ42 burden after six months of treatment with 3D6 was similar to that seen with 10D5 (86% versus 80%; both $p = 0.003$ versus controls), whereas 16C11 had no effect (Figure 7). As expected, the mice in these experiments did not exhibit a T cell proliferative response.

Examination of brain sections of mice involved in these studies revealed that 10D5 and 3D6 entered the brain and partially bound to the Aβ plaques. Subsequent experiments demonstrated that these antibodies triggered microglial cells to clear Aβ plaques through Fc receptor-mediated phagocytosis and subsequent degradation (121). However, DeMattos et al. (122, 123) have reported that a mAb

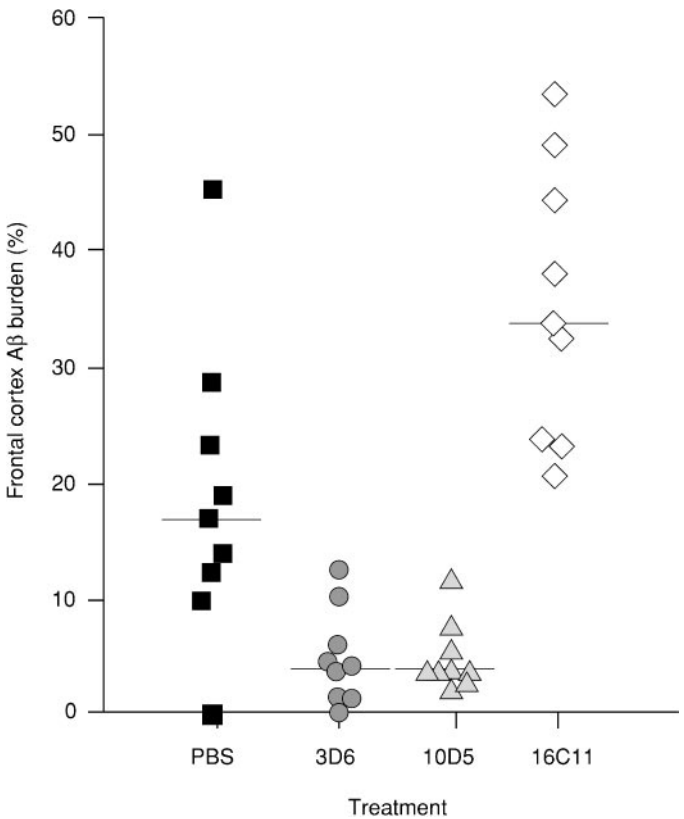

Figure 7 Effect of six months of treatment with monoclonal antibodies directed against Aβ on the Aβ burden in the frontal cortex of PDAPP mice. PBS: phosphate-buffered saline; 3D6: anti-Aβ1-5; 10D5: anti-Aβ1-16; 16C11: anti-tau. Horizontal lines indicate median values. Reproduced with permission from Reference (121).

(m266) directed against the central domain (residues 13–28) of Aβ does not bind to the Aβ plaques, yet still reduces cerebral Aβ burden. Their findings in a series of in vitro and in vivo experiments suggest that intravenous administration of m266 shifts the CNS-plasma Aβ equilibrium by acting as a peripheral sink (122, 123). They also showed that 3D6 and 10D5 [two of the mAb used by Bard et al. (121)] had a similar, although smaller, effect in their in vitro studies (122). These two mechanisms are not mutually exclusive, and both could be operative. A further potential mechanism by which Aβ immunization could work is suggested by the studies of Solomon et al. (124, 125), in which antibodies raised against the hydrophilic N-terminal region (1–28) of Aβ prevented the fibrillar aggregation of Aβ in vitro.

Moving from Mouse to Man

Following the successful outcomes of the studies of murine Aβ42 immunization in several laboratories, clinical trials were initiated. The immunogen used in these studies, AN-1792 (Elan Pharmaceuticals/Wyeth), contained synthetic Aβ42 in combination with an adjuvant (QS-21). Two Phase I studies were undertaken to evaluate the safety and tolerability of AN-1792 in patients with AD. The first was a single-dose study with three escalating Aβ42 dosage levels, but with a fixed adjuvant concentration. There were eight patients per dose level and a six-week period between escalations. The second Phase I study was a multi-dose (for both Aβ42 and QS-21 adjuvant), dose-escalation study, with a two to three month interval between each dose level.

As AN-1792 was well tolerated and a subset of patients developed an immunological response in these two studies, Phase IIa studies began in the United States and Europe. The Phase IIa program recruited 375 AD patients, and those in the active treatment group ($n = 300$) received multiple doses of the highest dose of Aβ42 combined with the lowest dose of QS-21 used in the Phase I trials. However, the program was suspended in January, 2002 after some patients in the active treatment group developed the signs and symptoms of aseptic meningo-encephalitis. All affected patients had received one to three doses of AN-1792, and their symptoms developed five days to five months after their last dose. Although the clinical development of AN-1792 has been discontinued, the study blind for the remaining patients has not been broken, and these patients will continue to be monitored for safety, CNS changes, cognitive decline, and immune function for a further year.

These reactions were unexpected, as toxicological testing in five different animal species, including primates, did not reveal any evidence of encephalitis or other adverse effects, and at the time of commencement of the Phase IIa study, none of the 64 patients receiving active treatment in the Phase I trials had developed any signs of brain inflammation. The reactions in some of the patients in the Phase IIa program do appear to be clearly linked to immunization, although the underlying mechanism has not yet been established. Ongoing tests are investigating whether the reactions in some of the patients in the Phase IIa program may be

linked to immunization, for example, through a T cell–mediated immune response to AN-1792.

Although AN-1792 has been discontinued, the development of safe and effective immunotherapies for AD is being actively pursued, based on the dramatic preclinical data. Several second-generation immunotherapeutic products with properties very different to those of AN-1792 are currently in active development.

STIMULATING α-SECRETASE CLEAVAGE OF APP TO LOWER Aβ

Neurotransmitter-Mediated Enhancement of α-Secretase Activity

Those APP molecules that undergo processing by α-secretase release p3 rather than Aβ. Although p3 has been detected in some plaques in the AD hippocampus, cortex, and cerebellum (126, 127), Aβ appears to be a far more abundant constituent of neuritic plaques. Consequently, enhancing the flux of APP molecules through the α-secretase, rather than β-secrease, pathway may be beneficial in AD. The proteolytic activities of ADAM 10 and ADAM 17 (TACE), which appear to be α-secretases for APP, can be up-regulated via stimulation of several neurotransmitter systems. The first to be recognized were the M_1 and M_3 muscarinic acetylcholine receptors acting through the phospholipase C/protein kinase C pathway (128). Agonists acting at these receptors were shown not only to increase APP_s-α levels in cultured cell medium, but also to reduce Aβ secretion, suggesting a competition for substrate between α- and β-secretases (129, 130). Moreover, systemic administration of the acetylcholinesterase inhibitor physostigmine for 10 days lowered cortical Aβ levels by ~30%–40% without altering full-length APP concentrations in guinea pigs (131). Similarly, 30%–45% and 15%–40% reductions in CSF levels of Aβ40 and Aβ42, respectively, were reported in rabbits treated for five days with one of three different M_1-selective muscarinic agonists (132). In clinical trials, two studies have reported approximately 25% reductions in CSF Aβ levels after administration of selective M_1 agonists to small numbers of AD patients (133).

Several other neurotransmitters that act through phosphatidylinositol hydrolysis and activation of protein kinases can increase APP_s-α secretion. Agonists of metabotropic glutamate receptors did so in cultured cortical astrocytes, and this was reversed by dibutryl cyclic AMP (134). Moreover, serotonin produced a three- to four-fold, dose-dependent increase in APP_s-α release in cells overexpressing 5-HT_{2A} or 5-HT_{2C} receptors (135), and stimulation of the G-protein-coupled receptors for bradykinin and vasopressin with their respective neuropeptides increased α-secretase processing of APP in PC-12 cells (136). In addition, electrical depolarization increased APP_s-α release in hippocampal and cortical slices (137).

Although such data suggest that cholinergic treatments could decrease Aβ levels while ameliorating acetylcholine deficits, there are theoretical concerns about chronic stimulation of α-secretase. As the APP ectodomain has several pharmacological activities, prolonged treatment could have adverse effects. Moreover, α-secretases have numerous substrates, making biological effects independent of those of APP_s-α likely. However, as β- and γ-secretase inhibitors are not yet available, further clinical trials of prolonged stimulation of α-secretase processing should be undertaken.

Cholesterol-Lowering Agents

Several lines of evidence lend increasing support to the concept that altering cholesterol homeostasis can change the production of Aβ in vitro and in vivo. The genetic linkage of apoE4 to both AD risk and Aβ burden first raised this issue, as the ε4 allele is associated with higher circulating cholesterol levels (138). There is also a complex body of literature suggesting that atherosclerosis may be associated with an increased risk of developing AD, although this could be explained, at least in part, by the co-occurrence in elderly patients of two independent processes: multi-infarct dementia and AD. A more direct mechanistic link between cholesterol and AD comes from the recognition that a fraction of neuronal APP is present in low-density, Triton-insoluble glycosphingolipid-cholesterol rafts (139), which appear to be one site for the conversion of APP to Aβ (140, 141). Reducing cholesterol levels in cultured hippocampal neurons by ~70% with lovastatin and methyl-β-cyclodextrin markedly inhibits Aβ formation without affecting neuronal viability or holoAPP and APP_s-α levels (142). The latter authors also obtained data suggesting that β-secretase-mediated processing of APP (not α-secretase) was affected by such treatment, as C99 levels were lowered and p3 levels were unchanged. However, another group treated non-neural and neural cells lines with either lovastatin or methyl-β-cyclodextrin and found a marked increase in APP_s-α production, including that generated by ADAM 10, with a concomitant decrease in Aβ secretion (143). In this study, cholesterol depletion in the non-neural cells inhibited internalization of surface APP, allowing more α-secretase processing. Clearly the precise mechanisms by which cholesterol-lowering agents reduce Aβ production require further study.

In vivo experiments have demonstrated that a three-week administration of very high doses of simvastatin markedly and reversibly decreased de novo brain cholesterol synthesis and lowered CSF levels of Aβ40 by ~50% and Aβ42 by ~40% in guinea pigs (144). Moreover, a similar reduction in total brain Aβ levels was observed. Treatment of double mutant PS1/APP transgenic mice for five weeks with the cholesterol-lowering drug BM15.766, which is brain-penetrant and inhibits the last step in cholesterol biosynthesis, decreased brain Aβ levels and plaque burden by ~50% (145). Brain levels of APP_s-α were increased, and those of C99 were reduced. Conversely, administration of a high fat/high cholesterol diet to this mouse line for seven weeks led to a significant 50% increase in total brain Aβ levels, which

correlated strongly with plasma and brain total cholesterol content (146). This diet increased both the number and size of Aβ deposits. The hypercholesterolemic mice showed decreased APP_s-α levels and increased C99 levels.

Three retrospective epidemiological analyses of statin usage and AD have been reported to date. One study examined records from three hospitals and found that the prevalence of a diagnosis of probable AD was approximately 60%–70% lower in patients taking lovastatin or pravastatin than in patients receiving other cardiovascular medications (147). The second study included 284 patients with dementia and 1080 matched controls (all aged >50 years) drawn from a general practice patient database in the United Kingdom (148). The adjusted relative risk of a diagnosis of dementia in the statin-treated patients was 0.29 of that in the controls ($p = 0.002$), whereas the risk in patients receiving other lipid-lowering agents was 0.96. The records did not allow a distinction between AD and other dementias. The third report was a case-control study of 492 patients with dementia, 326 of whom had clinically probable AD, and 823 controls in relation to the use of lipid-lowering agents (149). It was found that the use of statins and other lipid-lowering agents reduced the risk of AD in patients aged <80 years old (odds ratio: 0.26).

These collective data provide considerable circumstantial evidence that chronically lowering cholesterol levels, particularly with statins, may decrease cerebral Aβ concentrations, perhaps by enhancing α-secretase cleavage at the expense of the amyloidogenic processing of APP. However, no prospective randomized treatment trials of statins in AD have been reported. Until this occurs, chronic statin administration solely for the purpose of treating or preventing AD cannot be recommended.

CONCLUSIONS: THE FUTURE OF THE Aβ HYPOTHESIS AND ITS ROLE IN ALZHEIMER'S DISEASE

As with any medically driven scientific hypothesis, the ultimate value of the amyloid hypothesis is directly proportional to its clinical utility. A myriad of scientific achievements have occurred since George Glenner first identified the Aβ peptide in amyloidotic vessels in 1984 (150). Biochemical prowess combined with elegant genetics and careful neuropathological analyses have advanced the field to a point where there are numerous anti-amyloid molecules undergoing, or close to, therapeutic evaluation in AD patients.

The various treatment targets arising from research related to the amyloid hypothesis include inhibition of γ- or β-secretase activity and Aβ-related immunotherapeutic approaches. An additional approach not covered in this review involves strategies aimed at reducing the aggregation properties of the Aβ peptide. Thus far, γ-secretase inhibitors and Aβ42 peptide immunization have begun to be tested clinically, although neither approach has extended much beyond safety studies. Thus, clinical trials have not advanced to the point of actually testing the

amyloid hypothesis. It is probable, nevertheless, that modulators of γ- or β-secretase and refinements of Aβ immunotherapy (both passive antibody administration and Aβ-peptide conjugate immunization) will enter the clinic before long. In short, we currently stand on the threshold of convincingly testing the amyloid hypothesis.

Questions Remaining

Aβ NEUROTOXICITY Many questions remain regarding the relationship of Aβ to the cellular alterations underlying dementia in AD. The most notoriously difficult one to answer concerns the nature of the neurotoxic effect of the peptide itself in the brain. Both old and new work in this area has refined and clarified this issue considerably. For example, it has recently been directly shown that soluble oligomers of Aβ alter the electrophysiology of neurons in the brain at very low concentrations when microinjected in vivo, whereas monomers of the peptide do not (112). This and other related findings (108–111) have resulted in a growing consensus that it is probably small oligomers, rather than large fibrillar plaques, that principally cause the synaptotoxicity associated with the disease. Nonetheless, the complex equilibria associated with the interconversion of monomers, oligomers, protofibrils, fibrils, and very large aggregates of the Aβ peptide, together with the difficulty of measuring real-time concentrations of these various species in vivo, means that the debate about precisely which species of Aβ injures neurons and their processes in vivo will continue for some time.

γ-SECRETASE Compelling data have been reviewed here regarding the argument that presenlins mediate γ-secretase cleavage activity. In addition, a number of potent inhibitors of γ-secretase activity have been identified, and several of these have been chemically crosslinked directly to presenilins. Still unresolved, however, is exactly how many other proteins are required for full γ-secretase activity, and what their respective functions are. These are important questions, not just from the perspective of the basic science of this signaling hub, but also from the viewpoint that some compounds may be capable of selectively interacting with such proteins and thereby have an allosteric effect on γ-secretase activity. The final answer in this area will come from reconstitution studies in which various proteins are brought together in purified form and in detergents to generate γ-secretase activity, much like what has been done with β-secretase. Such an experiment could provide undeniable proof that presenilin is the authentic active site component of γ-secretase.

Also of great importance is the development of safe, orally active γ-secretase inhibitors. The observation that many general inhibitors of APP γ-secretase activity also inhibit Notch cleavage has concerned the pharmaceutical industry because Notch signaling is likely to be required for numerous normal processes in adults. The recent finding that certain NSAIDs can inhibit APP γ-secretase activity by shifting cleavage of Aβ42 to Aβ38, apparently without significantly perturbing

Notch cleavage (114), has provided encouraging results. Although the inhibitory potency of NSAIDs as a class (micromolar) does not recommend them as clinical candidates, they represent a pre-clinical proof of concept that it may be possible to design highly specific Aβ42 inhibitors.

VALIDITY OF ANIMAL MODELS The various APP and tau transgenic mouse models of AD have now been studied for several years. Nevertheless, much remains to be learned from these models. For example, it is clear from many studies that the Aβ deposits in these models are strikingly similar, though not identical, to those seen in AD. In addition, the neuritic dystrophy, gliosis, perikaryal changes, and even behavioral and electrophysiological phenotypes continue to suggest the utility of such mice for modeling the process of AD. Even neurofibrillary tangles can been seen in certain tau-APP "bigenic" mice. Though not entirely absent, neuronal loss in these animal models is subtle and not widespread. Finally, a central question remains: Will compounds that appear highly effective in APP transgenic mice, such as Aβ antibodies or γ-secretase inhibitors, prove to be useful in treating AD? Unfortunately there is little to be gained here from experience with other animal models of disease, as some have been highly predictive of human efficacy, and others not at all.

MECHANISM OF ACTION OF Aβ IMMUNOTHERAPY Immunization with Aβ and passive administration of anti-Aβ antibodies have both shown profound efficacy by many criteria in APP transgenic mice. What these results leave unanswered, however, is precisely how the antibodies are achieving their effect. Do they stimulate microglial phagocytosis of existing plaques (121), or do they achieve clearance by sequestering free Aβ in plasma, CSF, and brain extracellular fluid (122, 151). Experiments from many laboratories continue to attempt to address this question, as it may provide insight into the nature of Aβ peptide accumulation and toxicity and help direct future therapeutic approaches.

DESIGN OF CLINICAL TRIALS AIMED AT TESTING THE AMYLOID HYPOTHESIS As it is hoped that modulation or reduction of Aβ burden and associated cytopathology in AD will affect the course of the disease process, the primary outcome measures of current and future clinical trials based on the hypothesis will need to reflect this assumption. In practice, there are two main limitations of such trials. The first is that there is currently no validated method of detecting Aβ burden non-invasively in a living patient. Hence, it is not possible to know if an amyloid-lowering agent is achieving its desired immediate result. The second practical problem is the necessary length of such trials—typically a minimum of 18 months is needed to detect a sufficient change in the cognitive status of a cohort of AD patients in order to demonstrate a meaningful statistical difference. Thus, such trials require large numbers of patients and very significant time commitments to complete. Symptomatic agents, such as acetylcholinesterase inhibitors, likely have no effect on the underyling pathology but exert their pharmacological effects relatively rapidly,

thus circumventing one of the key limitations in assessing disease-modifying therapies. Ultimately, brain imaging modalities, such as functional magnetic resonance imaging and "amyloid scans," should prove useful as meaningful surrogates for testing the efficacy of anti-amyloid agents in AD.

Aβ and the Treatment of Alzheimer's Disease: The Future

Despite the formal delineation of the amyloid hypothesis well over a decade ago, we are only now beginning to test its application for treating AD. Unlike some medical hypotheses, this one appears to lend itself to many different approaches—from enzyme inhibitors to immunotherapy, to anti-aggregation compounds, to metal chelators. Each one of these approaches has its potential strengths and weaknesses. We can anticipate that meaningful treatment of this debilitating disease will involve more than a single therapeutic approach. Thus, despite the numerous approaches being actively pursued at present, still more are likely to be entertained in the future. As scientists, we eagerly await the outcome of these efforts; for patients, they cannot come soon enough.

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PROGRESS TOWARD CLINICAL APPLICATION OF THE NITRIC OXIDE–RELEASING DIAZENIUMDIOLATES[1]

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Key Words restenosis, thromboresistance, vasospasm, impotence, respiratory distress

■ **Abstract** Diazeniumdiolates, compounds of structure $R^1R^2NN(O)=NOR^3$, which have also been called NONOates, have proven useful for treating an increasing diversity of medical disorders in relevant animal models. Here, I review the chemical features that make them such excellent starting points for designing materials capable of targeting reliable and controllable fluxes of bioactive NO for in vitro and in vivo applications. This is followed by a consideration of recent proof-of-concept studies that underscore what I believe to be the substantial clinical promise of such materials. Examples covered include progress toward inhibiting restenosis after angioplasty, preparing thromboresistant medical devices, reversing vasospasm, and relieving pulmonary hypertension. Together with a very recent report describing the beneficial effects of diazeniumdiolate therapy in a patient with acute respiratory distress syndrome, the results of the animal experiments support the prediction that a broad selection of problems in clinical medicine can be solved by judiciously mining the enormous variety of possible $R^1R^2NN(O)=NOR^3$ structures.

INTRODUCTION

> *. . . Our current strategy . . . is quite simple: first bring NONO-2 to the market; second, start working on the next generation of products; and, third, explore some of the other therapeutic possibilities. . . . We could become the nitric oxide powerhouse of the pharmaceutical industry!*
>
> *Musings of corporate executive Renu Krishnan in "NO" (1, p. 116)*

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It seems from the quotation above that Renu Krishnan, the entrepreneurial protagonist of pharmaceutical innovator Carl ("Father of the Pill") Djerassi's recent novel *NO*, sensed early in her career that the nitric oxide (NO)-releasing diazeniumdiolates provide an unusual opportunity for rationally designing broad-spectrum drugs and biomedical devices. In Djerassi's story line (1), Renu parlays her knowledge of diazeniumdiolate chemistry into a blockbuster drug called "NONO-2" for treating male erectile dysfunction, ultimately earning her both international acclaim and enough personal wealth to fund her own basic research aimed at conquering other challenging medical problems, including pulmonary hypertension, cancer, and infectious disease.

I think Renu is right. Here, I review the growing body of in vivo proof-of-concept reports demonstrating the diazeniumdiolates' efficacy in relevant animal models for treating a variety of clinical disorders. These reports have convinced me that within this chemistry lurks a vast opportunity for designing solutions to a host of biomedical problems.

WHAT ARE DIAZENIUMDIOLATES AND WHAT MAKES THEM SO USEFUL FOR RATIONALLY DESIGNING NEW DRUGS AND BIOMEDICAL DEVICES?

Although Renu, her company, and presumably also her ability to fund her own wide-ranging basic research program in this day and age are all fictional, the compound class at the heart of her success is not. Diazeniumdiolates (or NONOates, as she also calls them) are compounds having the generic structure shown in Figure 1. They are blessed with three attributes that make them an especially attractive starting point for designing solutions to important clinical problems, namely structural diversity, dependable rates of NO release, and a rich derivatization chemistry that facilitates targeting of NO to specific sites of need—a critical goal for therapeutic uses of a molecule with natural bioeffector roles in virtually every organ.

Regarding structural diversity, all three R groups of Figure 1 can be varied over a wide range to produce isolable materials. Salts in which R^3 is ionically bound to the diazeniumdiolate oxygen have proven especially useful for generating controlled fluxes of NO spontaneously in aqueous media. Depending on the identity of the R^1R^2N group of Figure 1, reproducible half-lives of NO generation ranging from 2 s to 20 h have been documented for the simple diazeniumdiolate salts characterized thus far. The structures of ionic diazeniumdiolates discussed in this review are shown in Figure 2 along with their half-lives at pH 7.4 and 37°C.

Importantly, these solution half-lives tend to correlate very well with their pharmacological durations of action. This suggests that they are minimally affected by metabolism and are thus fundamentally different from currently available

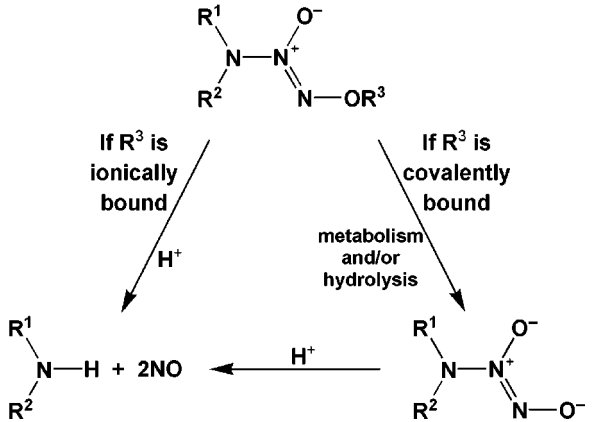

Figure 1 Mechanisms of NO release for the diazeniumdiolates discussed in this chapter. If R^3 of the generic structure shown at the top is a cation, NO is generated spontaneously on protonation of the anionic portion's R^1R^2N nitrogen (2), as shown at the left. If R^3 is covalently bound, it must be removed to free the anion (as shown on the right) before spontaneous NO generation can begin; some examples of masking the diazeniumdiolate ion in this way for pharmacological advantage are listed toward the end of the chapter, along with some specific mechanisms by which such a cleavage can be effected.

clinical nitrovasodilators that require redox activation before NO is released. Even SPER/NO, whose half-life has proven to be the most variable (2, 3) among the diazeniumdiolates investigated thus far (see Figure 2), has consistently shown pharmacological effects expected of a spontaneous NO-releaser intermediate in half-life between the rapid and slow examples of Figure 2. Of course, as ions of low molecular weight, these agents should be subject to excretion after in vivo administration, even in the absence of metabolism; thus, the 20-h in vitro half-life of DETA/NO (Figure 2) does not translate to a 20-h half-life for its in vivo biological effects, as is illustrated below.

The enormous diversity of isolable materials that can be synthesized by varying R^1 and R^2 of Figure 1 can be extended even further by covalently binding different R^3 groups to the terminal oxygen of the ionic diazeniumdiolate moiety. This can lead to a variety of well-defined outcomes. For example, when R^3 is a methoxymethyl (MOM) group and R^1R^2N is a piperazine ring, the diazeniumdiolate (MOM-PIPERAZI/NO; see Figure 3) is still a spontaneous NO releaser at physiological pH, but the rate-limiting step is hydrolytic cleavage of the MOM group; in this case, the MOM derivative's half-life for NO generation is 17 days (4). Most other diazeniumdiolates with covalently bound R^3 groups have proven effectively stable toward spontaneous hydrolysis, but by choosing R^3 groups that

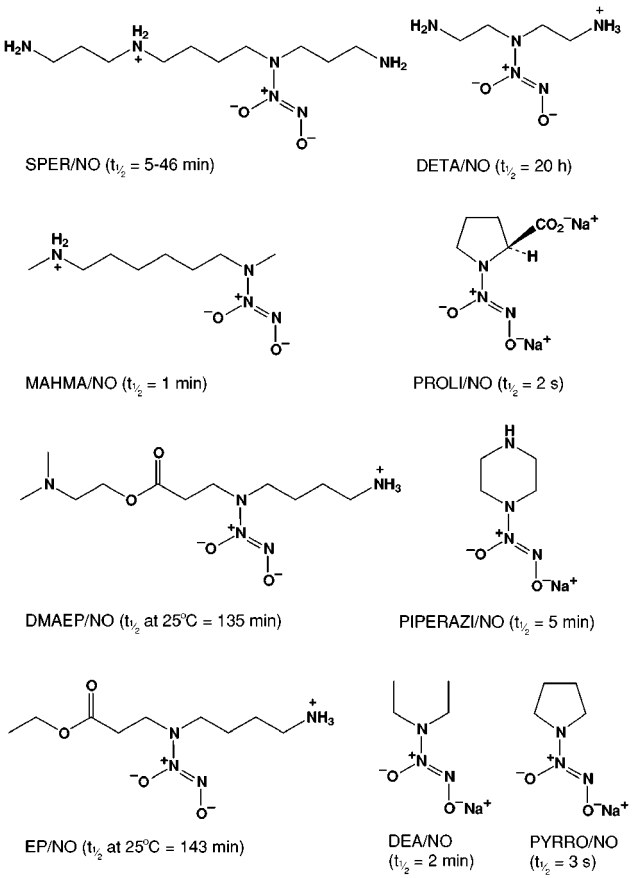

Figure 2 Structures and half-lives ($t_{1/2}$) in pH 7.4 phosphate buffer at 37°C of ionic diazeniumdiolates discussed in this chapter.

are vulnerable to cleavage by specific enzymes, agents capable of cell- or organ-selective NO generation after system-wide administration can be prepared. A case in point is AcOM-PYRRO/NO (see Figure 3), a cell-permeant neutral molecule that has proven stable in cell culture medium but undergoes hydrolysis upon catalysis by intracellular esterases. This feature was exploited in an in vitro test of NO's antileukemic activity (5) in which the ability of AcOM-PYRRO/NO to concentrate NO release inside the cell made it orders of magnitude more cytotoxic than the ion produced on esterase-induced AcOM-PYRRO/NO hydrolysis (PYRRO/NO, which generates extracellular NO homogeneously throughout the medium with a half-life of 3 s; see Figure 2). The structures of AcOM-PYRRO/NO and other neutral diazeniumdiolates bearing covalent R^3 groups that are discussed in this

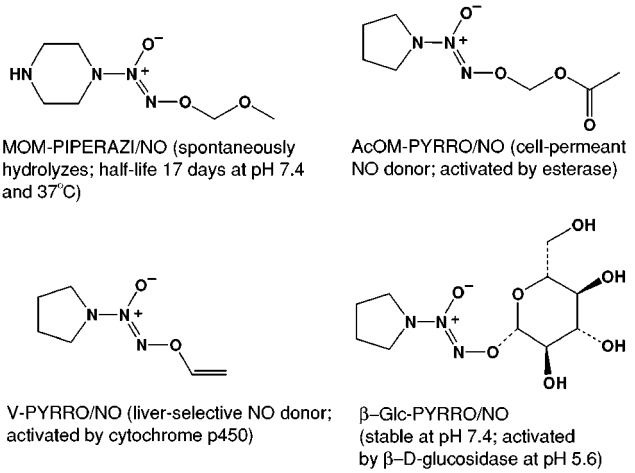

Figure 3 Structures of O^2-substituted diazeniumdiolates discussed in this chapter, along with conditions reported to convert them to NO-releasing form.

chapter are shown in Figure 3, together with the conditions under which they are known to release NO.

WHAT CLINICAL PROBLEMS HAS DIAZENIUMDIOLATE CHEMISTRY SHOWN THE POTENTIAL TO SOLVE THROUGH PROOF-OF-CONCEPT STUDIES IN RELEVANT ANIMAL MODELS?

The ease of preparing an exceptional breadth of diazeniumdiolate structures and the wide array of well-defined spontaneous and enzymatically induced NO generation patterns they provide make this class of compounds an ideal starting point for targeting NO selectively to bodily sites of need. This is a critical requisite for successful NO donor drug design, given the fact that NO affects the function of essentially every organ system we own. Several strategies for achieving such therapeutically useful targeting have been introduced, including local administration of spontaneous NO releasers having the appropriate half-life and duration of action, incorporation of the diazeniumdiolate group into insoluble polymers whose NO-derived effects are limited to the cells and physiological fluids in the immediate vicinity of the polymer surface, and design of prodrugs that are stable in the systemic circulation but activated for NO release by enzymes concentrated in the target organ or cell type. Examples of each strategy are described below.

Restenosis After Angioplasty

Blood vessel disorders are a leading cause of incapacity and death. Among the most common, and serious, of these is coronary artery disease, in which blood flow through the vessels supplying the heart muscle becomes restricted. When this happens through buildup of atheromatous plaque, compounded in some cases by acute thrombosis, a thickening of the vessel wall results; a procedure known as angioplasty has come into wide use as a means of restoring normal coronary blood flow. As illustrated in Figure 4, the procedure involves insertion of a balloon at the diseased site via a catheter. Inflating the balloon expands the artery beyond its ability to recoil, opening the vessel to its proper diameter for immediate restoration of proper perfusion.

The problem with this procedure is that the arterial overstretch that is crucial to its short-term success often gives rise to a long-term complication called restenosis. Overstretch is accompanied by extensive tearing and fracturing of the affected vessel that the body immediately seeks to heal, but in a substantial percentage of patients the healing mechanisms overreact, producing more vascular smooth muscle cells than are destroyed. This leads to further thickening of the vessel wall

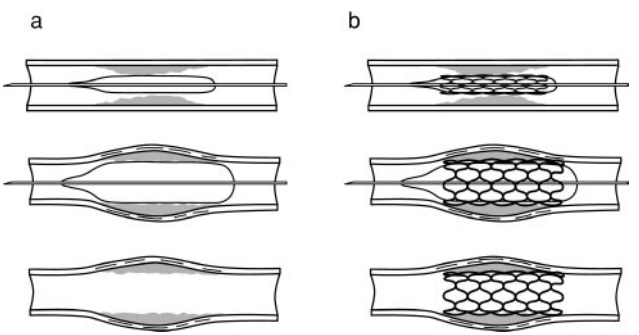

Figure 4 Balloon angioplasty with and without stent deployment. (*a*) In balloon angioplasty, a thin catheter is threaded through the circulatory system until the uninflated balloon at its tip penetrates the diseased artery at the point of blockage, as shown in the top diagram. The balloon is then inflated to expand the artery, as shown in the middle, before being deflated and withdrawn to allow blood flow to resume (*bottom panel*). (*b*) An increasingly common feature of angioplasty involves deployment of an expandable wire structure to help keep the artery from collapsing after the balloon is withdrawn. The procedure is the same as in (*a*), except that a wire stent is placed over the balloon before insertion (*top*). The stent expands when the balloon is inflated (*middle*) and retains its expanded form after the balloon and catheter are withdrawn (*bottom*), remaining in place after the procedure is complete to provide a permanent structural support for the arterial wall.

and thus renewed restriction of blood flow. It is reported that approximately 40% of the more than 1,000,000 coronary angioplasties performed each year in the United States and Europe show angiographic evidence of restenosis within six months after the procedure (6).

A possible approach to reducing the risk of restenosis following such surgical interventions has been suggested by the work of Kaul et al. (7). Recognizing that NO is an efficacious inhibitor of vascular smooth muscle cell proliferation, they hypothesized that exposing the outer (adventitial) side of the vessel to NO during and after inflation of the balloon may reduce the excessive production of neointimal cells on the inside of the vessel. To test this hypothesis, they surgically exposed a portion of a rat's iliofemoral artery and covered its adventia with a gel containing SPER/NO (an adduct of the physiological polyamine spermine with NO that regenerates NO and spermine on contact with sources of hydrogen ion; see Figure 2) before expanding the balloon and examined the vessel cross-section two weeks later. As shown in Figure 5, the SPER/NO-treated artery was similar in appearance to the normal, healthy, uninjured control, but the artery that was exposed to the carrier molecule spermine alone closely resembled the injured but untreated control.

This result clearly supported a potentially beneficial role for advential delivery of NO as an approach to promoting vascular healing after angioplasty, but it did not, at first blush, seem applicable to the problem of restenosis in the coronary arteries. If, to apply it, one would have to physically expose and directly contact a vessel on the surface of the heart, one might just as well circumvent the blockage using the time-tested approach of bypass surgery.

How, then, to localize NO delivery to the outer surface of a coronary artery? It happens that the human heart is largely enveloped by a flexible sac called the pericardium. The coronary arteries are seen as bulges on the portion of the heart within this sac and are bathed more or less continuously by the fluid it contains. With this in mind, Baek et al. (8) tested the hypothesis that injection of a long-lasting NO donor into the pericardial fluid immediately before angioplasty would minimize neointima formation after balloon overstretch. They started by considering the longest-acting spontaneous NO donor of Figure 2, DETA/NO. Unfortunately, its 20-h chemical half-life, as measured in 0.1 M phosphate buffer at 37°C, was partially canceled as an advantage by its fairly rapid clearance from the incompletely isolated pericardial compartment; experiments with a radioiodinated surrogate of this very low molecular weight (163 g/mol) agent pointed to a pericardial residence half-life of less than 5 h. Reasoning that a much larger molecule might be better retained in the pericardial space, they then considered a diazeniumdiolated bovine serum albumin derivative (D-BSA) prepared by joining 22 MOM-PIPERAZI/NO (see Figure 3) moieties to each protein molecule using standard linker chemistry. Consistent with the molecular size hypothesis, this high-mass protein derivative (74,000 g/mol) exhibited an intrapericardial residence half-life of over 22 h. Combining the chemical and biological kinetic data indicated that the initial rates (in

moles of NO per h per mole of agent dosed) of NO release from equimolar boluses of DETA/NO versus D-BSA would be almost identical, but that the flux 24 h later would be more than 30-fold greater for the diazeniumdiolated protein.

The protein derivative was thus chosen over DETA/NO for further work in which the researchers injected it into the pericardial fluid of the pig immediately before angioplasty and examined the treated vessel segment two weeks later. At a dose of 400 mg in 10 ml of buffered saline per pig, a concentration similar to that of normal albumin in serum, D-BSA reduced intimal hyperplasia by half, relative to that seen in controls receiving underivatized bovine serum albumin. Very importantly, a substantial portion of the neointimal cells produced in the healing process were seen to have concentrated themselves in the crack resulting from balloon-induced rupture of the arterial wall. This positive remodeling effect greatly enlarged the vessel diameter relative to controls treated with underivatized albumin, as illustrated in Figure 6.

These results strongly support the hypothesis that controlled dosage of NO over the outside of a coronary artery undergoing angioplasty can substantially benefit the healing process, but the procedure requires insertion of two catheters, one for delivering drug into the pericardium and a separate one for balloon deployment. To avoid this complication, one might envision dosing the NO into the inside of the artery during and for a therapeutically relevant length of time after the overstretch. One way to accomplish this in principle is to employ an NO-releasing stent. As illustrated in Figure 4, a stent is a semiflexible metallic mesh that is placed over the balloon such that inflation expands it into a rigid structure designed to oppose any tendency of the vessel to re-constrict to its pre-angioplasty diameter. Stent use has cut the restenosis rate from 40% in balloon-only patients to 20%–25% (6). Coating the stent surface with an NO-generating material could be an ideal way to reduce the restenosis risk even further by dosing the diseased portion of the artery undergoing angioplasty with NO.

Here again, the diazeniumdiolates' chemical versatility provides a convenient approach to localizing NO's effects at the site of need. Choosing selected silicon-bearing R groups from the effectively infinite variety of Figure 1 molecules, one investigation has focused on the silane-based strategy illustrated in Figure 7. In this structure, DETA/NO (the long-lived NO generator of Figure 2) molecules are covalently attached via conventional silanization reactions to the surface of the metallic stent. The spontaneous release of NO that was seen when the silanized, diazeniumdiolated metal surface was immersed in aqueous buffer (9) would be expected to occur when it is exposed to the aqueous conditions surrounding the fresh wound that is created as the balloon-driven stent struts push out sharply against the arterial wall; NO's rapid reaction with oxyhemoglobin would be expected to keep it from traveling very far downstream, and the fact that the NO carrier portion is covalently anchored to the metal surface should combine to minimize systemic effects. Diazeniumdiolated metal surfaces, such as that shown in Figure 7, have yet to be tested in a human-relevant animal model of balloon angioplasty, but further research and development may result in significant benefits with respect to postintervention healing, including the potential for reestablishment of a

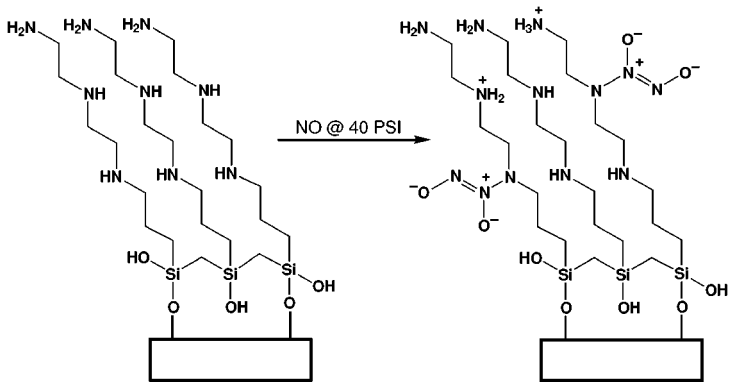

Figure 7 Preparation of NO-releasing metal surfaces (9).

functioning endothelium (the layer of cells that line the normal artery and synthesize essential vasoactive substances, such as NO, but that is often irreparably damaged in conventional vascular surgery procedures).

Thrombogenicity of Medical Devices

Foreign objects implanted in the body tend to induce a physiological response that includes adhesion of platelets to the implant surface as an unwanted, potentially life-threatening side effect (10). As one example, thrombus formation at the site of stent deployment is an occasional cause of acute angioplasty failure. Concurrent dosage with systemic anticoagulants is used to minimize this complication, but this necessarily increases the risk of uncontrolled bleeding at the point of catheter insertion or at potentially unrecognized sites in the brain or elsewhere that may be vulnerable to hemorrhage.

It happens that NO potently inhibits not only vascular smooth muscle cell proliferation, but also the adhesion, aggregation, and activation of platelets. With this in mind, it will be interesting to see whether the diazeniumdiolated metal stents derivatized as in Figure 7 will both inhibit acute thrombotic failure via NO's antiplatelet activity as well as reduce restenosis risk by way of its cytostatic effect in vascular smooth muscle.

But a stent is only one of many devices whose surfaces may be advantageously diazeniumdiolated, and covalent silanization of a metal is only one way to accomplish it. In the following paragraphs, I illustrate three different chemical approaches to diazeniumdiolating implant surfaces for three different in vivo applications.

VASCULAR GRAFTS When dealing with diseased or damaged blood vessels, surgeons often need to replace, rather than repair, the affected segment. This is usually done via transplants from elsewhere in the patient's own circulatory system, but artificial blood vessels (preferably known as synthetic vascular grafts) are often

used instead. Unfortunately, currently approved synthetic grafts are prone to clogging with thrombus, especially when their diameter is small (2–6 mm) (10).

With the goal of strictly confining the antithrombotic effect to the surface of the graft, thereby obviating the need for systemic anticoagulants, Smith et al. dipped small (4 mm)-diameter porous Teflon graft segments into a freshly prepared solution of both polyethylenimine (PEI) and a cross-linking agent such that the cross-linked PEI chains became intimately interwoven with, and thus securely fastened to, the fabric of the graft (11). Half of the segments were then exposed to NO to establish a multitude of diazeniumdiolate groups at secondary amine sites on the PEI, whereas the other half were left to serve as untreated controls. The segments were then placed into the unheparinized baboon circulatory system. The results are shown in Figure 8.

The diazeniumdiolated grafts experienced very little accumulation of thrombus during the 1-h observation period, whereas, as expected, platelet deposition steadily increased in the control grafts (11). In vitro measurement of the grafts' capacity for NO generation revealed a slowly declining rate that was observable by chemiluminescence methods for several weeks, suggesting that they might have remained thromboresistant far beyond the 1-h observation period of Figure 8. If so, and if the PEI-based coating remains biocompatible throughout the lifetime of the device, significant improvements in vascular graft technology could be forthcoming.

EXTRACORPOREAL MEMBRANE OXYGENATION (ECMO) CIRCUITS ECMO is a procedure of last resort for certain patients whose lungs do not function properly. It involves pumping their blood out of the body and through an oxygenating chamber, then back into the patient's circulatory system. A major complication of this invasive procedure is loss of platelets through adhesion to the inner surface of the tubing used to conduct the blood through its extracorporeal circuit. As with the abovementioned vascular surgery applications, systemic anticoagulant treatment with all its attendant disadvantages is a necessary part of conventional ECMO therapy (12).

M. Meyerhoff and his colleagues at the University of Michigan have exploited diazeniumdiolation chemistry by engineering significant thromboresistivity into ECMO circuitry. For one such formulation, they simply blended MAHMA/NO (structure shown in Figure 2) into poly(vinyl chloride) (PVC) to use as a coating for the internal surface of the tubing. After applying a topcoat of unblended PVC, the tubing was tested in an unheparinized rabbit model of ECMO. As shown in Figure 9, a significant reduction in platelet loss compared to heparinized, PVC-only controls was seen throughout the 4-h observation period (13).

CHEMICAL SENSORS The Meyerhoff group has demonstrated even longer periods of thromboresistivity for implantable devices that are central to their mainline research effort. As analytical chemists, they have long focused on developing electrodes that can give real-time readouts for clinically significant analytes in a patient's blood. However, platelet deposition on the electrode surface has been a major source of inaccuracy in the observed readings.

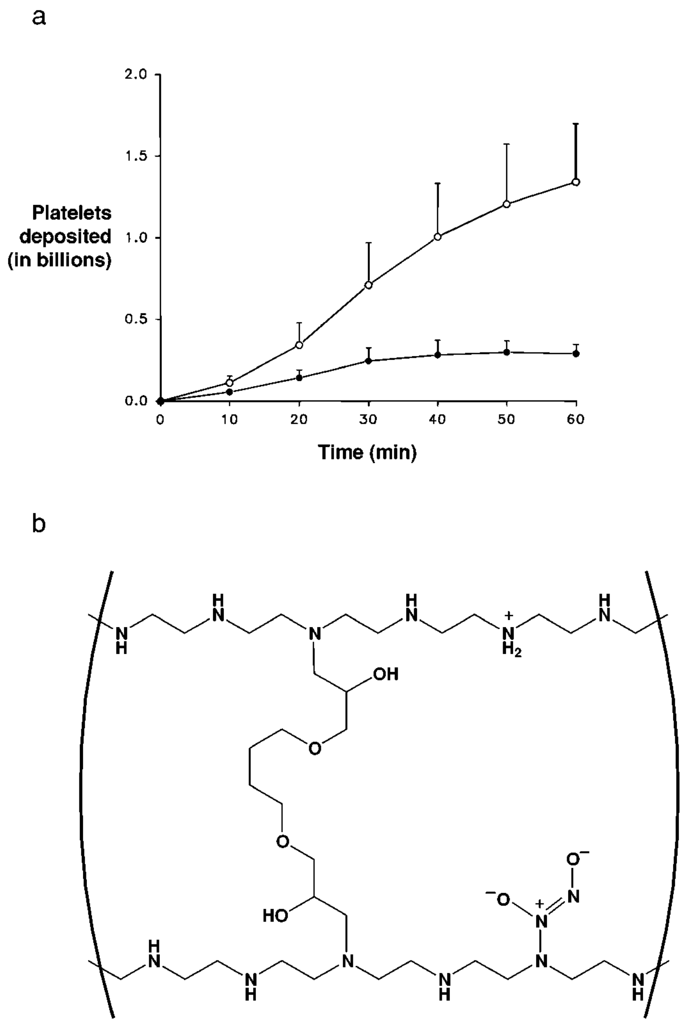

Figure 8 (*a*) Inhibition of platelet deposition in poly(tetrafluoroethylene) vascular grafts coated with cross-linked poly(ethylenimine) that was either (•) diazeniumdiolated to generate NO at a rate estimated to be 1–2 nmol/min/mg or (○) generating no NO. Data were collected by quantifying accumulation of radioactivity in grafts placed in arteriovenous shunts in baboons whose platelets had been labeled with ^{111}In but who were not treated with heparin. Data are means ± SEM (n = 5). (*b*) Representative structural unit of the crosslinked poly(ethylenimine) after incorporating it into the Teflon vascular graft and diazeniumdiolating it to produce the NO-releasing graft segments whose antiplatelet activity is summarized in (*a*). [Adapted from Reference 11 with permission.]

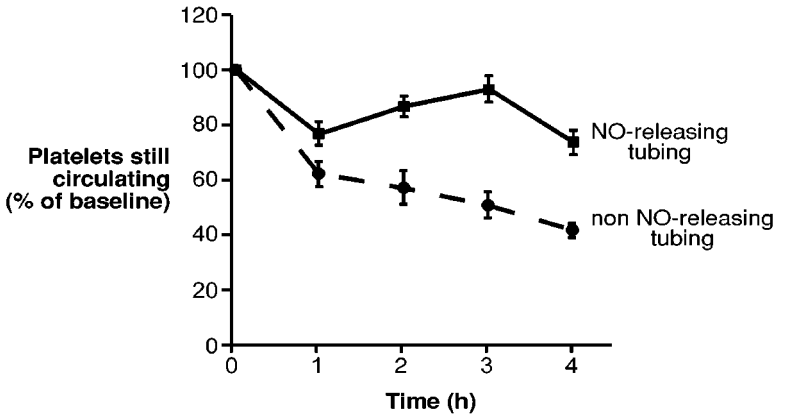

Figure 9 Degree of platelet retention in unheparinized rabbits undergoing simulated extracorporeal membrane oxygenation (ECMO) whose extracorporeal blood conduits are lined with MAHMA/NO-containing PVC to inhibit platelet adhesion (■) as compared with controls whose tubing is made of undiazeniumdiolated PVC (●). [Adapted from Reference 13 with permission.]

To address this problem, the researchers have designed a polymeric sleeve that they fit over the portion of the electrode tip that contacts blood before insertion into the blood stream. To overcome the additional problem that the MAHMA/NO in the PVC blend used for the above-mentioned ECMO study tended, as a small, hydrophilic molecule, to leach out of the polymer once deployed, they designed a sleeve made of diazeniumdiolated silicone rubber (14). As shown in Figure 10, the diazeniumdiolated sleeve not only inhibited platelet adhesion/activation and gross thrombus formation at the sensor tip, but also generated continuous readings of blood oxygen levels that are very close to those produced by standard clinical chemistry methods obtained using discrete arterial blood samples; by contrast, measurements collected with the undiazeniumdiolated control sleeve during the 16-h observation period tended to be much lower than the reference values (Figure 10).

Given the extensive variety of biomedical devices whose function may be significantly improved by fabricating them with NO-releasing surfaces, the market for insoluble polymers bearing covalently bound diazeniumdiolate groups could be very large indeed. The list of possibilities includes not only the stents, vascular grafts, ECMO circuitry, and chemical sensors mentioned above, but also dialysis shunts, penile implants, dermal patches, surgical supplies, wound dressings, and more.

Vasospasm

People reporting to hospital emergency rooms with what they describe as the worst headaches they have ever experienced often turn out to have suffered a

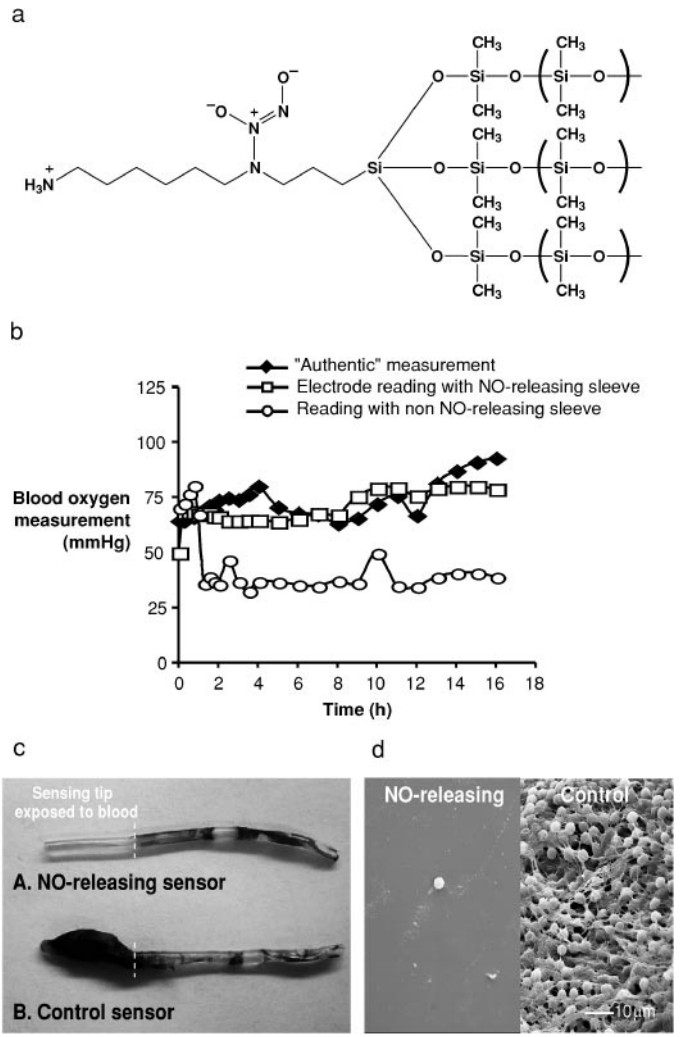

Figure 10 Improved biosensor accuracy using a diazeniumdiolated silicone rubber sleeve to cover the blood contact surface of the electrode. (*a*) Representative structural unit of the polymer. (*b*) Comparison of blood oxygen levels continuously monitored over a 16-h period with the NO-releasing intravascular electrode (□) versus both standard measurements (♦) and data collected with a control sleeve incapable of NO generation (○). (*c*) Gross view of sensor tips bearing (*top*) and devoid of (*bottom*) the NO-releasing sleeve after 16 h in the blood stream of a dog and (*d*) closeup view thereof obtained by scanning electron microscopy. $n = 1$ in all cases. (Courtesy of M. Frost & M. Meyerhoff, University of Michigan.)

subarachnoid hemorrhage, bleeding within the brain caused by rupture of a cranial artery. Neurosurgeons are able to clip off the ruptured aneurysm with remarkable proficiency, and many patients go on to full recovery and long, healthy lives thereafter.

The problem is that for a period of up to two weeks after the surgery these patients are at risk of one of the most debilitating forms of stroke, cerebral vasospasm. This is thought to occur because hemoglobin in the residual blood surrounding a cranial artery reacts with and destroys the vessel-produced NO; if this "steal" effect is sufficiently extensive, the NO that remains unreacted may be unable to oppose the vasoconstrictor factors that are normally present, allowing the artery to narrow dangerously or even collapse. This slows the flow of blood to downstream regions of the brain and can lead to serious impairment. Of the more than 28,000 Americans who suffer subarachnoid hemorrhage each year (15), 22%–40% experience the symptoms of vasospasm; of these, roughly one third are left with long-term neurological deficits and another third fail to survive the risk period (16).

Fortunately, the risk period for cerebral vasospasm lasts only a week or two after the bleeding has been stopped, ending as soon as the residual interstitial blood has been cleared by natural processes. Not so fortunately, none of the methods currently approved for treating cerebral vasospasm has proven very effective.

A very promising approach to addressing this unanswered medical need relies on the potent vasodilatory effect of NO. In a pioneering study, Pluta et al. reasoned that if the spasms are really caused by a local deficiency of NO, NO replacement therapy may be all that is needed to prevent them. To test this, they chose to infuse the ultrafast NO donor of Figure 2, PROLI/NO, immediately upstream from the affected vessel with the assumption that its 2-s half-life for NO release at the pH of blood, combined with the even shorter lifetime in the presence of red blood cells of the free NO produced via PROLI/NO dissociation, should limit the drug's vasodilatory effect to the site of the deficiency. They then prepared alkaline solutions of PROLI/NO and infused them into the carotid arteries of monkeys with induced cerebral vasospasm in such a way that the infusate was neutralized by the blood and began generating copious NO into the spasm but not much beyond that site. The degree of arterial spasm in the monkey with the least effective PROLI/NO response was less than 25% in the PROLI/NO-dosed animals, whereas all the saline-treated controls were more than 50% spastic. Very importantly, there was no observable effect on systemic blood pressure (15).

A second successful strategy employed the long-lived agent DETA/NO, whose half-life under physiological conditions is 20 h. If this material were to be infused as the PROLI/NO was, systemic effects would be unavoidable. For this reason, Wolf et al. injected the compound instead into the cerebrospinal fluid of the dog, where the blood/brain barrier kept it reasonably contained, concentrating NO release at the exteriors of the blood vessels in the brain. Normal vessels that were properly dilated were not overly dilated, but the spastic vessel was opened dramatically. Illustrating the latter result in Figure 11, part (*a*) shows the arteriogram of a normal

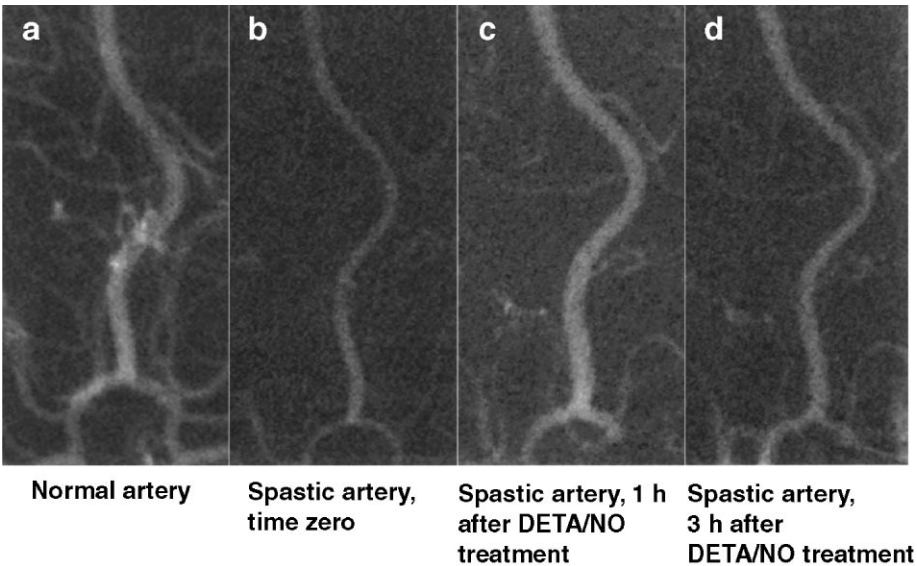

Figure 11 Reversal of cerebral vasospasm by diazeniumdiolates. Panels (*a*)–(*d*) show the results of injecting NO-releasing DETA/NO (structure in Figure 2) into the cerebrospinal fluid of a dog. Panel (*a*) shows the normal basilar artery. Lightening of the arteriogram indicative of severely restricted blood flow, the signature of a spastic vessel, is seen in (*b*). Panel (*c*) confirms that a 2-μmol dose of DETA/NO restores blood flow in the affected vessel; the photograph illustrates the dilation of the artery 1 h after DETA/NO was injected. Panel (*d*) shows the same artery 3 h after bolus intrathecal injection, demonstrating a lasting effect consistent with the compound's prolonged half-life for NO release. [Adapted from Reference 17 with permission.]

artery next to the same vessel in spasm (*b*). The next picture (*c*) shows the same vessel 1 h after administration of the DETA/NO. Finally, the same artery 3 h after injection shows that there is still a significant opening of the vessel after a single bolus administration (*d*) (17).

A third successful approach involved surgically introducing an ethylene/vinyl acetate copolymer blended with 20% DETA/NO around the artery at risk of collapse (18). In this rat femoral artery model of vasospasm, the DETA/NO-polymer blend kept the lumen of the affected vessel segment patent throughout the nine-day risk period, even when it was first implanted seven days after extra-arterial blood deposition. Although such polymeric NO sources may not be capable of therapeutically benefiting all regions of the brain at risk of spasm after subarachnoid hemorrhage, the method has the advantage of continuous, controlled NO release following a single treatment conveniently applied during the initial surgical intervention to repair the aneurysm (18).

Pulmonary Hypertension

Proper lung function depends on high-volume, low-resistance blood flow through the lung to promote adequate gas exchange. Pulmonary hypertension is a life-threatening condition in which the resistance (pulmonary vascular resistance, or PVR) and blood pressure in the lung increase to pathological levels. When this leads to sufficiently poor oxygenation, the ECMO procedure described above and other heroic interventions may be indicated.

An ingenious therapy for pulmonary hypertension involves mixing low concentrations of gaseous NO with the air these patients breathe. The concentration is kept low enough that the oxidation of NO to toxic NO_2 is negligible, and the vasodilatory effect is limited to the site of need—the pulmonary vascular bed. The procedure has been widely tested and is now approved by the FDA for treating hypoxic respiratory failure associated with persistent pulmonary hypertension of the newborn (19).

There are, however, several problems with this procedure. One is the danger of a rebound effect, in which abrupt cessation of NO inhalation leads to a rapid increase of pulmonary arterial pressure (PAP) to levels higher than those seen before treatment was begun. In addition, rigorous control of gas flows is needed to keep the NO_2 level acceptably low and the NO concentration within its narrow therapeutic range; the assortment of gas cylinders, meters, hoses, and other equipment involved in the procedure is so intricate to manage that it is not yet established in many hospitals, and in most cases demands that the patient remain on a respirator in the intensive care unit.

An important advance in NO inhalation therapy would be to develop an ambulatory treatment regime that allows the patient to leave the hospital and resume normal activities without being burdened by cumbersome gas handling equipment. With this in mind, Hampl et al. tested the ability of periodically administered DETA/NO (Figure 2) to relieve pulmonary hypertension in rats whose lungs had been injured by monocrotaline exposure (20). They found that once-a-day inhalation of aerosolized DETA/NO lowered these animals' PAP to normal levels with no observed effect on overall blood pressure (i.e., mean systemic arterial pressure, or MAP) or other signs of toxicity.

Similar results have been reported in a pig model of pulmonary hypertension with (21, 22) and without (23, 24) accompanying acute lung injury. These studies showed that intratracheally aerosolizing zwitterionic diazeniumdiolates EP/NO and DMAEP/NO (structures in Figure 2) consistently lowered PAP and PVR with little or no significant effect on MAP or SVR in animals treated intravenously with either U46619 or oleic acid. Addition of surfactant to the aqueous dosing solutions did not affect the rate of spontaneous NO generation or DMAEP/NO's in vitro vasodilatory activity, but surfactant pretreatment did improve the diazeniumdiolate's ability to lower PAP and increase oxygenation in the lung injury model (22). Other than mild systemic hypotension, no differences between diazeniumdiolate-treated animals and the saline controls were reported

in these studies (i.e., in methemoglobin levels or histological parameters). Of considerable interest from the mechanistic point of view, the researchers also reported a consistently greater degree of lung selectivity for DMAEP/NO than for EP/NO, a finding they attributed to the presence of two versus one basic amino groups in the former versus the latter as the structural feature that inhibited absorption of net cationic DMAEP/NO across the mucosal barrier into the systemic circulation (23, 24). This rationale may also explain the lung selectivity observed for cationic DETA/NO and the total lack thereof for aerosolized DEA/NO (25) (structure in Figure 2), an obligatory anion devoid of other polar functional groups.

Very recently, Lam et al. (26, 27) reported the results of administering aerosolized DETA/NO to a patient with acute respiratory distress syndrome (ARDS). As shown in Figure 12, the PVR index and the mean PAP were reduced by 8%–40% (mean 26) and 8%–29% (mean 18), respectively, as a result of nebulizing 5 ml of 0.03 M DETA/NO into the patient's airways over a 20-min period. Intrapulmonary shunting and alveolar-arterial oxygen partial pressure gradient were also temporarily reduced by DETA/NO treatment, and no significant effect on systemic hemodynamics, pulmonary artery wedge pressure, or serum methemoglobin levels was observed. The authors indicate that they are currently pursuing a Phase II clinical trial with aerosolized DETA/NO in ARDS patients.

WHERE DO WE GO FROM HERE?

Clinical studies such as the one just mentioned that was reported by Lam et al., as well as trials corresponding to protocols currently being prepared that propose to treat cerebral vasospasm using diazeniumdiolate therapy, will of course offer the most direct insights into the real clinical promise of this class of NO-releasing molecule; however, in my view, there is an equally urgent incentive to, as Renu put it in the Djerassi novel, "explore some of the other therapeutic possibilities" not mentioned above.

What of Renu's own golden focus, drugs useful for helping men achieve erections sufficiently tumescent for sexual success, for example? It happens that the ability of several different ionic diazeniumdiolates to induce erections in male cats has already been described by Champion et al. (28), and the techniques employed included transurethral administration, the same one Renu used for applying her commercially successful NONO-2. One may think that the demand for new erection-inducing drugs would have disappeared with the introduction of the oral phosphodiesterase (PDE) inhibitor sildenafil (Viagra®), which acts by slowing the enzymatic degradation of cyclic 3′,5′-guanosine monophosphate (cGMP), the agent that keeps the corpus cavernosum relaxed in a sustained erection. Because cGMP is synthesized by soluble guanylyl synthase when NO coordinates with its heme iron center, an absence of NO could result in a deficiency of cGMP, even in the presence of abundant PDE inhibitor. NO replacement therapy may, therefore,

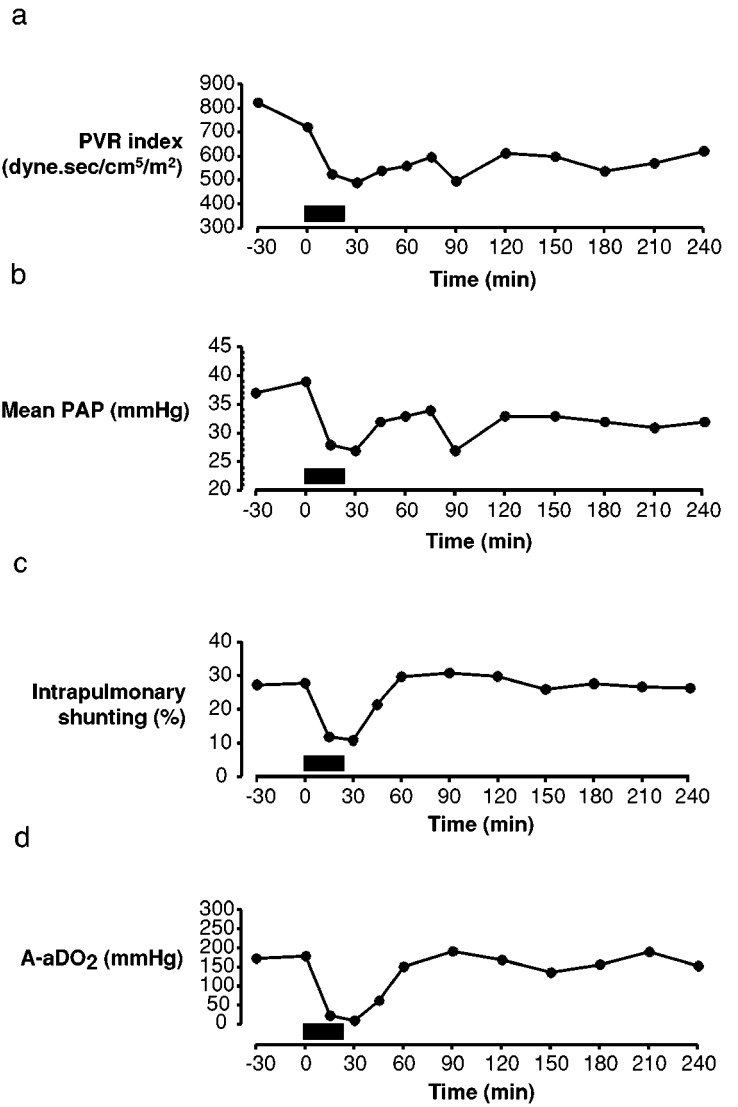

Figure 12 Changes in pulmonary hemodynamic and oxygenation parameters for an ARDS patient before and after aerosolized DETA/NO administration: (*a*) pulmonary vascular resistance (PVR) index, (*b*) mean pulmonary arterial pressure (PAP), (*c*) intrapulmonary shunting, and (*d*) alveolar-arterial oxygen partial pressure gradient (A-aDO_2). The 20-min period of DETA/NO aerosol administration is indicated by (▬). [Adapted from Reference 27 with permission.]

be an effective treatment option for the substantial proportion of impotent men who do not respond adequately to sildenafil. The experiments of Champion et al. suggest that it would not take much NO to do the trick; one of the diazeniumdiolates they tested was PROLI/NO, which generates NO with a half-life of 2 s at physiological pH, but that had a total duration of erectile response of more than 15 min per administration.

The Pro-Prodrug Approach

Although designing a penis-selective oral formulation of inherently acid-sensitive diazeniumdiolate ions poses a most difficult, perhaps insurmountable, challenge, chances of success would be greatly improved by covalently attaching a protecting group to the ion's terminal oxygen to stabilize it for delivery to that organ. Even if this "pro-prodrug" strategy does not yield an oral drug for treating erectile dysfunction, it may enable other routes of administration that are more palatable to the patient than transurethral injection. For example, it would be very interesting to see whether the cell- (and presumably tissue-) permeant pro-prodrug AcOM-PYRRO/NO (structure in Figure 3) would induce erections in animals on topical administration, presaging the possibility of a noninvasive treatment via local cream application.

Another established diazeniumdiolate pro-prodrug is V-PYRRO/NO, designed and demonstrated to be liver selective in its NO release via metabolism by enzymes concentrated in that organ. Its structure is illustrated in Figure 3. V-PYRRO/NO has been shown to be hepatoprotective in at least three settings: in rodent models of fulminant liver failure [induced on treating rats with tumor necrosis factor-α and galactosamine (29) or mice with bacterial endotoxin and galactosamine (30)]; in regional ischemia induced on preparing the livers of pigs for transplant (31); and in acetaminophen toxicity in mice (32). The drug is designed for activation as an NO donor by cytochrome P450-induced oxidative removal of the vinyl group to generate the free PYRRO/NO ion (29), whose half-life for NO release is very short (3 s; see Figure 2), a mechanism that would be consistent with similar selectivity for other organs rich in this enzyme.

Circumventing Toxicity

Just as the V-PYRRO/NO example of the previous section gave us the opportunity to illustrate the pro-prodrug concept of designing appropriately derivatized diazeniumdiolates for activation as NO donors by specific enzymes, it also serves as an excellent starting point for discussing the critical issue of toxicity. True, its hepatoprotective effects could be achieved without significant systemic hypotension, a major dose-limiting toxicity that must be avoided in any therapy in which physiological NO levels are increased. But V-PYRRO/NO also has the potential to be converted to *N*-nitrosopyrrolidine, one of the most potent experimental hepatocarcinogens known. It is not the goal of this work to cure someone's acute liver disorder one day only to increase the risk of liver cancer later.

Because conversion to N-nitroso compounds is possible for any compound having a Figure 1 structure with $R^1 \neq H \neq R^2$, it is crucial that the potential for conversion to carcinogens be explicitly addressed in any attempt to translate this work from bench to bedside. Here again, the chemical flexibility of the diazeniumdiolates provides us with a variety of strategies for doing this. One that could be applicable to PYRRO/NO derivatives, such as V-PYRRO/NO, is to use an R^1R^2N moiety whose N-nitroso derivative is not carcinogenic. In the case of V-PYRRO/NO, adding a carboxyl group to the 2-carbon of the pyrrolidine ring would convert the corresponding nitrosamine to *N*-nitrosoproline, a compound that is continuously produced in our bodies but that has been screened for carcinogenicity numerous times without once showing positive results (33). Anchoring the R^1R^2N moiety covalently to a stationary solid, as discussed in a previous section, would also make carcinogenicity of the derived N-nitroso functions a non-issue. Infusing PROLI/NO [the fast-acting, spontaneous NO releaser formed on 2-carboxylating PYRRO/NO ion that has already been shown to reverse cerebral vasospasm in monkeys (see above)] at the site of need is another approach to circumventing toxicity in general and systemic hypotension in particular. Another possibility would be to exploit carbon-bound diazeniumdiolates [$R_3C\text{-}N(O)=NOR^3$ analogues of the Figure 1 structure] that have been shown to generate NO under certain conditions, or primary amine derivatives [$R^1HN\text{-}N(O)=NOR^3$] that do not decompose to stable N-nitroso derivatives. Of course, no compound should ever be used for clinical purposes without first having been thoroughly evaluated for possible toxicity. There is no substitute for proper preclinical testing, no matter how benign the structure is predicted to be.

Chemistry is the Key

Perhaps the most general and appealing way to limit toxicity is to target NO release so efficiently to sites of therapeutic benefit that collateral effects on other tissues become insignificant. The ability to convert proteins and polysaccharides to NO-releasing form by diazeniumdiolation may offer the possibility of exploiting the homing capacity of antibodies and lectin-binding carbohydrates for this purpose. Further development of the (pro-)prodrug approach should also lead to a variety of important advances; an intriguing example recently introduced to the chemical literature involves O^2-glycosylated diazeniumdiolates such as β-Glc-PYRRO/NO (structure in Figure 3), a pro-prodrug shown to be activated for NO release by β-D-glucosidase (34). One-electron oxidation of C-bound diazeniumdiolates such as alanosine (35) and cupferron (36, 37) offers the possibility of generating NO on redox activation, and a pro-prodrug class that is activated for NO release by proteases, such as α-chymotrypsin and prostate-specific antigen, has also been characterized (38). It will be most interesting to see whether any of the strategies mentioned in this paragraph, or others yet to be described, will confirm their promise when their hypothetical activities are tested in vivo.

In closing this consideration of potential clinical applications for the diazeniumdiolates, I think it appropriate to revisit their fundamental chemical advantages

as a basis for further progress. Renu herself, when contemplating how to devote her newfound wealth to the conquest of cancer and infectious disease, started with the chemical basics. For example, if NONOates are good, would NONONOates be better? How about the hexazeniumhexolates? Although Renu did not say what specific structures these terms were meant to include, my own view is that clinical potential can best be maximized through an exhaustive understanding of fundamental diazeniumdiolate chemistry—spectral characteristics; structural considerations; acid-base behavior; coordination chemistry; analytical aspects; and reactivity studies, including mechanisms by which they dissociate to NO, their photochemistry, and their redox behavior. A growing number of laboratories are actively engaged in expanding this knowledge base by synthesizing new diazeniumdiolates and reporting on their properties. It remains only for industrious clinical innovators to convert the growing structural diversity of these compounds, their dependable rates of NO release, and their rich derivatization chemistry into means of pharmacologically targeting NO to specific sites of physiological need.

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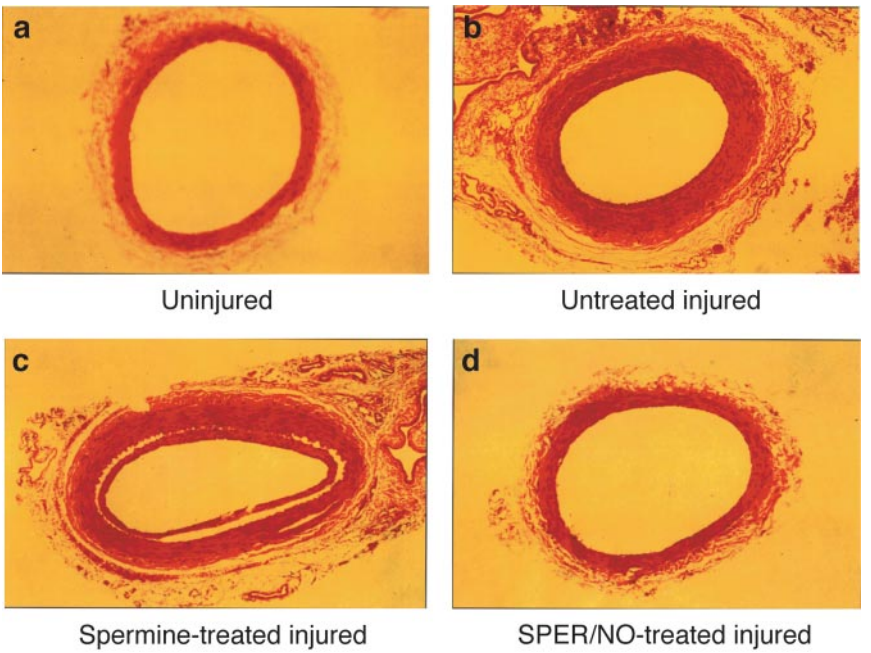

Figure 5 Beneficial effect of NO-releasing SPER/NO on healing of the rat iliofemoral artery after balloon angioplasty. Panel (*b*) shows the extensive stenosis (narrowing caused by excessive thickening of the vessel wall) of a balloon-injured artery two weeks after overinflation, relative to the normal cross-section in panel (*a*). Packing a spermine-containing gel around the outside of the vessel immediately before balloon treatment has no effect on injury-induced stenosis (*panel c*), but replacing the spermine with its diazeniumdiolate SPER/NO (structure shown in Figure 2) led to a nearly normal-looking vessel at the two-week time point (*panel d*). [Courtesy of Sanjay Kaul, Cedars Sinai Medical Center. Figure reprinted from Reference 39 with permission.]

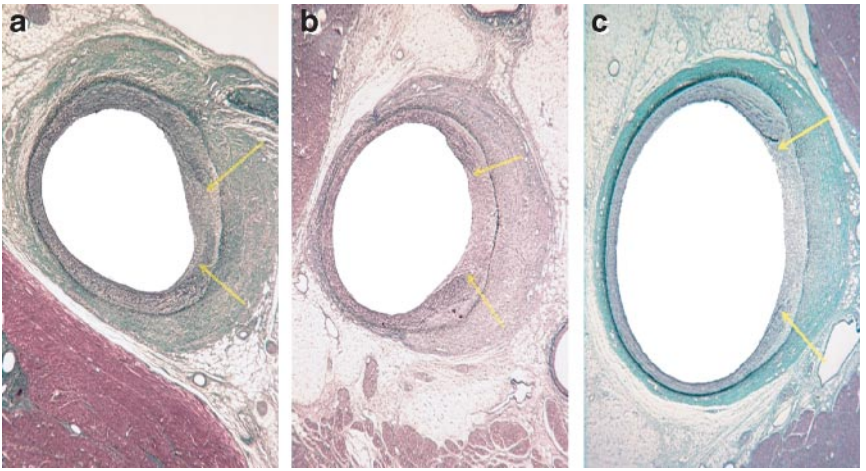

Figure 6 Effect of D-BSA, a diazeniumdiolated bovine serum albumin derivative, in promoting healing of balloon-injured pig coronary arteries. Shown are representative cross-sections of arteries taken two weeks after angioplasty from pigs dosed immediately before 30% balloon overstretch via intrapericardial instillation with 400 mg of underivatized BSA (*a*), 40 mg of D-BSA (*b*), and 400 mg of D-BSA (*c*). Arrows denote ruptured ends of the internal elastic lamina for each vessel shown. [Reprinted from Reference 8 with permission.]

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INNATE IMMUNE RESPONSES TO MICROBIAL POISONS: Discovery and Function of the Toll-Like Receptors

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Key Words LPS, lipopolysaccharide, TNF, Toll-like receptors, TLR4, innate immunity

■ **Abstract** There are many circumstances under which a toxin exploits an endogenous receptor or another protein of host origin to work its untoward effects. In most instances, the receptor normally fulfills a function that has nothing to do with the toxin per se; that is, the toxin is not the "natural" ligand. The situation with endotoxin, however, is a remarkable one. The endotoxin receptor evolved to detect endotoxin. Why have mammals maintained a gene that can undermine their survival? The search for the endotoxin receptor answered this question and also revealed the essential function and biological strategy of the Toll-like receptors: principal sensors of the innate immune system.

INTRODUCTION

Vertebrates are endowed with two fundamental types of immunity. The more ancient system was discovered by Metchnikoff, close on the heels of the foundation of the germ theory of disease by Pasteur and Koch. Metchnikoff observed the engulfment of fungal spores by specialized cells of invertebrate organisms and correctly concluded that this must comprise an important means of host defense. Not long after, phagocytic cells were observed in mammals, including humans, and the general importance of phagocytosis was widely confirmed. Ehrlich, a contemporary of Metchnikoff, discovered antibodies in the blood of animals that had been infected or inoculated with deadly bacteria or bacterial toxins. Many years would elapse before it was realized that antibodies were produced by lymphocytes and before the cooperative nature of the adaptive and innate immune systems was well understood. In the fullness of time, it has become evident that adaptive immunity is, in many ways, subordinate to innate immunity. Without the vital antigen-presenting function of mononuclear phagocytic cells, including macrophages and dendritic cells, and without individual molecules of myeloid origin, adaptive immune responses cannot be initiated. Moreover, immunodeficiencies that involve defects of the

myeloid lineage are often more severe than those that involve defects of the lymphoid lineage. But for much of the twentieth century the identity of receptors utilized by the innate immune system remained a puzzle.

The discovery of the afferent (sensing) system used by innate immune cells to perceive infection has been a major advance in immunology. The roots of this discovery were firmly anchored in genetics, toxicology, and microbial pathogenesis. A focused investigation into the mechanism of endotoxin (lipopolysaccharide) toxicity ultimately led to the understanding that the Toll-like receptors (TLRs), ten of which are encoded in the human genome, are the essential sensors that activate both innate and adaptive immune responses.

In this review, the critical events and discoveries leading to the elucidation of TLR function are presented, along with inferences that have since been drawn concerning signal transduction, genetic diversity, and pharmacotherapeutic opportunities that have arisen from the study of this class of proteins.

HISTORY

As reviewed by Rietschel & Cavaillon (1–3), the term endotoxin was coined by Richard Pfeiffer, a student of Robert Koch. Pfeiffer first identified endotoxin as an agent responsible for fever and shock in animals that were injected with heat-killed preparations of *Vibrio cholerae*, or organisms that had been neutralized with antibodies (4). Until the end of his career, he was unaware that the O-antigens of gram-negative bacteria were covalently attached to the substance he had called endotoxin. It fell to Boivin, Staub, Lüderitz, and others to demonstrate the lipopolysaccharide nature of endotoxin, to show that it was the principal glycolipid component of the outer membrane of gram-negative bacteria, and ultimately, to solve its chemical structure (5–8).

Endotoxin then became a toxin in search of a mechanism. As often happens, the first steps to be taken in finding the mechanism were partly descriptive. A great deal of early work was directed toward understanding precisely what endotoxin did within the mammalian host. As Pfeiffer had observed, much of the toxicity of a gram-negative infection seemed ascribable to endotoxin itself. But perhaps not all of the toxicity can be so ascribed, and it is worth noting that Pfeiffer mistakenly remarked on the presence of "endotoxin" in certain gram-positive bacteria (1). This bespeaks the similarity between the biological effects of true endotoxin and other microbial substances, including peptidoglycan, lipopeptides, and unmethylated DNA.

Endotoxin (from this point forward used interchangeably with LPS: lipopolysaccharide) is, for the most part, poisonous only to mammals. In embryo, birds are also sensitive to LPS (9). But in late embryonic life, a high state of resistance is acquired. Adult birds are scarcely sensitive to LPS at all (10). Among mammals, a haphazard pattern of sensitivity is observed (10). Ungulates, rabbits, anthropoid

apes, and humans are all extremely sensitive to LPS, whereas lower primates, rats, and mice are quite resistant. In all mammalian species, infusion of LPS causes fever, and immediate neutropenia produced as a result of margination of neutrophils within the vascular tree (11–14). Many neutrophils marginate in the lungs, and some extravagate into the air spaces, producing acute respiratory distress (11). Endotoxin is also known to modify the anticoagulant surface of vascular endothelium, permitting the deposition of fibrin and actually depleting fibrin from the plasma (15, 16). A prompt fall in blood pressure, caused partly by vasodilatation and partly by diminished cardiac output, leads to hypoperfusion of vital organs and ischemic injury (15–17). Ischemia of the intestinal tract can lead to the influx of still more endotoxin, sealing the fate of the affected individual (18). Endotoxin also sets in motion long-term effects that are still ill understood: It may influence the character of adaptive immune responses that far outlast the innate immune response.

Many poisons ultimately create a complex picture of deranged physiology so that an investigator is hard pressed to assign culpability to an initial event. Hence, it was once suspected that LPS might intercalate into the membranes of cells, creating ionophoric effects or signaling in the absence of a receptor. It was also felt that LPS might interact with many receptors. Or it might have a primary effect on blood coagulation.

The power of a pure genetic approach to the question of mechanism was made clear in 1965, when it was observed that mice of the C3H HeJ strain were highly resistant to the biological effects of LPS (19). In time, it became apparent that the phenotype of these mice was quite specific for LPS; other bacterial toxins provoked a normal response in these animals. Mice of the C3H/HeJ strain sustained a mutation that became fixed in the population within the first years of the 1960s. They were hypersusceptible to authentic gram-negative infections (20, 21), and a single gene was shown to be involved in the phenotypic difference (22, 23). A second strain of mouse was also found to be LPS resistant and to have an allelic disorder (24, 25). In aggregate, two very important lessons were absorbed.

First, however complex the interaction between LPS and cells and proteins of the host, the product of a single gene was required for all of the toxicity of LPS. This observation spoke strongly in favor of a single protein receptor target for LPS.

Second, it began to be quite clear that LPS was “intentionally” sensed. In other words, sensing LPS was advantageous to the host. Infection with gram-negative bacteria could not be eliminated effectively in the absence of LPS sensing.

The stage was then set for the solution to two of the central questions in the LPS field: Why does the host maintain a system that can produce such fearsome injury? Why is it advantageous to sense LPS at all? The inescapable conclusion has been that LPS sensing is a phenomenon intended to deal with small inocula of gram-negative organisms. More detailed discussion is given to this notion below.

THE ROLE OF HEMATOPOIETIC CELLS IN ENDOTOXIN SENSING

Although endotoxin interacts with many cells throughout the body, and probably does intercalate into membranes in a fairly nonspecific fashion, the lethal effect of LPS seems to be conferred by cells of hematopoietic origin. This fact was revealed by adoptive transfer studies in which crosstransplantation of C3H/HeJ and C3H/HeN hematopoietic stem cells was carried out after lethal irradiation (26). The phenotype of the donor determined the phenotype of the radiation chimera. In separate experiments, it was shown that macrophages were of principal importance in LPS toxicity (27, 28). As hematopoietic derivatives, macrophages therefore seem to be the most important responder cells, ultimately conferring the lethal effect of LPS.

In 1985, tumor necrosis factor (TNF) was shown to be one of the major secretory products of endotoxin-activated macrophages (29, 30). Passive immunization against TNF caused substantial inhibition of the lethal effect of LPS in vivo (31). In separate studies, TNF was revealed as a strongly proinflammatory mediator, capable of mimicking many of the end effects of endotoxin (32). It would, in fact, provoke shock, coagulation, and widespread tissue injury if administered to laboratory animals. In vitro, it was shown to be capable of activating neutrophils (33), stimulating the release of proteolytic enzymes and prostanoids from diverse cell types (34), and altering endothelial surfaces so as to favor coagulation (35, 36). Although TNF certainly does not work alone and was not the only endogenous mediator of endotoxicity, it seemed that the central sequence of events had been illuminated. LPS triggers the activation of macrophages; macrophages release TNF and other cytokine mediators; these mediators act on many tissues throughout the body to cause inflammation and shock.

This general scheme of events did not address questions as to how LPS might be recognized in the first instance. Separate lines of inquiry began to bring this issue into sharp focus.

THE LPS SIGNALING COMPLEX

In 1990, CD14 was shown to be a biologically relevant receptor for LPS on the surface of mononuclear cells (37). When transfected to express CD14, 70Z3 cells acquired sensitivity to LPS, and antibodies against CD14 blocked the LPS response (38). At the same time, a plasma protein called LBP was revealed as an LPS carrier protein (39), conveying LPS to the surface of cells where it became bound to CD14. CD14 could not, however, transduce a signal across the plasma membrane by itself. Being a GPI (glycosylphosphoinositol)-anchored protein, it lacked a cytoplasmic domain. Hence, a missing coreceptor for LPS was assumed to exist.

There seemed to be some hope of identifying such a receptor from the inside out. TNF, by this time taken as an important endpoint of LPS responses, was synthesized as a result of separate transcriptional and translational activation events. Enhanced TNF gene transcription in myeloid cells followed LPS activation as a result of translocation of NF-κB to the nucleus (40). Translational activation depended upon de-repression of a UA-rich element in the 3′-untranslated region of the TNF mRNA (41). Subsequently, this event required activation of p38 (43), a protein that was first identified because it became phosphorylated in endotoxin-activated macrophages (44). In addition, LPS activated the MAP kinase pathway (45) and PI3 kinase pathway (46–49). The added importance of a tyrosine kinase in LPS signaling was suggested by the fact that tyrosine kinase inhibitors could block signal transduction (50). All attempts to find the critical transmembrane receptor that initiated these events were unsuccessful.

Between 1993 and 1998, the LPS gene, which was defective in C3H/HeJ mice, was positionally cloned by Poltorak et al. (51, 52) and shown to encode the Toll-like receptor 4 (TLR4). Prior to this effort, the Toll-like receptors were known only for their similarity to Toll, a bifunctional plasma membrane protein involved in both development and innate immune responses in *Drosophila melanogaster*. Of critical importance, TLR4 was found to be one member of a family of paralogous proteins in mammals (53–57), now known to include ten members in humans (58–60). The fact that one member of the family, TLR4, was highly specific as a mediator of endotoxin responses suggested that each member of the family might recognize a separate set of microbial products.

This supposition proved to be the case when, in 1999, gene knockout work revealed that TLR2 was required for biological responses to bacterial lipopeptides and peptidoglycan (61). In 2000, TLR9 was shown to be required for responses to unmethylated bacterial DNA (62). Later, TLR5 was shown to carry the flagellin signal (63), whereas TLR3 was associated with signaling initiated by double-stranded RNA (64). Collectively, the Toll-like receptors seem to sense much of the microbial world.

Where the endotoxin receptor is concerned, a third component (at least) seems to be required: a small exteriorized protein known as MD-2. The role of MD-2 in LPS signaling was revealed by transfection studies, in which 293 cells made to express CD14 and TLR4 were refractory to LPS signaling (65). However, coexpression of MD-2 would confer an ability to signal to the level of NF-κB activation. Moreover, MD-2 has been shown to be tightly associated to the ectodomain of TLR4, through analysis of interactions between specific monoclonal antibodies that recognize only the complex of the two proteins (66). Although no other components of the receptor complex are known to exist, it is possible that they do. Because TLR4 exists at a very low concentration on the surface of macrophages (67), tremendous signal amplification must occur to convey the lethal effect of LPS. From a practical standpoint, it is difficult to identify new components of the LPS receptor complex, should they exist, using conventional biochemical methods.

THE EVOLUTION OF TOLL-LIKE RECEPTORS AND THE TIR DOMAIN

Whence Toll? As already mentioned, there are ten TLR paralogs in humans, whereas in flies, a set of nine such receptors exists (58). The namesake of the family, Toll, has an immune function in flies, responding to signals initiated by fungi (68) or gram-positive organisms (69, 70). A proteolytic cascade is triggered in response to these stimuli, leading to the cleavage of pro-spätzle, a prohormone, generating the ligand spätzle, which engages Toll. Toll then signals by way of at least three proteins: tube (a protein of unknown function), MyD88 (a conserved adapter protein with homology to Toll itself), and pelle (a serine kinase) to initiate a signal. The production of an antimicrobial polypeptide, drosomycin, is triggered by translocation of dif (71), an NF-κB homologue, to the nucleus after activation of the upstream signaling components just mentioned.

The discovery that Toll has an antimicrobial function in *Drosophila* was the product of pure genetic work carried out by Hoffmann and colleagues (68), who earlier recognized that the diptericin and drosomycin genes respond to NF-κB-like signals (72–75), and who were aware of the potential for Toll to activate NF-κB. At the time they performed their studies, it had already been shown that in mammals, a protein of immunologic importance also signals by way of a Toll-related receptor (76). The receptor in question was one of two that recognized the inflammatory cytokine IL-1 (77). On its cytoplasmic side, both chains of the Type I IL-1 receptor are now known to be homologous to Toll. IL-1 signals traverse MyD88, IRAK, and NF-κB (78), leading to the activation of many genes involved in the inflammatory response.

The first mammalian Toll-like receptor was cloned in 1994 by Nomura and colleagues (53). In 1996, Taguchi et al. mapped the gene encoding this protein, later known as TLR1, to chromosome 4 in humans (54). Because it was not yet known that Toll had an immunologic function, Taguchi and colleagues did not suspect that the protein was involved in immune responses; rather they suspected that it might play a role in mammalian development (54). The 1996 discovery that Toll protects flies against fungal infection (68) foreshadowed the 1998 discovery that TLR4, one of the mammalian homologues of Toll, played a role in the containment of gram-negative infection (51, 52). Prior to the determination that TLR4 was the mammalian LPS receptor, it was shown that the protein was capable of activating NF-κB translocation to the nucleus of cells, much as Toll and IL-1R had been shown to do (55). However, this finding could not enlighten understanding of function. Were the mammalian TLRs developmentally or immunologically important? Or perhaps both?

It is now believed that the developmental function of the Tolls in flies (so-called to distinguish them from the mammalian TLRs) is something of an evolutionary digression. This belief is predicated largely on the fact that in still more divergent species (notably plants), the conserved TIR domain (Toll/IL-1 receptor/resistance), which comprises most of the cytoplasmic domain of all of the Toll-like receptors,

has a defensive function (79). Therefore, in the fly, it seems most probable that the TIR domain was co-opted to serve a developmental purpose. This type of adaptation has not yet been observed elsewhere in the phylogenetic tree. A hint that such adaptations may be possible comes from the work of Weinmann and colleagues, who observed that TLR4 signaling may cause changes in chromatin structure, assessed by nucleosome placement (80). Hence, it may be that some organisms find it a short leap from NF-κB signaling to genuine developmental change.

All of the Toll-like receptors have a series of leucine-rich repeat motifs scattered throughout the ectodomain region and have a cytoplasmic domain that is composed mostly of a conserved TIR domain. The TIR domain can be used for evolutionary studies by constructing hidden Markov models (81). Moreover, a reasonable calibration standard for divergence can be produced based on the measurement of genetic distance between such orthologs as fish and mammalian TLR3, bird and reptilian TLR2, and bird and mammalian TLR2. On this basis, it has been calculated that TLR4, the endotoxin sensor, diverged from other TLRs near the dawn of vertebrate evolution (82). However, as of this writing, TLR4 has been identified only in mammals. Correspondingly, only mammals are highly susceptible to LPS. Some TLRs are clearly lost from the genome over a relatively short period of time: For example, no TLR10 sequence can be found in the mouse genome as currently represented in the Celera database or in the sequences captured by the public consortium. Only a single TLR, TLR3, is currently represented among *Danio rerio* (zebrafish) sequences. These evolutionary choices may reflect liabilities of certain TLRs that become evident with speciation, as vertebrates adapt to new and different pathogens.

TLR STRUCTURE

It is believed that TLR4 is a homodimer because enforced dimerization of TLR4 creates constitutive signaling activity (55). It is likely that TLR4, MD-2, and CD14 form a fairly tight complex with one another during endotoxin signaling because all can be labeled with photochemically activated lipid A derivatives. Furthermore, fluorescence resonance energy transfer (FRET) analyses suggest that LPS brings CD14 into close contact with the TLR4 MD-2 complex (83).

Though Toll does not come into direct contact with any product of fungi, TLR4 does have direct contact with LPS. This has been demonstrated through genetic techniques. Whereas human cells are induced to make TNF in response to lipid A but not tetra-acyl lipid A, mouse mononuclear cells respond to both stimuli (84). Transfection studies have revealed that the species-dependent difference in response is solely attributable to structural difference between human and mouse TLR4 (85, 86). In this sense, human TLR4 is able to make a distinction as to whether secondary acyl chains are present on the agonist molecule. To do so, it must be in very close proximity to the agonist, which is to say that it most likely engages in direct physical contact with it. The nature of the complex that is formed and the conformational changes that occur following engagement of LPS is a

subject of great interest and will probably only be resolved by crystallographic studies.

Crystallography has already shown the basic protein fold of the TIR domain, and it is interesting that modification of the TIR domain of TLR2 by "engrafting" the *Lps*^{d} mutation of the C3H/HeJ mouse does not change its tertiary structure in a major way (87). The modified protein is still crystallizable and retains its overall fold. Based on in vitro mutagenesis studies (67), it seems likely that the modification imposed by the *Lps*^{d} mutation leads to a change in the association between subunits of the molecule. Deleting the entire TIR domain does not have a codominant effect on LPS signaling as does the *Lps*^{d} mutation (67). Although it might be argued that the *Lps*^{d} mutation sequesters a downstream signaling molecule like MyD88, this seems unlikely because the mutation does not suppress signals through any of the other Toll-like receptors, as would happen if MyD88 were bound up in an association with TLR4.

SIGNAL TRANSDUCTION FROM THE ENDOTOXIN RECEPTOR

As with the activation of IL-1 (78), the activation of TLR4 leads to recruitment of MyD88 (88), a TIR domain–bearing protein that also has N-terminal death domains. By death domain interaction, MyD88 forms a complex with IRAK, or IRAK4, both of which are capable of transducing the LPS signaling. Knockout work suggests that IRAK4 is of primary importance (89). Mice lacking IRAK4 show almost complete insensitivity to LPS. IRAK (and presumably IRAK4) activates TRAF6 (90), which in turn activates NIK (90) and TAK1, the latter in a process that depends upon TAB1 and TAB2 (91). TAB1 may also signal toward activation of MAPK, permitting activation of TNF mRNA translation (92). TAK1 phosphorylates signalosome proteins, which in turn phosphorylate IκB, which permits NF-κB translocation to the nucleus.

Recently, MAL (93) [Tirap (94)] has been identified as a TIR domain-containing protein that also seems to participate in LPS signaling. MAL/Tirap also engages the TLR4 receptor and signals the activation of MAP kinase, p38, and NF-κB. The relative contribution of MAL/Tirap and MyD88 to activation can be properly assessed only with the knockout of MAL/Tirap. It is known that MyD88 is important in endotoxin signaling because targeted deletion of the MyD88 gene creates strong insensitivity to LPS (95).

Other signaling proteins may exist. In humans, a mutation is known to abolish sensitivity to LPS and MyD88. However, this mutation has not been traced to either the MyD88 gene, the IRAK4 gene, or to any other known component of the LPS-specific signaling pathway (95a).

Similarities between TLR signals seem to exceed differences. Although selected endpoints of signaling do show specificity with regard to the receptors that initiate them (96), many of the cytokines that transduce the LPS effect are shared in

common with those that transduce the lipopeptide effect or the effects of unmethylated DNA. TNF provides a ready example: Its synthesis is induced by TLRs 2, 4, and 9. Moreover, certain phenomena that have long been studied in the LPS field seem to apply to ligands that transduce their effects through TLRs other than TLR4.

One such phenomenon is endotoxin tolerance. It is known that LPS stimulation is associated with a prompt response (NF-κB translocation to the nucleus and cytokine production) followed by a refractory state, wherein a second challenge is far less effective at provoking such a response (97, 98). Cross-tolerance has been observed when a primary stimulus with lipopeptides is used in place of LPS (99). Although some have attributed tolerance to the production of antiinflammatory cytokines such as TGFβ and/or IL-10 (100), it is more widely held that tolerance reflects the activation of a feedback pathway within cells, causing paralysis of the LPS response. One example of tolerance at the cellular level involves the production of NF-κB p50 homodimers, which can bind to diverse promoters within the cell and prevent activation by p50/p65 heterodimers (101). Other levels of blockade are also possible and are currently under investigation.

A number of agents sensitize to endotoxin. Some are hepatotoxic agents, including lead acetate and D-galactosamine, that seem to sensitize by encouraging TNF-mediated apoptosis of cells in the liver (102). On the other hand, cytokines including interferon-γ sensitize to LPS by lowering the activation threshold of the macrophage population and increasing the amount of TNF that is produced in response to a given LPS challenge (103, 104). Agents of the latter class are more interesting in the sense that their mechanism of action within macrophages remains to be discovered. It is possible that they hold some clinical relevance insofar as priming states [the infection of mice with *Bacillus Calmette-Guerin* (BCG) or *P. acnes*] seem to depend on the production of endogenous cytokine mediators. When primed in this manner, mice may be 10,000-fold more sensitive to challenge with LPS than normal animals (105).

THE EFFECT OF MUTATIONS AT THE TLR4 LOCUS

TLR4 has been very heavily sequenced in an effort to determine how polymorphic it might be. Analyses of synonymous and nonsynonymous substitution within the TLR4 coding region have led to the conclusion that the gene is subject to weak purifying selection (106). That is, it seems to resist structural change and does not undergo promiscuous modification along the lines of some immune proteins that have direct contact with the microbial world (e.g., the MHC antigens). Most changes in the TLR4 coding sequence are weakly deleterious. For this reason, mutational changes have not risen to a high frequency in human populations. Among Caucasians, a double amino acid substitution within the midectodomain of the TLR4 molecule has been observed. This mutation seems to diminish responses to LPS in vivo (107). However, it has not yet been found at high frequency in any human disease.

Rare mutations of TLR4 are observed at higher frequency among patients with severe gram-negative infection (meningococcal sepsis). Such mutations are probably of etiologic importance, and their higher frequency among patients with meningococcal disease cannot be ascribed to linkage disequilibrium with another locus that is of authentic importance (I. Smirnova, N. Mann, M. Hibberd, M. Levin, B. Beutler, manuscript in preparation). The TLRs probably should be regarded as potential susceptibility loci in most infectious diseases, but it is likely that each locus makes only a small contribution to susceptibility for most pathogens.

EVOLUTIONARY CALCULATIONS: THE "SET POINT" OF LPS RESPONSES

As has been emphasized in this review, LPS is an unusual poison in that its mechanism of toxicity has been preserved by evolution. In effect, the host "realizes" that LPS is toxic and accepts the risk of toxicity for the greater good of combating infection. A single mutation would suffice to remove the threat of LPS toxicity, and many species seem to have chosen this option. In mammals, however, LPS sensing is acute, and with this faculty has come the burden of LPS toxicity. What is the nature of the tradeoff in mathematical terms? Can it be calculated?

It must be assumed that the LPS sensing mechanism of mammals was retained to detect small inocula (which might be overcome by innate immune defenses) rather than inocula that are large enough to trigger an injurious or lethal response through the same system. Sensitivity to LPS sets the vigor of the protective response to a small inoculum. But it also limits the microbial burden that can be tolerated without lethality. LPS tolerance, as a general phenomenon, may be viewed as an attempt to buffer the latter effect and extend the flexibility with which the organism may cope with infection.

For a given gram-negative organism (or for all gram-negative organisms that the host will ever encounter) and a given host species—for any and all routes of inoculation—several mathematical relationships might be considered. First, the lethal effect of an inoculum is related to inoculum size (Figure 1*A*), and the mean lethal inoculum (MLI) will generally be found at the point of maximum slope. A given host species will exhibit an acute survivable sensitivity to LPS, indicative of the maximum amount of LPS that can be tolerated. If acute survivable LPS sensitivity is low, then a substantial inoculum might be tolerated acutely. If acute survivable LPS sensitivity is enormous, then even one microbe might kill the host acutely (Figure 1*B*). The acute survivable LPS sensitivity can be estimated for a given species by determining the lethality curve for LPS in vivo. At the same time, the probability of receiving an inoculum per unit time is related to inoculum size as well: Small inocula are much more common than large inocula (Figure 1*C*), but it is also improbable that an organism will sustain no inocula with

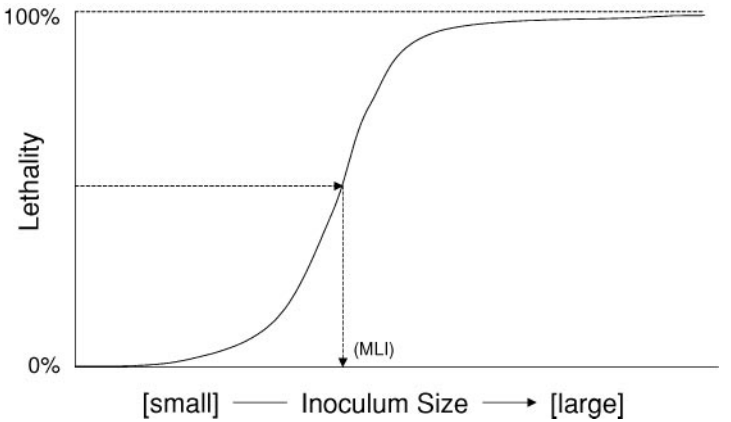

Figure 1*A* The relationship between inoculum size and lethal effect. A sigmoid curve can be expected in experimental analyses and most likely applies under all circumstances.

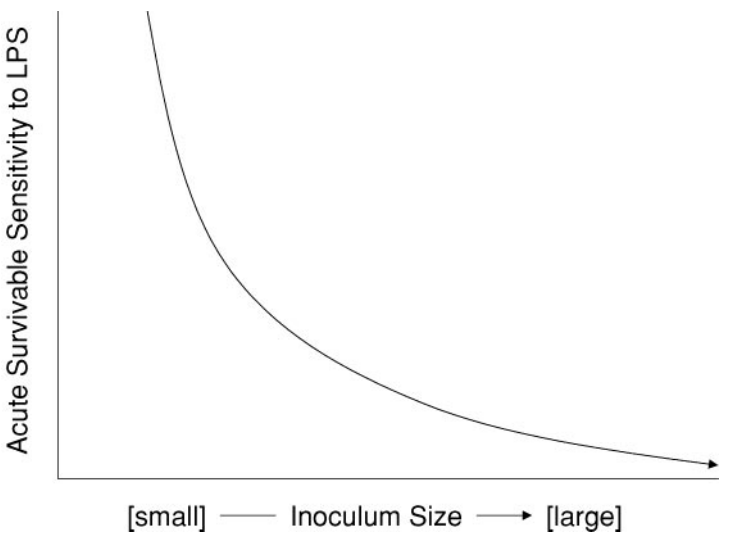

Figure 1*B* The relationship between inoculum size and acute survivable sensitivity to LPS. For any mammalian species, a lethal effect will attend the inoculation of gram-negative organisms, given that the inoculum is large enough. The less sensitive the host is to LPS, the larger the lethal inoculum will be. If sensitivity to LPS is exquisite, even minute inocula may prove lethal acutely.

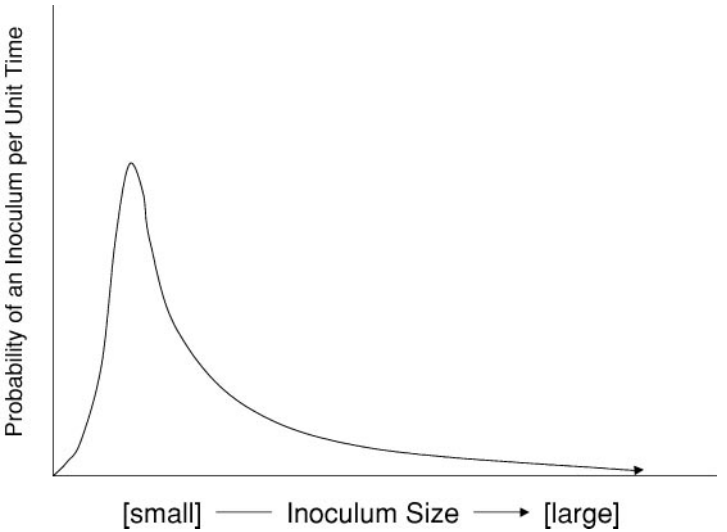

Figure 1*C* The relationship between the likelihood that inoculation will occur within a given length of time and the size of that inoculum. All individuals are exposed to small inocula very frequently, and it is therefore improbable that no inocula will occur per unit time. It is also improbable, however, that very large inocula will occur. Hence, the curve traverses a maximum.

LPS-bearing organisms. The probability of sustaining an infection of a given inoculum size undoubtedly influences the survivable sensitivity to LPS at the same inoculum size because highly probable events must not be lethal events. Therefore, species that are exposed to high concentrations of LPS in the course of life must not have exquisite sensitivity to LPS. In the end, survival at a given inoculum size (for example, at the mean lethal inoculum) can be expressed as a function of LPS-sensing competence: Extremely low LPS sensitivity leads to overwhelming infection; extremely high sensitivity leads to acute death (Figure 1*D*; also see Figure 2).

Given sufficient time, mutation and selection undoubtedly calculate the optimum response of a species to all microbial inducers. However, the relatively rapid shifts that are known to occur in the microbial world may cause a departure from equilibrium. Where our own species is concerned, we cannot conclude that we live in "the best of all possible worlds." An epidemic may alter realities within the space of a few days, confronting the host with microbial challenges that are beyond the established routine, in terms of the route of inoculation, LPS toxicity, and the efficacy of the LPS response. For this reason, it is not clear how one ought to proceed in moderating the LPS response during infection, assuming that one has the means to do so. And certainly, what applies for one infectious agent at one particular moment may not apply universally.

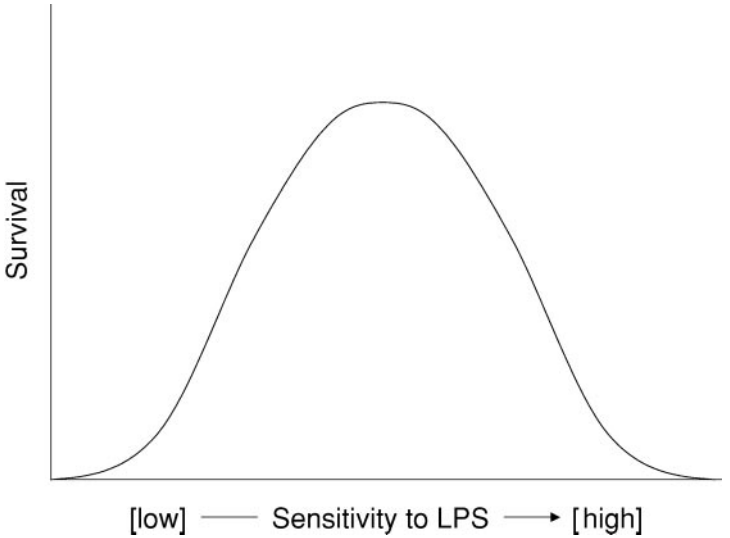

Figure 1*D* Survival as a function of LPS sensitivity. Animals that cannot perceive LPS are at high risk for mortality following gram-negative inoculation. In principle, animals that respond too vigorously to LPS would also be at risk.

PHARMACOLOGIC BLOCKADE OF TLR4 SIGNALING: IS IT POSSIBLE AND WOULD IT BE WISE?

Before our present understanding of LPS signaling was attained, many attempts to interdict the LPS signal were made nonetheless. The first such attempts involved antibodies against LPS, an approach that may be traced to the beginning of the LPS story itself and to the pioneering work of Besredka (1, 2). But attempts to mitigate the toxicity of infections with anti-LPS antibody, though widely publicized (108), have in no instance been widely accepted as successful. There have also been attempts to interdict the signal at the level of CD14—an approach that is still in progress. Anti-TNF antibodies have not shown a beneficial effect during sepsis, despite their ability to protect against LPS in animals (31), perhaps because intervention is too late.

The fact that TLR4, MD-2, CD14, MyD88, and IRAK4 are all critical LPS signaling proteins suggests that it should be possible to fashion small molecular antagonists that will impede signaling, perhaps sparing the host needless injury once an infection has been identified. The belief that antagonists might be produced artificially was encouraged by the fact that tetra-acyl lipid A (84, 109) and certain natural lipid A molecules, like the lipid A of *Rhodopseudomonas sphaeroides* (110), are indeed capable of blocking signal transduction from toxic LPS species. Indeed, it has been possible to create small molecular antagonists that block TLR4 signaling. Among these, E5531 has been tested most extensively (111). It remains

to be seen whether it will be clinically effective, though it does block LPS responses in humans in vivo (112).

Global blockade of TLR signaling might also be achieved, particularly at the level of MyD88 or IRAK4. The latter molecule is a particularly appealing target because it is an enzyme and is probably responsible for the bulk of signal amplification that occurs during LPS activation. Would this be a good idea? Possibly so, though at some point it must be recognized that immune paralysis is detrimental to the host. Even if high doses of antibiotics are administered, sterilizing immunity requires the integrity of myeloid cells, particularly neutrophils, which may, like their mononuclear relatives, rely upon TLRs for detection of pathogens.

SUMMARY AND FUTURE HURDLES

The nature of the LPS sensor was revealed by a spontaneous mutation, and as it happened, the long-cherished belief that LPS was an excellent model for infectious processes turned out to be correct. The LPS sensor was but one member of a paralogous family. When it was revealed, reverse genetic tools (chiefly gene targeting) were swiftly applied to determine the precise function of the other paralogs in the family. Some of the functions of these paralogs have now been deciphered. But certain facts must be borne in mind. Among the remaining TLRs, genuine microbial ligands have yet to be identified for TLRs 1, 6, 7, 8, and 10. TLR10 is not represented in mice and cannot be approached by means of a knockout. And for the others, there will be many phenotypes to test once knockouts are made. Most important, many of the essential molecular participants in signaling may remain to be identified.

The TLRs have occupied center stage for a time, but they attracted notice only because of pioneering forward genetic work—genetic work that begins with phenotype. The key events were the discovery of the immune function of Toll in *Drosophila* and the positional cloning of a spontaneous mutation first observed in mice 37 years ago. There may yet be a long way to go. The essential function of most mammalian genes remains undiscovered, the full complement of genes that subserve most complex functions are mostly undiscovered, and there is no reason to think that innate immune sensing pathways are particularly privileged in this regard.

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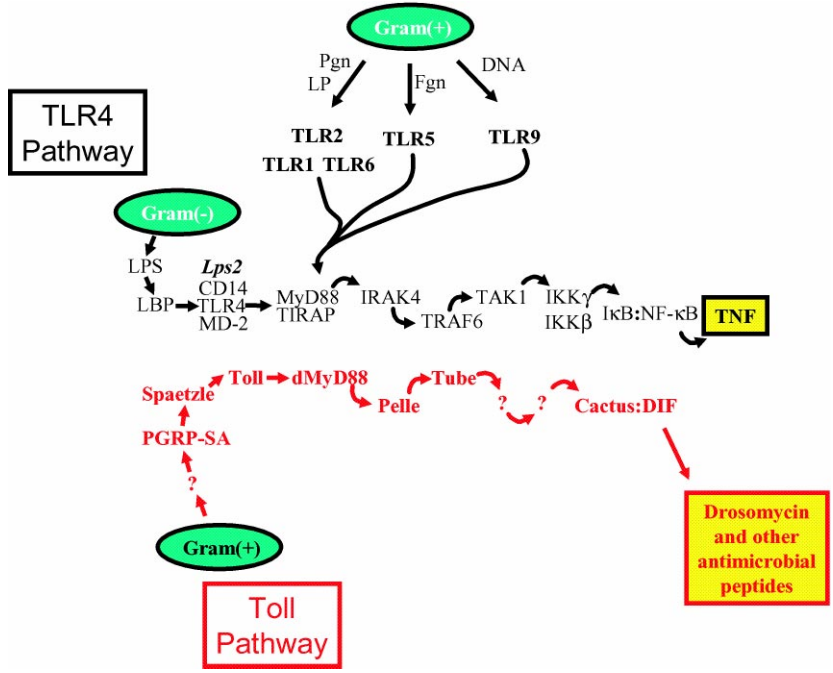

Figure 2 Core elements of the LPS signaling pathway. LPS, released from Gram-negative bacteria, triggers a sequence of events (black) that are largely mirrored by an ancestrally conserved pathway in *Drosophila* (red). At the apex of the LPS signaling apparatus is TLR4, which perceives LPS presented by CD14, and does so in conjunction with MD-2. *Lps2*, a still-unidentified protein known to exist on the basis of forward genetic studies, may also participate in the initial events of signaling. In *Drosophila*, Gram-positive bacteria and fungi are sensed via Toll, but there is no evidence of direct interaction between any molecule of microbial origin and the Toll protein itself. TLRs 1, 2, and 6 are involved in sensing peptidoglycan (Pgn), lipopeptides (LP), and probably other microbial molecules; TLR5 senses flagellin (Fgn); and TLR9 senses microbial DNA. All of these molecules feed signals into the core-sensing pathway at the level of MyD88 interaction. TNF production is probably the single most important terminal event where LPS toxicity is concerned, and all activators of TLR proteins elicit this response to one degree or another.

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THE ROLE OF DRUG TRANSPORTERS AT THE BLOOD-BRAIN BARRIER

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Key Words P-glycoprotein, multidrug-resistance proteins, transferrin receptor, scavenger receptors, large amino acid transporters, nucleoside transporters, anion transporters

■ **Abstract** The blood-brain barrier (BBB) is a dynamic interface between the blood and the brain. It eliminates (toxic) substances from the endothelial compartment and supplies the brain with nutrients and other (endogenous) compounds. It can be considered as an organ protecting the brain and regulating its homeostasis. Until now, many transport systems have been discovered that play an important role in maintaining BBB integrity and brain homeostasis. In this review, we focus on the role of carrier- and receptor-mediated transport systems (CMT, RMT) at the BBB. These include CMT systems, such as P-glycoprotein, multidrug-resistance proteins 1–7, nucleoside transporters, organic anion transporters, and large amino-acid transporters; RMT systems, such as the transferrin-1 and -2 receptors; and the scavenger receptors SB-AI and SB-BI.

INTRODUCTION AND SCOPE

Ehrlich (1) and Goldman (2) were the first to observe the existence of the blood-brain barrier (BBB) after the injection of the hydrophilic compound trypan blue in a rat did not distribute into and out of the brain. It is now known that the cerebral capillary regulates the influx and efflux of biologically important molecules both by preventing passive hydrophilic diffusion and by providing transport processes whose activity can be regulated in accordance with the metabolic and homeostatic requirements of the brain.

Drug transport to the brain depends on various parameters. The amount of drug available for transport across the BBB depends upon its systemic pharmacokinetics [represented by absorption, distribution, metabolism, and elimination (ADME); see also Figure 1]. For drugs that can easily pass the BBB, blood flow is a limiting factor, whereas for other drugs, BBB-permeability is restrictive. In addition, the cardiac output to the brain seems not to be the main determinant for blood flow, but rather the local blood flow and the capillary flow area. In vivo capillary flow was

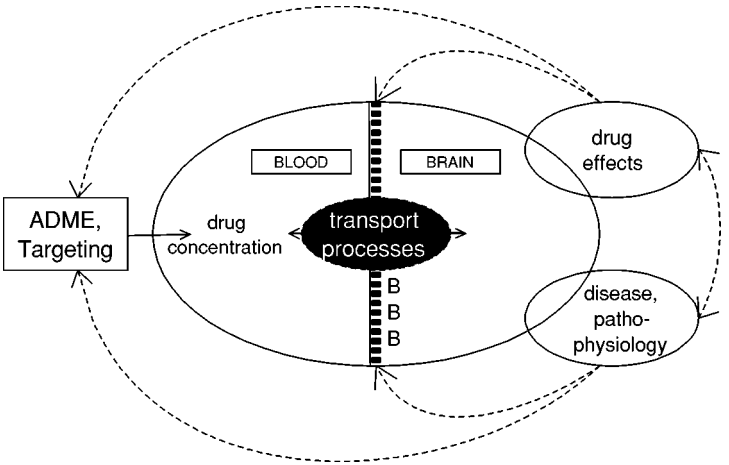

Figure 1 An illustration of the BBB as an organ protecting and regulating the homeostasis of the brain. In addition, the influence of pharmacokinetics (ADME) and drug targeting on the amount of drug available for transport to the brain and the influence of drug effects (pharmacodynamics) and disease/pathophysiology on BBB functionality and integrity is illustrated. The broken lines indicate the influence of drug effects and disease/pathophysiology on the systemic pharmacokinetics (ADME, targeting) and the various transport processes at the BBB [modified from (154)].

shown to be very low because the capillary pressure was approximately 17 mm Hg and not continuous (3). Another factor, protein binding, controls the free (unbound) fraction of drugs/compounds and their distribution in blood that both influence the amount that will be able to pass the BBB. Presently, it is assumed that only the free fraction of drug in plasma/blood is able to pass the BBB. This view may be too limited because many binding proteins (albumin, alpha-acid-glycoprotein, globulins, HDL (high-density lipoprotein), LDL (low-density lipoprotein), insulin-like growth factor binding proteins, etc.) present in plasma could "donate" ligands to the BBB.

Systemic metabolism of drugs occurs mainly in the liver; however, metabolism can also occur during BBB transport because many enzymes are present in the brain capillaries (4). These can transform drugs, before entering the central nervous system (CNS), into metabolites that can be more or less effective or even toxic.

The physico-chemical characteristics of drugs (e.g., hydrophilicity, lipophilicity, hydrogen bonding potential) largely determine the passive transport of drugs across the BBB. This comprises hydrophilic paracellular and lipophilic transcellular transport. Passive hydrophilic transport is mainly restricted by the tight junctions of the BBB endothelial cells. This paracellular permeability is, next to size (5), further dependent on the charge of the molecules and the possibility to form

hydrogen bonds. Passive transcellular processes are mainly dependent on the log-P (log-partition coefficient) or log-D (log-P at pH 7.4) values and their hydrogen bonding potential. There is a linear relationship between log-P and BBB transport in vivo (6). In addition, passive drug transport can be predicted in silico by application of the dynamic polar surface area method, which shows a very good correlation between the polar surface of a molecule and its in vitro transport across a Caco-2 epithelial monolayer (7). Similarly, data from our laboratory showed that the in vitro, in situ, and in vivo BBB transport of adenosine analogues correlate well with their calculated BBB permeability based on hydrogen donor and hydrogen acceptor properties and the lipophilicity of the compounds.

The physical barrier properties of the BBB are related to its narrow tight junctions, the absence of intercellular clefts, minor pinocytotic activity, a nonfenestrated endothelium, a continuous basement membrane, many mitochondria (9), a high electrical resistance (1500–2000 Ohm cm^2) (10), and anionic sites at the luminal membrane. Several of these BBB characteristics are induced by astrocytes (11). However, in small parts of the brain, particularly the circumventricular organs (CVOs), the BBB is physically leaky due to a fenestrated endothelium, but the contribution of drug transport to the brain from these regions is rather limited. Because of its physical barrier properties and to maintain BBB homeostasis, various transport processes, including influx and efflux transporters, operate at the BBB to supply and eliminate substances to and from the endothelial compartment, and subsequently, the brain. These include fluid phase–mediated- (FMT), adsorptive-mediated- (AMT), receptor-mediated- (RMT), and carrier-mediated transport (CMT). AMT depends on the negative charge of the membrane and is therefore rather unspecific and suited for cationic or cationized compounds (see Figure 2). FMT is nonsaturable and also unspecific, and the amount of compound that can be internalized depends upon its extracellular concentration. Both processes occur to a very low extent at a healthy BBB.

CMT occurs by membrane-fixed transporters that transport substances of relatively small size. In contrast, RMT occurs by receptors that are internalized with their ligand(s). These systems can internalize larger substances/particles. Both can be saturated or inhibited, competitively or noncompetitively. In addition, CMT and RMT are selective or specific processes and can be used to target drugs/substances to the BBB/brain. However, internalization or endocytosis is only the first step in the transcytosis of drugs/substances across the BBB endothelial cells, resulting in transport to the brain. As stated earlier, metabolism, but also degradation in the lysosomes can occur, reducing the amount of drug that reaches the brain.

All of these features, including their regulatory systems, provide the physiological basis to understand changed BBB functionality and permeability and therefore the transport of compounds into and out of the brain. Moreover, these influx and efflux systems also maintain BBB functionality by transporting substances in and out of the endothelial compartment. Therefore, the BBB can be considered an

organ that protects the brain and regulates its homeostasis. In this review, we focus on the role of transporters at the BBB. Particularly, we discuss in detail the efflux carriers P-glycoprotein (Pgp) and to a smaller extent the multidrug-resistance proteins (MRPs). Further, we discuss other CMT systems, including the nucleoside-, organic anion-, and organic anion polypeptide-transporter, the large amino acid carrier, and the RMT systems, including the transferrin- and the scavenger receptors. For an overview of transporters at brain barriers, the reader is referred to recent reviews (12–15).

BLOOD-BRAIN BARRIER HOMEOSTASIS AND DISEASE

It has been well recognized that diseases and pathophysiology influence BBB functionality and/or permeability. BBB permeability is changed under various disease conditions, e.g., multiple sclerosis (16), Alzheimer's disease (17), AIDS (18), AIDS-related dementia (19), inflammation-like encephalitis and meningitis (20), and hypertension and seizures, but also in psychiatric disorders (21). These diseases can influence BBB permeability directly or indirectly; directly via changed paracellular permeability, indirectly via changed functionality of BBB transport processes. This can occur at therapeutic concentrations, but also following accumulation of these drugs in BBB endothelial cells when transport systems are inhibited or become less effective, which can result in toxic intracellular concentrations. Ultimately, these changes can lead to CNS disturbances. Therefore, drugs developed to treat these diseases can also influence BBB permeability directly or indirectly. This is schematically illustrated in Figure 1.

There are various examples of drugs/compounds and diseases that influence BBB functionality. The influence of BBB pathology and the influence of disease mediators, such as LPS, NO, radical oxygen species/radical nitrogen species (ROS/RNS), etc., on BBB permeability is well-known, particularly with respect to paracellular permeability (22). Further, disease can up- or downregulate active transcellular transport systems (transporters and transcytosis mechanisms). Lesser known is the influence of the pharmacodynamic effects of drugs at the BBB, e.g., glucocorticoids and interferons that are able to make the BBB tighter (23, 24).

Very interesting experiments have demonstrated the influence of beta-amyloid$_{1\text{-}42}$ on BBB functionality (25). Intracarotid infusions of beta-amyloid$_{1\text{-}42}$ increase BBB permeability in rats. A dose-dependent increase in albumin-bound Evans blue is observed following administration of this peptide. In addition, histological studies indicated an almost complete disappearance of lectin binding sites at the affected endothelial cells. These results indicate that changes occur in the BBB endothelium, suggesting a role in the development of brain pathologies that are associated with Alzheimer's disease. These data are supported by earlier findings that aortic and pulmonary endothelial cells were severely damaged following exposure to beta-amyloid$_{1\text{-}42}$ (26).

Other experiments reveal the influence of apolipoprotein (apo) E on maintaining the integrity of the BBB in rats (27). Apo E in the brain is synthesized by astrocytes, and it was shown that apo E (E_2, E_3, and E_4) plays an important role in the development of Alzheimer's disease (28). Further, apo E knockout mice develop severe artheriosclerosis that is enhanced by a high-fat diet (29). Here, it was demonstrated that apo E3–Leiden mice, which develop severe artheriosclerosis that is enhanced by a high-fat diet, did not show signs of BBB disturbances, whereas apo-E-knockout mice did when put on a high-fat high-cholesterol diet (27). The results indicate a severe extravasation of IgG in the hippocampus and cerebellum of apo E–knockout mice and not in C57B1/6 control mice, which points to increased BBB transport. In addition, the transport of the vascular marker fluorescein into the brain of male and female knockout mice showed a tendency to increase compared to C57b1/6 control mice, which points to increased paracellular BBB transport. These data were supported by behavioral analysis, indicating that apo E–knockout mice were less efficient in acquiring the spatial Morris water maze task compared to C57B1/6 mice. These interesting observations relate cognitive functions with BBB functionality. It is hypothesized that apo E plays an important role in maintaining the integrity of the BBB and affects neurodegenerative processes as seen in Alzheimer's disease.

These experiments illustrate the importance of transport processes at the BBB to maintain CNS homeostasis for optimal CNS performance. However, it still has to be revealed if BBB disfunctionality will be the cause or the result of CNS diseases.

IN VITRO AND IN VIVO METHODS TO STUDY BLOOD-BRAIN BARRIER TRANSPORT

Research on drug transport across the BBB and its functionality has been very much enhanced by the availability of in vitro BBB endothelial/astrocyte (co)-culture systems. The use of such systems allows a detailed investigation of BBB-related phenomena at the (sub)-cellular level and in the absence of feedback systems from the rest of the body. This makes it much easier to study in vitro BBB transport and BBB functionality by (pharmacological) intervention techniques, such as the application of receptor agonists and antagonists, blockers of transporters and enzymes, antisense and (anti)gene approaches, and the influence of disease. Recently, BBB (co)-culture systems have been reviewed and discussed with respect to their use in BBB-related research (30, 31).

Various methods have been developed to estimate in vivo BBB drug transport also. The applicability of these methods depends on their sensitivity and selectivity to measure drug concentrations in the brain, the estimation of local concentrations in the brain (spatial resolution), and the measurement of single-time concentrations versus concentration-time profiles (time resolution). Because an extended discussion is beyond the scope of this review, the reader is referred to the literature (32).

TRANSPORT PROCESSES AT THE BLOOD-BRAIN BARRIER

Carrier Mediated Transport

Transporters comprise carriers and receptors that are located in the plasma membrane of the endothelial cells of the BBB. Carriers are membrane-restricted systems suited to generally transport compounds with a rather fixed size and a molecular mass smaller than 500–600 Da (33). Most of these systems are ATP-driven and therefore possess at least one intracellular ATP-binding domain. Others are equilibrative systems and do not require ATP. The activity of these transporters is temperature sensitive and they can be saturated at higher concentrations of ligands. Their activity can be influenced by competitive and noncompetitive inhibitors and by interfering with their phosphorylation by protein kinases.

Receptor Mediated Transport

Receptors may also be located in the plasma membrane of the endothelial cells of the BBB, but are not restricted to this location. They can be internalized and transported via the early endosome to the lysosomes or even transcytosed and shuttled back to the plasma membrane again. Internalization occurs via an endocytotic process. Endocytosis is used in this context to indicate vesicular transport pathways in eukaryotic cells to internalize extracellular fluid and particles (<500 nm) as well as plasma membrane molecules (34). Endocytosis may be very fast; in some cultured mammalian cells, 50% of the entire cell surface may be internalized every hour (35).

With receptor-mediated endocytosis, the uptake of particles or ligands is saturable because it is dependent upon the extracellular availability of receptors. Subsequently, following binding, the ligand-receptor complex is internalized. This process requires energy and is also temperature sensitive. In addition, the internalization process is time dependent. The half-life time for internalization of several proteins by coated vesicles under optimal conditions varies from 2–5 min (34). Further, the rate of internalization may be dependent on ligand binding (regulated endocytosis), whereas other receptors may be internalized equally fast with or without ligand [constitutive endocytosis; (36)]. In contrast to carriers, receptors are able to internalize relatively large compounds and systems and are therefore more suited for targeted drug delivery (peptides, proteins, etc.) to the brain (37).

Endocytosis

Endocytosis can occur via two distinct pathways, e.g., via coated or noncoated invaginations in the membrane of cells. Coated invaginations, or coated-pits, have shown the presence of clathrin, and the influence of adaptor-protein 2 (AP-2) complexes and dynamin (a GTPase required for budding of clathrin coated vesicles) as major components of the coated-pits have been demonstrated (34). The AP-2

adaptor complex plays an important role in the recognition of internalization motifs of extracellular receptors. Tyrosine-based motifs, such as NPXY and YXXO (the characters indicate single amino acids where X is any amino acid and O is a bulky hydrophobic amino acid), and the dileucine (LL) motif have been recognized (34). Various receptors, like the low-density lipoprotein, the transferrin-, and the mannose 6-phophate receptors, are internalized via these coated-pits.

Noncoated omega, smooth-coated, or flask-shaped invaginations (35) were initially discovered by Palade et al. (38). They are called caveolae and have a maximal diameter of 70 nm. They start from the plasmalemma and have a neck-like structure. By electron microscopy, caveolae can be distinguished from clathrin-coated pits because they are not associated with an electron-dense cytoplasmic coat. Caveolae are abundantly present in endothelial and smooth muscle cells, but are also present in epithelial cells, adipocytes, fibroblasts, type 1 pneumocytes, and striated muscle cells (39). Caveolae are coated with proteins that belong to the family of caveolins [caveolin-1(alpha,1beta), caveolin-2(alpha, beta, gamma), and caveolin-3; (39)], but other proteins, such as flotillin-1, MAL1, and MEC-2/stomatin proteins, may also be involved in caveolae formation (40).

The function of caveolae includes the transport of molecules across these cells, but recently, it was shown that caveolae are also involved in potocytosis (the internalization of small molecules without the merging of an endocytotic vesicle with endosomes), signal transduction regulation, and cholesterol transport (41).

In conclusion, it may be clear that RMT and CMT are important processes for the influx and efflux of substances to and from the BBB endothelial compartment. Changed activity of these processes due to disease, for example, can have serious consequences for functionality and integrity of the BBB. On one hand, this can result in increased para- and transcellular BBB permeability, and therefore, in changed CNS homeostasis. Ultimately, this can lead to CNS diseases, e.g., Alzheimer's or other neurodegenerative diseases (25–27). On the other hand, this offers opportunities for site-specific or targeted drug delivery to the brain when transport processes are selectively upregulated under disease conditions.

ROLE OF TRANSPORT PROCESSES AT THE BLOOD-BRAIN BARRIER: CARRIER-MEDIATED TRANSPORT OF DRUGS

Pgp Efflux Pump

One of the most important efflux transporters identified at the BBB is the Pgp efflux system. This system is discussed in more detail because its presence has been demonstrated at the luminal site of the BBB endothelium (42) and it has been extensively studied in past decades. The Pgp efflux system is responsible for the occurrence of multidrug resistance (MDR), and today, Pgp is considered as an amphipatic cationic efflux pump.

Pgp is a 170-kDa membrane-fixed glycoprotein and comprises two almost identical halves within 12 alpha-helical transmembrane-spanning domains and two intracellular ATP-binding sites. It belongs to the ABC (ATP-binding cassette) superfamily, which consists of more than 30 families transporting a tremendous variety of substrates.

In humans, the MDR1 and the MDR2 genes encode for the two different isotypes of Pgp (43). The MDR1-Pgp is mainly found in the apical membrane of epithelial tissues from the intestine, kidney, pancreas, and adrenal gland. Further, it has been found in the endothelium from the endocervix, endometrium, esophagus, glomeruli, intestine, lung, lymph nodes, myometrium, placental trophoblasts, ovarian cortex, papillary dermis, prostate, spleen, stomach, testes, blood-inner ear, and the BBB (42, 44, 45). Recently, Pgp was demonstrated to be partially localized in the caveolae of resistant (CH^RC5) cells and of drug-sensitive Chinese hamster ovary (CHO) (AuxB1) cells. A similar localization of Pgp was found in caveolae of rat brain capillary cells (46).

In rodents, there are three Pgp genes encoding for the mdr1a-, the mdr1b-, and the mdr2-Pgp (47). The mdr1a- and the mdr1b-gene products fulfill the same function as the MDR1-gene product in humans.

MDR2- and mdr2-Pgp do not play an important role in the transport of drugs. They are abundantly expressed in the liver and their function has been demonstrated by the transport of phospholipids across the canicular membranes in hepatocytes into the bile (48).

Physiological Role of Pgp

It has been postulated that Pgp acts as a so-called vacuum cleaner (49), moving compounds from the lipid bilayer into the extracellular space. A second hypothesis has been postulated where the transporter acts as a flippase (50), either moving the substrate from the inner to the outer leaflet of the membrane or locally altering membrane lipid composition such that the substrate detaches. These mechanisms support the observation that Pgp effluxes amphipatic peptides, proteins lacking signal sequences, or lipid-modified proteins from biological membranes (51).

The location of Pgp, particularly at blood-tissue and air-tissue interfaces, and its broad range of substrates, indicates that it limits the influx and diffusion of compounds and, subsequently, the exposure of cells to high (toxic) concentrations of compounds. In addition, it has been suggested that Pgp has several physiological functions in mammals (52). There is also evidence that Pgp transports steroid hormones. Therefore, the increased expression of Pgp during pregnancy in the placenta, embryo, and uterus may explain the need to protect the fetal tissue against these hormones (53, 54). Furthermore, Karssen et al. (55) demonstrated in mdr1a(+/+) and -(−/−) mice that Pgp was involved in limiting the access of the naturally occurring glucocorticoid cortisol rather than corticosterone to the mouse as well as human brain, particularly to the hippocampal area. In addition, similar

data were found for the glucocorticoid dexamethason (56). Because glucocorticoids influence behavior, it was suggested that Pgp may play an important role in the regulation of the behavioral response of glucocorticoids in the hippocampus.

The hypothesis that Pgp regulates volume-activated chloride channels was developed by the observation that Pgp was highly related to the cystic fibrosis transmembrane regulator protein (CFTR). CFTR belongs, like Pgp, to the ABC superfamily of transporters and is a plasma membrane chloride channel that is dysfunctional in cystic fibrosis (50). However, the role of Pgp in volume-regulatory processes has been controversial, and a current hypothesis is that Pgp does not have channel activity itself, but it may regulate swelling-induced anion channels (57).

It has been suggested that Pgp plays a role in the transport of prenylcysteinmethyl esters or cholesterol (58). In addition, esterification of cholesterol and triacylglycerol-rich lipoprotein secretion was inhibited by inhibitors of Pgp (59), whereas cholesterol seemed to be transported by Pgp also (60).

Recently, it was demonstrated that beta-amyloid$_{1\text{-}42}$ was transported by Pgp (61). Beta-amyloid$_{1\text{-}42}$ is an amphipatic peptide comprised of 28 hydrophilic and 12–14 hydrophobic amino acids. These peptides are rapidly released from both neuronal and nonneuronal cells; however, the sequence of the 12–14 hydrophobic amino acids is the reason that the peptide remains associated with the membrane following gamma-secretase cleavage. Therefore, it has been postulated that transport systems are required to efflux these peptides. In vitro binding studies show that addition of synthetic human beta-amyloid$_{1\text{-}40}$ and beta-amyloid$_{1\text{-}42}$ peptides to hamster mdr1-enriched vesicles results in saturated quenching. This suggests that both peptides interact directly with the transporter. Inhibition studies with the MDR1 inhibitors RU-486 and RU-49953 in MDR1-transfected cells reduced the secretion of the peptides. It was concluded that these data were strongly suggestive for Pgp-mediated efflux of beta-amyloid peptides.

In conclusion, Pgp is involved in the regulation of various physiological processes. Furthermore, the fact that mdr1a(−/−) and mdr1a/1b(−/−) mice appear normal suggests that Pgp does not have an essential role in life, although it should be taken into account that due to the knockout of mdr1a and mdr1b genes, Pgp function may be compensated by other transporters.

Pgp and Pharmacodynamic Effects of Drugs in the Brain

Important evidence for the role of Pgp at the BBB was obtained from experiments with mdr1a(−/−) and mdr1a/1b(−/−) mice. The significantly higher accumulation of several drugs in the brains of these mice in comparison to most other tissues and plasma demonstrated its important role (62). In addition, increased accumulation of these drugs in various tissues can affect their pharmacodynamics (63). This is best illustrated by centrally acting drugs. Morphine is often used as a narcotic analgesic for the treatment of pain. It acts at the opioid receptors within the CNS at both the spinal and supraspinal levels. In vitro and in vivo studies have

demonstrated that morphine is a weak Pgp substrate (64). Pgp influence on the pharmacodynamics of morphine was studied in mdr1a(−/−) and wild-type mice (65). The tail-flick response to radiant heat was taken as the pharmacodynamical endpoint to determine the antinociceptive effect. Morphine was administered subcutaneously to both mdr1a(−/−) and wild-type mice and it was found that antinociception was indeed increased in the mdr1a(−/−) mice. The ED_{50} of morphine was more than twofold lower in mdr1a(−/−) mice (3.8 +/− 0.2 mg/kg) compared to FVB (wild-type) mice (8.8 +/− 0.2 mg/kg), whereas EC_{50} in brain tissue was similar. Pgp inhibition in wild-type mice with R-verapamil resulted in an antinociceptive effect similar to that in mdr1a(−/−) mice. A comparable study was performed in rats, where GF120918 was used as a Pgp inhibitor (66). Both the antinociceptive effect was measured and the concentrations of morphine and its main metabolite morphine-3-glucuronide (M3G) in blood and brain extracellular fluid (ECF) were measured by intracerebral microdialysis. Inhibition of Pgp resulted in increased concentrations of morphine and M3G in the brain, whereas in the blood, only M3G concentrations were changed. The pharmacodynamic effect of morphine was increased in the presence of GF120918 and could be described using a pharmacokinetic/pharmacodynamic model based on morphine concentrations in the ECF.

It was suggested that pharmacoresistance for the antiepileptic drug phenytoin was caused by Pgp at the BBB (67). Phenytoin concentrations in the ECF of the cerebral cortex or rat brain were measured by microdialysis. Pgp inhibitors, such as sodium cyanide, verapamil, and PSC 8333, were directly administered by a 15–60 min infusion via a microdialysis probe in the right frontal cortex before intraperitoneal administration of phenytoin. Phenytoin concentrations in the ECF were significantly enhanced by Pgp inhibitors, indicating that Pgp limited the distribution of phenytoin into the brain. Interestingly, a similar effect was found for Cremophor, the vehicle used to administer the PSC 833. In addition, based on in vitro experiments in bovine BCEC and a Pgp-overexpressing cell line (MCF-7/Adr) and in vivo experiments in mdr1a(+/+) and (−/−) mice, similar results were found for some enaminone anticonvulsants (68).

The influence of Pgp on the therapeutic effects of drugs in the CNS is particularly illustrated by the effect of Pgp at the BBB in limiting the treatment of brain tumors. The presence of Pgp in tumors causes MDR, but Pgp at the BBB is also responsible for MDR in the case of brain tumors. Several anticancer drugs are Pgp substrates and poorly pass the BBB. Consequently, these drugs will not reach tumors in sufficient concentrations. Using Pgp inhibitors in cancer therapy can therefore be beneficial in two ways. First, the pharmacokinetics of the anticancer drugs can change; particularly, CNS drug concentrations can increase. Second, the intracellular drug concentration in brain tumors can increase (provided that the inhibitor also distributes to the brain tumor).

An increase in in vitro BBB permeability was observed following concomitant administration of the Pgp substrate vinblastine and the Pgp inhibitor PSC 833 (69, 70). A low concentration of vinblastine had only a small effect on the

trans-endothelial-electrical resistance (TEER). However, when given together with PSC 833, TEER dropped to very low levels, indicating that paracellular permeability had increased considerably. This was confirmed by experiments measuring the transport of the paracellular marker fluorescein. Others have shown similar data following administration of doxorubicin and a Pgp inhibitor in an in vitro BBB model (71). In addition, in vivo drug-drug interactions at the level of Pgp can also lead to side effects, as has been suggested for the interaction between diltiazem and tacrolimus (72).

Recently, it was shown that the 5-HT_{1A} receptor antagonist flesinoxan was effluxed by Pgp in vitro as well as in vivo in rats and mdr1a(+/+) and -(−/−) mice (73). In monolayers of MDR1-transfected LLC-PK1 cells, the transport of flesinoxan could be inhibited by PSC 833, LY 335979, and verapamil. In addition, transport could be saturated at concentrations higher than 10 ug/ml, and in the in vitro BBB system, transport polarity was also observed. Following administration of 3 mg flesinoxan/kg in the tail artery of mdr1a(−/−) mice, a brain plasma ratio of 27.0 was observed, whereas in mdr1a(+/+) mice, this ratio was 12.6. In addition, the ratios in other tissues (heart, kidney, liver, lung, and spleen) were much lower in the mdr1a(−/−) as well as in the mdr1a(+/+) mice. Moreover, intracerebral microdialysis experiments were performed in rats following administration of flesinoxan. The C_{max} and the area under the curve (AUC) of the concentrations in the ECF were increased by a factor of 5–6 following co-administration of PSC 833, whereas the transport of the extracellular marker compound fluorescein to the brain was unchanged (73).

HIV infection is another disease where Pgp at the BBB limits its treatment. Besides immunological cells, HIV also affects the CNS. In 40% of AIDS patients, serious neurological disorders, such as AIDS-dementia complex, are developed, particularly at late stages of the disease (74). Furthermore, the infected brain can continuously re-infect the periphery by serving as a reservoir for the virus (75). Therefore, it is important that an anti-HIV agent passes the BBB and achieves effective concentrations in the CNS. However, due to Pgp, the concentration of these drugs in the CNS may be too low to be effective to stop HIV replication and re-infection (76). A suitable strategy to overcome these problems would be to increase the concentrations of the HIV protease inhibitors in the CNS by inhibiting Pgp at the level of the BBB. However, although it was suggested that ritonavir could be used for this purpose, it did not increase the transport of other HIV protease inhibitors in vitro or in vivo (77, 78).

The function of Pgp to limit the access of drugs to the brain, and therefore their pharmacodynamic effects in the brain, is even more clearly demonstrated for drugs that are supposed to act peripherally. The opioid receptor agonist asimadoline, which is in development as a peripherally acting analgesic, and loperamide, an antidiarrheal drug, do not enter the CNS and normally have no central effects. However, administration of these drugs to mdr1a(−/−) or mdr1a/1b(−/−) mice leads to analgesic and morphine-like effects (79). This demonstrates that Pgp in the BBB is responsible for the selective peripheral effects of those drugs in humans.

Similarly, second-generation antihistaminics were excluded from the brain by Pgp, whereas first-generation compounds were not (80).

Thus, one could say that, currently, many CNS active drugs have been shown to be Pgp substrates. Inhibition of Pgp or drug-drug interactions at the level of Pgp can therefore have serious consequences for drug therapy of CNS disorders and can even lead to CNS toxicity (81).

Modulation of Pgp-Activity

Several compounds effectively inhibit Pgp, competitively or noncompetitively. These include verapamil, R-verapamil, cyclosporin-A, PSC 833, LY 335979, GF 120918, S 9788, and RU-486 (82). Another possibility for interaction with Pgp is at its glycosylation sites. Pgp has three glycosylation sites; however, blocking of these sites with tunicamycin did not change its efflux function (83). In addition, there are various phosphorylation sites at Pgp that are phosphorylated by protein kinase A and C (84). Application of protein kinase C (PKC) inhibitors resulted in increased accumulation of Pgp substrates (85), whereas phorbol esters stimulated its phosphorylation and increased drug resistance (86). However, the problem with PKC inhibitors is that these compounds are not specific for one PKC isoenzyme. In addition, the PKC inhibitor bryostatin leads first to activation of PKC and later to a downregulation of PKC. For these reasons, PKC modulation has led to many contradicting results (87).

Recently, it was shown that oxidative stress changes Pgp expression in primary rat BCEC (brain-capillary-endothelial cells) (88). Particularly, this may have consequences for the transport of substrates in and out of the brain under disease conditions like ischemia.

Another method of Pgp regulation has been demonstrated by adrenomedullin (AM) (89). It is produced by endothelial cells in the brain and acts as a vasodilator in the cerebral circulation. It was shown that AM antisense decreased the transendothelial electrical resistance across endothelial monolayers. Treatment of these cells with AM activated Pgp, suggesting that AM acts as an autocrine mediator in the regulation of the properties of BBB endothelial cells. In addition, AM incubation decreased BBB permeability for sodium fluorescein (376 Da) but not for Evan's blue albumin (67 kDa). An interesting observation was that it also attenuated fluid-phase endocytosis.

An approach to enhance Pgp inhibition was applied by Matsuo et al. (90). They used liposomes with a covalently bound monoclonal antibody against an extracellular epitope of Pgp (MRK-16). The binding of these liposomes to K-562/ADM cells (adriamycin-resistant human myelogenous leukemia cell line) was higher than that of IgG2A-modified liposomes and liposomes without modification. In addition, when vincristin was encapsulated in all types of liposomes, it was demonstrated that the cytotoxicity of MRK-16-modified liposomes was higher than that of IgG2a and nonmodified liposomes.

There are various ways to modulate Pgp activity. Effective Pgp inhibitors have been developed; however, their therapeutic use is often limited.

MRP-EFFLUX SYSTEMS

In addition to Pgp-mediated MDR, there is also a non-Pgp-mediated MDR phenomenon. This comprises another ABC transporter subfamily that is called the MRP-family. At least seven members have been identified, and five (MRP1, -3, -4, -5, and -6) of them are expressed at the BBB [(44, 91); reviewed by Borst (92, 93)]. The MRPs are membrane-fixed systems that vary in size from 1325 to 1545 amino acids (92). They comprise two transmembrane domains of six alpha helices, a cytoplasmic linker region, and two intracellular ABCs. The linker region is essential for its transport function (94, 95). In addition, MRP1, -2, -3, and -6 have an extra domain structure comprising five additional transmembrane-segments at the animo-end (92, 96). Today, MRPs are considered amphipatic anion efflux pumps.

The MRPs transport mainly anions, but can also transport cations and neutral compounds. Two mechanisms have been proposed. One is that the anions are directly transported, and the second is that the cationic and neutral compounds are cotransported with glutathion (GSH). It has been suggested that MRPs contain dual binding sites for the direct binding of drug-GSH complexes or sequential binding of GSH. In addition, one of these binding sites may have a higher affinity for drugs and a lower affinity for GSH, whereas the second binding site is of the opposite conformation (92).

Thus, the role of MRPs at the BBB is only partially known. They play a major role in the elimination of amphipatic anions (many of them being phase II metabolites) from the endothelial compartment. This may also be their main physiological function. Based on the limited knowledge about their physiological substrates, one can only speculate about their role in disease processes at the level of the BBB.

ORGANIC ANION INFLUX- AND EFFLUX TRANSPORTERS

Due to the negative charge on the cell membrane, negatively charged compounds have difficulty entering or exiting cells. Therefore, transporters have appeared at the BBB that influx and/or efflux such compounds. Many of these transporters are called multispecific, indicating that they are able to transport several substrates. There are two main families of multispecific anion transporters: the organic anion transporter (OAT) and the organic anion transporter polypeptide (oatp) family (97). They are all membrane-fixed transporters.

Presently, eight members of the oatp-family, i.e., oatp1, oatp2, oatp3, OAT-K1, OAT-K2, OATP(A), the prostaglandin transporter (PGT), and the liver-specific transporter-1 (LST), have been identified (97). Oatp2 is localized at the apical and basolateral side of BCEC and the basolateral side of the epithelial cells of the choroid plexus (98). It transports anions like bile acids, taurocholate, cholate, estrogen conjugates, ouabain, and digoxin (99). The human OATP transporter [OATP(A)] has been identified in human BCEC and was found to transport opioid peptides [deltorphin II and (D-Pen(2),D-Pen(5))enkephalin] (100).

Presently, four OATs have been identified, comprising three subfamilies of organic anion transporters: the sodium-dependent OATs, the sodium-independent facilitators or exchangers, and active OATs that require ATP. The sodium-dependent OATs have a narrow substrate specificity and are involved in the reabsorption of anionic substances from the proximal tubules in the kidney. The active and sodium-independent OATs have a broad substrate specificity and are involved in the efflux of organic anions in the kidney and the liver (97). OAT1 is a multispecific organic anion/dicarboxylate exchanger of various organic anions [para-aminohippurate, dicarboxylates, cyclic nucleotides, prostaglandin E, beta-lactam antibiotics, nonsteroidal antiinflammatory drugs, and diuretics; (97)]. Its expression in the brain is very low. OAT3 mRNA has been identified in human and rat brain, and the transporter is involved in the efflux of (endogenous) anionic compounds (para-aminohippurate, dehydroepiandosterone sulfate) from the brain (101).

In conclusion, OATPs and sodium-independent OATs transport anionic compounds with a rather broad substrate specificity, whereas the sodium-dependent OATs have a narrow substrate specificity. Considering their substrates, it can be concluded that they have a similar physiological function as the MRPs in eliminating anionic compounds from the endothelial compartment. This may also be their major role at the BBB. Little is known about diseases related to malfunction of these transporters.

NUCLEOSIDE TRANSPORT SYSTEMS

The brain needs the influx of nucleosides because the brain is deficient in de novo nucleotide synthesis (102). Purine and pyrimidine nucleosides are necessary for the synthesis of DNA and RNA, but nucleosides also influence many other biological processes. In addition, nucleosides play an important role in the treatment of diseases, such as cardiac diseases, brain cancers, and infections [parasitic and viral; (103)]. Nucleosides are hydrophilic compounds, and the influx and efflux of these compounds is therefore mediated by a number of distinct transporters (104). Nucleoside transporters are membrane-fixed transporters and are classified by their transport mechanisms (e = equilibrative, c = concentrative), their sensitivity to the transport inhibitor nitrobenzylmercaptopurine riboside (NBMPR; s = sensitive, i = insensitive), and their substrates. Presently, there are two equilibrative transporters (ENTs: *es* and *ei*) and six concentrative nucleoside transporters [CNTs: *cif* (concentrative, NBMPR insensitive, broad specificity; N1), *cit* (concentrative, NBMPR insensitive, common permeant thymidine; N2), *cib* (concentrative, NBMPR insensitive, broad specificity; N3), *cib* (concentrative, MBMPR insensitive, broad specificity; N4), *cs* (concentrative, NBMPR sensitive; N5), and *csg* (concentrative, NBMPR sensitive, accepts guanosine as permeant; N6); (104)]. The equilibrative *es* and *ei* nucleoside transporters are widely expressed in mammalian cells and are present at cultured endothelial cells and brain capillaries (105). In these cells, the expression of concentrative transporter *cit* (N2) was demonstrated also. In other parts of the rat brain, *ei* and *es* nucleoside transport systems have

been identified, but not their localization (106, 107). Other research indicates that a sodium-dependent nucleoside transport system is present at the BBB that is not involved in the transport of nucleoside analogues (108). Recently this transporter has been cloned from a rat brain cDNA library and shown to be similar to the rat concentrative sodium nucleoside cotransporter [CNT2; (104, 109)]. In addition, very recently, the *es*-NT transporter was demonstrated at bovine BCEC by RT-PCR (8).

In conclusion, the equilibrative nucleoside transporters are particularly widely expressed in mammalians, whereas until now, the concentrative transporter (CNT2) has been identified at the BBB. Although nucleosides play a role in many biological processes and various diseases, their role at the BBB in relation to brain diseases is not clear.

LARGE NEUTRAL AMINO ACID TRANSPORTER

The large neutral amino acid transporter (LAT) is expressed at the bovine BBB. It is analogous to the L-preferring system in peripheral tissues and a membrane-fixed transport system. Large amino acids are transported via this system, comprising a heterodimer of the 4F2hc heavy chain and the LAT1 light chain, which is similar to other amino acid transporters (110). From cloning experiments and full-length cDNA, it can be derived that the bovine LAT1 comprises 505 amino acids and has a predictive molecular mass of 55 kDa (111). Using Northern blotting experiments, it was estimated that LAT1 was profoundly upregulated in brain capillaries. The amount of LAT1 mRNA in bovine BCEC was very high compared to other tissues, such as lung, spleen, testes, and heart (111). However, the K_m of the L-system at the BBB was much smaller (10–100 μM) than of those in peripheral tissues [1–10 mM; (112, 113)]. In addition, the K_m of LAT1 at the BBB is similar to the plasma concentration of circulating large amino acids, which means that this transporter is saturated under normal conditions. This makes the brain vulnerable to pathological effects of hyperaminoacidmias (114).

cDNAs of rat and human LAT2 have also been cloned, and it was suggested that LAT2 was expressed at the BBB (115). The K_m of the LAT2 transporter for leucine was shown to be 120 ± 34 μM (115) and approximately 10 times greater than the K_m of leucine by the LAT1 transporter (112). Recently, it was shown that LAT1 is the predominant functional active LAT isoform at the microvascular endothelium of rat brain (116).

The LAT system has been used for the transport of various compounds to the brain. Variations in the cerebellum to plasma ratio at late times in 6-[18F]fluoro-L-DOPA studies are consistent with competitive binding of large neutral amino acids (LNAAs) for the LAT at the BBB (117). In addition, it was shown that oral administration of phenylalanine inhibited the uptake of an artificial amino acid [(11C)-aminocyclohexanecarboxylate] in human brain (118). Melphalan, a nitrogen mustard derivative of the neutral amino acid L-phenylalanine, was transported to the brain via the LAT system at the rat BBB. In addition, it was shown that melphalan competed with phenylalanine for the LAT system (119).

Other data show that under disease state, transport systems may be less available to transport compounds into the brain. It was demonstrated that the transport of phenylalanine by LAT into the brain of patients with phenylketonuria was blocked (120). In addition, EEG analysis revealed that brain activity was acutely disturbed when phenylalanine was given orally without other LNAAs. Following administration with LNAAs, phenylalanine influx was completely blocked and no influence on EEG could be observed.

On the other hand, it was demonstrated that 7-chlorokynurenic acid and 5,7-dichlorokynurenic acid were efficiently taken up by the LAT in the brain when they were administered as their amino acid precursors L-4-chlorokynurenine and L-4,6-dichlorokynurenine (121). In the brain, these precursors were converted again to the parent compounds.

It can be concluded that the LAT1 and -2 transporters are present at the BBB. The LAT1 seems to be more expressed than the LAT2, whereas its K_m is approximately a factor of 10 less. The applicability of this transporter for targeted drug transport to the brain has been demonstrated. In addition, its role at the BBB in diseases has been demonstrated with phenylketonuria where the transport of phenylalanine into the brain was blocked.

ROLE OF TRANSPORT PROCESSES AT THE BLOOD-BRAIN BARRIER: RECEPTOR-MEDIATED TRANSPORT OF DRUGS

Transferrin-Receptor-1 and -2

The brain needs iron-III (Fe) for processes like storage and transport of oxygen, electron transport, DNA synthesis, oxidation-reduction reactions, and cell division (122). Fe-containing Tf (holo-Tf) is transported into the cell by the transferrin-receptor (Tf-R). The Tf-R is heterogeneously distributed within the brain. It is a homodimer of two identical transmembrane subunits, each of 90–95 kDa (123). The receptor subunits are linked by two disulfide bonds (124) and each receptor subunit consists of three domains: a large extracellular C terminus consisting of 671 amino acids, a 28-residue intramembrane part, and an intracellular N-terminal domain of 61 residues (123). The extracellular part of the receptor contains a trypsin-sensitive site and cleavage leads to loss of Tf-binding activity. The Tf-binding site is located at the extracellular domain of the receptor, and each receptor subunit binds one Tf molecule. Tf-R has been identified in capillary endothelial cells in the brain (125).

A second Tf-R (Tf-R2) has been identified that shares 45% identity and 66% similarity in its extracellular domain with Tf-R (126). However, there are currently no indications that the Tf-R2 is expressed at BCEC.

The internalization of Tf occurs via an endocytotic clathrin-coated process, and the internalization signal YTRF is also recognized by clathrin lattices in the trans-Golgi (127). In vitro studies have indicated that there are two different endocytic pathways of clathrin-coated vesicles in cells: a short-term (10–20 min) recycling

pathway and a long-term (2–3 h) recycling pathway involving the movement of Tf-R from endosomes to the Golgi complex (128).

The expression of Tf-R in brain cells is dependent on the developmental stage and it varies with region, cell type, and age (129). In addition, it was shown that Tf-R expression in endothelial cells increased under conditions of Fe deficiency (130), and that oxidative stress leads to a rapid alteration of Tf-R trafficking and a downregulation in K562 and HL60 cells (131).

Recently, several genes have been discovered that encode for proteins that regulate transmembrane iron transport. These are the HFE gene (132), associated with hereditary hemochromatosis, and the DMT1/Nramp2 gene, which encodes for a divalent metal transporter (133). The HFE-protein binds Tf-R tightly at the pH of the cell surface and negatively regulates Tf-mediated iron uptake in transfected cells (134). DMT1 is an iron transporter that is also essential for the transport of iron from the endosomal membrane to the cytosol. In addition, it seems that the regulation of iron homeostasis in the brain is different or more complicated than in other tissues, and that other proteins, like the lactoferrin receptor, melanotransferrin, ceruloplasmin, and the DMT1-transporter, play an important role (135).

It is known that only part of the Tf-Rs are available at the cell surface. At porcine BCEC (129) and bovine BCEC (136), approximately 10% Tf-R was found at the surface of the cells. In our laboratory, we have found a similar percentage at the surface of bovine BCEC, whereas others found that extracellular expression was lower by a factor of three (138).

Internalization via the Tf-R has been demonstrated for various compounds, including systems comprising a compound that has been coupled via a spacer to rat Tf-R monoclonal antibodies [MAb-Tf-R; (139)]. In addition, pegylation of such systems prolonged the circulation time in plasma and resulted in increased brain uptake of brain-derived neurotrophic factor (BDNF). With these systems, it was possible to get neuroprotection against transient focal brain ischemia (139). Further, liposomal drug reservoirs coupled to MAb-Tf-R have been used to deliver plasmid DNA encoding beta-galactosidase (140) or the Photimus pyralis luciferase gene (pGL2 plasmid) to the brain (141). Following intravenous administration in pegylated liposomes without Ab or with the OX-26 MAb-Tf-R, the ^{32}pGL2 plasmid was preferentially taken up by liver and brain, and to a lower extent, by kidney and heart.

Another iron-transporting protein is the melanotransferrin (MTf), or the P97 protein. Its expression has been demonstrated in human brain capillary endothelial cells and it is bound to the plasma membrane via a glycosylpyosphatidylinositol (GPE) anchor. It transports iron independently from the Tf-R route. A role for MTf has been suggested in Alzheimer's disease because its expression has been shown in reactive microglia from amyloid plaques (142).

In conclusion, Tf-Rs are interesting systems. They can internalize relatively large substances or particulate systems, and are therefore suitable for drug targeting. However, Tf-Rs are also present in the liver and in bone marrow, which limits their selectivity and applicability for drug targeting to the brain. The expression of Tf-R is strongly regulated and influenced by many factors.

SCAVENGER RECEPTORS

The presence of the scavenger receptor was demonstrated in bovine and porcine BCECs (143, 144). It is an internalizing membrane-located multifunctional receptor that can transport ligands to the lysosomes where they can be degraded. There are at least nine distinct scavenger receptors that have been cloned (145). These have been organized into classes (A, B, D, etc.). Further subclassification has been done into various types. Particularly, the SR-BI receptor is expressed at BCEC. SR-BI is a member of the CD36 superfamily of proteins. It comprises 509 amino acids, and the rodent SR-BI is identical to the human SR-BI (146). The glycoprotein is heavily N-glycosylated and palmitoylated at the cysteins in the C-terminal cytoplasmic and transmembrane domains. It has a large extracellular loop that is anchored to the plasma membrane and short extension in the cytoplasm. The receptor clusters in caveolae cholesterol-rich lipid domains (147) and there is evidence that SR-BI undergoes rapid endocytosis from the plasma membrane of primary mouse hepatocytes and CHO cells expressing SR-BI (148). The receptor recognizes a broad variety of substrates, particularly chemically modified lipoproteins like acetoacetyl low-density lipoprotein, oxidized LDL, malondialdehyde-conjugates of either LDL or BSA, some polyanions, apoptotic cells, unmodified LDL, and VLDL (145). In addition, it was shown that this receptor binds HDL with high affinity and removes cholesteryl esters from the HDL particle, which is resecreted out of the cell (149). Further, it was shown that acetylated-LDL (AcLDL) was saturably taken up by BCEC, and that the rate of degradation of AcLDL was 20-fold lower in BCEC than in peripheral endothelial cells. In addition, association and degradation could not be influenced by 100 μM chloroquine or 10 mM ammonium chloride, indicating that the lysosomal pathway was at least a minor intracellular route following internalization (143).

The binding of other negatively charged compounds by the scavenger receptor at bovine BCECs was demonstrated by Nakamura et al. (150). They found that binding of naked plasmid DNA was saturable at 4°C and was inhibited by polyinosinic acid and dextran sulphate, which are typical ligands for the macrophage scavenger receptor. In addition, polycytidylic acid or EDTA could not inhibit binding.

Recently, it was shown that compounds that are bound to HDL have the possibility to be internalized by a "piggy-back"-like mechanism. HDL-associated alpha-tocopherol was selectively taken up by SR-BI by porcine BCECs (144). It was shown that the alpha-tocopherol uptake exceeded the uptake of HDL(3) particles up to 13-fold, suggesting a selective uptake of this compound without the concomitant internalization of the lipoprotein particle.

Apolipoprotein (apo) A-I expression was demonstrated in porcine brain capillaries, suggesting an independent lipid metabolism in the brain (151). Apo A-I is the major protein component of HDLs, which are responsible for reverse cholesterol transport from various tissues to the liver via the SR-BI receptor. Further research indicated that apo A-I was effluxed by porcine BCEC, whereas aortic endothelial cells did not. In addition, apo A-I–inducing compounds, such as cholesterol,

insulin, and retinoic acid, could upregulate apo A-I in these cells. These data indicate that at the porcine BCEC apo A-1 is effluxed apparently by the SR-BI receptor.

Other research demonstrated the uptake of the soluble beta-amyloid peptide sAß, which is the major part of Alzheimer's neuritic plaques. sAß was shown to be complexed to ApoJ in HDL(3)- and VHDL (very-high-density-lipid)-particles, and it was suggested that these particles were involved in the delivery of sAß across the BBB (152). Makic et al. (153) found binding of the soluble monomeric 1–40 amino acid peptide Alzheimer amyloid-beta (sA-beta$_{1\text{-}40}$) at the class A, type I scavenger receptor (SB-AI) and the receptor for advanced glycosylation products (RAGE) at human BCECs. Binding was polarized and could be inhibited by anti-RAGE antibodies and acetylated low-density lipoprotein for 63% and 33%, respectively. Transfected CHO-cells overexpressing the SR-AI or RAGE receptor internalized sA-beta$_{1\text{-}40}$, which remained intact. In addition, transcytosis occurred for more than 94%. It was temperature and time dependent and could be partially blocked by anti-RAGE antibodies (36%), but not by 100-fold excess of cold (sA-beta$_{1\text{-}40}$). These observations indicate the importance of the SR-AI receptor at the BBB. It functions as a multiligand receptor, and in that respect, looks similar to the MDR1-, the various MRP transporters, and the various cytochromes P450 (144, 154). However, scavenger receptors also bind particles and pathogens. The latter has lead to the suggestion that these receptors participate in the innate immune system by serving as pattern recognition receptors (155) that bind to a variety of components of pathogens (156).

In conclusion, scavenger receptors are multifunctional receptors with a wide substrate specificity. Particularly, the SR-AI and SR-BI are expressed at BCECs. In addition, these receptors are widely expressed in mammalian tissues, particularly in liver, macrophages, endothelial cells, etc. This makes these receptors less suitable for targeting drugs to the brain. Their role at the BBB seems to be a very important one because the SR-AI receptor seems to be involved with neurodegenerative diseases. In addition, the SR-BI receptor has been shown to play a role in the transport of cholesteryl esters at the BBB. Therefore, malfunction of this receptor can also result in atherosclerotic events leading to neurodegenerative processes in the brain.

CONCLUSIONS

It can be concluded that the role of drug transporters at the BBB can be a critical one. Next to the transport of nutrients like amino acids, glucose, and nucleotides, other systems are present to influx or efflux substances that otherwise cannot be eliminated from the BBB endothelial compartment. Particularly, charged substances will accumulate in the cell when efflux transporters are inhibited or less functional. This may result in toxic concentrations of these substances that can ultimately influence BBB functionality and integrity. Such a situation can occur under acute inflammatory conditions but also with diseases like multiple sclerosis and Alzheimer's disease. Under such conditions, CNS homeostasis can be disturbed, resulting in reduced (cognitive) performance and behavior. It is therefore

very important to understand the role of drug transporters at the BBB and to interfere with their functionality under disease or pathophysiological conditions to restore CNS homeostasis.

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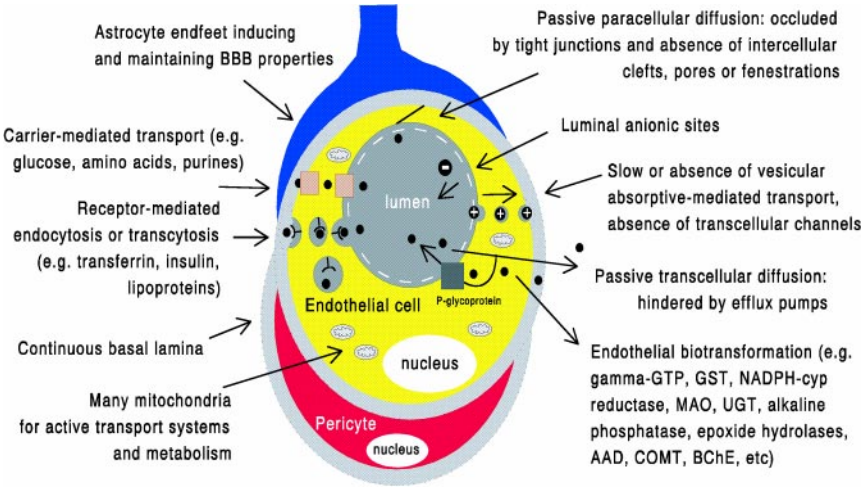

Figure 2 Schematic illustration of the (transport) properties of the blood-brain barrier. Shown is the influence of astrocyte endfeet at the brain capillary endothelial cell. This cell has narrow tight junctions, low pinocytotic activity, many mitochondria, and luminal anionic sites that hinder the transport of negatively charged compounds. Passive hydrophilic transport occurs via paracellular diffusion (tight junctions), whereas passive lipophilic transport is a transcytotic process. Adsorptive-, receptor-, and carrier-mediated transport has been indicated. The metabolic properties of the BBB are illustrated by the various enzymes at the BBB [from (157), with permission].