

Enzyme-Catalyzed Activation of Anticancer Prodrugs

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Abstract—The rationale for the development of prodrugs relies upon delivery of higher concentrations of a drug to target cells compared to administration of the drug itself. In the last decades, numerous prodrugs that are enzymatically activated into anti-cancer agents have been developed. This review describes the most important enzymes involved in pro-drug activation notably with respect to tissue distribution, up-regulation in tumor cells and turnover rates. The following endogenous enzymes are discussed: aldehyde oxidase, amino acid oxidase, cytochrome P450 reductase, DT-diaphorase, cytochrome P450, tyrosinase, thymidylate synthase, thymidine phosphorylase, glutathione *S*-transferase, deoxycytidine kinase, carboxylesterase, alkaline phosphatase, β -glucuronidase and cysteine conjugate β -lyase. In relation to each of these enzymes, several prodrugs are discussed regarding organ- or tumor-selective activation of clinically relevant prodrugs of 5-fluorouracil,

azaxaphosphorines (cyclophosphamide, ifosfamide, and trofosfamide), paclitaxel, etoposide, anthracyclines (doxorubicin, daunorubicin, epirubicin), mercaptopurine, thioguanine, cisplatin, melphalan, and other important prodrugs such as menadione, mitomycin C, tirapazamine, 5-(aziridin-1-yl)-2,4-dinitrobenzamide, ganciclovir, irinotecan, dacarbazine, and amifostine. In addition to endogenous enzymes, a number of nonendogenous enzymes, used in antibody-, gene-, and virus-directed enzyme prodrug therapies, are described. It is concluded that the development of prodrugs has been relatively successful; however, all prodrugs lack a complete selectivity. Therefore, more work is needed to explore the differences between tumor and nontumor cells and to develop optimal substrates in terms of substrate affinity and enzyme turnover rates for prodrug-activating enzymes resulting in more rapid and selective cleavage of the prodrug inside the tumor cells.

I. Introduction

A. General Introduction

For over 50 years chemotherapy has been used with varying success in the treatment of metastatic cancers.

¹Abbreviations: ADEPT, antibody-directed enzyme prodrug therapy; GDEPT, gene-directed enzyme prodrug therapy; VDEPT, virus-directed enzyme prodrug therapy; DPD, dihydropyrimidine dehydrogenase; 5-FU, 5-fluorouracil; 5-FP, 5-fluoro-2-pyrimidione; IUdR, 5-iodo-2'-deoxyuridine; SeCys conjugates, selenocysteine *Se*-conjugates; GSH, glutathione; GS⁻, glutathione anion; tirapazamine (SR 4233), 3-amino-1,2,4-benzotriazine-1,4-dioxide; EO9, 3-hydroxymethyl-5-aziridinyl-1-methyl-2-[1H-indole-4,7-dione]prop-2-en-1-ol; NQO1, NADPH-quinone oxidoreductase-1; NQO2, NADPH-quinone oxidoreductase-2; CB 1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide; dacarbazine (DTIC), 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; HMMTIC, 5-(3-hydroxymethyl-3-methyl-triazen-1-yl)imidazole-4-carboxamide; MTIC, 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide; AQ4N, 1,4-bis-[[2-(dimethylamino-*N*-oxide)ethyl]amino]-5,8-dihydroxyanthracene-9,10-dione; AQ4, 1,4-bis-[[2-(dimethylamino)ethyl]amino]-5,8-dihydroxyanthracene-9,10-dione; 4-*S*-CAP, 4-*S*-cysteaminylphenol; *N*-Ac-4-*S*-CAP, *N*-acetyl-4-*S*-cysteaminylphenol; GHB, γ -L-glutaminy-4-hydroxybenzene; I-GHB, γ -L-glutaminy-4-hydroxy-3-iodobenzene; BVdUMP, (*E*)-5-(2-bromovinyl)-2'-deoxyuridine 5'-monophosphate; NB1011, (*E*)-5-(2-bromovinyl)-2'-deoxy-5'-uridy-phenyl-L-methoxyalaninylphosphor

Most chemotherapeutic agents were discovered empirically with no pre-existing knowledge of the biochemical

amidate; 5'-DFUR, 5'-deoxy-5-fluorouridine; GST, glutathione *S*-transferase; TER286, γ -glutamyl- α -amino- β -(2-ethyl-*N,N,N',N'*-tetrakis(2-chloroethyl)phosphorodiamidate)-sulfonyl-propionyl)-(R)-(-)-phenylglycine; *S*-CPHC-glutathione, *S*-(*N*-*p*-chlorophenyl-*N*-hydroxycarbamoyl)glutathione; PTA, *cis*-3-(9H-purin-6-ylthio)acrylic acid; 6-MP, 6-mercaptopurine; CPT-11 (irinotecan), 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin; SN-38, 7-ethyl-10-hydroxycamptothecin; amifostine (WR-2721), *S*-2-(3-aminopropylamino)-ethylphosphorothioic acid; WR-1065, ; 3-AP, 3-amino-pyridine-2-carboxaldehyde thiosemicarbazone; HMR 1826, ; DNR-GA3, *N*-[4-daunorubicin-*N*-carbonyl-(oxymethyl)-phenyl]O- β -glucuronyl carbamate; DOX-GA3, *N*-[4-doxorubicin-*N*-carbonyl-(oxymethyl)-phenyl]O- β -glucuronyl carbamate; PC, *S*-(6-puriny-1-yl)-L-cysteine; GC, *S*-(guanin-6-yl)-L-cysteine; MeP-dR, 9-(β -2-deoxyerythropentofuranosyl)-6-methylpurine (6-methylpurine-2'-deoxyribonucleoside); HSV-TK, herpes simplex virus type 1 thymidine kinase; CMDA, 4-[*N*-(2-chloroethyl)-*N*-[2-(mesyloxy)ethyl]amino]benzoyl-L-glutamic acid; DPO, doxorubicin-*N*-*p*-hydroxyphenoxyacetamide; MelPO, melphalan-*N*-*p*-hydroxyphenoxyacetamide; NHPAP, *N*-(4'-hydroxyphenylacetyl)palytoxin; LY 266070, ; C-DOX (BMJ 46633), ; CM, 7-(phenylacetamido)-cephalosporin mustard; CCM, 7-(4-carboxybutanamido)-cephalosporin mustard; 5-FC, 5-fluorocytosine; WR 1065, *S*-2-(3-aminopropylamino)ethane thiol; HMR 1826, *N*-[(4'-*R,S*)-4'-ethoxy-4''-(1''-*O*- β -D-glucopyranuronate)-butyl]daunorubicin in sodium salt.

mechanisms of action. More recently, a more rational approach to design prodrugs has been used, which is based on molecular targets that are responsible for cell transformation. However, this approach has been relatively ineffective against malignancies because knowledge about the responsible molecular targets for initiation and progression of cancer is still incomplete (Huang and Oliff, 2001). A major problem with the use of many chemotherapeutic agents is their unacceptable damage to normal cells and organs, a narrow therapeutic index, a relatively poor selectivity for neoplastic cells, and multidrug resistance upon prolonged treatment due to up-regulation of efflux pumps, increased glutathione *S*-transferase expression, and enhanced DNA repair (Lowenthal and Eaton, 1996; Nielsen et al., 1996; Stavrovskaya, 2000).

A potential strategy to overcome the limitations of chemotherapeutic agents is the use of prodrugs. Prodrugs are compounds that need to be transformed before exhibiting their pharmacological action. The term prodrug was introduced in the late 1950s by Albert (1958). Prodrugs are often divided into two groups: 1) prodrugs designed to increase the bioavailability to improve the pharmacokinetics of antitumor agents and 2) prodrugs designed to locally deliver antitumor agents.

B. Prodrugs Designed to Increase the Bioavailability of Antitumor Drugs

Numerous antitumor drugs possess a limited bioavailability due to low chemical stability, a limited oral absorption, or a rapid breakdown in vivo (i.e., by first-pass metabolism) (Connors, 1986). To overcome these problems, various prodrugs that can be activated into antitumor drugs have been designed. In this case it is preferred if prodrugs are activated relatively slowly in the blood or liver, for example, thereby preventing acute toxic effects due to high peak concentrations of the antitumor drug (Connors, 1986). Therefore, prodrugs activated by enzymes with high catalytic efficiencies (k_{cat}/K_m), resulting in a rapid activation of the prodrug, are less attractive than prodrugs activated by enzymes with moderate catalytic efficiencies. An ideal prodrug designed to increase the bioavailability of an antitumor drug is slowly released. After this activation, the drug has to be transported via the bloodstream to the tumor site where it can execute its mode of action. However, due to the reactivity of most antitumor drugs, a limitation of this slow-releasing prodrug concept is that frequently nontumor tissues are also affected. Another drawback of the use of prodrugs activated by enzymes with low catalytic efficiencies may be the metabolism of these prodrugs by competing enzymes into inactive metabolites (Boddy and Yule, 2000).

C. Prodrugs Designed to Increase the Local Delivery of Antitumor Drugs

In this approach prodrugs are designed to achieve a high local concentration of antitumor drugs and to de-

crease unwanted side effects (Connors, 1986; Lowenthal and Eaton, 1996; Dubowchik and Walker, 1999). By this concept, referred to as targeting, organ-specific and tumor-specific prodrug activation can be achieved. To specifically activate prodrugs into a certain organ, either the enzyme involved in the prodrug activation must be selectively present in the target organ or the target organ should selectively take up the prodrug. In the case of tumor-specific targeting, the enzyme responsible for prodrug activation should be uniquely present in the tumor cell. Another possibility for tumor-specific targeting is by making use of hypoxic environments of solid tumors that can be treated with bioreductive prodrugs as described below (Lin et al., 1972; Begleiter, 2000). For both organ- and tumor-specific targeting, enzymes with high catalytic efficiencies are beneficial, enabling a rapid activation of the prodrug. A problem with tumor-specific targeting of prodrugs is that unlike bacteria and viruses, cancer cells do not contain molecular targets completely foreign to the host (Dubowchik and Walker, 1999).

D. Prodrugs Activated by Enzyme Immunoconjugates and by Gene Therapy

An alternative strategy to achieve local activation of prodrugs is the use of enzyme immunoconjugates. In this strategy, which is called antibody-directed enzyme prodrug therapy (ADEPT) or antibody-directed catalysis, antigens expressed on tumors cells are used to target enzymes to the tumor site (Fig. 1A). First, an enzyme-antibody conjugate is administered and allowed sufficient time to bind to tumor cells and to be cleared from the circulation. Subsequently, a prodrug is administered and selectively activated extracellularly at the tumor site. This concept was originally demonstrated by Philpott et al. (1973) and concerned generation of hydrogen peroxide from glucose catalyzed by glucose oxidase.

Meanwhile, significant progress has been made with ADEPT approaches, since it is possible to design and use prodrugs that are not activated by human enzymes by using enzymes of nonhuman origin (Deonarain and Epenetos, 1994; Dubowchik and Walker, 1999; Syrigos and Epenetos, 1999). However, the scarcity of tumor-selective antigens is still a limitation in the applicability of ADEPT. Also, adverse immune effects may cause unwanted results. Another problem with this strategy is that the prodrug is activated extracellularly, and therefore, the antitumor drug that is released still must cross the cell membrane. Furthermore, the lack of effectiveness of ADEPT in humans so far is disappointing in view of the high efficacy observed in rodent models with immunoconjugates (Dubowchik and Walker, 1999).

Alternative approaches designed to circumvent the limitations of ADEPT are gene-directed enzyme prodrug therapy (GDEPT) and virus-directed enzyme prodrug therapy (VDEPT) approaches (Deonarain et al., 1995; Singhal and Kaiser, 1998; Aghi et al., 2000; Smythe,

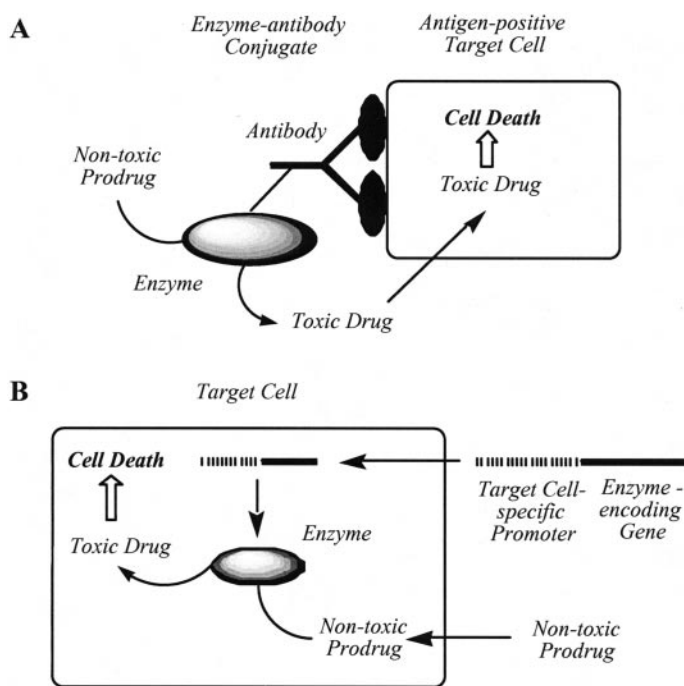


FIG. 1. Principles of drug targeting, using ADEPT, VDEPT, and GDEPT. A, in ADEPT an enzyme antibody is administered that binds to the surface of antigen-presenting target cells. Subsequently, a prodrug is administered that is activated by the enzyme, resulting in the formation of a toxic drug. In most cases the toxic drug has to penetrate the cell membrane to enable cell death. In some cases the drug can cause cell death without penetration through the cell membrane (e.g., palytoxin). B, a DNA construct containing an enzyme-encoding gene is delivered to the tumor cells using nonviral (GDEPT) or viral (VDEPT) vectors. The gene is transcribed, and the generated mRNA is translated to yield the functional enzyme. The enzyme that is subsequently expressed activates intracellularly a nontoxic prodrug into a toxic drug that causes cell death.

2000; Greco and Dachs, 2001). In these approaches, genes encoding prodrug-activating enzymes are targeted to tumor cells followed by prodrug administration (Fig. 1B). In GDEPT, nonviral vectors that contain gene-delivery agents, such as cationic lipids, peptides, or naked DNA, are used for gene targeting. In VDEPT, gene targeting is accomplished using viral vectors, with retroviruses and adenoviruses being the most often used viruses. For both GDEPT and VDEPT, the vector has to be taken up by the target cells, and the enzyme must be stably expressed in tumor cells. This process is called transduction. In addition, the prodrug must penetrate the cell membrane to be activated intracellularly. Because it is generally stated that gene targeting to every cell is impossible, the locally activated drug must also be able to kill nonexpressing cells, a phenomenon known as the “bystander effect.”

GDEPT and VDEPT effectiveness has been limited to date by insufficient transduction of tumor cells *in vivo*; further research is needed to increase transduction. To overcome the common problems in ADEPT, protein engineering to humanize immunoconjugates, optimize their pharmacokinetics, and remove fractions that cause unwanted side effects is essential (Dubowchik and Walker, 1999).

E. Aim and Scope of This Review

Currently, much effort is made in the development of prodrugs that are activated by organ- or tumor-selective human enzymes, and because of the existing limitations of ADEPT, GDEPT, and VDEPT approaches, as illustrated above. To date, many enzymes have been evaluated for their ability to activate prodrugs of antitumor agents. This review provides a comprehensive inventory of these enzymes regarding their tissue distribution and presence in tumor cells. The enzyme kinetic parameters of the enzymes and their respective prodrugs will also be presented to classify each enzyme-prodrug system as a slow-release or organ-/tumor-specific strategy.

Section II describes the endogenous enzymes that are capable of activating antitumor prodrugs, their tissue distribution, and relative presence in tumor and normal tissue. Section III summarizes the most important enzymes and their respective prodrugs that are used in ADEPT, GDEPT, and VDEPT. Because several recent, detailed, and comprehensive reviews on ADEPT, GDEPT, and VDEPT have been published, this review will only give a brief overview of ADEPT, GDEPT, and VDEPT approaches to be able to put the former approaches into an appropriate perspective (Deonarain and Epenetos, 1994; Deonarain et al., 1995; Singhal and Kaiser, 1998; Dubowchik and Walker, 1999; Syrigos and Epenetos, 1999; Aghi et al., 2000; Smythe, 2000; Greco and Dachs, 2001; Huang and Oliff, 2001). In Section IV, the concluding remarks and future perspectives of enzymatic bioactivation of antitumor prodrugs are presented.

II. Prodrugs Activated by Endogenous Enzymes

Numerous enzymes have been used to activate prodrugs of antitumor agents. These enzymes belong to four International Union of Pure and Applied Chemistry classes. Enzymes from class 1 are the oxidoreductases, enzymes from class 2 represent the transferases, enzymes from class 3 are hydrolases, and enzymes from class 4 represent the lyases. The enzyme characteristics, their localization in normal and tumor tissue, and their prodrugs will be discussed in upcoming paragraphs.

A. Class 1 Oxidoreductases

1. Aldehyde Oxidase

a. Enzymology of Aldehyde Oxidase. Aldehyde oxidases (EC 1.2.3.1) are FAD-, molybdenum-, and heme iron-containing enzymes, oxidizing aldehydes to the corresponding acids using molecular oxygen (Schomburg and Stephan, 1990–1998; Klaassen, 1996; Moriwaki et al., 1997). During this redox reaction, superoxide anions are also generated. In addition to aldehydes, these enzymes also catalyze the oxidation of pyrroles, pyridines, purines, pterins, and pyrimidines. Turnover numbers up to 4100 min⁻¹ (2-methyl-butylaldehyde) and greatly varying *K_m* values, i.e., from 0.002 mM (methylene blue)

to 1.3 mM (*N*-methylnicotinamide), have been reported (Schomburg and Stephan, 1990–1998). Aldehyde oxidases are homodimeric proteins with a mol. wt. of 270 to 300 kDa depending on the species (Moriwaki et al., 1997).

b. Localization of Aldehyde Oxidase. Aldehyde oxidase is widely distributed and is mainly located in the cytosolic fraction, although small amounts have been observed in the mitochondria of guinea pig liver (Moriwaki et al., 1997). Based on immunohistochemical staining of aldehyde oxidase in rat tissues, high concentrations of aldehyde oxidase were observed in the liver, esophagus, and lungs, whereas no staining was found in spleen and adrenal (Table 1) (Moriwaki et al., 1996). In human tissues, high immunostaining of aldehyde oxidase in liver and lung was observed. However, in contrast to the rat, aldehyde oxidase in humans was also present in high amounts in adrenal, testis, and prostate tissue (Table 2) (Moriwaki et al., 2001). In human tissues, aldehyde oxidase is not present in bladder, pancreas, ovary, thyroid, brain, skin, and heart. Although significant differences in tissue distribution of aldehyde oxidase occur among humans, rats, mice, and guinea, pig highest levels are present in the liver for all species (Beedham et al., 1987; Moriwaki et al., 1996, 2001; Kurosaki et al., 1999).

Although little is known about the differences of aldehyde oxidase levels between normal and malignant tissues, it has been shown that the specific aldehyde oxidase activity in rat hepatoma cells is 3-fold higher than that observed in normal rat liver tissue (Harvey and Lindahl, 1982).

c. Activation of Prodrugs by Aldehyde Oxidase. 5-Ethynyluracil is a mechanism-based inhibitor of dihydropyrimidine dehydrogenase (DPD), thereby preventing the rapid breakdown of 5-fluorouracil (5-FU). The bioavailability of 5-ethynyluracil is greater than 60%, and the compound lacks organ selectivity. To improve the organ selectivity, 5-ethynyl-2(1H)-pyrimidinone was designed as a liver-specific prodrug (Fig. 2). 5-Ethynyl-2(1H)-pyrimidinone was activated to 5-ethynyluracil by aldehyde oxidase purified from rabbit liver. The prodrug itself did not affect DPD activity (Porter et al., 1994) (Table 3). The catalytic efficiency (k_{cat}/K_m) for 5-ethynyl-2(1H)-pyrimidinone oxidation was 60-fold higher than for *N*-methylnicotinamide, a well known aldehyde oxidase model substrate, and the K_m value of aldehyde oxidase for 5-ethynyl-2(1H)-pyrimidinone was 50 μ M. After oral administration of 5-ethynyl-2(1H)-pyrimidinone to rats (2 or 20 μ g/kg), DPD activity was inhibited to a similar extent in liver, intestine, lung, spleen, and brain (Porter et al., 1994). Whether the lack of liver selectivity was due to a rapid distribution and/or clearance of 5-ethynyluracil or that other enzymes are involved in the bioactivation of 5-ethynyl-2(1H)-pyrimidinone remains unclear.

To overcome the rapid breakdown of 5-FU in the gastrointestinal tract, 5-fluoro-2-pyrimidinone (5-FP) was synthesized as a 5-FU prodrug (Table 3, Fig. 2) (Guo et al., 1995). 5-FP is activated by rat liver aldehyde oxidase with a K_m value of 220 μ M and a V_{max} of 8 nmol/min/mg. After oral or intravenous administration to mice, 5-FP was shown to be rapidly activated to 5-FU by aldehyde oxidase in the liver, whereas aldehyde oxidase activity was not present in the gastrointestinal tract. The half-life of 5-FP in plasma was at least 2-fold higher than that of 5-FU. Despite this interesting tissue selectivity, oral administration of 5-FP showed a similar cytostatic activity as 5-FU toward colon 38 tumor cells and P388 leukemia cells in mice (Guo et al., 1995).

5-Iodo-2'-deoxyuridine (IUdR) has been reported to be an effective radiosensitizer *in vitro* and *in vivo* (Kinsella, 1996). However, IUdR is rapidly metabolized by hepatic and extrahepatic enzymes, thereby limiting its bioavailability (Kinsella, 1996). Therefore, 5-iodo-2-pyrimidinone-2'-deoxyribose was developed as a prodrug and was activated by rat, mouse, and human hepatic aldehyde oxidase to IUdR (Table 3, Fig. 2). Allopurinol, a selective inhibitor of xanthine oxidase, did not alter bioactivation (Kinsella et al., 1994, 1998). The oxidation of 5-iodo-2-pyrimidinone-2'-deoxyribose in other rodent tissues including intestine, bone marrow, lung, brain, and kidney was more than 10-fold lower. The prodrug was further studied in rhesus monkeys and ferrets after oral and intravenous administration (Kinsella et al., 2000). Although its pharmacokinetics was satisfying, significant weight loss and gastrointestinal side effects were observed. However, no biochemical liver function abnormalities were demonstrated in serum. Based on these promising results, initial phase I clinical studies are in progress.

d. Discussion of Aldehyde Oxidase as a Prodrug-Activating Enzyme. Aldehyde oxidase has been used to activate prodrugs. However, the wide distribution of this enzyme does not make it an ideal candidate for organ-selective targeting. This is illustrated by the fact that after oral administration of 5-ethynyl-2(1H)-pyrimidinone to rats' DPD activity, inhibited by released 5-ethynyluracil, was inhibited to a similar extent in liver, intestine, lung, spleen, and brain (Porter et al., 1994). Another problem with aldehyde oxidase is the large difference in substrate specificity between species (Johns, 1967; Guo et al., 1995). Therefore, animal models used to test the efficacy of aldehyde oxidase prodrugs might not be good models for humans. The best strategy is to first optimize prodrug activation by human aldehyde oxidase *in vitro* before testing the efficacy *in vivo* in animals expressing the human enzyme.

2. Amino Acid Oxidase

a. Enzymology of Amino Acid Oxidase. Amino acid oxidases catalyze stereoselectively the oxidative deamination of amino acids to the corresponding α -keto acids ammonia and hydrogen peroxide (Hamilton, 1985;

TABLE 1
Tissue distribution of rat enzymes involved in prodrug activation

	Liver	Kidney	Intestine	Brain	Lung	Adrenal	Testis	Skin	Spleen	Heart	Serum	Stomach	Lymph node	Muscle
Aldehyde oxidase ^a	++ (4.0)	+	(1.3)	++ (-)	++ (0.4)	ND (-)	+	+	ND (0.6)	± ND	- (-)	+	- (-)	ND (-)
Amino acid oxidase ^b														
1-AAO														
d-AAO	(16.2)	(17.1)	(-)	(21.5)	()	(-)	(-)	(-)	(-)	(ND)	(ND)	(-)	(-)	(-)
Cytochrome P450 ^c	0.22-0.92	0.05-0.21	0.02-0.13	0.025-0.051	0.035	0.50	0.05-0.10	0.05	0.025	-	-	-	-	-
P450 reductase ^d	37.1	25.1	47.4	6.1	16.4	53.1	7.1	-	-	-	-	-	-	-
DT-diaphorase ^e	389	28	76	-	117	-	-	-	-	-	-	-	-	-
Thymidylate synthase ^f	0.18	0.07	0.04	0.11	0.39	-	1.42	-	3.32	0.14	-	-	-	ND
Thymidine phosphorylase ^f	14	2.3	25	-	-	-	-	-	ND	-	-	-	-	-
Glutathione S-transferase ^g														
Cytosolic	1400	336	429	190	79	253	3850	-	56	93	-	-	-	-
Microsomal	126	8.5	60	7.9	15	52	129	-	9	7.2	-	-	-	-
Deoxycytidine kinase ⁱ	0.032	-	-	-	-	-	-	-	0.091	-	-	-	-	-
Cysteine conjugate β-lyase ^j	2.2	23.0	0.6	0.8	0.2	-	-	-	0.7	0.9	-	-	-	-

^a Based on immunohistochemical staining; ±, equivocal staining; ++, low staining; +, moderate staining (Moriwaki et al., 1996). Values in parentheses indicate specific activities in guinea pig tissues expressed as nmol/min/mg of protein with phthalazine (Beedham et al., 1987). Intestine value represents sum of ileum, jejunum, and duodenum (Beedham et al., 1987).

^b Expressed as μmol/min/mg of protein with d-alanine as a substrate in hog tissues (Katagiri et al., 1991). In lung tissue the enzyme was detected with an enzyme immunoassay; however, enzyme activity could not be determined.

^c Expressed as nmol/mg of microsomal protein (Vainio, 1980).

^d Expressed as nmol/min/mg of protein with cytochrome c as a substrate (Benedetto et al., 1976).

^e Expressed as nmol/min/mg of protein with menadiol as a substrate (Schlager and Powis, 1990).

^f Expressed as nmol/h/mg of protein with dUMP as a substrate (Hashimoto et al., 1988). High activities were observed in thymus (11.33) and bone marrow (2.70).

^g Expressed as μmol/h/g of tissue with thymidine as a substrate (Zimmerman and Seidenberg, 1964).

^h Expressed as nmol/min/mg of protein with 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate (DePierre and Morgenstern, 1983).

ⁱ Expressed as pmol/min/mg of protein with deoxycytidine as a substrate (Chan et al., 1983). In another study, high activity is present in thymus, bone marrow, spleen, and skeletal muscle (Harkrader et al., 1980).

^j Expressed as nmol/min/mg of protein with S-(2-chloro-1,1,2-trifluoroethyl)-l-cysteine as a substrate (Rooseboom et al., 2002). Similar tissue distribution was observed with various SeCys conjugates and S-(1,2-dichlorovinyl)-l-cysteine (Jones et al., 1988; Rooseboom et al., 2002). Intestine activity represents the sum of large and small intestine.

ND, no detectable enzyme activity or no staining observed; -, no data available.

TABLE 2
Tissue distribution of human enzymes involved in prodrug activation

	Liver	Kidney	Intestine	Brain	Lung	Adrenal	Testis	Skin	Spleen	Heart	Serum	Stomach	Lymph node	Muscle
Aldehyde oxidase ^a	++	+ / + +	+	ND	++	++	++	ND	-	ND	-	±	-	ND
Amino acid oxidase ^b														
L-AAO	0.33	0.35	-	ND	ND	-	-	-	ND	ND	ND	-	-	-
d-AAO	4.51	5.94	-	0.48	ND	-	-	-	ND	ND	ND	-	-	-
Cytochrome P450 ^c	0.26-1.02	0.03	-	-	-	0.23-0.54	0.005	-	-	-	-	-	-	-
DT-diaphorase ^d	17	117	17	-	10	-	-	-	-	-	-	387	-	-
Thymidine phosphorylase ^e	1.37	0.22	0.42	-	1.44	-	-	-	1.12	0.35	-	0.23	1.51	ND
Glutathione S-transferase ^f														
Cytosolic	1.80	1.41	0.66	-	0.61	-	-	-	-	-	-	-	-	-
Microsomal	100	34	23	24	15	-	-	1	14	36	-	-	3	
Deoxytydine kinase ^g	-	-	37	14	-	-	-	-	-	-	-	-	-	11
Cysteine conjugate β-lyase ^h	0.43	0.13	-	-	-	-	-	-	-	-	-	-	-	-

^a Based on immunohistochemical staining. ±, equivocal staining; +, low staining; ++, moderate staining (Moriwaki et al., 2001).

^b Expressed as μmol/min/mg for four individuals with d-alanine as a substrate (Holme and Goldberg, 1982).

^c Expressed as nmol/mg of microsomal protein (Vainio, 1980).

^d Expressed as nmol/mg of protein with dichlorodophenol as a substrate (Schlager and Powis, 1990). A similar tissue distribution was observed with menadione as a substrate.

^e Expressed as nmol/h/mg of protein with thymidine as a substrate; intestine activity represents the sum of colon and small intestine (Yoshimura et al., 1990).

^f Cytosolic GST expressed as nmol/min/mg of protein with benzo[*a*]pyrene-4,5-oxide as a substrate (Pacifci et al., 1988). Microsomal GST expressed as percentage of liver mRNA expression that is known to closely correlate with GST protein and enzyme activity (Buetler et al., 1995; Estonius et al., 1999).

^g Expressed as pmol/min/mg of protein with deoxycytidine as a substrate (Armer et al., 1992, #559). Intestine value represents colon, and heart value represents ventricle.

^h Expressed as nmol/min/mg of protein with S-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine as a substrate (Rooseboom et al., 2000, 2002). Similar tissue distribution was observed with various SeCys conjugates (Rooseboom et al., 2002).

ND, no detectable enzyme activity or no staining observed; -, no data available.

Schomburg and Stephan, 1990–1998; Curti et al., 1992). In addition, amino acid oxidase from rat can dehydrogenate α-hydroxy acids to their corresponding α-keto acids. Amino acid oxidases have also been reported to catalyze β-elimination reactions of some substrates, including β-chloroalanine, β-cyanoalanine, and selenocysteine Se-conjugates (SeCys conjugates), resulting in the formation of chloride, cyanide, and selenols, respectively, and concomitant production of pyruvate and ammonia (Walsh et al., 1971; Miura et al., 1980; Rooseboom et al., 2001b). Amino acid oxidases are flavoproteins that contain FAD as a cofactor, and they are involved in amino acid catabolism and inflammatory responses. L-Amino acid oxidase (EC 1.4.3.2) is a homodimeric flavoprotein with a mol. wt. between 85 and 150 kDa, whereas D-amino acid oxidase (EC 1.4.3.3) is somewhat smaller (mol. wt. 38–125 kDa). The latter has been purified both as a monomer (pig, *Candida tropicalis*) and a homodimer (*Trigonopsis variabilis*, *Rhodotorula gracilis*). Turnover numbers for L-amino acid oxidases up to 11,000 min⁻¹ (L-arginine) and for D-amino acid oxidases up to 43,250 (D-alanine) have been reported. *K_m* values are in the millimolar range, although lower *K_m* values have been measured as well for some amino acids (50–100 μM) (Hamilton, 1985; Schomburg and Stephan, 1990–1998; Curti et al., 1992).

b. Localization of Amino Acid Oxidase. Amino acid oxidases occur in many species and are mainly located in peroxisomes. These cytosolic enzymes are present in various organs, and the tissue distribution of D- and L-amino acid oxidase is very similar (Tables 1 and 2). In mammals, amino acid oxidases are mainly present in the kidney, with liver containing somewhat fewer. However, the enzyme is not present in mouse liver (Konno et al., 1997). Significant levels have also been found in brain, nerve, leukocytes, adrenal cortex, intestine, heart, lung, tongue, skin, stomach, spleen, muscle, and fat tissue (Hamilton, 1985). The ability of L-amino acid oxidase to also oxidize α-hydroxy acids is peculiar to the rat kidney, where the enzyme is frequently designated α-hydroxy acid oxidase. Amino acid oxidase activity in hog has been observed in kidney, liver, and brain (Table 2) (Katagiri et al., 1991). Based on an enzyme immunoassay, the enzyme was detected in low amounts in lung, although no enzyme activity could be detected. In heart tissue from hog amino acid oxidase, activity was not observed (Katagiri et al., 1991). In humans amino acid oxidase is less widely distributed than in rat and hog and was found to a similar extent in kidney and liver, whereas low levels have been observed in the brain (Table 2) (Holme and Goldberg, 1982). Amino acid oxidase activity was not observed in human lung, spleen, heart, and serum (Holme and Goldberg, 1982). Differences in amino acid oxidase concentrations between normal and tumor cells have not been investigated so far.

c. Activation of Prodrugs by Amino Acid Oxidase. D-Alanine was used as a prodrug to induce oxidative stress

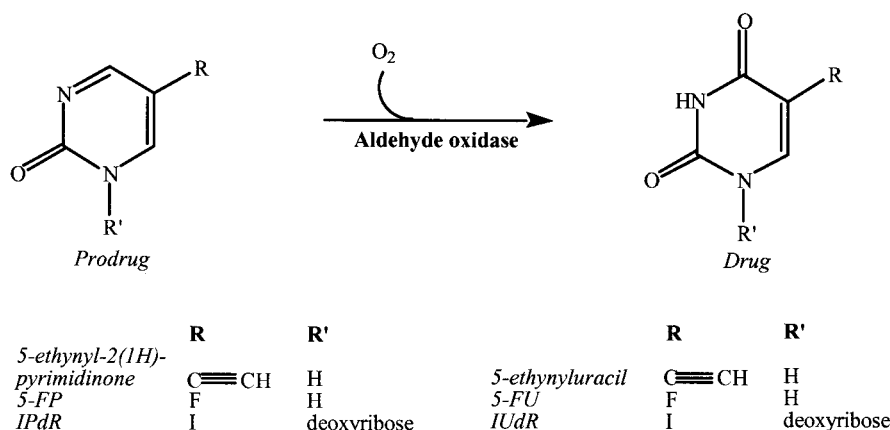


FIG. 2. Activation of prodrugs by aldehyde oxidase.

TABLE 3
Prodrugs activated by aldehyde oxidases

Prodrug	Drug	Pharmacology	Reference
5-Ethynyl-2(1H)-pyrimidinone IPdR	5-Ethynyluracil IUdR	Mechanism-based inhibitor of DPD Radiosensitizer	Porter et al., 1994 Kinsella et al., 1994
5-FP	5-FU	Thymidylate synthase inhibitor/incorporated into DNA and RNA	Guo et al., 1995

in brain tumor cells in vitro based on local bioactivation to hydrogen peroxide by D-amino acid oxidase (Stegman et al., 1998) (Table 4, Fig. 3). cDNA encoding D-amino acid oxidase of *R. gracilis* was mutated to remove the peroxisomal targeting sequence to prevent a possibly rapid breakdown of hydrogen peroxide by peroxisomal catalase. Exposure of brain tumor cells to D-alanine resulted in an elevated cytotoxicity mediated by oxidative stress when compared with parental cells. The K_m value for oxidative deamination of D-alanine for the mutated protein was 0.7 mM, which is comparable with the wild-type protein (0.8 mM) (Stegman et al., 1998).

SeCys conjugates were recently proposed as kidney-selective prodrugs of pharmacologically active selenols (Andreadou et al., 1996; Rooseboom et al., 2000). These compounds have been shown to be potent chemopreventive and antitumor agents (Ip, 1998; Ip et al., 1999) (Table 4). The compounds induce apoptosis in cell lines with wild-type or nonfunctional p53; these effects were not attributable to DNA damage (Ip et al., 2000; Zhu et al., 2000). Although the precise molecular mechanism of apoptosis induction remains to be elucidated, bioactivation to selenols is thought to be critical (Ip, 1998). SeCys conjugates were recently reported to be bioactivated by mammalian amino acid oxidases from rat and hog kidney and L-amino acid oxidase from snake venom to hydrogen peroxide and the corresponding α -keto acid

(Rooseboom et al., 2001b) (Fig. 3). K_m values were in the micromolar range, and the catalytic efficiencies (k_{cat}/K_m) were comparable with that of L-phenylalanine, known as a good substrate for amino acid oxidases.

In addition to oxidative deamination, amino acid oxidases also catalyze β -elimination of SeCys conjugates similarly, resulting in the formation of selenols, pyruvate, and ammonia (Fig. 3) (Rooseboom et al., 2001b). The bioactivation of SeCys conjugates by amino acid oxidase is stereoselective, indicating that the corresponding enantiomers might be used in antitumor and chemopreventive experiments, based on the presence of L-amino acid oxidase or D-amino acid oxidase in those systems. Currently, only racemates are used in such studies (Ip et al., 1999). The concomitant production of selenols and hydrogen peroxide from SeCys conjugates may have an advantage over the above-described hydrogen peroxide generation from D-alanine, because selenols also possess antitumor activity.

In addition to amino acid oxidases, two other enzymes, i.e., cysteine conjugate β -lyases (EC 4.4.1.13; see Section II.D.1.) and flavin-containing monooxygenases (EC 1.14.13.8) are involved in the β -elimination of SeCys conjugates (Commandeur et al., 2000; Rooseboom et al., 2001a). Therefore, the relative contribution of these enzymes will determine the organ-selective activation of these prodrugs. From in vitro studies, the kidney seems

TABLE 4
Prodrugs activated by amino acid oxidases

Prodrug	Drug	Pharmacology	Reference
d-alanine	Hydrogen peroxide	Oxidative stress	Stegman et al., 1998
SeCys conjugates	Selenols and hydrogen peroxide	Apoptosis inducer and cancer preventive agent	Rooseboom et al., 2001b

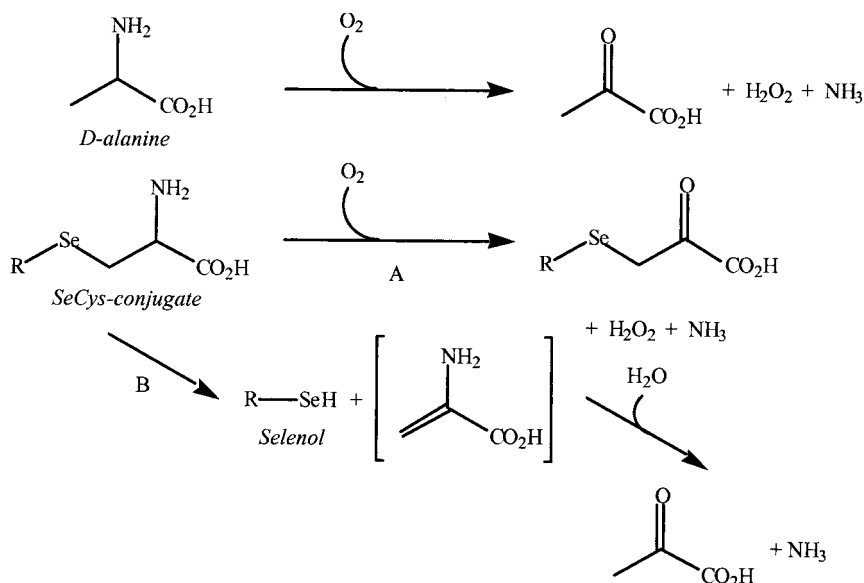


FIG. 3. Activation of prodrugs by amino acid oxidase. Reaction of oxidative deamination of SeCys conjugates (A), and reaction of β -elimination of SeCys conjugates (B). Reaction (B) is also catalyzed by cysteine conjugate β -lyase enzymes (Section II.D.1.).

to be the major organ of prodrug activation, and the organ selectivity was comparable with *S*-(1-chloro-1,2,2-trifluoroethyl)-L-cysteine, which is known to cause selective nephrotoxicity in rodents (Commandeur et al., 1995; Rooseboom et al., 2000, 2002).

d. Discussion of Amino Acid Oxidase as a Prodrug-Activating Enzyme. Amino acid oxidase has been used to activate some prodrugs. Amino acid oxidases in humans are mainly present in liver and kidney with low levels in the brain, thus implicating possible organ-selective targeting. Interestingly, the enzyme is not present in lung, spleen, heart, and serum. Amino acid oxidases demonstrate high turnover numbers toward appropriate substrates and are not genetically polymorphic in European populations (Barker and Hopkinson, 1977), in contrast to many other enzymes. The activation and toxicity of amino acid oxidase-dependent prodrugs have so far only been evaluated in vitro; the in vivo efficacy remains to be established.

3. Cytochrome P450 Reductase

a. Enzymology of Cytochrome P450 Reductase. Cytochrome P450 reductase (EC 1.6.2.4; NADPH-ferrihemoprotein oxidoreductase, cytochrome *c* reductase) is localized in the endoplasmic reticulum and catalyzes the reduction of cytochrome P450s (P450s) using NADPH (Schomburg and Stephan, 1990–1998; Klaassen, 1996). This flavoprotein functions as an electron donor for P450, because electrons are transferred from NADPH to P450 via its FMN and FAD cofactors. The enzyme is able to reduce aldehydes and quinones directly or via P450s. Aldehydes are reduced to the corresponding alcohols, whereas in the case of quinones the one-electron reduction results in the formation of semiquinone free radicals. Semiquinone radicals are readily auto-oxidizable in the presence of oxygen, resulting in the formation of the

parent quinone and superoxide anion, of which the latter can be converted to hydrogen peroxide and hydroxyl radicals, thereby initiating lipid peroxidation. Typical K_m values are in the micromolar range, and turnover numbers up to 6100 min^{-1} (cytochrome *c*) have been reported (Schomburg and Stephan, 1990–1998; Klaassen, 1996).

b. Localization of Cytochrome P450 Reductase. Cytochrome P450 reductase is located in many tissues (Table 1). In rat tissues the highest activity was found in adrenal gland followed by intestine (89% of adrenal activity), liver (70% of adrenal activity), kidney (47% of adrenal activity), and lung (31% of adrenal activity) (Benedetto et al., 1976). Rat testis and brain register relatively low activity, which is only 13% of the adrenal cytochrome P450 reductase activity. The lower cytochrome P450 reductase activity in lung and kidney than in liver (29% and 28%, respectively, of hepatic activity) was also observed by others (Litterst et al., 1975).

The distribution of cytochrome P450 reductase in humans is less well established than that in the rat. Based on immunological staining, the enzyme was shown to be present in a variety of human tissues (Baron et al., 1983; Hall et al., 1989). Strong staining was observed in the liver, lung, and small intestine, whereas the intensity of staining in the stomach and colon was considerably less (Hall et al., 1989). Presence of cytochrome P450 reductase was also shown in pancreas, gall bladder, appendix, adrenal gland, skin, breast, and prostate (Baron et al., 1983; Hall et al., 1989). The tissue distribution in human liver, lung, pancreas, adrenal gland, and gastrointestinal tract is similar to laboratory animals (Hall et al., 1989). However, cytochrome P450 reductase in the human kidney is more widely distributed than in the kidney of rat, rabbit, and minipig (Hall et al., 1989). The

distribution is thought to be correlated with P450s, an enzyme system that is widely distributed in mammals (see Section II.A.5.b. and Tables 1 and 2).

Cytochrome P450 reductase is present in a variety of tumor cell lines including cells from leukemia and melanoma and central nervous system, breast, colon, lung, ovarian, prostate, and renal tumors (Yu et al., 2001). However, the level of activity in these tumor cells does not necessarily reflect that of the corresponding tumor tissue due to loss of enzyme activity as a result of cell culturing. Data on the levels of cytochrome P450 reductase between normal and tumor cells are diverse. In general, cytochrome P450 reductase activity is lower in tumor tissue than in the corresponding normal tissue and correlates with P450 activity (Forkert et al., 1996). Based on a study performed with human lung and breast tumors, only a small variation in cytochrome P450 reductase activity in tumor tissues versus normal tissues was observed (Lopez de Cerain et al., 1999). Recently, it was shown that the specific activity of cytochrome P450 reductase may increase up to 1.8-fold in human liver cancer tissue compared with normal tissue (Plewka et al., 2000).

c. Activation of Prodrugs by Cytochrome P450 Reductase. Several bioreductive prodrugs have been developed as anticancer agents (Table 5, Fig. 4) (Ross et al., 1996; Begleiter, 2000). The rationale for the development of bioreductive prodrugs is the presence of low levels of oxygen in solid tumors compared with normal tissues (Lin et al., 1972). Bioreductive prodrugs are being activated into their radical intermediates, which are oxidized to the prodrug under aerobic conditions. However, under the hypoxic conditions (e.g., solid tumors), this oxidation is much slower and thus usually results in higher levels of toxic radical intermediates, resulting in a solid tumor-selective therapy. In general, hypoxic cells in low oxygen tension regions are more resistant to treatment with radiotherapy, requiring a 2- to 3-fold higher radiation dose, indicating the importance of bioreductive drugs (Denny and Wilson, 2000).

Menadione (vitamin K3; 2-methyl-1,4-naphthoquinone), a synthetic derivative of vitamin K1, displays antitumor activity against a variety of tumor cells, such as tumors from liver, colon, lung, stomach, and prostate and leukemia (Nutter et al., 1991; Jamison et al., 2001). Menadione also significantly reduced the growth rate of solid prostate tumors in nude mice, without any significant toxicity to bone marrow, changes in organ weight, or pathological changes to these organs (Jamison et al.,

2001). Menadione is activated by cytochrome P450 reductase via a one-electron reduction resulting in the formation of a semiquinone radical, which subsequently may reduce molecular oxygen to superoxide anion (Table 5) (Misaka and Nakanishi, 1965). This oxidative stress induces a variety of effects, including depletion of glutathione (GSH), induction of single-stranded DNA breaks, and apoptosis. Menadione is also metabolized via a two-electron reduction by DT-diaphorase and carbonyl reductase (Jamison et al., 2001). In this case the nontoxic hydroquinone is generated, and therefore, the balance between the one- and two-electron reduction determines the extent of oxidative stress response (Nutter et al., 1991; Jamison et al., 2001).

Mitomycin C is a naturally occurring, clinically used, bioreductive alkylating prodrug that is activated by several enzymes, including cytochrome P450 reductase and DT-diaphorase (Bachur et al., 1979; Keyes et al., 1984; Tomasz, 1995; Hargreaves et al., 2000) (Table 5 and Section II.A.4.c.). After reductive activation of the quinone moiety, a series of spontaneous rearrangements results in the opening of the aziridine ring and the formation of a quinone methide intermediate, which alkylates the DNA (for a review, see Tomasz, 1995). Other DNA-alkylating agents have also been identified (Spannick et al., 1998). Mitomycin C has been shown to kill preferentially hypoxic tumor cells (Rockwell and Sartorelli, 1990) and increases cure rates in head and neck cancer patients when used as an adjuvant to radiotherapy (Haffty et al., 1993). Enzymes involved in the activation of mitomycin C and its analog porfiromycin are cytochrome P450 reductase, DT-diaphorase, xanthine dehydrogenase, and xanthine oxidase. However, based on studies in Chinese hamster ovary cell lines, cytochrome P450 reductase plays a predominant role in the hypoxic activation of these prodrugs (Belcourt et al., 1998). In this study Chinese hamster ovary cells were transfected with cDNAs of cytochrome P450 reductase or DT-diaphorase. In comparison with the parental cell line, the cells transfected with either cDNA-encoding cytochrome P450 reductase or DT-diaphorase were more sensitive toward mitomycin C and its analog porfiromycin, indicating an important role for both enzymes. However, no difference in cytotoxicity of mitomycin C and porfiromycin was observed under both hypoxic and aerobic conditions for DT-diaphorase-expressing cells, whereas for cytochrome P450 reductase-expressing cells, cytotoxicity was greater under hypoxic conditions than under aerobic conditions (Belcourt et al., 1998). In

TABLE 5
Prodrugs activated by cytochrome P450 reductase

Prodrug	Drug	Pharmacology	Reference
Menadione	Semiquinone radical	DNA alkylation/oxidative stress	Misaka and Nakanishi, 1965
Mitomycin C	Quinone methide intermediate	DNA alkylation/ oxidative stress	Bachur et al., 1979
Tirapazamine	Nitroxide radical	DNA alkylation/ oxidative stress	Walton et al., 1989
EO9	Unidentified semiquinone radical	DNA alkylation/ oxidative stress	Saunders et al., 2000a

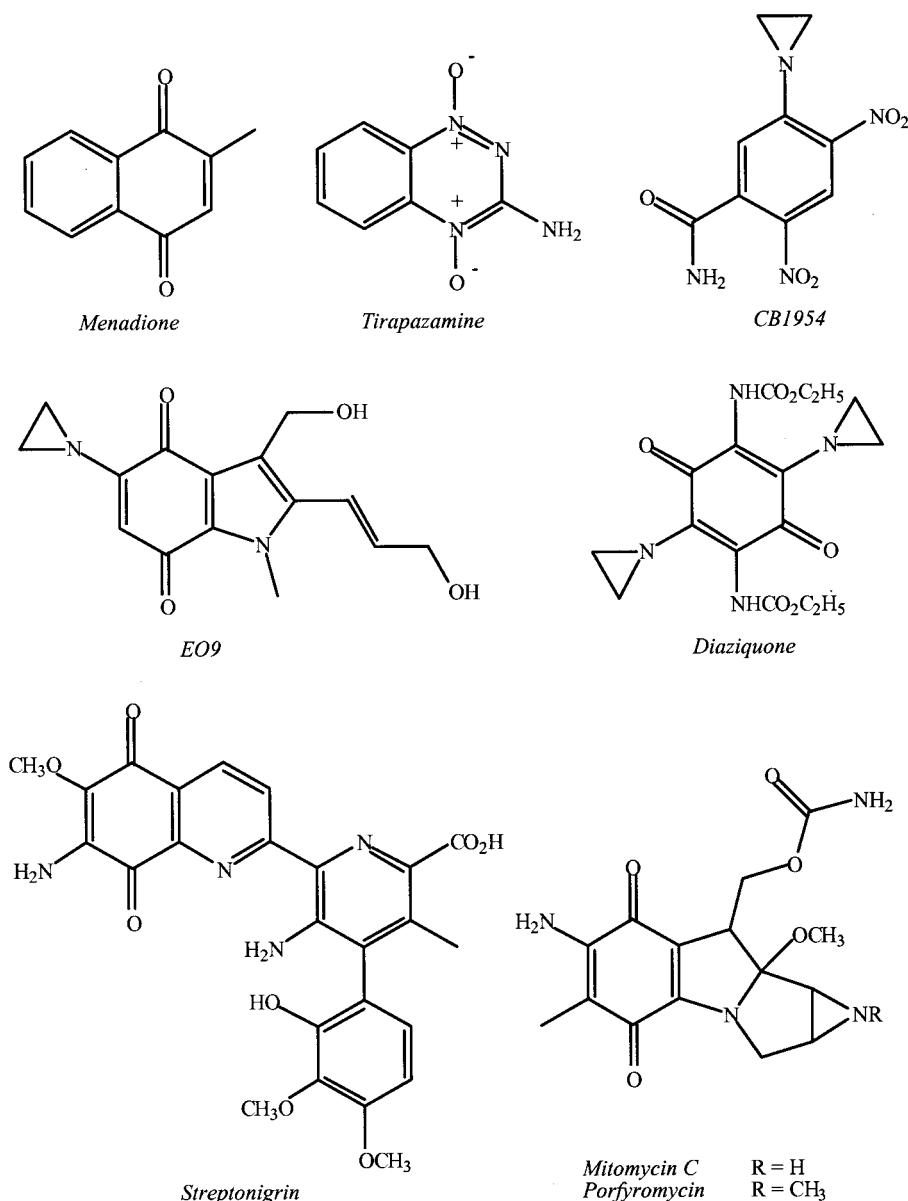


FIG. 4. Bioreductive drugs that are activated by cytochrome P450 reductase and DT-diaphorase.

another study, it was also shown that mitomycin C is mainly activated by cytochrome P450 reductase to DNA binding adducts in COS1 cells expressing human cytochrome P450 reductase. Only at high concentrations of mitomycin C did DT-diaphorase play a role in the activation (Joseph et al., 1996).

Another clinically used prodrug, tirapazamine, is a bioreductive agent that is activated by a one-electron reduction by cytochrome P450 reductase to a cytotoxic nitroxide radical intermediate before further reduction to the nontoxic metabolite SR 4317 (3-amino-1,2,4-benzotriazine-1-oxide) (Walton et al., 1989; Brown, 1993; Denny and Wilson, 2000) (Table 5). Because the nitroxide radical is quenched by molecular oxygen under aerobic conditions, it has been shown to cause selective toxicity by both DNA alkylation and formation of hydroxyl radicals in hypoxic cells (Brown, 1993). Although

tirapazamine appears to be predominantly bioactivated by cytochrome P450 reductase, other enzymes such as P450 and DT-diaphorase may play a role as reviewed by Patterson et al. (1998). Tirapazamine is reduced by DT-diaphorase via four-electron reduction to the inactive benzotriazine SR 4330 (3-amino-1,2,4-benzotriazine) (Walton et al., 1992). Tirapazamine was active against human breast tumor cells and human nonsmall-cell lung cancer cells; cytochrome P450 reductase activity correlated with cytotoxicity in both kinds of cells (Patterson et al., 1995a; Chinje et al., 1999). Furthermore, cells overexpressing human cytochrome P450 reductase were more sensitive toward tirapazamine than parental cells, and cells with a lower cytochrome P450 reductase activity were less sensitive (Patterson et al., 1997; Saunders et al., 2000b). These promising preclinical studies with tirapazamine have resulted in clinical evaluation for

treatment against lung, head, and neck cancer (Rodríguez et al., 1996; Lee et al., 1998), and as such this prodrug is currently in phase II and III clinical trials as an adjunct to cisplatin-based chemotherapy or radiation.

The activation of another clinically used prodrug EO9 (3-hydroxymethyl-5-aziridinyl-1-methyl-2[1H-indole4,7-dione]prop-2-en-1-ol) has been shown to be catalyzed by cytochrome P450 reductase and DT-diaphorase (see *Section II.A.4.c.*) (Walton et al., 1991). Based on studies performed with breast cancer cells expressing cytochrome P450 reductase, it was shown that both under aerobic and hypoxic conditions cytochrome P450 reductase contributes to the bioactivation of EO9 (Saunders et al., 2000a) (Table 5). Furthermore, using purified rat cytochrome P450 reductase it was shown that the formation of a free radical- and DNA-damaging species from EO9 was catalyzed by this enzyme (Bailey et al., 2001). The relative contributions of the enzymes cytochrome P450 reductase and DT-diaphorase remain unclear, however.

d. Discussion of Cytochrome P450 Reductase as a Prodrug-Activating Enzyme. Because several bioreductive drugs (e.g., mitomycin C) are activated by both cytochrome P450 reductase and DT-diaphorase, the use of cytochrome P450 reductase as an enzyme to activate prodrug will be discussed in *Section II.A.4.d.*

4. DT-diaphorase

a. Enzymology of DT-Diaphorase. DT-diaphorase [EC 1.6.99.2; NAD(P)H dehydrogenase (quinone)] catalyzes the two-electron reduction of several substrates, including quinones such as vitamin K, using NAD(P)H (Schomburg and Stephan, 1990–1998; Ross et al., 1994; Klaassen, 1996). DT-diaphorase is a flavoprotein containing FAD and FMN as cofactors, and it reduces quinones to the corresponding hydroquinones (diols). This reductive pathway is considered a detoxification pathway since it is generally not associated with oxidative stress, unlike one-electron reduction reactions of quinones by cytochrome P450 reductase, although there are exceptions (see below) (Ross et al., 1994). DT-diaphorase is a homodimeric protein with subunits of 27 kDa. Mouse, rat, and human DT-diaphorase appear to possess two, three, and four forms of this enzyme, respectively (Riley and Workman, 1992). The enzyme NADPH-quinone oxidoreductase-1 (NQO1) accounts for the majority of DT-diaphorase activity present in human tissues. Another DT-diaphorase enzyme known as NADPH-quinone oxidoreductase-2 (NQO2) is polymorphic expressed in humans (Jaiswal et al., 1990). Turnover numbers for DT-diaphorase are often high and have been reported to be up to $700,000 \text{ min}^{-1}$ (menadione), and K_m values are often in the micromolar range (Maerki and Martius, 1960).

b. Localization of DT-Diaphorase. DT-diaphorase, which is located in the cytosol, is present in virtually all mammalian tissues, including liver, brain, heart, lung, kidney, small intestine, and mammary gland (Tables 1

and 2). In the rat the highest DT-diaphorase activity is with menadione as a substrate present in the liver, followed by lung, colon, and kidney (Schlager and Powis, 1990). However, using dichloroindophenol as a substrate, the highest activity was observed in the lung closely followed by the liver (90% of lung activity), whereas colon (64% of lung activity) and kidney (16% of lung activity) were of less importance (Schlager and Powis, 1990). Whether this is due to the involvement of other enzymes in the metabolism of menadione compared with dichloroindophenol is not known.

The tissue distribution of DT-diaphorase in humans is significantly different from that in rats (Table 2) (Schlager and Powis, 1990). Highest DT-diaphorase activity levels in humans are found in the stomach with lower activity levels in kidney (30% of stomach activity), breast (13% of stomach activity), colon (4% of stomach activity), liver (4% of stomach activity), and lung (3% of stomach activity) (Schlager and Powis, 1990). High mRNA levels of NQO1 in humans have been found in skeletal muscle, whereas lung, liver, and kidney contain lower amounts, and a very low expression is observed in heart tissue (Jaiswal, 1994). Expression of NQO2 mRNA is highest in kidney. Lower amounts of NQO2 mRNA have been found in skeletal muscle, liver, and lung, whereas heart and brain amounts are even lower (Jaiswal, 1994).

Interestingly, DT-diaphorase levels are often strongly elevated in tumor tissues compared with normal tissues, making it an interesting target enzyme (Schlager and Powis, 1990; Riley and Workman, 1992; Spanswick et al., 1998). Elevated levels of DT-diaphorase in human tissues have been observed in primary tumors from lung (12- to 18-fold), liver (4- to 19-fold), colon (3- to 4-fold), and breast (3-fold) compared with normal tissue. However, 8- to 12-fold lower DT-diaphorase activity was found in kidney tumors, compared with nontumor tissue and stomach tumors having 2- to 3-fold lower DT-diaphorase activity, compared with nontumor tissue (Schlager and Powis, 1990).

c. Activation of Prodrugs by DT-Diaphorase. Streptonigrin is an aminoquinone antibiotic isolated from cultures of *Streptomyces flocculus* and has been shown to possess antitumor activity against lymphomas, leukemias, and melanomas and is activated by DT-diaphorase (Kremer and Laszlo, 1966; Bolzan and Bianchi, 2001) (Table 6, Fig. 4). Although the active metabolite has not been identified so far, the quinone moiety is essential since the antitumor activity is lost when the quinone moiety is absent as in isopropylidene azastreptonigrin (Kremer and Laszlo, 1967). Streptonigrin was also cytotoxic toward human colon carcinoma cells, and this cytotoxicity was correlated to DT-diaphorase activity (Beall et al., 1996; Winski et al., 2001). Activation of streptonigrin causes cytotoxicity via DNA strand breaks and inhibits DNA and RNA synthesis as secondary effects to ATP depletion and formation of hydroxyl radi-

TABLE 6
Prodrugs activated by DT-diaphorase

Prodrug	Drug	Pharmacology	Reference
Streptonigrin	Unidentified	DNA alkylation/oxidative stress	Kremer and Laszlo, 1966
Mitomycin C	Quinone methide intermediate	DNA alkylation/oxidative stress	Keyes et al., 1984
CB 1954	5-(Aziridin-1-yl)-4-hydroxyl-amino-2-nitrobenzamide	DNA alkylation/oxidative stress	Knox et al., 1988
Diaziquone	Semiquinone radical ^a	DNA alkylation/oxidative stress	Ross et al., 1990

^a DT-diaphorase activates diaziquone to its hydroquinone via a two-electron reduction, which auto-oxidates to a semiquinone radical (Fisher and Gutierrez, 1991).

cals and hydrogen peroxide (Kremer and Laszlo, 1966; Beall et al., 1996). The genotoxic effects of this antitumor prodrug have been reviewed recently (Bolzan and Bianchi, 2001).

As described above, mitomycin C is a naturally occurring, clinically used, bioreductive alkylating prodrug that is activated by DT-diaphorase, xanthine oxidase, xanthine dehydrogenase, and cytochrome P450 reductase (Table 6 and Section II.A.3.c.) (Bachur et al., 1979; Keyes et al., 1984; Tomasz, 1995; Cummings et al., 1998; Spanswick et al., 1998; Hargreaves et al., 2000). The role of DT-diaphorase in the bioactivation of mitomycin C has been somewhat controversial. However, the use of cell lines with high and low DT-diaphorase activity showed a role for this enzyme, and DT-diaphorase from rat liver carcinoma cells was able to bioactivate mitomycin C to the DNA-alkylating intermediates (Suresh Kumar et al., 1997; Cummings et al., 1998; Spanswick et al., 1998). Human DT-diaphorase was shown to be much less effective than the rat enzyme, and it has been proposed to be less efficient than other mitomycin C-metabolizing enzymes (Cummings et al., 1995; Chen et al., 1997). Mitomycin C acts not as a substrate but as an inhibitor of purified human kidney DT-diaphorase, and mitomycin C is not a substrate for purified rat liver DT-diaphorase (Workman et al., 1989). On the other hand, most tumors overexpress DT-diaphorase, making it an interesting target enzyme for novel bioreductive drugs (Workman et al., 1989; Schlager and Powis, 1990; Spanswick et al., 1998).

5-(Aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) is activated to the cytotoxic metabolite 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide by both *Escherichia coli* nitroreductase and rat DT-diaphorase isolated from Walker cells (Knox et al., 1988, 1992) (Table 6, Fig. 4). The rat DT-diaphorase was able to reduce the 4-nitro group of CB 1954, whereas the *E. coli* nitroreductase could reduce either of the 2-nitro groups. CB 1954 has been shown to be reduced with four electrons by both rat and human DT-diaphorase, preferentially to the highly cytotoxic 4-hydroxylamine (Walton et al., 1992). However, the bioactivation of CB 1954 is too slow in human tumor cells (Boland et al., 1991). A novel antitumor prodrug therapy was developed (Knox et al., 2000). It was shown that the DT-diaphorase enzyme NQO2 was able to bioactivate CB 1954. This enzyme is present in human tumor cells but appeared to be inactive. Upon cell exposure to reduced nicotinamide riboside or other

reduced pyridinium compounds, the enzyme NQO2 was activated, resulting in a 100- to 3000-fold increase in the cytotoxicity of CB 1954. NQO2 activity appeared to be related to the expression of NAD(P)H dehydrogenase (quinone) (DT-diaphorase; NQO1) (Knox et al., 2000). As described later (Section III.A.), ADEPT and GDEPT approaches have been developed for CB 1954 in combination with *E. coli* nitroreductase.

Diaziquone [2,5-bis(carboethoxyamino)-3,6-diaziridinyl-1,4-benzoquinone] is an antitumor prodrug (Fig. 4) that is bioactivated by DT-diaphorase purified from human colon carcinoma cells (HT-29). The cytotoxicity and DNA strand breakage induced by diaziquone in these cells were inhibited by the specific DT-diaphorase inhibitor dicoumarol (Ross et al., 1990; Siegel et al., 1990) (Table 6). The bioactivation of diaziquone via two-electron reduction catalyzed by DT-diaphorase results in the formation of the corresponding hydroquinone, which auto-oxidized in the presence of molecular oxygen leading to the semiquinone radical, superoxide, hydrogen peroxide, and hydroxyl radical, thereby inducing oxidative stress (Fisher and Gutierrez, 1991). Cytochrome P450 reductase also activates diaziquone to the semiquinone radical, superoxide, hydrogen peroxide, and hydroxyl radical but by a one-electron reduction pathway (Fisher and Gutierrez, 1991). Several analogs of diaziquone were synthesized and tested as reviewed recently (Hargreaves et al., 2000).

d. Discussion of DT-Diaphorase and Cytochrome P450 Reductase (Section II.A.3.) as Prodrug-Activating Enzymes. Hypoxia, which often occurs in solid tumors, appears to be an attractive target as evidenced by the development of successful bioreductive prodrugs such as tirapazamine. Bioreductive prodrugs are activated to cytotoxic agents (often radicals' intermediates) that are rapidly oxidized back to the parent nontoxic prodrugs in tissues with high levels of oxygen, whereas in hypoxic tissues (e.g., solid tumors) quenching is less efficient, resulting in a solid tumor-selective therapy (Lin et al., 1972). This selectivity is of special interest, since hypoxic solid tumors are more resistant to treatment with radiotherapy, requiring a 2- to 3-fold higher radiation dose (Denny and Wilson, 2000). Cytochrome P450 reductase and DT-diaphorase are enzymes that activate bioreductive drugs as discussed in this review. However, these enzymes are widely distributed, and therefore, bioreductive drugs, activated by these enzymes, often suffer from side effects. Furthermore, DT-diaphorase is

inducible up to 10-fold by various compounds, such as alcohol and cigarette smoke, thus resulting in possible differences in drug response between individuals (Schlager and Powis, 1990; Klaassen, 1996). Cytochrome P450 reductase activity is generally lower in tumor tissue than in the corresponding normal tissue (Forkert et al., 1996). On the other hand, most tumors overexpress DT-diaphorase, making it an interesting target enzyme for bioreductive drugs (Workman et al., 1989; Schlager and Powis, 1990; Spanswick et al., 1998). However, the role of DT-diaphorase in the bioactivation of bioreductive drugs seems questionable, because the human enzyme is not very active (Riley and Workman, 1992). Additionally, the enzyme normally catalyzes two-electron reduction reactions that generate stable hydroquinones and can therefore be classified as a detoxifying enzyme. Overexpression of this enzyme in NIH 3T3 cells to levels found in human tumors did not increase the cytotoxicity of mitomycin C, diaziquone, and menadione (Powis et al., 1995).

Both cytochrome P450 reductase and DT-diaphorase activate many bioreductive prodrugs, and the relative contribution to prodrug activation depends on the respective enzyme levels. Therefore, tumor enzyme profiling to determine individual levels of cytochrome P450 reductase and DT-diaphorase may be beneficial to the treatment of patients. This concept is known as enzyme-directed bioreductive drug therapy and can increase individual drug responses (Spanswick et al., 1998).

5. Cytochrome P450

a. Enzymology of Cytochrome P450. The superfamily of P450 enzymes (EC 1.14.14.1) catalyze the mono-oxygenation of substrates in which one atom of molecular oxygen is incorporated into a substrate and the other is reduced to water and uses NADPH as a cofactor (Schomburg and Stephan, 1990–1998; Klaassen, 1996; Shou et al., 2001). The enzyme system has a broad substrate specificity and catalyzes several types of oxidation reactions, including hydroxylations, epoxidations, heteroatom oxidations, heteroatom dealkylations, oxidative group transfer, cleavage of esters, and dehydrogenations. Mammalian P450s, which are heme-containing microsomal proteins, are involved in the biosynthesis or catabolism of steroid hormones, bile acids, fat-soluble vitamins, fatty acids, and eicosanoids. The P450 enzymes are divided into families based on their homology in amino acid sequence identity. The most important human isoenzymes for metabolism of drugs and other xenobiotics are CYP1A1/2, 2C9/19, 2D6, 2E1, and 3A4 (Wormhoudt et al., 1999; Lin and Lu, 2001). A number of P450 enzymes are genetically polymorphic in Caucasians (Wormhoudt et al., 1999). Deficiency of functional protein is occurring at the following frequencies: CYP1A <1%, 2C9/19 2 to 5%, and 2D6 5 to 10%. The concentration of individual P450 enzymes in liver microsomes differs greatly, i.e., from 5 ± 4 pmol/mg for 2D6 to $96 \pm$

51 pmol/mg for 3A determined from 60 individuals (Shimada et al., 1994).

b. Localization of Cytochrome P450. P450 enzymes are the most important drug-metabolizing enzymes of mammals. The enzyme system is present in virtually all mammalian tissues, with highest concentrations in the liver (Tables 1 and 2). In liver the enzyme system is located on the endoplasmic reticulum (microsomes). Similar P450 contents compared with the liver can be found in the adrenal gland (Tables 1 and 2). P450 contents in the kidney, intestine, brain, lung, testis, skin, and spleen are lower than those in liver tissue (Vainio, 1980). The tissue distribution between humans and rodents is very much comparable (Tables 1 and 2).

It has been shown that P450 is present in a variety of tumors, including those from the central nervous system, breast, colon, lung, ovarian, prostate, and kidney; however, their relative levels compared with normal tissue is less well established (Murray et al., 1995; Yu et al., 2001). Immunohistochemical staining showed that primary and secondary liver tumors from patients can express CYP1A1, 2A6, 2B6, 2C8/9, 3A4, and 4A, although the levels are much lower than those present in normal liver tissue (Philip et al., 1994). Also, renal cell tumors express CYP3A4/5/7. Other tumor cells that contain CYP3A enzymes are tumor cells from colon adenocarcinoma and stomach, breast, bladder, prostate, and lung cancers (Murray et al., 1995). Expression of P450s in colon and lung tumors is lower than in the corresponding normal tissue. In contrast, several reports show higher levels of CYP1A and CYP3A in tumors than in their corresponding normal tissue as reviewed by Patterson et al. (1999).

c. Activation of Prodrugs by Cytochrome P450. Although most P450 prodrugs were not designed as prodrugs, these chemotherapeutic agents have been shown to be activated by P450s. 4-Ipomeanol (1-(3-furyl)-4-hydroxy-1-pentanone) is a naturally occurring furanoterpenoid isolated from common sweet potatoes infected with the fungus *Fusarium solani* (Fig. 5) (Boyd and Wilson, 1972; Boyd, 1977). Although not designed as a prodrug, 4-ipomeanol was shown to be metabolized by P450s to a highly reactive electrophilic compound, speculated to be a furan epoxide, that binds covalently to macromolecules including DNA (Table 7, Fig. 5). Both piperonyl butoxide and GSH completely abolished the covalent binding of ipomeanol, indicating the involvement of P450 in the formation of electrophilic metabolites (Gram, 1997). Bioactivation is catalyzed by CYP4B1, which is found in most mammalian pulmonary Clara cells and type II pneumocytes. Metabolic activation was studied using 12 human and 2 rodent P450s that were expressed in Hep G2 cells (Czerwinski et al., 1991). 4-Ipomeanol was shown to be metabolized to DNA-reactive metabolites by human CYP1A2, 3A3, and 3A4, which are not constitutively expressed in human lung cells, whereas CYP2F1 and CYP4B1, which are expressed in lung, displayed

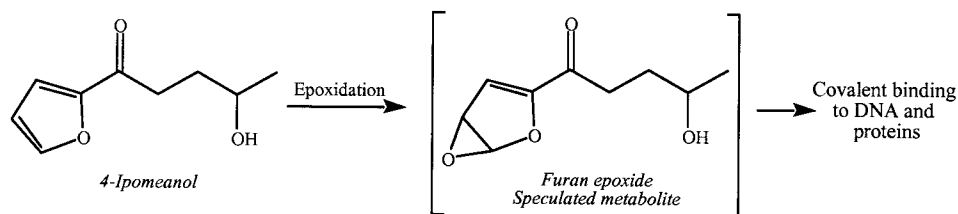


FIG. 5. Structure of 4-ipomeanol, which is activated by cytochrome P450.

TABLE 7
Prodrugs activated by cytochrome P450

Prodrug	Drug	Pharmacology	Reference
4-Ipomeanol	Unidentified (furan epoxide is speculated)	DNA alkylation	Boyd, 1977
Ftorafur (tegafur)	5-FU	Thymidylate synthase inhibitor/incorporated into DNA and RNA	Kawata et al., 1984
Dacarbazine	HHMTIC	DNA alkylation	Meer et al., 1986
Trofosfamide	Trofosfamide mustard	DNA alkylation	Della Morte et al., 1986
Ifosfamide	Isophosphamide mustard	DNA alkylation	Della Morte et al., 1986
Cyclophosphamide	Phosphoramidate mustard	DNA alkylation	Roy et al., 1999
AQ4N	AQ4	Topoisomerase II inhibitor	Raleigh et al., 1998

only modest activity toward 4-ipomeanol. From all 14 P450s tested, rabbit CYP4B1 was the most active generator of DNA-reactive metabolites from 4-ipomeanol. In phase I clinical trials that were conducted with 4-ipomeanol in lung cancer patients, however, no anti-tumor response was observed, although hepatocellular toxicity, indicated by elevation of hepatic transaminases such as alanine amino transferase, was reported (Kasturi et al., 1998). Because 4-ipomeanol was not active toward human lung cancer but did induce hepatotoxicity, it was studied also in phase II clinical trials in liver cancer patients. However, no clinical activity against hepatocellular carcinoma was observed in 19 patients treated with 4-ipomeanol (Lakhanpal et al., 2001). 4-Ipomeanol was also used in a GDEPT approach to treat hepatocellular carcinoma using cDNA-encoding rabbit CYP4B1 as described below (Section III.B.).

Ftorafur [tegafur; 5-fluoro-1-(tetrahydro-1-furyl)uracil] is a slow-releasing prodrug of 5-FU that has been used for over 20 years as an anticancer drug (Table 7, Fig. 6). Ftorafur avoids the rapid breakdown of 5-FU in the gastrointestinal tract. 5-FU inhibits the growth of cancer cells by thymidylate synthase inhibition and by incorporation into DNA and RNA (Au and Sadee, 1981;

Katona et al., 1998). Ftorafur is activated to 5-FU by both cytosolic thymidine phosphorylase and microsomal P450s (Kawata et al., 1984; Sugata et al., 1986; Komatsu et al., 2001). The activation of ftorafur occurs mainly in the liver. The bioactivation of ftorafur to 5-FU by human liver microsomes was 5- to 2-fold higher than that in human liver cytosol (containing thymidine phosphorylase). The apparent K_m value in human liver microsomes was also 9 times lower than that in human liver cytosol, indicating that ftorafur is mainly bioactivated by P450s (Komatsu et al., 2001). Bioactivation of ftorafur to 5-FU by human P450 isoenzymes has been investigated (Ikeda et al., 2000; Komatsu et al., 2000). The prodrug was shown to be activated by CYP1A2, 2A6, 2E1, and 3A5, and the highest catalytic efficiency (k_{cat}/K_m) was observed for CYP1A2 ($10 \text{ ml} \cdot \text{min}^{-1} \cdot \text{nmol}^{-1}$) (Komatsu et al., 2000). In studies with 12 human liver microsomal samples, large differences in 5-FU formation from ftorafur were not observed. However, using human anti-P450 antibody differences in the contribution of P450 isoenzymes in ftorafur bioactivation by human liver microsomes was reported. Based on these experiments, ftorafur appears to be mainly metabolized by human 1A2, 2A6, and 2C8 (Komatsu et al., 2000). In another study, it

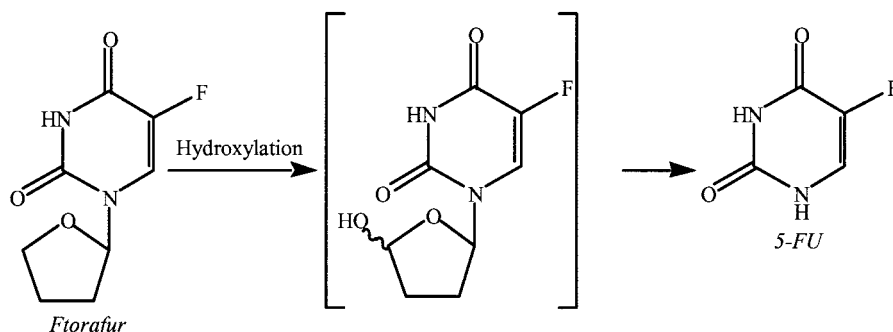


FIG. 6. Activation of ftorafur by cytochrome P450 into 5-FU.

was shown that ftorafur is almost exclusively metabolized by CYP2A6; formation of 5-FU was inhibited by 90% in human liver microsomes using human anti-CYP2A6 antibody (Ikeda et al., 2000). Based on this study, approximately 10% of ftorafur bioactivation is catalyzed by CYP2C9 (Ikeda et al., 2000). Additionally, intracellular expression of CYP2A6, by transfection of cDNA construct encoding CYP2A6, has been shown to sensitize colon cancer cells to ftorafur (Murayama et al., 2001). Oral administration of ftorafur resulted in the induction of CYP1A, 2B, and 3A (Yamazaki et al., 2001). UFT is a combination of ftorafur and uracil in a molar ratio of 1:4 that has been used in the treatment of colorectal and breast cancer as reviewed elsewhere (Sun and Haller, 2001). Uracil is used in this therapy to concomitantly inhibit dihydropyrimidine dehydrogenase, thereby increasing the half-life of 5-FU.

Dacarbazine (DTIC) [5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide] is a P450-activated prodrug used in the treatment of malignant melanoma and Hodgkin's lymphoma (Dollery, 1991; Kopf et al., 2001). The prodrug is activated by hydroxylation to produce 5-(3-hydroxymethyl-3-methyl-triazen-1-yl)imidazole-4-carboxamide (HMMTIC) (Table 7, Fig. 7). Formaldehyde is subsequently eliminated from HMMTIC nonenzymatically, resulting in 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC), which rapidly decomposes to aminoimidazole carboxamide, N_2 , and CH_3^+ . P450-mediated DTIC bioactivation induces apoptosis and mutagenicity via the formation of O^6 -alkylguanine-DNA adducts (Dollery, 1991). The limited antitumor effect of dacarbazine in humans compared with rodents has been attributed to lower P450 activity levels in humans. Additionally, pretreatment of mice with the P450 inhibitor metyrapone inhibited dacarbazine-induced apoptosis in intestinal tumors (Toft et al., 2000). Dacarbazine bioactivation by human liver microsomes, resulting in the formation of MTIC, was shown to be catalyzed by

CYP1A1, 1A2, and 2E1 (Reid et al., 1999). Furthermore, DTIC bioactivation was inhibited by 75% in human liver microsomes using human anti-CYP1A2 antibody, indicating a predominant role for this P450 enzyme. Using cDNA-expressed human P450 isoenzymes, it has been shown that CYP1A2 is the predominant P450 catalyzing DTIC hepatic metabolism ($k_{cat} = 14 \text{ min}^{-1}$; $K_m = 0.66 \text{ mM}$), CYP2E1 contributes to hepatic DTIC metabolism at higher substrate concentrations ($K_m > 2.8 \text{ mM}$), and CYP1A1 activates DTIC extrahepatically ($k_{cat} = 10 \text{ min}^{-1}$; $K_m = 0.60 \text{ mM}$) (Reid et al., 1999). Human CYP2C9/19, 2D6, and 3A4 were not capable of DTIC prodrug activation.

Cyclophosphamide, ifosfamide, and trofosfamide, belonging to the oxazaphosphorines, are nonspecific alkylating agents that have been shown to undergo bioactivation catalyzed by P450 enzymes (Boddy and Yule, 2000) (Table 7, Fig. 8). These nitrogen mustard prodrugs are activated through 4-hydroxylation followed by spontaneous degradation to the ultimate cytotoxins, acrolein and oxazaphosphorine mustards. The generation of acrolein accounts for the observed side effects. Oxazaphosphorines can also be oxidatively metabolized at the chloroethyl side chains, resulting in pharmacologically inactive dechloroethyl metabolites that simultaneously release chloroacetaldehyde, thereby causing neurotoxicity (Fig. 8). The metabolism and pharmacokinetics of oxazaphosphorines have been reviewed recently (Boddy and Yule, 2000).

Cyclophosphamide is the most commonly used alkylating agent with broad application in cancer chemotherapy (Kwon, 1999). Cyclophosphamide is activated by 4-hydroxylation catalyzed by CYP2B6, 2C, and 3A4 and is deactivated by *N*-dechloroethylation by CYP3A4 (Roy et al., 1999; Boddy and Yule, 2000). To overcome the problem that P450 expression is generally high in liver, resulting in a first-pass effect, and low in tumor cells, P450-based cancer gene therapy has been demon-

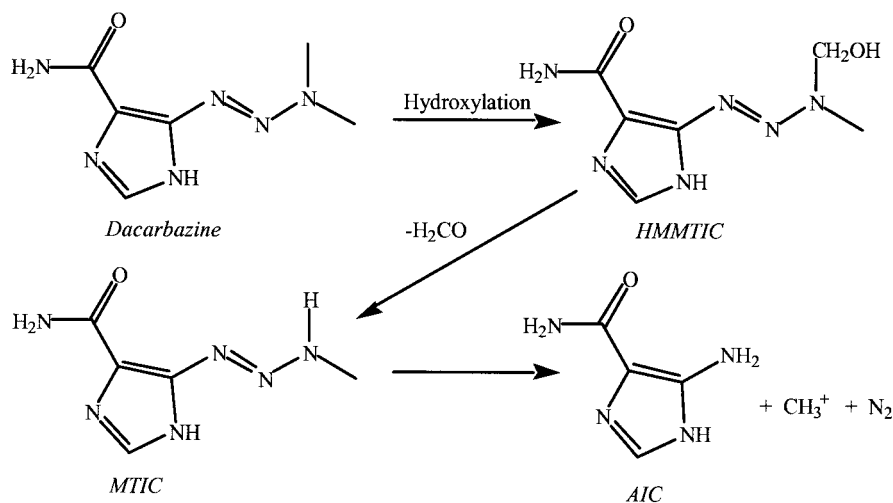


FIG. 7. Activation of dacarbazine by cytochrome P450. Hydroxylation of dacarbazine by cytochrome P450 results in the formation of HMMTIC. MTIC is generated nonenzymatically by loss of formaldehyde. MTIC rapidly decomposes into aminoimidazole carboxamide, CH_3^+ , and N_2 .

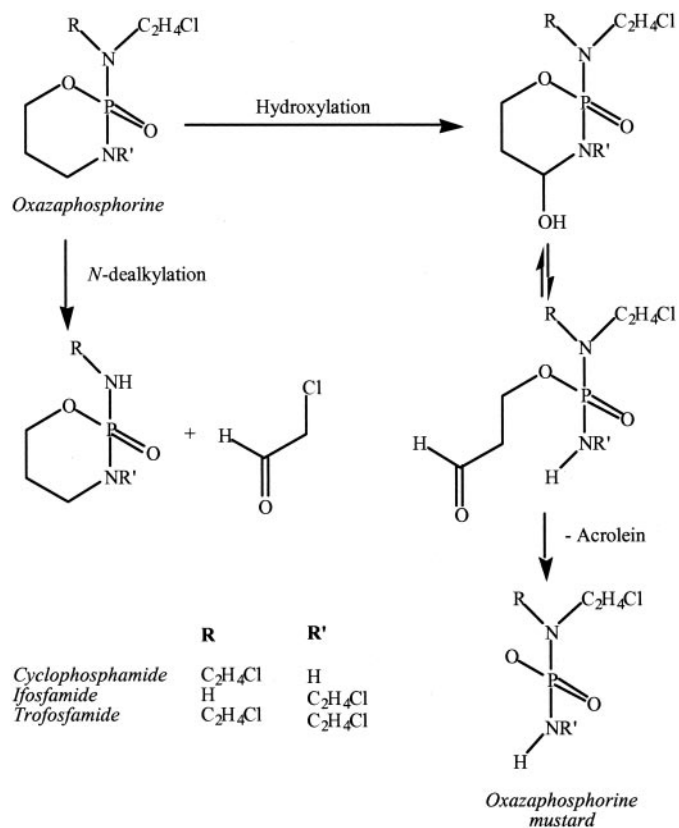


FIG. 8. Activation of the oxazaphosphorines (cyclophosphamide, ifosfamide, and trofosfamide) by cytochrome P450 into oxazaphosphorine mustards. *N*-dealkylation by cytochrome P450s results in the formation of inactive metabolites and chloroacetaldehyde.

strated with cyclophosphamide and ifosfamide as described below (*Section III.B.*).

Trofosfamide, which is another oxazaphosphorine, has been commercially available since 1973 and is metabolized by 4-hydroxylation and by *N*-dechloroethylation. It is metabolized predominantly by recombinant human CYP3A4 and to a lesser extent by recombinant human CYP2B6, whereas recombinant human CYP1A1/A2, 2A6, 2D6, and 2E1 are not active (Della Morte et al., 1986; May-Manke et al., 1999).

Another nitrogen mustard prodrug, ifosfamide, is activated in humans by 4-hydroxylation catalyzed by CYP3A4 with a minor contribution of CYP2A6, 2B6,

3A5, and 2C9/18/19 and is deactivated by the *N*-dechloroethylation pathway by CYP3A4 and CYP2B6 (Della Morte et al., 1986; Roy et al., 1999; Boddy and Yule, 2000). The total turnover of 4-hydroxylation in human liver microsomes is 2.4 min⁻¹ and 0.21 min⁻¹ for *N*-dechloroethylation (Preiss et al., 2002). Because CYP2B6 does contribute significantly to the *N*-dechloroethylation of ifosfamide (10–70%), which is responsible for the toxic side effects but only minor to the 4-hydroxylation of ifosfamide, inhibition of CYP2B6 in patients treated with ifosfamide has been postulated to reduce the toxic effects of this prodrug (Huang et al., 2000). Nephrotoxicity is a limiting factor in the use of ifosfamide, whereas the congener cyclophosphamide, which is predominantly metabolized by 4-hydroxylation, is not associated with renal toxicity (Woodland et al., 2000). This has been attributed to a higher production of chloroacetaldehyde from ifosfamide than from cyclophosphamide. In addition, human liver microsomes from a female catalyzed *N*-dechloroethylation of ifosfamide more rapidly than human liver microsomes from a male, implicating a higher risk for renal and neurotoxicity in female patients (Schmidt et al., 2001).

The alkylaminoanthraquinone 1,4-bis-[[2-(dimethylamino-*N*-oxide)ethyl]amino]-5,8-dihydroxyanthracene-9,10-dione (AQ4N) was designed as a tumor-specific prodrug by making use of low levels of oxygen (hypoxia) in solid tumors. This bioreductive prodrug is activated into the high-affinity DNA-binding agent and potent topoisomerase II inhibitor 1,4-bis-[[2-(dimethylamino)ethyl]amino]-5,8-dihydroxyanthracene-9,10-dione (AQ4) by P450 (Raleigh et al., 1998, 1999) (Table 7, Fig. 9). AQ4N is relatively nontoxic toward V79 cells under aerobic conditions ($EC_{50} \gg 10 \mu\text{M}$), although toxicity is increased up to 1000-fold in absence of air and presence of rat liver microsomes (Patterson, 1993). The apparent K_m value for bioactivation in rat liver microsomes is 30 μM , which is comparable with the kinetic parameters reported for P450-dependent reduction of other aliphatic amine *N*-oxides (Raleigh et al., 1999). Bioactivation of AQ4N to AQ4 in rat liver microsomes is catalyzed by CYP2E and 2B, but not by 3A. However, human CYP3A enzymes, known to be present in tumors of the colon,

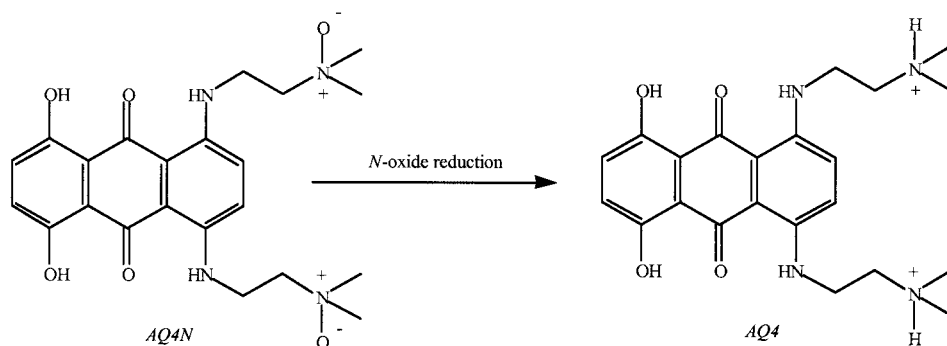


FIG. 9. Activation of AQ4N into AQ4 by cytochrome P450.

lung, liver, esophagus, breast, bladder, prostate, stomach, and kidney, are able to bioactivate AQ4N to AQ4 (Raleigh et al., 1998). Recently, the pharmacokinetic properties of AQ4N were studied in mice (Loadman et al., 2001), and AQ4N was shown to potentiate the anti-tumor effect of cisplatin and cyclophosphamide in the T50/80 murine tumor model and male BDF mice implanted with the T50/80 mammary carcinoma, respectively (Friery et al., 2000; Gallagher et al., 2001). Soon AQ4N will be entered in phase I clinical trials in the United Kingdom (Raleigh et al., 1999).

d. Discussion of Cytochrome P450 as a Prodrug-Activating Enzyme. In addition to cytochrome P450 reductase and DT-diaphorase, P450 enzymes have also been shown to activate bioreductive prodrugs. The relatively novel prodrug AQ4N is activated by P450s into the topoisomerase inhibitor AQ4. P450s have also been shown to activate several other prodrugs including ftorafur, dacarbazine, and oxazaphosphorines. The fact that these compounds are metabolized by P450s is not really surprising since virtually all drugs marketed today are being metabolized by hepatic P450s (Spatzenegger and Jaeger, 1995). However, most drugs are inactivated by P450s, whereas these prodrugs are activated.

There are several limitations in the use of P450-mediated prodrugs. The expression of P450s is often highly variable among individuals. Furthermore, as a result of genetic, physiological, and environmental factors, inter-individual differences in therapy responses may occur (Klaassen, 1996; Iyer and Ratain, 1998; Wormhoudt et al., 1999). P450s are also widely distributed over the body with high activity levels in the adrenal gland and liver, which may result in side effects and first-pass effects. However, the P450s' isoenzymes in adrenal gland are usually not involved in drug metabolism but rather in the catabolism of steroids. Turnover rates of P450s are usually relatively low. This results in a relatively slow release of the anti-tumor drug and is thus a disadvantage for organ- or tumor-selective targeting. Activation of prodrugs by P450s may be inhibited or induced by concomitant drug treatment, which is another problem with P450 prodrugs (Lin and Lu, 2001). Although P450 activity is present in a variety of tumor tissues, the activity is generally lower than in the corresponding normal tissue, which also results in prodrug activation in nontarget cells. Despite these negative aspects regarding prodrugs activated by P450s, it should be notified that several P450 prodrugs, including ftorafur,

dacarbazine, and oxazaphosphorines, are successfully used in the clinic.

6. Tyrosinase

a. Enzymology of Tyrosinase. Tyrosinase (EC 1.14.18.1) catalyzes the oxidation of L-tyrosine to the corresponding *o*-quinone dopaquinone, which is the first step in melanin pigment biosynthesis (Schomburg and Stephan, 1990–1998; del Marmol and Beermann, 1996). The polymerization of these dopaquinones results in the formation of melanin pigment. The enzyme also oxidizes other phenols and catechols to their corresponding *o*-diphenols (creolase activity) and *o*-quinones (catecholase activity), respectively. This copper protein is involved in the formation of melanin pigments. The enzyme is present in mammals, bacteria, and fungi. Turnover numbers have been reported up to 19,000 min⁻¹ (L-dopa), and *K_m* values are in the millimolar range (Schomburg and Stephan, 1990–1998).

b. Localization of Tyrosinase. Tyrosinase is uniquely located in melanocytes and melanoma cells both in the cytosolic and membrane part of the cell of mammals (Pawelek et al., 1980). Based on studies in platyfish, the degree of malignancy of pigment cells was shown to be correlated with tyrosinase activity (Vielkind et al., 1977). Because tyrosinase is only present in melanoma cells, it has been used as a target enzyme to treat malignant melanoma, a cancer with increasing worldwide incidence. This strategy is also known as melanocyte-directed enzyme prodrug therapy.

c. Activation of Prodrugs by Tyrosinase. The first tyrosinase-dependent drug was discovered by coincidence (Dewey et al., 1977). *p*-Hydroxyanisole inhibited the incorporation of ³H-thymidine in melanoma cells. Because the cultured cells had lost part of their pigment-forming capacity, the enzyme tyrosinase was added to the cells. This sensitized the cells toward the toxicity of *p*-hydroxyanisole, strongly suggesting that cells expressing this enzyme are preferentially killed by the drug (Dewey et al., 1977).

2,4-Dihydroxyphenylalanine was the first tyrosinase-dependent prodrug that was shown to be activated to the cellular toxin 6-hydroxydopa (2,4,5-trihydroxyphenylalanine) (Morrison et al., 1985), which is further oxidized by tyrosinase into 5-(2-carboxy-2-aminoethyl)-4-hydroxy-1,2-benzoquinone (Rodriguez-Lopez et al., 1992) (Table 8, Fig. 10). 2,4-Dihydroxyphenylalanine was cytotoxic to both B-16 and Cloudman melanoma cells, and the prodrug was more cytotoxic toward mela-

TABLE 8
Prodrugs activated by tyrosinase

Prodrug	Drug	Pharmacology	Reference
2,4-Dihydroxyphenylalanine	6-Hydroxydopa	Inhibition of DNA, RNA, and protein synthesis	Morrison et al., 1985
4-S-CAP	BQ	Thymidylate synthase and DNA synthase inhibition	Pankovich et al., 1990
GHB	GBQ	Inhibition of DNA synthesis	Prezioso et al., 1993
Substituted phenols	Orthoquinones	Inhibition of DNA synthesis	Riley et al., 1997
Phenyl mustards	Phenol mustard	DNA-alkylating	Jordan et al., 1999
Urea mustards	Unidentified	DNA-alkylating	Jordan et al., 2001

BQ, dihydro-1,4-benzothiazine-6,7-dione; GBQ, γ -l-glutaminy-3,4-benzoquinone.

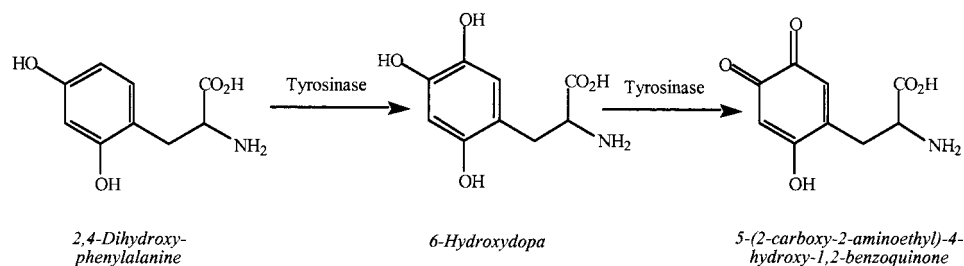


FIG. 10. Activation of 2,4-dihydroxyphenylalanine by tyrosinase.

notic cells (tyrosinase-containing) than toward amelanotic cells (tyrosinase-lacking). The cytotoxicity, measured as inhibition of DNA, RNA, and protein synthesis, was blocked by the tyrosinase inhibitor, phenylthiourea (Morrison et al., 1985). The potential of 2,4-dihydroxyphenylalanine as a melanoma-specific prodrug was not further evaluated, however.

Two other prodrugs, 4-*S*-cysteaminyphenol (4-*S*-CAP) and *N*-acetyl-4-*S*-cysteaminyphenol (*N*-Ac-4-*S*-CAP), were shown to inhibit thymidylate synthase and DNA synthesis in various melanoma cells, although the compounds were essentially nontoxic to nonmelanoma cells (Miura et al., 1990; Pankovich et al., 1990; Prezioso et al., 1992a,b) (Table 8). Partial inhibition of the cytotoxicity of these prodrugs was caused by the tyrosinase inhibitor phenylthiourea, indicating an important role for tyrosinase (Prezioso et al., 1992a). 4-*S*-CAP is metabolized by tyrosinase to 4-*S*-cysteaminyocatechol, which auto-oxidates to the ultimate toxin dihydro-1,4-benzothiazine-6,7-dione (Fig. 11) (Pankovich et al., 1990; Hasegawa et al., 1997). *N*-Ac-4-*S*-CAP was shown to induce tyrosinase-dependent apoptosis, but also non-tyrosinase-mediated cytostatic cytotoxicity (Thomas et al., 1999). More recently, *N*-Ac-4-*S*-CAP was demonstrated to be a useful prodrug in nonmelanotic cells transfected with tyrosinase (Simonova et al., 2000). This system was effective against tumor cells derived from gliosarcoma, adenocarcinoma, and fibrosarcoma. Sev-

eral analogs of *N*-Ac-4-*S*-CAP have been prepared to increase the lipophilicity, and some of them were as effective toward melanoma cells as cisplatin (Gili et al., 2000; Lant et al., 2000).

γ -L-Glutaminy-4-hydroxybenzene (GHB) was shown to be a prodrug of γ -L-glutaminy-3,4-benzoquinone (Prezioso et al., 1993) (Table 8, Fig. 12). The iodinated analog of GHB γ -L-glutaminy-4-hydroxy-3-iodobenzene (I-GHB), was 3-fold more cytotoxic in human and murine melanoma cells than GHB. GSH significantly inhibited the cytotoxicity of the prodrugs, indicating the involvement of quinones. Prodrug activation was catalyzed by tyrosinase. Both prodrugs displayed growth inhibitory activity and inhibition of thymidylate synthase to both highly metastatic B16-BL6 melanoma cells and weakly metastatic B16-F1 melanoma cells with approximately equal tyrosinase activity. However, GHB and I-GHB were more cytotoxic toward B16-BL6 cells than B16-F1 cells.

Because B16-BL6 cells had a 20-fold higher γ -glutamyltransferase activity, the involvement of this enzyme in the bioactivation of these prodrugs was also considered (Prezioso et al., 1994). This alternative prodrug activation mechanism results in the formation of 4-aminophenols, products of γ -glutamyltransferase-catalyzed conversion of GHB and I-GHB. 4-Aminophenols can subsequently be oxidized by tyrosinase and are known to inhibit thymidylate synthase (Fig. 12) (Prezi-

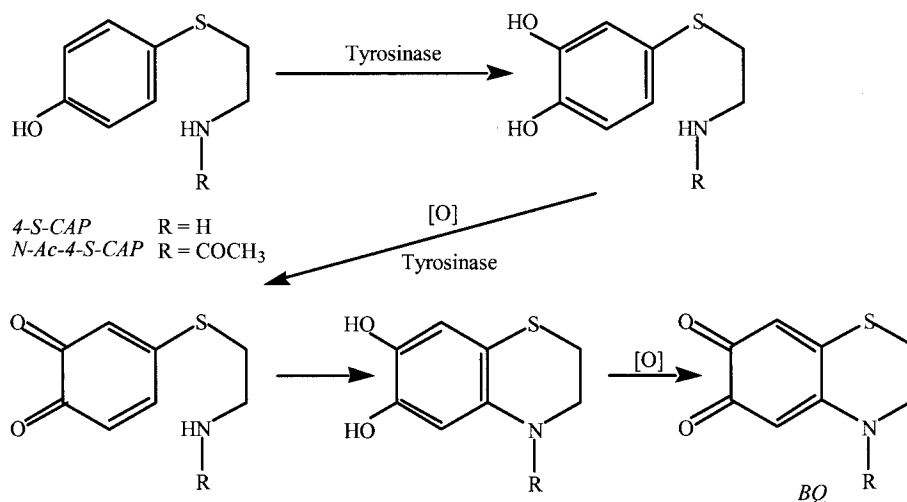


FIG. 11. Activation of 4-*S*-CAP and *N*-Ac-4-*S*-CAP into their corresponding quinones by tyrosinase.

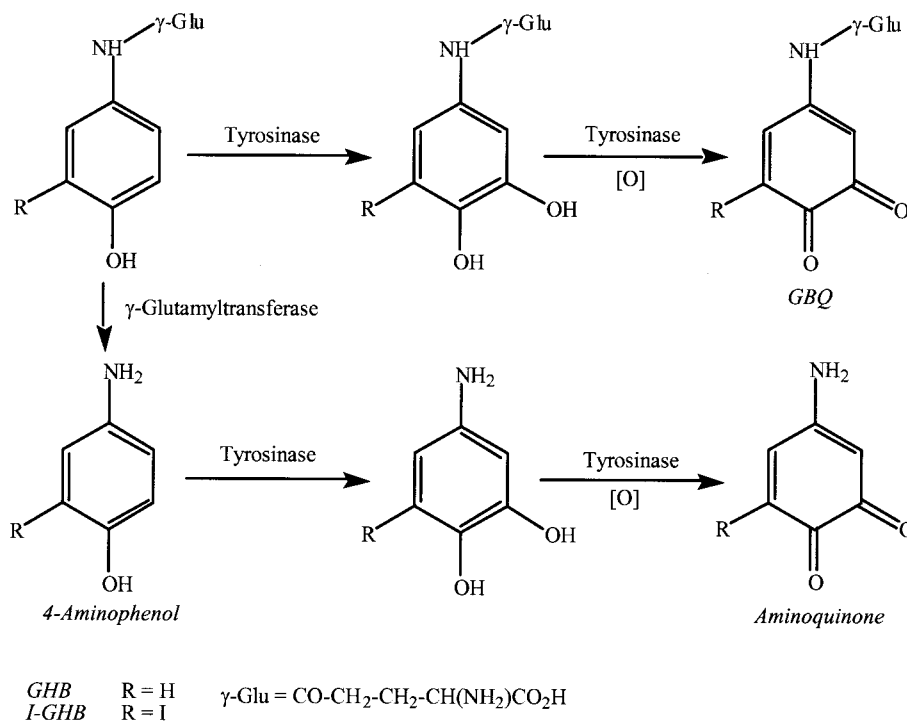


FIG. 12. Activation of GHB or I-GHB by tyrosinase. Two mechanisms of action have been proposed: 1) oxidation by tyrosinase into γ -L-glutaminy-3,4-benzoquinone; or 2) hydrolysis by γ -glutamyltransferase into 4-aminophenol and subsequent oxidation by tyrosinase into aminoquinones.

oso et al., 1993, 1994). Although the combined data strongly suggest that the formation of 4-aminophenols from GHB and I-GHB mediates the antitumor effect, this remains to be proven. The possible involvement of γ -glutamyltransferase in the activation of GHB and I-GHB is of interest since malignant hepatocytes, human breast cancer, squamous-cell carcinomas, lung adenocarcinomas, and melanomas have increased γ -glutamyltransferase activity compared with the corresponding normal tissue (Prezioso et al., 1994).

A series of 26 substituted phenols were tested as tyrosinase-activated prodrugs, and the cytotoxicity was measured by thymidine incorporation (Riley et al., 1997) (Table 8). Generally, the cytotoxicity reflected the rate of oxidation and was ascribed to the formation of toxic *o*-quinones. However, for some prodrugs this correlation was not obtained. This might be due to the lack of uptake of the respective quinones. Based on this study, it was concluded that a lipophilic side chain is an important structural feature of potent melanocytotoxic phenols (Riley et al., 1997).

Recently, prodrugs for phenyl and urea mustards were also synthesized as substrates for tyrosinase (Jordan et al., 1999, 2001) (Table 8). These compounds are oxidized by tyrosinase into the corresponding *o*-quinones, which rearrange into *o*-diols followed by hydrolysis, thereby releasing the active drug (Fig. 13). The drugs released were phenyl mustards, bis-ethyl amine mustards, and daunorubicin (daunomycin), all of which have been clinically used as anticancer agents. Shortening of the dopamine chain length from two carbons to

one carbon resulted in a reduced enzyme affinity as well as nitrogen methylation. As illustrated by the prodrug of daunorubicin, sterically hindered bulky groups also resulted in a decreased rate of oxidation (Jordan et al., 2001). Interestingly, in preliminary cell studies using cell lines with high and low tyrosinase activity, the cytotoxicity of these prodrugs seems to be correlated with the tyrosinase activity (Jordan et al., 1999).

d. Discussion of Tyrosinase as a Prodrug-Activating Enzyme. As illustrated, tyrosinase is a promising target enzyme for prodrug activation. The enzyme is uniquely located in melanocytes and melanoma cells, and tyrosinase activity appears to be correlated with the degree of malignancy (Vielkind et al., 1977; Pawelek et al., 1980). In addition, turnover numbers are high for tyrosinase resulting in a rapid prodrug activation. Despite these promising *in vitro* data, the *in vivo* efficacy of tyrosinase prodrugs remains to be established. Because tyrosinase catalyzes oxidation of several phenolic substrates into the corresponding quinones, which are by definition cytotoxic, it might be worthwhile to focus initially on the development of better substrates for human tyrosinase.

B. Class 2 Transferases

1. Thymidylate Synthase

a. Enzymology of Thymidylate Synthase. Thymidylate synthase (EC 2.1.1.45) catalyzes the methylation of dUMP to dTMP using 5,10-methylenetetrahydrofolate as a cofactor, which is converted into 7,8-dihydrofolate (Schomburg and Stephan, 1990–1998; Carreras and

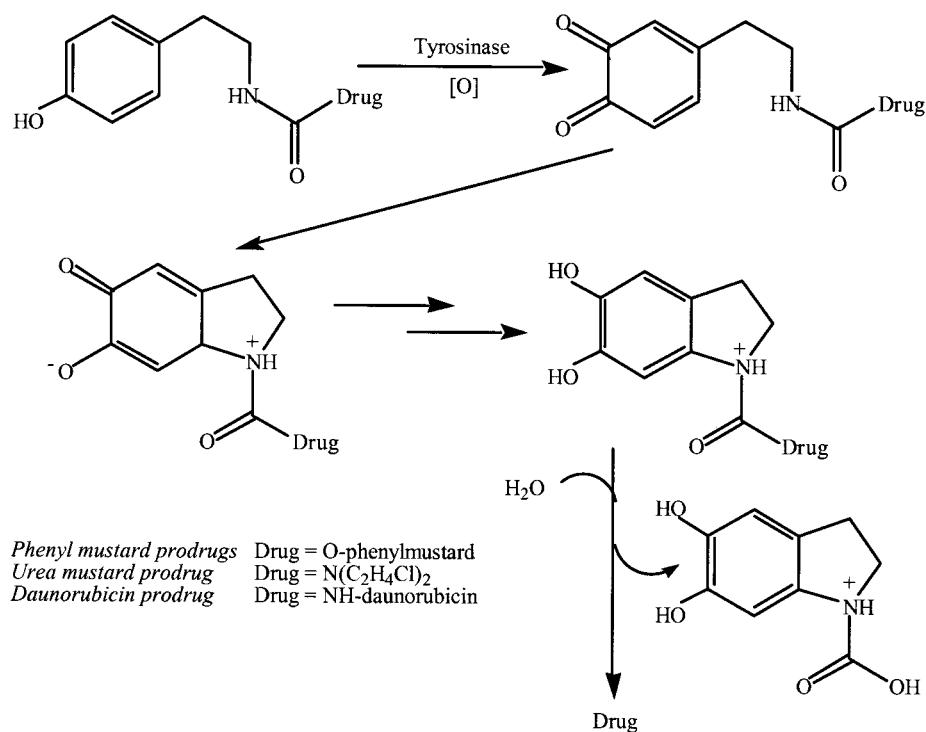


FIG. 13. Activation of prodrugs by tyrosinase.

Santi, 1995). In this two-step process, first one carbon unit is transferred from the cofactor to the 5-position of dUMP and subsequently the one carbon unit is reduced to a methyl group. Thymidylate synthase, which can be activated by thiols such as 2-mercaptoethanol, is the key enzyme in the biosynthesis of DNA-thymine and is essential for cell proliferation (Danenberget al., 1999). Turnover numbers are low, i.e., 380 min⁻¹ for dUMP and 5,10-methylenetetrahydrofolate, and *K_m* values are in the micromolar range. The human enzyme is a dimer consisting of two identical subunits of 33 kDa (Schomburg and Stephan, 1990–1998).

b. Localization of Thymidylate Synthase. Thymidylate synthase is present in various tissues (Table 1). The highest thymidylate synthase activity was observed in thymus followed by spleen, bone marrow, and testis (Hashimoto et al., 1988). Other organs including liver and kidney are of minor importance. In general, thymidylate synthase activity in mammals is high in organs of high cell renewal and low in organs with low cell proliferation (Hashimoto et al., 1988). An exception may be the small intestine that has a low thymidylate synthase activity (Hashimoto et al., 1988), although in another study the activity in small intestine was relatively high (26% of thymus activity) (Herzfeld and Raper, 1980). Although it has been shown that human colon tissue, stomach tissue, and leukocytes contain thymidylate synthase activity, a comprehensive profile of tissue distribution in humans has not been reported (Hashimoto et al., 1988; Iqbal et al., 1993).

Several thymidylate synthase inhibitors, such as fluoropyrimidines (i.e., 5-FU), have been used successfully

to treat tumors (Danenberget al., 1999). However, continuous exposure of tumors to these inhibitors can lead to increased levels and activity levels of thymidylate synthase, thus resulting in a decreased clinical response and a more rapidly progressing disease (Bathe et al., 1999). It has been shown that highly increased expression of thymidylate synthase is common in tumor tissue as a result of the loss of tumor suppression function. Thymidylate synthase activity was much higher in rat hepatoma (up to 125-fold), human colon carcinomas (up to 8-fold), and leukemic leukocytes (up to 11-fold) compared with normal tissue (Hashimoto et al., 1988). Thymidylate synthase mRNA levels were also much higher in human colon tissue samples than in the corresponding normal tissue (Li et al., 2001b).

c. Activation of Prodrugs by Thymidylate Synthase. Based on the observation that thymidylate synthase is frequently overexpressed in tumor cells that are resistant to thymidylate synthase inhibitors, several tumor-selective prodrugs have been designed that are activated by this enzyme to cytotoxic agents. To use the resistance of tumor cells toward thymidylate synthase inhibitors as a biomarker for the use of thymidylate synthase prodrugs is also known as the enzyme-catalyzed therapeutic agent approach (Lackey et al., 2001; Li et al., 2001b). In this approach, a lack of clinical response toward thymidylate inhibitors can be used as a marker of susceptibility to tumor-selective prodrugs that can be activated by thymidylate synthase.

(*E*)-5-(2-bromovinyl)-2'-deoxyuridine-5'-monophosphate (BVdUMP) was shown to be activated by cloned human thymidylate synthase in the presence of thiols,

and the catalytic efficiency was shown to be 60-fold lower than that obtained for the natural substrate dUMP (Li et al., 2001b) (Fig. 14). The enzyme was reversibly inhibited by BVdUMP, and two metabolites resulting from nucleophilic substitution of bromide have been identified (Lackey et al., 2001). Because BVdUMP is a charged molecule, it cannot efficiently enter the cells. Therefore, a phosphoramidate derivative, (*E*)-5-(2-bromovinyl)-2'-deoxy-5'-uridyl phenyl-L-methoxyalanylphosphoramidate (NB1011), was developed (Fig. 14) (Lackey et al., 2001; Li et al., 2001b). Although the active metabolite was not yet identified, incubation of cells with NB1011 resulted in increased intracellular accumulation of BVdUMP. Furthermore, NB1011 was converted into the same metabolites as observed in cells treated with BVdUMP (Lackey et al., 2001; Li et al., 2001b). NB1011 was shown to be at least 10-fold more cytotoxic to 5-FU-resistant thymidylate synthase overexpressing human colorectal tumor cells than to normal cells. Raltitrexed, a specific inhibitor of thymidylate synthase, blocked the cytotoxicity of NB1011 (Li et al., 2001b). Exposure of MCF7/TDX cells, resistant to raltitrexed, to NB1011 resulted in recovery of cell populations and clones with diminished thymidylate synthase levels as well as restored sensitivity to raltitrexed (Li et al., 2001b). Despite these promising results, the clinical efficacy of thymidylate synthase prodrugs remains to be established.

d. Discussion of Thymidylate Synthase as a Prodrug-Activating Enzyme. Thymidylate synthase is frequently overexpressed in tumors, resulting in a decreased clinical response toward thymidylate synthase

inhibitors such as 5-FU. The resistance against thymidylate synthase can be used as a biomarker for the sensitivity against prodrugs that are activated by thymidylate synthase (Lackey et al., 2001; Li et al., 2001b). Although thymidylate synthase inhibitors have been developed for a long time, the development of thymidylate prodrugs BVdUMP and NB1011 is of very recent date (Lackey et al., 2001; Li et al., 2001b). Based on the tissue distribution of thymidylate synthase in rat tissues, it is clear that tissues with rapid cell proliferation contain high levels of thymidylate synthase, whereas slowly proliferating cells contain low levels of this enzyme. However, to further evaluate the clinical potential of this enzyme-prodrug system, the tissue distribution in humans should also be studied.

2. Thymidine Phosphorylase

a. Enzymology of Thymidine Phosphorylase. Thymidine phosphorylase (EC 2.4.2.4) catalyzes the formation of thymine and deoxyribose-1-phosphate from thymidine and orthophosphate (Schomburg and Stephan, 1990–1998). This pentosyl transfer reaction is reversible for the natural substrates thymidine and thymine. Thymidine phosphorylase is part of the salvage pyrimidine nucleic acid pathway by recycling thymine, which can be reincorporated into DNA. The enzyme also metabolizes uridine and uridine analogs. K_m values are in the millimolar range for the above-mentioned natural substrates. Human thymidine phosphorylase is a homodimeric protein with subunits of 55 kDa, is identical to platelet-derived endothelial cell growth factor, and plays a role in nucleotide salvage and angiogenesis (Griffiths and Stratford, 1997; Brown and Bicknell, 1998;

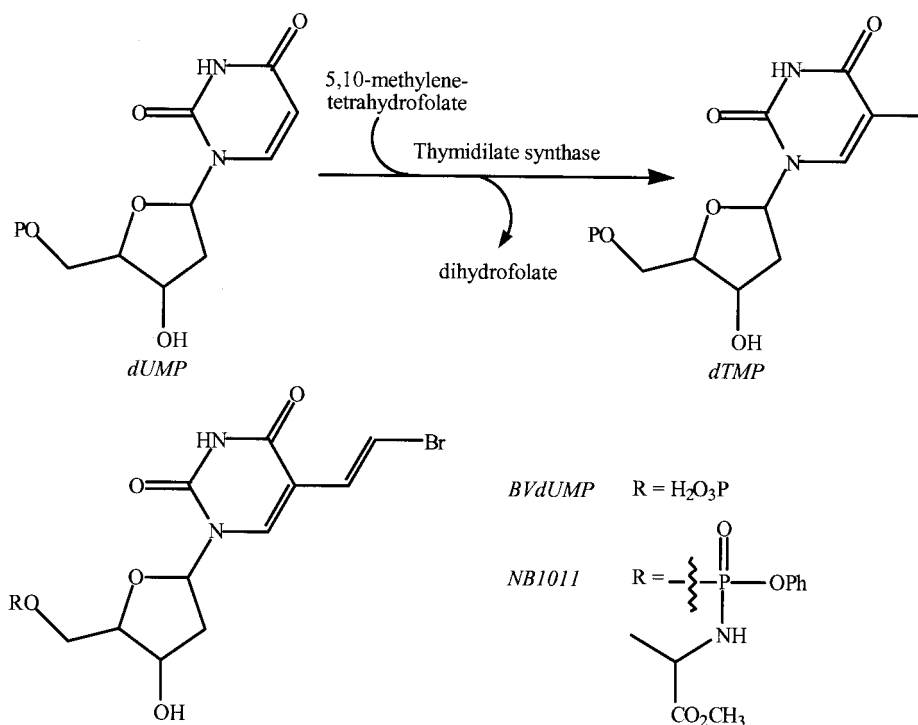


FIG. 14. Activation of natural substrate dUMP and prodrugs BVdUMP and NB1011 by thymidylate synthase.

Cole et al., 1999). There are two distinct pyrimidine nucleoside phosphorylase enzymes present in normal and neoplastic cells, namely thymidine phosphorylase and uridine phosphorylase (EC 2.4.2.4). However, thymidine phosphorylase is the predominant pyrimidine nucleoside phosphorylase enzyme present in human tumor cells (Eda et al., 1993).

b. Localization of Thymidine Phosphorylase. Thymidine phosphorylase is present in a variety of tissues (Zimmerman and Seidenberg, 1964; Yoshimura et al., 1990). Highest levels are present in liver, lung, and lymph node both in rat and humans (Tables 1 and 2). In human spleen the activity was found to be comparable with that of liver, lung, and lymph node. Thymidine phosphorylase activity was not observed in spleen tissue of the rat. Other tissues contain lower activity levels of thymidine phosphorylase, i.e., up to 30% of human lung tissue (Yoshimura et al., 1990). Thymidine phosphorylase is also high in the rat and human intestine, although the activity in human intestine is lower than in other human tissues when compared with rats (Zimmerman and Seidenberg, 1964).

Thymidine phosphorylase activity is up to 10-fold elevated in tumors compared with normal tissue. Overexpression of thymidine phosphorylase is mainly found in the hypoxic areas of solid tumors (Cole et al., 1999). Up to 2.8-fold higher levels of thymidine phosphorylase have also been observed in human gastrointestinal cancer tissues compared with normal tissues (Choong et al., 1988) and in colorectal tumors and metastasis, in breast, mammary, stomach, ovary, esophagus, and pancreas of human, rat, and mouse (Maehara et al., 1989; Yoshimura et al., 1990; Nio et al., 1992; Mimori et al., 1999; Collie-Duguid et al., 2001). The presence of thymidine phosphorylase is a negative prognostic indicator in cancers such as breast, liver, renal, and colorectal and has been associated with invasiveness and malignancy (Cole et al., 1999).

c. Activation of Prodrugs by Thymidine Phosphorylase. Based on the knowledge that thymidine phosphorylase activity is often higher in tumor tissue than in normal tissue, tumor-selective prodrugs have been de-

veloped. 5'-deoxy-5-fluorouridine (5'-DFUR) is a fluoro-pyrimidine nucleoside agent that can be bioactivated into the antitumor drug 5-FU by thymidine phosphorylase and uridine phosphorylase (Fig. 15) (Armstrong and Diasio, 1980; Patterson et al., 1995b). MCF-7 breast cancer cells transfected with thymidine phosphorylase cDNA showed increased sensitivity to 5'-DFUR compared with parental cells (Patterson et al., 1995b). Similar results were obtained in other in vitro studies. Human colon carcinoma cells transfected with thymidine phosphorylase showed a 40-fold increase in the cytotoxicity of 5'-DFUR compared with the parental cell line, which indicates an important role for this enzyme (Evrard et al., 1999). However, only a 3- to 10-fold increase in 5'-DFUR cytotoxicity was obtained in a rat carcinoma cell line, transfected with cDNA encoding thymidine phosphorylase and expressing levels comparable with that obtained in human tumors, compared with the parental cells (Ishikawa et al., 1998; Marchetti et al., 2001). The bioactivation of 5'-DFUR by thymidine phosphorylase in human head and neck and rat carcinoma cell lines correlated with the cytotoxicity of 5'-DFUR, and apparent K_m values between 1 and 2 mM were obtained (Peters et al., 1989; Marchetti et al., 2001).

Up-regulation of pyrimidine nucleoside phosphorylases by the cytokine interferon- α was shown to increase the antitumor activity of 5'-DFUR in colorectal cancer cells both in vitro and in vivo (Eda et al., 1993; Laurent et al., 1994). Recently, thymidine phosphorylase activity appeared also to be correlated with the effect of 5'-DFUR on tumors in a clinical study with 216 breast cancer patients (Hata et al., 2000).

Although 5'-DFUR is clinically used for treatment of breast, colorectal, gastric, and other cancers, a major drawback is the gastrointestinal toxicity of 5'-DFUR. Therefore, the 5'-DFUR prodrug capecitabine (*N*4-pentyloxycarbonyl-5'-deoxy-5-fluorocytidine) was developed (Fig. 16). Capecitabine is activated to 5-FU by a cascade of three enzymes (Budman, 2000). Capecitabine is metabolized by carboxylesterase to 5'-deoxy-5-fluorocytidine (see Section II.C.1.b.) and then by cytidine deami-

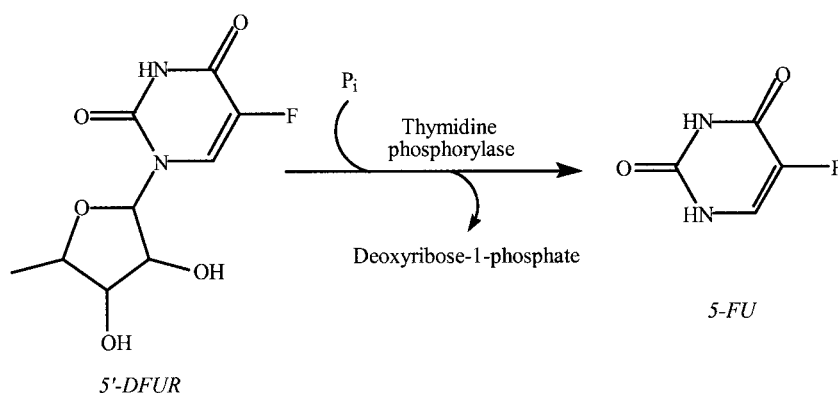


FIG. 15. Activation of 5'-DFUR into 5-FU by thymidine phosphorylase.

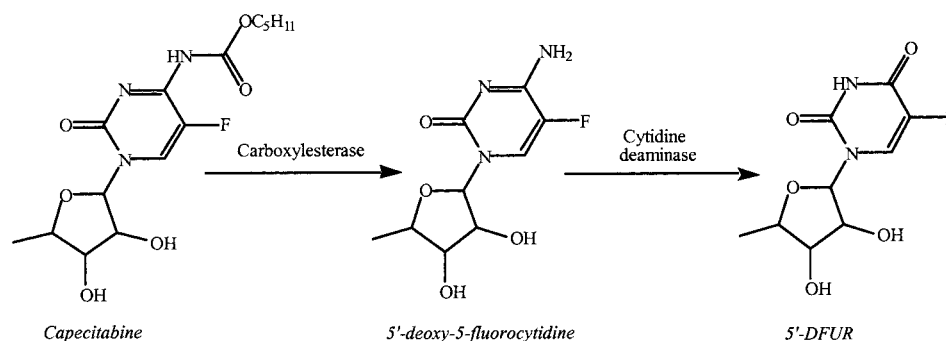


FIG. 16. Activation of the 5-FU prodrug capecitabine. Capecitabine is metabolized by carboxylesterase into 5'-deoxy-5-fluorocytidine, which is subsequently metabolized by cytidine deaminase into 5'-DFUR. 5'-DFUR is activated by thymidine phosphorylase into 5-FU as shown in Fig. 15.

nase to 5'-DFUR, which is subsequently activated to 5-FU by thymidine phosphorylase (Miwa et al., 1998). Pharmacokinetic studies showed satisfying gastrointestinal absorption of capecitabine and a high conversion to the main metabolite 5'-DFUR. The pharmacokinetics and antitumor activity of capecitabine in clinical trials have been reviewed recently (Budman, 2000).

d. Discussion of Thymidine Phosphorylase as a Pro-drug-Activating Enzyme. Although up to 10-fold levels of thymidine phosphorylase in some breast, ovarian, colorectal, and gastric cancers implicate the effectiveness of the 5-FU prodrug 5'-DFUR, the success has been limited due to the low frequency of tumors displaying sufficient thymidine phosphorylase activity (Cole et al., 1999). Another problem with 5'-DFUR, which is clinically used for treatment of breast, colorectal, gastric, and other cancers, is the significant gastrointestinal toxicity. As described above, the intestine contains considerable thymidine phosphorylase activity; thus, capecitabine was developed that is activated by carboxylesterase, cytidine deaminase, and thymidine phosphorylase (Budman, 2000).

3. Glutathione S-Transferase

a. Enzymology of Glutathione S-Transferase. Glutathione S-transferases (GST; EC 2.5.1.18) constitute a family of dimeric proteins that catalyze the conjugation of the tripeptide GSH to electrophiles resulting in the formation of the corresponding GSH conjugates (Schomburg and Stephan, 1990–1998; Hayes and Pulford, 1995). GSTs play an important role in the detoxification of xenobiotics; however, depending on the properties of the substrate, bioactivation to toxic compounds has also been described (Commandeur et al., 1995; van Bladeren, 2000). The mechanism by which GSTs increase the rate of GSH conjugation involves deprotonation of GSH to GS^- by a tyrosine residue, which functions as a base catalyst. GST isoenzymes have been divided into at least seven classes based on amino acid sequence similarity, five of which are cytosolic (designated alpha, mu, pi, theta, and kappa), and two are membrane-bound. One of the two microsomal GST enzymes is identical with leukotriene C_4 synthase. Several isoenzymes, including those from mu, pi, and theta, have been shown to be

polymorphic in humans (Wormhoudt et al., 1999). GSTP1 and GSTT1 genotypes have been associated with occurrence of asthma and cutaneous basal cell carcinoma (Strange et al., 2001).

b. Localization of Glutathione S-Transferases. GSTs are present in all species and in virtually all tissues (Tables 1 and 2). In rats, high activity levels of both cytosolic and membrane-bound GST activity levels have been found in the liver (DePierre and Morgenstern, 1983). The testes also possess a high GST activity level exceeding hepatic activity for cytosolic GST enzyme activity. Other tissues are of minor importance in this regard (DePierre and Morgenstern, 1983). In humans, the highest cytosolic GST activity level is present in the liver, whereas the kidney (78% of liver), lung (34% of liver), and intestine (37% of liver) have lower activity levels (Pacifci et al., 1988). For microsomal GST tissue distribution in humans, only mRNA levels have been reported. These are known to closely correlate with GST protein expression and enzyme activity (Buetler et al., 1995). GST mRNA expression in human tissues is highest in liver followed by the kidney and colon that express 34% and 35% of the hepatic mRNA levels, respectively. High levels are also found in brain, lung, testis, and small intestine (Estonius et al., 1999).

Class pi GST (P1-1) is frequently overexpressed in rat and human tumors, including carcinoma of the colon, lung, kidney, ovary, pancreas, esophagus, and stomach (Hayes and Pulford, 1995; O'Brien and Tew, 1996). Overexpression of class pi GST in breast cancer and renal cell carcinoma can be used as a significant prognostic factor of these diseases. The overexpression of GSTs in tumor cells may result in a more rapid detoxification of antitumor drugs, thereby diminishing the effectiveness of these agents. This is illustrated by the fact that several drug-resistant cell lines overexpress GSTs (O'Brien and Tew, 1996). In addition to class pi GST, classes alpha and mu GSTs are also overexpressed in certain drug-resistant cell lines. However, it should be noted that decreased levels of classes pi and mu GSTs have also been observed in drug-resistant cell lines (O'Brien and Tew, 1996).

c. Activation of Prodrugs by Glutathione S-Transferase. Based on the fact that GSTs are frequently over-expressed in tumor tissue when compared with normal tissue, several prodrugs have been designed that can be activated by GSTs. γ -Glutamyl- α -amino- β -(2-ethyl-*N,N,N',N'*-tetrakis(2-chloroethyl)phosphoro-diamidate)-sulfonyl-propionyl-(*R*)-(-)-phenylglycine (TER286) is a nitrogen mustard prodrug that is activated by GSTA1-1 and GSTP1-1, which are often elevated in human tumors (Table 9) (Lyttle et al., 1994; O'Brien and Tew, 1996). This prodrug is activated by tyrosine-7 from the GST enzyme that abstracts a proton from TER286, resulting in a β -elimination reaction, subsequently generating a phosphorodiamidate (Fig. 17) (Lyttle et al., 1994; Satyam et al., 1996). The phosphorodiamidate spontaneously rearranges to an aziridinium agent that alkylates DNA. Cytotoxicity of TER286 correlated with GSTP1-1 activity in human colon carcinoma and human breast carcinoma cell lines (Lyttle et al., 1994; Morgan et al., 1998). Furthermore, mouse fibroblast GSTP1-1^{-/-} cells exhibited a 2-fold resistance to TER286 compared with GSTP1-1^{+/+} cells (Rosario et al., 2000).

S-(*N*-*p*-Chlorophenyl-*N*-hydroxycarbamoyl)glutathione (*S*-CPHC-glutathione) is a powerful mechanism-based inhibitor of the cancer target enzyme glyoxalase I. However, this agent does not readily cross cell membranes. Therefore, the lipophilic prodrug *S*-(*N*-*p*-chlorophenyl-*N*-hydroxycarbamoyl)ethylsulfoxide was developed and shown to be activated intracellularly by GSH conjugation yielding *S*-CPHC-glutathione (Table 9, Fig. 18) (Hamilton et al., 1999). This reaction was shown to be catalyzed by class pi GST from human placenta with a catalytic efficiency of 54 mM⁻¹ · min⁻¹, whereas the nonenzymatic GSH conjugation was only 1.8 mM⁻¹ · min⁻¹. Rapid uptake was observed in murine leukemia cells, and the IC₅₀ for growth inhibition was 0.5 μ M, which is much lower than for ester prodrugs of *S*-CPHC-glutathione (Hamilton et al., 1999). Despite these interesting results, further studies have so far not been reported.

Cis-3-(9H-Purin-6-ylthio)acrylic acid (PTA) is a prodrug of the antitumor and immunosuppressive agent 6-mercaptopurine (6-MP), which is activated by GSH conjugation by Michael addition to the acrylic acid moiety of PTA (Table 9, Fig. 19) (Gunnarsdottir and Elfarra, 1999). Although bioactivation of PTA also occurs nonenzymatically, 6-MP formation was enhanced 2- and 7-fold by liver and kidney homogenates, respectively. The 6-MP formation was not affected by the γ -glutamyl-transferase inhibitor acivicin and by the cysteine conju-

gate β -lyase inhibitor aminooxyacetic acid, indicating that the 6-MP formation was not a result of the enzymatic degradation of the GSH conjugate but resulted from a chemical rearrangement of the GSH conjugate. GST-mediated 6-MP formation was catalyzed preferentially by GSTA1-1, whereas GSTM1-1 and GSTP1-1 played a minor role. After administration of PTA to rats, 6-MP was detected in urine; apparent liver and kidney toxicity was not observed (Gunnarsdottir and Elfarra, 1999). However, the antitumor activity of PTA in comparison with 6-MP remains to be established.

d. Discussion of Glutathione S-Transferase as a Prodrug-Activating Enzyme. As thymidylate synthase increased drug resistance of tumor cells, a higher expression of GSTs, especially class pi GST, can be used as a marker for the sensitivity toward prodrugs that are activated by GSTs. The number of GST prodrugs that have been developed is limited, however. Elevated levels of this enzyme in tumor cells indicate the clinical potential of this approach. TER286 seems an interesting prodrug for such an approach since it is activated by GSTP1-1 and GSTA1-1 (Lyttle et al., 1994; O'Brien and Tew, 1996). A problem with GST prodrugs, however, is the wide tissue distribution of GSTs in normal tissues. The liver, kidney, and testis might therefore be sensitive to GST prodrugs; however, this remains to be proven. Genetic polymorphisms have also been shown for GST enzymes which will influence individual clinical responses toward GST prodrugs (Iyer and Ratain, 1998; Wormhoudt et al., 1999).

4. Deoxycytidine Kinase

a. Enzymology of Deoxycytidine Kinase. Deoxycytidine kinase (EC 2.7.1.74) catalyzes the phospho group transfer from ATP to 2'-deoxycytidine resulting in the formation 2'-deoxycytidine-5-phosphate (dCMP) and ADP (Schomburg and Stephan, 1990–1998; Arner and Eriksson, 1995). The enzyme also phosphorylates deoxyguanosine, deoxyuridine, deoxythymidine, deoxyadenosine, analogs, and several antitumor drugs. The enzyme is present in various organisms and various tissues. Turnover numbers of 4,700,000 min⁻¹ have been reported for 2'-deoxycytidine as a substrate.

b. Localization of Deoxycytidine Kinase. The cytosolic deoxycytidine kinase is present in various tissues of the rat (Table 1). High activity levels have been found in thymus, bone marrow, spleen, and skeletal muscle. Lower activity levels have been observed in brain, lung, kidney, and liver, whereas levels in small intestine, testis, plasma, and heart are even lower (Harkrader et al.,

TABLE 9
Prodrugs activated by glutathione S-transferase

Prodrug	Drug	Pharmacology	Reference
TER286	Aziridinium agent	DNA alkylating	Lyttle et al., 1994
<i>S</i> -CPHC-ethylsulfoxide	<i>S</i> -CPHC-glutathione	Glycolase I inhibition	Hamilton et al., 1999
PTA	6-MP	Antimetabolite	Gunnarsdottir and Elfarra, 1999

S-CPHC-ethylsulfoxide, *S*-(*N*-*p*-chlorophenyl-*N*-hydroxycarbamoyl)ethylsulfoxide.

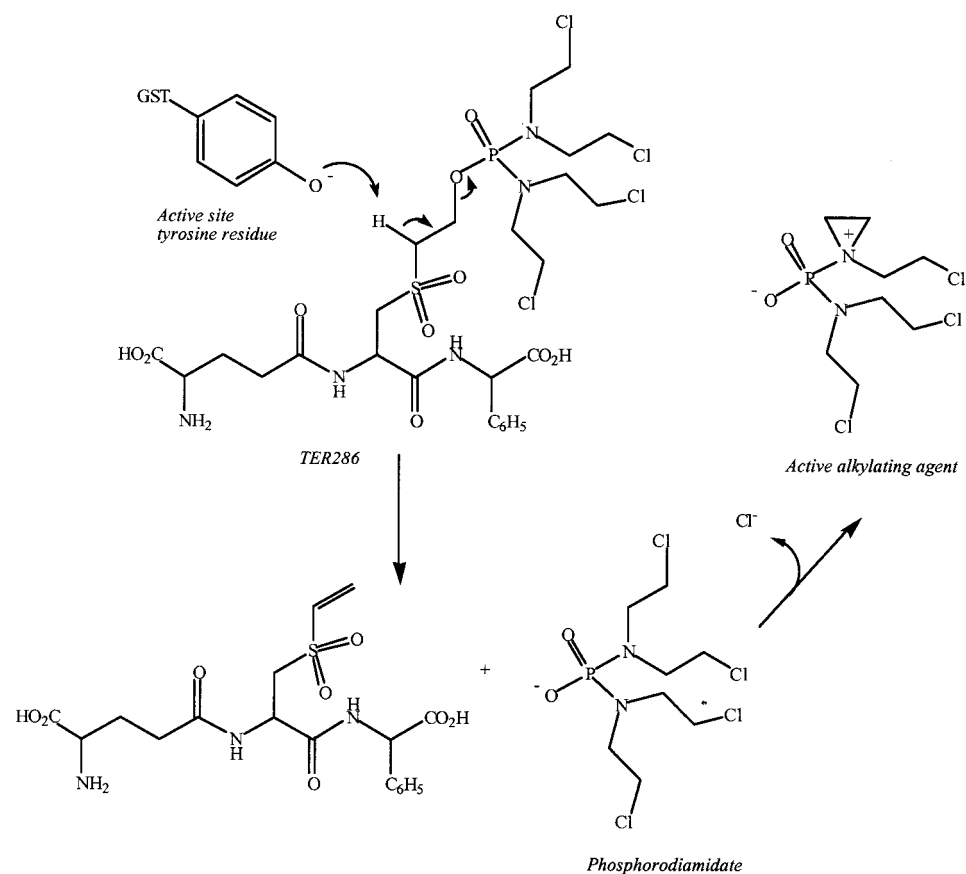


FIG. 17. Activation of TER286 by an active site tyrosine residue from glutathione S-transferase.

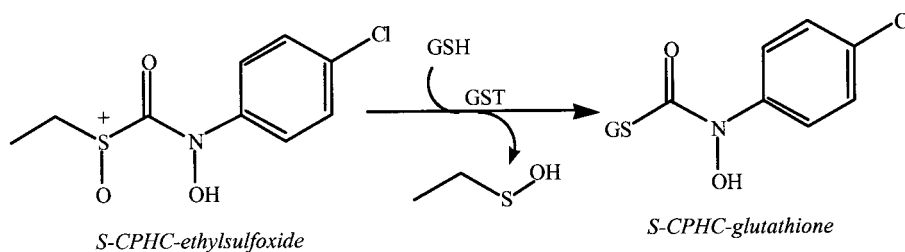


FIG. 18. Activation of *S*-(*N*-*p*-chlorophenyl-*N*-hydroxycarbonyl)ethylsulfoxide by glutathione S-transferase into *S*-CPHC-glutathione.

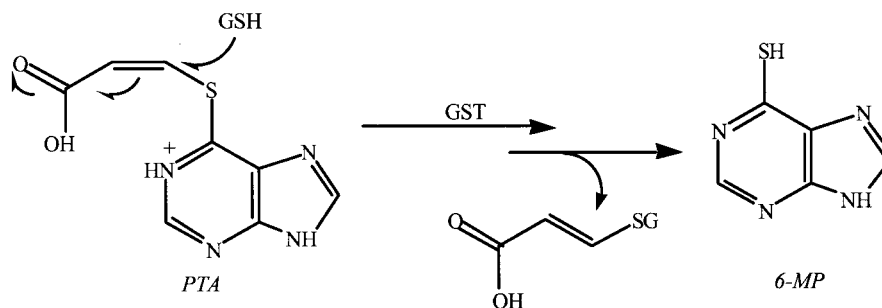


FIG. 19. Activation of PTA by glutathione S-transferase into 6-MP.

1980). In another study, it was shown that deoxycytidine kinase activity in rat spleen is higher than in rat liver (Chan et al., 1983). Deoxycytidine kinase was also observed in plasma of the rat but was undetectable in plasma of mouse and human (Chan et al., 1983). In

humans, high levels of deoxycytidine kinase have been reported in lymphocytes and infant thymus. Lower levels were found in brain, muscle, ventricle, lymphoid, and colon, whereas the enzyme appeared to be absent in liver (Table 2) (Arner et al., 1992; Arner and Eriksson, 1995).

Deoxycytidine kinase is expressed in tumor cells, although in low and varying levels (Arner and Eriksson, 1995; Johnson, 2000). In particular, brain and colon tumors have very low levels of deoxycytidine kinase. Interestingly, in rat hepatomas and kidney tumors, deoxycytidine kinase activity is correlated with growth rates (Harkrader et al., 1980).

c. Activation of Prodrugs by Deoxycytidine Kinase. Cytarabine (arabinosylcytosine), pentostatin (2'-deoxycoformycin), fludarabine (9- β -D-arabinofuranosyl-2-fluoroadenine), cladribine (2-chlorodeoxyadenosine), and gemcitabine (difluorodeoxycytidine) are clinically used prodrugs that are activated into their respective triphosphate metabolites by phosphorylation catalyzed by deoxycytidine kinase (Fig. 20) (Johnson, 2000). After bioactivation the phosphorylated drugs are incorporated into DNA which results in cell death, although the mechanism of action of pentostatin results from inhibition of adenosine deaminase. Tumor cells transfected with a gene encoding deoxycytidine kinase, expressing 1.7- to 16-fold higher levels of deoxycytidine kinase, were 20- to 106-fold more sensitive toward cytarabine, cladribine, and fludarabine than the parental cell lines (Hapke et al., 1996).

Cytarabine and fludarabine are active against a wide range of hematological malignancies, and cytarabine is one of the main agents used in the treatment of leukemia. Pentostatin and cladribine possess clinical efficacy against lymphoid malignancies. Gemcitabine is being highly acti-

vated in solid tumors (Johnson, 2000). A general problem with these deoxycytidine kinase prodrugs is their damage to normal lymphoid cells resulting in significant and long-lasting immunosuppression. These prodrugs and their mechanisms of bioactivation have been reviewed recently (Dubowchik and Walker, 1999; Johnson, 2000).

d. Discussion of Deoxycytidine Kinase as a Prodrug-Activating Enzyme. Deoxycytidine kinase prodrugs have been developed for the treatment of hematological malignancies. Although these prodrugs are being used clinically, a general problem is their damage to normal lymphoid cells resulting in significant and long-lasting immunosuppression (Johnson, 2000). Furthermore, tumor cells contain low and varying levels of deoxycytidine kinase activity, and cytarabine is rapidly inactivated by deamination catalyzed by cytidine deaminase. Because of these limitations, GDEPT and VDEPT approaches have been developed as described (Dubowchik and Walker, 1999; Agbi et al., 2000).

C. Class 3 Hydrolases

1. Carboxylesterase

a. Enzymology and Localization of Carboxylesterase. Carboxylesterases (EC 3.1.1.1) catalyze the conversion of a carboxylic ester to an alcohol and a carboxylic acid (Schomburg and Stephan, 1990–1998). In addition to carboxylic acid hydrolysis, the enzyme also hydrolyzes

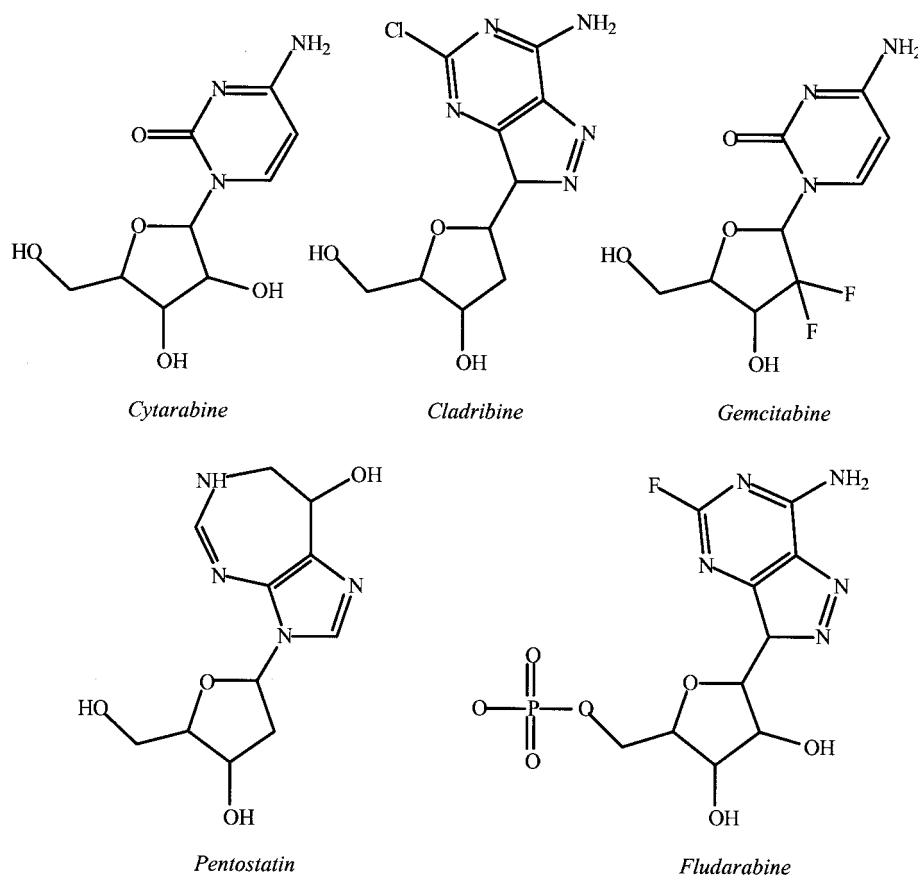


FIG. 20. Deoxycytidine kinase prodrugs.

amides, thioesters, phosphoric acid esters, and acid anhydrides. Carboxylesterases are widely distributed throughout the body, with high levels in the liver, kidney, testis, lung, and plasma, both in normal and tumor tissues. The carboxylesterase enzyme is present in both microsomes and cytosol. Because numerous carboxylesterases have been reported, turnover numbers and K_m values differ to a large extent. The differences between human carboxylesterase from liver and carboxylesterases from animals have been reviewed (Satoh and Hosokawa, 1995). Although a systematic nomenclature for classifying carboxylesterases has not yet been made, these enzymes are classified based on their interaction with organophosphates (Aldridge, 1953). Carboxylesterases that hydrolyze organophosphates are designated A-esterases, and organophosphates that inhibit carboxylesterases are classified as B-esterases. C-esterases do not interact with organophosphates.

b. Activation of Prodrugs by Carboxylesterase. Because of the widespread distribution of carboxylesterases, these enzymes cannot be used to activate prodrugs locally, except when targeted to tumors by ADEPT or GDEPT approaches. Therefore, carboxylesterase prodrugs have been developed mainly to increase the bioavailability of cytostatic drugs (Fig. 21).

The clinically used camptothecin analog 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11; irinotecan) was shown to be activated by carboxylesterase enzymes from rat and human to the potent DNA topoisomerase I inhibitor 7-ethyl-10-hydroxycamptothecin (SN-38) (Satoh et al., 1994) (Table 10, Fig. 21). Comparing carboxylesterase isoenzyme activity levels in different species, the highest activity level was present in guinea pig liver, and the human isoenzyme showed relatively low activity (Satoh et al., 1994). This was recently rationalized by computer modeling studies that indicated that bioactivation of CPT-11 by carboxylesterases is constrained by size-limited access of the prodrug to the active site of the isoenzymes (Wadkins et al., 2001). As a side effect, CPT-11 causes severe forms of

diarrhea, which was attributed to the intestinal breakdown of the glucuronic acid conjugate of SN-38 by β -glucuronidases (Takasuna et al., 1996; Iyer et al., 1998). Coadministration of CPT-11 with antibiotics, thereby inhibiting β -glucuronidase in intestinal microflora, markedly reduced diarrhea and damage in the cecum (Takasuna et al., 1996). CPT-11 is also metabolized by CYP3A4 to yield a number of inactive oxidation products (Rivory, 2000). Because the activation of CPT-11 by human carboxylesterases is relatively inefficient, several GDEPT strategies mostly using rabbit carboxylesterase were developed and shown to be successful (Crystal, 1999; Aghi et al., 2000; Rivory, 2000; Wierdl et al., 2001).

Paclitaxel-2-ethylcarbonate was shown to be activated by rat serum carboxylesterase to the microtubule-binding agent paclitaxel (taxol) (Senter et al., 1996) (Table 10, Fig. 21). Paclitaxel-2-ethylcarbonate is 8-, 300-, and 35-fold less toxic than paclitaxel to human colon cancer, lung adenocarcinoma, and melanoma cells, respectively (Ueda et al., 1994; Senter et al., 1996). After exposure of these cell lines to the prodrug and purified rat serum carboxylesterase, the cytotoxicity was comparable with paclitaxel, indicating efficient prodrug activation (Senter et al., 1996).

To overcome the significant gastrointestinal toxicity of 5'-DFUR, the prodrug capecitabine (*N*4-pentyloxycarbonyl-5'-deoxy-5-fluorocytidine) was developed. Capecitabine was shown to be activated by carboxylesterase to 5'-deoxy-5-fluorocytidine (Table 10, Fig. 16) (Miwa et al., 1998). The largest formation of 5'-deoxy-5-fluorocytidine after incubation of human tissue homogenates with capecitabine was observed in the liver for both normal and tumor tissues. 5'-Deoxy-5-fluorocytidine is subsequently activated by cytidine deaminase to 5'-DFUR, which is then activated to 5-FU by thymidine phosphorylase (see Section II.B.2. and Fig. 16) (Miwa et al., 1998). Pharmacokinetic studies showed good gastrointestinal absorption of capecitabine and several of its analogs as well as a high conversion to the main metabolite 5'-DFUR (Shimma et al., 2000). The pharmacokinetics and

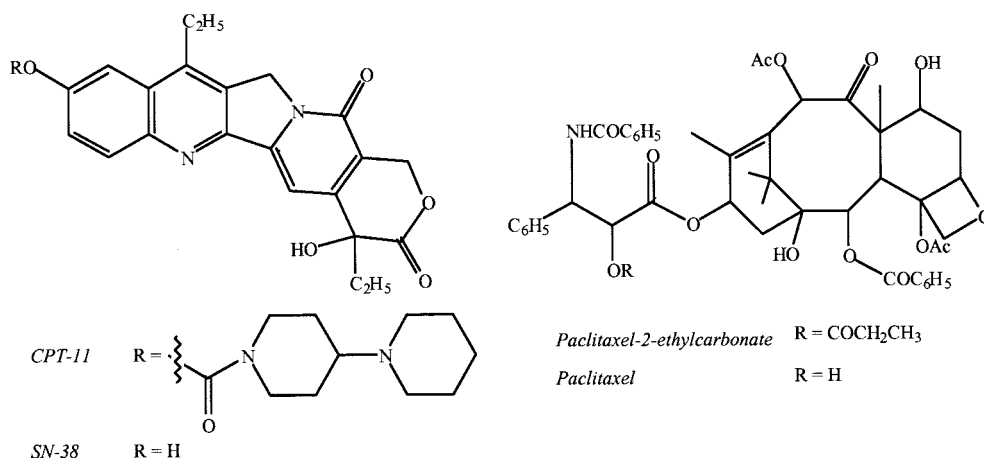


FIG. 21. Activation of CPT-11 and paclitaxel-2-ethylcarbonate by carboxylesterase into SN-38 and paclitaxel, respectively.

TABLE 10
Prodrugs activated by carboxylesterase

Prodrug	Drug	Pharmacology	Reference
CPT-11 Paclitaxel-2-ethylcarbonate	SN-38 Paclitaxel	Topoisomerase I inhibitor Microtubule-binding agent	Sato et al., 1994 Senter et al., 1996
Capecitabine	5'-Deoxy-5- fluorocytidine (5-FU)	Thymidylate synthase inhibitor/ incorporated into DNA and RNA	Miwa et al., 1998
Tertiary amidomethyl esters	Carboxylic acids and amines ^a	Not reported	Iley et al., 1999

^a These product were obtained after carboxylesterase-mediated cleavage of esters; however, pharmacology of the carboxylic acids and amines is not reported.

antitumor activity of capecitabine in clinical trials have been reviewed recently (Budman, 2000).

A series of amidomethyl ester prodrugs were synthesized (Iley et al., 1999). The esters were shown to be activated by amide hydrolysis catalyzed by rat liver homogenates, resulting in the corresponding carboxylic acids and amines, and this prodrug activation was inhibited by the carboxylesterase inhibitor eserine (Table 10). The hydrolysis rate in liver was up to 120-fold that of plasma. Although no pharmacological rationale was given for these prodrugs, new pharmacologically relevant prodrugs might be developed based on the structure activity relationships reported (Iley et al., 1999).

2. Alkaline Phosphatase

a. Enzymology and Localization of Alkaline Phosphatase. Alkaline phosphatases (EC 3.1.3.1) catalyze the hydrolysis of phosphoric esters to the corresponding alcohols and phosphate (Schomburg and Stephan, 1990–1998). This metalloenzyme contains zinc and magnesium in the active center and has a broad substrate specificity. The enzyme is present in nearly all living organisms (Van Hoof and De Broe, 1994). It is both soluble and membrane-bound and is present in liver, bone, kidney, and many other tissues in several species, including humans. The highest activity is present in the liver (Holmberg et al., 1986; Van Hoof and De Broe, 1994). Differences in isoenzyme patterns of liver and bone isoforms have been found in malignancies and renal diseases and can be used as a diagnostic parameter (Van Hoof and De Broe, 1994; Fishman, 1995). Many alkaline phosphatase placental, intestinal,

and tissue-nonspecific isoenzymes have been cloned and sequenced (Moss, 1992).

b. Activation of Prodrugs by Alkaline Phosphatase. Because of the wide distribution of alkaline phosphatases, these enzymes cannot be used to locally activate prodrugs except when targeting the enzyme by ADEPT, GDEPT, or VDEPT approaches as described below (*Section III.E.*). Therefore, alkaline phosphatase prodrugs have been developed mainly to increase the bioavailability of antitumor drugs.

S-2-(3-Aminopropylamino)-ethylphosphorothioic acid (amifostine, WR-2721; Fig. 22) is a clinically used prodrug that is dephosphorylated by membrane-bound alkaline phosphatases to the chemoprotective thiol WR-1065 (Calabro-Jones et al., 1985) (Table 11). This thiol drug has been shown to scavenge radicals that are generated by radiation and by drugs such as anthracyclines, platinum analogs, and alkylating agents. The membrane-bound alkaline phosphatase that activates amifostine is highly active in the cell membrane of normal endothelial cells but not in the cell membranes and neovascular capillaries of tumors, enabling selective protection of nontumor cells (Yuhás, 1980; Orditura et al., 1999). The chemoprotective properties, bioactivation, and clinical use of amifostine have recently been reviewed (Orditura et al., 1999; Santini and Giles, 1999; Santini, 2001).

To increase the bioavailability of 3-amino-pyridine-2-carboxaldehyde thiosemicarbazone (3-AP), an inhibitor of ribonucleotide reductase that plays a crucial role in the biosynthesis of DNA by reductive conversion of ribo-

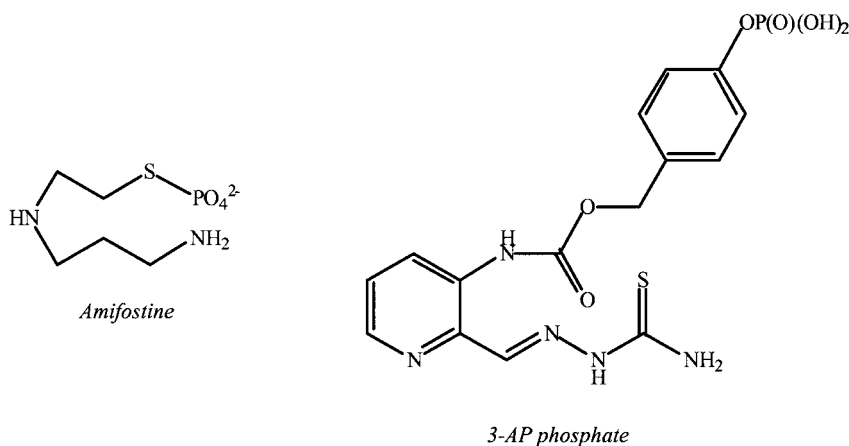


FIG. 22. Alkaline phosphate prodrugs.

nucleotides to deoxyribonucleotides, a phosphate prodrug was developed (Li et al., 1998) (Table 11, Fig. 22). 3-AP phosphate showed improved water solubility, was chemically stable, and rapidly converted to 3-AP by alkaline phosphatase from bovine intestine and rat liver S9 (Li et al., 1998, 2001a). In mice bearing murine lung carcinoma or murine melanoma xenografts, the prodrug 3-AP phosphate, although used at a higher concentration than 3-AP, showed a lower mortality and improved antitumor efficacy compared with 3-AP (Li et al., 1998, 2001a). Tissue-selective activation was not examined.

3. β -Glucuronidase

a. *Enzymology and Localization of β -Glucuronidase.* β -Glucuronidase (EC 3.2.1.31) is a group of isoenzymes that catalyze the hydrolyzes of β -D-glucuronides to the corresponding alcohol and D-glucuronate (Schomburg and Stephan, 1990–1998). This O-glycosyl bond hydrolysis reaction has been observed with numerous glucuronides. The enzyme is present in virtually all tissues and can be found in microsomes (endoplasmic reticulum) and lysosomes (Fishman, 1995). Although considerable β -glucuronidase activity is present in a variety of tissues, the highest concentrations can be found in the liver and spleen (Holmberg et al., 1986). In humans, considerable β -glucuronidase activity is also present in intestinal microflora. Because levels of β -glucuronidase are elevated in necrotic areas of tumors, the enzyme has been used extensively in prodrug approaches (Fishman, 1995; Sperker et al., 1997a; Huang and Oliff, 2001).

b. *Activation of Prodrugs by β -Glucuronidase.* Because β -glucuronidase enzymes are widely distributed, these enzymes cannot be used to locally activate prodrugs without ADEPT or GDEPT approaches as described below (Section III.F.). β -Glucuronidase prodrugs have been developed to improve the bioavailability of antitumor agents. Most prodrugs consist of the general structure drug-spacer-glucuronic acid (Figs. 23 and 24). Upon prodrug hydrolysis by β -glucuronidase, fragmentation of the self-immolative spacer occurs and the drug is released.

The tetra-*n*-butyl ammonium salt of (*p*-di-2-chloroethylaminophenyl- β -D-glucopyranoside) uronic acid (BHAMG) is a nitrogen-mustard prodrug that is activated by β -glucuronidase to *N,N*-di-(2-chloroethyl)-*p*-hydroxyaniline mustard (Roffler et al., 1991) (Table 12, Fig. 23). This work has been reviewed recently elsewhere (Dubowchik and Walker, 1999).

Many glucuronidated prodrugs of anthracyclines have been developed and were shown to be successful in i.v. therapy (Haisma et al., 1992, 1994; Houba et al., 1996; Bakina et al., 1997; Desbene et al., 1998; Florent et al.,

1998; Papot et al., 1998; Leenders et al., 1999) as reviewed extensively (Deonarain and Epenetos, 1994; Dubowchik and Walker, 1999; Huang and Oliff, 2001). These include glucuronidated prodrugs of doxorubicin, daunorubicin, and epirubicin, and these prodrugs have the general structure of anthracycline-spacer-glucuronic acid. The prodrugs were developed to improve bioavailability and to overcome the dose-related cardiotoxicity of anthracyclines. In this review only the most promising and new agents will be discussed.

Epirubicin-glucuronide is an anthracycline prodrug, isolated from patients treated with epirubicin, that was shown to be activated by β -glucuronidase (Haisma et al., 1992) as reviewed (Deonarain and Epenetos, 1994; Dubowchik and Walker, 1999) (Table 12). Another anthracycline prodrug, HMR 1826, is converted to doxorubicin by β -glucuronidase (Bakina et al., 1997); this work has been reviewed recently (Table 12, Fig. 23) (Huang and Oliff, 2001). HMR 1826 is particularly effective in ADEPT. The antitumor effect of HMR 1826 and a β -glucuronidase immunoconjugate in mice bearing human carcinoembryonic antigen-expressing tumor xenografts was much better than that observed for doxorubicin administration alone (Bosslet et al., 1994). From a group of glucuronidated daunorubicin prodrugs *N*-[4-daunorubicin-*N*-carbonyl-(oxymethyl)-phenyl]O- β -glucuronyl carbamate (DNR-GA3) was selected for preclinical evaluation (Table 12, Fig. 23) (Houba et al., 1996). The bioactivation of DNR-GA3 by β -glucuronidase resulting in the formation of daunorubicin has been reviewed elsewhere (Dubowchik and Walker, 1999). The pharmacokinetics was more recently studied in mice bearing human ovarian cancer xenografts (Houba et al., 1999). Bioactivation of DNR-GA3 in mice by β -glucuronidase was considerably higher in tumor tissue than in plasma, indicating a tumor-selective therapy. In other tissues the concentration of daunorubicin was 5- to 23-fold lower than in plasma. More recently, *N*-[4-doxorubicin-*N*-carbonyl-(oxymethyl)-phenyl]O- β -glucuronyl carbamate (DOX-GA3) was synthesized and found to be stable upon incubation with human serum (Leenders et al., 1999) (Fig. 23). The prodrug DOX-GA3 was slightly better activated by human β -glucuronidase with a K_m value of 1.1 mM and a V_{max} value of $25 \mu\text{mol}^{-1} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ than obtained for DNR-GA3, and the IC_{50} for antiproliferative activity against human ovarian cancer cells was 10 μM , which was also observed for DNR-GA3 (Table 13) (Leenders et al., 1999; Houba et al., 2001a). In studies in mice bearing human ovarian cancer xenografts, DOX-GA3 was shown to be even more effective than doxoru-

TABLE 11
Prodrugs activated by alkaline phosphatase

Prodrug	Drug	Pharmacology	Reference
Amifostine 3-AP phosphate	WR-1065 3-AP	Chemoprotection Ribonucleotide reductase inhibitor	Calabro-Jones et al., 1985 Li et al., 1998

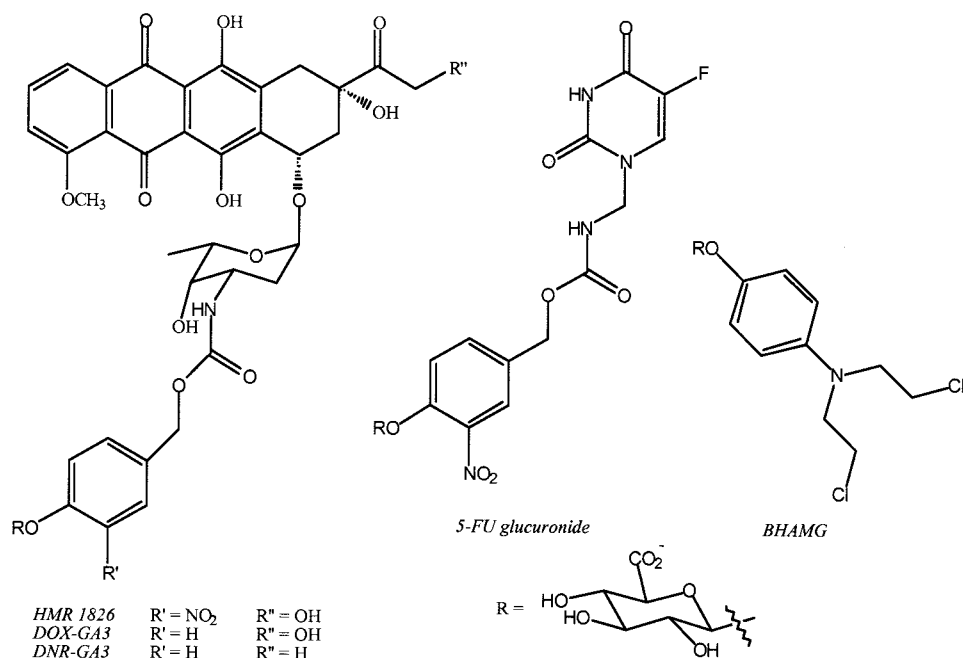


FIG. 23. β -Glucuronidase prodrugs that can be activated into doxorubicin (HMR 1826 and DOX-GA3), daunorubicin (DNR-GA3), 5-FU (5-FU glucuronide), and *N,N*-di-(2-chloroethyl)-*p*-hydroxyaniline mustard.

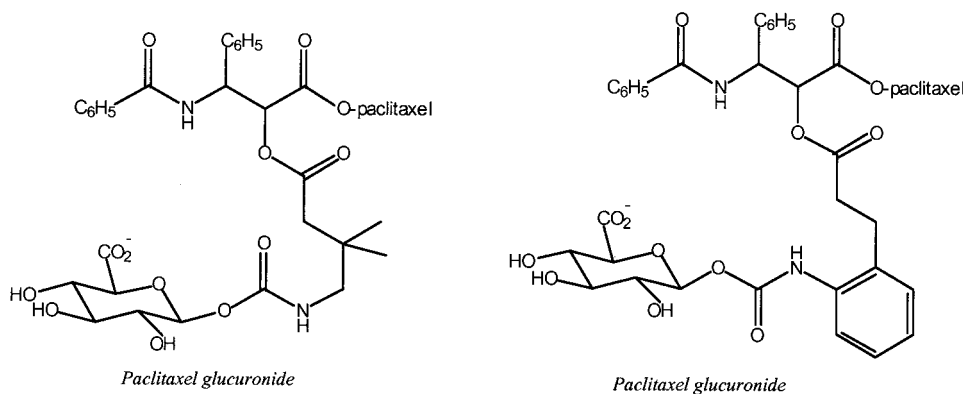


FIG. 24. Paclitaxel prodrugs that are activated by β -glucuronidase. Paclitaxel is coupled as shown in Fig. 21.

TABLE 12
Prodrugs activated by β -glucuronidase

Prodrug	Drug	Pharmacology	Reference
BHAMG	pHAM	Diverse ^a	Roffler et al., 1991
Epirubicin-glucuronide	Epirubicin	Diverse ^a	Haisma et al., 1992
HMR 1826	Doxorubicin	Diverse ^a	Bakina et al., 1997
DNR-GA3	Daunorubicin	Diverse ^a	Houba et al., 1996
DOX-GA3	Doxorubicin	Diverse ^a	Leenders et al., 1999
Paclitaxel glucuronide	Paclitaxel	Microtubule-binding	de Bont et al., 1997
5-FU glucuronide	5-FU	Thymidylate synthase inhibitor/incorporated into DNA and RNA	Guerquin-Kern et al., 2000

^a DNA intercalation, inhibition of topoisomerase II, and redox cycling (oxidative stress).

BHAMG, (*p*-di-2-chloroethylaminophenyl- β -*D*-glucopyranoside) uronic acid; pHAM, *N,N*-di-(2-chloroethyl)-*p*-hydroxyaniline mustard.

bicin (Houba et al., 2001a). An almost 5-fold higher doxorubicin peak concentration in the tumor was observed after DOX-GA3 administration to mice compared with doxorubicin administration, whereas the doxorubicin concentration in the heart (target organ for dose-limiting toxicity) were up to 5-fold lower after administration of DOX-GA3 compared with doxorubicin (Houba

et al., 2001a). An ADEPT approach with an enzyme immunoconjugate prepared from β -glucuronidase and a carcinoma-specific monoclonal antibody showed improved antitumor activity in mice compared with DOX-GA3 administration alone (Houba et al., 2001b).

Two β -glucuronyl carbamate-based prodrugs of paclitaxel (paclitaxel glucuronide) were synthesized and

TABLE 13
Prodrugs activated by cysteine conjugate β -lyase

Prodrug	Drug	Pharmacology	Reference
PC	6-MP	Antimetabolite	Hwang and Elfarra, 1989
GC	6-Thioguanine	Antimetabolite	Elfarra et al., 1995
SeCys conjugate	Selenol	Apoptosis inducer and cancer preventive agent	Commandeur et al., 2000

shown to be activated to the antitumor agent paclitaxel by human β -glucuronidase (Table 12, Fig. 24) (de Bont et al., 1997). The prodrugs were 2 orders of magnitude less cytotoxic than paclitaxel in human ovarian cancer cells, although after activation of these prodrugs with human β -glucuronidase, a similar cytotoxicity as with paclitaxel was observed. An ADEPT approach was developed, and the IC₅₀ values of cytotoxicity were approximately 2-fold lower than after prodrug administration alone; however, the amount of enzyme bound to cells was too low to complete the activation (de Bont et al., 1997).

Recently, the 5-FU prodrug 5-fluoro-*N*-[4-*O*-(β -D-glucopyranosyluronic acid)-3-nitrobenzyl-oxycarbonyl]-2,4-di-oxo-1,2,3,4-tetrahydropyrimidin-1-yl-methylamine (5-FU glucuronide) was developed, and the bioactivation was studied in vivo with ¹⁹F NMR (Guerquin-Kern et al., 2000) (Table 12, Fig. 23). Because of the very fast elimination of the prodrug, despite being stable in plasma, intratumor injection in mice was required. Thirty minutes after intratumor injection of the prodrug into mice, 5-FU became apparent in the tumor as determined by ¹⁹F NMR. In human colon cancer cell lines, the prodrug was also converted to 5-FU by β -glucuronidase (Guerquin-Kern et al., 2000).

4. Discussion of Hydrolase Enzymes as Prodrug-Activating Enzymes. Three enzyme systems have been used to activate prodrugs of antitumor agents, i.e., carboxylesterases, alkaline phosphatases, and β -glucuronidases. Because of the wide distribution of carboxylesterases, these enzymes can only be used to activate slow-release prodrugs as shown for paclitaxel-2-ethylcarbonate. To obtain a tumor-specific activation of carboxylesterase prodrugs, ADEPT and GDEPT need to be developed, as has been shown for the camptothecin analog CPT-11 (Crystal, 1999; Aghi et al., 2000; Rivory, 2000; Wierdl et al., 2001).

Using alkaline phosphatase and β -glucuronidase tumor selectivity can also only be accomplished using ADEPT and GDEPT strategies (described in *Sections III.E.* and *III.F.*, respectively), because both enzymes are widely distributed. Furthermore, interindividual differences in liver β -glucuronidase activity and protein expression (up to 6-fold) and kidney β -glucuronidase activity and protein expression (up to 14-fold) limit the use of β -glucuronidase prodrugs without making use of ADEPT or GDEPT (Sperker et al., 1997b). An interesting observation is that β -glucuronidase activity is higher in inflammatory necrotic tumor areas, thus in solid tumors; however, glucuronidated prodrugs are very poorly taken up by cells (Senter, 1990; Huang and Oliff, 2001).

Therefore, ADEPT strategies, as have been shown, appear to be a promising strategy to target glucuronidated prodrugs to tumor cells, because the prodrug is activated extracellularly in ADEPT (Dubowchik and Walker, 1999; Huang and Oliff, 2001).

D. Class 4 Lyases

1. Cysteine Conjugate β -Lyase

a. Enzymology of Cysteine Conjugate β -Lyase. Cysteine conjugate β -lyases (EC 4.4.1.13) catalyze the β -elimination of various cysteine *S*-conjugates and SeCys conjugates to the corresponding thiols and selenols, pyruvate, and ammonia (Schomburg and Stephan, 1990–1998; Cooper, 1998; Commandeur et al., 2000). The thiols released can be highly toxic or pharmacologically active depending on their structure (Commandeur et al., 1995), and the selenols released can be pharmacologically active as well (see below). The enzyme, which contains pyridoxal 5'-phosphate, also catalyzes the transamination of various amino acids, concomitantly using α -keto acids as a cofactor. For both routes only L-isomers are substrates. The biochemistry of cysteine conjugate β -lyase and mechanisms of action has been reviewed elsewhere (Cooper, 1998).

b. Localization of Cysteine Conjugate β -Lyase. Cysteine conjugate β -lyase activity was initially shown in rat liver cytosol (Tateishi et al., 1978). This enzyme appeared to be identical with kynureninase (EC 3.7.1.3) (Stevens, 1985). In addition to cytosol, cysteine conjugate β -lyase is also present in mitochondria (Commandeur et al., 1995). However, the highest activity of cysteine conjugate β -lyase was demonstrated in the proximal tubule of the kidney (Jones et al., 1988). Renal cytosolic and mitochondrial cysteine conjugate β -lyase appears to be identical to glutamine transaminase K (EC 2.6.1.64) (Stevens et al., 1986). In addition to the liver and kidney, the enzyme is also present in various other tissues but to a lower extent (Tables 1 and 2) (Jones et al., 1988; Rooseboom et al., 2002). Gastrointestinal bacteria also possess cysteine conjugate β -lyase activity and may contribute to bioactivation of substrates (Commandeur et al., 1995). In humans the highest specific activity is present in the liver, and the activity in the kidney is 2- to 5-fold lower depending on the substrate used (Tomisawa et al., 1986; Rooseboom et al., 2000, 2002). For both rat and human, the organ selectivity of cysteine conjugate β -lyase substrates may even be increased in the kidney due to active uptake of cysteine *S*-conjugates and SeCys conjugates (Commandeur et al., 1995).

Cysteine conjugate β -lyase activity is also present in renal carcinoma tissue; however, the activity varies between various patients with the same kind of carcinoma up to 50-fold (Nelson et al., 1995). Although considerable heterogeneity in cysteine conjugate β -lyase activity was observed in normal and tumor tissue, a high proportion of tumor samples had an enzyme activity that was at least 50% of that in normal tissue. For several patients a higher cysteine conjugate β -lyase activity was observed in tumoral tissue than in normal renal tissue (Nelson et al., 1995).

c. Activation of Prodrugs by Cysteine Conjugate β -Lyase. The high activity of cysteine conjugate β -lyase in proximal tubular cells has been associated with selective nephrotoxicity by cysteine *S*-conjugates of halogenated alkenes when administered to rodents (Commandeur et al., 1995). This finding and the presence of cysteine conjugate β -lyase in renal carcinoma have led to the development of kidney-selective prodrugs that can be activated by cysteine conjugate β -lyases (Table 13).

S-(6-Puriny)-L-cysteine (PC) was shown to be activated to the antitumor drug 6-MP in rats and by renal subcellular fractions (Hwang and Elfarra, 1989) (Table 13, Fig. 25). This bioactivation was shown to be inhibited by aminoxyacetic acid, indicating the involvement of cysteine conjugate β -lyase enzymes (Hwang and Elfarra, 1989, 1991). After administration of PC to rats, metabolite concentrations in kidney were 2.5- and 100-fold higher than concentrations in liver and plasma, respectively (Hwang and Elfarra, 1991).

The prodrug, *S*-(guanin-6-yl)-L-cysteine (GC), was shown to be activated to 6-thioguanine by renal cysteine conjugate β -lyases from mitochondria and cytosol with comparable K_m values (Elfarra et al., 1995) (Table 13, Fig. 25). After administration of GC to rats, renal concentration of 6-thioguanine was 4-fold higher than hepatic 6-thioguanine, whereas plasma concentrations of 6-thioguanine were undetectable. The renal 6-thioguanine concentration obtained was higher than IC_{50} values for cytotoxicity of 6-thioguanine in human renal carci-

noma cell lines (0.5–1.0 μ M), supporting the potential use of GC as a kidney-selective antitumor prodrug (Elfarra et al., 1995).

Another group of prodrugs that are activated by cysteine conjugate β -lyases are SeCys conjugates (see Table 13 and Section II.A.2.c.). These prodrugs were shown to be bioactivated by rat and human renal cytosolic enzymes to their pharmacologically active selenols, pyruvate, and ammonia (Fig. 3) (Andreadou et al., 1996; Rooseboom et al., 2000). The pharmacology of selenium compounds including SeCys conjugates and their selenols has been reviewed elsewhere (Ip, 1998; Mughesh et al., 2001). Using purified rat renal cytosolic cysteine conjugate β -lyase/glutamine transaminase K, it was shown that some substrates were activated at higher activity levels by this enzyme than *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine, which was until then the best substrate known for this enzyme. It was also shown that the SeCys conjugates were activated up to 200-fold faster by cysteine conjugate β -lyase than their corresponding sulfur conjugates, making them more suitable for prodrug delivery (Commandeur et al., 2000). As described before (Section II.A.2.c.), these SeCys conjugates were also activated by amino acid oxidases and FMOs, enzymes that are highly expressed in the kidney; however, in vivo kidney selectivity remains to be established (Rooseboom et al., 2001a,b).

d. Discussion of Cysteine Conjugate β -Lyase as a Prodrug-Activating Enzyme. Cysteine conjugate β -lyases have been used to activate prodrugs. Cysteine conjugate β -lyases are mainly located in the kidney, implying a kidney-selective antitumor therapy. This is of special interest since no effective chemotherapy is available to treat renal cell carcinoma to date, and the golden rule is still a radical nephrectomy. As described above, administration of PC and GC to rats resulted in much higher levels of 6-MP and 6-thioguanine in the kidney than in liver and plasma (Hwang and Elfarra, 1991; Elfarra et al., 1995). Interestingly, SeCys conjugates are activated by cysteine conjugate β -lyases into selenols, possessing

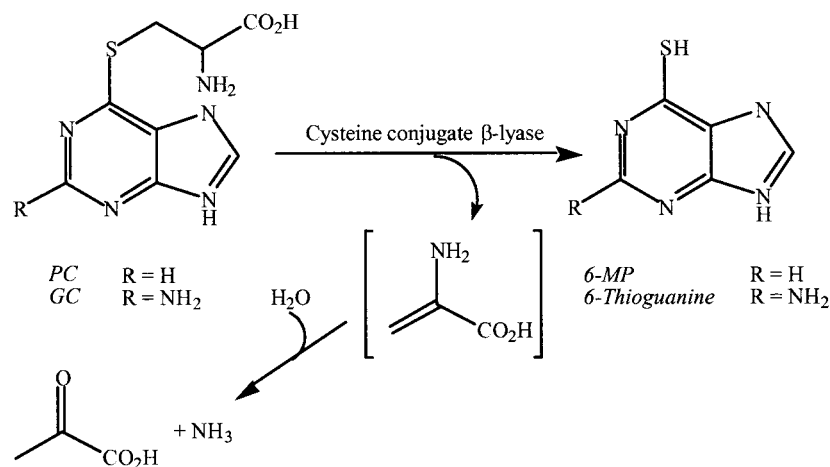


FIG. 25. Activation of PC and GC by cysteine conjugate β -lyase into 6-MP and 6-thioguanine, respectively.

antitumor activity, much more rapidly than their corresponding cysteine *S*-conjugates (up to 200-fold) (Ip, 1998; Commandeur et al., 2000).

Interindividual differences in renal and hepatic cysteine conjugate β -lyase activity are small (3-fold); however, in contrast to the rat, human renal cysteine conjugate β -lyase activity was 2- to 5-fold lower than human hepatic cysteine conjugate β -lyase (Rooseboom et al., 2000, 2002). This might result in a first-pass effect, although the kidney selectivity of cysteine conjugate β -lyase substrates may even be increased in the kidney due to active uptake of cysteine *S*-conjugates and SeCys conjugates (Commandeur et al., 1995). Therefore, in vivo studies are needed to further elucidate the kidney selectivity of cysteine conjugate β -lyase prodrugs.

III. Prodrugs Activated by Antibody-, Gene-, and Virus-Directed Enzyme Prodrug Therapy Approaches

In the past, several prodrugs have been developed that were shown to be inefficiently activated by human enzymes. These prodrugs have therefore been used in ADEPT strategies using nonhuman enzymes (Fig. 1A). The availability of the genes encoding for the respective enzymes have led to the development of GDEPT and

VDEPT approaches (Fig. 1B). In the next paragraphs, the most important examples of ADEPT, GDEPT, and VDEPT enzyme-prodrug approaches will be discussed (Table 14).

A. Nitroreductase

1. *Enzymology of Nitroreductase.* Nitroreductase catalyzes the reduction of nitro groups to hydroxylamino groups (Grove et al., 1999). In addition to the reduction of nitro compounds, the enzyme also reduces quinones such as menadione. The enzyme has been isolated from *E. coli*, uses NADH or NADPH as a cofactor, and is inhibited by the DT-diaphorase inhibitor dicoumarol. The molecular weight of nitroreductase is 24 kDa. Turnover numbers for this enzyme are 42,000 min⁻¹ for menadione and 360 min⁻¹ for CB 1954. The *K_m* of nitroreductase for menadione is 80 μ M, and the *K_m* for CB 1954 is 862 μ M (Anlezark et al., 1992; Grove et al., 1999).

2. *Activation of CB 1954 by Nitroreductase.* As described above (Section II.A.4. and Fig. 4), the antitumor prodrug CB 1954 is activated to the cytotoxic metabolite 5-aziridin-1-yl-4-hydroxylamino-2-nitrobenzamide by both rat DT-diaphorase and *E. coli* nitroreductase (Knox et al., 1988, 1992). However, *E. coli* nitroreductase is

TABLE 14
Bioactivating enzymes and prodrugs used in ADEPT, GDEPT, and VDEPT

Enzyme	Prodrug	Drug	Reference
Nitroreductase	CB 1954	5-(Aziridin-1-yl)-4- hydroxyl-amino-2-nitro- benzamide	Knox et al., 1992
Cytochrome P450	4-Ipomeanol	Unidentified (furan epoxide is speculated)	Mohr et al., 2000a
Cytochrome P450	Ifosfamide	Isophosphoramidate mustard	Waxman et al., 1999
Cytochrome P450	Cyclophosphamide	Phosphoramidate mustard	Waxman et al., 1999
Purine-nucleoside phosphorylase	Fludarabine	2-Fluoroadenine	Huang and Plunkett, 1987
Purine-nucleoside phosphorylase	MeP-dR	MeP	Sorscher et al., 1994
Thymidine kinase	Ganciclovir	Ganciclovir-triphosphate nucleotide	Moolten, 1986
Alkaline phosphatase	Etoposide phosphate	Etoposide	Senter et al., 1988
Alkaline phosphatase	Mitomycin C phosphate	Mitomycin C	Senter et al., 1989
Alkaline phosphatase	POMP	POM	Wallace and Senter, 1991
Alkaline phosphatase	<i>N</i> -(4-phosphonoxy)-phenylacetyl)doxorubicin	Doxorubicin	Vrudhula et al., 1993a
β -Glucuronidase	Glucuronidated <i>Nornitrogen</i> mustard	Oxazolidinone	Papot et al., 2000
β -Glucuronidase	Glucuronidated 9-amino-camptothecin	9-Aminocamptothecin	Leu et al., 1999
β -Glucuronidase	Glucuronide mustard	Mustard	Lougerstay-Madec et al., 1998
Carboxypeptidase	Methotrexate-amino acids	Methotrexate	Dubowchik and Walker, 1999
Carboxypeptidase	CMDA	Benzoic acid mustard	Blakey et al., 1993
Penicillin amidase	DPO	Doxorubicin	Kerr et al., 1990
Penicillin amidase	MelPO	Melphalan	Kerr et al., 1990
Penicillin amidase	NHPAP	Palytoxin	Bignami et al., 1992
Penicillin amidase	<i>N</i> -(phenylacetyl) doxorubicin	Doxorubicin	Vrudhula et al., 1993a
Penicillin amidase	<i>N</i> -(phenylacetyl) melphalan	Melphalan	Vrudhula et al., 1993a
β -Lactamase	LY 266070	DAVLBHYD	Jungheim et al., 1992
β -Lactamase	C-DOX	Doxorubicin	Hudyma et al., 1993
β -Lactamase	PRODOX	Doxorubicin	Jungheim et al., 1993
β -Lactamase	CM	Phenylenediamine mustard	Svensson et al., 1992
β -Lactamase	CCM	Phenylenediamine mustard	Vrudhula et al., 1993b
β -Lactamase	Cephalosporin-DACCP	DACCP	Hanessian and Wang, 1993
β -Lactamase	PROTAX	Taxol	Rodrigues et al., 1995a
β -Lactamase	Cephalosporin mitomycin C	Mitomycin C	Vrudhula et al., 1997
β -Lactamase	C-Mel	Melphalan	Kerr et al., 1998
Cytosine deaminase	5-Fluorocytosine	5-Fluorouracil	Senter et al., 1991
Methionine γ -lyase	Selenomethionine	Methylselenol	Miki et al., 2001
Methionine γ -lyase	Trifluoromethionine	CSF ₂	Coombs and Mottram, 2001

MeP, 6-methylpurine; POMP, *p*-[*N,N*-bis(2-chloroethyl)amino]phenyl phosphate; POM, *p*-[*N,N*-bis(2-chloroethyl)amino]phenol; DAVLBHYD, 4-desacetylvinblastine-3-carboxylic acid hydrazide; DACCP, 4'-carboxyphthalato(1,2-cyclohexanediamine) platinum; CSF₂, carbonothionic difluoride.

much more efficient than rat DT-diaphorase in activating CB 1954, with k_{cat} values of 360 min^{-1} and 4 min^{-1} , respectively (Anlezark et al., 1992). Human DT-diaphorase is less active than rat DT-diaphorase, and CB 1954 appears to inhibit the enzyme rather than being a substrate for this enzyme (Boland et al., 1991). In combination with purified *E. coli* nitroreductase, CB 1954 was cytotoxic to V79 cells that were insensitive toward CB 1954 only. Cloning of *E. coli* nitroreductase led to its use in GDEPT. Infection of mammalian cells with a recombinant retrovirus containing the nitroreductase cDNA enhanced CB 1954 sensitivity with a bystander effect. The use of ADEPT and GDEPT approaches with CB 1954/nitroreductase has been reviewed elsewhere (Dubowchik and Walker, 1999; Aghi et al., 2000; Greco and Dachs, 2001).

B. Cytochrome P450

1. Activation of Prodrugs by Nonhuman Cytochromes P450. The enzymology of P450s (EC 1.14.14.1) is described above (Section II.A.5.a.). To overcome the fact that P450 expression is generally high in liver, often resulting in a first-pass effect, and low in tumor cells, a P450-based cancer gene therapy was recently developed to target P450 to tumor cells.

Brain tumors cannot be treated effectively with cyclophosphamide (Fig. 8) due to the high expression of P450s in the liver, a low P450 activity in brain, and a poor transport of active metabolites of cyclophosphamide over the blood-brain barrier. A GDEPT has been developed using CYP2B1 and cyclophosphamide to treat tumors of the central nervous system. Cyclophosphamide and ifosfamide have been used in a GDEPT approach using rat CYP2B1 gene both in vitro and in vivo. Both prodrugs were much more cytotoxic toward 9L gliosarcoma cells stably transfected with cDNA-encoding CYP2B1 than toward the parental gliosarcoma cells. Cyclophosphamide was also much more cytotoxic toward gliosarcoma cells expressing CYP2B1 implanted in rats than after implantation with parental gliosarcoma. These and other GDEPT approaches have been reviewed recently by others (Dubowchik and Walker, 1999; Waxman et al., 1999; Aghi et al., 2000).

The naturally occurring furanoterpenoid 4-ipomeanol (Fig. 5) has been used for gene therapy of hepatocellular carcinoma cell lines using cDNA-encoding rabbit CYP4B1, the most active bioactivating enzyme, and human hepatocellular carcinoma cell lines (Czerwinski et al., 1991; Mohr et al., 2000a). This in vitro system rapidly induced cell death by apoptosis with a bystander effect and was independent of the p53 functional gene (Mohr et al., 2000a).

C. Purine-Nucleoside Phosphorylase

1. Enzymology of Purine-Nucleoside Phosphorylase. Purine-nucleoside phosphorylase (EC 2.4.2.1) catalyzes the conversion of purine nucleoside in the presence of P_i

to purine and α -D-ribose-1-phosphate (Schomburg and Stephan, 1990–1998). Adenosine, guanosine, inosine, and analogs, including their deoxy analogs, are substrates for this enzyme. For the natural substrates the reaction is reversible. The phosphorylase enzyme is found in bacteria, such as *E. coli*, and mammals. In mammals it is located especially in the liver, brain, thyroid, kidney, and spleen (Bzowska et al., 2000). The highest concentrations of the enzyme are found in the kidney, peripheral lymphocytes, and granulocytes. Turnover numbers of pentosyl group transfer have been reported up to 9480 min^{-1} (guanine), and K_m values for the natural substrates are in the micromolar range. The biochemistry and clinical aspects of purine-nucleoside phosphorylase have been reviewed comprehensively elsewhere (Bzowska et al., 2000).

2. Activation of Prodrugs by Purine-Nucleoside Phosphorylase. Because of the large differences in specificity of *E. coli* and human purine-nucleoside phosphorylase, the enzyme can be used in ADEPT, GDEPT, and VDEPT approaches using prodrugs that cannot be activated by human purine-nucleoside phosphorylases. The antitumor agent 9- β -D-arabinofuranosyl-2-fluoroadenine (fludarabine; Fig. 20) is activated by purine-nucleoside phosphorylase from *E. coli* to 2-fluoroadenine with a K_m of 1.35 mM and a V_{max} of 7.7 nmol/min/mg of protein (Huang and Plunkett, 1987). Although the mechanism by which 2-fluoroadenine exerts its antitumor effect is unknown, 2-fluoroadenine has to be phosphorylated to the toxic 2-fluoroadenine-triphosphate. Fludarabine is not activated by mammalian purine-nucleoside phosphorylases as has been shown for other adenine and adenine nucleosides (Parks and Agarwal, 1972). Therefore, VDEPTs were developed to treat hepatocellular carcinoma using fludarabine and purine-nucleoside phosphorylase (Mohr et al., 2000b). Adenoviral delivery of a gene-encoding *E. coli* purine-nucleoside phosphorylase followed by fludarabine administration prevented subcutaneous and intrahepatic tumor formation in nude mice and was also effective for the treatment of established tumors (Mohr et al., 2000b).

Another prodrug that is activated by *E. coli* purine-nucleoside phosphorylase is 9-(β -2-deoxy-erythropentofuranosyl)-6-methylpurine (MeP-dR). This prodrug was shown to be 20-fold less cytotoxic to Hep-2 cells than 6-methylpurine (Montgomery and Hewson, 1968). The prodrug MeP-dR is activated by *E. coli* purine-nucleoside phosphorylase to its active metabolite 6-methylpurine, whereas the corresponding mammalian enzymes cannot catalyze this reaction (Sorscher et al., 1994). Therefore, ADEPT/GDEPT approaches were developed and shown to be successful in the treatment of human colon carcinoma, melanoma, and breast and prostate cancer in vitro as reviewed elsewhere (Lockett et al., 1997; Dubowchik and Walker, 1999; Aghi et al., 2000; Gadi et al., 2000).

D. Thymidine Kinase

1. *Enzymology of Thymidine Kinase.* Using ATP, thymidine kinase (EC 2.7.1.21) phosphorylates thymidine, resulting in the formation of thymidine-5'-phosphate and ADP (Schomburg and Stephan, 1990–1998; Wintersberger, 1997). This phospho group transfer has also been observed with uridine and cytidine analogs. The enzyme is involved in the salvage pathway of nucleotide biosynthesis. K_m values of natural ligands are generally in the low micromolar range (Schomburg and Stephan, 1990–1998). The cytosolic enzyme is present in bacteria such as *E. coli*, viruses such as herpes simplex viruses, and mammals. Thymidine kinase is present in all tissues containing proliferating cells, and high activity levels are present in thymus and spleen (Herzfeld and Raper, 1980; Arner and Eriksson, 1995). The thymidine kinase activity level is high in some leukemias, lymphomas, and other tumors, and increased serum levels have been observed in malignancies (Arner and Eriksson, 1995; Wintersberger, 1997). Whether there exists a correlation between the high thymidine kinase activity and rapid tumor proliferation is unknown. The regulation and biological functions of thymidine kinase have been reviewed (Wintersberger, 1997).

2. *Activation of Ganciclovir by Thymidine Kinase.* Ganciclovir is an antiherpetic prodrug that is activated by thymidine kinase (Moolten, 1986 and Fig. 26). The resulting ganciclovir monophosphate is subsequently converted by cellular kinases into the toxic ganciclovir triphosphate nucleotide. Ganciclovir is activated by herpes simplex virus type 1 thymidine kinase (HSV-TK), 3 orders of magnitude more efficient than any human kinase. Several gene therapy approaches have been developed with HSV-TK and ganciclovir as has been reviewed recently (Aghi et al., 2000; Greco and Dachs, 2001).

E. Alkaline Phosphatase

1. *Activation of Prodrugs by Nonhuman Alkaline Phosphatase.* The enzymology of alkaline phosphatase (EC 3.1.3.1) is described above (Section II.C.2.a.). Because of the wide distribution of alkaline phosphatases, these enzymes cannot be used to locally activate pro-

drugs except by using ADEPT, GDEPT, or VDEPT approaches.

During initial applications of the ADEPT approaches using alkaline phosphatase, it was shown that the prodrug etoposide phosphate (etopophos) is efficiently activated to the topoisomerase II inhibitor etoposide by a monoclonal antibody/alkaline phosphatase (L6-AP) immunoconjugate that binds to the surface of antigen-positive tumor cells (Senter et al., 1988). The prodrug was more than 100-fold less toxic than etoposide to human colon carcinoma cells, H3347 cells, whereas equal toxicity was observed upon preincubation with the L6-AP conjugate, which binds to the surface of H3347 cells. Similar results were obtained in vivo after implantation of the H3347 cell line in nude mice and subsequent administration of L6-AP conjugate and etoposide phosphate. A strong antitumor activity was observed compared with etoposide phosphate or etoposide administration alone (Senter et al., 1988). Pharmacodynamics of etoposide was studied in a phase I clinical trial in cancer patients. Etoposide phosphate was rapidly converted to etoposide (main metabolite), although myelosuppression appeared to be a dose-limiting toxicity (Kaul et al., 1996). The clinical status of etoposide phosphate and etoposide has been reviewed elsewhere (Hande, 1998).

The ADEPT strategy using the L6-AP conjugate developed by Senter et al. (1989) was also applied to study activation of the prodrug mitomycin C phosphate, *p*-[*N,N*-bis(2-chloroethyl)amino]phenyl phosphate, a prodrug of *p*-[*N,N*-bis(2-chloroethyl)amino]phenol, and *N*-(4-phosphonooxy)phenylacetyl)-doxorubicin (Wallace and Senter, 1991; Vrudhula et al., 1993a). The cytotoxicity of these prodrugs was largely enhanced upon activation by dephosphorylation catalyzed by alkaline phosphatase.

F. β -Glucuronidase

1. *Activation of Prodrugs by Nonhuman β -Glucuronidase.* The enzymology of β -glucuronidase (EC 3.2.1.31) is described above (Section II.C.3.a.). Although β -glucuronidase is present in a variety of human tissues, problems with β -glucuronidase prodrugs include their rapid

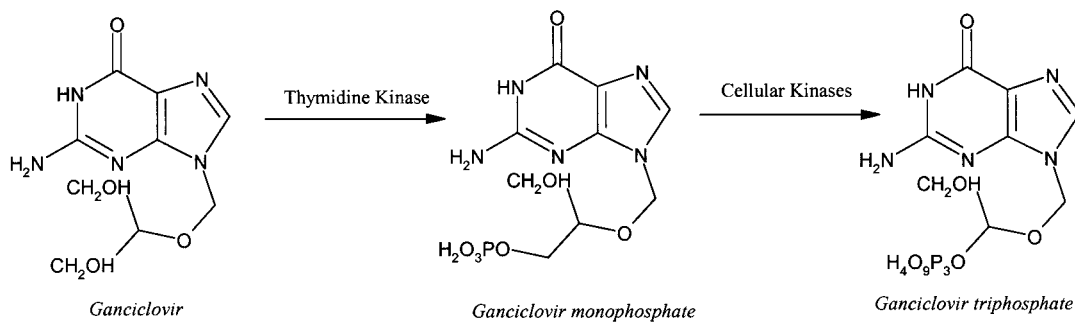


FIG. 26. Activation of ganciclovir into ganciclovir monophosphate by thymidine kinase and subsequent activation by cellular kinases into toxic ganciclovir triphosphate.

clearance, predominant activation in inflammatory necrotic tumor areas, and the lack of intracellular activation since glucuronidated prodrugs are very poorly taken up by cells (Senter, 1990; Huang and Oliff, 2001). To overcome these limitations, ADEPT and GDEPT approaches have been developed and might be more effective (Dubowchik and Walker, 1999).

A glucuronidated prodrug of the *nor*nitrogen mustard bis-(2-chloroethyl)amine was prepared using the same spacer as used for HMR 1826 (Fig. 23) (Papot et al., 2000). The prodrug was shown to be 27-fold less cytotoxic to colon cancer cells than the parent drug. Upon activation of the prodrug by *E. coli* β -glucuronidase, cytotoxicity of the prodrug was only 6-fold lower than the cytotoxicity of the drug itself. However, the *nor*nitrogen mustard prodrug was not activated to the expected drug bis-(2-chloroethyl)amine, but an oxazolidinone was observed generated by cyclization of the intermediate carbamic acid (nucleophilic substitution). This oxazolidinone has also been shown in patients treated with cyclophosphamide as a result of carboxylation of bis-(2-chloroethyl)amine (Papot et al., 2000).

N-[9-(β -D-glucuronyl)benzyloxycarbonyl]aminocamptothecin (glucuronidated 9-aminocamptothecin) was prepared by coupling of 9-aminocamptothecin to glucuronic acid via a carbamate spacer to use in an ADEPT approach and was shown to be activated by β -glucuronidase into the topoisomerase I inhibitor 9-aminocamptothecin (Leu et al., 1999). The prodrug was 20-fold less cytotoxic toward human carcinoma cells than 9-aminocamptothecin and was stable in human serum. In the presence of β -glucuronidase, the cytotoxicities of the prodrug and 9-aminocamptothecin in human carcinoma cells, measured by thymidine incorporation, were similar (Leu et al., 1999).

A mustard prodrug, designed to be used in ADEPT, was recently synthesized consisting of a glucuronic acid group connected to the drug via a carbamate spacer (Lougerstay-Madec et al., 1998). The prodrug was activated by *E. coli* β -glucuronidase and was 50-fold less toxic than the parent mustard drug toward human colon adenocarcinoma cells. After treatment with β -glucuronidase, cytotoxicity of the prodrug toward human colon adenocarcinoma cells was comparable with the parent mustard drug (Lougerstay-Madec et al., 1998).

G. Carboxypeptidase

1. Enzymology of Carboxypeptidase. Carboxypeptidases catalyze the hydrolysis of peptides, resulting in the formation of a shortened peptide and an amino acid (Schomburg and Stephan, 1990–1998). In GDEPT and ADEPT approaches, carboxypeptidase A (EC 3.4.17.1) and carboxypeptidase G (EC 3.4.19.9, formerly EC 3.4.22.12 and EC 3.4.12.10) have been most extensively studied. Carboxypeptidase A requires zinc as a cofactor, whereas carboxypeptidase G does not require a cofactor but is stimulated by sulfhydryl compounds, such as

2-mercaptoethanol, and by urea (Ludwig and Lipscomb, 1973; Rao and Noronha, 1977). Carboxypeptidases A and G are widely distributed over the body and are present in a variety of organisms including bovine, rat, and human.

2. Activation of Prodrugs by Carboxypeptidase. Successful carboxypeptidase prodrugs using ADEPT and GDEPT approaches have been developed, and this work has been reviewed extensively (Dubowchik and Walker, 1999; Schally and Nagy, 1999; Huang and Oliff, 2001). The antifolate drug methotrexate has been coupled to various amino acids (i.e., Ala, Asp, or Arg). These prodrugs are up to 100-fold less cytotoxic than methotrexate in vitro itself and can be activated efficiently by enzyme immunoconjugates of carboxypeptidase A. This approach has been converted to a GDEPT strategy as has been reviewed (Aghi et al., 2000). Various ADEPT approaches with bacterial carboxypeptidase G in combination with 4-[*N*-(2-chloroethyl)-*N*-[2-(mesyloxy)ethyl]amino]benzoyl-L-glutamic acid (CMDA) have been described (Blakey et al., 1993; Dubowchik and Walker, 1999; Aghi et al., 2000; Greco and Dachs, 2001). The first ADEPT phase I clinical trial was performed with CMDA and bacterial carboxypeptidase G showing that the prodrug was well tolerated, cyclosporin could be used to counteract the immune response to the conjugate, and the antitumor response with this ADEPT protocol was successful (Bagshawe and Begent, 1996; Dubowchik and Walker, 1999).

H. Penicillin Amidase

1. Enzymology of Penicillin Amidase. Penicillin amidase (EC 3.5.1.11) catalyzes the carboxylic acid amide hydrolysis of penicillin to 6-aminopenicillanate (Schomburg and Stephan, 1990–1998). The enzyme is present in several bacteria such as *E. coli* and occurs both intracellularly (*E. coli*) and extracellularly (*Bacillus megaterium*). Penicillin amidases are used in the semisynthesis of penicillin antibiotics. Because these enzymes are not present in humans, ADEPT approaches can be used to target penicillin amidase-based prodrugs.

2. Activation of Prodrugs by Penicillin Amidase. Doxorubicin-*N*-*p*-hydroxyphenoxyacetamide (DPO) and melphalan-*N*-*p*-hydroxyphenoxyacetamide (MelPO) were synthesized as prodrugs for doxorubicin and melphalan, respectively, and were shown to be activated by bacterial penicillin V amidase (Kerr et al., 1990). Both prodrugs are stable in mouse serum. The ADEPT approach in this study was developed using anticarcinoma and anti-B-cell lymphoma monoclonal antibodies conjugated to penicillin V amidase. The prodrugs DPO and MelPO were 20- to 80-fold and 100- to 1000-fold, depending on the cell type studied, less cytotoxic than their corresponding drugs, respectively. In the case of DPO, pretreatment of lung carcinoma cell line and B-cell lymphoma cell line with the corresponding enzyme immunoconjugates resulted in a 12.5-fold and 7.5-fold en-

hancement in the cytotoxicity compared with prodrug administration alone. However, cytotoxicity was still lower than after doxorubicin treatment alone, indicating only partial prodrug activation. Similar results were obtained for MelPO (Kerr et al., 1990). More recently, DPO was used in another ADEPT approach by targeting penicillin V amidase to folate receptor-positive cells (Lu et al., 1999). DPO was as toxic to folate receptor-positive cells as doxorubicin, whereas DPO was nontoxic toward folate receptor-negative cells. Preliminary in vivo studies also suggest that this ADEPT approach acts specifically on folate receptor-positive cells (Lu et al., 1999).

The prodrug *N*-(4'-hydroxyphenylacetyl)palytoxin (NHPAP) is activated by penicillin G amidase to the highly cytotoxic marine natural agent palytoxin (Bignami et al., 1992). The parent drug palytoxin is at least 5 orders of magnitude more toxic toward human lung adenocarcinoma cells than doxorubicin, mitomycin C, or 5-FU. The prodrug NHPAP was at least 1000-fold less cytotoxic than palytoxin toward human lung adenocarcinoma, ovarian carcinoma, and colon carcinoma, whereas in the presence of penicillin G amidase equal toxicities were observed. The prodrug was also effective against two drug-resistant cell lines, whereas doxorubicin, mitomycin C, or 5-FU was not effective against these cells. NHPAP has been studied in an ADEPT approach using enzyme immunoconjugates to target penicillin G amidase to human lung adenocarcinoma cells and human T-lymphoma cells. An advantage in the use of palytoxin prodrugs in ADEPT is that palytoxin exerts its cytotoxicity extracellularly, i.e., the place of activation of prodrugs by enzyme immunoconjugates in ADEPT. In this ADEPT approach, NHPAP was very effective toward human lung adenocarcinoma cells, whereas the prodrug was less effective against human T-lymphoma cells, both in vitro (Bignami et al., 1992).

Two other prodrugs of doxorubicin and melphalan namely *N*-(phenylacetyl)doxorubicin and *N*-(phenylacetyl)melphalan, respectively, are also activated by penicillin G amidase (Vrudhula et al., 1993a). *N*-(Phenylacetyl)doxorubicin and *N*-(phenylacetyl)melphalan were 10- and 20-fold less cytotoxic toward lung adenocarcinoma cells than doxorubicin and melphalan, respectively, whereas in the presence of penicillin G amidase the prodrugs were equitoxic with the corresponding parent drugs (Vrudhula et al., 1993a).

I. β -Lactamase

1. *Enzymology of β -Lactamase.* β -Lactamase (EC 3.5.2.6) catalyzes the hydrolysis of β -lactams to substituted β -amino acids (Schomburg and Stephan, 1990–1998). The enzyme, which is present in various bacteria, also hydrolyzes cephalosporins and penicillins. β -Lactamase is not present in human cells, and therefore only ADEPT approaches are suitable to target prodrugs that needs β -lactamase-mediated activation. An advantage of the lack of β -lactamase activity in humans is the

absence of unfavorable activation of β -lactamase-activated prodrugs.

2. *Activation of Prodrugs by β -Lactamase.* LY 266070, a cephalosporin-derived prodrug of 4-desacetylvinblastine-3-carboxylic acid hydrazide, was prepared and shown to be activated by β -lactamase with a catalytic efficiency (k_{cat}/K_m) of $11 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ (K_m , 160 μM ; k_{cat} , 1700 s^{-1}) (Jungheim et al., 1992). LY 266070 was 5-fold less cytotoxic toward a colon tumor cell line than the parent drug, whereas in the presence of an enzyme immunoconjugate of β -lactamase, enhanced cytotoxicity was observed. The prodrug LY 266070 was further studied in colon carcinoma xenografts in nude mice using β -lactamase from *Enterobacter cloacae* conjugated to antibodies (Meyer et al., 1993). Treatment with these antibodies and the prodrug LY 266070 resulted in a significant reduction of tumor mass compared with treatment with the parent drug 4-desacetylvinblastine-3-carboxylic acid hydrazide alone (Meyer et al., 1993).

The cephalosporin doxorubicin prodrug C-DOX (BMY 46633) was prepared and shown to be activated by β -lactamase from *E. cloacae* with a K_m value of 200 μM (Hudyma et al., 1993). In a subsequent study, a K_m value of 46 μM was reported for C-DOX with the same enzyme (Svensson et al., 1995). K_m values and catalytic efficiencies for C-DOX and β -lactamase and enzyme immunoconjugates of β -lactamase were similar. C-DOX was less toxic than the parent doxorubicin in three different tumor cell lines. In mice C-DOX was at least 7-fold less toxic than doxorubicin based on the maximum tolerated dose. In mice bearing lung adenocarcinoma cells, intratumor levels of doxorubicin were higher after treatment with C-DOX and an enzyme immunoconjugate consisting of β -lactamase and the monoclonal antibody L6 that selectively binds to antigens on lung adenocarcinoma cells, than after treatment with doxorubicin. Similar results were obtained for tumor volume reduction, indicating beneficial effects of C-DOX in an ADEPT approach over doxorubicin only (Svensson et al., 1995). In an ADEPT approach in mice bearing human melanoma tumor cells, similar results were obtained for C-DOX (Kerr et al., 1995). Several analogs of C-DOX were prepared, but none of them were more effective than C-DOX (Meyer et al., 1995; Vrudhula et al., 1995).

The doxorubicin prodrug PRODOX was shown to be activated by β -lactamase and was 20-fold less cytotoxic toward human breast cancer cells than doxorubicin (Jungheim et al., 1993; Rodrigues et al., 1995b). Using an ADEPT approach with an antibody that recognizes breast cancer cells overexpressing the p185^{HER2} product of proto-oncogene *HER2* and β -lactamase, it was shown that PRODOX was equitoxic to breast cancer cells with elevated levels of p185^{HER2} with doxorubicin, indicating a complete prodrug activation (Rodrigues et al., 1995b).

7-(Phenylacetamido)-cephalosporin mustard (CM) was developed as a prodrug of the DNA-alkylating agent

phenylenediamine mustard (Svensson et al., 1992). CM is activated by β -lactamase from *Bacillus cereus* and *E. coli*, resulting in the formation of phenylenediamine mustard with K_m values of 6 and 43 μM , respectively. CM was 50-fold less cytotoxic toward human lung adenocarcinoma cells than phenylenediamine mustard, whereas after activation by a monoclonal antibody- β -lactamase conjugate, cytotoxicity was comparable (Svensson et al., 1992). The CM analog 7-(4-carboxybutanamido)-cephalosporin mustard (CCM) was also shown to be activated to phenylenediamine mustard by β -lactamases with K_m values in the micromolar range (Vrudhula et al., 1993b). CCM was less toxic than the parent drug phenylenediamine mustard toward human lung cancer cells, and prodrug activation was observed after coadministration with an enzyme immunoconjugate consisting of β -lactamase and the monoclonal antibody L6 that binds to antigens on lung adenocarcinoma cells. In mice bearing lung adenocarcinoma cells, phenylenediamine mustard was ineffective, whereas a significant antitumor activity was observed using CCM and an enzyme immunoconjugate containing β -lactamase (Vrudhula et al., 1993b). In a subsequent study, it was shown that CCM was even more effective than C-DOX in mice bearing human melanoma tumor cells (Kerr et al., 1995), and CCM was more recently shown to be effective in renal cell carcinoma and human lung carcinomas using ADEPT approaches (Svensson et al., 1998; Kerr et al., 1999).

A cephalosporin prodrug of the potent antitumor agent 4'-carboxyphthalato(1,2-cyclohexanediamine) platinum was synthesized (Hanessian and Wang, 1993). The release of 4'-carboxyphthalato(1,2-cyclohexanediamine) platinum from the prodrug upon incubation with β -lactamase from *E. cloacae* was demonstrated in vitro using ^1H NMR previously used for cephaloridine hydrolysis; however, kinetic parameters were not reported. In the same study another cephalosporin-platinum prodrug containing a hydrophobic ester linking arm was synthesized, but β -lactamase-mediated bioactivation was not studied (Hanessian and Wang, 1993).

A cephalothin-derived prodrug of taxol, PROTAX was shown to be rapidly activated by β -lactamase to taxol with a catalytic efficiency (k_{cat}/K_m) of $1.4[\text{zmd}]10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Rodrigues et al., 1995a). PROTAX was approximately 10-fold less cytotoxic than taxol in breast tumor cells in vitro. Although using the same ADEPT approach as used by PRODOX, PROTAX showed activity approaching taxol, indicating the potential use of PROTAX as a taxol prodrug (Rodrigues et al., 1995a, b).

The cephalosporin mitomycin C prodrug [7'-[(N-9'-methoxymitosanyl)methyl]-7-(δ -carboxybutanamido)5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid] is bioactivated by β -lactamase from *E. cloacae* to mitomycin C with a K_m value of 119 μM and a k_{cat} value of 248 s^{-1} (Vrudhula et al., 1997) (Table 14). The IC_{50} value of inhibition of thymidine incorporation by the mitomycin

C prodrug was 10- and 20-fold lowered by the addition of β -lactamase for mouse melanoma cells transfected with a human melanoma-associated antigen and a human lung adenocarcinoma cell line, respectively. Furthermore, the prodrug was approximately 18-fold less toxic to both cell lines than the parent agent mitomycin C. Using the mitomycin C prodrug in an ADEPT approach, it was demonstrated that the prodrug was activated in both cell lines resulting in equitoxicity compared with mitomycin C.

The cephalosporin carbamate derivative of melphalan, C-Mel, was prepared and shown to be bioactivated by β -lactamase to the nitrogen mustard DNA-alkylating agent melphalan with a K_m of 218 μM and a k_{cat} of 980 s^{-1} (Kerr et al., 1998) (Table 14). The prodrug was 40-fold less toxic than the parent drug toward human melanoma cells. C-Mel in combination with an enzyme immunoconjugate consisting of β -lactamase and a melanoma-specific antibody was more effective than melphalan alone in mice bearing human melanoma cells. The therapeutic effect of C-Mel was comparable with the phenylenediamine mustard prodrug CCM (see above); however, the effectiveness of melphalan against malignant melanoma is clinically approved (Kerr et al., 1998).

J. Cytosine Deaminase

1. *Enzymology of Cytosine Deaminase.* Cytosine deaminase (EC 3.5.4.1) catalyzes the amidine hydrolysis of cytosine to uracil and ammonia (Schomburg and Stephan, 1990–1998). Several cytosine analogs, such as 5-methylcytosine and halogenated cytosines, are also substrates. The enzyme is present in several bacteria and fungi but not in mammalian cells. Therefore, ADEPT and GDEPT prodrug approaches have been developed.

2. *Activation of 5-Fluorocytosine by Cytosine Deaminase.* The prodrug 5-fluorocytosine (5-FC) is activated by cytosine deaminase to 5-FU (Fig. 27), which kills cells by incorporation into DNA and RNA and by inhibition of thymidylate synthetase (Nishiyama et al., 1985). Because cytosine deaminase is not expressed in human cells, several ADEPT and GDEPT approaches have been developed as initially reported by Senter et al. (1991) and Mullen et al. (1992), respectively. Cytosine deaminase from yeast was coupled to a tumor-specific antibody, and the combination of this immunoconjugate and 5-FC was equitoxic as 5-FU to human lung adenocarcinoma cells, although 5-FC itself was nontoxic (Senter et al., 1991). In the GDEPT approach, transfected murine fibroblast cells expressing *E. coli* cytosine deaminase were selectively killed by incubation with 5-FC, whereas control cell lines were not affected (Mullen et al., 1992). The use of 5-FC as a 5-FU prodrug using a GDEPT approach has been tested in cancer patients. The approach has been reviewed extensively and will thus not be further described in this review (Dubowchik and

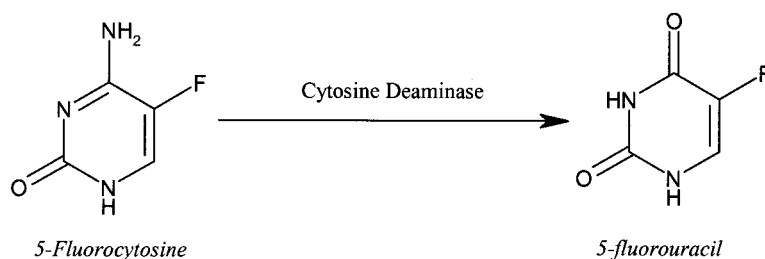


FIG. 27. Activation of 5-fluorocytosine into 5-FU by cytosine deaminase.

Walker, 1999; Aghi et al., 2000; Cunningham and Nemunaitis, 2001; Greco and Dachs, 2001).

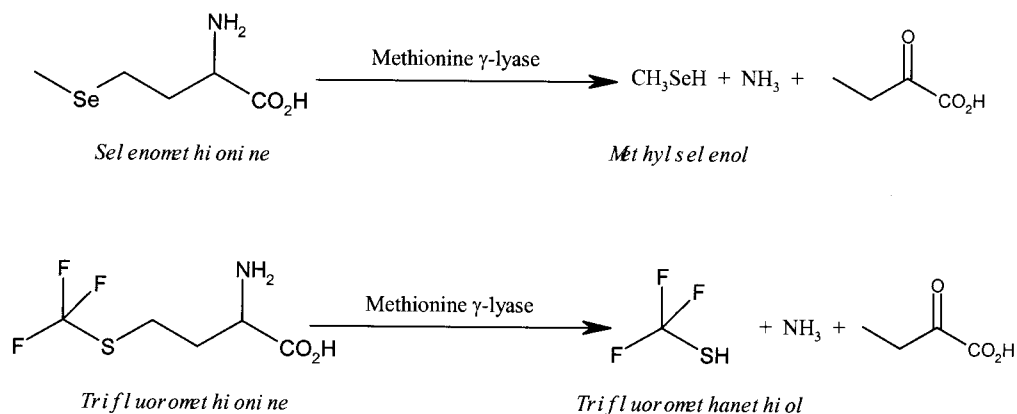
K. Methionine γ -Lyase

1. Enzymology of Methionine γ -Lyase. Methionine γ -lyase (EC 4.4.1.11) catalyzes the stereoselective α,γ -elimination of L-methionine to methanethiol, ammonia, and α -ketobutyrate (2-oxobutanoate) (Esaki et al., 1979; Esaki and Soda, 1987; Schomburg and Stephan, 1990–1998). The pyridoxal 5'-phosphate-dependent enzyme also catalyzes the elimination of several other amino acids such as L-ethionine, L-homocysteine, and selenomethionine (Esaki et al., 1979; Soda, 1987). In addition, methionine γ -lyase catalyzes the γ -replacement reaction between these substrates and thiols, such as 2-mercaptoethanol. The enzyme occurs in anaerobic microorganisms, whereas mammalian tissues do not possess methionine γ -lyase activity. This has led to the development of gene therapy approaches (GDEPT) using methionine γ -lyase.

2. Activation of Prodrugs by Methionine γ -Lyase. Selenomethionine is activated by methionine γ -lyase to α -ketobutyrate, ammonia, and toxic methylselenol via an α,γ -elimination mechanism (Fig. 28). Selenomethionine is a better substrate than natural methionine (Esaki et al., 1979). Because selenomethionine cannot be converted into methylselenol by mammalian cells, a GDEPT approach has recently been developed (Miki et al., 2001). Human lung, ovarian, pancreatic, and head and neck cancer cells were transduced with recombinant adenovirus-encoding methionine γ -lyase gene from

Pseudomonas putida. When selenomethionine was added, this resulted in a 1000-fold increased cytotoxicity compared with nontransduced cells. Superoxide anion radical was produced by methylselenol in ovarian carcinoma cells, resulting in mitochondrial damage, and cytochrome *c* release was observed, thereby activating the caspase cascade and apoptosis. In vivo experiments showed that selenomethionine in combination with recombinant adenovirus-encoding methionine γ -lyase gene were effective against rat hepatoma tumor cells implanted in mice; 4 of 5 mice were still alive by day 24, whereas all five mice treated with either the recombinant adenovirus or selenomethionine were dead by day 24 (Miki et al., 2001).

The anti-infective prodrug trifluoromethionine was also recently shown to be activated by methionine γ -lyase from parasite *T. vaginalis* to α -ketobutyrate, ammonia, and trifluoromethanethiol (Coombes and Mottram, 2001 and Fig. 28). The latter compound is known to be activated nonenzymatically to the potent cross-linker of primary amine groups, carbonothionic difluoride. The K_m value of trifluoromethionine was 13-fold lower than that of methionine. Propargylglycine, an inhibitor of methionine γ -lyase that does not affect the parasite, greatly reduced the toxicity of trifluoromethionine against *T. vaginalis*. Trifluoromethionine also protected mice against subcutaneous lesions by a *T. vaginalis* parasite. Because mammals do not contain methionine γ -lyase activity, this agent and this enzyme might be considered a lead compound in the develop-


 FIG. 28. Activation of selenomethionine and trifluoromethionine by methionine γ -lyase.

ment of anti-infective prodrugs with potential as chemotherapeutic agents (Coombs and Mottram, 2001).

IV. Concluding Remarks and Future Perspectives

As described in this review, numerous prodrugs have been developed for the delivery of higher concentrations of a drug to target cells than could otherwise be obtained by the administration of the drug itself. Although this goal is conceptually simple, the execution appears to be very complex. Nevertheless, several prodrugs are currently used to treat patients successfully. However, a general problem in designing prodrug strategies still concerns the minor biochemical differences between target and nontarget cells, often resulting in toxic side effects. More basic research is still needed to elucidate the phenotypic differences between target and nontarget cells. Another problem is that several human enzymes are relatively inefficient in the activation of the respective prodrugs. In an attempt to overcome this problem, ADEPT, GDEPT, and VDEPT strategies, enabling the use of exogenous enzymes (e.g., from bacteria and fungi), have been explored. The potential of these strategies is illustrated by the fact that the turnover of CB 1954 by *E. coli* nitroreductase is 90-fold higher than that of rat DT-diaphorase, whereas the human enzyme is less active than rat DT-diaphorase (Boland et al., 1991; Anlezark et al., 1992). Also, HSV-TK activates ganciclovir more efficiently than any human thymidine kinase (Aghi et al., 2000; Greco and Dachs, 2001). ADEPT, GDEPT, and VDEPT strategies are also used to increase tumor or organ selectivity as shown for treatment of brain tumors with cyclophosphamide. Using GDEPT, the low P450 activity in brain and poor transport of active metabolites of cyclophosphamide over the blood-brain barrier were overcome (Dubowchik and Walker, 1999; Aghi et al., 2000). ADEPT, GDEPT, and VDEPT can be used to activate prodrugs in humans using enzyme activities that are not present in humans as described in this review for penicillin amidase, β -lactamase, cytosine deaminase, and methionine γ -lyase.

The scarcity of tumor-specific antigens, adverse immune effects, and the need for penetration of the released drug through the cell membrane are clear limitations in ADEPT. Furthermore, the effectiveness of ADEPT in humans so far is disappointing in view of the high efficacy observed in rodent models with immunoconjugates (Dubowchik and Walker, 1999). GDEPT and VDEPT effectiveness has been limited to date by insufficient transduction of tumor cells in vivo, and further research is needed to increase transduction of gene-encoding bioactivating enzymes. To overcome the common problems in ADEPT, GDEPT, and VDEPT, novel protein-engineering strategies to humanize immunoconjugates and vectors are essential.

Because of the limitations in ADEPT, GDEPT, and VDEPT, it is still important to develop prodrugs that can be activated by tumor-selective endogenous enzymes, not only into established antineoplastic agents but also as initial targets for drug development. The successful clinical use of many antitumor prodrugs underlines the effectiveness of this concept, which was already introduced in the late 1950s by Albert (1958). A problem with several endogenous enzymes is their relatively low turnover, and it is therefore often a question whether a high enough steady influx of the desired drug in the target cells can be accomplished.

For two established antitumor agents, 5-FU and doxorubicin, a relatively wide variety of prodrug systems have been evaluated, enabling a comparison between several prodrugs and the bioactivating enzymes involved, based on their efficiency of activation, tissue selectivity of activation, and their antitumor activity (Table 15). 5-FU prodrugs that have been studied are 5-FP activated by aldehyde oxidase (Guo et al., 1995), ftorafur activated by P450s (Kawata et al., 1984), 5'-DFUR activated by thymidine phosphorylase (Armstrong and Diasio, 1980), 5-FU glucuronide (5-fluoro-*N*-[4-*O*-(β -D-glucopyranosyluronic acid)-3-nitrobenzyl-oxycarbonyl]-2,4-di-oxo-1,2,3,4-tetrahydropyrimidin-1-yl-methylamine) activated by β -glucuronidase (Madec-Lougerstay et al., 1999; Guerquin-Kern et al., 2000), and 5-FC activated by cytosine deaminase (Senter et al., 1991). All these prodrugs were designed to overcome the rapid breakdown of 5-FU in the gastrointestinal tract and the gastrointestinal toxicity of 5-FU. 5-FP is activated by rat liver aldehyde oxidase in vitro and by mice liver aldehyde oxidase in vivo, although no activity was present in mice intestine homogenates (Guo et al., 1995). Although the half-life of 5-FP in mice was at least twice that of 5-FU, it had no improved antitumor activity compared with 5-FU (Guo et al., 1995). A major problem with aldehyde oxidase is its wide distribution and the interspecies differences between substrate specificities. Therefore, aldehyde oxidase appears not to be the ideal candidate for the development of prodrugs. In analogy with aldehyde oxidase, P450 and β -glucuronidase are also widely distributed. Although ftorafur is activated by P450s, avoids the rapid breakdown of 5-FU in the gastrointestinal tract, and is clinically used, the problem with P450-dependent prodrugs is their activation in nontumor tissues because the activity is generally lower in tumor tissue than in the corresponding normal tissue (Philip et al., 1994; Murray et al., 1995). Another problem with P450s concerns the fact that large interindividual differences occur that may result in different clinical responses among patients treated with P450-dependent prodrugs (Iyer and Ratain, 1998; Wormhoudt et al., 1999). As recently illustrated for cyclophosphamide, an improvement of the tumor-selective activation of P450-activated prodrugs may be accomplished by using GDEPT, although there are still several drawbacks

TABLE 15
Prodrugs of 5-FU and doxorubicin, their respective enzymes, their activation, their antitumor activity, and their limitations

Drug	Side effect	Prodrug	Enzyme	Activation Investigated		Antitumor Effect Investigated		Limitations
				Vitro	Vivo	Vitro	Vivo	
5-FU	Rapid breakdown in gastrointestinal tract and gastrointestinal toxicity	5-FP	Aldehyde oxidase	Yes	Yes (Mice)	No	Yes (Mice)	No improvement in antitumor activity compared with 5-FU
		Ftorafur	P450	Yes	Yes (human)	Yes	Yes (human)	Non-tumor-specific activation, clinically used prodrug
		5'-DFUR	Thymidine phosphorylase	Yes	Yes (human)	Yes	Yes (human)	Causes gastrointestinal toxicity, ^a clinically used prodrug
		5-FU glucuronide	β -Glucuronidase	Yes	Yes (mice)	Yes	Yes (mice)	Rapid clearance after i.v. or i.p. injection, intratumoral injection necessary
		5-FC	Cytosine deaminase	Yes	Yes (mice)	Yes	Yes (mice)	General ADEPT limitations (see text), promising candidate, in clinical trials
Doxorubicin	Cardiotoxicity, acute myelo suppression, nausea, and vomiting	<i>N</i> -(4-phosphono-oxy-phenylacetyl) doxorubicin	Alkaline phosphatase	Yes	No	No	No	Widespread distribution, few data available
		HMR 1826	β -Glucuronidase	Yes	Yes (mice)	Yes	Yes (mice)	Only activated in necrotic sites of the tumor
		DOX-GA3	β -Glucuronidase	Yes	Yes (mice)	Yes	Yes (mice)	Only activated in necrotic sites of the tumor
		DPO	Penicillin amidase	Yes	No	Yes	No	Partial prodrug activation, adverse immune effects due to bacterial enzyme
		<i>N</i> -(phenylacetyl) doxorubicin	Penicillin amidase	Yes	No	No	No	Poor solubility in aqueous solution, Adverse immune effects due to bacterial enzyme
		C-DOX	β -Lactamase	Yes	Yes	Yes	Yes (mice)	Adverse immune effects due to bacterial enzyme, few data available
		PRODOX	β -Lactamase	Yes	No	Yes	No	Adverse immune effects due to bacterial enzyme

^a To overcome this gastrointestinal toxicity, capecitabine was developed (see text).

regarding this gene therapy, such as insufficient transduction of tumor cells, as discussed above (Waxman et al., 1999). Another 5-FU prodrug, 5-FU glucuronide, is activated by β -glucuronidase (Guerquin-Kern et al., 2000). This enzyme is also widely distributed and possesses high substrate turnover rates, resulting in a rapid clearance of glucuronidated prodrugs. Indeed, after i.v. or i.p. injection in mice, a very rapid clearance from the blood was observed. Therefore, intratumoral injection was necessary, although it eliminates the advantage of a prodrug approach (Guerquin-Kern et al., 2000). β -Glucuronidase seems only suitable for using ADEPT, GDEPT, or VDEPT approaches. Cytosine deaminase in combination with 5-FC has also been used in ADEPT and GDEPT approaches since the enzyme is not present in humans. The results seem promising, and the system is in clinical trial for metastatic colon carcinoma of the liver and metastatic breast cancer (Aghi et al., 2000), although the general ADEPT and GDEPT problems, illustrated above, need to be resolved. Thymidine phosphorylase might be a promising enzyme system since it is up-regulated in some breast, ovarian, colorectal, and gastric cancers (Cole et al., 1999). Thymidine phosphorylase activates the clinically used prodrug 5'-DFUR into 5-FU. However, the clinical success has been limited due to a low frequency of tumors displaying high levels of thymidine phosphorylase (Cole et al., 1999). Therefore, tumor-specific profiling of thymidine phosphorylase activity needs to be further elucidated. As a side effect, 5'-DFUR causes gastrointestinal toxicity, thereby limiting its use. Capecitabine has been developed to overcome this toxicity (Miwa et al., 1998). This prodrug is sequentially activated into 5-FU by three enzymes, i.e., carboxylesterase, cytidine deaminase, and thymidine phosphorylase. Capecitabine is a promising 5-FU prodrug that is more effective against murine and human tumors than 5-FU, and clinical trials are in progress (Punt, 1998).

In addition to 5-FU prodrugs, several doxorubicin prodrugs have been developed, i.e., *N*-(4-phosphonoxy)-phenylacetyl)doxorubicin that is activated by alkaline phosphatase (Vrudhula et al., 1993a), HMR 1826 and DOX-GA3 activated by β -glucuronidase (Bakina et al., 1997; Leenders et al., 1999), DPO and *N*-(phenylacetyl)doxorubicin activated by penicillin amidase (Kerr et al., 1990), and C-DOX and PRODOX activated by β -lactamase (Hudyma et al., 1993; Jungheim et al., 1993) (Table 15). These prodrugs have been designed to overcome the cardiotoxicity of the widely used antitumor drug doxorubicin.

For the targeting of doxorubicin only, enzyme systems have been used for enzymes that are either widely distributed (alkaline phosphatase and β -glucuronidase) or not expressed in humans (penicillin amidase and β -lactamase). In addition to the wide distribution of alkaline phosphatase and β -glucuronidase, these enzymes possess high turnover rates, resulting in a rapid clearance

of their respective prodrugs, therefore requiring high amounts of prodrugs. Very few data are available for the only doxorubicin prodrug that is activated by alkaline phosphatase, i.e., *N*-(4-phosphonoxy)-phenylacetyl)doxorubicin (Vrudhula et al., 1993a), and the antitumor activity of this prodrug remains to be evaluated. An advantage of β -glucuronidase is that the activity is higher in necrotic tumor areas, i.e., solid tumors, compared with normal tissue (Fishman, 1995). Indeed, for the promising prodrug DOX-GA3, an almost 5-fold higher doxorubicin peak concentration in the tumor was observed after administration to mice bearing human ovarian cancer xenografts when compared with doxorubicin exposure (Houba et al., 2001a). Furthermore, the doxorubicin concentration in the heart (target organ for dose-limiting toxicity) were up to 5-fold lower after administration of DOX-GA3 compared with doxorubicin exposure alone (Houba et al., 2001a). It should be realized, however, that glucuronidated prodrugs are not activated in small non-necrotic tumors due to low levels of β -glucuronidase (Houba et al., 2001b). Therefore, the therapeutic potential of these prodrugs seems limited. To overcome this limitation, ADEPT approaches can be used (Houba et al., 2001b). In this case the glucuronidated prodrugs do not have to pass the cell membrane, which is an advantage since glucuronidated prodrugs are very poorly taken up by cells. Both HMR 1826 and DOX-GA3 have shown their antitumor efficacy in mice bearing human tumors, although the efficacy in humans remains to be established (Bosslet et al., 1994; Houba et al., 2001b).

The enzymes penicillin amidase and β -lactamase are both of bacterial origin. Therefore, these enzymes can only be used in ADEPT or GDEPT approaches. For most of the doxorubicin prodrugs that are activated by penicillin amidase and β -lactamase, few data are available. A general problem with this approach still is adverse immune effects of the bacterial immunoconjugates. C-DOX is the most promising candidate (Svensson et al., 1995). This prodrug was more effective than doxorubicin against mice bearing human lung and melanoma tumor cells in ADEPT. In mice C-DOX was at least 7-fold less toxic than doxorubicin, based on the maximum tolerated dose (Svensson et al., 1995). However, it should be realized that the general problems in ADEPT should be resolved.

In conclusion, several clinically successful prodrugs have been developed; however, all prodrugs developed so far lack full tumor selectivity. This can be rationalized by the fact that, unlike bacteria and viruses, cancer cells do not contain molecular targets that are completely foreign to the host. Therefore, more research is needed to elucidate the phenotypic differences between normal and tumor cells, especially the presence of enzymes that may activate prodrugs. By evaluating this, tumors may be treated more selectively. Enzymes which can be used for local activation of prodrugs include tyrosinase (*Sec-*

tion II.A.6.) due to a selective localization in melanoma cells, thymidylate synthase (Section II.B.1.), thymidine phosphorylase (Section II.B.2.), and GST (Section II.B.3.) due to overexpression in tumors, cysteine conjugate β -lyase (Section II.D.1.) due to the relatively selective presence in kidney, and enzyme-activating bioreductive drugs, i.e., cytochrome P450 reductase (Section II.A.3.) and DT-diaphorase (Section II.A.4.). As illustrated in this review, for several prodrug-activating enzymes the tissue distribution in humans and levels between normal and tumor tissues has to be further investigated. In addition to this, more work needs to be performed with a series of prodrug analogs to determine optimal substrates in terms of high substrate affinity and fast enzyme turnover rates for prodrug-activating enzymes resulting in fast cleavage of the prodrug inside the tumor cells.

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