

## Symposium Report

### CANCER CHEMOTHERAPY AND DRUG METABOLISM

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#### ABSTRACT:

Drug-metabolizing enzymes and drug transporters are key determinants of the pharmacokinetics and pharmacodynamics of many antineoplastic agents. Metabolism and transport influence the cytotoxic effects of antineoplastic agents in target tumor cells and normal host tissues. This article summarizes several state-of-the-art approaches to enhancing the effectiveness and safety of cancer therapy based on recent developments in our understanding of antineoplastic drug metabolism and transport. Advances in four interrelated research areas presented at a recent symposium sponsored by the Division for Drug Metabolism of the American Society for Pharmacology and Experimental Therapeutics (Exper-

imental Biology 2004; Washington D.C., April 17–21, 2004) are discussed: 1) interactions of anthracyclines with drug-metabolizing enzymes; 2) use of hypoxia-selective gene-directed enzyme prodrug therapy (GDEPT) in combination with bioreductive prodrugs; 3) synergy between glutathione conjugation and conjugate efflux in conferring resistance to electrophilic toxins; and 4) use of cytochromes P450 as prodrug-activating enzymes in GDEPT strategies. A clear theme emerged from this symposium: drug metabolism and transport processes can be modulated and exploited in ways that may offer distinct therapeutic advantages in the management of patients with cancer.

Chemotherapy involving the use of cytotoxic antineoplastic agents remains an important strategy in the overall management of patients with malignant tumors. As with most therapeutic agents, drug-metabolizing enzymes and drug transporters play key roles in determining the pharmacokinetics and overall disposition of antineoplastic agents in the body. In addition, metabolism and transport can be important contributors to 1) the toxicity produced by antineoplastic agents in

normal host tissues and 2) the delicate balance between drug sensitivity and resistance displayed by target tumor cells.

Several features of antineoplastic drugs make the metabolism of these agents particularly significant. Many antineoplastics display steep dose-response curves and low therapeutic indices, and the toxicities that they produce can be severe and life-threatening. Since cancer chemotherapy often involves the use of multiple antineoplastic agents and supportive drugs in combination regimens, drug interactions represent a real clinical concern (Kivisto et al., 1995). Of particular importance are pharmacokinetic drug interactions that result in clinically significant alterations in host toxicity or therapeutic response. Pharmacokinetic drug interactions in cancer chemotherapy often go unrecognized because toxic reactions to these drugs are common and unpredictable, individual

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**ABBREVIATIONS:** GDEPT, gene-directed enzyme prodrug therapy; ABC, ATP-binding cassette; CDNB, 1-chloro-2,4-dinitrobenzene; 15-d-PGJ<sub>2</sub>, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub>; E09, 3-hydroxymethyl-5-aziridin-1-yl-methyl-2-[1H-indole-4,7-dione]prop-2-en-1-ol; RSU1069, 1-[3-aziridinyl-2-hydroxypropyl]-2-nitroimidazole; CB1954, 5-[aziridin-1-yl]-2,4-dinitrobenzamide; AQ4N, 1,4-bis-[[2-(dimethylamino-N-oxide)ethyl]amino]5,8-dihydroxyanthracene-9,10-dione; GSH, reduced glutathione; GST, glutathione S-transferase; GS-X, glutathione conjugate; HIF, hypoxia-inducible factor; HRE, hypoxia-response element; LDH, lactate dehydrogenase A; MRP, multidrug resistance-associated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NOS, nitric oxide synthase; iNOS or NOSII, inducible nitric oxide synthase; NOSR, reductase activity of nitric oxide synthase; NQO, 4-nitroquinoline 1-oxide; NQO1, NAD(P)H:quinone oxidoreductase 1; P450, cytochrome P450; P450R, NADPH-cytochrome P450 reductase; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-activated receptor-responsive element; QO-SG, 4-S-glutathionyl quinoline 1-oxide; ROS, reactive oxygen species; RTV4, four-times treatment size; SR4317, 3-amino-1,2,4-benzotriazine-1-N-oxide.

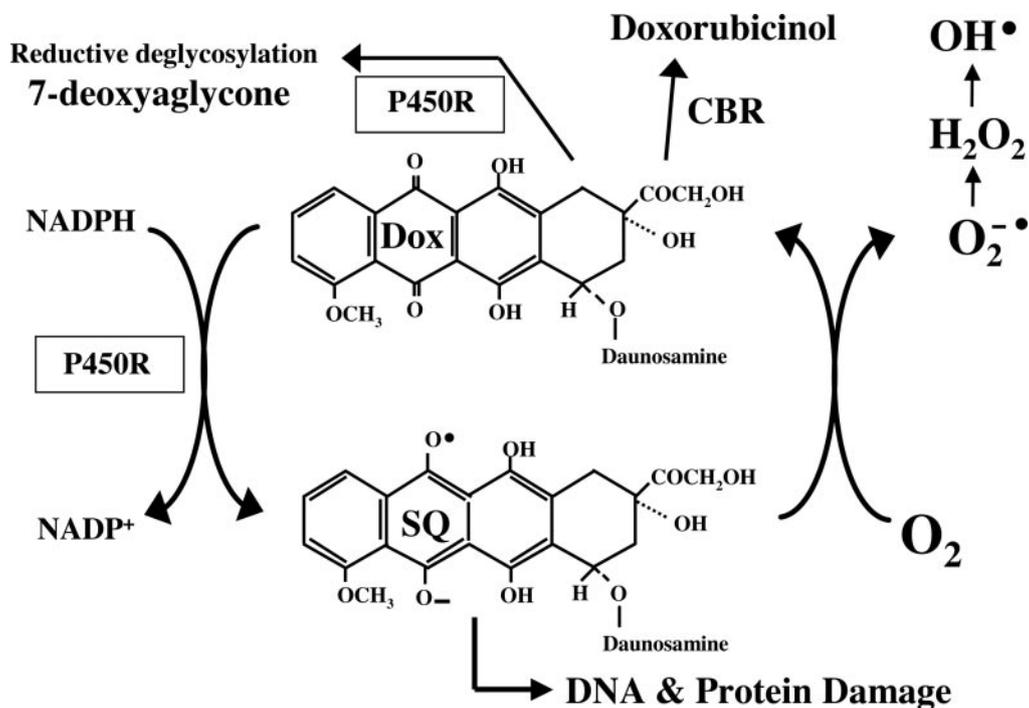


FIG. 1. Selected aspects of doxorubicin biotransformation. The major pathway of doxorubicin (Dox) metabolism in humans is conversion to doxorubicinol catalyzed by carbonyl reductase (CBR). P450R contributes to the detoxification of doxorubicin by catalyzing the reductive deglycosylation to a 7-deoxyaglycone metabolite. In addition, P450R catalyzes the NADPH-dependent one-electron reduction of doxorubicin to the reactive and cytotoxic semiquinone radical (SQ). This radical can react with oxygen to initiate redox cycling and ROS production.

agents may display overlapping toxicities, and drug resistance and therapeutic failure in cancer management is common. Since most pharmacokinetic drug interactions involve drug metabolism and/or transport as a mechanistic basis, it is important to understand two general aspects of the interactions between antineoplastic agents and drug-metabolizing enzymes and drug transporters: 1) the role of human enzymes and transporters in the metabolism and disposition of existing and new antineoplastics; and 2) the mechanisms by which antineoplastics modulate the expression and activity of human enzymes and transporters.

The goal of this symposium was to critically examine current state-of-the-art approaches by which our understanding of antineoplastic drug metabolism and transport can be used to enhance the safety and effectiveness of cancer therapy. With drug-metabolizing enzymes as the central focus of the symposium, five interrelated and complementary themes were developed.

1) Gene-directed enzyme prodrug therapy (GDEPT) is a promising means to promote local intratumoral drug bioactivation (Rooseboom et al., 2004). 2) The balance between bioactivation and detoxification pathways can be an important determinant of normal tissue toxicity and drug resistance in tumor cells. 3) The modulation of the expression and activity of drug-metabolizing enzymes, especially cytochrome P450 (P450) proteins, by antineoplastic agents is a significant contributor to drug pharmacokinetics and potential for drug interactions. 4) An integrated cytoprotective network between drug conjugation and conjugate transport can contribute to tumor drug resistance. 5) Genetic polymorphisms in drug-metabolizing enzymes and drug transporters impact antineoplastic drug pharmacokinetics and pharmacodynamics in clinically significant ways (Petros and Evans, 2004). These and other aspects of antineoplastic drug metabolism were explored in this symposium.

#### Interactions of Anthracyclines with Drug-Metabolizing Enzymes (D.S.R., C.L., S.R.)

The anthracycline antibiotic doxorubicin is one of the most useful antineoplastic agents, displaying a broad range of clinical activity against several solid and hematological malignancies. Several mechanisms contribute to doxorubicin's cytotoxic effects: 1) initiation of DNA damage via inhibition of topoisomerase II, 2) DNA intercalation, 3) direct membrane effects, and 4) redox cycling leading to the generation of reactive oxygen species (ROS) and subsequent lipid peroxidation and DNA damage (Gewirtz, 1999). Our laboratory has been particularly interested in the metabolism of doxorubicin via reductive pathways that can lead to the generation of the semiquinone radical and ROS.

A simplified scheme showing only the relevant aspects of doxorubicin metabolism is presented in Fig. 1. The major doxorubicin metabolite in humans is doxorubicinol, produced via cytosolic carbonyl reductase-catalyzed reduction of the ketone at C-13 of the parent drug. Our main enzyme of interest has been NADPH:cytochrome P450 reductase (P450R). This flavoprotein catalyzes the one-electron reduction of doxorubicin to the semiquinone radical, which can be directly cytotoxic in hypoxic environments via covalent modification of cellular macromolecules. Under aerobic conditions, the semiquinone radical undergoes redox cycling leading to the generation of ROS including superoxide anion, hydrogen peroxide, and hydroxyl radical. This oxidative stress mechanism is a contributor to doxorubicin's ability to cause cell death, and this aspect seems to be particularly important in the dose-limiting cardiotoxicity associated with this and other anthracyclines. Interestingly, P450R also plays a role in a doxorubicin detoxification pathway involving reductive deglycosylation to a 7-deoxyaglycone metabolite (Niitsu et al., 2000).

One of our areas of interest has been the effect of doxorubicin on the hepatic P450 and P450R biotransformation system. Treatment of

rodents with doxorubicin results in lowered hepatic P450 content and decreases in a variety of P450-catalyzed enzyme activities (Marchand and Renton, 1981). This response has traditionally been attributed to a combination of direct inhibition of P450 enzymes by doxorubicin and/or its metabolites and destruction of P450 heme via redox cycling and lipid peroxidation (Mimnaugh et al., 1981). The observations that both P450R and specific rodent P450 enzymes (e.g., rat CYP2B1) can catalyze the one-electron reduction of doxorubicin to the semiquinone radical (Goepfert et al., 1993) and that doxorubicin-derived species bind covalently to hepatic microsomal protein suggested that this antineoplastic agent may function as a mechanism-based inactivator of P450 and/or P450R.

We asked the following research question: does doxorubicin function as a mechanism-based inactivator of hepatic microsomal P450 and/or P450R under conditions in which lipid peroxidation is minimized (Di Re et al., 1999)? We conducted a series of *in vitro* studies using hepatic microsomes prepared from untreated and phenobarbital-treated male rats. Microsomes were incubated for 30 min at 37°C in the absence or presence of NADPH and a high, but pharmacologically relevant, concentration (10  $\mu$ M) of doxorubicin. Inclusion of 1.5 mM EDTA, an iron chelator, in the incubation buffer minimized doxorubicin-stimulated, NADPH-dependent lipid peroxidation. In contrast, carbon tetrachloride caused significant stimulation of NADPH-dependent lipid peroxidation under these conditions. Doxorubicin did not cause NADPH-dependent loss of microsomal P450 or heme content, whereas these parameters were decreased markedly by the positive control 1-aminobenzotriazole, a heme-destructive mechanism-based inactivator of several P450s. Neither doxorubicin nor 1-aminobenzotriazole caused NADPH-dependent loss of P450R catalytic activity, assessed as the rate of cytochrome *c* reduction under aerobic conditions. Using the regio- and stereoselective hydroxylation of androstenedione as a probe for the catalytic activity of multiple rat hepatic P450s, we found that 1-aminobenzotriazole caused pronounced NADPH-dependent loss of CYP2A, CYP2B, CYP2C11, and CYP3A function; doxorubicin had no effect on these marker activities. Neither doxorubicin nor 1-aminobenzotriazole altered the levels of immunoreactive protein of CYP2B, CYP2C11, CYP3A, and P450R. The results of this investigation (Di Re et al., 1999) demonstrated that doxorubicin does not cause direct mechanism-based inactivation of hepatic P450 or P450R. Future studies in this area should address whether the observed *in vivo* effects of doxorubicin on the hepatic P450 system are due to 1) heme destruction via lipid peroxidation, or 2) modulation of the expression of genes encoding specific P450 enzymes. Our earlier work with antimetabolites (Afsar et al., 1996; Cheung et al., 1996) examined the ability of cancer chemotherapeutic agents to alter the expression of hepatic P450 genes following *in vivo* administration to rats.

Another area of interest in our laboratory has been the reductive bioactivation of doxorubicin and subsequent redox cycling under aerobic conditions and whether these events can be manipulated to make human cancer cells more sensitive to cytotoxicity. We first asked the following research question: does human P450R catalyze the reductive bioactivation of doxorubicin (Ramji et al., 2003)? Doxorubicin reduction was measured indirectly under aerobic conditions as the rate of doxorubicin-stimulated microsomal oxidation of NADPH, a validated surrogate indicator for conversion of doxorubicin to its semiquinone radical (Goepfert et al., 1993). As the first component of our reaction phenotyping studies, we showed that heterologously expressed human P450R displayed 17-fold higher P450R catalytic activity and 11-fold higher doxorubicin reduction activity compared to vector transfectants. In a panel of 17 human liver microsome samples, we found that doxorubicin reduction activity correlated

TABLE 1

*Effect of carbon monoxide on P450R activity and doxorubicin reduction in rat and human liver microsomes*

P450R activity was determined spectrophotometrically as the rate of cytochrome *c* reduction. Doxorubicin reduction was assayed spectrophotometrically as the rate of doxorubicin-stimulated microsomal NADPH consumption. Rat liver microsomes were prepared from untreated adult male Fischer 344 rats. Human liver microsome sample K15, from a tissue bank maintained by Dr. Tadanobu Inaba (University of Toronto, Toronto, ON, Canada), was used in this experiment. Data are expressed as mean  $\pm$  S.D. of triplicate or sextuplicate determinations.

Sample	P450R Activity	Doxorubicin Reduction
<i>nmol/min/mg microsomal protein</i>		
Rat liver microsomes		
– Carbon monoxide	253 $\pm$ 13	11.0 $\pm$ 0.4
+ Carbon monoxide	274 $\pm$ 3	7.0 $\pm$ 0.8**
Human liver microsomes		
– Carbon monoxide	103 $\pm$ 2	4.8 $\pm$ 0.3
+ Carbon monoxide	101 $\pm$ 9	6.1 $\pm$ 0.7**

\*\* Significantly different ( $p \leq 0.01$ ) from activity in the absence of carbon monoxide, based on Student's *t* test.

significantly with both P450R catalytic activity and P450R immunoreactivity. Diphenyliodonium chloride, a mechanism-based inactivator of P450R, inhibited P450R catalytic activity and doxorubicin reduction in human liver microsomes with similar concentration dependence. Finally, we also used carbon monoxide as a universal P450 inhibitor to examine the role of P450 enzymes in microsomal doxorubicin reduction (Table 1). As expected, carbon monoxide did not inhibit P450R activity in rat and human liver microsomes. Consistent with earlier findings (Goepfert et al., 1993), carbon monoxide caused significant inhibition of doxorubicin reduction in rat liver microsomes. In human liver microsomes, carbon monoxide did not inhibit, but apparently stimulated, doxorubicin reduction. Although this finding suggests that P450s may play a more important role in doxorubicin reduction in rat liver than in human liver, understanding the mechanistic basis for the apparent stimulation of doxorubicin reduction activity by carbon monoxide in human liver microsomes will require further investigation. Overall, the results of this investigation (Ramji et al., 2003) demonstrate that human P450R is an important contributor to the reductive biotransformation of doxorubicin.

Carrying on from this finding, we then asked the following research question: does enhanced P450R expression sensitize human breast cancer cells to the cytotoxic effects of doxorubicin (Ramji et al., 2003)? Our initial cell model consisted of a series of stably transfected clones of MDA231 human breast cancer cells overexpressing human P450R immunoreactive protein and catalytic activity (Patterson et al., 1997). Elevated P450R expression was associated with enhanced sensitivity to the aerobic cytotoxicity of tirapazamine, a bioreductive prodrug known to be activated by P450R. However, the MDA231 transfectants overexpressing P450R were not sensitized to the aerobic cytotoxicity of doxorubicin (Ramji et al., 2003).

As a second cell model, we turned to the wild-type estrogen receptor-positive human breast cancer cell line MCF-7/WT. We also obtained from Dr. Gerald Batist (Sir Mortimer B. Davis-Jewish General Hospital, Montreal, QC, Canada) a derived doxorubicin-resistant cell line known to overexpress P-glycoprotein (Cowan et al., 1986). This cell line was named MCF-7/ADR in the original literature (Cowan et al., 1986) and in our earlier publication (Wang et al., 1999); however, DNA fingerprinting data prompted a change in the designation of this cell line to NCI/ADR-RES (Scudiero et al., 1998). In our hands, this cell line is about 30- to 65-fold more resistant to doxorubicin cytotoxicity than the MCF-7/WT cells and shows pronounced overexpression of glutathione *S*-transferase (GST) P1 mRNA, protein, and catalytic activity (Wang et al., 1999). Elevated GST expression is

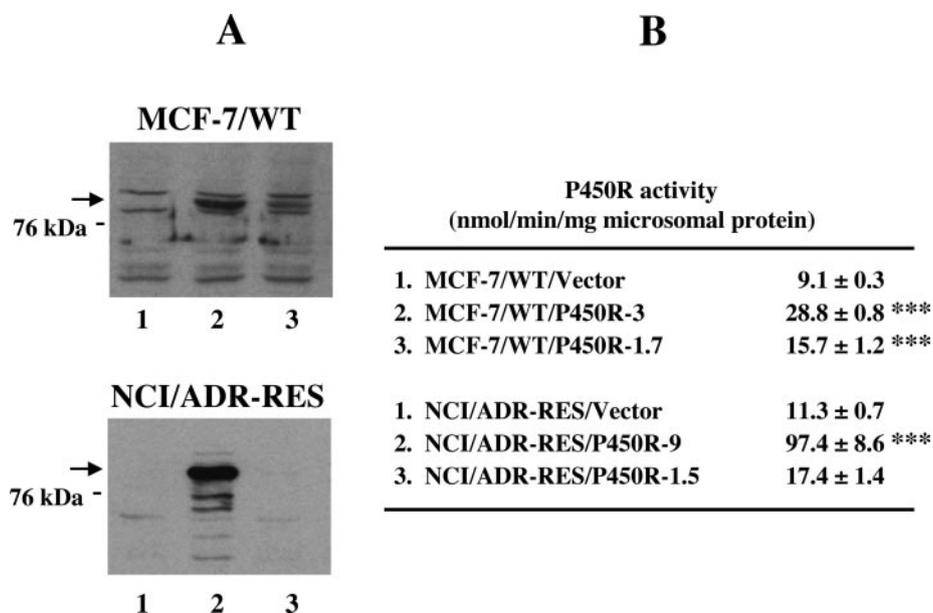


FIG. 2. Overexpression of P450R immunoreactive protein (A) and catalytic activity (B) in microsomes from human breast cancer cell lines stably transfected with human P450R cDNA. MCF-7/WT and NCI/ADR-RES human breast cancer cell lines were either transfected with empty vector (Vector) or transfected with vector containing the human P450R cDNA (P450R). A, immunoblot analysis of microsomal protein (3 or 5  $\mu$ g) using a polyclonal antibody directed against rat P450R. The number on the left indicates the size of a molecular mass marker, and the arrow indicates the position of immunoreactive P450R protein (~77 kDa). Lanes 1, 2, and 3 contain microsomes from the specific cell lines identified by number in panel B of the figure. Note that the film exposure time for the upper MCF-7/WT blot is 5 times longer than that for the lower NCI/ADR-RES blot. B, microsomal P450R catalytic activity was determined spectrophotometrically as the rate of cytochrome *c* reduction. Data are expressed as mean  $\pm$  S.D. of triplicate determinations. \*\*\*, significantly different ( $p \leq 0.001$ ) from Vector, based on a randomized design one-way analysis of variance and post hoc Newman-Keuls test.

commonly associated with doxorubicin resistance (Beaumont et al., 1998). We obtained a full-length human P450R cDNA from Dr. Frank Gonzalez (National Cancer Institute, Bethesda, MD), subcloned it into the pcDNA3.1(+) mammalian expression vector, and then selected geneticin-resistant stably transfected clones of MCF-7/WT and NCI/ADR-RES cells overexpressing microsomal P450R protein and catalytic activity. As shown in Fig. 2, we obtained MCF-7/WT transfectants with 3-fold and 1.7-fold elevations in microsomal P450R catalytic activity compared to vector-transfected cells. Overexpression of P450R protein was detectable by immunoblot. We also obtained NCI/ADR-RES transfectants with 9-fold and 1.5-fold elevations in microsomal P450R catalytic activity, and overexpression of P450R protein was detectable by immunoblot, particularly in the NCI/ADR-RES/P450R-9 line (Fig. 2). Similar to our results with the MDA231 cells with marked P450R overexpression (Ramji et al., 2003), a more modest elevation of human P450R expression also did not enhance the aerobic cytotoxicity of doxorubicin in MCF-7/WT and NCI/ADR-RES transfectants (Fig. 3).

Taken together, we conclude that although human P450R catalyzes doxorubicin reduction, overexpression of this enzyme does not confer enhanced sensitivity of human breast cancer cells to the aerobic cytotoxicity of doxorubicin. Future studies in this area should address the following issues: 1) P450R-mediated doxorubicin reduction under anaerobic conditions, 2) doxorubicin sensitivity of breast cancer cells overexpressing P450R under hypoxic conditions, and 3) the relative importance of the doxorubicin free radical mechanism and topoisomerase II inhibition in normal and tumor cells with different proliferative states.

#### Bioreductive Prodrugs: Routes of Activation and Potential Application in Gene Therapy (E.C.C., R.L.C., K.J.W., A.V.P., I.J.S.)

Regions of hypoxia are present in the majority of solid primary and secondary human tumors (Vaupel et al., 2001). Chronic hypoxia

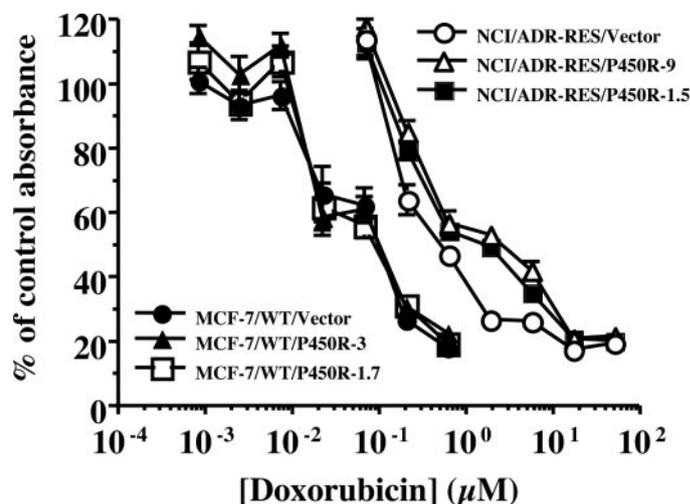


FIG. 3. Concentration-dependent doxorubicin cytotoxicity in human breast cancer cell lines stably transfected with human P450R cDNA. MCF-7/WT and NCI/ADR-RES human breast cancer cell lines were either transfected with empty vector (Vector) or transfected with vector containing the human P450R cDNA (P450R). Cells were treated for 72 h with the concentrations of doxorubicin indicated on the abscissa. Cytotoxicity was assessed using a microplate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Fractional absorbance is expressed as a percentage of the absorbance displayed by cells not exposed to doxorubicin. Each point represents the mean  $\pm$  S.D. of duplicate 96-well microplates with sextuplicate determinations per plate. Similar results were obtained in a total of two to four independent experiments.

results because of rapid tumor growth before an adequate blood supply can be recruited such that tumor cells lie beyond the diffusion distance of oxygen from a functioning blood vessel. Once established, tumor vasculature is also poorly organized, exhibiting convoluted vessels prone to temporary occlusion, giving rise to acute hypoxia. The level of hypoxia in tumors is of clinical significance since

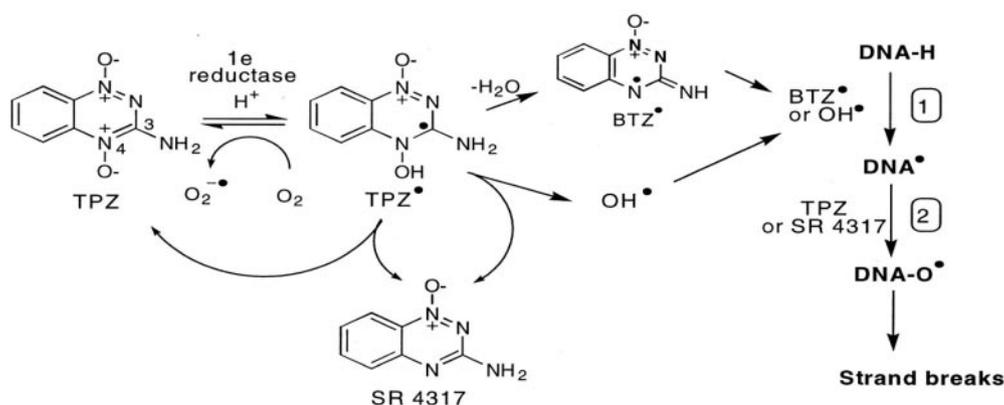


FIG. 4. Proposed mechanism of the bioactivation of tirapazamine (TPZ). Modified from: Siim et al., 2004.

hypoxic cells show resistance to cancer chemo- and radiotherapy, and hypoxic tumors are predisposed to a more malignant phenotype (Brizel et al., 1996). Therefore, researchers have endeavored to circumvent the therapeutic resistance induced by hypoxia and to exploit it as a tumor-specific physiological abnormality. To this end, chemotherapy drugs that are preferentially toxic to hypoxic cells (bioreductive drugs) have been developed and, more recently, this has been combined with hypoxia-selective gene therapy strategies.

Four main classes of bioreductive prodrugs have been developed for use in cancer treatment and diagnosis and are at varying stages of preclinical and clinical development (Stratford et al., 2003). The prototype bioreductive drug is mitomycin C, which is a quinone. This drug class also comprises E09 (3-hydroxymethyl-5-aziridin-1-ylmethyl-2-[1H-indole-4,7-dione]prop-2-en-1-ol). The aromatic and heterocyclic nitro compounds include RSU1069 (1-[3-aziridinyl-2-hydroxypropyl]-2-nitroimidazole) and CB1954 (5-[aziridin-1-yl]-2,4-dinitrobenzamide) and the hypoxic marker pimonidazole. The third class, the aliphatic *N*-oxides, is exemplified by AQ4N (1,4-bis-[[2-(dimethylamino-*N*-oxide)ethyl]amino]5,8-dihydroxyanthracene-9,10-dione), and the fourth class is the benzotriazine di-*N*-oxides that include the lead bioreductive drug, tirapazamine.

The principle of bioreductive drug activation is that in the reductive environment created by hypoxia, endogenous reductases will transfer electrons to a nontoxic prodrug to generate a reduced cytotoxic metabolite. Hence, this provides for differential killing in hypoxic tumors versus normal tissues. Several factors will determine the therapeutic index and potency of bioreductive drugs. The electron affinity of the prodrug will determine the oxygen concentration requirements for activation and the ease with which it is reduced. Furthermore, reduction is mediated by intracellular reductases. Of the large complement of endogenous reductases, both oxygen-dependent and -independent reductases have been shown to be able to activate bioreductive drugs. These include the oxygen-independent enzyme DT-diaphorase or NAD(P)H:quinone oxidoreductase 1 (NQO1) and the oxygen-dependent enzymes P450, P450R, xanthine oxidase, cytochrome *b*<sub>5</sub> reductase, and nitric-oxide synthase (NOS).

Tumor levels of each of these enzymes can govern their sensitivity to bioreductive drug treatment. Therefore, knowledge needs to be gained to determine which are the key enzymes involved in the activation of each of the different bioreductive drugs and what is their substrate specificity. When combined with an assessment of the hypoxic fraction in tumor biopsies, this could enable tumors that are likely to respond to a class/subclass of bioreductive drug to be identified, paving the way for tailored individualized treatment regimens. It will also enable rational drug design to be used in the synthesis of second-generation bioreductive drugs. Potential strategies

include selecting against oxygen-independent reduction and/or targeting toward activation by a particular cellular reductase up-regulated in tumor cells or one that can be easily delivered in a gene therapy.

We have focused on identifying the key route of bioactivation for tirapazamine. Tirapazamine exhibits a high specificity for hypoxic cells over a broad oxygen concentration range at clinically relevant partial oxygen pressures. This hypoxic selectivity results from tirapazamine being a prodrug that is bioactivated in hypoxic conditions via one-electron reduction to a highly reactive free radical intermediate. The fate of this tirapazamine free radical is dependent upon the level of oxygen. In hypoxia, it has been postulated that the free radical spontaneously decays, generating hydroxyl radical that abstracts hydrogen from DNA causing single- and double-strand breaks with the concomitant production of the nontoxic two-electron metabolite SR4317 (3-amino-1,2,4-benzotriazine-1-*N*-oxide) (Siim et al., 2004). However, oxygen inhibits the metabolic activation of tirapazamine and the free radical species undergoes rapid back oxidation to the prodrug (futile cycling), with the release of the less toxic superoxide anion species that can be deactivated by cellular defense mechanisms (Fig. 4) (Siim et al., 2004). Preclinical studies have demonstrated that tirapazamine can significantly enhance the antitumor effect of radiotherapy and chemotherapy, in particular, platinum-based drugs and taxanes, in both murine and human xenograft models. These encouraging data have initiated phase I, II, and III clinical trials with tirapazamine in combination with cisplatin or radiotherapy (reviewed in Brown and Wilson, 2004).

We have evaluated the relative importance of the oxygen-dependent, one-electron reductases, P450R and inducible NOS (iNOS, NOSII) in the activation of tirapazamine. Unlike P450R, a high incidence of tumor NOSII expression has been reported in many clinical studies in which the level of expression has been shown to correlate with tumor grade (Thomsen and Miles, 1998). In addition, the presence of a hypoxia-response element (HRE) within the NOSII promoter (Melillo et al., 1995) suggests that its expression may be markedly increased within the hypoxic regions of tumors. Hence, NOSII presents an attractive target for bioreductive drug design. NOSII functions as a dimer with two distinct catalytic domains, an oxidative and a reductive domain, that bioactivate a broad repertoire of established and novel chemotherapeutic prodrugs. The reductase domain shares a high degree of sequence homology with P450R (Fig. 5A). However, these two enzymes differ in their subcellular localization because P450R resides in the endoplasmic reticulum and NOSII is cytoplasmic (Fig. 5B).

Early studies by Patterson et al. (1995) identified P450R as a key player in tirapazamine bioactivation. This evidence came from studying the *in vitro* sensitivity of breast tumor cell lines to tirapazamine

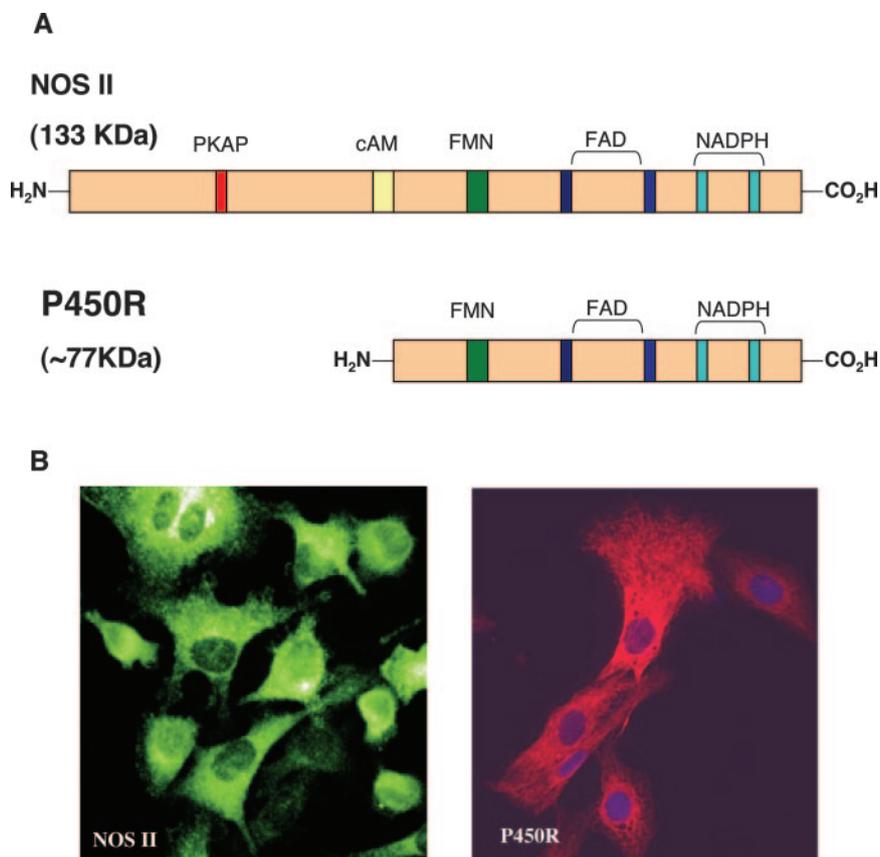


FIG. 5. Comparison of the structure and subcellular localization of NOSII and P450R. A, structural comparison between NOSII and P450R showing the important cofactor binding sites. Consensus sequences for the binding of the cofactors NADPH, FAD, FMN, calmodulin (cAM), and protein kinase A phosphorylation (PKAP) are labeled. The N-terminal region of NOSII also contains putative L-arginine binding regions and probably the binding site for tetrahydrobiopterin and heme (not shown). B, micrographs showing the different subcellular localization of NOSII (cytoplasm, green staining) and P450R (endoplasmic reticulum, red staining).

that exhibited an inherent 6-fold range in P450R activity. Using short exposures (3 h) to tirapazamine, the level of P450R correlated strongly with the extent of tirapazamine toxicity in hypoxic conditions. However, aerobic sensitivity was also measured following both short-term (3 h) and prolonged (96 h) exposures to tirapazamine and was also shown to be dependent on P450R activity.

In an MDA231 human breast cell line background, we have directly compared tirapazamine toxicity following genetic engineering to constitutively overexpress P450R (Patterson et al., 1997) or NOSII (Chinje et al., 2003). For the P450R clones, elevation in P450R activity ranged from 5.6- to 53-fold above the endogenous level measured in parental and vector control MDA231 cells. Similarly in the NOSII-overexpressing clones, NOSII reductase (NOSR) activity increased by 2- to 6-fold.

The dependence of the  $IC_{50}$  for tirapazamine on either P450R or NOSR activity was determined using a short (3-h) drug exposure in aerobic and hypoxic MDA231 clonal tumor cells overexpressing P450R or NOSII (Fig. 6). Under hypoxic conditions, there was a highly significant relationship between the  $IC_{50}$  for tirapazamine and both P450R activity and NOSR activity. Clones with increased reductase activity were more sensitive than parental and vector control cells. However, elevation of P450R also sensitized the cells to tirapazamine in aerobic conditions. This is in contrast to the  $IC_{50}$  data obtained for the NOSII-expressing clones, where the intracellular activity of NOSR predicted for cytotoxicity specifically under hypoxic, but not aerobic, exposure conditions (Fig. 6).

In an earlier study (Garner et al., 1999), we compared the kinetic parameters of tirapazamine metabolism under hypoxic conditions in a

cell-free system. Using purified recombinant mouse NOSII, kinetic analysis revealed that its  $K_m$  was significantly lower than that for P450R (Fitzsimmons et al., 1994) [ $K_m = 283 \mu\text{M}$  (NOSII) and  $1260 \mu\text{M}$  (P450R)]. This finding suggests that NOSII may have a greater affinity for tirapazamine than P450R has. The  $k_{cat}$  for SR4317 formation was also marginally lower compared to that for P450R [ $k_{cat} = 1.9 \text{ s}^{-1}$  (NOSII) and  $6.34 \text{ s}^{-1}$  (P450R)]. Thus, NOSII, like P450R, is able to carry out the one-electron reduction of tirapazamine to toxic products.

From this work, it is clear that both P450R and NOSII are important enzymes for the reductive activation of tirapazamine. Data on levels of P450R in tumors versus normal tissue vary; if anything, P450R expression tends to be lower in tumors (Forkert et al., 1996). In contrast, tumor NOSII levels are elevated compared to that measured in normal tissue. Furthermore, our data suggest that nontumor NOSII expression in aerobic tissues would be of little consequence because cytotoxicity is restricted by the presence of oxygen.

We hypothesized that if P450R is to be used to sensitize tumor cells to bioreductive drugs such as tirapazamine, a gene therapy must be used to overexpress its activity. However, overexpression of exogenous P450R in a gene therapy approach, unlike high endogenous levels, would potentiate drug toxicity not only in hypoxic conditions but also in aerobic conditions, increasing the potential for healthy tissue toxicity. Consequently, tumor-specific overexpression of P450R will be required. We therefore have developed a hypoxia-selective gene therapy in which the expression of P450R and tirapazamine drug toxicity are dependent upon hypoxia, simultaneously

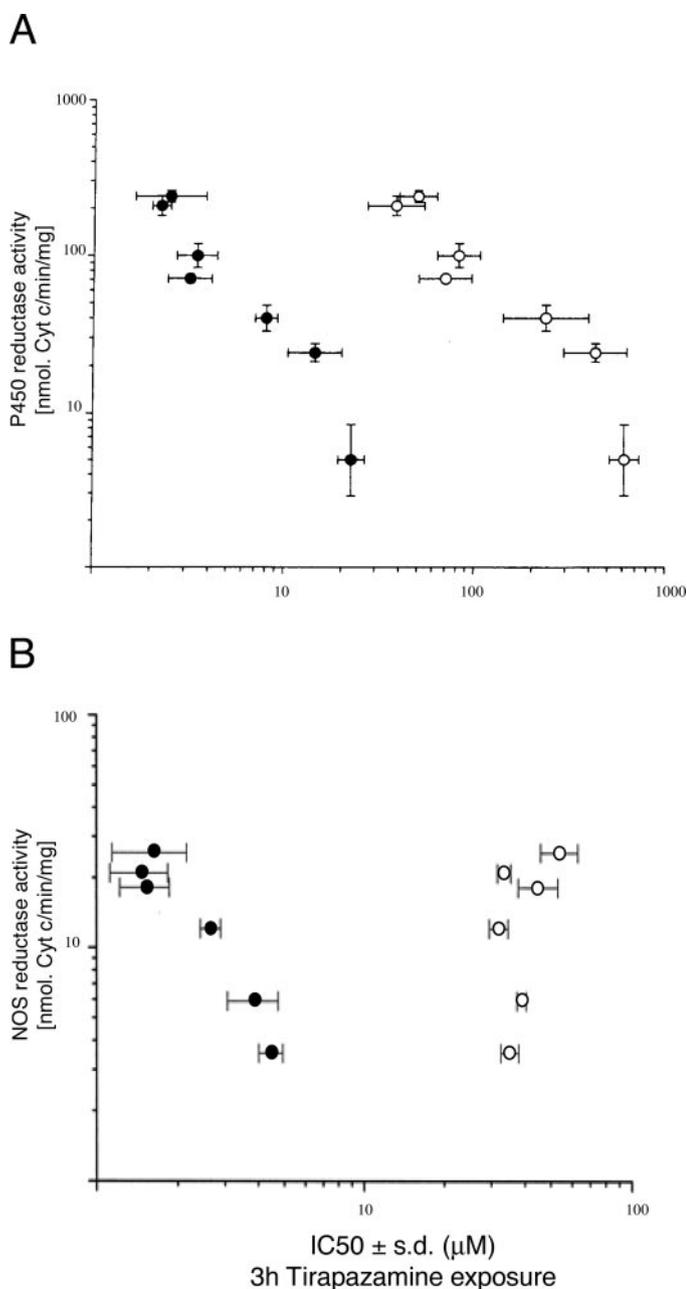


FIG. 6. Dependence of the  $IC_{50}$  of tirapazamine in MDA231 wild-type and transfected clones on the activity of P450R (A) and NOSR (B). Drug exposures were for 3 h in air (○) or hypoxia (●). Values of  $IC_{50}$  were determined using the MTT assay.

increasing the therapeutic index of the bioreductive chemotherapy without increasing systemic toxicity (Fig. 7) (Cowen et al., 2004). This dual hypoxic specificity is achieved by incorporation of a hypoxia-responsive promoter (Dachs et al., 1997) into the P450R expression cassette. HREs within the promoter bind the transcription factor hypoxia-inducible factor (HIF)-1, resulting in the transcriptional activation of the reductase gene alongside the endogenous up-regulation of HIF target genes under hypoxic conditions. A comparison of hypoxia-responsive promoter function within the tumor microenvironment and in normal healthy tissues has shown good tumor specificity and a considerable reduction in nonspecific expression in physiologically normal tissue compared to strong viral promoters routinely used in gene therapy (Binley et al., 2003).

To deliver the hypoxia-responsive cassette to the hypoxic tumor

fraction, the innate ability of adenovirus to infect dividing and quiescent human tumor cells has been used. We have engineered a replication-defective adenoviral vector Ad LDH HRE P450R, encoding for P450R with expression driven from a hypoxia-responsive promoter containing HREs from lactate dehydrogenase A (LDH). We evaluated this viral-mediated hypoxia-targeted gene therapy in the human HT1080 fibrosarcoma tumor model that is resistant to tirapazamine when given as a single agent or in combination with radiotherapy (Siim et al., 1997).

Monolayer cultures were efficiently transfected with Ad LDH HRE P450R virus, resulting in increased P450R activity when infected cells were cultured in low oxygen conditions (40-fold higher than infected aerobic tumor cells). Consistent with the MDA231 stable cell line work, the rise in P450R level in HT1080 cells directly correlated with an increase in sensitivity to tirapazamine. At the highest viral dose of Ad LDH HRE P450R, the hypoxic toxicity of tirapazamine was 400-fold higher compared to untransduced aerobic HT1080 tumor cells.

Ad LDH HRE P450R was then administered to established HT1080 tumors by intratumoral injection. Immunohistochemical analysis of transduced tumors 2 days after viral injection showed that the virus could disseminate throughout the tumor and into hypoxic tumor regions. This resulted in the overexpression of P450R colocalizing with the hypoxic marker pimonidazole. Having established that our viral-mediated gene therapy results in the focal elevation of P450R specifically at the site of bioreductive drug action, we evaluated its impact on the tumor response to tirapazamine either alone or in combination with radiotherapy (Fig. 8). As expected, HT1080 tumors are refractory to single-agent tirapazamine treatment (50 mg/kg) and, as a fast growing tumor model, reach 4 times treatment size (RTV4) by day 9 after treatment. Viral gene therapy had no effect on tumor growth and did not enhance tumor response to tirapazamine alone. A delay in HT1080 tumor growth results following 10-Gy radiotherapy (RTV4 at day  $32 \pm 4$ ), and in concordance with other studies (Siim et al., 1997), administration of tirapazamine immediately after 10-Gy radiation does not significantly enhance this response (RTV4 at day  $36 \pm 6$ ). However, when Ad LDH HRE P450R is used to presensitize tumors to the combined tirapazamine and 10-Gy radiation treatment, 85% of tumors completely regressed and were tumor-free 90 days after therapy (Fig. 8).

In conclusion, hypoxia-selective enzyme prodrug therapy has great promise in cancer treatment. By selectively targeting hypoxic tumor cells that are resistant to radiation and some forms of chemotherapy, it has the ability to enhance treatment efficacy. The validity of this approach has been proven using an adenovirus that expresses P450R specifically under hypoxic conditions. As a consequence, hypoxic cells are sensitized to the cytotoxic effects of the bioreductive agent and, when combined with radiotherapy, yields a curative regimen. Both P450R and NOSII are clearly important enzymes for the reductive activation of tirapazamine and a number of other bioreductive drugs (Garner et al., 1999; Patterson et al., 2002; Chinje et al., 2003; Cowen et al., 2003; McCarthy et al., 2003). Another issue that has been demonstrated is that NOS itself may be important for consideration in the individualization of bioreductive drug treatment that could be achieved together with a measure of hypoxia.

#### Role of Glutathione Conjugation and Conjugate Efflux in Cellular Resistance to Alkylating Agents and Other Reactive Electrophiles (C.S.M., A.J.T.)

Chemotherapy with cytotoxic antitumor drugs remains an essential component in the treatment of cancers. This is particularly true for the treatment of disseminated and recurrent cancers but also for the

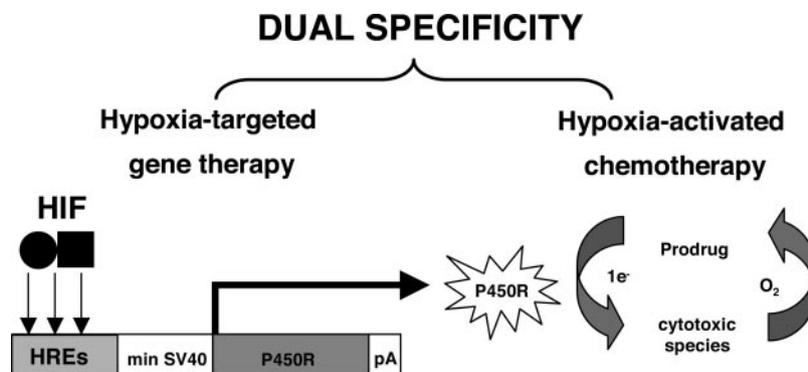


FIG. 7. Schematic representation of the oxygen-dependent GDEPT strategy. Dual specificity is achieved using a hypoxia-regulated promoter to drive expression of the activating enzyme P450R combined with a hypoxia-selective cytotoxic prodrug.

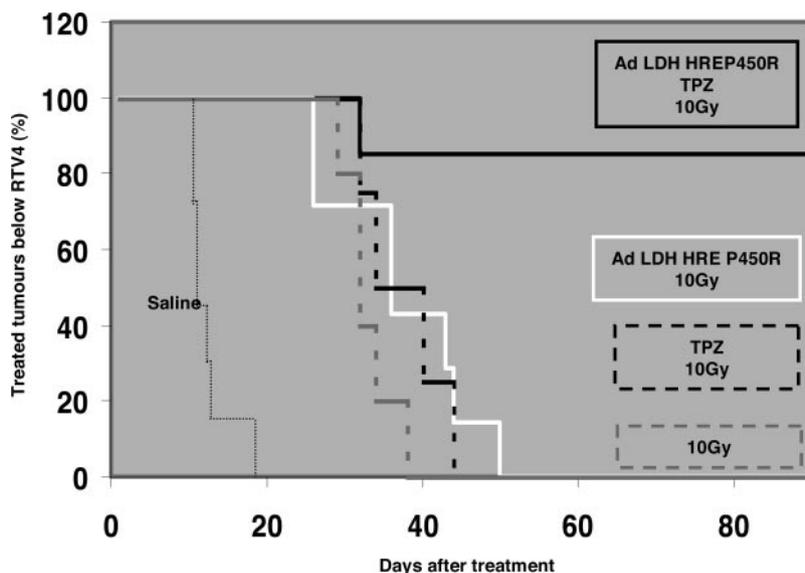


FIG. 8. Kaplan-Meier plot depicting the in vivo response of HT1080 tumors to a hypoxia-selective GDEPT strategy combined with radiation. The time taken to reach RTV4 is shown for treatment with saline (dotted line), 10-Gy radiation (gray dashed line), tirapazamine (TPZ) plus 10-Gy radiation (black dashed line), Ad LDH HRE P450R plus 10-Gy radiation (white solid line), and Ad LDH HRE P450R plus TPZ plus 10-Gy radiation (black solid line).

treatment, as an adjuvant to surgery or radiation, of cancers with a high risk of recurrence. The emergence of acquired or de novo resistance of tumor cells to the cytotoxicities of cancer drugs is a major obstacle to effective therapy. Hence, an understanding of the cellular factors that determine, or are associated with, anticancer drug resistance has the potential to identify patients at particular risk of treatment failure and to suggest the application of alternative strategies to reverse or circumvent cancer drug resistance (Moscow et al., 2003).

At the cellular level, numerous mechanisms for drug resistance have been described, including reduced drug accumulation due to decreased uptake or increased efflux, reduced drug activation or increased drug inactivation, altered drug targets, increased repair of drug damage, and altered apoptotic thresholds (Moscow et al., 2003). This laboratory has been particularly interested in the early, or proximal cellular processes involved in drug detoxification and the emergence of drug resistance; namely, the so-called phase II and phase III detoxification pathways (Ishikawa, 1992). In phase II detoxification, chemically reactive cytotoxic drugs are rendered less reactive and, hence, generally less toxic, often via conjugation with reduced glutathione (GSH), glucuronide, or sulfate. Whereas the conjugations of reactive nucleophilic toxins to glucuronide or sulfate are invariably

enzyme-dependent, conjugations of reactive electrophiles to the thiol of GSH may often proceed nonenzymatically as well as in GST-catalyzed reactions (Hayes and Pulford, 1995). Phase III detoxification involves the energy-dependent, transporter-mediated efflux of drugs or drug-conjugates from the cell. An important feature of phase II and III detoxification pathways is that they serve not only as the basis for cellular resistance to cytotoxic drugs but also as a means to inactivate a variety of other xeno- and endobiotic compounds including carcinogens, mutagens, and bioactive fatty acid derivatives. Thus, knowledge of the components and substrate specificities of the phase II and III processes offers the opportunity to understand and influence cancer drug sensitivity, carcinogen detoxification, and lipid signaling.

It has been long recognized that GSTs catalyze the conjugation with GSH of a number of electrophilic cancer drugs and carcinogens (Hayes and Pulford, 1995). However, the relationship between increased cellular levels of GST and drug resistance has been, at best, inconsistent. Indeed, work in our laboratory and others using transgenic expression of GST isozymes showed that enforced overexpression of GST conferred little, if any, resistance to a variety of cancer drugs, including drugs known to be substrates of the particular GST isozyme expressed. When in 1994 it was recognized that multidrug resistance-associated protein 1 (MRP1) was a GSH-conjugate (GS-X) efflux transporter (Jedlitschky et al., 1994), we hypothesized that

effective GST-mediated detoxification of cancer drugs may require the concomitant expression of the GS-X efflux pump to remove the drug-conjugates formed. MRP1 (ABCC1) is a member of a multigene family of ATP-binding cassette (ABC) transmembrane proteins that mediate energy-dependent efflux of various substrates (Holland et al., 2003). In addition to their roles in transport of GS-X, MRP1 and the related isoform, MRP2, are intimately involved in GSH homeostasis: they transport both oxidized glutathione and GSH. Moreover, for the transport of some MRP1/2 substrates, GSH cotransport is required. For many substrates, GSH augments transport presumably by effecting conformational changes in MRP1/2 (Holland et al., 2003). Because our model MCF-7 cell lines, in which GSTs were transgenically overexpressed, lacked MRP1 expression, this hypothesis would explain the failure to observe GST-mediated drug resistance in these cell lines. Indeed, subsequent work in our laboratory has confirmed that various isozymes of cytosolic GST can operate in combination with MRP1 to confer resistance to toxic electrophiles including the anticancer drug chlorambucil, the model carcinogen 4-nitroquinoline 1-oxide (NQO), and other cellular toxins (Morrow et al., 1998a,b; Paumi et al., 2001). In many cases, MRP and GST acted synergistically in that overexpression of MRP or GST alone had little effect on cellular sensitivity to the toxins, whereas combined expression of MRP and GST conferred relatively high-level resistance (Morrow et al., 1998a,b).

As additional MRP isoforms have been characterized, it has become apparent that several others including MRP2 (ABCC2), MRP3 (ABCC3), and MRP7 (ABCC10) also support GS-X efflux, albeit with variable substrate specificities and transport efficiencies (Holland et al., 2003). Accordingly, we have examined alternative MRP isoforms and have shown that they also can act together with GSH/GST conjugations to confer resistance to chlorambucil (GSTA1-1 + MRP1 or MRP2), NQO (GSTP1-1 + MRP1 or MRP2), and the electrophilic prostaglandin, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (15-d-PGJ<sub>2</sub>) (nonenzymatic GSH conjugation + MRP1 or MRP3) (Morrow et al., 2000; Paumi et al., 2003; Smitherman et al., 2004).

**MRP and Chlorambucil.** Work from our laboratory showed that combined expression of GSTA1-1 and MRP1 confers resistance to the alkylating anticancer drug chlorambucil (Morrow et al., 1998b). Remarkably, the expression of neither GSTA1-1 alone nor MRP1 alone had any effect on chlorambucil sensitivity. Subsequent studies demonstrated that MRP1 mediates efficient efflux of chlorambucil-SG (Paumi et al., 2001). We observed that chlorambucil-SG was a potent product inhibitor of GSTA1-1 ( $K_i = 0.14 \mu\text{M}$ ). Thus, MRP1-mediated efflux of chlorambucil-SG is necessary for GSTA1-1-associated resistance to relieve product inhibition and allow sustained, detoxifying GSTA1-1 catalysis of chlorambucil-SG production (Paumi et al., 2001). More recently, we showed that MRP2 is also an effective transporter of chlorambucil-SG and that this transport supports GSTA1-1-mediated resistance to chlorambucil in HepG2 cells (Smitherman et al., 2004). These results establish the generality of MRP/GST resistance synergy for multiple isoforms of MRP. Moreover, differences in the plasma membrane localization of MRP1 and MRP2 have implications for the overall disposition of chlorambucil and other drugs that form GS-X. MRP1, ubiquitously expressed and localized on the basolateral surface of polarized cells, is important for secretion of chlorambucil-SG and other GS-Xs into the interstitial space and distribution into the general circulation. MRP2, uniquely expressed on the apical, secretory surface of tissues such as kidney tubular epithelium and hepatocyte canaliculi, is likely to play an important role in the overall detoxification and excretion of chlorambucil and other alkylating agents that can also form conjugates with GSH (Holland et al., 2003).

**MRP and NQO.** NQO is a potent model carcinogen and cytotoxic electrophile. NQO is an excellent substrate of GSTP1-1, and we have shown that its glutathione conjugate, 4-S-glutathionyl quinoline 1-oxide (QO-SG) is efficiently transported by MRP1. Using MCF-7 and HepG2 model cell lines, we showed that MRP1 and MRP2 act in synergy with GSTP1-1 to protect cells from the genotoxicity (DNA adduct formation) and cytotoxicity of NQO (Morrow et al., 1998a; Smitherman et al., 2004). The data demonstrate that 1) MRP1 and MRP2 alone confer modest protection from NQO cytotoxicity; 2) MRP1 or MRP2 in combination with GSTP1-1 confers considerably more protection from cytotoxicity and genotoxicity than does MRP1 or MRP2 alone; and 3) expression of functional MRP1 or MRP2 is absolutely required for GSTP1-1-dependent protection from cytotoxicity. These results underscore the importance of both the phase II and phase III pathways of electrophile detoxification and suggest that the balance between GST/MRP-mediated protection from cytotoxicity versus carcinogenic genotoxicity has important implications for the outcome of carcinogenic exposure in a variety of cells and tissues.

**Role of MRP in GS-X Detoxification.** Our work has established the validity of the notion that combined actions of GSH/GST and MRP can cooperate to confer cellular resistance to some electrophilic toxins. An implicit concept resulting from these findings is that the formation of the GS-X is insufficient to fully detoxify the electrophile, suggesting that MRP-mediated efflux is required to alleviate a persistent, or acquired, direct or indirect toxicity of the GS-X. The toxicities of the parent electrophiles are believed to reside in their chemical reactivities with cellular nucleophiles. Formation of GS-X at the reactive, electrophilic centers would be expected to greatly attenuate their chemical reactivities and hence their toxicities. Thus, the need to remove GS-X is not immediately apparent. The conjugates may retain or acquire chemical reactivity or other biological activities. Alternatively, GS-X toxicity may be indirect. For example, GS-X may inhibit GST catalysis or other GSH-dependent processes, thereby limiting the ability of the cell to deal with other toxins. Lastly, the conjugation reaction may be reversible, as is well described in Michael addition reactions. In the latter case, formation of GS-X in the absence of MRP or other GS-X transporters will lead to accumulation of GS-X, a depot of conjugate that can regenerate the toxic parent electrophile by the reverse reaction.

**Conjugate Accumulation.** In several studies, we have shown that, in the absence of efficient MRP-dependent GS-X transporter activity, cells treated with electrophiles can accumulate extraordinarily high levels of the corresponding GS-X. Indeed, MRP-deficient cells treated with as little as  $1 \mu\text{M}$  NQO in the medium can accumulate  $\geq 0.8 \text{ mM}$  intracellular QO-SG (Morrow et al., 1998a, 2000). Similarly, MRP-deficient cells treated with  $1 \mu\text{M}$  1-chloro-2,4-dinitrobenzene (CDNB) rapidly accumulated up to a  $0.4 \text{ mM}$  concentration of the corresponding conjugate, dinitrophenyl glutathione. For both compounds, CDNB and NQO, toxicity was correlated with intracellular accumulation of conjugate, and MRP-mediated protection was associated with efficient conjugate efflux and maintenance of low intracellular conjugate levels (Morrow et al., 1998a, 2000). Although these studies did not directly address the mechanism of GS-X toxicity, they emphasize that, because conjugates can accumulate to such extreme (millimolar) levels in MRP-deficient cells, even conjugates of modest toxicity can have a profound effect on cell survival.

**Product Inhibition of GST.** Although GSTA1-1 is among the best catalysts of chlorambucil conjugation with GSH, expression of GSTA1-1 alone in MRP-deficient MCF-7 cells failed to confer resistance to chlorambucil (Morrow et al., 1998b; Smitherman et al., 2004). Only when coexpressed with MRP1 or MRP2 did GSTA1-1 confer resistance to chlorambucil. To account for the requirement of

MRP for chlorambucil resistance, we examined chlorambucil-SG transport and the effect of chlorambucil-SG on GST activity. Studies showed that chlorambucil-SG is transported very efficiently by MRP1 ( $K_m = 0.37 \mu\text{M}$ ). Kinetic analysis of enzyme inhibition revealed that chlorambucil-SG is a potent competitive inhibitor of GSTA1-1 (Paumi et al., 2001). Indeed, chlorambucil-SG inhibits GSTA1-1 with a  $K_i$  of  $0.14 \mu\text{M}$ . From these results, we concluded that in the absence of MRP, chlorambucil-SG rapidly accumulates to levels that essentially completely inhibit further GSTA1-1 catalysis of chlorambucil conjugation. MRP is required to relieve this potent product inhibition, thereby maintaining GSTA1-1 catalysis of chlorambucil detoxification. It is remarkable that the  $K_i$  of GSTA1-1 inhibition and the  $K_m$  for MRP1-mediated transport are similar, indicating that the kinetic properties of MRP1 transport are well suited to eliminate chlorambucil-SG at pharmacologically relevant drug concentrations. These studies demonstrate how determinations of quantitative kinetic properties of phase II and phase III detoxification components can be used to predict and explain sensitivities of cells to electrophilic toxins.

**Detoxification of the Electrophilic Fatty Acid, 15-d-PGJ<sub>2</sub>.** The cyclopentenone prostaglandins are arachidonic acid metabolites that influence a variety of cellular processes including induction of apoptosis and inhibition of cell growth, induction of adipocyte differentiation, and inhibition of inflammatory signaling pathways (Kliwer et al., 1995; Shibata et al., 2002). 15-d-PGJ<sub>2</sub>, a terminal product of prostaglandin D<sub>2</sub> metabolism, is among the most potent cyclopentenone prostaglandins. Its biological effects are mediated by both peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ )-independent and -dependent mechanisms. Many PPAR $\gamma$ -independent effects are believed to involve the formation of 15-d-PGJ<sub>2</sub>/protein adducts via reactions between protein thiols and the  $\alpha,\beta$ -unsaturated ketone on 15-d-PGJ<sub>2</sub>. Formation of these adducts inactivates or modifies the function of the target protein resulting in the observed biological effects.

15-d-PGJ<sub>2</sub> is also a ligand for the nuclear receptor, PPAR $\gamma$ . Activation of PPAR $\gamma$  by binding of this ligand or others facilitates PPAR $\gamma$ /retinoid X receptor heterodimer formation, recruitment of transcriptional cofactors, and binding of the PPAR $\gamma$ /retinoid X receptor complex to its PPAR-responsive elements (PPRE) (Chawla et al., 2001). Consequently, ligand binding to PPAR $\gamma$  is associated with the altered expression of a variety of genes including those involved in adipocyte differentiation, cell proliferation, and lipid and glucose homeostasis (Hihi et al., 2002). 15-d-PGJ<sub>2</sub> has attracted considerable attention because of its antitumor activities, including the ability to inhibit growth and induce apoptosis in several cancer cell lines.

With this background, we determined that 15-d-PGJ<sub>2</sub> forms a GS-X, 15-d-PGJ<sub>2</sub>-SG, efficiently but nonenzymatically under physiological conditions (Paumi et al., 2003, 2004). Next we asked whether 15-d-PGJ<sub>2</sub>-SG was a substrate of MRP and, if so, whether GSH conjugation plus MRP-dependent conjugate efflux would attenuate the biological effects of exogenously added 15-d-PGJ<sub>2</sub>. Indeed, 15-d-PGJ<sub>2</sub>-SG was transported by MRP1 and MRP3. Moreover, expression of MRP1 or MRP3 conferred a 2-fold GSH-dependent resistance to 15-d-PGJ<sub>2</sub> cytotoxicity, as was demonstrated in GSH-replete and -depleted cells. Expression of these transporters obliterated 15-d-PGJ<sub>2</sub> activation of PPAR $\gamma$ -dependent transcription of a PPRE-containing reporter gene. Although cytosolic GSTs were ineffective in catalysis of 15-d-PGJ<sub>2</sub>-SG formation, they bound both 15-d-PGJ<sub>2</sub> and 15-d-PGJ<sub>2</sub>-SG avidly and, in so doing, significantly inhibited 15-d-PGJ<sub>2</sub> transactivation of the PPAR-dependent PPRE-containing reporter gene (Paumi et al., 2004).

These results established that GSH conjugation and MRP-mediated

conjugate efflux can attenuate the cytotoxic and transcription-activating effects of the potent lipid electrophile, 15-d-PGJ<sub>2</sub>. The findings that MRP-mediated efflux of 15-d-PGJ<sub>2</sub>-SG is associated with reduced transcriptional activation and that cytosolic GST can attenuate transcriptional activation via noncatalytic sequestration of 15-d-PGJ<sub>2</sub> and 15-d-PGJ<sub>2</sub>-SG raised the intriguing possibility that the GS-X, 15-d-PGJ<sub>2</sub>-SG, like its parent compound, 15-d-PGJ<sub>2</sub>, was also a ligand of PPAR $\gamma$ , a possibility supported by preliminary data from our laboratory. The concept that the GS-X of bioactive lipids may retain or acquire receptor-binding activity is likely to be relevant for other GS-Xs.

In summary, work in this laboratory has examined how GSH and the processes of GSH conjugation and MRP-mediated efflux interact to influence the sensitivities of cells to anticancer drugs, carcinogens, and other cellular toxins. In particular, we have tested the hypothesis that GSTs, enzymes that catalyze the conjugation of electrophilic compounds with GSH, and MRP1–3, members of a family of ABC transporters that mediate GS-X efflux, act in synergy to confer resistance to electrophilic toxins. Indeed, we have shown that combined expression of isozymes of GST with isoforms of MRP confers resistance to the cytotoxicities and genotoxicities of some electrophilic cancer drugs and carcinogens. GSH conjugation generally renders these electrophiles less chemically reactive and hence less toxic. However, our results show the added benefit of MRP expression, findings that indicate that GSH conjugation is insufficient to completely detoxify these and many other electrophilic compounds. In the absence of efficient efflux, the GS-X of the electrophiles can accumulate to high levels. The requirement for MRP to fully potentiate GST-mediated resistance to these compounds suggests that their GS-Xs retain or acquire important biological activities. We have provided evidence that MRP-dependent GS-X efflux can potentiate GSH conjugation-mediated resistance by 1) relieving potent product inhibition of GST by some GS-X, and 2) removing GS-Xs that have measurable biological activities such as nuclear receptor ligand-binding properties.

#### **P450-Based Gene-Directed Enzyme Prodrug Therapy (Y.J., C.-S.C., T.S., H.L., P.S.S., D.J.W.)**

Several novel gene-based treatments for cancer have been introduced over the past few years. In one approach, genes encoding prodrug-activation enzymes are used as “suicide” genes to sensitize tumor cells to drugs that are otherwise not cytotoxic (Springer and Niculescu-Duvaz, 1996). This strategy, referred to as GDEPT, confers on tumor cells the genetic capacity to activate a prodrug locally, within the tumor, and is designed to increase antitumor activity while minimizing toxic side effects to critical host tissues.

Several P450 prodrug-activating enzymes have been evaluated for GDEPT applications in cancer treatment (Chen and Waxman, 2002). The sensitivity of tumors to cyclophosphamide and several other anticancer prodrugs can be dramatically increased by introduction of a P450 gene, which confers the capability to activate the anticancer prodrug directly within the tumor (Fig. 9) and is associated with a strong bystander cytotoxic effect. The efficacy of this P450 GDEPT strategy is manifest in a wide range of rodent and human tumor xenograft models and can be enhanced in several ways: 1) by coexpression of P450 with the flavoenzyme P450R, which substantially increases P450 metabolic activity; 2) by combination of cyclophosphamide with the P450/P450R-activated bioreductive drug tirapazamine; and 3) by the selective inhibition of liver prodrug activation using P450 inhibitors or antithyroid drugs, which suppress hepatic but not tumor cell P450R (Chen and Waxman, 2002). Other studies have shown that P450 GDEPT can be facilitated by 1) localized, polymer-

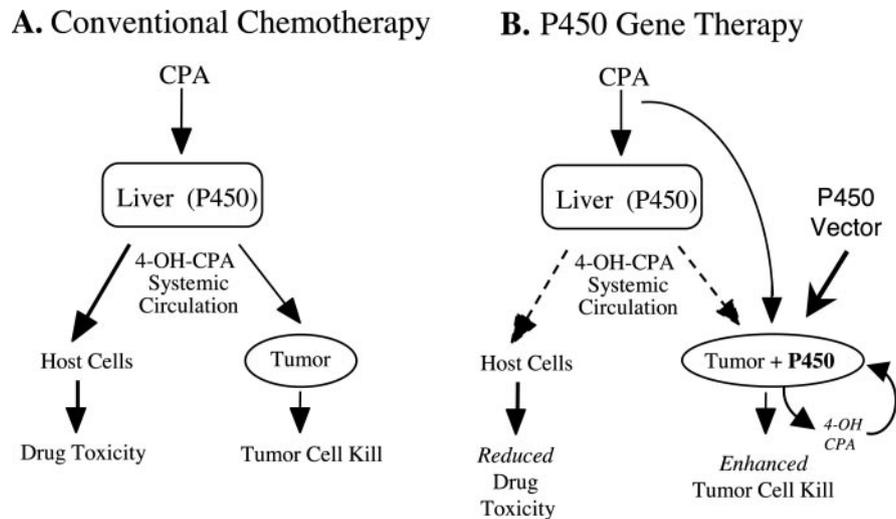


FIG. 9. Strategy for P450 GDEPT compared to conventional chemotherapy using cyclophosphamide (CPA). In conventional chemotherapy (A), CPA is primarily activated via liver P450 enzymes, which release the activated 4-hydroxy metabolite into the systemic circulation with associated host cell toxicity. In P450 GDEPT (B), a prodrug-activating P450 gene is delivered to the tumor cell using a suitable gene therapy vector, providing for localized, intratumoral prodrug activation and enhanced tumor cell kill.

based delivery of the prodrug (Ichikawa et al., 2001); 2) using an antiangiogenic schedule of cyclophosphamide administration (Jounaidi and Waxman, 2001); and 3) combination with other prodrug activation gene therapies (Aghi et al., 1999). Tumor-selective P450 gene delivery can be achieved in a variety of ways, including: 1) intratumoral injection of plasmid DNA, or retrovirus encoding P450 (Kan et al., 2001); 2) localized delivery of P450-expressing cells encapsulated in cellulose sulfate (Lohr et al., 2002); 3) use of human macrophages transduced with a hypoxia-regulated P450 cDNA (Griffiths et al., 2000); and 4) intratumoral delivery of conditionally replicating oncolytic herpes virus encoding P450 (Pawlik et al., 2002). Tumor cell-replicating adenovirus may also be used to facilitate P450 gene expression, thereby enhancing the chemosensitivity of tumor cells that are relatively resistant to adenovirus infection. This has been exemplified in our recent studies using the E1b-55k-deleted adenovirus Onyx-017 as a helper virus, which substantially enhances tumor-selective replication and tumor cell spread of Adeno-P450, a replication-defective human type 5 adenovirus that delivers CYP2B6 linked to P450R by an internal ribosome entry site sequence to tumor cells both in vitro and in vivo (Jounaidi and Waxman, 2004). Together, these studies establish the potential of P450 GDEPT to enhance the efficacy of P450-activated anticancer prodrugs, including novel investigational agents (Lu and Waxman, 2005), without increasing host toxicities associated with the systemic distribution of active drug metabolites formed by the liver.

Recent reports of phase I/II gene therapy trials using P450 provide strong support for the therapeutic potential of this GDEPT strategy. In one study, treatment of inoperable pancreatic carcinoma patients with ifosfamide in combination with encapsulated cells expressing CYP2B1 led to a 3-fold increase in 1-year survival (Lohr et al., 2003). In a separate study in advanced breast cancer patients who failed to respond to all available treatments, retroviral delivery of CYP2B6 to surface nodules in combination with cyclophosphamide treatment elicited an apparent systemic antitumor response, as indicated by the marked improvement of lesions distal to the treatment site and by the induction of a specific antitumor antigen immune response that correlated with a 60% decline in circulating levels of the breast cancer marker CA-153 [personal communication from S. Kingsman (2002) and <http://www.oxfordbiomedica.co.uk/metxia.htm>]. This “systemic

bystander effect” may be of great therapeutic value in treating disseminated metastatic disease.

P450-activated cyclophosphamide induces tumor cell death by a mechanism that involves mitochondrial transition stimulated by drug-induced DNA damage (Fig. 10). That, in turn, leads to activation of a caspase 9-dependent apoptotic pathway (Schwartz and Waxman, 2001). The overall pathway of cyclophosphamide-induced cell death is blocked in tumor cells that overexpress the mitochondrial antiapoptotic factor Bcl-2, which inhibits the release of cytochrome *c* from mitochondria, thereby conferring drug resistance to cyclophosphamide-treated tumor cells. By contrast, when the caspase 9 pathway is blocked in P450-expressing tumor cells at a step that is downstream of the critical stage of mitochondrial transition/cytochrome *c* release, cyclophosphamide-induced cell death is delayed but, ultimately, is not blocked (Schwartz et al., 2002). Thus, caspase activation is not a prerequisite for cyclophosphamide-induced tumor cell death. Proapoptotic factors, such as Bax, p53, and various caspases, stimulate tumor cells to undergo apoptosis, and are presently being explored in gene therapies designed to enhance tumor cell death. As discussed elsewhere (Waxman and Schwartz, 2003), this strategy is *not* suitable for GDEPT, however, insofar as enhanced apoptosis of a tumor cell transduced with a prodrug-activating enzyme, such as P450, will *decrease* the net formation of active drug metabolites and thereby compromise the bystander effect that is so critical to the success of the overall GDEPT strategy. Recent studies demonstrate, however, that *antiapoptotic* factors can be combined with P450 GDEPT to prolong the longevity of prodrug-activating tumor cells. In particular, retroviral expression of the pan-caspase inhibitor p35 has been shown to substantially delay the death of P450-expressing tumor cells treated with cyclophosphamide, and thereby increases the bystander killing effect of the P450-activated prodrug. Importantly, the introduction of p35 delays, but does not completely block the ultimate death of the tumor cells (Schwartz et al., 2002). Moreover, the introduction of p35 may enable tumor cells to undergo necrotic cell death instead of apoptotic death, potentially enhancing systemic (immune system-based) bystander activity (Waxman and Schwartz, 2003). Inclusion of an antiapoptotic factor, such as p35, may facilitate the transmission of replicating adenovirus bearing P450 genes, in view of the role played

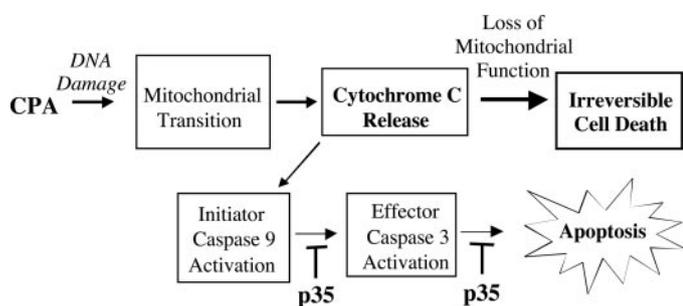


FIG. 10. Role of p35 in inhibiting apoptosis without blocking tumor cell death. DNA damage induced by P450-activated cyclophosphamide (CPA) leads to mitochondrial transition, release of cytochrome *c*, and activation of caspase 9 and downstream apoptotic events. The baculovirus protein p35 is a pan-caspase inhibitor that blocks caspase activity and the ensuing apoptotic response downstream of mitochondrial transition. The p35 protein acts in a manner that does not interrupt drug-induced loss of mitochondrial function, which ultimately leads to irreversible tumor cell death.

by viral antiapoptotic factors, such as adenoviral E1b-19k, in increasing viral replication and spread.

P450-based GDEPT presents several important, practical advantages that may facilitate its incorporation into existing cancer treatment regimens (Chen and Waxman, 2002). 1) P450 GDEPT may be considered as an adjuvant gene therapy that can be implemented in the clinic as an “add-on” to standard care regimens using established anticancer agents, such as cyclophosphamide. By contrast, the prodrugs used for most other cancer GDEPT protocols are investigational agents whose ultimate clinical utility for cancer remains untested. 2) P450 GDEPT can be carried out using human P450 enzymes, such as CYP2B6, an efficient catalyst of cyclophosphamide activation. 3) The active metabolites generated by P450-transduced tumor cells (“P450 factory cells”) diffuse freely from cell to cell by nonfacilitated mechanisms, conferring a strong tumor cell-directed bystander effect that does not require cell-cell contact. 4) P450 prodrug substrates are diverse in their structure, mechanism of action, and optimal prodrug-activating P450 gene; they include both established and investigational anticancer prodrugs, as well as bioreductive drugs that can be activated by P450/P450R in a hypoxic tumor environment (Jounaidi and Waxman, 2000; McCarthy et al., 2003). Finally, 5) replication of the oncolytic viral vectors used to deliver therapeutic genes is often strongly inhibited by activated nucleoside prodrugs (e.g., ganciclovir triphosphate) but not by P450-activated cyclophosphamide.

P450 GDEPT may enhance tumor cell killing by three distinct, but interrelated, “bystander” mechanisms (Fig. 11). These are: 1) a localized *tumor cell bystander effect* that results from diffusion (or gap junctional transfer) of the active drug metabolite to bystander tumor cells; 2) an *endothelial cell bystander effect* that derives from the intrinsic antiangiogenic potential that is associated with cyclophosphamide and certain other chemotherapeutic agents, which, in turn, leads to increased tumor cell kill (Fig. 11); and 3) a *systemic bystander effect* that involves a cell-mediated immune response that targets the drug-treated tumor cells and may extend the gene therapeutic effect to include distant, metastatic tumor cells. Antiangiogenic activity characterizes anticancer drugs that preferentially kill tumor-associated endothelial cells at low doses. This antiangiogenic potential is manifest in the case of cyclophosphamide when the drug is administered using a regularly repeating (“metronomic”) schedule. This may involve either continuous, low-dose cyclophosphamide treatment or, alternatively, repeated cyclophosphamide treatment on a 6-day schedule (Browder et al., 2000; Man et al., 2002). Both metronomic cyclophosphamide schedules eliminate the extended rest periods between treatments that characterize traditional maximum tolerated dose

## Multiple Bystander Killing Mechanisms

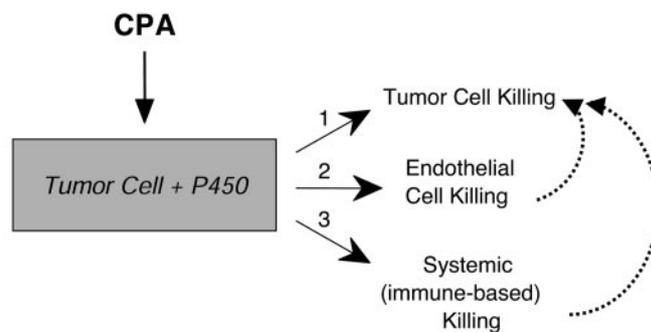


FIG. 11. P450 GDEPT stimulates antitumor responses to cyclophosphamide (CPA) by multiple, interrelated bystander killing mechanisms.

schedules. Under these conditions, tumor-associated endothelial cells do not have sufficient time to recover from cyclophosphamide-induced sublethal DNA damage and are, therefore, exquisitely sensitive to cyclophosphamide toxicity. Tumors transduced with P450 are especially sensitive to metronomic cyclophosphamide treatment, which can induce total, or near-total regression of even very large P450-containing tumors in a way that cannot be achieved with a more traditional schedule (Jounaidi and Waxman, 2001). However, the mechanism for this dramatic antitumor effect, including the potential that P450 GDEPT may contribute to the antiangiogenic action of cyclophosphamide through enhanced killing of tumor endothelial cells in close proximity to the P450-expressing tumor cells, is not known.

## Concluding Remarks

This symposium summarized recent investigations into some of the important ways in which antineoplastic agents interact with drug-metabolizing enzymes and drug transporters. Each of the preceding sections has highlighted how we have arrived at the current state of knowledge in four distinct, but interrelated, areas of research concerning cancer chemotherapy and drug metabolism. The salient findings discussed in each section of the symposium can be summarized as follows. 1) Although P450R is an important catalyst of doxorubicin reduction, the hepatic P450/P450R system is not targeted for mechanism-based inactivation by this anthracycline, and P450R overexpression does not enhance the aerobic cytotoxicity of doxorubicin in human breast cancer cell lines. 2) By selectively targeting hypoxic tumor cells, hypoxia-selective GDEPT strategies with bioreductive prodrugs have great promise in enhancing cancer treatment efficacy. 3) GST-dependent conjugation of electrophiles with GSH and MRP-dependent conjugate efflux act in synergy to confer resistance to specific anticancer drugs, carcinogens, and other cellular toxins. 4) P450-based GDEPT strategies have the potential to enhance the efficacy of P450-activated anticancer drugs by augmenting the local intratumoral generation of cytotoxic metabolites while minimizing toxicities in critical host tissues.

The dominant unifying theme that emerged from this symposium is that drug metabolism and drug transport are key determinants of antineoplastic drug pharmacokinetics and pharmacodynamics, and these processes can be modulated and exploited in ways that offer therapeutic advantage. Enhanced understanding of antineoplastic drug metabolism and transport is contributing to our ability to circumvent tumor drug resistance and to enhance the safety and effectiveness of cancer therapy. The work presented in this symposium generates considerable optimism that basic research on drug metabolism and transport conducted in *in vitro*, cell culture, and animal models will have positive impacts on the pharmacological management of patients with cancer.

## References

- Afsar A, Lee C, and Riddick DS (1996) Modulation of the expression of constitutive rat hepatic cytochrome P450 isozymes by 5-fluorouracil. *Can J Physiol Pharmacol* **74**:150–156.

- Aghi M, Chou TC, Suling K, Breakefield XO, and Chiocca EA (1999) Multimodal cancer treatment mediated by a replicating oncolytic virus that delivers the oxazaphosphorine/rat cytochrome P4502B1 and ganciclovir/herpes simplex virus thymidine kinase gene therapies. *Cancer Res* **59**:3861–3865.
- Beaumont PO, Moore MJ, Ahmad K, Payne MM, Lee C, and Riddick DS (1998) Role of glutathione S-transferases in the resistance of human colon cancer cell lines to doxorubicin. *Cancer Res* **58**:947–955.
- Binley K, Askham Z, Martin L, Spearman H, Day D, Kingsman S, and Naylor S (2003) Hypoxia-mediated tumor targeting. *Gene Ther* **10**:540–549.
- Brizel DM, Scully SP, Harrelson JM, Layfield LJ, Bean JM, Prosnitz LR, and Dewhirst MW (1996) Tumor oxygenation predicts for the likelihood of distant metastases in human soft tissue sarcoma. *Cancer Res* **56**:941–943.
- Browder T, Butterfield CE, Kraling BM, Shi B, Marshall B, O'Reilly MS, and Folkman J (2000) Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. *Cancer Res* **60**:1878–1886.
- Brown JM and Wilson WR (2004) Exploiting tumour hypoxia in cancer treatment. *Nat Rev Cancer* **4**:437–447.
- Chawla A, Repa JJ, Evans RM, and Mangelsdorf DJ (2001) Nuclear receptors and lipid physiology: opening the X-files. *Science (Wash DC)* **294**:1866–1870.
- Chen L and Waxman DJ (2002) Cytochrome P450 gene-directed enzyme prodrug therapy (GDEPT) for cancer. *Curr Pharm Des* **8**:1405–1416.
- Cheung RLH, Lee C, Jones EJ, and Riddick DS (1996) Lack of effect of methotrexate on the expression of constitutive hepatic cytochromes P450 in the male rat. *Xenobiotica* **26**:503–514.
- Chinje EC, Cowen RL, Feng J, Sharma SP, Wind NS, Harris AL, and Stratford IJ (2003) Non-nuclear localized human NOSII enhances the bioactivation and toxicity of tirapazamine (SR4233) in vitro. *Mol Pharmacol* **63**:1248–1255.
- Cowan KH, Batist G, Tulpule A, Sinha BK, and Myers CE (1986) Similar biochemical changes associated with multidrug resistance in human breast cancer cells and carcinogen-induced resistance to xenobiotics in rats. *Proc Natl Acad Sci USA* **83**:9328–9332.
- Cowen RL, Patterson AV, Telfer BA, Airley RE, Hobbs S, Phillips RM, Jaffar M, Stratford IJ, and Williams KJ (2003) Viral delivery of P450 reductase recapitulates the ability of constitutive overexpression of reductase enzymes to potentiate the activity of mitomycin C in human breast cancer xenografts. *Mol Cancer Ther* **2**:901–909.
- Cowen RL, Williams KJ, Chinje EC, Jaffar M, Sheppard FCD, Telfer BA, Wind NS, and Stratford IJ (2004) Hypoxia targeted gene therapy to increase the efficacy of tirapazamine as an adjunct to radiotherapy: reversing tumor radioresistance and effecting cure. *Cancer Res* **64**:1396–1402.
- Dachs GU, Patterson AV, Firth JD, Ratcliffe PJ, Townsend KM, Stratford IJ, and Harris AL (1997) Targeting gene expression to hypoxic tumor cells. *Nat Med* **3**:515–520.
- Di Re J, Lee C, and Riddick DS (1999) Lack of mechanism-based inactivation of rat hepatic microsomal cytochromes P450 by doxorubicin. *Can J Physiol Pharmacol* **77**:589–597.
- Fitzsimmons SA, Lewis AD, Riley RJ, and Workman P (1994) Reduction of 3-amino-1,2,4-benzotriazine-1,4-di-N-oxide (tirapazamine, WIN 59075, SR4233) to DNA-damaging species: a direct role for NADPH:cytochrome P450 oxidoreductase. *Carcinogenesis* **15**:1503–1510.
- Forkert PG, Lord JA, and Parkinson A (1996) Alterations in expression of CYP1A1 and NADPH-cytochrome P450 reductase during lung tumor development in SWR/J mice. *Carcinogenesis* **17**:127–132.
- Garner AP, Paine MJI, Rodriguez-Crespo I, Chinje EC, Ortiz de Montellano P, Stratford IJ, Tew DG, and Wolf CR (1999) Nitric oxide synthases catalyze the activation of redox cycling and bioreductive anticancer agents. *Cancer Res* **59**:1929–1934.
- Gewirtz DA (1999) A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics Adriamycin and daunorubicin. *Biochem Pharmacol* **57**:727–741.
- Goeptar AR, Koppele JMT, Lamme EK, Pique JM, and Vermeulen NPE (1993) Cytochrome P450-2B1-mediated one-electron reduction of Adriamycin: a study with rat liver microsomes and purified enzymes. *Mol Pharmacol* **44**:1267–1277.
- Griffiths L, Binley K, Iqbal S, Kan O, Maxwell P, Ratcliffe P, Lewis C, Harris A, Kingsman S, and Naylor S (2000) The macrophage: a novel system to deliver gene therapy to pathological hypoxia. *Gene Ther* **7**:255–262.
- Hayes JD and Pulford DJ (1995) The glutathione S-transferase supergene family: regulation of CRT and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit Rev Biochem Mol Biol* **30**:445–600.
- Hibi AK, Michalik L, and Wahli W (2002) PPARs: transcriptional effectors of fatty acids and their derivatives. *Cell Mol Life Sci* **59**:790–798.
- Holland I, Cole S, Kuchler K, and Higgins C (2003) *ABC Proteins From Bacteria to Man*. Academic Press, Amsterdam.
- Ichikawa T, Petros WP, Ludeman SM, Fangmeier J, Hochberg FH, Colvin OM, and Chiocca EA (2001) Intraneoplastic polymer-based delivery of cyclophosphamide for intratumoral bioconversion by a replicating oncolytic viral vector. *Cancer Res* **61**:864–868.
- Ishikawa T (1992) The ATP-dependent glutathione S-conjugate export pump. *Trends Biochem Sci* **17**:463–468.
- Jedlitschky G, Leier I, Buchholz U, Center M, and Keppler D (1994) ATP-dependent transport of glutathione S-conjugates by the multidrug resistance-associated protein. *Cancer Res* **54**:4833–4836.
- Jounaidi Y and Waxman DJ (2000) Combination of the bioreductive drug tirapazamine with the chemotherapeutic prodrug cyclophosphamide for P450/P450-reductase-based cancer gene therapy. *Cancer Res* **60**:3761–3769.
- Jounaidi Y and Waxman DJ (2001) Frequent, moderate-dose cyclophosphamide administration improves the efficacy of cytochrome P-450/cytochrome P-450 reductase-based cancer gene therapy. *Cancer Res* **61**:4437–4444.
- Jounaidi Y and Waxman DJ (2004) Use of replication-conditional adenovirus as a helper system to enhance delivery of P450 prodrug-activation genes for cancer therapy. *Cancer Res* **64**:292–303.
- Kan O, Griffiths L, Baban D, Iqbal S, Uden M, Spearman H, Slingsby J, Price T, Esapa M, Kingsman S, et al. (2001) Direct retroviral delivery of human cytochrome P4502B6 for gene-directed enzyme prodrug therapy of cancer. *Cancer Gene Ther* **8**:473–482.
- Kivisto KT, Kroemer HK, and Eichelbaum M (1995) The role of human cytochrome P450 enzymes in the metabolism of anticancer agents: implications for drug interactions. *Br J Clin Pharmacol* **40**:523–530.
- Kliwer SA, Lenhard JM, Willson TM, Patel I, Morris DC, and Lehmann JM (1995) A prostaglandin J<sub>2</sub> metabolite binds peroxisome proliferator-activated receptor-γ and promotes adipocyte differentiation. *Cell* **83**:813–819.
- Lohr M, Hummel F, Faulmann G, Ringel J, Saller R, Hain J, Gunzburg WH, and Salmons B (2002) Microencapsulated, CYP2B1-transfected cells activating ifosfamide at the site of the tumor: the magic bullets of the 21st century. *Cancer Chemother Pharmacol* **49**:S21–S24.
- Lohr M, Kroger JC, Hoffmeyer A, Freund M, Hain J, Holle A, Knofel WT, Liebe S, Nizze H, Renner M, et al. (2003) Safety, feasibility and clinical benefit of localized chemotherapy using microencapsulated cells for inoperable pancreatic carcinoma in a phase I/II trial. *Cancer Ther* **1**:121–131.
- Lu H and Waxman DJ (2005) Anti-tumor activity of methoxymorpholinyl doxorubicin: potentiation by cytochrome P450 3A metabolism. *Mol Pharmacol* **67**:1–8.
- Man S, Bocci G, Francia G, Green SK, Jothy S, Hanahan D, Bohlen P, Hicklin DJ, Bergers G, and Kerbel RS (2002) Antitumor effects in mice of low-dose (metronomic) cyclophosphamide administered continuously through the drinking water. *Cancer Res* **62**:2731–2735.
- Marchand DJ and Renton KW (1981) Depression of cytochrome P-450-dependent drug biotransformation by Adriamycin. *Toxicol Appl Pharmacol* **58**:83–88.
- McCarthy HO, Yakkundi A, McErlane V, Hughes CM, Keilty G, Murray M, Patterson LH, Hirst DG, McKeown SR, and Robson T (2003) Bioreductive GDEPT using cytochrome P450 3A4 in combination with AQ4N. *Cancer Gene Ther* **10**:40–48.
- Melillo G, Musso T, Sica A, Taylor LS, Cox GW, and Varesio LA (1995) A hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter. *J Exp Med* **182**:1683–1693.
- Minnaugh EG, Trush MA, Ginsburg E, Hirokawa Y, and Gram TE (1981) The effects of Adriamycin *in vitro* and *in vivo* on hepatic microsomal drug-metabolizing enzymes: role of microsomal lipid peroxidation. *Toxicol Appl Pharmacol* **61**:313–325.
- Morrow CS, Diah S, Smitherman PK, Schneider E, and Townsend AJ (1998a) Multidrug resistance protein and glutathione S-transferase P1-1 act in synergy to confer protection from 4-nitroquinoline 1-oxide toxicity. *Carcinogenesis* **19**:109–115.
- Morrow CS, Smitherman PK, Diah K, Schneider E, and Townsend AJ (1998b) Coordinated action of glutathione S-transferases (GSTs) and multidrug resistance protein 1 (MRP1) in antineoplastic drug detoxification: mechanism of GST A1-1 and MRP1-associated resistance to chlorambucil in MCF7 breast carcinoma cells. *J Biol Chem* **273**:20114–20120.
- Morrow CS, Smitherman PK, and Townsend AJ (2000) Role of multidrug-resistance protein 2 in glutathione S-transferase P1-1-mediated resistance to 4-nitroquinoline 1-oxide toxicities in HepG2 cells. *Mol Carcinog* **29**:170–178.
- Moscow JA, Morrow CS, and Cowan KH (2003) Drug resistance and its clinical circumvention. In *Cancer Medicine*, 6th ed (Kufe D, Pollock R, Weischelbaum R, Bast R, Gansler T, Holland J, and Frei E eds) pp 711–725. BC Decker Inc, Hamilton, ON, Canada.
- Niitsu N, Kasukabe T, Yokoyama A, Okabe-Kado J, Yamamoto-Yamaguchi Y, Umeda M, and Honma Y (2000) Anticancer derivative of butyric acid (pivaloyloxymethyl butyrate) specifically potentiates the cytotoxicity of doxorubicin and daunorubicin through the suppression of microsomal glycosidic activity. *Mol Pharmacol* **58**:27–36.
- Patterson AV, Barham HM, Chinje EC, Adams GE, Harris AL, and Stratford IJ (1995) Importance of P450 reductase activity in determining sensitivity of breast tumour cells to the bioreductive drug, tirapazamine (SR 4233). *Br J Cancer* **72**:1144–1150.
- Patterson AV, Saunders MP, Chinje EC, Talbot DC, Harris AL, and Stratford IJ (1997) Overexpression of human NADPH:cytochrome c (P450) reductase confers enhanced sensitivity to both tirapazamine (SR 4233) and RSU 1069. *Br J Cancer* **76**:1338–1347.
- Patterson AV, Williams KJ, Cowen RL, Jaffar M, Telfer BA, Saunders M, Airley R, Honess D, van der Kogel AJ, Wolf CR, et al. (2002) Oxygen-sensitive enzyme-prodrug gene therapy for the eradication of radiation-resistant solid tumours. *Gene Ther* **9**:946–954.
- Paumi CM, Ledford DG, Smitherman PK, Townsend AJ, and Morrow CS (2001) Role of multidrug resistance protein 1 (MRP1) and glutathione S-transferase A1-1 in alkylating agent resistance: kinetics of glutathione conjugate formation and efflux govern differential cellular sensitivity to chlorambucil versus melphalan toxicity. *J Biol Chem* **276**:7952–7956.
- Paumi CM, Smitherman PK, Townsend AJ, and Morrow CS (2004) Glutathione S-transferases (GSTs) inhibit transcriptional activation by the peroxisomal proliferator-activated receptor γ (PPAR γ) ligand, 15-deoxy-Δ<sup>12,14</sup>-prostaglandin J<sub>2</sub> (15-d-PGJ<sub>2</sub>). *Biochemistry* **43**:2345–2352.
- Paumi CM, Wright M, Townsend AJ, and Morrow CS (2003) Multidrug resistance protein (MRP) 1 and MRP3 attenuate cytotoxic and transactivating effects of the cyclopentenone prostaglandin, 15-deoxy-Δ<sup>12,14</sup>-prostaglandin J<sub>2</sub> in MCF7 breast cancer cells. *Biochemistry* **42**:5429–5437.
- Pawluk TM, Nakamura H, Mullen JT, Kasuya H, Yoon SS, Chandrasekhar S, Chiocca EA, and Tanabe KK (2002) Prodrug bioactivation and oncolysis of diffuse liver metastases by a herpes simplex virus 1 mutant that expresses the CYP2B1 transgene. *Cancer* **95**:1171–1181.
- Petros WP and Evans WE (2004) Pharmacogenomics in cancer therapy: is host genome variability important? *Trends Pharmacol Sci* **25**:457–464.
- Ramji S, Lee C, Inaba T, Patterson AV, and Riddick DS (2003) Human NADPH-cytochrome P450 reductase overexpression does not enhance the aerobic cytotoxicity of doxorubicin in human breast cancer cell lines. *Cancer Res* **63**:6914–6919.
- Rooseboom M, Commandeur JNM, and Vermeulen NPE (2004) Enzyme-catalyzed activation of anticancer prodrugs. *Pharmacol Rev* **56**:53–102.
- Schwartz PS, Chen CS, and Waxman DJ (2002) Enhanced bystander cytotoxicity of P450 gene-directed enzyme prodrug therapy by expression of the antiapoptotic factor p35. *Cancer Res* **62**:6928–6937.
- Schwartz PS and Waxman DJ (2001) Cyclophosphamide induces caspase 9-dependent apoptosis in 9L tumor cells. *Mol Pharmacol* **60**:1268–1279.
- Scudiero DA, Monks A, and Sausville EA (1998) Cell line designation change: multidrug-resistant cell line in the NCI anticancer screen. *J Natl Cancer Inst* **90**:862.
- Shibata T, Kondo M, Osawa T, Shibata N, Kobayashi M, and Uchida K (2002) 15-Deoxy-Δ<sup>12,14</sup>-prostaglandin J<sub>2</sub>: a prostaglandin D<sub>2</sub> metabolite generated during inflammatory processes. *J Biol Chem* **277**:10459–10466.
- Siim BG, Menke DR, Dorie MJ, and Brown JM (1997) Tirapazamine-induced cytotoxicity and DNA damage in transplanted tumors: relationship to tumor hypoxia. *Cancer Res* **57**:2922–2928.
- Siim BG, Pruijn FB, Sturman JR, Hogg A, Hay MP, Brown JM, and Wilson WR (2004) Selective potentiation of the hypoxic cytotoxicity of tirapazamine by its 1-N-oxide metabolite SR 4317. *Cancer Res* **64**:736–742.
- Smitherman PK, Townsend AJ, Kute TE, and Morrow CS (2004) Role of multidrug resistance protein 2 (MRP2, ABCC2) in alkylating agent detoxification: MRP2 potentiates glutathione

- S-transferase A1-1-mediated resistance to chlorambucil cytotoxicity. *J Pharmacol Exp Ther* **308**:260–267.
- Springer CJ and Niculescu-Duvaz I (1996) Gene-directed enzyme prodrug therapy (GDEPT): choice of prodrugs. *Adv Drug Deliv Rev* **22**:351–364.
- Stratford IJ, Williams KJ, Cowen RL, and Jaffar M (2003) Combining bioreductive drugs and radiation for the treatment of solid tumors. *Semin Radiat Oncol* **13**:42–52.
- Thomsen LL and Miles DW (1998) Role of nitric oxide in tumor progression: lessons from human tumors. *Cancer Metastasis Rev* **17**:107–118.
- Vaupel P, Thews O, and Hoeckel M (2001) Treatment resistance of solid tumors: role of hypoxia and anemia. *Med Oncol* **18**:243–259.
- Wang K, Ramji S, Bhatena A, Lee C, and Riddick DS (1999) Glutathione S-transferases in wild-type and doxorubicin-resistant MCF-7 human breast cancer cell lines. *Xenobiotica* **29**:155–170.
- Waxman DJ and Schwartz PS (2003) Harnessing apoptosis for improved anticancer gene therapy. *Cancer Res* **63**:8563–8572.

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