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Efficient Hypoxic Activation of the Anticancer Agent AQ4N by CYP2S1 and CYP2W1

Clinton R. Nishida, Melody Lee, and Paul R. Ortiz de Montellano

Department of Pharmaceutical Chemistry, University of California, San Francisco, California

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ABSTRACT

AQ4N [1,4-bis{[2-(dimethylamino-*N*-oxide)ethyl]amino}-5,8-dihydroxyanthracene-9,10-dione], a prodrug with two dimethylamino *N*-oxide groups, is converted to the topoisomerase II inhibitor AQ4 [1,4-bis{[2-(dimethylamino)ethyl]amino}-5,8-dihydroxy-anthracene-9,10-dione] by reduction of the *N*-oxides to dimethylamino substituents. Earlier studies showed that several drug-metabolizing cytochrome P450 (P450) enzymes can catalyze this reductive reaction under hypoxic conditions comparable with those in solid tumors. CYP2S1 and CYP2W1, two extrahepatic P450 enzymes identified from the human genome whose functions are unknown, are expressed in hypoxic tumor cells at much higher levels than in normal tissue. Here, we demonstrate that CYP2S1, contrary to a published report (*Mol Pharmacol* **76:**1031–1043, 2009), is efficiently reduced by NADPH–P450 reductase. Most importantly, both CYP2S1 and CYP2W1 are better catalysts for the reductive activation of AQ4N to AQ4 than all previously examined P450 enzymes. The overexpression of CYP2S1 and CYP2W1 in tumor tissues, together with their high catalytic activities for AQ4N activation, suggests that they may be exploited for the localized activation of anticancer prodrugs.

Introduction

Anticancer agents are designed to exploit, in one way or another, the differences that exist between tumor and normal cells. Anticancer agents can directly interfere with a cellular process that occurs at different rates or is differentially important in the two types of cells, or they can be preferentially delivered to the cancer cells. One property of cancer cells in solid tumors that can be used to preferentially target them instead of normal cells is their relatively hypoxic nature (Lee et al., 2007; Tanabe et al., 2007). The poor vascularization resulting from the rapid growth of tumors means that cells within the tumor are less well oxygenated than normal cells. This hypoxic environment is the basis for the selective activation in tumors of agents such as tirapazamine (Brown, 1999), mitomycin (Tomasz, 1995), NLCQ-1 [4-[3-(2-nitro-1-imidazolyl)-propylamino]-7-chloroquinoline hydrochloride] (Papadopoulou and Bloomer, 2003), KS119 [1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine] (Seow et al., 2005), and AQ4N [1,4-bis{[2-(dimethylamino-*N*-oxide)ethyl]amino}-5,8-dihydroxyanthracene-9,10-dione] (Patterson, 2002; Lalani et al., 2007). A second property of cancer cells that can be recruited for selective targeting is the overexpression in tumor cells of specific enzymes that can be used to activate anticancer prodrugs.

AQ4N is the di-*N*-oxide derivative of AQ4 [1,4-bis{[2-(dimethylamino)ethyl]amino}-5,8-dihydroxy-anthracene-9,10-dione] (Fig. 1), a potent topoisomerase II inhibitor (Patterson et al., 1994; Smith et al., 1997). AQ4 itself is not suitable as an anticancer drug because it is highly toxic to normal cells. In contrast, AQ4N has negligible topoisomerase inhibitory activity (Patterson et al., 1994; Smith et al., 1997). Thus, reduction of AQ4N to the mono *N*-oxide intermediate AQ4M [1-{[2-(dimethylamino-*N*-oxide)ethyl]amino}-4-{[2-(dimethylamino) ethyl]amino}-5,8-dihydroxyanthracene-9,10-dione] and finally to AQ4 provides a mechanism for selectively generating the active topoisomerase inhibitor within hypoxic cancer cells. Previous studies of a panel of well characterized drug-metabolizing cytochrome P450 (P450) isoforms found that several have sig-

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ABBREVIATIONS: 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; AQ4M, 1-{[2-(dimethylamino-*N*-oxide)ethyl]amino}-4-{[2-(dimethylamino)ethyl]amino}-5,8-dihydroxyanthracene-9,10-dione; P450, cytochrome P450; CPR, cytochrome P450 reductase; iNOS, inducible nitric-oxide synthase; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; NLCQ-1, 4-[3-(2-nitro-1-imidazolyl)-propylamino]-7-chloroquinoline hydrochloride; KS119, 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

nificant activity for the reduction of AQ4N to AQ4 (Raleigh et al., 1998; Yakkundi et al., 2006). Among these, CYP3A4 was the most effective (Raleigh et al., 1998). We have further shown that the inducible nitric-oxide synthase (iNOS) can also reduce AQ4N to AQ4 (Nishida and Ortiz de Montellano, 2008). AQ4N is in clinical trials as a potential anticancer drug (Lalani et al., 2007).

Determination of the human genome revealed that it encodes 57 full-length cytochrome P450 enzymes, of which approximately a quarter had not previously been identified (Guengerich, 2005). Of those previously unknown P450 enzymes, several are denoted as orphan proteins because their properties, substrates, and physiological functions remain obscure. Two of the orphan P450 enzymes are CYP2S1 and CYP2W1. CYP2S1 is expressed in the epithelial cells of trachea, small intestine, stomach, spleen, lung, and colon, but is found at much lower levels in liver, kidney, and most other tissues (Wu et al., 2006; Jang et al., 2007; Rivera et al., 2007). The protein is apparently overexpressed in psoriatic skin (Karlgren et al., 2005) and hypoxic tumors (Downie et al., 2005; Kumarakulasingham et al., 2005). Remarkably, the presence of CYP2S1 in tumors was associated with poor prognosis (Downie et al., 2005; Kumarakulasingham et al., 2005). Selective expression of CYP2S1 in hypoxic tumors results from the presence in its promoter of three hypoxia response elements that respond to the hypoxia sensor hypoxia-inducible factor 1 (Rivera et al., 2007).

Little is known about the catalytic activity of CYP2S1 beyond the fact that the heterologously expressed, purified enzyme did not oxidize any of 30 or so substrates tested, including naphthalene, arachidonic acid, and retinoic acid (Wu et al., 2006; Jang et al., 2007). This contradicts earlier reports that the unpurified enzyme oxidized retinoic acid (Smith et al., 2003) and naphthalene (Karlgren et al., 2005). It was recently reported that CYP2S1 is not reduced by cytochrome P450 reductase (CPR) and instead uses fatty acid hydroperoxides as cosubstrates in the oxidation of a variety of polycyclic aromatic hydrocarbons (Bui and Hankinson, 2009; Bui et al., 2009). However, the oxidations supported by fatty acid hydroperoxides were completely inhibited by antioxidants, a hallmark of cooxidation reactions that occur outside of the enzyme active site. At this time, no substrate has been reproducibly identified for CYP2S1.

CYP2W1 is not significantly expressed in liver and kidney, but is highly expressed in human colon and adrenal tumors (Aung et al., 2006; Karlgren et al., 2006; Gomez et al., 2007). The protein seems to be regulated epigenetically by methylation of a CpG island at the exon 1/intron 1 junction (Karlgren et al., 2006). In contrast to CYP2S1, CYP2W1 has been shown to oxidize a diversity of substrates, although its specificity remains ill defined because the available data come primarily from a study of the activation of procarcinogens in a cell culture assay (Wu et al., 2006).

In this study, we report that CYP2S1 and CYP2W1 are not only readily reduced by CPR, but both enzymes catalyze the hypoxic reduction of AQ4N to AQ4 at faster rates than either CYP3A4, the previously identified best P450 catalyst, or iNOS. The high AQ4N reduction activities of these two enzymes, combined with their preferential overexpression in certain populations of cancer cells, provide a dual-selectivity mechanism for the preferential activation of AQ4N in tumors. These AQ4N results, which identify the first substrate for CYP2S1, provide a paradigm for the design of future anticancer agents that are selectively activated in cancer tissues.

Materials and Methods

Materials. AQ4N, AQ4M, and AQ4 were generous gifts from Novacea, Inc. (Richmond, CA). Human CYP3A4 and cytochrome P450 reductase were donated by Drs. Jed Lampe and Sylvie Kandel (University of California, San Francisco), respectively. CYP2S1 and CYP2W1 pCWori plasmids and pGro12 expression plasmids encoding the chaperone protein GroEL/ES were generous gifts from Dr. F. P. Guengerich (Vanderbilt University, Nashville, TN). *Escherichia coli* DH5$_\alpha$ F′1Q competent cells were purchased from Invitrogen (Carlsbad, CA). α-Dithiothreitol (DTT), phenylmethylsulfonyl fluoride, lysozyme, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and mitoxantrone were purchased from Sigma-Aldrich (St. Louis, MO).

Bacterial Expression and Purification of CYP2S1 and CYP2W1. The experimental procedures were adapted from those of a previous report (Wu et al., 2006). *E. coli* DH5α F′1Q competent cells were transformed with both pGro12 ES/EL, for coexpression of GroES/L, and the CYP2S1 or CYP2W1 plasmid. Ampicillin- and kanamycin-resistant transformants were used to inoculate overnight 75-ml Luria-Bertani small-scale cultures, followed by large-scale expression in 2.8-liter Fernbach flasks as follows. A 7-ml aliquot of the overnight culture was transferred to 1 liter of terrific broth medium containing 50 μg/ml kanamycin, 100 μg/ml ampicillin, and 0.1% of L-arabinose for the induction of GroES/L and supplemented with trace elements. The cultures were grown at 37°C and 220 rpm and 30 min before isopropyl β-D-1-thiogalactopyranoside induction of P450 expression, at which time 0.5 mM 5-aminolevulinic acid was added. After approximately 5 h, when the optical density reached 0.8, 1 mM isopropyl β-D-1-thiogalactopyranoside was added, and the temperature and shaking speed were reduced to 29°C and 190 rpm, respectively. These conditions were continued for 40 h before harvest.

The culture was harvested by centrifugation at 6000*g* for 15 min, frozen, and stored at −80°C. Subsequent steps were performed at 4°C. The frozen pellet was broken into smaller pieces and mixed with 5 ml of lysis buffer [100 mM potassium phosphate buffer, pH 7.4, with 20% (v/v) glycerol, 0.1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 50 μg/ml lysozyme] per 1 mg of pellet dry weight. The

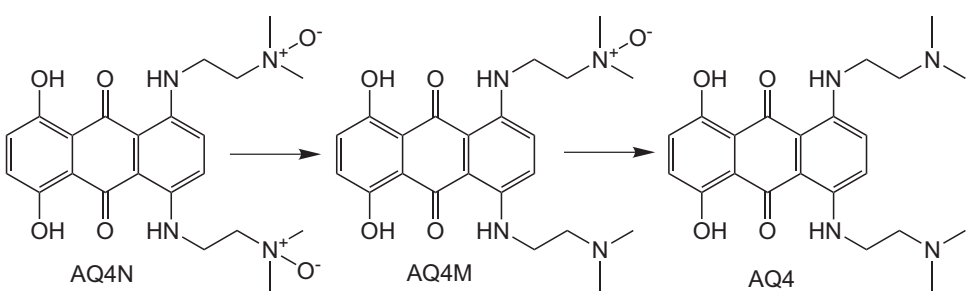

Fig. 1. Scheme showing the reduction of AQ4N (left) to AQ4 (right) via the singly reduced intermediate AQ4M (center).

cells were lysed by sonication to generate the P450-containing lysate, which was centrifuged at 10,000*g* for 20 min. The supernatant was transferred to a chilled tube and centrifuged at 100,000*g* for 1 h. The pellet containing the P450 membrane fractions was collected and stored at −80°C. The P450 membrane fractions were thawed on ice and resuspended in 15 ml per 1 g of wet-weight membrane fractions of 50 mM Tris-acetate buffer, pH 7.4, containing 250 mM sucrose and 0.25 mM EDTA. The mixture was then diluted with four parts of 300 mM potassium phosphate buffer, pH 7.6, containing 20% (v/v) glycerol, 1.25% (w/v) CHAPS, 0.1 mM DTT, and 0.1 mM EDTA. This mixture was stirred for 2 h and then centrifuged at 100,000*g* for 1 h.

All purification steps were performed at 4°C. The supernatant generated above was collected and 20 mM imidazole was added before loading onto a Ni^{2+}-nitriloacetic acid agarose column that had been equilibrated with eight column volumes of buffer A [300 mM potassium phosphate buffer, pH 7.6 with 20% (v/v) glycerol, 1% (w/v) CHAPS, 0.1 mM DTT, and 0.1 mM EDTA] followed by 1 column volume of buffer A with 20 mM imidazole. After washing with eight column volumes of buffer A with 20 mM imidazole, 2 column volumes of 300 mM potassium phosphate buffer, pH 7.6, containing 20% (v/v) glycerol, 0.1 mM DTT, and 0.1 mM EDTA were added to remove CHAPS. The P450 protein was eluted at a flow rate of 0.5 ml/min on an eight-column volume gradient from 10 to 100% buffer B [300 mM potassium phosphate buffer, pH 7.6, containing 20% (v/v) glycerol, 0.1 mM DTT, 0.1 mM EDTA, and 200 mM imidazole] followed by 100% buffer B for 2 column volumes. The eluted P450s were pooled and loaded onto an SP-Sepharose column that had been equilibrated with 4 column volumes of buffer C (100 mM potassium phosphate, pH 7.4 containing 20% glycerol) and 50 mM NaCl. After washing with 3 column volumes of buffer C with 50 mM NaCl then three column volumes of buffer C with 150 mM NaCl, the P450 protein was eluted at a flow rate of 0.5 ml/min on a four-column volume gradient from 20 to 100% buffer D (buffer C with 500 mM NaCl) followed by 100% buffer D for two additional column volumes.

Spectroscopic Analysis of P450 Reduction by CPR. In an anaerobic cuvette, CYP2S1 or CYP2W1 was added to 100 mM potassium phosphate, pH 7.4, 100 mM NaCl, 10% glycerol, 3.5 mM glucose 6-phosphate, 3 mM $MgCl_2$, 1.3 mM $NADP^+$, and 20 U/ml of glucose-6-phosphate dehydrogenase. Argon was flushed through the cuvette with frequent agitation followed by a 2-min flush with carbon monoxide, After a baseline spectrum was obtained, 0.25 equivalents of purified recombinant rat CPR were added anaerobically by gas-tight syringe, and a difference spectrum was measured every 2 min on a Cary 1E UV-visible spectrophotometer (Varian, Inc., Palo Alto, CA).

Kinetic Parameter Determination. Reactions were carried out in duplicate in 1.5-ml Eppendorf tubes at 37°C in an anaerobic chamber. AQ4N was diluted to the following concentrations (10, 25, 50, 100, 300, and 600 μM) and incubated in 200-μl reaction volumes containing 100 nM purified CYP2S1 or CYP2W1 and 100 nM purified CPR in Chelex-treated, deoxygenated 100 mM potassium phosphate, pH 7.4, buffer containing 1 mM deferoxamine. The reaction was started by the addition of 2 mM NADPH, and at time points of 0, 5, 10, and 15 min, 40-μl aliquots were terminated by combination with 40 μl of quenching solution containing 1:1 50 mM ammonium formate, pH 3.6 and acetonitrile. Samples were prepared and analyzed as described previously (Nishida and Ortiz de Montellano, 2008). The integrated AQ4M peaks gave calculated areas under the curve that were used to determine the rate of AQ4M formation. These rates were then analyzed in Prism 4 (GraphPad Software Inc., San Diego, CA) with a nonlinear regression curve fit to a one-site binding (hyperbola) with the equation $Y = B_{max} \times X/(K_m + X)$ used to determine the K_m of AQ4N turnover by CYP2S1 and CYP2W1.

AQ4N Assays. Reactions were carried out in duplicate in 1.5-ml Eppendorf tubes at 37°C in an anaerobic chamber. AQ4N at concentrations of 10, 25, 50, 100, 300, and 600 μM was incubated in 200-μl reaction volumes containing 100 nM purified CYP2S1 and 100 nM purified CPR in Chelex-treated, deoxygenated 100 mM potassium phosphate, pH 7.4 containing 1 mM deferoxamine. The reaction was started by the addition of 2 mM NADPH and at 0-, 5-, 10-, and 15-min time points, 40-μl aliquots were terminated by the addition of 40 μl of quenching solution containing 1:1 50 mM ammonium formate, pH 3.6, and acetonitrile. The samples were prepared and analyzed by HPLC as described previously (Nishida and Ortiz de Montellano, 2008) using authentic metabolites and mitoxantrone as an internal standard. The anaerobic reactions were performed in an Unilab anaerobic glove box (MBRAUN, Garching Germany).

For oxygen-dependence studies, air-saturated and anaerobic buffers were mixed in various proportions to achieve the desired oxygen concentrations (Gevantman, 2007) within sealed vials. Reactions were initiated by the addition of NADPH with a gas-tight syringe.

Results

Reduction of CYP2S1 and CYP2W1 by CPR. CPR is the endogenous agent that transfers reducing equivalents from NADPH to all nonmitochondrial mammalian P450 enzymes that can catalyze the activation of molecular oxygen. Upon reduction, the ferrous P450 enzyme rapidly binds CO if it is present to give the characteristic 450-nm absorbance spectrum of the ferrous carbonmonoxy [Fe(II)-CO] complex. As shown in Fig. 2, the expected spectrum is obtained when a mixture of CYP2S1, NADPH, and CPR is incubated under a CO atmosphere. CPR and NADPH were used in all subsequent turnover studies.

Is Retinoic Acid a CYP2S1 Substrate? As noted earlier, it has been reported that naphthalene (Karlgren et al., 2005) and retinoic acid (Smith et al., 2003) are substrates for CYP2S1. However, it has also been reported that neither of these compounds is a detectable substrate (Wu et al., 2006). In the present investigation, we have been unable to detect any metabolites in aerobic incubations of 1 μM purified

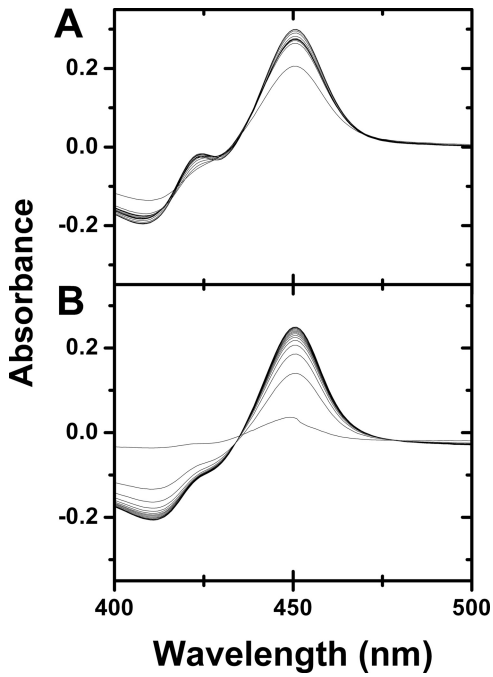

Fig. 2. UV-visible spectrophotometric monitoring of ferrous-carbonmonoxy P450 formation after the addition of NADPH and CPR. A solution of 3 μM purified CYP2S1 (A) or CYP2W1 (B) and 1.3 mM NADPH in a stopper-sealed cuvette was made anaerobic by argon and CO flushing. Difference spectra were recorded every 2 min after the addition of 1.2 μM CPR.

CYP2S1 with 1 μM retinoic acid for 120 min, at 37°C. Although we do not have authentic standards for all possible retinoic acid metabolites, the detection limit for retinoic acid is 5 pmol, or 1% turnover over 2 h.

***N*-Oxide Reduction of AQ4N to AQ4M and AQ4 by CYP2S1 and CYP2W1.** Efforts to detect the binding of AQ4N to CYP2S1 or CYP2W1 by difference UV-vis spectroscopy have not been successful. Addition of AQ4N to either enzyme in one cuvette with the same buffer solution in a reference cuvette did not give rise to a reliable difference spectrum, although the sensitivity of the assay was impaired by the fact that AQ4N has significant absorption at the wavelengths used to monitor substrate binding and this absorption is sensitive to slight protein-dependent changes in buffer conditions. A type I difference spectrum is normally observed if the ligand displaces the distal water ligand to the ferric iron atom. The absence of such a spectrum suggests that the ligand does not cause this water displacement, or that the oxygen of the *N*-oxide substitutes as an iron ligand, giving no detectable spectroscopic change.

Despite the absence of observable binding spectra, CYP2S1 and CYP2W1 were incubated with AQ4N, and the products of the reactions were analyzed by HPLC as reported previously for studies with iNOS (Nishida and Ortiz de Montellano, 2008). Authentic samples of AQ4N, AQ4M, and AQ4 were used as metabolite standards, and mitoxantrone was used as an internal standard. As shown in Fig. 3, AQ4N was converted into AQ4M and AQ4 by both CYP2S1 and CYP2W1.

Kinetic studies indicate that CYP2S1 converted AQ4N to AQ4M with a K_m of 27 μM and V_{max} of 12 min^{-1} (moles of AQ4M formed per mole of enzyme) (Fig. 4). The 95% confidence intervals of nonlinear regression were 13 to 40 μM and 10 to 13 min^{-1}. The kinetics for the reduction of AQ4N by CYP2W1 were similar, with K_m = 30 μM and V_{max} = 12 min^{-1}. For CYP3A4 in a previously described reconstitution system (Shaw et al., 1997), K_m = 28 μM and V_{max} = 7.2 min^{-1}, with 95% confidence intervals of 19 to 39 μM and 6.6 to 7.7 min^{-1}. The nonlinear regression fit of the data was done by using Prism version 4.03. In comparison, reactions from which either CYP2S1 or CYP2W1 was omitted resulted in a rate of 0.7 min^{-1} (Table 1). NADPH was required for any AQ4M or AQ4 production. Thus, although reduced CPR is capable of reducing AQ4N, it does so at a background rate less than 1/10 that of CYP2S1 and CYP2W1.

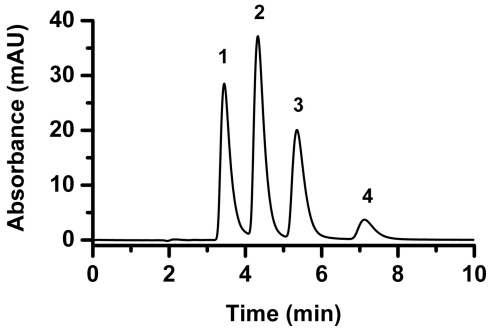

Fig. 3. HPLC chromatogram of the anthraquinone reaction products produced from AQ4N by CYP2W1. The reaction contained 0.05 μM purified CYP2W1, 0.05 μM CPR, 100 μM AQ4N, and 2 mM NADPH. The peaks from lowest to highest elution time are mitoxantrone (1), AQ4 (2), AQ4M (3), and AQ4N (4), all of which were identified by spectroscopic and chromatographic comparison with authentic standards. Detection was at 600 to 650 nm.

The two-step reduction of AQ4N to AQ4 theoretically can occur with or without release of the AQ4M intermediate. To help differentiate between these two pathways, the time course of the reduction of AQ4N by CYP2S1 and CYP2W1 was examined. As shown in Fig. 5, the AQ4M intermediate accumulated during the reaction, and there was a lag period in formation of the final product AQ4.

Because the reaction with AQ4N is a reduction, it is to be expected that oxygen would interfere with the reaction. We therefore determined the effect of increasing oxygen concentrations on the reaction. The specific oxygen dependence of each isoform is shown in Fig. 6. The first-order exponential fit of the data was done by using Origin version 7 (OriginLab Corp., Northampton, MA).

Discussion

One of the intriguing features of CYP2S1 is that, to date, no substrate for the enzyme has been identified. The reports that CYP2S1 oxidizes naphthalene and retinoic acid (Smith et al., 2003; Karlgren et al., 2005) were not confirmed by a subsequent publication (Wu et al., 2006) or now by our inability to detect any metabolites in incubations of CYP2S1 with retinoic acid. If retinoic acid is a substrate for CYP2S1, it is a very poor one.

One requirement for catalytic turnover of P450 enzymes is reduction of the heme iron atom by electrons transferred from an electron donor partner, in the case of CYP2S1 and CYP2W1 ostensibly NAPDH-CPR. However, Bui and Hankinson (2009) reported that they were unable to reduce recombinant CYP2S1 with CPR, as judged by the failure to observe a ferrous-CO absorption spectrum when the two

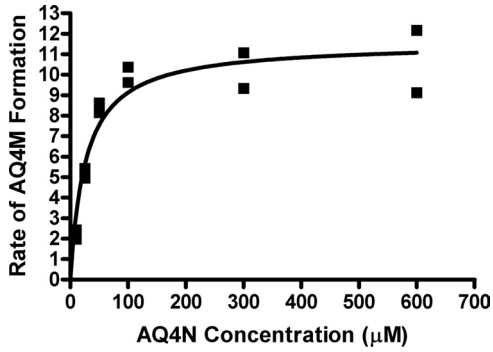

Fig. 4. Determination of the kinetic parameters of AQ4M production from AQ4N by CYP2S1. Nonlinear regression fit of data is shown (line). A solution of 0.1 μM purified CYP2S1, 0.1 μM CPR, 100 μM AQ4N, and 2 mM NADPH was incubated for 30 min, and the products were analyzed by HPLC.

TABLE 1

Rate of AQ4M production

Reaction conditions are described in the text.

Reaction	k_{cat}
	mol AQ4M/min/mol P450
2S1 + CPR	12
2W1 + CPR	12
3A4 + CPR	7
-P450	0.7[a]
-NADPH	N.D.

N.D., not detectable.

[a] For comparison purposes, calculated assuming the same amount of P450 had it been added.

proteins were incubated together with NADPH under a CO atmosphere. In contrast to that report, we have found that reduction of both CYP2S1 and CYP2W1 occurs readily and gives rise to the expected ferrous-CO spectrum (Fig. 2). It is unclear why this was not observed in the earlier study of CYP2S1, particularly as the authors reported successful reduction under the same conditions of CYP3A4. Nevertheless, repeated trials in our hands clearly establish that CYP2S1 is reduced normally by CPR.

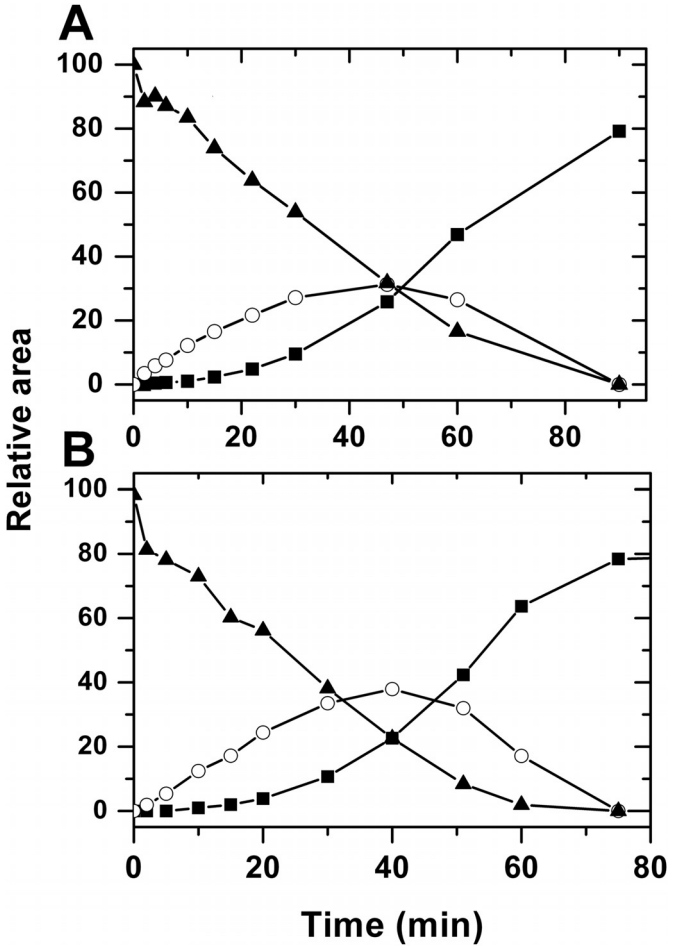

Fig. 5. The time course of AQ4N consumption and metabolite formation by CYP2S1 (A) and CYP2W1 (B). The amounts of AQ4N (▲), AQ4M (○), and AQ4 (■) were determined by HPLC analysis and are proportional to the relative peak areas shown. Purified CYP2S1 and CYP2W1 were used.

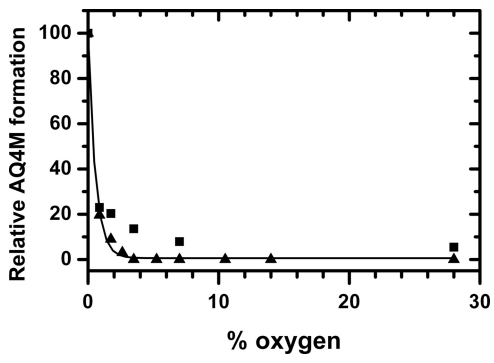

Fig. 6. Oxygen dependence of AQ4M formation from AQ4N by CYP2S1 (▲) and CYP2W1 (■). Purified CYP2S1 and CYP2W1 were used. The line indicates the first-order exponential fit of the CYP2S1 data.

One conclusion that follows from this observation is that the failure to oxidize retinoic acid is not caused by a failure to provide electrons to the CYP2S1 heme iron atom. A second conclusion is that the basis for the postulate that CYP2S1 normally uses lipid peroxides for its catalytic function, as suggested previously (Bui and Hankinson, 2009; Bui et al., 2009), is invalid. It is likely that CYP2S1 reacts with lipid hydroperoxides, as do many P450 enzymes, through a shunt reaction that generates lipid alkoxy and peroxy radicals (Dix et al., 1985; Rota et al., 1997). These radicals, in turn, can enter into cooxidation reactions outside of the confines of the enzyme active site (Eling et al., 1983). The oxidation of polycyclic aromatic hydrocarbons observed under such conditions is therefore unlikely to be any more physiologically significant for CYP2S1 than for other P450 enzymes.

The evidence that CYP2S1 and CYP2W1 are overexpressed in tumors led us to investigate whether they might be able to activate AQ4N (Patterson et al., 1994), an anticancer prodrug. CYP3A4 has been shown to reductively activate AQ4N to its active form AQ4 in liver homogenates (Raleigh et al., 1998). Here, we demonstrate that purified CYP2S1 and CYP2W1, together with recombinant CPR, readily reduce AQ4N to AQ4M and AQ4 at a rate of approximately 12 mol of substrate per mole of enzyme per minute. The K_m values for AQ4N were 27 and 30 μM for CYP2S1 and CYP2W1, respectively, and the V_{max} values for both of approximately 12 min^{-1} are representative of useful affinities and relatively efficient turnover rates. We measured the rate of AQ4N turnover by CYP3A4 using a previously described reconstitution system (Shaw et al., 1997) and obtained a K_m value of 28 μM and a rate of 7.2 min^{-1} (mole of AQ4M formed per mole of CYP3A4), an increase over the 0.5 min^{-1} we observed previously by using baculovirus supersomes or a simple reconstitution of CYP3A4 into dilauroylphosphatidylcholine liposomes (Nishida and Ortiz de Montellano, 2008). In comparison, the nitric-oxide synthases have rates of 5, 2, and 0.5 min^{-1} for the inducible, neuronal, and endothelial isoforms, respectively (Nishida and Ortiz de Montellano, 2008). Thus, CYP2S1 and CYP2W1 exhibit the highest turnover rates among the enzymes examined to date.

For a two-step reduction such as AQ4N to AQ4, the intermediate AQ4M may either be released from the enzyme active site, followed by rebinding and reduction to produce AQ4, or it may reorient within the active site without dissociating from it. Our reaction time course reveals a buildup of the AQ4M intermediate, the amount of which reaches a maximum approximately halfway to completion of the reaction. Conversely, AQ4 exhibits a lag in its formation. Both CYP2S1 (Fig. 5A) and CYP2W1 (Fig. 5B) exhibit this time course profile. Clearly, AQ4M is released from both CYP2S1 and CYP2W1 before its subsequent reduction to AQ4.

AQ4N reductions by CYP2S1 and CYP2W1 were inhibited by oxygen (Fig. 6). The half-maximal inhibition of CYP2S1 occurs at approximately 0.5% oxygen. For CYP2W1 this point is reached at or below 0.5% oxygen. It is expected that the activation of AQ4N by both CYP2W1 and CYP2S1 would be restricted to the more hypoxic regions of the tumor.

No substrates for CYP2S1, and few for CYP2W1, have been discovered so far (Wu et al., 2006). Here, we have demonstrated that both isoforms reduce the prodrug AQ4N at significant rates to the toxic metabolites AQ4M and AQ4. Indeed, CYP2S1 and CYP2W1 are more active in this regard

than CYP3A4, the best previously identified AQ4N-activating enzyme (Raleigh et al., 1998). AQ4N has been demonstrated to be taken up by tumor tissues, where it is converted to AQ4 at significant levels (Albertella et al., 2008). This activity by tumor-related P450s CYP2S1 and CYP2W1 can form the basis for the selective bioreductive activation of AQ4N within an anaerobic tumor environment.

References

Albertella MR, Loadman PM, Jones PH, Phillips RM, Rampling R, Burnet N, Alcock C, Anthoney A, Vjaters E, Dunk CR, et al. (2008) Hypoxia-selective targeting by the bioreductive prodrug AQ4N in patients with solid tumors: results of a phase I study. *Clin Cancer Res* **14:**1096–1104.

Aung PP, Oue N, Mitani Y, Nakayama H, Yoshida K, Noguchi T, Bosserhoff AK, and Yasui W (2006) Systematic search for gastric cancer-specific genes based on SAGE data: melanoma inhibitory activity and matrix metalloproteinase-10 are novel prognostic factors in patients with gastric cancer. *Oncogene* **25:**2546–2557.

Brown JM (1999) The hypoxic cell: a target for selective cancer therapy—eighteenth Bruce F. Cain Memorial Award lecture. *Cancer Res* **59:**5863–5870.

Bui PH and Hankinson O (2009) Functional characterization of human cytochrome P450 2S1 using a synthetic gene-expressed protein in *Escherichia coli. Mol Pharmacol* **76:**1031–1043.

Bui PH, Hsu EL, and Hankinson O (2009) Fatty acid hydroperoxides support cytochrome P450 2S1-mediated bioactivation of benzo[a]pyrene. *Mol Pharmacol* **76:** 1044–1052.

Dix TA, Fontana R, Panthani A, and Marnett LJ (1985) Hematin-catalyzed epoxidation of 7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene by polyunsaturated fatty acid hydroperoxides. *J Biol Chem* **260:**5358–5365.

Downie D, McFadyen MC, Rooney PH, Cruickshank ME, Parkin DE, Miller ID, Telfer C, Melvin WT, and Murray GI (2005) Profiling cytochrome P450 expression in ovarian cancer: identification of prognostic markers. *Clin Cancer Res* **11:**7369–7375.

Eling T, Boyd J, Reed G, Mason R, and Sivarajah K (1983) Xenobiotic metabolism by prostaglandin endoperoxide synthetase. *Drug Metab Rev* **14:**1023–1053.

Gevantman LH (2007) Solubility of selected gases in water, in *CRC Handbook of Chemistry and Physics*, 88th ed (Lide DR ed) pp 80–83, CRC Press, Boca Raton, FL.

Gomez A, Karlgren M, Edler D, Bernal ML, Mkrtchian S, and Ingelman-Sundberg M (2007) Expression of CYP2W1 in colon tumors: regulation by gene methylation. *Pharmacogenomics* **8:**1315–1325.

Guengerich FP (2005) Human cytochrome P450 enzymes, in *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 3rd ed (Ortiz de Montellano PR ed) pp 377–530, Kluwer Academic/Plenum, New York.

Jang YJ, Cha EY, Kim WY, Park SW, Shon JH, Lee SS, and Shin JG (2007) CYP2S1 gene polymorphisms in a Korean population. *Ther Drug Monit* **29:**292–298.

Karlgren M, Gomez A, Stark K, Svärd J, Rodriguez-Antona C, Oliw E, Bernal ML, Ramón y Cajal S, Johansson I, and Ingelman-Sundberg M (2006) Tumor-specific expression of the novel cytochrome P450 enzyme, *CYP2W1. Biochem Biophys Res Commun* **341:**451–458.

Karlgren M, Miura S, and Ingelman-Sundberg M (2005) Novel extrahepatic cytochrome P450s. *Toxicol Appl Pharmacol* **207:**57–61.

Kumarakulasingham M, Rooney PH, Dundas SR, Telfer C, Melvin WT, Curran S, and Murray GI (2005) Cytochrome p450 profile of colorectal cancer: identification of markers of prognosis. *Clin Cancer Res* **11:**3758–3765.

Lalani AS, Alters SE, Wong A, Albertella MR, Cleland JL, and Henner WD (2007) Selective tumor targeting by the hypoxia-activated prodrug AQ4N blocks tumor growth and metastasis in preclinical models of pancreatic cancer. *Clin Cancer Res* **13:**2216–2225.

Lee KA, Roth RA, and LaPres JJ (2007) Hypoxia, drug therapy and toxicity. *Pharmacol Ther* **113:**229–246.

Nishida CR and Ortiz de Montellano PR (2008) Reductive heme-dependent activation of the N-oxide prodrug AQ4N by nitric oxide synthase. *J Med Chem* **51:**5118–5120.

Papadopoulou MV and Bloomer WD (2003) NLCQ-1 (NSC 709257): exploiting hypoxia with a weak DNA-intercalating bioreductive drug. *Clin Cancer Res* **9:**5714–5720.

Patterson LH (2002) Bioreductively activated antitumor *N*-oxides: the case of AQ4N, a unique approach to hypoxia-activated cancer chemotherapy. *Drug Metab Rev* **34:**581–592.

Patterson LH, Craven MR, Fisher GR, and Teesdale-Spittle P (1994) Aliphatic amine *N*-oxides of DNA binding agents as bioreductive drugs. *Oncol Res* **6:**533–538.

Raleigh SM, Wanogho E, Burke MD, McKeown SR, and Patterson LH (1998) Involvement of human cytochromes P450 (CYP) in the reductive metabolism of AQ4N, a hypoxia activated anthraquinone di-*N*-oxide prodrug. *Int J Radiat Oncol Biol Phys* **42:**763–767.

Rivera SP, Wang F, Saarikoski ST, Taylor RT, Chapman B, Zhang R, and Hankinson O (2007) A novel promoter element containing multiple overlapping xenobiotic and hypoxia response elements mediates induction of P4502S1 by both dioxin and hypoxia. *J Biol Chem* **282:**10881–10893.

Rota C, Barr DP, Martin MV, Guengerich FP, Tomasi A, and Mason RP (1997) Detection of free radicals produced from the reaction of cytochrome P450 with linoleic acid hydroperoxide. *Biochem J* **328:**565–571.

Seow HA, Penketh PG, Shyam K, Rockwell S, and Sartorelli AC (2005) 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine: an anticancer agent targeting hypoxic cells. *Proc Natl Acad Sci USA* **102:**9282–9287.

Shaw PM, Hosea NA, Thompson DV, Lenius JM, and Guengerich FP (1997) Reconstitution premixes for assays using purified recombinant human cytochrome P450, NADPH-cytochrome P450 reductase, and cytochrome b_5. *Arch Biochem Biophys* **348:**107–115.

Smith G, Wolf CR, Deeni YY, Dawe RS, Evans AT, Comrie MM, Ferguson J, and Ibbotson SH (2003) Cutaneous expression of cytochrome P450 CYP2S1: individuality in regulation by therapeutic agents for psoriasis and other skin diseases. *Lancet* **361:**1336–1343.

Smith PJ, Blunt NJ, Desnoyers R, Giles Y, and Patterson LH (1997) DNA topoisomerase II-dependent cytotoxicity of alkylaminoanthraquinones and their *N*-oxides. *Cancer Chemother Pharmacol* **39:**455–461.

Tanabe K, Zhang Z, Ito T, Hatta H, and Nishimoto S (2007) Current molecular design of intelligent drugs and imaging probes targeting tumor-specific microenvironments. *Org Biomol Chem* **5:**3745–3757.

Tomasz M (1995) Mitomycin C: small, fast and deadly (but very selective). *Chem Biol* **2:**575–579.

Wu ZL, Sohl CD, Shimada T, and Guengerich FP (2006) Recombinant enzymes overexpressed in bacteria show broad catalytic specificity of human cytochrome P450 2W1 and limited activity of human cytochrome P450 2S1. *Mol Pharmacol* **69:**2007–2014.

Yakkundi A, McErlane V, Murray M, McCarthy HO, Ward C, Hughes CM, Patterson LH, Hirst DG, McKeown SR, and Robson T (2006) Tumor-selective drug activation: a GDEPT approach utilizing cytochrome P450 1A1 and AQ4N. *Cancer Gene Ther* **13:**598–605.

Address correspondence to: Paul R. Ortiz de Montellano, University of California, 600 16th Street, N572D, San Francisco, CA 94158-2517. E-mail: ortiz@cgl.ucsf.edu