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Human Cytochrome P450 Oxidation of 5-Hydroxythalidomide and Pomalidomide, an Amino Analogue of Thalidomide

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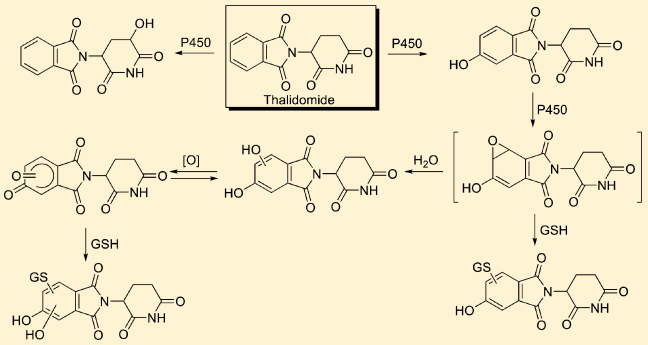
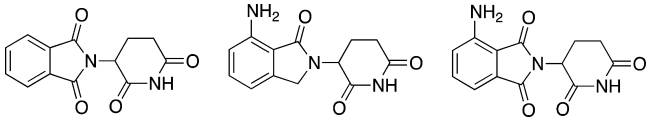
Supporting Information

ABSTRACT: The sedative and antiemetic drug thalidomide [α-(*N*-phthalimido)glutarimide] was withdrawn in the early 1960s because of its potent teratogenic effects but was approved for the treatment of lesions associated with leprosy in 1998 and multiple myeloma in 2006. The mechanism of teratogenicity of thalidomide still remains unclear, but it is well-established that metabolism of thalidomide is important for both teratogenicity and cancer treatment outcome. Thalidomide is oxidized by various cytochrome P450 (P450) enzymes, the major one being P450 2C19, to 5-hydroxy-, 5′-hydroxy-, and dihydroxythalidomide. We previously reported that P450 3A4 oxidizes thalidomide to the 5-hydroxy and dihydroxy metabolites, with the second oxidation step involving a reactive intermediate, possibly an arene oxide, that can be trapped by glutathione (GSH) to GSH adducts. We now show that the dihydroxythalidomide metabolite can be further oxidized to a quinone intermediate. Human P450s 2J2, 2C18, and 4A11 were also found to oxidize 5-hydroxythalidomide to dihydroxy products. Unlike P450s 2C19 and 3A4, neither P450 2J2, 2C18, nor 4A11 oxidized thalidomide itself. A recently approved amino analogue of thalidomide, pomalidomide (CC-4047, Actimid), was also oxidized by human liver microsomes and P450s 2C19, 3A4, and 2J2 to the corresponding phthalimide ring-hydroxylated product.



INTRODUCTION

Thalidomide [α-(*N*-phthalimido)glutarimide] is a sedative and antiemetic drug originally introduced in the clinic in the 1950s. Because of its potent teratogenicity, it was withdrawn in the early 1960s.[1] However, owing to its clinical properties, it was approved in 1998 for the treatment of lesions associated with leprosy and in 2006 for multiple myeloma.[2−4] In addition, thalidomide has been tested for the treatment of many diseases including refractory esophageal Crohn's disease, recurrent bleeding resulting from gastric angiodysplasia in hereditary hemorrhagic telangiectasia, and oral lesions in the course of myelofibrosis.[4−6] Two analogues of thalidomide with increased potency and reduced toxicity, lenalidomide and pomalidomide, have also entered the clinic for the treatment of refractory multiple myeloma.[7−9]



Thalidomide Lenalidomide Pomalidomide

Because of the recent emergence of thalidomide as a drug with clinical potential, there is renewed interest in both its toxicity and pharmacological mechanisms. The teratogenicity of thalidomide is severe and also very species-specific, being teratogenic in primates and rabbits but not in rats and mice. It was initially believed that the R isomer is sedative, whereas the S isomer is teratogenic;[10,11] however, the two enantiomers are readily interconvertible.[12] Although the teratogenicity of thalidomide is well-established, the mechanism still remains unclear. Various hypotheses have been proposed including generation of reactive oxygen species, generation of reactive arene oxide and acylating intermediates, inhibition of angiogenesis, and inhibition of the protein cereblon.[13−17]

Metabolism of thalidomide is important for both teratogenicity and anticancer efficacy.[18,19] Thalidomide is metabolized by two major pathways: hydrolysis and P450-mediated oxidation (Scheme 1). Various P450s oxidize thalidomide to 5-hydroxy-, 5′-hydroxy-, and dihydroxythalidomide metabolites, with the major one being P450 2C19.[20,21] Recently, we reported that P450 3A4 and 3A5 also oxidize thalidomide to the 5-hydroxy and dihydroxy metabolites.[22,23] The second oxidation step in the P450 3A4 pathway generates a reactive

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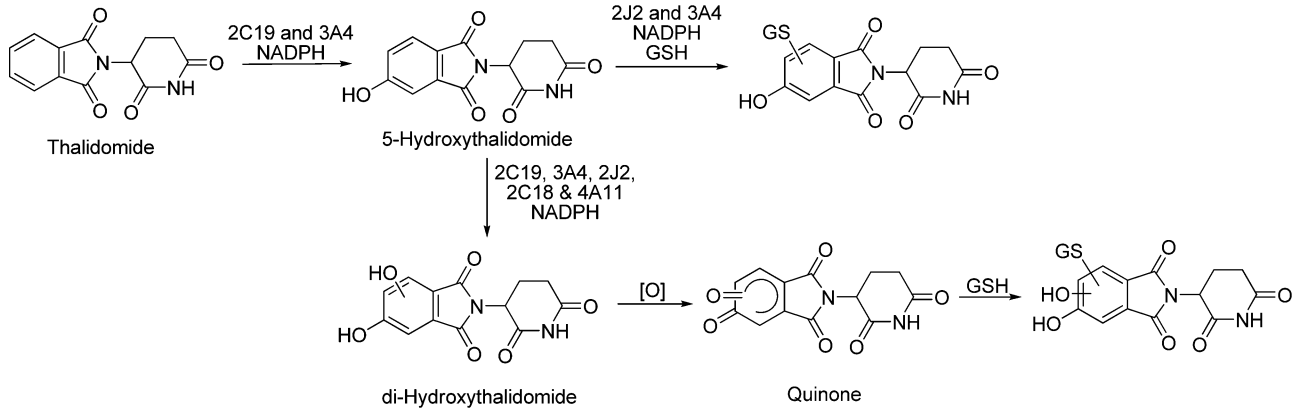
Scheme 1. Proposed Metabolic Pathway of Thalidomide



intermediate, possibly an arene oxide (as initially suggested by Gordon et al.[15]) that can be trapped by GSH to give GSH adducts.[22,23] The formation of GSH adducts of 5-hydroxythalidomide was also confirmed in humanized mouse models.[24] Interestingly, lenalidomide is minimally metabolized to the 5-hydroxy and *N*-acetyl lenalidomide metabolites (each produced at <5% of the total dose), with the majority of the dose being excreted unchanged in humans.[25] Pomalidomide has been shown to be extensively metabolized to various products, including two pthalidimide ring-hydroxylated, one N-acetylated, and various hydrolysis products.[26]

The P450s (EC 1.14.14.1) constitute a large superfamily of enzymes found throughout nature and function primarily as monooxygenases.[27] P450s are involved in the oxidation of a large number of compounds including fatty acids, sterols, fat-soluble vitamins, and xenobiotics. Drugs are metabolized primarily by P450s, and metabolism contributes to many of their pharmacological and toxicological actions.[28] The human liver is the major site of P450s, although other organs including intestine, lung, kidney, heart, skin, and brain also have P450s. P450 3A4 is the major P450 enzyme in human liver and small intestine; P450 2C19 is a relatively minor P450 in the liver, in terms of the amount of the protein.[28] The major P450 in the heart and cardiovascular system is P450 2J2,[29] 2C18 is a major P450 in the skin,[30,31] and 3A7 is the dominant P450 in the fetus.[32] Although the liver is the major site of drug metabolism, P450s in other organs can also metabolize drugs. For example, P450 2J2 oxidizes terfenadine and ebastine,[33] and P450 2C18 oxidizes phenytoin.[34] Thus, extrahepatic bioactivation of xenobiotics to toxic products may be responsible for organ-specific toxicity.

In our continuing efforts to understand the metabolism of thalidomide and its possible role in teratogenicity,[22,23] we studied the biotransformation of thalidomide, its 5-hydroxy product, and recently approved amino analogue pomalidomide by P450s that are preferentially expressed in specific tissues. These three P450s were selected for several reasons. P450 2J2 is primarily expressed in the endothelial cells of the intestine and the cardiovascular systems and also the myocardiocytes in the heart.[35,36] It is also expressed in the liver, kidney, and lung.[29] The major function of P450 2J2 is apparently epoxidation of arachidonic acid to the four regioisomers of epoxyicosatrienoic acid.[37] P450 2J2 has also been shown to also metabolize drugs including ebastin, terfenidine, and artemezole.[33] P450 2C18 is significantly expressed in the skin, although it is also expressed in other tissues.[28,30,31] The antiepileptic drug phenytoin is oxidized by 2C18 to hydroxylated and (toxic) quinone products.[34] P450 4A11 is expressed in various tissues including the liver, kidney, and intestine.[28] The primary function of 4A11 is ω- and ω-1 hydroxylation of arachidonic acid.[38] Interestingly, each of these three P450s has been reported to be expressed in fetal tissues as well.[39,40]

We found that P450s 2J2, 2C18, and 4A11 all oxidize 5-hydroxythalidomide to the corresponding dihydroxy product, although they are unable to metabolize thalidomide itself. The dihydroxythalidomide product is further oxidized to a potentially toxic quinone intermediate. We also report on the metabolism of the amino analogue pomalidomide by several P450s. Pomalidomide (4-amino α-(*N*-phthalimido)glutarimide, Actimid, CC-4047) is an amino analogue of thalidomide with increased efficacy and reduced toxicity. It has recently been approved for the treatment of relapsed or refractory multiple myeloma.[8] Pomalidomide is considered to be the most potent immunomodulatory drug in this class, 100 times more potent than thalidomide and 10 times more potent than lenalidomide. The mechanism of action, however, is not fully understood but involves antiangiogenic effects, immunomodulation, effects on the myeloma tumor microenvironment, and inhibition of the protein cereblon.[7] In a recent report,[26] pomalidomide was shown to be metabolized in humans to the *ortho*- and *para*-phthalidimide ring-hydroxylated metabolites along with N-acetylated and hydrolyzed products. However, using in vitro assays with purified enzymes, the authors failed to detect any *para*-hydrothalidomide product.[26] With P450s 1A2, 2C19, 2D6, and 3A4, *ortho*-hydroxythalidomide was detected as a metabolite, although the apparent rate of oxidation was very low. Because of our interest in tissue-specific metabolism (especially with respect to the antiangiogenic effect), we examined the metabolism of pomalidomide by P450 2J2 and compared it with P450s 2C19 and 3A4 and human liver microsomes.

EXPERIMENTAL PROCEDURES

Chemicals. (*R*)-(+)-Thalidomide and (*S*)-(−)-thalidomide (Sigma-Aldrich, St. Louis, MO) and racemic pomalodomide (Selleck Chemicals, Houston, TX) were purchased from the indicated sources. 5-Hydroxythalidomide was synthesized as reported previously.[41] Other chemicals and reagents used in this study were obtained from the sources described previously[22−24] or were of the highest quality commercially available.

Enzyme Preparations. P450 2J2 and 2C18 Supersomes (microsomes with P450s expressed in insect cells using baculovirus vectors)

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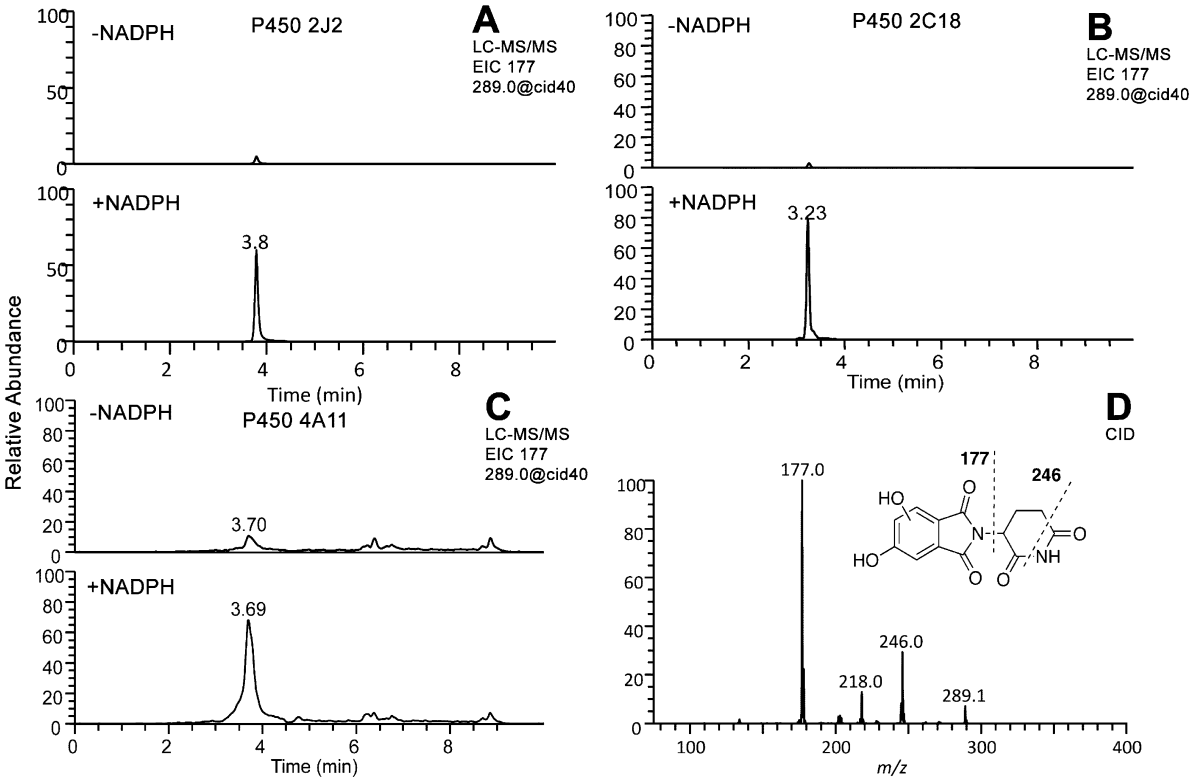

Figure 1. LC−MS/MS chromatograms and CID spectra showing the oxidation of 5-hydroxythalidomide to dihydroxythalidomide by human P450s 2J2, 2C18, and 4A11. Extracted ion chromatograms of the product ion using the m/z 289 → 177 transition of dihydroxythalidomide. (A) P450 2J2, (B) P450 2C18, (C) P450 4A11, and (D) MS/MS spectrum of dihydroxythalidomide ($[M - H]^-$ 289). 5-Hydroxythalidomide (100 μM) was incubated with the human P450 (1.0 μM) in the presence or absence of an NADPH-generating system, as indicated, for 1 h.

were obtained from BD Biosciences (San Jose, CA). Pooled human liver microsomes were prepared in 10 mM Tris-HCl buffer (pH 7.4) containing 0.10 mM EDTA and 20% (v/v) glycerol, as described previously.[42] Recombinant human P450s, coexpressed with human NADPH-P450 reductase (EC 1.6.2.4) in *Escherichia coli* membranes, were prepared as described earlier.[22,43] In some cases, purified P450 proteins were reconstituted with purified (rat) recombinant NADPH-P450 reductase.[44] Microsomal protein concentrations were estimated using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). P450 4A11 was heterologously expressed and purified as described elsewhere.[45] Concentrations of total P450[46] and NADPH-P450 reductase[42] were estimated spectrally as described previously.

Hydroxylation of Thalidomide and Pomalidomide. Thalidomide and pomalidomide hydroxylation activities were determined using LC−MS and LC−MS/MS. Briefly, a typical incubation mixture (total volume of 200 μL) contained microsomal protein (1.0 mg mL^{-1}) or recombinant P450 (0.10 μM, in bacterial membranes or in Supersomes from insect cells) or reconstituted purified proteins (0.1−1 μM in P450), an NADPH-generating system (0.25 mM $NADP^+$, 2.5 mM glucose 6-phosphate, and 0.25 unit mL^{-1} of yeast glucose 6-phosphate dehydrogenase),[42] and thalidomide or pomalidomide (0.1−0.2 mM) in 0.10 M potassium phosphate buffer (pH 7.4), unless otherwise specified. For P450 activity determinations, incubations were carried out at 37 °C for 30−60 min. Incubations were terminated by adding 0.20 mL of ice-cold CH_3CN or 10 μL of acetic acid. The samples were centrifuged at 2000*g* for 10 min, and the aqueous supernatant was analyzed using a LC−MS or LC−MS/MS systems, vide infra.

Oxidation of 5-Hydroxythalidomide by P450s 2J2, 2C18, and 4A11. A typical incubation mixture (total volume of 200 μL) contained recombinant P450 2J2 or 2C18 (1.0 μM) in Supersomes or purified P450 4A11 protein (0.2 μM) in a reconstituted system, an NADPH-generating system (0.25 mM $NADP^+$, 2.5 mM glucose 6-phosphate, and 0.25 unit mL^{-1} of yeast glucose 6-phosphate dehydrogenase),[42] and (*R*)- or (*S*)-5-hydroxythalidomide (0.1−0.2 mM) in 100 mM potassium phosphate buffer (pH 7.4). When indicated, GSH was added at 5 mM. Incubations were carried out at 37 °C for 60 min and terminated by adding 10 μL of glacial CH_3CO_2H. The solution was centrifuged at 2000*g* for 10 min, and the supernatant was analyzed using LC−MS/MS, vide infra.

LC−MS/MS Assays. LC−MS and LC−MS/MS analyses of the oxidation products were performed on a Waters Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA) connected to a Thermo LTQ mass spectrometer (Thermo Fisher, Waltham, MA) using an Aquity UPLC BEH octadecylsilane (C18) column (2.1 mm × 50 mm). LC conditions were as follows: buffer A contained 10 mM $NH_4CH_3CO_2$ (pH 4.0) in 5% CH_3CN and 95% H_2O (v/v), and buffer B contained 10 mM $NH_4CH_3CO_2$ (pH 4.0) in 95% CH_3CN and 5% H_2O (v/v). The following gradient program was used with a flow rate of 0.3 mL min^{-1}: 0−5 min, linear gradient from 100% A to 75% A (v/v); 5−5.5 min, linear gradient to 100% B; 5.5−7.5 min, hold at 100% B; 7.5−8 min, linear gradient to 100% A; 8−10 min, hold at 100% A. The temperature of the column was maintained at 40 °C. Samples (20 μL) were infused with an autosampler. MS analyses were performed in the electrospray ionization (ESI) negative ion mode. The mass spectrometer was tuned using 5-hydroxythalidomide for thalidomide oxidation products or with pomalidomide for pomalidomide oxidation products. Product ion spectra were acquired over the range m/z 100−500 using an Aquity UPLC BEH octadecylsilane (C18) column (2.1 mm × 50 mm). For initial characterization of the oxidation products, a Thermo LTQ mass spectrometer was used; for kinetic analysis, a Thermo Ultra Quantum mass spectrometer (Thermo Fisher, Waltham, MA) was used. Both instruments were connected to a Waters Acquity UPLC system (Waters, Milford, MA) and were operated in the negative ESI mode. Hydroxypomalidomide was quantified using the m/z 288 → 176 transition compared with the m/z 273 → 161 transition of 5-hydroxythalidomide because of the lack of an authentic standard. 5-Hydroxythalidomide was also used as an internal standard.

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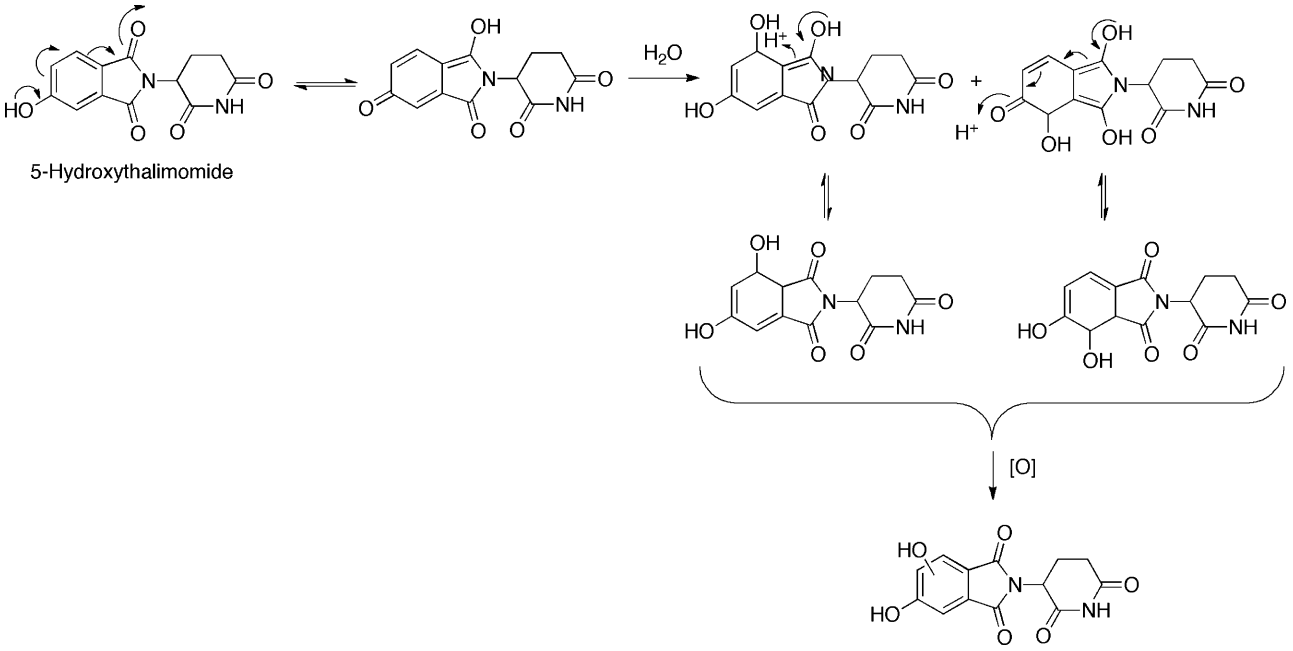
Scheme 2. Proposed Mechanism for Nonenzymatic Oxidation of 5-Hydroxythalidomide



LC–MS and LC–MS/MS analyses of the GSH conjugates of hydroxythalidomide and dihydroxythalidomide were performed on a Waters Acquity UPLC system connected to a Thermo LTQ mass spectrometer using an Acquity UPLC BEH octadecylsilane (C18) column (2.1 mm × 50 mm). LC conditions were as follows: buffer A contained 2% CH_3CN in H_2O (v/v), and buffer B contained 95% CH_3CN (v/v), with each containing 0.1% HCO_2H. For the UPLC column, the following gradient program was used with a flow rate of 0.3 mL min^{-1}: 0–5 min, linear gradient from 100% A to 75% A (v/v); 5–5.5 min, linear gradient to 100% B; 5.5–7.5 min, hold at 100% B; 7.5–8 min, linear gradient to 100% A; 8–10 min, hold at 100% A. The temperature of the column was maintained at 40 °C. Samples (10–20 μL) were infused with an autosampler. For GSH adduct detection the MS analyses were performed in the positive ion mode, and the mass spectrometer was tuned using GSH.

LC–high-resolution mass spectrometry (HRMS) was performed on a Waters Acquity UPLC system connected with a Waters Synapt hybrid quadropole/OA-TOF mass spectrometer equipped with a dual chemical ionization/ESI source. LC conditions were the same as mentioned in the previous section. MS analyses were performed in the positive ion mode for GSH adducts and negative ion mode for hydroxypomalidomide. ESI conditions were as follows: capillary voltage, 2.59 V; sampling cone, 30; extraction cone, 4.1; source temp, 125 °C; desolvation temperature, 325 °C; and Trap CE parameter, 6. Ion spectra were acquired over the range m/z 50–500 using the waters MassLynx V4.1 software.

Kinetic Analysis. Kinetic analysis was done using nonlinear regression analysis (Prism, GraphPad Software, La Jolla, CA). The Michaelis constant K_m and k_{cat} values were determined using hyperbolic substrate concentration-dependent velocity curves.

RESULTS

Biotransformation of Thalidomide and 5-Hydroxythalidomide by P450s 2J2, 2C18, and 4A11. In vitro metabolism studies of thalidomide with P450s 2J2, 2C18, and 4A11 revealed that none of these enzymes are capable of metabolizing thalidomide (e.g., Supporting Information Figure S1). However, when (*R*)-5-hydroxythalidomide was used as a substrate, all three of these P450 enzymes were able to metabolize it to a dihydroxythalidomide product, as detected by LC–MS and LC–MS/MS analysis (Figure 1). LC–MS analysis in the negative ion mode revealed formation of a peak at t_R 3.2–3.8 min with m/z 289, 16 amu higher than (*R*)-5-hydroxythalidomide. Collision-induced dissociation (CID) of the parent ion (m/z 289) yielded fragment ions with m/z 177, 218, and 246, which are consistent with the presence of a dihydroxythalidomide product with the two hydroxyl groups being present on the phenyl ring of phthalimide moiety of thalidomide (Figure 1D). Surprisingly, in the control reactions where an NADPH-generating system was not added, a small but detectable and reproducible peak with a similar t_R and mass and fragmentation pattern to dihydroxythalidomide was observed. This trace reaction may be due to nonenzymatic addition of H_2O to a tautomer of (*R*)-5-hydroxythalidomide followed by oxidation to the dihydroxythalidomide product (Scheme 2). The formation of the dihydroxythalidomide metabolite was also observed for the (S) enantiomer (data not shown).

GSH Adduct Formation from 5-Hydroxythalidomide Oxidation Products. Oxidation of (*R*)-5-hydroxythalidomide by P450 2J2 in the presence of GSH (5 mM) resulted in the formation of 5-hydroxythalidomide-GSH adducts, similar to the case with P450 3A4.[22] LC–MS and LC–MS/MS analysis (positive ion mode) led to the detection of a peak at t_R ~3.6 min with m/z 580, which is consistent with the presence of a GSH conjugate of 5-hydroxythalidomide (Figure 2A). Using LC–HRMS, the mass of the $[M + H]^+$ ion of the t_R ~3.6 min peak was found to be 580.1344 (calculated $[M + H]^+$ 580.1344). CID of the peak at t_R ~3.6 min in the positive ion mode yielded fragment ions with m/z 505 (loss of 75) and 451 (loss of 129), typical of GSH conjugates (Figure 2B).[47] Together, these results clearly indicate that oxidation of (*R*)-5-hydroxythalidomide by P450 2J2 generates a reactive intermediate that can be trapped by GSH to form a 5-hydroxythalidomide–GSH conjugate. GSH conjugates were also detected for the (S) enantiomer (data not shown).

Oxidation of Dihydroxythalidomide to a Quinone Intermediate. Compounds containing the *ortho*- and *para*-hydroxyphenol functionality (i.e., catechols and hydroquinones,

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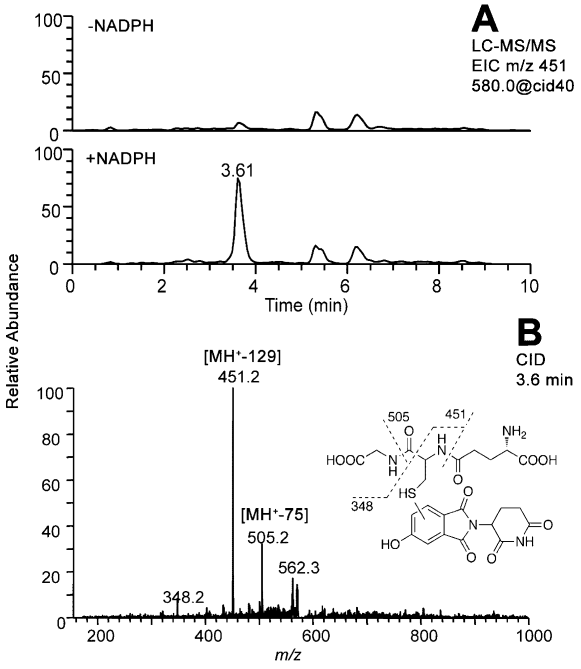

Figure 2. LC−MS/MS chromatograms and CID spectra showing the presence of the GSH conjugate of 5-hydroxythalidomide formed in the presence of recombinant human P450 2J2. Extracted ion chromatogram of the product ion using the m/z 580 → 451 transition of 5-hydroxythalidomide−GSH conjugate. (A) P450 2J2 in the presence and absence of NADPH. (B) MS/MS spectrum of GSH conjugate of 5-hydroxythalidomide ($[M + H]^+$ 580). 5-Hydroxythalidomide (100 μM) was incubated with human P450 2J2 (1.0 μM) and GSH (5 mM) in the presence or absence of an NADPH-generating system, as indicated, for 1 h.

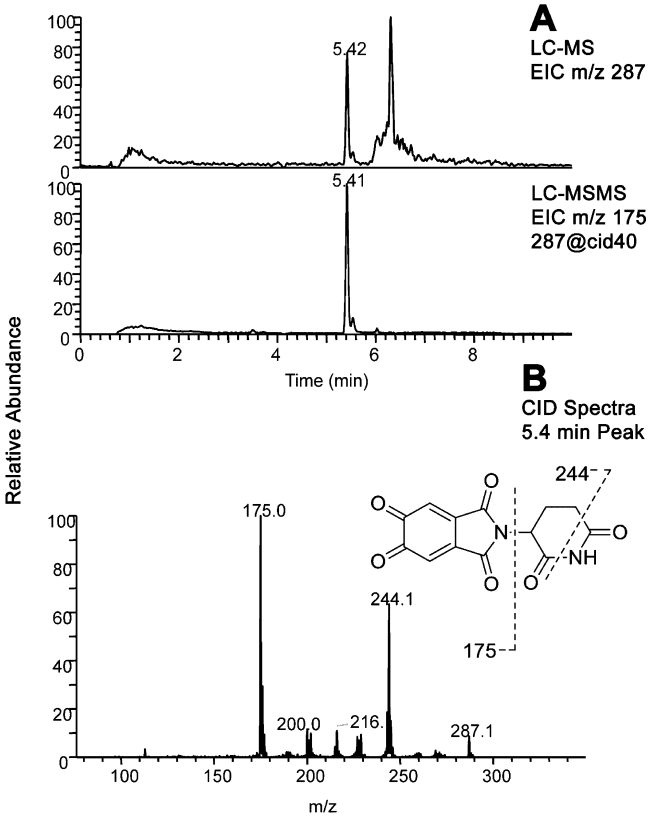

Figure 3. LC−MS/MS chromatograms and CID spectrum showing the oxidation of dihydroxythalidomide to the quinone product by P450 3A4. (A) Extracted ion chromatogram of the quinone metabolite using the molecular ion m/z 287 (top chromatogram) and the characteristic product ion m/z 175 (bottom chromatogram). (B) MS/MS spectrum of the quinone metabolite ($[M - H]^-$ 287) showing the characteristic m/z 175 and 244 fragment ions.

respectively) are known to undergo nonenzymatic or P450-catalyzed oxidation to potentially toxic quinone intermediates. The hypothesis that dihydroxythalidomide may be oxidized to quinones led us to look for possible thalidomide quinone metabolites in the incubation mixtures. Incubations of (*R*)-5-hydroxythalidomide with NADPH-fortified P450 3A4 *E. coli* membranes for 3 h followed by LC−MS analysis led to the detection of a major peak at t_R ∼5.4 min and a minor peak at t_R ∼5.5 min in the LC−MS chromatogram (m/z 287 in the negative ion mode) (Figure 3). LC−MS/MS analysis of the t_R ∼5.4 min peak in the negative ion mode gave fragment ions with m/z 244 and 175. These results are consistent with the presence of quinone metabolites of thalidomide. The quinone intermediates were also detected for the (S) enantiomer (data not shown).

Trapping of the Quinone Intermediate of Thalidomide with GSH. *Ortho*- and *para*-quinones are known to react with electrophiles to form various adducts including GSH and protein adducts.[48] To obtain further evidence for the presence of the quinone metabolites, an experiment was performed to detect possible GSH adducts of dihydroxythalidomide. (*R*)-5-Hydroxythalidomide was incubated with NADPH-fortified P450 3A4 *E. coli* membranes or P450 2J2 Supersomes in the presence of GSH for 3 h. LC−MS analysis of the reaction mixture in the positive ion mode revealed the presence of peaks at t_R ∼2.4 for P450 3A4 and t_R ∼2.4 and 3 min for P450 2J2 with m/z 596 (Figure 4). CID of the peaks at t_R ∼2.4 for P450 3A4 and t_R ∼ 3 min for P450 2J2 (positive ion mode) yielded fragment ions with m/z 521 (loss of 75) and 467 (loss of 129), typical of GSH conjugates.[47] On the basis of the mass and fragmentation data, it can be concluded that the t_R 2.4 and 3.0 min peaks correspond to dihydroxythalidomide−GSH conjugate(s). The enzyme tyrosinase oxidizes catechols to quinones, and its addition to the reaction mixture increased the yield of the GSH adducts (Figure S2, Supporting Information). GSH conjugates of the dihydroxythalidomide were also detected for the (S) enantiomer (data not shown).

Pomalidomide Hydroxylation Catalyzed by Human Liver Microsomes and Recombinant P450s 2C19, 2J2, and 3A4. Incubation of racemic pomalidomide with NADPH-fortified microsomes, purified P450 2C19, P450 3A4 bicistronic *E. coli* membranes, or P450 2J2 Supersomes at 37 °C for 1 h followed by LC−MS analysis gave a peak at t_R ∼3.1 min with m/z 288 (negative ion mode) (Figure 5 and Supporting Information Figure S3). CID of the m/z 288 ion (negative ion mode) gave characteristic fragment ions at m/z 176 and 245. On the basis of the fragmentation of dihydroxythalidomide, this compound corresponds to hydroxypomalidomde, with hydroxylation taking place either in the amino-phthilidimide ring or on the amino group (Scheme 3). To distinguish further between these two structural possibilities, the products were treated with a bathochromic reagent that forms a color in the presence of hydroxylamines, detected by its visible absorbance spectroscopy (ε_{595} 32 900 M^{-1} cm^{-1}).[49−51] In the case of pomalidomide, we were unable to detect absorbance above background. Together, these results suggest that pomalidomide is oxidized by P450

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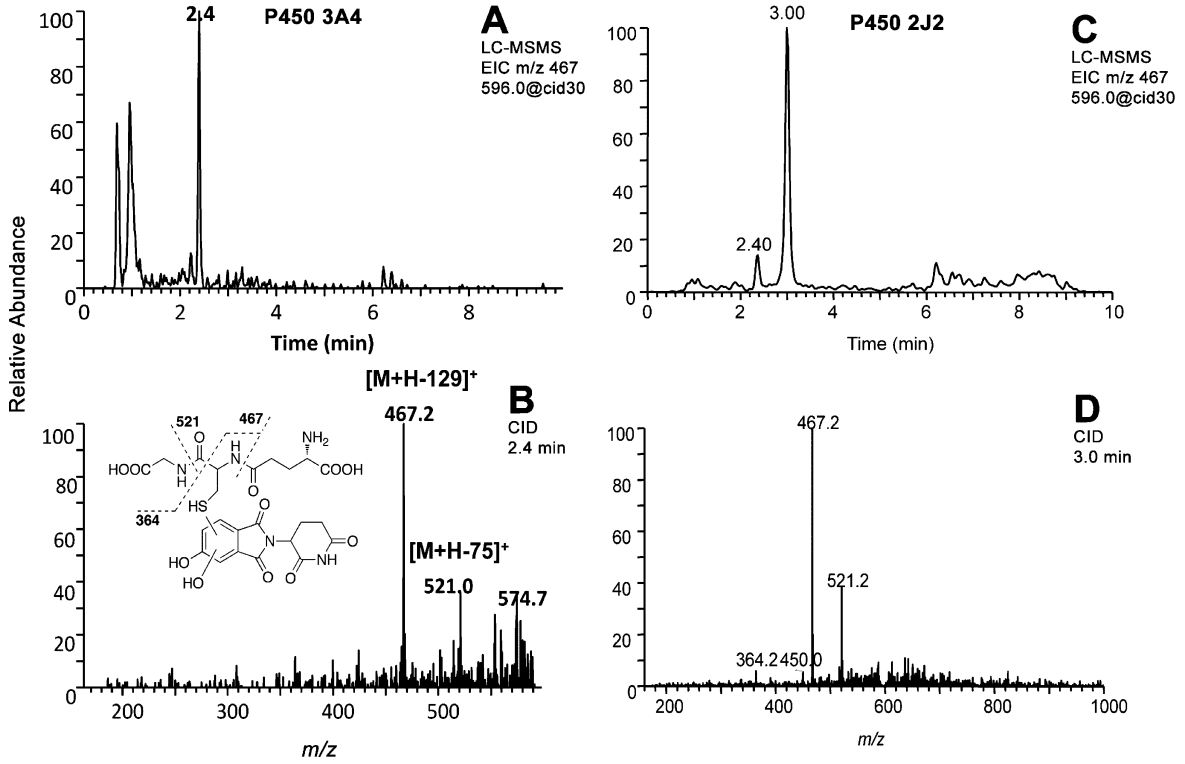

Figure 4. LC−MS/MS chromatograms and CID spectrum showing the presence of the GSH conjugates of 5-hydroxythalidomide formed in the presence of GSH. Extracted ion chromatogram of the product ion using the m/z 596 → 467 transition of the dihydroxythalidomide−GSH conjugate. (A) P450 3A4, (B) MS/MS spectrum of the dihydroxythalidomide−GSH conjugate ($[M + H]^+$ 596) eluting at t_R 2.4 min, (C) P450 2J2, and (D) MS/MS spectrum of the dihydroxythalidomide−GSH conjugate ($[M + H]^+$ 596) eluting at t_R 3.0 min. 5-Hydroxythalidomide (200 μM) was incubated with P450 3A4 bicistronic membranes (1.0 μM P450) or P450 2J2 Supersomes (1.0 μM P450) in the presence of GSH (5 mM) and an NADPH-generating system for 3 h.

2C19, 3A4, and 2J2 and human liver microsomes to the ring-hydroxylated metabolite.

Kinetic Analysis of Pomalidomide Hydroxylation Catalyzed by Recombinant P450 2C19, 2J2, and 3A4 Enzymes. Kinetic analyses of P450-mediated pomalidomide hydroxylation were conducted with recombinant human P450s 2C19, 3A4, and 2J2 using LC−MS/MS in the single reaction monitoring (SRM) mode. The m/z 288 → 176 transition was utilized for quantitation of the product formed. Because of the lack of authentic standards, 5-hydroxythalidomide was used as an internal standard and for quantitation. The apparent k_{cat} values for P450-mediated hydroxylation of racemic pomalidomide were found to be 0.5, 0.05, and 0.02 pmol/min/pmol P450 for P450s 2C19, 3A4, and 2J2, respectively (Table 1).

DISCUSSION

Renewed interest in thalidomide has followed its recent approval for the treatment of leprosy and multiple myeloma.[2,3] In addition, the potential use of thalidomide for other clinical conditions is being currently explored. However, the clinical use of thalidomide and other immunomodulatory drugs in this class has been tightly regulated because of their teratogenicity. The mechanism of teratogenicity of thalidomide is yet to be conclusively established. Various hypotheses have been proposed, including oxidative stress, generation of reactive intermediates, inhibition of angiogenesis, and inhibition of the protein cereblon.[13,14,17,52−54]

The involvement of reactive products has been entertained previously on the basis of indirect evidence and even thought to be the cause for the antiangiogenic effects of thalidomide. Using an in vitro activation/toxicity assay with purified epoxide hydrolase and epoxide hydrolase inhibitor 1,1,1-trichloropropylene oxide, the presence of an epoxide intermediate was proposed (although direct evidence of action of epoxide hydrolase on an epoxide was not demonstrated).[15] We have recently provided direct evidence for the P450 3A4-catalyzed formation of a reactive intermediate by detecting the GSH-conjugate of 5-hydroxythalidomide both in vitro and in vivo.[22,23] However, it is unlikely that a reactive intermediate capable of reacting with GSH (without conjugation catalyzed by GSH transferase) will travel from its site of formation in the liver to a potential site of action, the limb bud, or the endothelial cells at the site of angiogenesis. A recently proposed hypothesis, inhibition of the protein cereblon, currently seems to be the most popular proposal.[17] Ito et al.[17] reported noncovalent binding of thalidomide to the protein cereblon, part of a protein complex postulated to be important in development. Cereblon is a component of the E3 ubiquitin ligase complex (along with DDB1 and Cu14) involved in the downregulation of fibroblast growth factor and possibly the teratogenic effects associated with thalidomide.[54] Most of the experiments in the study by Ito et al.[17] were done with 300−400 μM concentrations of thalidomide, which is inconsistent with the fact that a single dose of thalidomide during a time-sensitive window is enough to cause teratogenicity.[17] However, cereblon expression has been reported to be required for the antimyeloma activity of lenalidomide and pomalidomide.[54]

Although the mechanism of teratogenicity of thalidomide remains to be conclusively established, considerable evidence suggests that metabolism of thalidomide is necessary for

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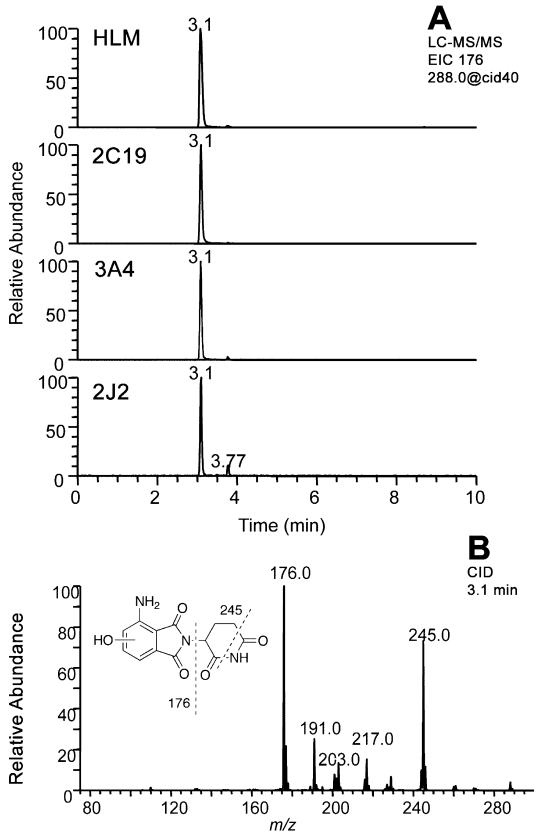

Figure 5. LC–MS/MS chromatograms and CID spectrum showing the oxidation of pomalidomide to the hydroxypomalidomide metabolite by human liver microsomes and P450s 2C19, 2J2, and 3A4. (A) Extracted ion chromatogram of the hydroxypomalidomide metabolite using the characteristic m/z 288 → 176 transition. (B) MS/MS spectrum of the hydroxypomalidomide product ($[M - H]^-$ 288) showing the characteristic m/z 176 and 245 fragment ions.

Scheme 3. Proposed Pathway of Metabolism of Pomalidomide

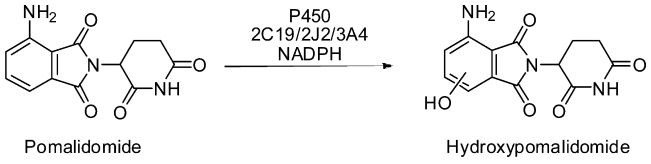

Table 1. Kinetic Parameters for Aminophthalidimide Ring Hydroxylation of Pomalidomide by P450s

P450	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})
2C19	0.52 ± 0.062	0.82 ± 0.17	0.63
3A4	0.05 ± 0.004	0.26 ± 0.06	0.19
2J2	0.02 ± 0.001	0.14 ± 0.03	0.14

teratogenicity.[18,19] However, previous metabolic studies of thalidomide did not provide any evidence to support this theory. In our effort to understand the metabolism of thalidomide and its possible association to teratogenicity and/or biological activity, we found that human P450s 2J2, 2C18, and 4A11 can oxidize 5-hydroxythalidomide to a corresponding and potentially toxic dihydroxythalidomide metabolite. Interestingly, none of these P450s metabolize thalidomide itself (e.g., Supporting Information Figure S3). Using a GSH-trapping experiment, we also found that the P450 2J2-catalyzed oxidation of 5-hydroxythalidomide involves the formation of a reactive intermediate that can be trapped with GSH to give a GSH–5-hydroxythalidomide conjugate. This observation is consistent with the results obtained with P450 3A4.[22] However, unlike P450 3A4, P450 2J2 does not oxidize thalidomide (Supporting Information Figure S3). Therefore, the presence of thalidomide should not interfere with the formation of the toxic metabolite or the reactive product by P450 2J2, 2C18, or 4A11.

Ortho- and *para*-hydroxyphenolic compounds undergo nonenzymatic or enzyme-catalyzed oxidation to the corresponding quinones, which can redox cycle to produce oxidative stress.[48] Because various P450s (including human P450s 3A4, 3A5, 2C19, 2J2, 2C18, and 4A11) can produce dihydroxythalidomide, we examined incubation mixtures for the presence of quinones using LC–MS/MS analysis. The presence of quinone products was further confirmed by detection of the GSH conjugate of dihydroxythalidomide. (We have been unsuccessful in establishing the sites of oxidation (in the catechol/quinones) because of the low yield and the need for NMR spectroscopy.) This thalidomide quinone metabolite resulting from the oxidation of the major metabolite, dihydroxythalidomide, may be the species responsible for the oxidative stress caused by thalidomide, as reported by Parman et al.[14] One issue is whether enough of this could be generated to have a large impact in oxidative stress in vivo.

P450 2J2 is an enzyme found mainly in human extrahepatic tissues, with predominant expression in the endothelial cells of the cardiovascular systems and in lower levels in the intestine, kidney, lung, pancreas, brain, and liver.[29,35,36] P450 2J2 has also been shown to be expressed in various human tumor cells and fetal tissue.[39,55] Because angiogenesis involves generation of endothelial cells to form blood vessels, this preferential expression and P450 2J2-catalyzed formation of reactive intermediates may be involved in the antiangiogenic and possibly teratogenic effect of thalidomide. A primary site of expression of P450 2C18 is the skin,[30,31] which might be related to the clinical outcome of the treatment of the skin lesions associated with leprosy. In addition, P450 2C18 has been shown to metabolize phenytoin to a quinone intermediate, which is believed to be responsible for its teratogenic effect.[34] Although additional direct evidence is required to draw a conclusion regarding the involvement of reactive products in both the clinical and teratogenic effects of thalidomide, the evidence presented here is consistent with this possibility.

Pomalidomide, a recently approved analogue of thalidomide, was also found to be metabolized by P450s 2C19, 2J2, and 3A4 to the corresponding hydroxy product (we were unable to determine rigorously the structure of the metabolite because of the lack of an authentic standard and (for NMR) the low yields). We were not able to detect any quinoneimine intermediate or GSH conjugate in our in vitro assays (data not shown). In a recently published human study,[26] pomalidomide was found to be metabolized primarily to two hydroxy metabolites and the glucuronide conjugate of the 5-hydroxy metabolite. With recombinant P450s 1A2, 2C19, 2D6, and 3A4, only the 5-hydroxypomalidomide metabolite was formed in very low amounts,[26] and the oxidation rates were significantly lower than in our work (Table 1) (the values reported are semiquantitative because of the lack of authentic standards and represent the relative efficacy of oxidation of pomalidimide by the various P450s). In accord with our observations, no GSH conjugate was detected in the human studies, although the hydroxy metabolites were formed in

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significant amounts. These results are consistent with our results on the metabolism of pomalidomide (Table 1) and support our hypothesis that extrahepatic P450s may be involved in the species-specific teratogenicity of thalidomide and its analogues. Pomalidomide has been shown to be nonteratogenic in chicken and zebrafish, and lenalidomide was found to be nonteratogenic in rabbits.[56,57] These findings are in contrast to thalidomide, which is teratogenic in all of these species. This species-specific teratogenicity of thalidomide and its analogues clearly contradicts the cereblon theory[17] because cereblon is ubiquitously expressed. The species-specific teratogeneicity does, however, support the metabolism and reactive intermediate theory.[13,15] The fact that pomalidomide is not teratogenic in thalidomide-sensitive species is consistent with our observation that no GSH adduct or reactive intermediate is formed during pomalidomide metabolism.

In conclusion, we have shown that 5-hydroxythalidomide is metabolized by P450s 2J2, 2C18, and 4A11 to dihydroxythalidomide. Metabolism by P450 2J2 involves formation of a reactive intermediate that may be responsible for the antiangiogenic and teratogenic properties of thalidomide. The major metabolite dihydroxythalidomide can be further oxidized to the quinone metabolite, which may be the species responsible for oxidative stress or protein binding, either of which could be a cause for teratogenicity. Although the data presented here provide evidence for the presence of reactive species that may be responsible for imparting toxicity and oxidative stress, a comprehensive metabolic study across species and tissues is required for a better understanding of the species-specific teratogenicity of thalidomide and its analogues.

■ ASSOCIATED CONTENT

Supporting Information

LC−MS/MS chromatograms showing lack of formation of hydroxythalidomide in the presence of P450 2J2 from (*R*)- or (*S*)-thalidomide, LC−MS/MS chromatogram and CID spectrum showing enhanced formation of the GSH conjugate of dihydroxythalidomide in the presence of P450 3A4 and tyrosinase, and LC−HRMS chromatogram and spectrum showing the presence and exact mass of hydroxypomalidomide formed in the presence of human liver microsomes. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

CID, collision-induced dissociation; ESI, electrospray ionization; HRMS, high-resolution mass spectrometry; UPLC, ultraperformance liquid chromatography

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