Mechanism-Based Inactivation of P450 2A6 by Furanocoumarins†

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ABSTRACT: Several furanocoumarins were tested for their ability to inhibit human P450 2A6 activity. The metabolites and conjugates formed from these furanocoumarins after incubation with reconstituted purified P450 2A6 in the absence and presence of exogenous nucleophiles were characterized by UV and LC/ESI-MS/MS analysis. The results suggest initial oxidation to form a furanoepoxide followed by hydrolytic attack, or attack of exogenous nucleophiles, to form dihydrofuranocoumarin products. Initial epoxidation is confirmed by the finding that a single ^{18}O atom is incorporated into the 8-methoxypsoralen (8-MOP) and psoralen (P) dihydrodiol metabolites when the incubations are performed in the presence of $H_2{}^{18}O$. In contrast, 19% of the dihydrodiol formed from 5-methoxypsoralen (5-MOP) involves incorporation of two ^{18}O atoms, implicating a γ-ketoenal intermediate in the formation of this metabolite. Thus, the structure of the reactive intermediate(s) formed is dictated by the intrinsic electronic properties of the parent compound. After exposure to [^{14}C]-8-MOP and [^{14}C]-5-MOP, SDS−PAGE and HPLC experiments, followed by radiometric detection, indicated that both P450 2A6 and P450 reductase were covalently modified in the purified system. In contrast, only P450 2A6 was covalently modified in a lymphablastoid cell line (GENTEST). With the purified system, partition ratios were higher (1.5−3.9X), and the ability to scavenge reactive intermediates with exogenous nucleophiles was greater. These results suggest that relative to the cell system, more reactive intermediates can escape, rather than bind to, the active site of purified reconstituted P450 2A6.

In vivo, cytochrome P450 (P450)[1] mediated oxidation of xenobiotics to more polar species is often the first step in the elimination of these compounds. In some cases, however, P450 catalysis can generate reactive species that covalently bind to and inactivate the P450 responsible for their formation as well as bind to other cellular proteins that may culminate in cell death. For example, P450 2A6 has been implicated in the oxidation of a number of compounds associated with toxicity and cell death including quinoline (*1*), 4,4′-methylenebis(2-chloroaniline) (*2*), aflatoxin B_1 (*3*), and various *N*-nitroso compounds present in cigarette smoke (*4*). Although these compounds were oxidized to reactive intermediates capable of binding to protein, the inactivation of P450 2A6 itself was not established. Mechanism-based inactivation of P450 is thought to occur by three different pathways (*5*): (a) binding of the inactivator to the apoprotein, (b) binding of the inactivator to the heme, and (c) inactivator-induced fragmentation of the heme to products which bind to the apoprotein.

8-Methoxypsoralen (8-MOP), a natural product present in many foodstuffs such as parsley, parsnips, figs, and celery (*6*), is used in the treatment of psoriasis, vitiligo, and T cell lymphoma in combination with long-wavelength UV light (*7, 8*). We and others have shown that 8-MOP is a potent mechanism-based inactivator (MBI) of P450 2A6 activity (*9, 10*) that selectively inactivates this enzyme in human liver microsomes (*9*) when used in low concentrations and for short exposure times. Since the concentration required to inactivate >90% of microsomal P450 2A6 activity (2.5 μM) is within the range of the plasma concentration of 8-MOP observed in vivo (*11*), administration of this drug could lead to loss of P450 2A6 activity in vivo, which could result in unexpected deleterious therapeutic consequences. Although 8-MOP-mediated inactivation of P450s, as well as the corresponding in vivo and in vitro metabolic profiles, has been reported for several mammalian species (*12*−*14*), including man (*15, 16*), the structure of the reactive intermediate is unknown. Thus, one goal of this investigation was to identify the reactive species generated from 8-MOP that is responsible for P450 2A6 inactivation.

To help elucidate the mechanism of 8-MOP-mediated P450 2A6 inactivation, several other furanocoumarins (Figure 1), selected on the basis of their potential as probes of different aspects of the mechanism of inactivation, were tested for their ability to inhibit P450 2A6 activity in human

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[1] Abbreviations: ACN, acetonitrile; CID, collisionally induced dissociation; cytochrome b_5, human cytochrome b_5; DH-8-MOP, 4′,5′-dihydro-8-methoxypsoralen; DLPC, L-α-dilauroylphosphatidylcholine; EtOH, ethanol; 8-OH-P, 8-hydroxypsoralen; 8-MOP, 8-methoxypsoralen; 5,8-diOH-P, 5,8-dihydroxypsoralen; 5-OH-P, 5-hydroxypsoralen; 5-MOP, 5-methoxypsoralen; GSH, reduced glutathione; OH-DH-8-MOP, hydroxylated 4′,5′-dihydro-8-methoxypsoralen; LC/ESI-MS/MS, liquid chromatography electrospray ionization tandem mass spectrometry; MBI, mechanism-based inactivator; MeOH, methanol; MOA, methoxylamine; NAC, *N*-acetylcytsteine; P, psoralen; P450, cytochrome P450; P450 reductase, NADPH−cytochrome P450 rat reductase; SOD, superoxide dismutase; TLC, thin-layer chromatography; TOX, trioxsalen.

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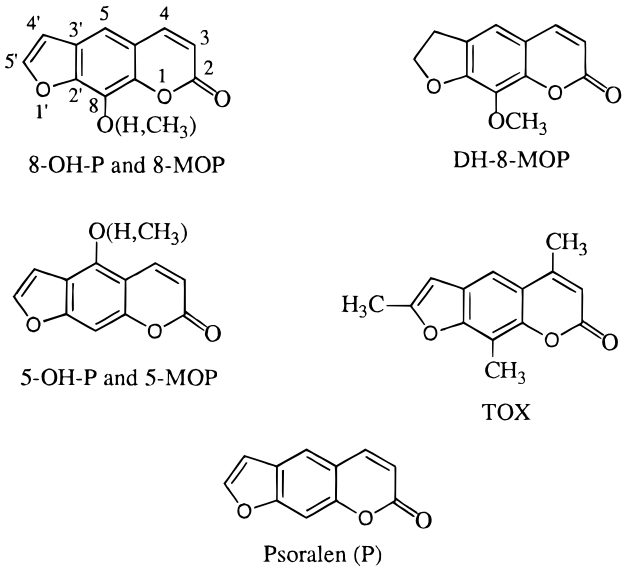

FIGURE 1: Structures of the furanocoumarins used in this investigation.

liver microsomes. The metabolites and conjugates of these furanocoumarins, formed in the absence and presence of various exogenous nucleophiles, were characterized using purified P450 2A6, and UV and LC/ESI-MS/MS analysis. The nature of inactivation was further characterized by analysis of covalent binding to P450, in the absence and presence of exogenous nucleophiles, using reconstituted purified or GENTEST P450 2A6.

MATERIALS AND METHODS

Materials

Chemicals. 7-Hydroxycoumarin, 8-MOP, 5-MOP, P, L-α-dilauroylphosphatidylcholine (DLPC), catalase, glutathione (GSH), deferoxamine mesylate, *N*-acetylcysteine (NAC), superoxide dismutase (SOD), $NADP^+$, and NADPH were purchased from Sigma (St. Louis, MO). 5-Hydroxypsoralen (5-OH-P) was purchased from Indofine (Somerville, NJ). Sodium cyanide, methoxylamine hydrochloride (MOA), benzofuran, dihydrobenzofuran, dihydrocoumarin, and semicarbazide hydrochloride were purchased from Aldrich (Milwaukee, WI). Glucose 6-phosphate and glucose-6-phosphate dehydrogenase (yeast, grade II) were purchased from Boehringer-Mannheim (Indianapolis, IN), and coumarin was from Merck (Rahway, NJ). Slide-A-Lyzers (molecular mass cutoff = 10 kDa) were from Pierce (Rockford, IL), and Centricon 30 concentrators were from Amicon (Bedford, MA). Cytoscint ES liquid scintillation cocktail, $^{14}CH_3I$ (50 Ci/mol), and hyamine hydroxide were from ICN (Costa Mesa, CA). Flo Scint II liquid scintillation cocktail was from Packard (Downers Grove, IL). $H_2{}^{18}O$ was from Cambridge (Andover, MA). HPLC solvents were of the highest grade commercially available and were used as received.

Enzymes. Human liver samples were from the NIH-supported human liver bank at the University of Washington, and microsomes from these liver samples were prepared as previously described (*17*). The baculovirus-mediated expression and characterization of P450 2A6 used in this study will be reported elsewhere (*18*), and the purification of P450 2A6 from the crude insect cell paste was accomplished according to published procedures for the purification of P450 2C9 (*19*), with minor modifications. cDNA-expressed P4502A6 in lymphoblastoid cells was obtained from GENTEST Corp. (Woburn, MA). Recombinant rat NADPH−cytochrome P450 oxidoreductase was purified according to published procedures (*20*), with minor modifications. Human cytochrome b_5 was expressed and purified from bacterial cultures according to previously published procedures (*21*). Experimental data are presented as the average of duplicate determinations which did not vary by greater than 10% with the exception of the gel slicing experiments which, because of the extensive sample handling involved, varied up to 15%. Each experiment was repeated at least twice, and consistent results were obtained.

Synthetic Procedures. (A) 8-Hydroxypsoralen (8-OH-P). The demethylation of 8-MOP was carried out according to a procedure previously described (*22*). An ice-cold solution of 2 mmol of boron tribromide (1 M in CH_2Cl_2) was added to a stirring CH_2Cl_2 solution of 1.7 mmol of 8-MOP in a round-bottomed flask fitted with a septum. This solution was allowed to come to room temperature and stirred overnight. Water, 10 mL, was added, the mixture was extracted 3 times with an equal volume of ether, and the extracts were combined and dried over anhydrous sodium sulfate. After ether evaporation under reduced pressure, preparative TLC (CH_2Cl_2) was used to purify 8-OH-P. Recrystallization from ethanol afforded the pure [>99% by TLC on silical gel and HPLC−UV (Hewlett-Packard 1050, Avondale, PA)] product in a 75% yield: mp 248−249 °C; ESI-MS 203.2 $[M+H]^+$ (Fisons Instrument VG Quattro II triple quadrupole mass spectrometer interfaced to a Shimadzu LC10AD solvent delivery module and fitted with a Megaflow Electrospray source); ^1H NMR (Varian XL300, Palo Alto, CA) (Me_2SO-d_6) δ 8.13 (d, 1H), 8.10 (d, 1H), 7.45 (s, 1H), 7.04 (d, 1H), 6.40 (d, 1H), 3.35 (br, 1H).

(B) 4′,5′-Dihydro-8-methoxypsoralen (DH-8-MOP). 8-MOP, 1 mmol, dissolved in 10 mL of boiling ethanol to which had been added 5 mL of glacial acetic acid and 10 mg of 30% Pd/C, was hydrogenated on a Paar hydrogenator at 40 psi for 3 h. TLC of the reaction mixture indicated complete conversion to a highly fluorescent product. Filtration through Celite and extensive washing with boiling ethanol were followed by evaporation under reduced pressure. Preparative TLC (10:0.25 CH_2Cl_2/EtOAc) of the residue was used for initial purification of DH-8-MOP. Recrystallization from ethanol afforded the pure (>99% by TLC on silical gel and HPLC−UV) product in 90% yield: mp 137−138 °C; ESI-MS 219.2 $[M+H]^+$; ^1H NMR ($CDCl_3$) δ 7.60 (d, 1H), 6.99 (s, 1H), 6.23 (d, 1H), 4.73 (t, 2H), 4.05 (s, 3H), 3.28 (t, 2H).

(C) [^{14}C]-5-MOP and [^{14}C]-8-MOP. $^{14}CH_3I$ (0.5 mCi of 50 Ci/mol, neat) was dissolved in 2 mL of ice-cold acetone and added to a tube containing 4.5 mg of hydroxypsoralen and a few grains of K_2CO_3 and KI. The tube was tightly sealed, heated to 45 °C, and allowed to mix overnight. Preparative TLC on silical gel (10:1 CH_2Cl_2/EtOAc for 5-MOP and CH_2Cl_2 for 8-MOP) was used to purify the methoxypsoralens. Yields exceeding 95% were obtained by this methodology. HPLC−UV and HPLC-radiometric detection (Packard Radiomatic 150TR Flow Scintillation Analyzer, Downers Grove, IL) indicated that the radiolabeled

products, stored as EtOH solutions at −80 °C, were >99% pure.

Methods

Inactivation Assays. The procedure for the inactivation assays using HL109 has been previously described (*9*). Preliminary experiments with various furanocoumarin concentrations and exposure times were performed to determine appropriate values for obtaining reliable kinetic constants for the observed inhibition. All furanocoumarins were added to each incubation as methanolic solutions such that the final concentration of MeOH in the incubate was less than 1%. Different concentrations of each furanocoumarin were used in order to determine inactivation kinetics: 5-MOP (0−25 μM), DH-8-MOP (0−100 μM), P (0−4.2 μM), 5-OH-P (0−133.4 μM), 8-OH-P (0−50 μM), and trioxsalen (TOX) (0−100 μM). At various time points after exposure to the furanocoumarin, a small aliquot of the inactivation assay (5%) was transferred to a tube containing a saturating amount of coumarin (80K_m). Different exposure times were also used for each furanocoumarin: 5-MOP (0−8 min), DH-8-MOP (0−8 min), P (0−4 min), 5-OH-P (0−20 min), 8-OH-P (0−20 min), and TOX (0 and 14 min). Residual P450 2A6 activity was monitored as coumarin 7-hydroxylase activity, and K_I and k_{inact} values were determined as previously described (*9*). A fresh solution of furanocoumarin was prepared daily, and no degradation was observed for any of the compounds under the conditions of these experiments; however, considerable degradation does occur if solutions of these compounds are stored at room temperature for longer than a few days.

Competitive Inhibition Experiments. Human liver microsomes (5 pmol, HL109) were preincubated with coumarin (0.25−5 μM) and 8-OH-P (0−15 μM) or TOX (0−100 μM) for 3 min at 37 °C. The reaction was initiated with NADPH (final concentration, 1 mM) and allowed to proceed for 10 min (final incubation volume, 1 mL). P450 2A6 activity was monitored as coumarin 7-hydroxylase activity as previously described (*9*). K_i values were estimated by Dixon plot ($1/V$ vs inhibitor concentration) and then determined by nonlinear regression analysis of the data.

Reconstitution Procedures. The reconstituted purified P450 2A6 system consisted of P450 2A6, P450 reductase, cytochrome b_5, and DLPC in 25 mM potassium phosphate buffer (pH 7.4) in the relative amounts required for optimal coumarin 7-hydroxylase activity as previously described (*9*). P450, reductase, and lipid were reconstituted in that order and allowed to mix for 60 min on ice. Cytochrome b_5 was added, and this mixture was allowed to interact for an additional 15 min. Buffer, furanocoumarins (100 μM, unless noted otherwise), and the nucleophilic trapping agents (when indicated) were added, and each mixture was preincubated at 30 °C for 2 min. The reactions were initiated by addition of NADPH (1 mM, final concentration) and were terminated by the addition of $HClO_4$ (0.2 N, final concentration). GENTEST P450 2A6 was supplemented and reconstituted as described above with P450 reductase and cytochrome b_5 in a ratio of 2:1, respectively, relative to P450 2A6 content. GENTEST P450 2A6 content was determined using the method of Omura and Sato (*23*) and found to be 90% of the amount stated on the package. The coumarin 7-hydroxylase activity for the uninhibited reaction was 19.4 and 20.0 nmol (nmol of P450)$^{-1}$ min^{-1} in the reconstituted and GENTEST P450 2A6 systems, respectively.

Metabolite Analysis by LC/ESI-MS/MS. A Digital DECpcLPx 466d2 computer, running Fisons Mass Lynx 0.1 software, controlled the Quattro II. Instrument settings were as follows: source temperature, 100 °C; N_2 drying gas = 150 L/h; nebulizing gas = 20 L/h; probe voltage = 3.8 kV; cone voltage = 30−35 kV; probe position, on axis. Acquisition was carried out from m/z 50−500 and 100−1000 Da in 2.5 and 4.5 s, respectively, in the CENTROID scanning mode, with unit resolution up to at least 1500 Da based on calibration and resolution optimization using poly(ethylene glycol) (PEG) 300/600/1000/1500. MS/MS spectra were acquired from m/z 50−500 or 100−1000 Da in 2.5 s using the CONTINUUM scanning mode. Argon was employed as the target gas for collisionally induced dissociation (CID) studies. The LC interface to the mass spectrometer consisted of Shimadzu LC10AD solvent delivery modules and a Shimadzu SPD-10AV module UV−Vis spectrophotometric detector monitoring the absorbance at 320 nm (Shimadzu Scientific Instruments, Inc., Columbia, MD). The solvent system consisted of solvent A (2.5% CH_3CO_2H) and solvent B (100% ACN). The flow rate was 1 mL/min with 50 μL directed to the ion source using a splitter. It was necessary to use different HPLC gradients of 0−45% B over 30 min for each particular furanocoumarin after exposure to NADPH and exogenous nucleophiles (when present) to separate metabolites, conjugates, and parent compound.

UV Analysis. Purified P450 2A6 (200 pmol) was reconstituted as described above and preincubated with 8-MOP, 5-MOP, or P (100 μM) for 3 min at 30 °C. The reaction was initiated with NADPH (final concentration, 1 mM), allowed to proceed for 45 min (final incubation volume, 250 μL), and quenched with 50 μL of 1.2 N $HClO_4$. The major metabolite generated from each furanocoumarin was purified by HPLC and redissolved in 100% EtOH. A UV spectrum was collected (Hewlett-Packard 8451A Diode Array Spectrophotometer, Avondale, PA) for each metabolite as well as for 8-MOP, DH-8-MOP, 5-MOP, P, coumarin, dihydrocoumarin, benzofuran, and dihydrobenzofuran in 100% EtOH using 100% EtOH as a reference.

$H_2{}^{18}O$ Experiments. Purified P450 2A6 (50 pmol) was reconstituted as described with P, 5-MOP, or 8-MOP (100 μM) in a total volume of 100 μL that was 50% $H_2{}^{18}O$. The incubations were carried out at 30 °C for 45 min and terminated by the addition of 50 μL of 1 N $HClO_4$. A 50 μL aliquot was injected onto the LC/ESI-MS system described and analyzed by single ion monitoring: m/z = 250.1−260.1 for 5-MOP and 8-MOP dihydrodiols and m/z = 220.1−230.1 for the P dihydrodiol. The percent ^{18}O incorporated into each dihydrodiol product from $H_2{}^{18}O$ was calculated by correcting each dihydrodiol for the percent $H_2{}^{18}O$ present in the incubation and for theoretical isotopic abundances (^{18}O, $^{13}C+{}^{2}H_1$, etc.) occurring at a mass of M+2.

Partition Ratio Determinations and Calculations. One measure of the efficiency of an inhibitor as a MBI is the partition ratio. It is defined as the fraction of all metabolic events which the inhibitior is subjected to by the enzyme, divided by the number of inactivating events (*24*). For the furanocoumarins, partition ratios were calculated in two ways. The first was based on total enzyme depletion, and

was calculated as (total substrate consumed)/(P450 2A6 present in the incubation), while the second was based on major metabolite formation and calculated as (amount of major metabolite formed + P450 2A6 present in the incubation)/(P450 2A6 present in the incubation). If nucleophiles capable of scavenging enzyme-generated reactive intermediates are added to the incubations, partition ratio values can be affected. Under these conditions, corrected partition ratios were calculated as described above except that the P450 2A6 present in the incubation was calculated as [(P450 2A6 added to the incubation) × (P450 2A6 bound in the presence of the nucleophiles)]/(P450 bound in the absence of the nucleophiles). The percent P450 2A6 activity in the presence of the nucleophiles was calculated as (uncorrected partition ratio based on total substrate depletion in the presence of the nucleophile)/(partition ratio based on total substrate depletion in the absence of exogenous nucelophiles).

Psoralen and DH-8-MOP. P (10 nmol) and DH-8-MOP (100 nmol) were incubated with reconstituted purified and GENTEST P450 2A6 (25 pmol) for 45 min at 30 °C. The parent compound was separated from its metabolites using the HPLC−UV assay described above.

[^{14}C]-8-MOP and 5-MOP. [^{14}C]-8-MOP and [^{14}C]-5-MOP in EtOH were added to a plastic microfuge tube and then evaporated to dryness to give a final concentration of 50 μM furanocoumarin. Purified and GENTEST P450 2A6 (25 pmol) were reconstituted as described above in a final volume of 30 μL. NADPH (1 mM, final concentration) was added to initiate the reaction which was allowed to proceed for 45 min. A small aliquot of this mixture (5 μL) was added to 0.2 N $HClO_4$ (100 μL) to terminate the reaction and briefly centrifuged on a tabletop microfuge, and 50 μL was injected onto the HPLC. Metabolites, conjugates, and parent compound were quantified using a radiometric detector coupled with the HPLC using Flo Scint II liquid scintillation cocktail.

Covalent Binding of [^{14}C]-8-MOP and [^{14}C]-5-MOP to Protein Determined by SDS−PAGE. The remaining 25 μL of the partition ratio incubation mixture described above was dissolved in 10 μL of sample buffer, boiled for 3 min, and used for analysis of covalent binding by 9% SDS−PAGE. The destained gels were sliced into 5 mm sections that were extracted with 75% hyamine hydroxide for 3 h at 60 °C. Cytoscint ES liquid scintillation fluid (10 mL) was added, and the samples were allowed to sit in the dark for at least 24 h prior to analysis by a liquid scintillation counter (Packard 2200CA Tricarb liquid scintillation counter, Downers Grove, IL).

Covalent Binding of [^{14}C]-8-MOP to Protein Determined by HPLC. Purified P450 2A6 (20 pmol) was reconstituted with [^{14}C]-8-MOP as described above and exposed to buffer or NADPH (1 mM, final concentration). This solution was then dialyzed extensively against 4 × 500 mL of potassium phosphate buffer (50 mM, pH 7.4) or concentrated and washed at least 10 × with potassium phosphate buffer (50 mM, pH 7.4) using a Centricon-30 concentrator. The protein mixture was injected onto an HPLC equipped with a POROS R1 perfusive-particle column (4.6 × 100 mm) from Perseptive Biosystems (Cambridge, MA) at a flow rate of 3 mL/min. The solvent gradient consisted of buffer A, 0.05% trifluoroacetic acid (pH 3.0), and buffer B, 0.05% trifluoroacetic acid in ACN (pH 3.0). Proteins were separated using a gradient elution of 35% buffer B to 100% buffer B over 12 min. Under these conditions, heme eluted at 2.4 min, P450 reductase eluted at 3.5 (short form) and 5 min (long form), and P450 2A6 eluted at 8 min. Fractions (0.5 min) were collected into scintillation vials, quenched with 2.5 mL of Cytoscint ES liquid scintillation cocktail (2.5 mL), and measured for radioactivity using a liquid scintillation counter. A standard curve comprised of various amounts of P450 2A6 was used to determine the amount of P450 injected onto the HPLC column.

Table 1: Effect of Furanocoumarins on P450 2A6 Activity in Human Liver Microsomes, Reconstituted Supplemented GENTEST P450 2A6, and Reconstituted Purified P450 2A6[a]

furanocoumarin	MBI	K_I (μM)	k_{inact} (min^{-1})	partition ratio (system)[b]
8-MOP	yes	1.9	2.0	11.2 (GENTEST) 23.9 (purified)
5-MOP	yes	12.0	0.36	25.5 (GENTEST) 57.4 (purified)
DH-8-MOP	yes	27.0	0.14	215 (GENTEST) 840 (purified)
P	yes	0.6	0.30	46.0 (GENTEST) 71.2 (purified)
5-OH-P	yes	11.6	0.02	−
8-OH-P	no	1.8[c]	−	−
TOX	no	>100[c]	−	−

[a] Each furanocoumarin was tested for its ability to act as a MBI, and in cases where inactivation was not observed, the compound was tested as a competitive inhibitor. Kinetic constants were determined by nonlinear regression analysis of the rate data. [b] Partition ratios based on total inactivator depletion were determined in reconstituted purified and supplemented GENTEST P450 2A6 systems. [c] These are K_i values since these compounds were not found to be MBIs.

RESULTS

Effects of Psoralen Analogues on P450 2A6 Activity. Of all the analogues tested, 8-MOP with a K_I of 1.9 μM, a k_{inact} of 2 min^{-1}, and a partition ratio of 23.8 was characterized as the most potent MBI of human liver microsomal P450 2A6 (Table 1). 5-MOP (Figure 2a,b), DH-8-MOP, and P were also found to be time-dependent inactivators of P450 2A6 activity as was 5-OH-P to a minor degree (Table 1). In contrast, 8-OH-P was found to be only a competitive inhibitor, albeit potent, of P450 2A6 (K_i of 1.8 μM) up to concentrations of 50 μM and 20 min exposure times whereas TOX was simply not an inhibitor even up to concentrations of 100 μM and exposure times of 14 min. The inactivation of P450 2A6 mediated by both 5-MOP and DH-8-MOP was NADPH dependent and was not prevented by the addition of nucleophilic trapping agents or scavengers of reactive oxygen species (Figure 3a,b). The presence of a free iron chelator in the 5-MOP experiment also had no effect on P450 2A6 inactivation. The presence of the P450 2A6 inhibitor pilocarpine in the DH-8-MOP incubation was able to partially prevent P450 2A6-mediated inactivation, presumably through competitive inhibition. P450 2A6 activity, after incubation with 8-MOP, could not be restored by the addition of H_2O_2, CuOOH, or P450 reductase, although in the absence of 8-MOP, addition of P450 reductase to the activity assay did increase P450 2A6 activity by 25%. Finally, the partition ratios calculated for all furanocoumarins were significantly lower (1.5−3.9 ×) when the reconstituted GENTEST system was used (Table 1).

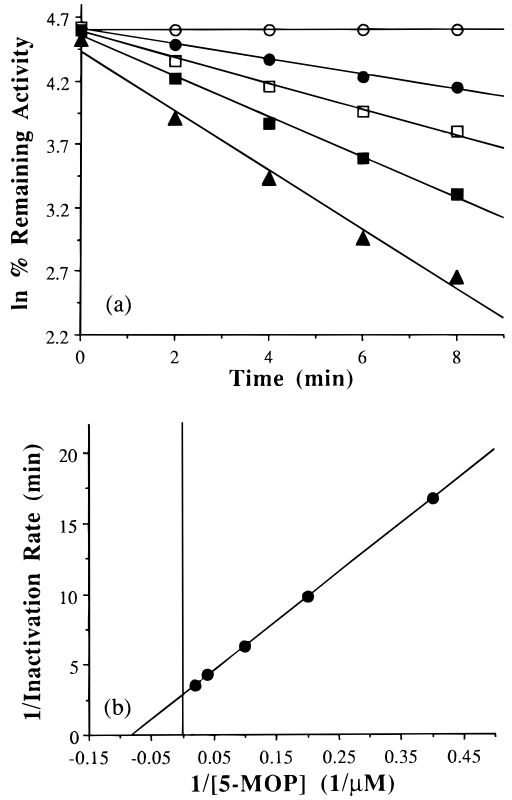

FIGURE 2: 5-MOP-mediated inactivation of human liver microsomal P450 2A6 (HL109) in the presence of an NADPH-generating system (a) and the double reciprocal plot of the relationship between inactivation rate and 5-MOP concentration (b). The concentrations of 5-MOP used in the inactivation assay were 0 μM (○), 2.5 μM (●), 5.0 μM (□), 10 μM (■), and 25 μM (▲). The reversible binding constant (K_I) and the rate constant for inactivation (k_{inact}) associated with microsomal P450 2A6 and 5-MOP were calculated (*24*) to be 12.0 μM and 0.36 min^{-1}, respectively, using nonlinear regression. The rate of coumarin 7-hydroxylation for the uninhibited reaction was 5.21 nmol (nmol of P450)$^{-1}$ min^{-1}.

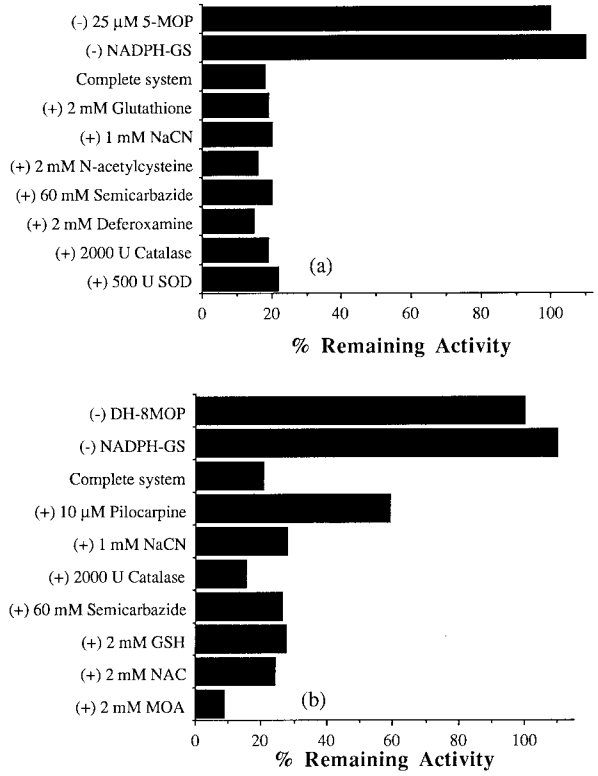

FIGURE 3: Effect of trapping agents on P450 2A6 inactivation by (a) 5-MOP and (b) DH-8-MOP. (a) Human liver microsomes (HL109) were exposed to 0 and 25 μM 5-MOP in the presence and absence of NADPH and various nucleophiles, reactive oxygen species trapping agents, and a free iron chelator. After 10 min exposure at 30 °C, the residual P450 2A6 activity was measured. (b) Human liver microsomes (HL109) were exposed to 0 and 50 μM DH-8-MOP in the presence and absence of NADPH and various nucleophiles, reactive oxygen species trapping agents, and pilocarpine, a P450 2A6 inhibitor. After 20 min exposure at 30 °C, the residual P450 2A6 activity was measured.

Analysis of Furanocoumarin Metabolites and Conjugates. LC/ESI-MS and HPLC-radiometric analysis indicated that, after exposure to NADPH and purified or GENTEST P450 2A6, 8-MOP, 5-MOP, and P were converted to the same major metabolites in both enzyme systems. The $[M+H]^+$ ions for the major metabolites of 8-MOP, 5-MOP, and P were found at $m/z = 251$, 251, and 221, respectively (Table 2). These results indicated that two hydrogen and two oxygen atoms had been incorporated into the parent furanocoumarin after incubation with P450 2A6. For the purposes of presentation, the most likely species, dihydrodiols, are assumed to be the major metabolites of 8-MOP, 5-MOP, and P. However, their structural identity will be more firmly established in the Discussion. The amount of monohydroxylated and demethylated product(s) formed from these furanocoumarins was negligible (<1% of total furanocoumarin conversion to product).

UV spectra of the HPLC-purified dihydrodiols formed from 8-MOP, 5-MOP, and P, in addition to those for a set of commercially available compounds selected for use as standards, are presented in Figure 4. The UV spectra of the presumed dihydrodiol metabolites, in the range of 300−375 nm, were remarkably similar to that of the DH-8-MOP standard, but very different from those of all the other standards. The major structural difference between DH-8-MOP and all the other standards, except for dihydrobenzofuran, is that DH-8-MOP is the only standard that contains a furan ring that has been reduced. Comparison of the dihydrodiol metabolites with the furanocoumarin standards reveals the appearance of a band at 325−335 nm and loss of a band at 290−310 nm that is present in the furanocoumarin standards. These spectral features are also observed in the UV spectrum of DH-8-MOP and mirrored in the UV spectrum of dihydrobenzofuran compared to benzofuran by a red shift from 245 to 275 nm.

In contrast to the furanocoumarins, the major metabolite of DH-8-MOP formed by P450 2A6 was a monohydroxylated product with an $[M+H]^+$ ion at $m/z = 235$ (Table 3). Two minor metabolites were also detected. The first had an $[M+H]^+$ ion at $m/z = 217$, and a MS/MS fragmentation pattern and HPLC retention time exactly matching those of the 8-MOP standard. The second had an $[M+H]^+$ ion at $m/z = 251$ and an MS/MS fragmentation pattern and HPLC retention time identical to the 8-MOP dihydrodiol metabolite. Contamination of the DH-8-MOP sample by residual 8-MOP could not account for the presence of 8-MOP and 8-MOP dihydrodiol in the incubations as the amounts of both compounds present in the incubations were found to increase with time. After 60 min, the concentration of 8-MOP in the incubation, formed from DH-8-MOP, reached 1.5 μM.

The inclusion of 10 mM GSH in the P450 2A6 and furanocoumarin incubations resulted in the formation of

Table 2: LC/ESI-MS/MS Information of the Furanocoumarin Standards and Furanocoumarin Metabolites and Conjugates Formed by P450 2A6 in the Absence and Presence of Various Nucleophiles[a]

compound	$[M+H]^+$	MS/MS fragmentation[b]
8-MOP standard	217	202, 174, 161, 146, 145, 131, 118, 90, 77
DH-8-MOP standard	219	204, 203, 176, 147, 131, 120, 119, 102, 92, 91, 77
5-MOP standard	217	202, 174, 146, 145, 131, 118, 100, 90, 89, 77
P standard	187	131, 115, 103, 89, 77
8-MOP dihydrodiol	251	233, 218, 205, 190, 162, 145, 117, 89
5-MOP dihydrodiol	251	233, 218, 205, 190, 175, 162, 147, 119, 91, 77
P dihydrodiol	221	203, 175, 147, 119, 117, 91, 77
8-MOP GSH conjugates	540	522, 447, 393, 308, 290, 247, 233, 177, 162, 130
5-MOP GSH conjugates	540	522, 447, 393, 308, 290, 247, 233, 179, 162, 130
P GSH conjugates	510	492, 417, 363, 278, 260, 217, 203, 177, 162, 130
8-MOP NAC conjugate	396	378, 336, 233, 179, 162, 146, 130, 102, 78
5-MOP NAC conjugate	396	378, 336, 233, 181, 162, 130, 102
P NAC conjugate	366	348, 306, 258, 240, 203, 178, 162, 130, 78
8-MOP MOA conjugate	280	262, 249, 248, 231, 230
5-MOP MOA conjugate	280	262, 249, 248, 231, 230
P MOA conjugate	250	232, 219, 218, 201, 200

[a] Reconstituted purified P450 2A6 was exposed to 100 μM furanocoumarin and NADPH (except for the standards which were not exposed to NADPH) for 45 min at 30 °C. The reactions were terminated and analyzed by LC/ESI-MS/MS as described. [b] Representative mass spectral fragmentation patterns are depicted in Figures 6a,b and 7a−c.

conjugates with $[M+H]^+$ ions at $m/z = 540$ for 8-MOP (Table 2), 5-MOP, and DH-8-MOP and at $m/z = 510$ for P. Addition of 10 mM NAC to the incubations led to the production of NAC conjugates with $[M+H]^+$ ions at $m/z = 396$ for 8-MOP (Table 2), 5-MOP, and DH-8-MOP and at $m/z = 366$ for P. Similarly, the inclusion of 10 mM MOA to the incubations generated MOA conjugates with $[M+H]^+$ ions at $m/z = 280$ for 8-MOP (Table 2), 5-MOP, and DH-8-MOP and at $m/z = 250$ for P. These $[M+H]^+$ ions are consistent with the opening of an epoxide ring by the nucleophile to generate a furanocoumarin adduct containing a hydroxyl group and the nucleophile. The reconstructed ion chromatograms for the putative GSH conjugates of an epoxide metabolite of these furanocoumarins revealed the presence of multiple adducts, whereas only a single NAC or MOA conjugate was formed.

The major metabolite formed at high concentrations of 5-OH-P (100 μM) had an $[M+H]^+$ ion at $m/z = 421$, indicating formation of a hydroxylated 5-OH-P dimer (Table 3). The minor metabolites had $[M+H]^+$ ions at $m/z = 219$, 237, and 251 corresponding to a hydroxylated product, dihydrodiol, and 5-MOP dihydrodiol (formed from 5-MOP, present as a minor impurity in 5-OH-P), respectively. The ratio of the hydroxylated dimer relative to 5-OH-P dihydrodiol fell from 5.2:1 to 1:1 by decreasing the concentration of 5-OH-P 10-fold. The dimer was not detected if GSH was present in the incubation, indicating that dimer formation was dependent on the concentration of 5-OH-P and that it had occurred in the bulk solvent. The major metabolite formed from 5-OH-P in the presence of GSH had an $[M+H]^+$ ion at $m/z = 526$, consistent with a hydroxylated GSH adduct. In addition, when 8-MOP and 5-OH-P were coincubated with P450 2A6, a hydroxylated mixed furanocoumarin dimer with an $[M+H]^+$ ion at $m/z = 435$ was observed (Table 3). A single monohydroxylated metabolite was produced from 8-OH-P with an $[M+H]^+$ ion at $m/z = 219$ and an HPLC retention time identical to the monohydroxylated product generated from 5-OH-P. This result indicates that P450 2A6 generated 5,8-dihydroxypsoralen (5,8-diOH-P) is a common metabolite that is formed from both 8-OH-P and 5-OH-P. Finally, it should be noted that while both the GENTEST system and the purified system produced identical metabolic profiles, the relative amount of metabolite produced using the GENTEST system was significantly less.

$H_2{}^{18}O$ Incorporation Experiments. LC/ESI-MS and single ion monitoring were used to measure the degree of ^{18}O incorporation into the product diols in the presence of $H_2{}^{18}O$. The amount of dihydrodiol formed by the addition of two ^{18}O atoms accounted for 19% of the 5-MOP dihydrodiol formed but was negligible for both 8-MOP and P.

Covalent Binding of [^{14}C]-8-MOP and [^{14}C]-5-MOP to Protein in Reconstituted Enzyme Systems. After separation of the reconstituted purified P450 2A6 and P450 reductase by SDS−PAGE (Figure 5a) and HPLC, liquid scintillation counting indicated that exposure to [^{14}C]-8-MOP and [^{14}C]-5-MOP and NADPH resulted in the covalent modification of both P450 2A6 and P450 reductase. In contrast, exposure of the two radiolabeled substrates to the GENTEST system resulted in covalent binding to P450 2A6 exclusively (Figure 5b). The amount of covalently modified P450 2A6 was 25% and 33% of the total spectrally detectable P450 loaded onto the gel for the purified and GENTEST systems, respectively. The amount of covalently modified P450 2A6 was 34% and 33% of the total P450 loaded onto the gel for the purified and GENTEST systems, respectively. Furthermore, HPLC-radiometric analysis indicated that there was no radioactivity associated with the heme, which was stripped from P450 2A6 under the acidic HPLC conditions. The inclusion of high concentrations (10 mM) of GSH and NAC was not able to prevent modification of P450 reductase without causing a concomitant decrease in the binding to P450 2A6.

Partition Ratio Determinations: Comparisons between Purified and GENTEST Systems. The partition ratios for 8-MOP and 5-MOP and P450 2A6 were determined using an HPLC-radiometric assay (Tables 4−7). The amount of covalent binding to P450 was determined as described above, and the reported partition ratios have been corrected for the relative amount of P450 bound in the presence of added nucleophile.

8-MOP metabolite formation and covalent binding to P450 2A6 was NADPH dependent (Table 4). It was necessary to

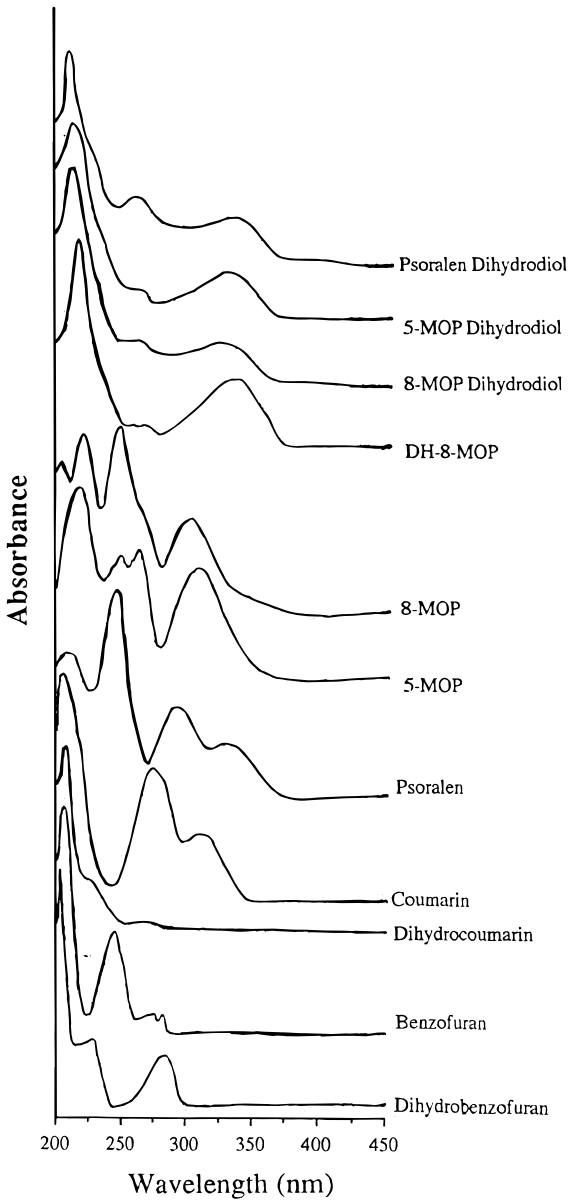

FIGURE 4: UV spectra of the furanocoumarin diols formed after incubation with purified reconstituted P450 2A6. Metabolites were purified by HPLC, lyophilized, and dissolved in 100% EtOH and compared to commercially available and synthesized standards also dissolved in 100% EtOH. The reference cuvette contained 100% EtOH.

add P450 reductase and cytochrome b_5 to the GENTEST system in a ratio of 2:1, relative to the amount of P450 2A6 present in the cells, to attain optimal enzymatic activity. Using this optimized GENTEST system, the ratio of total metabolic events (total substrate depletion) to P450 inactivating events (P450 present in the incubation) was 11.2. The partition ratio calculated based on just dihydrodiol formation exactly matched that calculated based on total 8-MOP depletion, consistent with the production of a single metabolite from 8-MOP, the dihydrodiol. P450 2A6 catalyzed demethylation of [^{14}C]-8-MOP to generate 8-OH-P would result in loss of the label; however, formation of this metabolite was negligible based on the partition ratio calculations and on HPLC−UV analysis. The addition of catalase did not affect the partition ratio or relative percent P450 bound. The addition of GSH, NAC, or MOA (2 mM) led to a 16, 2, and 31% decrease, respectively, in the relative

Table 3: LC/ESI-MS Information on the Metabolites and Conjugates Formed from Various Furanocoumarins by P450 2A6[a]

		[M+H]$^+$ ions of metabolites and conjugates		
furanocoumarin	additive[b]	major	minor	minor
8-MOP	5-OH-P	435.1		
DH-8-MOP	−	235.1 (53)	251.1 (1)	217.1 (1)
5-OH-P	−	421.1 (68)	237.4 (13)	251.1 (2)
5-OH-P (10 μM)	−	421.1 (3)	237.4 (3)	219.5 (1)
5-OH-P	GSH	526.3		
8-OH-P	−	219.1		

[a] Reconstituted purified P450 2A6 was exposed to 100 μM furanocoumarin (unless otherwise noted) and NADPH for 45 min at 30 °C. The reactions were terminated and analyzed by LC/ESI-MS as described. Major and minor metabolites were designated based on their LC/UV intensities, and the ratios are given in parentheses. [b] GSH and 5-OH-P were present at concentrations of 10 mM and 100 μM, respectively.

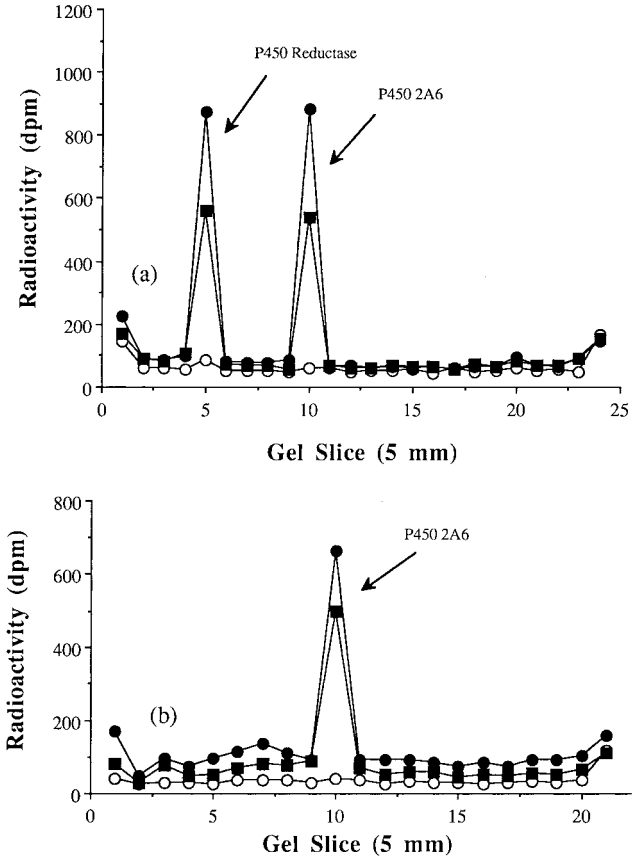

FIGURE 5: SDS−PAGE analysis of covalently modified proteins present in (a) the reconstituted P450 2A6 system and (b) the GENTEST P450 2A6 system after exposure to [^{14}C]-8-MOP. Incubations were performed in the absence of NADPH (○), presence of NADPH (●), and presence of NADPH and 10 mM GSH and NAC (■).

percent of P450 bound without affecting the total activity (amount of 8-MOP consumed). Increasing the concentration of GSH, NAC, and MOA (10 mM) produced a 23, 18, and 47% decrease, respectively, in the relative percent P450 bound; however, the total activity also decreased by 29, 25, and 26%, respectively.

A partition ratio of 23.9 was calculated for 8-MOP based on dihydrodiol formation and total inactivator depletion using the purified system. As with the GENTEST system, this result is consistent with production of a single 8-MOP metabolite, the dihydrodiol. The addition of GSH, NAC,

Table 4: 8-MOP and P450 2A6 Partition Ratio Determinations in the Supplemented GENTEST System[a]

	partition ratio calculations				
incubation components	diol formation	total depletion	difference	% P450 bound	% P450 activity
complete GENTEST system[b]	11.2 (100)	11.2 (100)	0	100[c]	100
(−) NADPH-generating system	0 (0)	0 (0)	0	0	0
(−) reductase and (−) b_5	12.8 (114)	12.8 (114)	0	50	57
(−) cytochrome b_5	12.4 (111)	12.4 (111)	0	80	89
(+) 2000 units of catalase	11.2 (100)	11.2 (100)	0	100	100
(+) 2 mM GSH	14.9 (133)	15.8 (141)	0.9	84	119
(+) 10 mM GSH	5.8 (52)	8.1 (72)	2.3	77	73
(+) 2 mM NAC	10.2 (91)	10.8 (97)	0.6	98	95
(+) 10 mM NAC	8.0 (71)	8.9 (79)	0.9	82	75
(+) 2 mM MOA	16.2 (145)	17.0 (152)	0.8	69	105
(+) 10 mM MOA	15.2 (136)	15.6 (139)	0.4	53	74

[a] Partition ratios were calculated based on diol (major metabolite) formation and total MBI depletion assuming 100% P450 2A6 activity loss over the course of the 45 min incubation. Values shown in parentheses are percentages relative to the complete GENTEST system. [b] The complete GENTEST system was comprised of P450 2A6 supplemented with purified P450 reductase and cytochrome b_5 in a 2:1 ratio, respectively, to P450 2A6 and an NADPH-generating system in 70 mM Tris-HCl buffer (pH 7.5). [c] 100% P450 bound corresponds to 25% of the total P450 loaded onto the gel.

Table 5: 8-MOP and P450 2A6 Partition Ratio Determinations in the Purified Reconstituted System[a]

	partition ratio calculations				
incubation components	diol formation	total depletion	difference	% P450 bound	% P450 activity
complete purified system[b]	23.9 (100)	23.9 (100)	0	100[c]	100
(−) NADPH-generating system	0 (0)	0 (0)	0	0	0
(+) 2000 units of catalase	23.9 (100)	23.9 (100)	0	100	100
(+) 2 mM GSH	24.9 (104)	28.1 (117)	3.2	93	109
(+) 10 mM GSH	11.4 (48)	36.1 (151)	24.7	77	116
(+) 2 mM NAC	26.7 (112)	26.8 (112)	0.1	99	111
(+) 10 mM NAC	19.4 (81)	31.6 (132)	12.2	82	108
(+) 2 mM MOA	22.6 (95)	24.2 (101)	1.6	100	101
(+) 10 mM MOA	27.7 (116)	47.1 (197)	19.4	52	102

[a] Partition ratios were calculated based on diol (major metabolite) formation and total MBI depletion assuming 100% P450 2A6 activity loss over the course of the 45 min incubation. Values shown in parentheses are percentages relative to the complete reconstituted system. [b] Complete reconstituted system was comprised of P450 2A6/P450 reductase/cytochrome b_5 in a ratio of 1:2:1, 25 μg of DPLC/mL, and an NADPH-generating system in 25 mM potassium phosphate buffer (pH 7.4). [c] 100% P450 bound corresponds to 33% of total P450 loaded onto the gel.

Table 6: 5-MOP and P450 2A6 Partition Ratio Determinations in the Supplemented GENTEST System[a]

	partition ratio calculations				
incubation components	diol formation	total depletion	difference	% P450 bound	% P450 activity
complete GENTEST system[b]	23.3 (100)	25.5 (100)	2.2	100[c]	100
(−) NADPH-generating system	0 (0)	0 (0)	0	0	0
(+) 2 mM GSH	27.6 (118)	33.3 (131)	5.7	87	114
(+) 10 mM GSH	29.7 (127)	41.1 (161)	11.4	65	105
(+) 2 mM NAC	17.8 (76)	20.9 (82)	3.1	86	83
(+) 10 mM NAC	19.7 (84)	23.7 (93)	3.0	63	59
(+) 2 mM MOA	15.0 (64)	18.5 (73)	3.5	60	59
(+) 10 mM MOA	16.8 (72)	18.7 (73)	1.9	47	49

[a] Partition ratios were calculated based on diol (major metabolite) formation and total MBI depletion assuming 100% P450 2A6 activity loss over the course of the 45 min incubation. Values shown in parentheses are percentages relative to the complete GENTEST system. [b] The complete GENTEST system was comprised of P450 2A6 supplemented with purified P450 reductase and cytochrome b_5 in a 2:1 ratio, respectively, to P450 2A6 and an NADPH-generating system in 70 mM Tris-HCl buffer (pH 7.5). [c] 100% P450 bound corresponds to 34% of total P450 loaded onto the gel.

and MOA (2 mM) led to a 7, 1, and 0% decrease in the extent of P450 bound, respectively, and a relatively small amount of conjugate formation (0.4−11%). The extent of conjugate formation increased dramatically in the presence of 10 mM GSH (68%), NAC (37%), and MOA (41%) with a concomitant decrease in the percent P450 bound (23−48%). There was no detectable effect on total activity in the purified system even at the higher concentration of nucleophile.

Using the GENTEST system, partition ratios of 23.3 and 25.5 were calculated for 5-MOP based on dihydrodiol formation and total inactivator depletion, respectively. The difference of 14% between the two methods of calculation indicates that a small amount of the parent compound was converted to metabolites other than the dihydrodiol (Table 6). The addition of 2 and 10 mM GSH led to a 13 and 35% decrease, respectively, in the amount of P450 bound without significantly affecting total activity. The addition of NAC

Table 7: 5-MOP and P450 2A6 Partition Ratio Determinations in the Purified Reconstituted System[a]

	partition ratio calculations				
incubation components	diol formation	total depletion	difference	% P450 bound	% P450 activity
complete purified system[b]	52.0 (100)	57.4 (100)	5.4	100[c]	100
(−) NADPH-generating system	0 (0)	0 (0)	0	0	0
(+) 2 mM GSH (99)	36.3 (70)	56.8 (99)	20.5	100	99
(+) 10 mM GSH (100)	33.3 (64)	75.9 (132)	42.6	76	100
(+) 2 mM NAC (98)	38.2 (73)	59.1 (103)	20.9	95	98
(+) 10 mM NAC (101)	46.0 (89)	79.3 (138)	33.3	73	101
(+) 2 mM MOA (60)	35.8 (69)	47.4 (83)	11.6	73	75
(+) 10 mM MOA (36)	38.2 (73)	41.6 (72)	3.4	50	50

[a] Partition ratios were calculated based on diol (major metabolite) formation and total MBI depletion assuming 100% P450 2A6 activity loss over the course of the 45 min incubation. Values shown in parentheses are percentages relative to the complete reconstituted system. [b] Complete reconstituted system was comprised of P450 2A6/P450 reductase/cytochrome b_5 in a ratio of 1:2:1, 25 μg of DPLC/mL, and an NADPH-generating system in 25 mM potassium phosphate buffer (pH 7.4). [c] 100% P450 bound corresponds to 33% of total P450 loaded onto the gel.

and MOA led to minimal conjugate formation, and the observed decreases in the relative amount of P450 bound (14−53%) correlated well with the decreases in total activity (17−51%).

For the 5-MOP-mediated inactivation of purified P450 2A6, partition ratios of 52.0 and 57.4 were calculated based on dihydrodiol formation and total inactivator depletion, respectively. In agreement with the GENTEST system, the small difference in partition ratios between the two methods of calculation is indicative of the production of minor metabolites (Table 7). No effort was made to characterize the minor 5-MOP metabolites as they were formed in minute quantities. The addition of GSH and NAC (2 mM) led to a substantial amount of conjugate formation, but a negligible decrease in the amount of P450 bound (0−5%). Increasing the concentration of GSH and NAC 5-fold increased the extent of conjugation by 160% and decreased the relative percent P450 bound by 22%, but did not affect total activity (1−2% decrease). The results from MOA addition (2 mM) were somewhat different, in that a 27% decrease in the relative percent of P450 bound due to conjugate formation was accompanied by a 25% decrease in the total activity. Furthermore, in the presence of 10 mM MOA, there was a 50% decrease in the relative percent P450 bound with a corresponding 50% decrease in the total activity.

DISCUSSION

8-MOP is a potent, isoform-selective MBI of human liver microsomal P450 2A6 at concentrations (*9*) observed in vivo when it is used therapeutically. As stated earlier, one goal of this investigation was to identify the reactive species of 8-MOP responsible for P450 2A6 inactivation. To that end, the inhibitory properties of structurally related furanocoumarins, 5-MOP, P, 5-OH-P, 8-OH-P, DH-8-MOP, and TOX, toward P450 2A6 were determined (Figure 1). DH-8-MOP was selected to test the possibility that the reactive intermediate might be a furanoepoxide, while the other compounds were selected to clarify the role of the 8-methoxy group. Of these six compounds, 8-OH-P was purely a competitive inhibitor while TOX simply did not inhibit the enzyme (K_i > 100 μM) at the concentrations used. The remaining four were MBIs, with 8-MOP being the most potent and 5-OH-P the least potent (Table 1). The fact that P (no substituent) and 8-MOP are potent MBIs while 8-OH-P is only a competitive, albeit potent, inhibitor, suggests that the 8-OH group must reorient substrate binding, perhaps through hydrogen bonding. This reorientation could relocate the structural element necessary for generation of the reactive intermediate, so that enzymatic attack at this site and the resulting inactivation are now disfavored. A similar explanation would account for the difference in inhibitory properties between 5-MOP and 5-OH-P (Table 1). The three methyl substituents of TOX apparently introduce sufficient steric hindrance so that this furanocoumarin can no longer enter the active site of P450 2A6 at the concentrations tested (Table 1).

Placement of the methoxy group is important as the K_I and k_{inact} of 5-MOP are almost 6-fold larger and 7-fold smaller, respectively, than those of 8-MOP. Apparently, the decrease in binding affinity of 5-MOP results in a decrease in its ability to affect enzyme inactivation. The smaller K_I of P relative to 8-MOP may be rationalized by the close structural resemblance of P to coumarin, a substrate which has a high affinity for P450 2A6 ($K_m \approx 0.5$ μM) (*9*). The additional groups (methoxy, methyl, or hydroxy) present in the other furanocoumarins apparently decrease their binding affinity to P450 2A6, relative to P and coumarin, through steric or other unfavorable interactions. The smaller k_{inact} of P in comparison to 8-MOP may be due to its ability to adopt both 8-MOP and 5-MOP binding modes, the latter of which leads to less potent inactivation.

The mass spectra of 8-MOP and 5-MOP under CID conditions are characterized by an initial loss of 15 mass units ($-CH_3$) followed by four consecutive losses of 28 mass units (Table 2). The latter fragmentation pathway indicates that the four oxygen atoms, present in each of the two compounds, are ejected from the $[MH-15]^+$ species in sequential losses of CO.[2] The mass spectrum of P is characterized by three consecutive losses of 28 mass units (-3 CO) followed by loss of 26 mass units ($-C_2H_2$) to generate a C_6H_5 species at *m*/*e* 77 (Table 2). Under electron impact conditions, consecutive losses of CO have been found to be characteristic of furanocoumarins (*25*). The major metabolites of 8-MOP, 5-MOP, and P produced after exposure to P450 2A6 were found by LC/ESI-MS analysis to possess $[M+H]^+$ ions consistent with the addition of two hydrogen and two oxygen atoms. CID conditions indicated

[2] L. L. Koenigs and W. F. Trager, unpublished ESI-MS/MS observations.

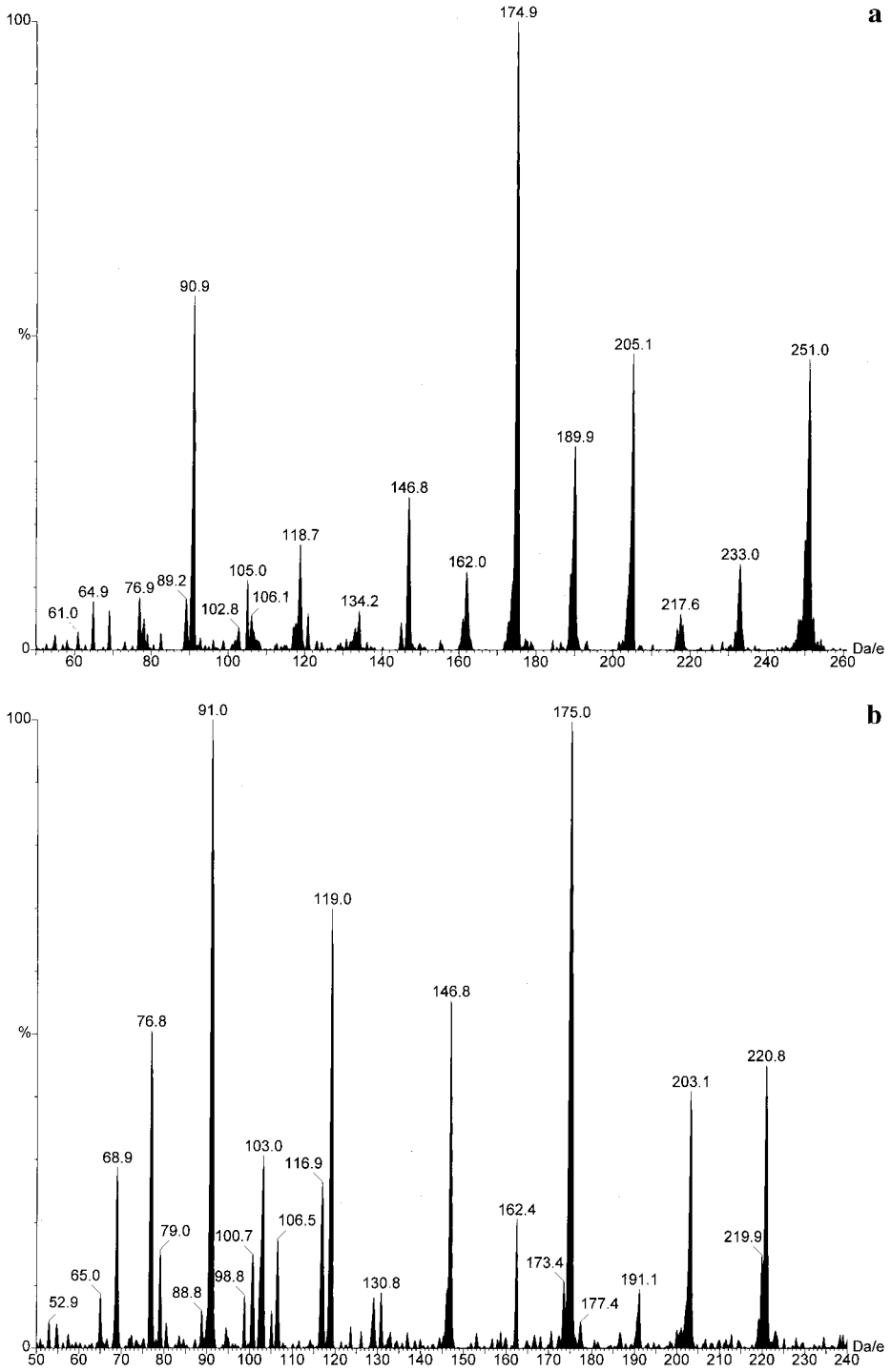

FIGURE 6: CID spectra of the 5-MOP (a) and P (b) dihydrodiols formed after incubation with reconstituted purified P450 2A6 and NADPH and analysis by LC/ESI-MS/MS. The collision energy used was 40 eV for the 5-MOP dihydrodiol and 30 eV for the P dihydrodiol.

facile loss of H_2O from all three metabolites, followed by the loss of a methyl group from the metabolites of 8-MOP and 5-MOP (Table 2 and Figure 6a,b). In general, these initial fragmentation steps were followed by multiple losses of CO. The addition of two hydrogen atoms plus two oxygen atoms to each parent, coupled to the facile mass spectral loss of H_2O, eliminates diphenolic metabolites as possible products and suggests that the major metabolite in each case is a dihydrodiol (either the 4′,5′-dihydrofuranocoumarin diol or the furano-3,4-dihydrocoumarin diol) formed by hydrolytic opening of an epoxide ring. The intermediacy of an epoxide is supported by the finding that the inclusion of GSH, NAC, and MOA in the incubations generated conjugates having MS/MS characteristics consistent with the addition of the nucleophile plus a hydroxyl group. A similar type of intermediate has been invoked to explain the formation of dihydrodiols by P450 from aflatoxin B_1 (*3*) and Merck's investigational HIV protease inhibitor, L-754,394 (*26*). The neutral loss of 129 Da (after initial loss of H_2O) in the furanocoumarin−GSH conjugates and fragmentation of the $[M+H]^+$ ion to afford protonated GSH ($m/z = 308$) (Table 2 and Figure 7a) are characteristic mass spectral features of GSH adducts (*27*). The product ion spectra of the NAC and MOA conjugates (Table 2 and Figure 7b,c) indicated CID-

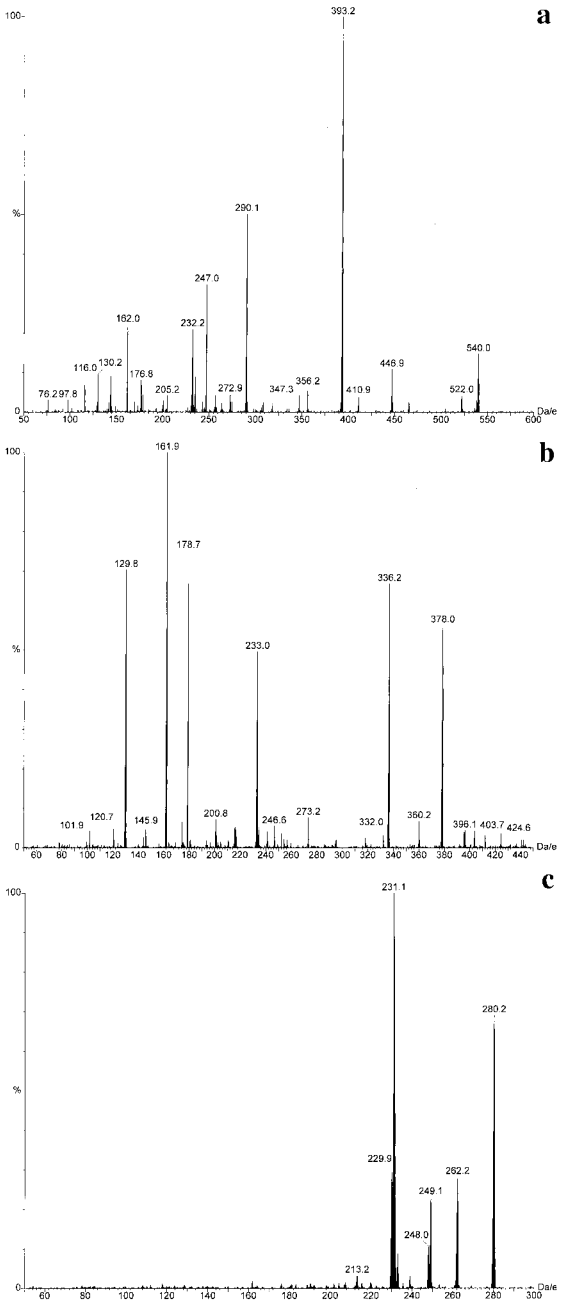

FIGURE 7: CID spectra of the (a) 8-MOP GSH, (b) NAC, and (c) MOA conjugates formed after incubation with reconstituted purified P450 2A6 and NADPH and analysis by LC/ESI-MS/MS. The collision energy used was 30 eV for the 8-MOP GSH conjugates, 15 eV for the NAC conjugate, and 10 eV for the MOA conjugate. The concentration of the nucleophiles in the incubation was 10 mM.

induced loss, from $[M+H]^+$, of H_2O (8-MOP, *m*/*z* 378; 5-MOP, *m*/*z* 378; P, *m*/*z* 348; 8-MOP, *m*/*z* 262; 5-MOP, *m*/*z* 262; P, *m*/*z* 232, respectively) or in the case of the NAC adduct loss of the entire added nucleophile (8-MOP, *m*/*z* 233; 5-MOP, *m*/*z* 233; P, *m*/*z* 203). Losses of *m*/*z* 58 (CH_3-CONH−) from $[M+H]^+$ and losses of *m*/*z* 31 (CH_3O−) from $[MH-18]^+$ were also characteristic of the NAC and MOA adducts, respectively. While these results alone do not allow a 4′,5′-dihydrofuranocoumarin diol to be distinguished from a furano-3,4-dihydrocoumarin diol, three independent lines of evidence strongly implicate a 4′,5′-dihydrofuranocoumarin diol as the stuctural core of the major metabolites formed from 8-MOP, 5-MOP, and P.

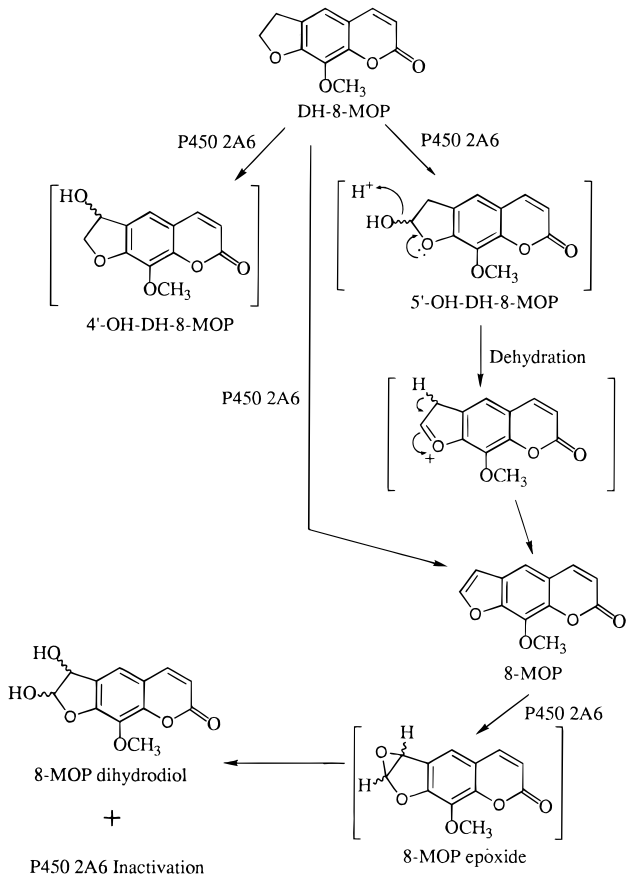

FIGURE 8: Postulated scheme of P450 2A6 mediated DH-8-MOP oxidation to form metabolites and cause inactivation.

The first is provided by comparison of the UV spectra of the furanocoumarin substrates, their major metabolites, and a set of standards. The similarity between the UV spectra of the major metabolites and that of DH-8-MOP, coupled with their lack of similarity to the UV spectra of the other standards (Figure 4), indicates that the aromaticity of the furan ring has been lost in the metabolites. This result establishes oxidative attack at the furan ring, rather than the coumarin ring, of the furanocoumarins. Second, coumarin itself is not a MBI of P450 2A6, but is an excellent substrate for the enzyme that is oxidized exclusively at the 7-position. If the binding orientation of the furanocoumarins is analogous to that of coumarin, as seems likely, the furan ring would be the expected site of oxidation. Finally, unlike 8-MOP, 5-MOP, and P which are almost exclusively converted to dihydrodiols, DH-8-MOP is predominantly monohydroxylated. The major change in metabolism caused by reduction of the furan to a dihydrofuran, as is found in DH-8-MOP, strongly suggests that dihydrodiol formation must involve the furan ring and not the coumarin ring. The probability of oxidative attack at the coumarin ring, because it is remote from the furan, should be largely independent of the structural state of the furan ring, which it clearly is not. Interestingly, reduction of the furan ring does not completely abolish inactivation potency. This is explained by the fact that a relatively minor fraction of DH-8-MOP is dehydrogenated to 8-MOP which upon further oxidation to the furanoepoxide can function as a MBI or be hydrolyzed to the dihydrodiol (Table 3 and Figure 8). Dehydrogenation presumably occurs through initial hydroxylation at the 5′- or possibly the 4′-position, followed by dehydration. We postulate that the

major monohydroxylated product of DH-8-MOP is formed by oxidation at the benzylic 4′-position to form 4′-OH-DH-8-MOP; however, rigorous characterization of this species is lacking. Attempts to isolate sufficient quantities of this metabolite and the dihydrodiols to obtain NMR spectra have thus far proved to be unsuccessful.

Conversion of the methoxyl group to a hydroxyl group severely decreased (5-OH-P) or completely abolished (8-OH-P) inactivation. The monohydroxylated products of 5-OH-P and 8-OH-P have identical mass spectral and HPLC characteristics; thus, it appears that both substrates form the same metabolite, 5,8-diOH-P. These observations suggest that a hydroxyl group in either the 5- or the 8-position reorients the substrate at the active site such that oxidative attack of the furan ring is greatly diminished in favor of monohydroxylation at some other site. As a minor pathway, 5-OH-P is also susceptible to oxidative attack at the furan ring as evidenced by a small amount of time-dependent inhibition and the presence of mass spectral species with $[M+H]^+$ ions corresponding to a 5-OH-P dihydrodiol and 5-OH-P dimer. The 5-OH-P dimer became particularly prevalent when incubations were run at high concentrations of 5-OH-P. In addition, a hydroxylated 5-OH-P/8-MOP heterodimer was formed when the two furanocoumarins were coincubated with P450 2A6 and NADPH. The formation of these heterogeneous dimers provides additional evidence for the furanoepoxide intermediate.

The literature is replete with examples of furan-containing compounds, including ipomeanol (*28*), (*R*)-methofuran (*29*), furosemide (*30*), aflatoxin B_1 (*3*), L-739,010 (*31*), teucrin A (*32*), L-754,394 (*33*), and coriandrin (*34*), that undergo oxidation by P450 to reactive intermediates capable of forming covalent adducts with proteins and nucleic acids. Figure 9a,b illustrates hypothetical models for the generation of reactive furanoepoxide and γ-ketoenal species from 8-MOP and 5-MOP. These intermediates are directly analogous to those that have been invoked to account for protein alkylation observed with (*R*)-methofuran (*29*). Since the compound in this case is an aromatic furan, the first step in the oxidative mechanism is addition of the active iron−oxo species to the molecule (*35*). This radical species can collapse to an epoxide (pathway a) or rearrange to a ring-opened allylic radical which then collapses to a γ-ketoenal (pathway b). The γ-ketoenal can also be formed by ring opening of the epoxide and, in the presence of $H_2{}^{18}O$, is capable of incorporating three atoms of ^{18}O (pathway c) prior to formation of the dihydrodiol. In contrast, furanocoumarin dihydrodiol formed exclusively from epoxide (pathway a) can only incorporate a single ^{18}O atom from $H_2{}^{18}O$ exchange. For 8-MOP and P, >99.5% of the dihydrodiol formed was the result of the addition of only one atom of ^{18}O, while 81% and 19% of the 5-MOP dihydrodiol arose via the addition of one and two atoms of ^{18}O, respectively. The difference in $H_2{}^{18}O$ incorporation into the dihydrodiols between 8-MOP and 5-MOP can be explained by a resonance effect where the methoxyl group electrons of 5-MOP assist ring opening of the epoxide to generate the γ-ketoenal. This additional resonance effect is not present in 8-MOP and P; therefore, the epoxide pathway is the only one possible for these substrates. Extension of these findings to the inactivation event(s) suggests that only the epoxides of 8-MOP and P (and DH-8-MOP) are responsible for the covalent modification of P450 2A6, whereas both the γ-ketoenal and epoxide of 5-MOP contribute to protein alkylation.

The irreversible nature of the inactivation caused by these furanocoumarins is supported by the inability of overnight dialysis to restore P450 2A6 catalytic activity after exposure to 8-MOP (*9*). Since none of the common nucleophilic trapping agents could prevent the 8-MOP-, 5-MOP-, and DH-8-MOP-mediated inactivation, the inactivating events are confined to the active site of the enzyme and involve the formation of a covalent bond between the reactive intermediate(s) and the enzyme. Mechanism-based inactivation of cytochrome P450 is thought to occur by three different pathways: (a) alkylation of the heme, (b) covalent modification of the apoprotein, and/or (c) covalent binding to the heme followed by a heme "explosion" into fragments which bind apoprotein. The presence of radiolabeled protein as established by SDS−PAGE and HPLC separation followed by radiometric analysis of the reconstituted P450 2A6 mixtures after exposure to radiolabeled 8-MOP and 5-MOP indicates that mechanism (b) is operative, while the lack of any radiolabeled heme, as demonstrated in the HPLC experiments, rules out mechanism (a) and probably mechanism (c) as some residual intact radiolabeled heme might be expected to be associated with mechanism (c).

The observation that only 25−34% of the total spectrally detectable P450 loaded onto the gel is associated with radioactivity, despite almost complete loss of catalytic activity, is perplexing. The apparent lack of any radiolabeled heme excludes heme modification and loss as a possible explanation. A lack of 1:1 stoichiometry between adducted protein and P450 has been noted previously for the inactivation of P450 2B1 by *N*-benzyl-1-aminobenzotriazole [(0.4−0.5):1], but was without explanation (*36*). One possibility might be that the protein adducts are unstable to the conditions used to analyze covalent binding. For example, in the case of the furanocoumarins and P450 2A6, covalent binding was evaluated using denaturing conditions of SDS−PAGE. However, since extensive dialysis does not restore P450 2A6 activity after incubation with 8-MOP (*9*), a 1:1, rather than the observed 0.3:1, stoichiometry in the subsequent HPLC experiments would have been expected. Perhaps, once covalent modification of P450 has occurred, it is impossible to restore enzyme activity even after removal of the inactivator from the active site.

Another possibility for the lack of 1:1 stoichiometry is that not all of the spectrally detectable P450 is catalytically active. This could arise from inefficient incorporation of P450 2A6 into the lipid environment and/or nonoptimal interactions with P450 reductase and cytochrome b_5. Indeed, exogenous P450 reductase and cytochrome b_5 were required in order for an equivalent amount of GENTEST P450 2A6, compared to purified P450 2A6, to be modified. Although the proteins in the GENTEST system are presumably incorporated into the lipid environment correctly, the amount of supporting enzyme activity is insufficient for optimal P450 activity. As noted in an earlier investigation, some purified P450 2A6 activity is lost in the absence of substrate, simply by exposure to NADPH. However, the loss in activity does not result in a corresponding loss of P450 2A6 spectral content (*9*) and may be due to a disruption in the electron-transfer pathways between P450, P450 reductase, and cytochrome b_5. In effect, this would decrease the catalytically

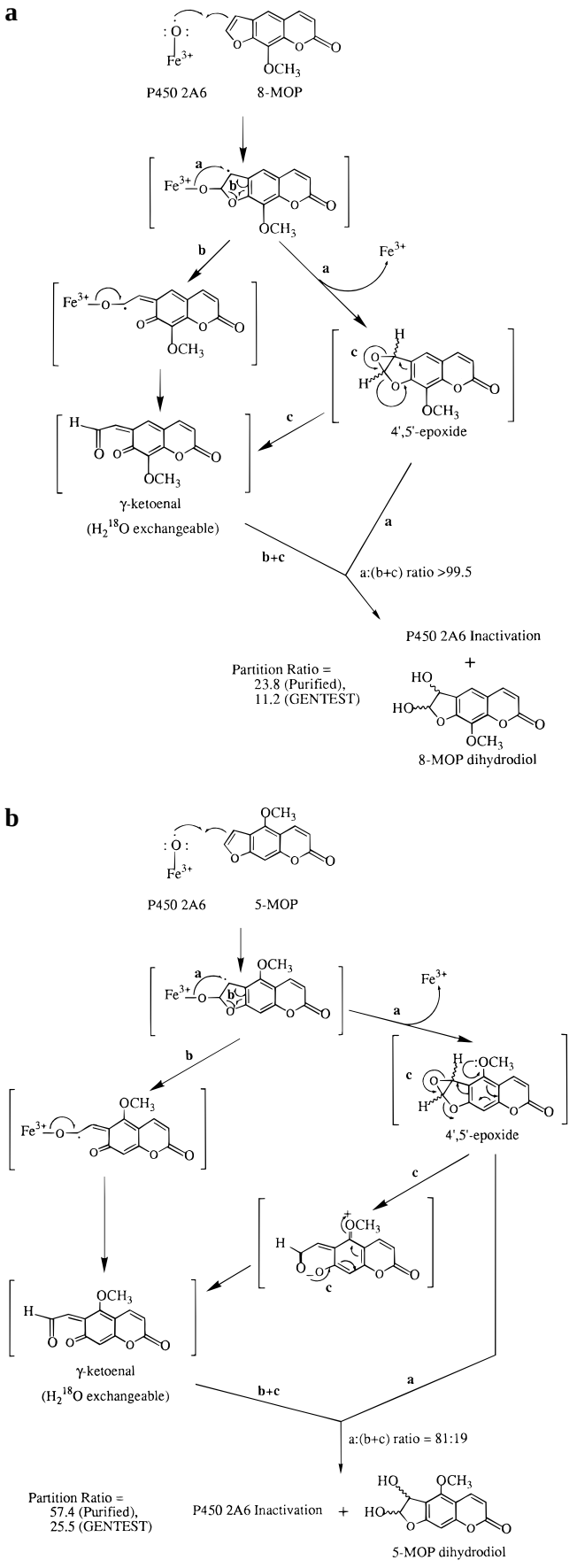

FIGURE 9: (a) Postulated mechanism of P450 2A6 inactivation by 8-MOP and P (only 8-MOP is shown). (b) Postulated mechanism of P450 2A6 inactivation by 5-MOP. Fe represents the heme iron atom of P450 2A6.

competent P450 without affecting spectrally detectable P450 2A6.

Still another explanation might be that in the presence of the furanocoumarin, significant uncoupling of the P450 2A6 system occurs and the inactivation of P450 2A6 results from the large amounts of reactive oxygen species that are generated. Support for this possibility can be found in recent studies using P450 2A6 coexpressed with P450 reductase that examined the relationship between the degree of uncoupling and the presence of a substrate (coumarin) (*37*). These studies demonstrated that the degree of NADPH that was uncoupled to form H_2O_2 increased significantly (16−18 ×) when substrate was present compared to when the substrate was absent. Therefore, it is possible that in the presence of an inactivating furanocoumarin only a small amount of P450 2A6 activity is utilized for formation of the reactive intermediate leading to covalent modification while the majority of the P450 is inactivated by the reactive oxygen species liberated via enzyme uncoupling. These reactive oxygen species would have to inactivate the enzyme by reacting with the active site of P450 2A6 (probably at the heme) as large amounts of catalase and superoxide dismutase were unable to enhance the amount of covalent binding to the enzyme (Figure 3). Substrate-induced uncoupling would explain our earlier observation that exposure of P450 2A6 to 8-MOP resulted in loss of the characteristic 450 nm species whereas in the absence of substrate this loss was relatively small (*9*). This explanation would also account for a lack of 1:1 stoichiometry between the furanocoumarins and any P450 2A6 preparation (e.g., purified or GENTEST) because it depends solely on the characteristics of the enzyme−substrate interaction. Which of the three possible reasons considered (unstable adducts; inactive, but spectrally detectable enzyme; or uncoupling) is responsible for the lack of 1:1 stoichiometry is unknown, but it may well be some combination of all three and remains an important issue to be resolved.

Partition ratios based on total inactivator depletion and major metabolite (dihydrodiols) formation were calculated for 8-MOP and 5-MOP and found to be approximately 2-fold lower after incubation with GENTEST P450 2A6 in comparison to purified P450 2A6. The total amount of covalently modified P450 2A6 remained the same, and, therefore, the observed differences do not appear to be due to a decrease in total P450 2A6 activity. This effect was also noted for the DH-8-MOP and P partition ratios which were 3.9 and 1.5 × lower, respectively, when using the GENTEST system. The addition of GSH, NAC, and MOA to the purified P450 2A6 incubations with 8-MOP and 5-MOP led to a substantial amount of conjugate formation and a corresponding decrease in the relative amount of P450 bound without significantly affecting the total catalytic activity. Similarly, addition of exogenous nucleophiles to the GENTEST incubations decreased the relative amount of P450 bound, but to a much smaller extent. However, unlike the purified system, the overall catalytic activity decreased. In addition, SDS−PAGE and HPLC analysis of the separated proteins revealed that binding to P450 and P450 reductase occurred in the purified system even in the presence of high concentrations of GSH and NAC (10 mM). In this regard, covalent binding to P450 2B1 and P450 reductase by [^{3}H]-phencyclidine that could not be prevented by 10 mM GSH has been observed by

others (*38*). In the GENTEST system, however, binding of 8-MOP and 5-MOP to P450 2A6 was exclusive. Interestingly, the binding to P450 reductase observed with the purified system was not responsible for inactivation, as addition of reductase to a P450 2A6 sample after exposure to 8-MOP did not restore activity. Overall, these results suggest that escape of the reactive intermediate(s) from the active site of P450 2A6 is more probable with the purified enzyme than with the GENTEST system. This could be due to differences in active site and/or protein orientations depending on whether the P450 is naturally (GENTEST) or artificially (purified) incorporated into the lipid bilayer.

In this investigation, a number of furanocoumarins were found to behave as MBIs or competitive inhibitors of P450 2A6. The mechanism of inactivation appears to be initial oxidation of the furan ring to generate a furanoepoxide (8-MOP, 5-MOP, and P) or γ-ketoenal (5-MOP) which then reacts either with H_2O to form a dihydrodiol or with a nucleophilic amino acid at the active site of P450 2A6. Mechanism-based inactivation accounts for approximately 33% of the spectrally determined P450 2A6 lost, based on covalently bound enzyme. The factor(s) responsible for the remaining 67% loss is (are) unknown, but may include unstable adducts, inactive, but spectrally detectable enzyme, and/or the liberation of reactive oxygen species through uncoupling. Interestingly, the P450 2A6 active site present in the purified system permits a greater amount of reactive intermediate(s) to escape than does the P450 2A6 active site present in the GENTEST system. This difference in active site integrity is attributed to differences in the enzyme−lipid environment of the two systems. The chemical nature of the covalent modification of P450 2A6 apoprotein after exposure to these furanocoumarins and NADPH is currently under investigation.

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