Catalysis of the Cysteine Conjugation and Protein Binding of Acetaminophen by Microsomes from a Human Lymphoblast Line Transfected with the cDNAs of Various Forms of Human Cytochrome P450

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ABSTRACT
We have previously found that for acetaminophen kinetic differences exist between the hepatic microsomal catalyzed protein binding and cysteine conjugation. We have also observed that the protein binding of acetaminophen is only to intraluminal proteins. Together these data suggested that two pools of the reactive metabolite, N-acetyl-p-benzoquinone imine (NABQI), are formed during the oxidative metabolism of acetaminophen: one on the cytosolic surface and the other within the lumen of the microsomes. This would indicate that some of the cytochrome P450 (CYP) catalyzing NABQI formation have their active site on the cytosolic surface and others on the luminal surface. We have examined this question by comparing the rates of cysteine conjugation and protein binding of acetaminophen by microsomes from lymphoblasts transfected with the cDNAs for human CYPs. We found that CYP2D6 catalyzed only cysteine conjugation; CYP1A2 and 3A4 catalyzed only protein binding; CYP2E1 catalyzed both; and CYP1A1, CYP2A6 and CYP2B6 catalyzed neither. These data suggest that CYP2D6 has its active site only on the cytosolic surface; CYP1A2 and CYP3A4 only on the luminal surface; and CYP2E1 has catalytic sites on both the luminal and cytosolic surfaces of the membrane. In mouse studies we have found that ethanol administration increased acetaminophen protein binding by 265% but cysteine conjugation by only 61%. CYP2E1 and CYP2B increased, whereas CYP3A decreased and the others did not change. These data suggest that in control mice CYP2E1 catalyzes the bulk of protein binding, whereas CYP2D catalyzes slightly more cysteine conjugation than does CYP2E1.

The initial step in the oxidative metabolism of acetaminophen is generally thought to be the cytochrome P450-catalyzed formation of the reactive metabolite NABQI. The bulk of the NABQI is reduced nonenzymatically by NADPH back to acetaminophen (Dahlin et al., 1984; Prasad et al., 1990). At low concentrations of NABQI, the remainder is quickly detoxified by reacting nonenzymatically with GSH. If the GSH is depleted, either by reacting with the metabolite or by other processes, then the NABQI binds to critical, cellular macromolecules, including both cytosolic and microsomal proteins.

Kinetic studies from our laboratory have suggested that two distinct pools of NABQI are formed during the in vitro, microsomal, oxidative metabolism of acetaminophen (Prasad et al., 1990; Zhou et al., in press). In these studies we determined the rate of its oxidative metabolism by isolated microsomes with two different procedures. In the first, we measured the overall metabolism by the addition of cysteine to the incubation medium. This reagent rapidly reacts nonenzymatically with the GSH and gives the product critical, cellular macro- molecules, including both cysteic and microsomal proteins. The product of this reaction was then determined by HPLC. Because the reaction with cysteine is very rapid, essentially all of the NABQI that is formed is trapped to give the conjugate. In the second procedure, we determined the rate of binding of radiolabeled acetaminophen to microsomal proteins in the

ABBREVIATIONS: CYP, Cytochrome P450 forms; NABQI, N-acetyl-p-benzoquinone imine; HPLC, high-performance liquid chromatography; GSH, glutathione.
Cysteine conjugation is catalyzed by a member of the CYP2D CYP2E1 whereas, in control animals, more than half of the found that the protein binding is primarily catalyzed by the rates of cysteine conjugation and protein binding. We concentrations of these CYPs in the hepatic microsomes with 1-butanol (Sinclair et al., 1993). We found that CYP2D6 and CYP2E1 catalyze the conjugate and protein adducts by microsomes from a human lymphoblast line that has been transfected with plasmids because the topology of the microsomes is lost during these procedures. In light of these problems, in the current investigation, we sought to determine whether there are apparent differences in the location of the catalytic site for the various CYPs by determining the relative rates of formation of the cysteine conjugate and protein adducts by microsomes from a human lymphoblast line that has been transfected with plasmids containing the cDNAs for several human CYPs (Penman et al., 1993). We found that CYP2D6 and CYP2E1 catalyze the cysteine conjugation of acetaminophen, whereas CYP1A2, CYP2E1 and CYP3A4 catalyze the protein binding. CYP1A1, CYP2A6 and CYP2B1 catalyzed neither reaction. These data suggest that CYP2E1 has catalytic sites both on the lumenal and cytosolic surfaces of the endoplasmic membrane, whereas CYP2D6 has its catalytic site only on the cytosolic side. Furthermore, these observations suggest that CYP1A2 and CYP3A4 have their catalytic sites only on the lumenal surface of the microsomal vesicles. Finally, we correlated the effect of the administration in mice of two inducers of cytochrome P450-dependent, oxidative metabolism and were induced to different concentrations after the administration of ethanol. Based on these observations and our finding that the protein binding appeared to be specific for intralumenal, and not membrane-bound, microsomal proteins, we concluded that some CYPs must have their catalytic site on the lumenal surface of the microsomal vesicle and others on the cytosolic surface (Prasad et al., 1990). Clearly, this model cannot be tested by purification and reconstitution studies, because the topology of the microsomes is lost during these procedures. In light of these problems, in the current investigation, we sought to determine whether there are apparent differences in the location of the catalytic site for the various CYPs by determining the relative rates of formation of the cysteine conjugate and protein adducts by microsomes from a human lymphoblast line that has been transfected with plasmids containing the cDNAs for several human CYPs (Penman et al., 1993). We found that CYP2D6 and CYP2E1 catalyze the cysteine conjugation of acetaminophen, whereas CYP1A2, CYP2E1 and CYP3A4 catalyze the protein binding. CYP1A1, CYP2A6 and CYP2B1 catalyzed neither reaction. These data suggest that CYP2E1 has catalytic sites both on the lumenal and cytosolic surfaces of the endoplasmic membrane, whereas CYP2D6 has its catalytic site only on the cytosolic side. Furthermore, these observations suggest that CYP1A2 and CYP3A4 have their catalytic sites only on the lumenal surface of the microsomal vesicles. Finally, we correlated the effect of the administration in mice of two inducers of cytochrome P450, ethanol (Peterson et al., 1980) and 3-methyl-1-butanol (Sinclair et al., 1989; Louis et al., 1994), on the concentrations of these CYPs in the hepatic microsomes with the rates of cysteine conjugation and protein binding. We found that the protein binding is primarily catalyzed by CYP2E1 whereas, in control animals, more than half of the cysteine conjugation is catalyzed by a member of the CYP2D family.

**Materials and Methods**

**Materials.** [1H(G)]Acetaminophen was obtained from DuPont/NEN (Boston, MA). Just before use it was purified by thin layer chromatography on silica gel GF plates (Analtech, Newark, DE) with methanol/water (70:30). The acetaminophen band was scraped and eluted with methanol. The methanol was removed in a stream of nitrogen. NADP+ glucose 6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were American Chemical Society reagent grade.

**Microsomal preparations.** Microsomes from a human lymphoblast line that had been transfected with the cDNAs for human CYP1A1, CYP1A2, CYP2A6, CYP2B1, CYP2D6, CYP2E1 and CYP3A4 were obtained from GenTest (Woburn, MA) (Penman et al., 1993). Microsomes from cells that had been transfected with the same plasmid but without the cDNA for a CYP were used as the background control. The cytochrome P450 contents of these preparations were determined by the supplier from the known turnover numbers of characteristic substrates for each of the CYPs (table 1). Hepatic microsomes were also prepared from 25-g CF1 male mice obtained from Harlan Laboratories (Madison, WI). The mice received either ethanol (10%) or 3-methyl-1-butanol (0.5%) in their drinking water for 4 weeks. The mice were sacrificed by guillotine. The livers were removed, quickly cooled on ice and homogenized in 3 ml/g liver in Tris-HCl (150 mM; pH 7.4, 4°C). The homogenates were centrifuged at 9000 × g for 15 min and the supernatants were centrifuged at 140,000 × g for 45 min. The pellets were resuspended in KCl-Tris and stored at −80°C until use.

**Determination of protein binding of acetaminophen.** The protein binding was determined as described previously (Peterson et al., 1980; Prasad et al., 1990). The samples containing microsomes (~3 mg/ml) were incubated with [1H(G)]acetaminophen (1 mM, 7.1 × 104 cpm), NADP+ (0.4 mM), glucose 6-phosphate (5.5 mM) and glucose-6-phosphate dehydrogenase (0.6 U/ml) in KCl-Tris-MgCl2 (3 ml) (150 mM; pH 7.5; 4°C) for 20 min at 37°C with constant shaking in air. The reaction was terminated by the addition of trichloroacetic acid (5% final concentration) to the incubate. The microsomes were then washed 14 times with increasing concentrations of methanol in water (Peterson et al., 1980). The final washes contained no radioactivity. The washed protein pellets were suspended in Solvable (0.5 ml) (DuPont/NEN). After incubation over night at room temperature, H2O2 (30%, 10 μl) was added to decolorize the solubilized proteins. This was followed by the addition of a scintillation fluid (10 ml) (Ultimata Gold, Packard Instrument, Des Plains, IL). The radioactivity was determined in a Tri-Carb 1900CA β-liquid scintillation counter (Packard Instrument). The rate of protein binding was taken as the difference between the activities of the transfected and control microsomes. These incubation conditions were linear with respect to time. The protein concentration was the lowest which consistently gave concentrations of products which were 10-fold greater than the minimum detectable concentration for both assays.

**Determination of the rate of cysteine conjugation.** The incubation conditions were the same as those used in the protein binding assay except that unlabeled acetaminophen was used. L-cysteine (1 mM) was included in the incubation mixture and the incubation time was decreased to 5 min to remain within the linear range (Zhou et al., in press). The concentration of the cysteine conjugate was determined by the HPLC method of Wilson et al. (1982). In this procedure the reaction was terminated by the addition of 30% trichloroacetic acid (0.5 ml) and the samples were centrifuged at 1500 × g for 10 min. The supernatant (1 ml) was neutralized with NaOH (0.5 M), 0.6 ml) and 20 μl were injected directly into a HPLC system consisting of an Altex 110a pump, a Rheodyne 7125 injection valve, an ODS-C18 column (Regis, Chicago, IL) and a Kratos 770 spectrophotometer set to 256 nm. The mobile phase consisted of methanol (7.0%) and glacial acetic acid (0.75%) in 0.1 M KH2PO4 (Wilson et al., 1982).

**Antibody studies.** Immunoblotting was performed according to the transblotting procedures described by Towbin et al. (1979). The blots were reacted with monoclonal antibodies which were specific for CYP2E1 (Mab 1–9–1) (Ko et al., 1987) and CYP1A1 and CYP1A2 (Mab 1–7–1) (Park et al., 1982), respectively. The latter antibody reacts with both CYP1A1 and CYP1A2, but the two can be distinguished by differences in their mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. They were also reacted with polyclonal antibodies to CYP2B1 (Gonzalez et al., 1987, 1988), CYP2D6 (Gonzalez et al., 1987, 1988) and to CYP3A4 (Wyatt et al., 1982).
The lymphoblast microsomes for the appropriate CYP were used as standards on each immunoblot to determine the concentration of each of the CYPs. An alkaline phosphatase reaction was used to develop the color. The indicator dye was a combination of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate was used to develop the color. The indicator dye was a combination of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. We next sought to determine the relative importance in mouse microsomes by immunoblotting, we found that microsomes for the appropriate CYP were transfected with CYP2E1 catalyzed cysteine conjugation (table 1). On the other hand, CYP1A2 and CYP3A4 with a turnover number about half of that observed for CYP3A4 (table 1), but we also found that CYP2D6 catalyzed this reaction (table 2). When we determined the content of the various CYPs in the mouse microsomes by immunoblotting, we found that inducing agents did not increase the protein binding (table 3).

**Results**

In line with the results of Raucy et al. (1989), Lee et al. (1991a, b) and Patten et al. (1993), we found that microsomes transfected with CYP2E1 catalyzed cysteine conjugation (table 1), but we also found that CYP2D6 catalyzed this reaction with a turnover number about half of that observed for CYP2E1 (table 1). On the other hand, CYP1A2 and CYP3A4 catalyzed protein binding but not cysteine conjugation (table 1). CYP2E1 also catalyzed the protein binding. CYP1A1, CYP2A6 and CYP2B6 did not catalyze either reaction.

We next sought to determine the relative importance in mouse microsomes of each isoform in these two oxidative pathways of acetaminophen metabolism. In this portion of the study we examined the overall rates of protein binding and cysteine conjugation after administration of one of two inducing agents, ethanol (Sinclair et al., 1989) or 3-methyl-1-butanol (Louis et al., 1994) (table 2). As in our previous studies, we found that ethanol induced protein binding by 265% (P < .05), but only increased cysteine conjugation by 61% (P < .05) (table 2). 3-Methyl-1-butanol had no significant effect on either pathway of acetaminophen metabolism.

When we determined the content of the various CYPs in the mouse microsomes by immunoblotting, we found that ethanol administration increased both CYP2B1 (159%) (P < .05) and CYP2E1 (289%) (P < .01) (table 3). Contrary to the observations of Sinclair et al. (1991) in isolated hepatocytes, we found that these inducing agents did not increase the CYP3A content in intact mice. Instead, after treatment for 4 weeks, the CYP3A content of the microsomes was 53% of control after both ethanol (P < .002) and 3-methyl-1-butanol (P < .02) administration. There was also a small, but non-significant decrease in the CYP1A2 content (table 3).

**Discussion**

We believe that our current data indicate that the active site of many CYPs is polarized to either the cytosolic or luminal surface of the microsomes, but not both. The sole exception to this in our study was CYP2E1, which clearly metabolizes acetaminophen on both surfaces of the membrane. This model is based on the acceptance of two assump-
tions. First, it is necessary that little or no NABQI can penetrate the membrane. If such is the case then, as we have noted in our kinetic studies, there will be two independent pools of NABQI with the membrane serving as a barrier between them. This assumption seems reasonable because this metabolite is highly reactive and is likely to form adducts with the free amino or thiol groups of the membrane proteins before it can reach the lumen. Furthermore, this assumption is supported by our observation that CYP2D6 has a turnover for the formation of NABQI which is only half of that of CYP2E1, but, unlike the latter CYP, it does not catalyze any protein binding. This total lack of protein binding is only likely if its catalytic site is on the cytosolic surface and the membrane itself is essentially impermeable to NABQI. Although the failure of CYP1A2 and CYP3A4 to catalyze cysteine conjugation lends support to this model, it is not strong evidence, because the turnover numbers of these CYPs for the formation of NABQI might have been low, hence the yields of the cysteine conjugate may have been below the detection limit of our current procedure. This seems unlikely because other workers have shown in studies with solubilized preparations that CYP1A2 has a high turnover number for the formation of the cysteine conjugate (Raucy et al., 1989; Lee et al., 1991a).

The second assumption is that the proteins forming the adducts are truly intraluminal (Zhou et al., 1996). This assumption is based on much stronger evidence. As noted above, we have found in our purification studies that acetaminophen forms adducts with only three microsomal proteins: calreticulin (Chen et al., 1996) and the two forms of the thiol:protein disulfide oxidoreductase, Q-2 and Q-5 (Srivastava et al., 1991; Zhou et al., 1996). Although low concentrations of the oxidoreductases are membrane-bound, the bulk of these two proteins are found within the lumen. Yet, even if the NABQI is reacting with membrane-bound oxidoreductases, we know some NABQI is formed within the microsomal lumen because calreticulin is restricted to this compartment. Hence, we feel that the body of evidence supports the assumption that acetaminophen forms adducts with only intraluminal proteins. In light of these considerations our data are consistent with the concept that some CYPs have their active site only on the cytosolic surface of the membrane, whereas others have it only on the luminal side and at least one has it on both.

These conclusions are supported by our inhibitor studies on the metabolism of acetaminophen through these two pathways (Prasad et al., 1990). Whereas CO, imidazole and metyrapone profoundly inhibited cysteine conjugation, they had little (CO and metyrapone) or a markedly reduced effect (imidazole) on protein binding. Because these inhibitors are relatively hydrophilic, they may not penetrate the membrane and therefore would not inhibit intraluminal, catalytic sites. On the other hand, SKF-525A, which is quite hydrophobic and would therefore readily penetrate the membrane, inhibited both pathways. The only anomalous inhibitor was KCN, which may be more ionic, but had similar effects on the rates of the two reactions. We can also use the turnover numbers we obtained for the microsomes from the human lymphoblasts to estimate in mouse microsomes the relative importance of each of the CYPs in the two pathways for the metabolism of acetaminophen. On the basis of this approach, we found that, in hepatic microsomes from control mice, CYP2E1 catalyzes only 44% of the cysteine conjugation (table 4). On the other hand, it catalyzes 83% of the protein binding. In microsomes from animals treated with ethanol, these percentages increased for both reactions, but, as would be expected from the observations of the relative role of each of the CYPs in the metabolism in control mice, the protein binding was much more sensitive to the increase in CYP2E1 than was the cysteine conjugation.

A problem with these estimates of the relative importance of each of the CYPs in the metabolism of acetaminophen is that the microsomes we used as standards for our immunooassays were derived from lymphoblasts transfected with cDNAs for human and not mouse CYPs. Because the relative turnover numbers and the reactivity with the various antibodies for the CYPs from the two species may be quite different, our data can only give a qualitative estimate of the relative importance of the various isoforms in the metabolism of acetaminophen. Unfortunately, comparable preparations from cells transfected with cDNAs for mouse CYPs are not available. Patten et al. (1993) had a similar problem because they used microsomes from Hep G2 which had been transfected with the same cDNAs for human CYPs. Furthermore, the Hep G2 line is not an ideal cell type to use because the expression of the CYPs is unstable. Hence, until the lymphoblast line, or a similar stable, in vitro cell system, is transfected with the cDNAs for mouse CYPs, we can only make qualitative estimates concerning the relative importance of the various CYPs in the two metabolic pathways for acetaminophen in mice.

Our studies are only in partial agreement with previous work from other laboratories (Raucy et al., 1989; Lee et al., 1991a, b). These groups identified both CYP2E1 and 1A2 as the major isoforms catalyzing the metabolism of acetaminophen. Yet, there are potentially significant differences in the methodology among the different studies which may explain these differences. First, both of these groups only determined the cysteine conjugation. Furthermore, in the bulk of their studies, they examined this metabolism in purified systems or they used antibodies to the CYPs in intact microsomes. It is possible that these antibodies may not have been able to penetrate into the lumen and therefore would not be able to block protein binding. Another possible reason for these differences is that, in the report by Patten et al. (1993), they used markedly different incubation conditions to determine the cysteine conjugation. In particular, they included 15 mM GSH in their incubation medium, whereas we added only 1 mM cysteine. Furthermore, they incubated for 40 min.

### TABLE 4

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<thead>
<tr>
<th>Treatment</th>
<th>Cysteine Conjugation</th>
<th>Protein Binding</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>44.3 ± 1.7</td>
<td>83.3 ± 2.2</td>
</tr>
<tr>
<td>3-Methyl-1-butanol</td>
<td>73.9 ± 1.6</td>
<td>95.3 ± 0.2</td>
</tr>
<tr>
<td>3-Methyl-1-butanol</td>
<td>44.6 ± 1.2</td>
<td>85.2 ± 1.0</td>
</tr>
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</table>

* Values are the average of three pools of three livers/pool ± S.E.M.
* Values calculated from the specific activities of expressed CYPs and their percentages in the mouse microsomes as determined by immunoblotting.
whereas we have found that the reaction was not linear for more than 5 min under our incubation conditions. The higher concentration of GSH may have increased the time for which this reaction is linear. These differences in the incubation conditions may also be the reason why we observed activities which were 1 to 2 orders of magnitude higher than those reported by these previous workers. Finally, neither of these groups examined the role of CYP2D6 in the metabolism of acetaminophen.

Yet in spite of these possible problems, our data do clearly indicate that CYP2E1 has a predominant role in the intraluminal metabolism of acetaminophen. If the toxicity of this drug is related to the formation of adducts with the intraluminal proteins, then our data suggest that CYP2E1 should be the primary enzyme catalyzing the synthesis of this critical pool of NABQI. This concept is supported by our studies on the effect of ethanol administration on acetaminophen toxicity. In these investigations we demonstrated that there is a strong correlation between the toxicity of acetaminophen and the in vitro formation of microsomal protein adducts (Peterson et al. 1980). This model has gained further support from the recent studies of Lee et al. (1996) in which they developed a mouse "knock-out" model lacking CYP2E1. In preliminary investigations they found that acetaminophen was significantly less toxic in the CYP2E1-deficient strain than in the parent strain, again supporting the role for CYP2E1 in catalyzing the formation of the critical pool of NABQI which mediates the toxicity of acetaminophen.

Another interesting aspect of our current observations is that if any of the CYPs do have their active site on the luminal surface of the microsomes, it might suggest that there is a coupling between the metabolism by the oxidative enzymes and the major conjugating enzymes, the UDPG-glucuronyltransferases. It is well known that the activity of these latter enzymes in intact microsomes is markedly increased by the addition of low concentrations of detergents. This observation has been interpreted to indicate that the catalytic sites of these enzymes are on the luminal surface of the microsomes. It has been thought that the addition of enough detergent to make the microsomes leaky, but not enough to totally disrupt the membrane, allows the two substrates for this enzyme, UDPG-glucuronic acid and the appropriate xenobiotics, to more readily penetrate the microsomal membrane to get to their active sites. Our observation that some CYPs also have their active site on the luminal surface of the microsomes suggests that the hydrophilic metabolic products of oxidative metabolism have ready access to the UDPG-glucuronyl transferases without having to traverse the membrane.

Yet one problem with this model is that some procarcinogens, such as the aromatic amines and estrogens, are thought to be activated by CYP1A2 and CYP3A4. If these CYPs have their catalytic sites on the luminal surface of the endoplasmic reticulum, then the question arises about how the active metabolites of these agents can penetrate the various membranes to form DNA adducts, yet all of these metabolites are relatively large molecules which may be sufficiently hydrophobic to penetrate the membranes to reach the nucleus. Furthermore, the nuclear membrane is contiguous with the endoplasmic reticulum membrane and may contain some of these same metabolic enzymes (Oesch et al., 1985; Cavaleri et al., 1990). Hence, these reactive metabolites may be formed in close proximity to the chromosomes.

Finally, the topology of the formation of NABQI may have profound implications for the elucidation of the mechanism of acetaminophen toxicity. To date the bulk of the studies in this process have centered on the formation of cytosolic adducts (Birge et al., 1987; Pumford et al., 1990), alterations in Ca++ homeostasis (Hardwick et al., 1992) and decreases in mitochondrial function (Harris and Hamrick, 1993). Our observation that acetaminophen forms adducts with three intraluminal proteins presents a fourth possible toxic mechanism for acetaminophen. A recent body of literature indicates that the proteins which we have found to be adducted play a critical role in the posttranslational modification of membrane and secretory proteins (for a review, see Holtzman, in press, 1997). Furthermore, studies in yeast have suggested that eukaryotic cells require these proteins for survival (La Mantia et al., 1991). Hence the critical event in the toxicity of acetaminophen may be the inactivation of this posttranslational system. As a result the cell is no longer able to replace plasma membrane proteins as they turnover. This could lead to the blebbing of these membranes which has been observed in hepatocyte cultures incubated with toxic concentrations of acetaminophen (Hardwick et al., 1992). Clearly, the validation of this model will have to await further studies.

In conclusion, our present data indicate that the microsomal metabolism of acetaminophen leads to the production of two pools of NABQI: one within the lumen of the microsomes and a second on the outer surface. Furthermore, the presence of these two pools seems to be caused by differences in the location of the catalytic sites of the various CYPs that can metabolize acetaminophen to NABQI. Our current data suggest that CYP2E1 catalyzes the formation of the bulk of the intraluminal pool and thereby mediates microsomal, protein, adduct formation. On the other hand, CYP2D6 shares a major role in catalyzing the metabolism on the cytosolic surface. Both of these CYPs probably catalyzed adduct formation to a large number of cytotoxic proteins (Birge et al., 1987; Pumford et al., 1990). To our knowledge, these are the first data to suggest that there are significant differences in the location of the active sites of the various CYPs.

References
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