CYTOCHROME P-450 1A1 EXPRESSION IN HUMAN SMALL BOWEL: INTERINDIVIDUAL VARIATION AND INHIBITION BY KETOCONAZOLE

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(Received September 30, 1998; accepted December 21, 1998)

This paper is available online at http://www.dmd.org

ABSTRACT:

Human cytochrome P-450 1A1 (CYP1A1) is located primarily in extrahepatic tissues. To begin the characterization of this enzyme in the small intestine, we screened a bank of 18 human small intestinal microsomal preparations for CYP1A1 catalytic [7-ethoxyresorufin O-deethylase (EROD)] activity and protein content. Although EROD activity was below detectable limits in 12 of the preparations, 6 exhibited measurable activity (1.4–123.5 pmol/min/mg), some exceeding that for 2 human liver microsomal preparations (11.0 and 26.4 pmol/min/mg). This variation was not due to variable quality of the preparations because each sample displayed readily detectable CYP3A4 catalytic activity and immunoreactive protein. We inadvertently found that intestinal EROD activity was inhibitable by ketoconazole at a concentration commonly believed to selectively inhibit CYP3A4. The possibility that CYP3A4 metabolizes 7-ethoxyresorufin was excluded because there was no correlation between intestinal CYP3A4 catalytic and EROD activity, and cDNA-expressed human CYP3A4 exhibited no EROD activity. Moreover, CYP1A1 immunoreactive protein was most abundant in the three intestinal preparations with the highest EROD activities, and the mean apparent K_i of ketoconazole observed for these three preparations (40 nM) was essentially identical with that for cDNA-expressed human CYP1A1 (37 nM). In summary, there is large interindividual variation in CYP1A1 expression in human small bowel, and ketoconazole is not a selective CYP3A4 inhibitor in in vitro metabolism studies involving intestinal tissue obtained from some individuals. These observations raise the possibility that in vivo drug interactions involving ketoconazole could result from CYP1A1 inhibition in the intestine in some individuals.

Human cytochromes P-450 (CYP)1 1A1, 1A2, and 3A4 bioactivate a number of procarcinogens that are ubiquitous in our environment. Such compounds include the polycyclic aromatic hydrocarbons found in cigarette smoke (e.g., benzo[a]pyrene), the heterocyclic aromatic amines found in cooked meat (e.g., aflatoxin B1), quinone-diazob[4,5-b]quinoline, and mycotoxins found in some grains (e.g., aflatoxin B1) (Gonzalez and Gelboin, 1995). These isozymes, particularly CYP3A4, also metabolize a myriad of commonly prescribed drugs. In adults, CYP3A4 is the major CYP isozyme expressed in both the small intestine and liver (Watkins et al., 1987; Shimada et al., 1994). CYP1A2, however, appears to be expressed only in the liver, whereas CYP1A1 is located primarily in extrahepatic tissues (Guengerich, 1995).

Our laboratory and others have characterized CYP3A along the entire digestive tract (Kolars et al., 1994; Paine et al., 1997) and have shown that the small intestine contributes significantly to the first-pass metabolism of several CYP3A substrates, including cyclosporin (Kolars et al., 1991; Hebert et al., 1992), midazolam (Paine et al., 1996; Thommel et al., 1996), verapamil (Fromm et al., 1996; Fromm et al., 1998), and nifedipine (Holtebocker et al., 1996). CYP1A1 has been well characterized in the placenta (Sesardic et al., 1990; Hakko et al., 1996) and lung (Wheeler et al., 1990; Shimada et al., 1992; Shimada et al., 1996). CYP1A1 immunoreactive protein, mRNA, and catalytic [7-ethoxyresorufin O-deethylase (EROD)] activity have also been detected at low levels in human small bowel in previous studies involving small numbers of human subjects (McDonnell et al., 1992) or microsomal preparations (Peters and Kremers, 1989; Prueksaritanont et al., 1996).

To further characterize intestinal CYP1A1, we examined a bank of 18 human small intestinal microsomal preparations and found great variability in both EROD activity and CYP1A1 immunoreactive protein content. We also serendipitously found that ketoconazole, at a concentration typically used to selectively inhibit CYP3A-mediated reactions, virtually abolished EROD activity. Subsequent studies revealed that small intestinal CYP1A1 is clearly inhibitable by ketoconazole, with a sensitivity comparable with that for CYP3A4. These observations may have important implications to investigators studying drug-drug interactions that occur at the level of the small intestine.

Materials and Methods

7-Ethoxyresorufin, resorufin, ketoconazole, quinidine, NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, 5,6-benzoflavon, and 7,8-benzoflavone were purchased from Sigma Chemical Co. (St. Louis, MO). Sulphaphenazole and fluvoxamine were kindly provided by our colleague, Dr. Michael E. Fitzsimmons. Insect cell microsomes containing cDNA-expressed human CYP1A1, CYP1A2, or CYP3A4 and control insect cell microsomes

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(P211, P203, P207, and P201, respectively) were purchased from Gentest (Woburn, MA). The polyvinylidene difluoride membrane (Hybond-P) and enhanced chemiluminescence kit were purchased from Amersham (Arlington Heights, IL). All other chemicals were of reagent grade or better.

**Human Tissue Preparation.** Liver microsomes and three different intestinal tissue preparations (microsomes, small bowel biopsy homogenate, and Caco-2 cell homogenate) were used for study. The liver microsomes had been previously prepared and characterized for midazolam 1'-hydroxylase activity as described (Paine et al., 1997). The small intestinal microsomes, a generous gift from our colleague, Dr. Michael E. Fitzsimmons, had been previously prepared from whole mucosal tissue, obtained by scraping the length of the organ, and characterized for saquinavir oxidase activity as described (Fitzsimmons and Collins, 1997). Two human liver (HL) and 18 intestinal (HG) preparations were examined. Medical and drug histories were available for the liver donors (HLS 103 and 152) and for only eight of the intestinal donors (HGs B1, B2, B4, B5, 19, 23, 24, and 29). Upper small bowel pinch biopsies (~5 mg) had been previously obtained by endoscopy from healthy volunteers in an unrelated study in which the subjects had received a diet rich in char-grilled meat for 1 week (Fontana et al., 1997). The biopsies were immediately homogenized in solution D (0.05 M Tris-HCl, 20% glycerol, 2 mM EDTA, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride using a conical ground glass tissue grinder (Woburn, MA). The polyvinylidene difluoride membrane (Hybond-P) and immunoreactive protein were readily detectable in all preparations with the highest EROD activities (HGs 19, 26, and 30). A 5 × 6 matrix of substrate and inhibitor concentrations was employed. Substrate solutions were serially diluted from an ethanol stock solution (0.3 M) in phosphate buffer to yield concentrations ranging from 0.16 to 4.0 μM; ketoconazole solutions were serially diluted from an ethanol stock solution (0.15 μM) in ethanol to yield concentrations ranging from 2.5 to 40 μM. Final substrate and inhibitor concentrations both ranged from 0 to 160 mM. Final ethanol concentrations were ~0.4%. Initial estimates of apparent Kᵢ and V_max were obtained from an Eadie-Hofstee plot; an initial estimate of Kᵢ was obtained from a Dixon plot. The mechanism of inhibition was determined from Dixon and Cornish-Bowden plots (Cornish-Bowden, 1974). Final apparent kinetic parameters were obtained by nonlinear least-squares regression using PCNONLIN (v4.2, SCI Software, Lexington, KY). Intrinsic clearance (microsomes) or turnover number (expressed enzyme, CYP1A1), CLₜwitter was calculated by dividing V_max by Kᵢ.

**Western Blot Analysis of CYP1A1 and CYP3A4.** Liver and intestinal microsomes were analyzed for CYP1A1 and CYP3A4 proteins as previously described (Paine et al., 1997), except that the proteins were transferred to a polyvinylidene difluoride membrane (overnight) and incubated with a polyclonal goat antibody that recognizes both rat CYP1A1 and CYP1A2 (Gentest, Woburn, MA) or a mouse monoclonal antibody that recognizes all major human CYP3A isoforms (3A4, 3A5, and 3A7) (Beaune et al., 1985). Purified human CYP3A4, used as a reference standard, was a gift from Dr. Steven A. Wrighton (Eli Lilly, Indianapolis, IN). Binding of the secondary antibody, horseradish peroxidase-conjugated mouse anti-goat IgG (Zymed Laboratories, San Francisco, CA), was visualized by enhanced chemiluminescence.

**EROD Assay.** The rate of resorufin formation was measured using a SPEX Model CM-1TII recording spectrophotofluorometer (SPEX Industries, Inc., Edison, NJ). The excitation and emission wavelengths were set at 530 and 585 nm, respectively (Burke et al., 1994). 7-Ethoxyresorufin was diluted from an ethanol stock solution (1 mM) in phosphate buffer (0.1 M potassium phosphate, 1 mM EDTA, 5 mM MgCl₂, pH 7.4) to yield a concentration of 25 μM. Microsomal protein was diluted in phosphate buffer so that 0.2 ml contained 0.02 to 0.1 mg protein. A NADPH-generating system (25 mM NADP, 0.25 M glucose 6-phosphate, and 25 U/ml glucose 6-phosphate dehydrogenase) was freshly prepared in phosphate buffer. The following were added to a disposable cuvette: 0.2 ml microsomal protein, 2.1 ml phosphate buffer, 0.1 ml NADPH-generating system, and a stir bar. The cuvette was placed into the spectrophluorometer, whose cuvette holder was heated to 37°C. Following establishment of a steady baseline under gentle mixing (~30 s), the reaction was initiated with 0.1 ml substrate. The fluorescence of resorufin was monitored continuously over 1 to 5 min; the rate of product formation was always linear during this interval and over the protein concentration range studied. Final substrate, NADPH, and ethanol concentrations were 1 μM, 1 mM, and 0.1% (v/v), respectively. The amount of metabolite formed during a 1- to 3-min interval was quantified by comparing the change in fluorescence to a standard curve prepared by the addition of known amounts of resorufin (0–10 pmol) to phosphate buffer. The inter- and intraday variability of the assay were 7.3% and <8%, respectively, with a limit of quantitation of 0.3 pmol.

**Chemical Inhibition Studies.** Intestinal microsomes with the highest EROD activity (HG-30) and one of the liver microsomal preparations (HL-103) were incubated with the following CYP isoform inhibitors: 3 μM fluvoxamine (CYP1A2), 1 μM 7,8-benzoflavone (CYP1A1), 0.3 μM quinidine (CYP2D6), 2 μM sulfaphenazole (CYP2C9) and 3 μM ketoconazole (CYP3A4). All inhibitors were dissolved in ethanol as 250-fold concentrated solutions. Incubations were performed in the same manner as described earlier except that the inhibitor (10 μL) was added to the cuvette first. Incubations with expressed enzymes and homogenate were performed in the same manner as described for microsomes except that 0.5, 2, or 10 pmol expressed enzyme (CYP1A1, CYP1A2, or CYP3A4, respectively), 0.1 mg small bowel biopsy homogenate, or 0.01 mg Caco-2 cell homogenate were added to the cuvette. Results were compared with control incubations containing 10 μl ethanol. The final ethanol concentration in these incubations was 0.5%.

**Determination of Apparent Kᵢ for Ketoconazole-CYP1A1.** The apparent Kᵢ for the inhibitor-enzyme pair, ketoconazole-CYP1A1, was determined using cDNA-expressed human CYP1A1 and the three intestinal microsomal preparations that exhibited the highest EROD activities (HGs 19, 26, and 30). A 5 × 6 matrix of substrate and inhibitor concentrations was employed. Substrate solutions were serially diluted from an ethanol stock solution (0.3 M) in phosphate buffer to yield concentrations ranging from 0.16 to 4.0 μM; ketoconazole solutions were serially diluted from an ethanol stock solution (0.15 μM) in ethanol to yield concentrations ranging from 2.5 to 40 μM. Final substrate and inhibitor concentrations both ranged from 0 to 160 mM. Final ethanol concentrations were ~0.4%. Initial estimates of apparent Kᵢ and V_max were obtained from an Eadie-Hofstee plot; an initial estimate of Kᵢ was obtained from a Dixon plot. The mechanism of inhibition was determined from Dixon and Cornish-Bowden plots (Cornish-Bowden, 1974). Final apparent kinetic parameters were obtained by nonlinear least-squares regression using PCNONLIN (v4.2, SCI Software, Lexington, KY). Intrinsic clearance (microsomes) or turnover number (expressed enzyme, CYP1A1), CLₜwitter was calculated by dividing V_max by Kᵢ.

**Statistical Analysis.** Correlation analyses were performed using Sigmaplot for Windows (v1.0; Jandel Corp., San Rafael, CA). Correlation coefficients (Pearson product-moment r) were considered significant if the p value was < 0.05.

**Results**

**Interindividual Variation in CYP1A1.** Western blot analysis of human small intestinal and control liver microsomes for CYP1A1 protein revealed the hepatic protein (CYP1A2) to migrate slightly faster than the intestinal protein (CYP1A1) (Fig. 1A). This is consistent with CYP1A2 having a lower molecular mass compared with CYP1A1 (54 versus 57 kD) (Sesaridic et al., 1990). Although CYP1A1 immunoreactive protein was readily detectable in only the three intestinal preparations with the highest EROD activities (HGs 19, 26, and 30), six exhibited measurable EROD activity (Table 1). In addition, the activities displayed by four of these were comparable with or exceeded those for the two liver microsomal preparations. Of the eight donors for which medical and drug histories were available, three were cigarette smokers, yet exhibited undetectable EROD activity (HGs 29, B1, and B5). Donor HG-19, who had high EROD activity, was known not to be a smoker.

In contrast to CYP1A1, CYP3A4 catalytic activity (saquinavir oxidation, sum of metabolites M-2 and M-7; Fitzsimmons and Collins, 1997) and immunoreactive protein were readily detectable in all samples tested (Fig. 1B). Moreover, there was a significant correlation between CYP3A4 activity and protein content (r = 0.80; p < .001). There was no correlation between intestinal EROD activity and CYP3A4 catalytic activity (r = 0.52; p = .29). Due to the high background on the CYP1A1 immunoblot, we were not able to obtain reliable estimates of CYP1A1 protein content.
Lanes 1–8 (donors B1, B2, B4, B5, 19–21, and 23, respectively) and 12–20 (donors 24–32, respectively) were loaded with 40 μg (A) or 5 μg (B) microsomal protein. Human liver microsomes (HL-103, 1 μg) and cDNA-expressed human CYP1A1 and CYP1A2 (50 pmol each) or purified human CYP3A4 (0.1, 0.5, and 1 pmol) were used as references.

**Effects of Chemical Inhibitors on CYP1A Catalytic Activity in Intestine and Liver.** The effects of 7,8-benzoflavone, quinidine, and sulfaphenazole on EROD activity (>95%, <10%, and <15% inhibition, respectively) were similar between human small intestinal and liver microsomes. Unexpectedly, fluvoxamine and ketoconazole exhibited discordant results between the two tissues. That is, fluvoxamine abolished EROD activity in liver microsomes but had little effect in intestinal microsomes, whereas ketoconazole abolished EROD activity in intestinal microsomes but had little effect in liver microsomes. Thus the effects of ketoconazole and fluvoxamine on EROD activity were further examined in cDNA-expressed human CYP1A1, CYP1A2, and CYP3A4; human small bowel biopsy homogenate; and Caco-2 cell homogenate and compared with results in microsomes. The effects of ketoconazole and fluvoxamine on EROD activity in the liver microsomes, the various intestinal preparations, and expressed CYP1A1 and CYP1A2 are shown in Fig. 2. Results from the intestinal preparations mirrored those from expressed CYP1A1, whereas results from liver microsomes mirrored those from expressed CYP1A2. Corresponding rates of resorufin formation in control incubations for expressed CYP1A1 and CYP1A2 were 5.7 and 1.4 pmol/min/pmol, respectively. Corresponding control rates for HG-19, HG-30, small bowel biopsy homogenate, Caco-2 cell homogenate, HL-103, and HL-152 were 30.6, 99.0, 12.6, 111.5, 22.0, and 8.0 pmol/min/mg, respectively. Expressed CYP3A4 and control insect cell microsomes exhibited no EROD activity.

**TABLE 1**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Rate of Resorufin Formation (pmol/min/mg)</th>
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<tbody>
<tr>
<td>HG-B2</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>HG-19</td>
<td>35.8 ± 0.7</td>
</tr>
<tr>
<td>HG-24</td>
<td>6.7 ± 0.5</td>
</tr>
<tr>
<td>HG-26</td>
<td>56.4 ± 3.2</td>
</tr>
<tr>
<td>HG-30</td>
<td>123.5 ± 7.5</td>
</tr>
<tr>
<td>HG-32</td>
<td>11.5 ± 0.4</td>
</tr>
<tr>
<td>HL-103</td>
<td>26.4 ± 1.7</td>
</tr>
<tr>
<td>HL-152</td>
<td>11.0 ± 0.4</td>
</tr>
</tbody>
</table>

Each value denotes mean (± S.D.) of triplicate incubations. HG, human small intestine; HL, human liver.

Results from this study demonstrate great interindividual variability in the expression of CYP1A1 in human small bowel. Using 7-ethoxyresorufin-O-deethylation as an indicator of CYP1A1 catalytic activity, resorufin formation was measurable in one-third of the intestinal microsomal samples tested. EROD activity in some of these samples was comparable with or exceeded that in liver microsomes observed in the current study and by others (Williams et al., 1986; Shimada et al., 1996). This variation in intestinal CYP1A1 was not accounted for by smoking habits nor concomitant medications because three donors known to be smokers exhibited undetectable EROD activity, while one nonsmoker exhibited high EROD activity. In addition, this nonsmoker had not been taking omeprazole, the only drug reported to induce CYP1A isoforms in humans (McDonnell et al., 1992).

Unfortunately, diet histories were not available for these donors because interindividual differences in the intake of food-containing CYP1A inducers (e.g., polycyclic aromatic hydrocarbons and heterocyclic aromatic amines) could potentially have accounted for at least some of the variation in intestinal CYP1A1. Indeed, our laboratory recently found that duodenal CYP1A1 mRNA and protein levels (Fontana et al., 1997) and EROD activity (our unpublished observations), all undetectable at baseline in 10 healthy subjects, became readily detectable following a 7-day diet rich in chargrilled meat.

**Fig. 1.** Western blots of human small intestinal microsomes (HG) showing presence of CYP1A1 protein in only three samples (A) or CYP3A4 protein in all samples (B).

**Fig. 2.** Comparative inhibitory effects of ketoconazole (3 μM) and fluvoxamine (3 μM) toward EROD activity in cDNA-expressed human CYP1A1 and CYP1A2, small intestinal microsomes (HGs 19 and 30), small intestinal biopsy homogenate (HG-bx), Caco-2 cell homogenate (Caco-2), and liver microsomes (HLS 103 and 152).

Each bar represents mean activity of triplicate incubations containing inhibitor, expressed as percentage of mean control activity (triplicate incubations containing ethanol); variation in all triplicate incubations was < 8%. ND, not detectable.

**EROD Kinetics and Inhibition by Ketoconazole.** The inhibitory potency (apparent \( K_i \)) of ketoconazole toward CYP1A1 was determined for the three intestinal microsomal preparations with the highest EROD activities and compared with that for expressed CYP1A1. Results from HG-26 and expressed enzyme are depicted in Figs. 3 and 4, respectively. For both microsomes and expressed enzyme, the apparent \( K_i \) of 7-ethoxyresorufin-O-deethylation increased, whereas the \( V_{max} \) decreased with an increase in inhibitor concentration, indicating that ketoconazole is a mixed-type inhibitor of CYP1A1. The data were well described by the simplest of mixed systems, linear mixed-type inhibition (Segel, 1975), and the apparent kinetic parameters (\( K_m \), \( V_{max} \), and \( K_i \)) for each preparation are summarized in Table 2.

**Discussion**

Results from this study demonstrate great interindividual variability in the expression of CYP1A1 in human small bowel. Using 7-ethoxyresorufin-O-deethylation as an indicator of CYP1A1 catalytic activity, resorufin formation was measurable in one-third of the intestinal microsomal samples tested. EROD activity in some of these samples was comparable with or exceeded that in liver microsomes observed in the current study and by others (Williams et al., 1986; Shimada et al., 1996). This variation in intestinal CYP1A1 was not accounted for by smoking habits nor concomitant medications because three donors known to be smokers exhibited undetectable EROD activity, while one nonsmoker exhibited high EROD activity. In addition, this nonsmoker had not been taking omeprazole, the only drug reported to induce CYP1A isoforms in humans (McDonnell et al., 1992).
Other commonly ingested foods, leafy vegetables for example, contain high levels of various flavone and indole derivatives that are known to induce and/or inhibit CYP1A isoforms (Yang et al., 1992; Zhai et al., 1998); their effects on intestinal CYP1A1 in humans, however, have not been rigorously tested.

Because ketoconazole is commonly believed to be a selective inhibitor of CYP3A4 (Baldwin et al., 1995; Bourrié et al., 1996), we were initially surprised to find that it potently inhibited the formation of resorufin in human intestinal tissues (microsomes, small bowel biopsy homogenate, and Caco-2 cell homogenate). Because CYP3A4 is the major CYP isoform expressed in the intestine (Watkins et al., 1987), one explanation for this finding was that CYP3A4 must also metabolize 7-ethoxyresorufin. However, CYP3A4 catalytic activity did not correlate with EROD activity in intestinal microsomes and cDNA-expressed human CYP3A4 displayed no EROD activity. Our conclusion that ketoconazole inhibited CYP1A1 was then confirmed by several observations. First, the three intestinal microsomal preparations with the highest EROD activities exhibited readily detectable CYP1A1 immunoreactive protein. Second, as expected, the apparent \( K_m \) for 7-ethoxyresorufin \( O \)-deethylation varied little among the three intestines, with a mean value near the apparent \( K_m \) for expressed enzyme (46 versus 24 nM). These values were within the range reported by others who used microsomes prepared from human CYP1A1-expressing yeast or B-lymphoblastoid cells (17–92 nM) (Eugster et al., 1990; Eugster and Sengstag, 1993; Penman et al., 1994). Finally, the mean apparent \( K_i \) observed for ketoconazole in intestinal microsomes was essentially identical with that observed for expressed CYP1A1 (22 nM). Our findings confirm these often overlooked earlier reports.

In conclusion, there is large interindividual variation in human small intestinal CYP1A1 expression that cannot be accounted for by cigarette smoking. Our observation that CYP1A1 is highly sensitive to inhibition by ketoconazole has implications concerning in vitro drug–drug interactions when using intestinal tissue obtained from some individuals. Catalytic activity that is inhibitable by ketoconazole can-
not be assumed to reflect only CYP3A4 unless the substrate is known not to be metabolized by CYP1A1, or the absence of active CYP1A1 in the tissue has been confirmed. Likewise, if intestinal CYP1A1 contributes significantly to the first-pass metabolism of drugs in some individuals, in vivo ketoconazole-drug interactions cannot be assumed to reflect only CYP3A4.

References


Eugster H-P and Sengstag C (1993) Xenobiotics are not to be metabolized by CYP1A1, or the absence of active CYP1A1 in intestinal epithelial cells. Drug Metab Dispos 21:931–937.


