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Short Communication

(+)-BUFURALOL 1′-HYDROXYLATION ACTIVITY IN HUMAN AND RHESUS MONKEY INTESTINE AND LIVER

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Abstract—(+)-Bufuralol 1′-hydroxylation, a commonly used marker of hepatic CYP2D6 activity, was investigated in human and rhesus monkey intestinal microsomes and compared with that in hepatic microsomes. The cumene hydroperoxide (CuOOH)-mediated metabolism of (+)-bufuralol suggested that at least two enzymes were responsible for bufuralol 1′-hydroxylation in both human and monkey intestinal microsomes. In contrast, the kinetics of the CuOOH-mediated metabolism in human and monkey livers were monophasic. The K_m values for the higher affinity component of the intestinal enzyme(s) of both species were similar to, while the corresponding V_{max} values were much lower than, those obtained with the livers. Bufuralol metabolism mediated by NADPH exhibited biphasic kinetics and was less efficient than that observed in the presence of CuOOH in both human and monkey intestines, in agreement with the observations in the livers. Inhibition of bufuralol hydroxylase activity in the intestine and liver preparations from the same species by known CYP2D6 inhibitors/substrates was qualitatively similar. Quinidine was the most potent inhibitor of (+)-bufuralol 1′-hydroxylation in all tissues studied. Western immunoblots using anti-CYP2D6 peptide antibody revealed a protein band in human and monkey intestinal microsomes of the same molecular weight as that observed in the liver preparations. The intestinal CYP2D protein content appeared to be much less than that of liver, and correlated with the (+)-bufuralol hydroxylase activity. Immunoinhibition studies indicated significant (up to 50%) inhibition of the CuOOH-mediated (+)-bufuralol metabolism in human and monkey intestines only by anti-CYP2D6, and not by anti-CYP2A6, or anti-CYP2E1. Inhibition of the bufuralol 1′-hydroxylase activity by anti-rat CYP3A1 was only slight (20%) in human, but marked (60–65%) in monkey intestinal microsomes. The hepatic metabolism of (+)-bufuralol in humans and monkeys was only inhibited (75%) by anti-CYP2D6, but not by anti-CYP3A1. Overall, the results suggest that (1) tissue and species differences exist in the catalysis of (+)-bufuralol 1′-hydroxylation, and (2) CYP2D6-related enzymes are partially or primarily responsible for the bufuralol hydroxylase activity in human and monkey intestines or monkey liver.

Key words: CYP2D6; bufuralol hydroxylation; human; rhesus monkey; intestine; liver

The intestine is the major route for exposure to xenobiotics administered orally. Such compounds may be activated or deactivated by intestinal enzymes before or during absorption, resulting in increased toxicity or diminished efficacy. Several enzyme systems, including cytochromes P450, have been identified in the small intestine [1–3]. Among the intestinal P450 enzymes, CYP3A, a major P450 subfamily in human intestine, has been studied extensively [4–6]. Although enzyme(s) immunologically related to CYP2D6 has been detected in human intestine [7], no detailed studies have been reported. This paper reports the catalytic activity of the human intestine towards (+)-bufuralol, a commonly used CYP2D6 marker substrate. The intestinal (+)-bufuralol 1′-hydroxylase activity also was compared with those in human liver. To date, only African green (*Cercopithecus aethiops*) and crab-eating (*Macaca fascicularis*) monkeys have been demonstrated to be good animal models for enzymatic studies of human hepatic CYP2D6 [8–10]. However, there have been no reports of the presence of enzymes of the CYP2D subfamily in the intestine of these monkeys, or in rhesus monkey (*Macaca mulatta*) intestine and liver. Because rhesus monkeys have been used extensively to study the metabolism and disposition of new drug candidates, we also carried out a comparative study using intestine and liver preparations from these species.

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† Abbreviation: CuOOH, cumene hydroperoxide.

Materials and Methods

Chemicals. (+)-Bufuralol was a gift from Hoffman–La Roche (Nutley, NJ). 1′-Hydroxybufuralol was purchased from Ultrafine Chemicals (Manchester, UK). NADPH, CuOOH†, quinidine, quinine, debrisoquine, ajmaline and dextromethorphan were purchased from the Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were of analytical grade. Antibodies recognizing human CYP2D6 were raised against a 20-amino acid peptide corresponding to residues 254–273 of the native protein [11]. In brief, the sequence of the synthetic peptide, (NH2-K)LLTEHRMTWDPAQPPRLTE, corresponds to a major 33-amino acid sequence recognized by human LKM-1 autoantibodies [12]. The antisera recognized recombinant CYP2D6, but not CYP2C9, CYP1A2, CYP3A4 or CYP2E1. The antibody was shown to inhibit only the CYP2D6-dependent *O*-demethylation, but not the CYP3A-mediated *N*-demethylation of dextromethorphan [11]. Monoclonal antibodies against human CYP2A6 and polyclonal antibodies against rat CYP2E1 were obtained from Gentest (Woburn, MA). These antibodies showed slight cross-reactivity to each other, but not to CYP1A, CYP2B6, CYP2C9, CYP2D6, or CYP3A4. Polyclonal antibodies against rat CYP3A1, obtained from Human Biologics, Inc. (Phoenix, AZ), cross-reacted with the human CYP3A subfamily. Antibodies conjugated with alkaline phosphatase, alkaline

phosphatase color reagents, and nitrocellulose membrane were obtained from Bio-Rad Laboratories (Richmond, CA).

Intestinal samples and preparation of microsomes. Human small intestines (mostly jejunum and ileum) from two subjects (males, 29 and 43 years old) were obtained from the International Institute for the Advancement of Medicine (Exton, PA). The intestinal tissue was rinsed with ice-cold 0.9% sodium chloride, and cut along the axis, and then the epithelial cells were scraped off. These processes were performed within 1 hr after removal of the tissues. The intestinal scrapings were snap frozen, placed at –70%, and shipped overnight on dry ice. Two human livers (male, 65 years old, and female, 21 years old) were provided by Dr. Judy Raucy of the University of New Mexico (Alberquerque, NM). Monkey small intestines and livers (male, N = 3) were obtained within 1–2 hr after the animals were killed. Monkey intestinal scrapings were obtained in a fashion similar to the human intestinal preparations. Microsomes were prepared from intestinal scraping or liver tissues, as described previously [13]. Protein concentrations were determined according to the method of Lowry *et al.* [14].

Incubation conditions. Microsomal protein (25–150 μg) was incubated, in a final volume of 100 μL, with (+)-bufuralol (0.5–1000 μM) in 0.1 M sodium phosphate buffer, pH 7.4. Following preincubation for a few minutes, the reaction was started with NADPH (1 mM) or CuOOH (125 μM). The reaction was allowed to proceed for 5 min (liver microsomes) or 10 min (intestinal microsomes) before being stopped with 10 μL of 60% $HClO_4$ (w/v). All incubations were performed, in duplicate, at 37° for NADPH-mediated reactions and at room temperature for CuOOH-mediated reactions as described by Zanger *et al.* [15]. Preliminary study indicated that the reactions were linear under the conditions used. The formation of 1′-hydroxybufuralol was determined by HPLC with fluorescence detection, using a previously reported method [16].

The inhibitory experiments with known CYP2D6 inhibitors or substrates (quinidine, quinine, debrisoquine, ajmaline and dextromethorphan) were performed at 10 and 100 μM (+)-bufuralol and 40 μM inhibitors, using CuOOH as a cofactor. For immunoinhibition studies, microsomes were first incubated at room temperature for 20 min with the antibodies or control sera, at the ratios of incubation mixture to antibody volume shown to have maximum inhibitory effects (5 mg IgG/mg microsomal protein for anti-2D6, anti-2E1, and anti-3A1, and 0.5 mg IgG/mg microsomal protein for anti-2A6). The studies were performed at 10 and 100 μM (+)-bufuralol, and the reactions were initiated by the addition of CuOOH (125 μM).

Immunoblot analysis. Intestinal and hepatic microsomal proteins (2–10 μg) were separated on 8% SDS–PAGE according to Laemmli [17], transferred electrophoretically to a nitrocellulose sheet [18], and probed with anti-CYP2D6. Antibody–antigen complexes were visualized using a second antibody conjugated with alkaline phosphatase and alkaline phosphatase color reagents.

Data analysis. Apparent K_m and V_{max} values were estimated using a nonlinear regression program (PCnonlin, Statistical Consultant, Lexington, KY).

Results

Kinetic studies. In human intestine, the kinetic characteristics of CuOOH- or NADPH-mediated (+)-bufuralol 1′-hydroxylation were biphasic (Table 1). K_m values obtained from both reactions, especially of the higher affinity enzyme, were similar; these values were 10 to 20-fold less than those of the lower affinity component. V_{max} values were much higher for the CuOOH-supported metabolism than those for the NADPH-mediated reaction.

The intestinal metabolism of (+)-bufuralol in rhesus monkeys, in the presence of CuOOH or NADPH, also was biphasic (Table 2). Unlike the intestine, the kinetics of (+)-bufuralol metabolism in monkey liver were monophasic when CuOOH served as cofactor, but were biphasic in the presence of NADPH (Table 2). K_m values of both tissues obtained with either CuOOH or NADPH were in the same range as those of human intestine. Similar to the observation in humans, bufuralol metabolism mediated by CuOOH appeared to be more efficient than that observed in the presence of NADPH.

Table 1. Kinetic parameters of microsomal bufuralol 1′-hydroxylation in human intestine

	K_{m1} (μM)	V_{max1} (pmol/min/mg)	K_{m2} (μM)	V_{max2} (pmol/min/mg)
CuOOH				
Subject 1	5.4	2.5	132.4	23.4
Subject 2	14.2	9.2	291.1	7.6
$NADPH/O_2$				
Subject 1	ND*			
Subject 2	10.5	0.7	99.0	5.6

* Not determined.

Effects of CYP2D6 inhibitors/substrates. Inhibition of the 1′-hydroxylation of bufuralol at substrate concentrations of 10 and 100 μM by CYP2D6 inhibitors/substrates showed similar patterns in all the microsomal preparations studied (Fig. 1). In general, the degree of inhibition was greater at the lower bufuralol concentration, especially with the intestinal microsomes, and was more pronounced in the liver than in the intestine from both species. In human intestine and liver, quinidine and ajmaline proved to be potent inhibitors, while dextromethorphan and debrisoquine were the least potent. In contrast, debrisoquine, but not dextromethorphan, least inhibited the CuOOH-mediated metabolism in both monkey intestine and liver. Quinine seemed to be more effective in inhibiting bufuralol metabolism in monkey than in human, while quinidine was more potent in human than in monkey. In the present study, the estimated IC_{50} values for inhibition of 1′-hydroxylation of bufuralol (100 μM) by quinidine, the most potent inhibitor in both species, were 6, 17 and 18 μM for human intestine, monkey intestine and monkey liver, respectively.

Immunoblots. Proteins of similar molecular weight in human and monkey intestine and monkey liver were recognized by the anti-CYP2D6 peptide antibody (Fig. 2). The electrophoretic mobility of these proteins was comparable to that of human hepatic 2D6. The protein content of the intestinal enzyme(s), as measured by densitometric intensity, was less than that of the hepatic CYP2D6 (15- and 100-fold in human and monkey, respectively).

Immunoinhibition studies. In human intestine, anti-CYP2D6, but not anti-CYP2A6 or anti-CYP2E1, significantly inhibited the CuOOH-mediated 1′-hydroxylation of bufuralol, at both low (50%) and high (34%) substrate concentrations (Fig. 3). Under the same conditions, anti-CYP2D6 inhibited bufuralol metabolism in human liver by about 75%, independent of bufuralol concentration. Control sera insignificantly affected the enzyme activity. Increasing the volume of the antisera used (up to 3-fold) did not result in increased inhibitory effects. Anti-CYP2D6 also inhibited (+)-bufuralol 1′-hydroxylase in monkey intestine (40–50%) and liver (~75%). Bufuralol hydroxylase activity was only slightly inhibited (20%) in human intestine by anti-rat CYP3A1, but was markedly inhibited (60–65%) in monkey intestinal microsomes (Fig. 3). Again, increasing the antibody/microsomal protein ratio did not increase inhibition. The hepatic metabolism of (+)-bufuralol in human and monkey was not affected by anti-CYP3A1 (Fig. 3). At the concentration ratio of IgG/microsomal protein used in the present study, anti-CYP3A1 was shown to completely inhibit 6β-hydroxytestosterone formation (CYP3A4 marker) in human liver microsomes (data not shown).

Discussion

Consistent with the previous report [7], the presence of enzymes of the CYP2D subfamily in human intestines from both

Table 2. Kinetic parameters of microsomal bufuralol 1′-hydroxylation in rhesus monkey intestine and liver in the presence of CuOOH or NADPH/O_2

Organ	K_{m1} (μM)	V_{max1} (pmol/min/mg)	K_{m2} (μM)	V_{max2} (pmol/min/mg)
Intestine				
CuOOH	12.8 ± 3.6	12.1 ± 0.8	320.3 ± 73.2	23.2 ± 3.9
NADPH/O_2	8.9 ± 3.1	3.2 ± 0.4	383.2 ± 99.0	12.4 ± 1.4
Liver				
CuOOH	6.7 ± 0.3	1389.0 ± 12.2	–*	–
NADPH/O_2	7.3 ± 0.3	860.9 ± 17.8	81.8 ± 7.2	840.2 ± 28.6

Results expressed as means ± SEM (N = 3).
* Monophasic kinetics.

subjects was detected immunologically using an anti-CYP2D6 peptide. This intestinal enzyme appeared active in catalyzing the 1′-hydroxylation of (+)-bufuralol, a commonly employed CYP2D6 functional marker. The catalytic activity in intestinal preparations from these two subjects was much less than that obtained from liver microsomes, corresponding to the relatively lower amount of immunoreactive protein in the intestine (Fig. 2). Kinetics of the NADPH-mediated (+)-bufuralol 1′-hydroxylation in human intestine were similar to, while those of the CuOOH-supported metabolism were different from, those reported previously for human liver [15]. The kinetics of NADPH-dependent reaction by human hepatic CYP2D6 exhibit biphasic characteristics (K_m = 5.3 ± 0.4 and 121.0 ± 22.0 μM, V_{max} = 23.0 ± 1.2 and 24.7 ± 1.0 nmol/mg/hr, respectively), whereas those of CuOOH-mediated metabolism have been shown to be monophasic (K_m = 7.3 ± 0.1 μM and V_{max} = 86.0 ± 0.3 nmol/mg/hr) [15]. Interestingly, K_m values for the intestinal enzyme(s), catalyzed either by CuOOH or NADPH, were comparable to those reported for human hepatic CYP2D6 [15]. The corresponding V_{max} values for the intestine, based on lim-

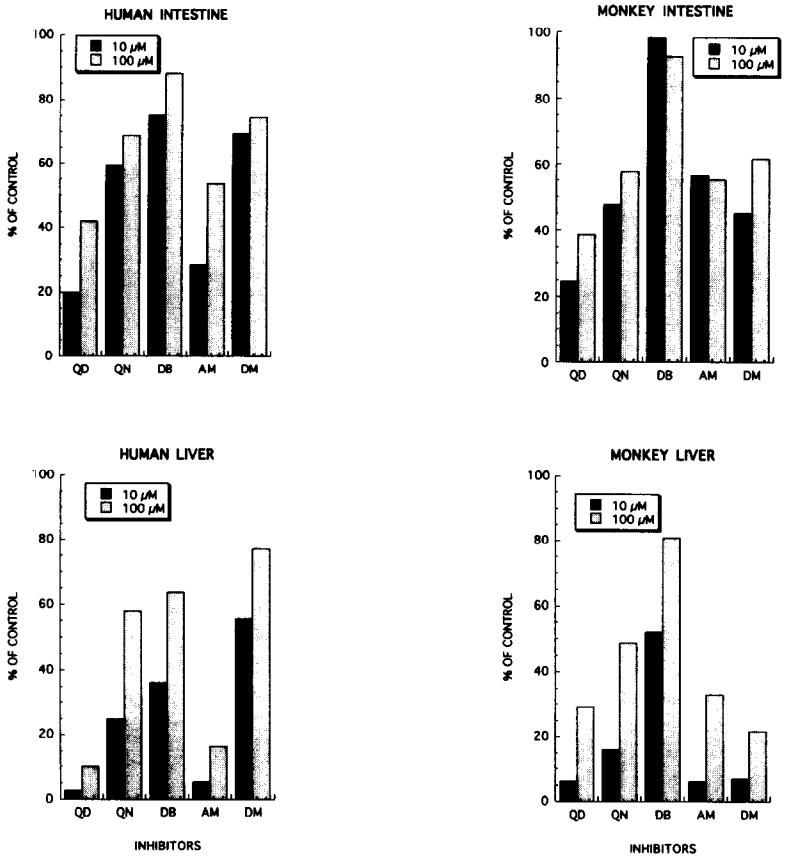

Fig. 1. Inhibition of CuOOH-mediated (+)-bufuralol 1′-hydroxylation by various compounds in human and monkey intestine and liver microsomes. Incubation conditions: (+)-bufuralol 10 μM and 100 μM, and inhibitors 40 μM. Results are from duplicate determinations expressed as percentages of control activity (in the absence of inhibitors). Control activities in human intestine (expressed as pmol/min/mg protein) = 0.9 and 5.4, monkey intestine = 1.2 and 8.3, human liver = 40.7 and 130.4, and monkey liver = 591 and 1119 at 10 and 100 μM (+)-bufuralol, respectively. Abbreviations: QD, quinidine; QN, quinine; DB, debrisoquine; AM, ajmaline; and DM, dextromethorphan.

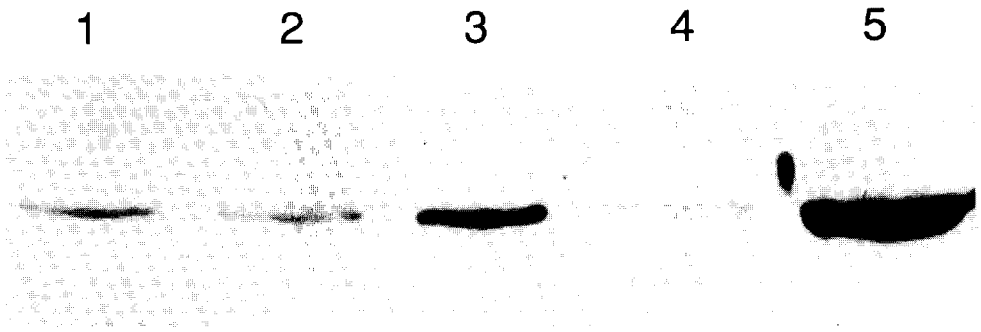

Fig. 2. Immunoblots of intestinal (10 μg) and hepatic (2 μg) microsomes from human and rhesus monkey developed with antisera that recognize human CYP2D6 (see Materials and Methods). Lanes 1 and 2 = human intestine from subjects 1 and 2, respectively; lane 3 = human liver; lane 4 = monkey intestine; and lane 5 = monkey liver.

ited data ($N = 2$), were much lower than those reported previously in human livers [15]. The intestinal metabolism supported by CuOOH in these two subjects appeared to be more efficient than that mediated by NADPH, also similar to the results reported earlier for the hepatic CYP2D6 [15].

The CuOOH-mediated (+)-bufuralol 1′-hydroxylation in human liver has been shown to be catalyzed exclusively by CYP2D6 [15]. The apparent biphasic metabolism of (+)-bufuralol in human intestine suggests the involvement of more than one enzyme, possibly of the CYP2D subfamily. The observation of similar inhibitory patterns of (+)-bufuralol metabolism in intestine and liver at bufuralol concentrations close to the K_m values of both enzymes is consistent with the suggestion that the responsible enzymes are probably related to one another, and may also be related to CYP2D6. The finding that anti-2D6 inhibited about 34–50% of the intestinal activity depending on bufuralol concentration, as compared with 75% inhibition in the liver at both substrate concentrations, supports the above conclusion. The more pronounced effect of anti-CYP2D6 at the lower substrate concentration (10 μM) further suggests that a closely related form of CYP2D6 is significantly involved in the high affinity component metabolism. Slight (20%) inhibition by anti-rat CYP3A1 in human intestinal microsomes suggests possibly minor involvement of CYP3A-related enzymes in the metabolism of bufuralol. Interestingly, differences in the expression of enzymes of the CYP3A subfamily and, consequently, in CYP3A catalytic activities also have been observed in human intestine and liver [4].

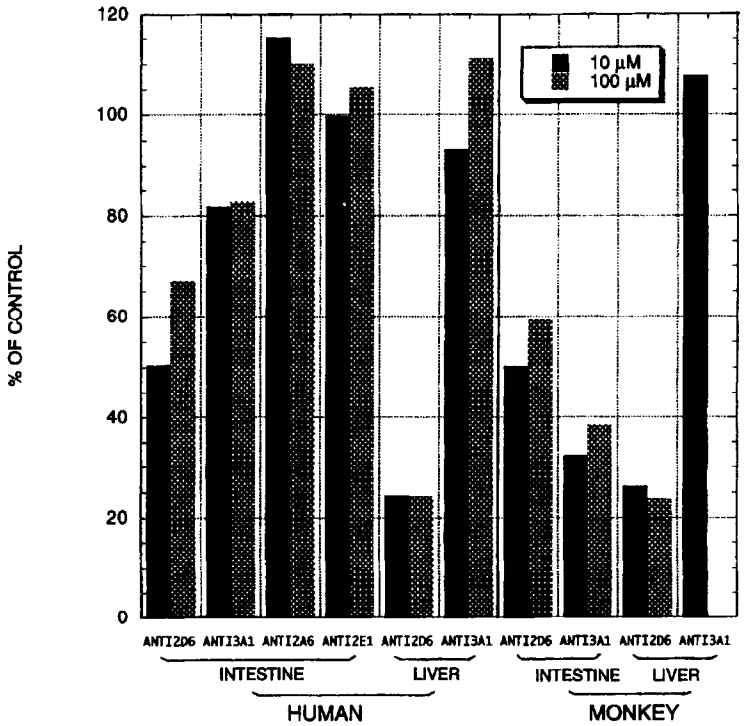

Fig. 3. Inhibition of (+)-bufuralol metabolism by anti-CYP2D6, anti-CYP3A1, anti-CYP2A6, and anti-CYP2E1 in human and monkey intestine and liver microsomes. Results are from duplicate determinations expressed as percentages of control activity (in the presence of preimmune sera). Control activities in human intestine (expressed as pmol/min/mg protein) = 1.8 and 10, monkey intestine = 4 and 14, human liver = 100 and 250, and monkey liver = 1250 and 1800 at 10 and 100 μM (+)-bufuralol, respectively. (Inhibition by anti-CYP3A1 in monkey liver microsomes was not done at 100 μM.)

Hepatic tissues of the rhesus monkey contained a relatively high content of CYP2D6-related protein, and exhibited high bufuralol 1-hydroxylase activity. Based on similarities in the kinetics of (+)-bufuralol metabolism and in the immunoinhibitory patterns between rhesus monkey and human livers obtained, it is likely that a CYP2D6-related enzyme present in rhesus monkey liver was primarily responsible for the metabolism. Although the kinetics of hepatic metabolism of (+)-bufuralol in the rhesus monkey were similar to those in humans, they were different from those reported in crab-eating monkeys. In crab-eating monkey liver, both CuOOH- and NADPH-mediated metabolism displayed biphasic kinetics [8]. The reported K_m values for crab-eating monkey [8], especially of the higher affinity enzymes, however, were in the same range as those obtained for rhesus monkey or human tissue preparations. The finding that liver microsomes from rhesus monkey appeared to be more sensitive to inhibition by quinine but was less affected by quinidine, than those from human, is in agreement with published results for hepatic dextromethorphan *O*-demethylase activity, another CYP2D6 marker, in the African green monkey [10].

Like the human, kinetics of (+)-bufuralol metabolism in rhesus monkey intestine were different from, but the effects of CYP2D6 inhibitors were similar to, those found in the liver of the rhesus monkey. The findings of biphasic kinetics of (+)-bufuralol 1′-hydroxylase in monkey intestine, with K_m values comparable to those observed in human intestine, and of similar effects of CYP2D6 inhibitors on 1′-hydroxybufuralol formation in monkey and human intestines suggest that enzymes of similar catalytic properties are involved. Unlike the human, inhibition of the COOH-mediated metabolism of (+)-bufuralol in monkey intestinal microsomes by anti-CYP3A1 was equal to or greater than that by anti-CYP2D6. These results suggest significant involvement of both CYP3A- and 2D6-related enzymes and/or possibly of enzymes having common epitopes to both CYP3A and CYP2D6 in the intestinal metabolism in the monkey. More detailed studies appear warranted.

Overall, the results demonstrate tissues and species differences in enzymes involved in the metabolism of (+)-bufuralol, a commonly known CYP2D6 marker, and indicate the presence of functionally active CYP2D6-related enzymes in human intestine, monkey liver and monkey intestine. The presence of CYP2D subfamily enzymes in liver from the rhesus monkey suggests that the rhesus monkey may be an animal model in studies of hepatic CYP2D6. In our preliminary experiments (data not shown), CYP2D subfamily enzymes were not detected immunologically or catalytically in the intestines from the Beagle dog or Sprague–Dawley rat, although they were functionally active in the livers of both species. Despite some differences in enzymes involved in the intestinal metabolism of (+)-bufuralol between monkey and human, the rhesus monkey may, to some extent, represent an animal model for studying the metabolism of drugs that are substrates for both intestinal and hepatic CYP2D subfamily enzymes.

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