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Short Communication

Improved Reliability of the Rapid Microtiter Plate Assay Using Recombinant Enzyme in Predicting CYP2D6 Inhibition in Human Liver Microsomes

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ABSTRACT:

A higher throughput method of screening for the inhibition of recombinant CYP2D6 using a microtiter plate (MTP) assay was evaluated using 62 new chemical entities and compared to data from the dextromethorphan *O*-demethylase assay in human liver microsomes (HLM). The IC_{50} values for the two assays closely matched for 53 compounds (85%). Six of the variant nine compounds had higher IC_{50} values with the recombinant enzyme, whereas three had lower IC_{50} values with the recombinant enzyme. When the inhibition with the recombinant enzyme was determined at various time points, the IC_{50} values increased as the duration of the incubation increased for the six compounds with higher IC_{50} values in the MTP assay. The IC_{50} values at 10 min matched more closely the IC_{50} values in HLM (95% compared with 85%). For three compounds that showed comparable IC_{50} values in the two assays, and the three compounds with lower IC_{50} values in the MTP assay, the IC_{50} values did not change over time. These results suggest that the six compounds that showed higher IC_{50} values in the MTP assay at 45 min are substrates for CYP2D6. Using known CYP2D6 substrates, a similar phenomenon was observed, i.e., inhibition curves shifted to higher IC_{50} values as incubation time increased. These results indicate that the higher throughput MTP assay is more comparable to HLM if the IC_{50} values are determined at 10 min rather than the recommended 45 min. Furthermore, data acquisition at multiple time points may indicate if a compound is a potential substrate or metabolism/mechanism-based inhibitor for the enzyme.

Xenobiotics are primarily oxidatively metabolized in the liver by a family of heme-containing enzymes, namely cytochrome P-450 (Gonzalez, 1989). In human liver, the five isoforms that are primarily responsible for the metabolism of most drugs include CYP3A4, CYP2D6, CYP2C9, CYP2C19, and CYP1A2 (Guengerich, 1995). It has been reported that CYP2D6 represents only 2% of total liver cytochrome P-450, although it is involved in the metabolism of 30% of marketed drugs (Shimada et al., 1994; Guengerich, 1996; Lewis et al., 1997). Therefore, it is important to evaluate the inhibition potential of new chemical entities (NCEs)[1] for CYP2D6 early in the discovery stage to avoid potential drug-drug interaction.

Recently, a 96-well microtiter plate (MTP) assay using microsomes from recombinant baculovirus-infected insect cells expressing a single cytochrome P-450 (Supersomes) has been reported (Crespi et al., 1997). The MTP assay is rapid, reproducible, and inexpensive, whereas the assessment of CYP2D6 inhibition in human liver microsomes (HLM) is much more time consuming. Our laboratory has used this method to estimate the IC_{50} values for CYP2D6 and compared them to values obtained for the inhibition of dextromethorphan *O*-demethylase activity in HLM using conventional HPLC techniques. We have compared the IC_{50} values obtained by both methods for a series of 62 structurally related NCEs and found that the values for 53 compounds were similar in both assays (Palamanda et al., 1998). For the other nine compounds, the IC_{50} values differed by 6- to 93-fold in both assays. The present study was initiated to address the differences between the two assays observed for these nine compounds. The objective was to minimize the discrepancy between the two methods and therefore reduce the number of false negatives and/or false positives.

[1] Abbreviations used are: NCE, new chemical entity; MTP, microtiter plate; HLM, human liver microsomes;

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Materials and Methods

Source of Chemicals. Human liver microsomes were obtained from the International Institute for the Advancement of Medicine (Exton, PA). The microsomes were thawed, pooled, aliquoted, and refrozen at −80°C before use. Insect cell microsomes containing cDNA-expressed CYP2D6-Val (Supersomes) were obtained from Gentest Corporation (Woburn, MA). Quinidine, dextromethorphan, and β-NADPH were purchased from Sigma Chemical Company (St. Louis, MO). Imipramine, trifluperidol, and propranolol were purchased from ICN Biomedicals (Aurora, OH). The substrate for the MTP assay, 3-cyano-7-ethoxycoumarin and its metabolite, 3-cyano-7-hydroxycoumarin, were obtained from Molecular Probes, Inc. (Eugene, OR). Pluronic F-68 was purchased from Life Technologies (Gaithersburg, MD). NCEs for the enzyme inhibition studies were synthesized at Schering-Plough Research Institute as part of a drug discovery program.

Enzyme Assays. The dextromethorphan *O*-demethylase assay for CYP2D6 in HLM was performed according to a modification of a previously published method (Chen et al., 1990; Palamanda et al., 1998). Initial studies in our laboratory have established a K_m of approximately 18 μM. The substrate concentration used in the inhibition studies is 16 μM, which approximates the K_m. Also, it is estimated that the reaction mixture contains 1 pmol of CYP2D6. Metabolite production was quantified by comparison to a standard curve of known concentrations of dextrorphan.

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TABLE 1

Comparison of the IC_{50} values from the human liver microsomes (HLM) assay and from selected time points in the MTP assay

Compound No.	IC_{50} μM				Fold Difference*	
	HLM Assay	MTP Assay			at 10 min	at 50 min
		10 min	30 min	50 min		
18	2.00	1.10	1.70	2.30	−2	+1
21	1.50	0.90	1.30	1.60	−2	+1
26	0.08	0.03	0.08	0.16	−3	+2
54	0.08	0.21	0.42	0.76	+3	+10
55	0.05	0.10	0.35	0.70	+2	+14
56	0.12	0.16	0.90	1.30	+1	+11
57	0.03	0.06	0.22	0.27	+2	+9
58	0.06	0.08	0.53	0.74	+1	+12
59	0.05	0.26	0.67	1.40	+5	+28
60	>30.00	3.40	3.00	3.10	>−9	>−10
61	40.00	0.43	0.45	0.43	−93	−93
62	0.61	0.10	0.08	0.10	−6	−6

* A positive value indicates that the IC_{50} estimate is greater in the MTP assay than in the HLM assay. A negative value indicates that the IC_{50} estimate is greater in HLM assay than in the MTP assay.

The microtiter plate assay for CYP2D6 was performed with a CytoFluor Series 4000 Multi-well Plate Reader (PerSeptive Biosystems, Framingham, MA) according to Crespi et al. (1997) with modifications. All compounds for analysis were initially dissolved in methanol. Stock solutions were made in 20% aqueous methanol and the final methanol concentration in the assay was 0.5%. A 20 mM 3-cyano-7-ethoxycoumarin solution was prepared in acetonitrile. After the addition of 5 μl of the test compound, 95 μl of a 100 mM potassium phosphate buffer, pH 7.4, containing 3 mM β-NADPH, 0.02% Pluronic F-68 (a nonionic surfactant of polyoxyethylene-polyoxypropylene copolymer used to help solubilize the substrate), and 100 μM 3-cyano-7-ethoxycoumarin, was added to each well. The plate was preincubated at 37°C for 5 min, then 100 μl of a 100 mM potassium phosphate buffer, pH 7.4, containing 50 pmol/ml of insect cell-expressed CYP2D6-Val (Supersomes) was added. Therefore, the reaction mixture contained 5 pmol CYP2D6 as reported by Crespi et al. (1997). Initial studies in our laboratory have shown that the K_m for 3-cyano-7-ethoxycoumarin was 45 μM, similar to a K_m of 67 μM reported by Crespi et al. (1997). The final substrate concentration used was 50 μM, which approximates the K_m. The plate was incubated for 45 min at 37°C followed by the addition of 100 μl of a reaction-stop solution containing 60% acetonitrile and 40% 0.1 M Tris, pH 9. In some experiments, after the addition of the recombinant enzyme to start the reaction, fluorescence data were acquired every 10 min for 50 min by scanning while the plate remained in the instrument.

Data Analysis. Relative CYP2D6 activity was calculated from the fluorescence data for each time point using CytoCalc Data Analysis Software (PerSeptive Biosystems, Framingham, MA). The IC_{50} values were estimated by comparison to control values, i.e., enzyme in the reaction mixture and 0.5% methanol.

Results and Discussion

We previously reported the IC_{50} values for 62 NCEs using Supersomes and human liver microsomes for CYP2D6 inhibition (Palamanda et al., 1998). Of these structurally related compounds, the IC_{50} values for the two assays closely matched for 53 of the 62 (85%) compounds (difference equal to or less than 5-fold). The remaining nine compounds had IC_{50} values in the MTP assay that differed from the IC_{50} values in HLM assay by more that 5-fold (compounds 54 to 62, Table 1). The IC_{50} values for six compounds were 9- to 28-fold higher in the MTP assay, whereas the IC_{50} values for the other three compounds were 6- to 93-fold higher in the HLM assay (Palamanda et al., 1998). A difference of 5-fold or less in the IC_{50} was considered acceptable because we were comparing data from a single cytochrome P-450 isoform (Supersomes) to data from a mixture of cytochrome P-450 enzymes (HLM) and the substrates were different. Also, literature IC_{50} values for quinidine inhibition of CYP2D6 using dextromethorphan (Rodrigues and Roberts, 1997) and bufuralol (Halliday et al., 1995) as substrates were 0.22 μM and 0.04 μM, respectively, a difference of slightly over 5-fold. However, it should be noted that the $[S]/K_m$ ratios for the two reactions may be different, which could have contributed to the difference in the IC_{50} values. In our studies, the $[S]/K_m$ ratio was approximately 1 for both the MTP and HLM assays.

To determine if the IC_{50} values observed at the 45-min endpoint in the MTP assay remained constant throughout the incubation, fluorescence data were acquired at 10, 30, and 50 min intervals. The IC_{50} values for the six compounds that showed higher IC_{50} in the MTP assay shifted markedly to higher values over time (compounds 54–59, Table 1), whereas the IC_{50} values for the three compounds that showed lower IC_{50} values in the MTP assay remained relatively stable (compounds 60–62, Table 1). Also, three additional compounds (compounds 18, 21, and 26; Table 1) whose IC_{50} values were within 5-fold of the IC_{50} values determined in HLM showed relatively stable IC_{50} (2- to 3-fold differences in the IC_{50} values; Table 1). Upon using the IC_{50} values observed at 10 min in the MTP assay, all but the three compounds that showed lower IC_{50} in the MTP assay were found to have IC_{50} values within a 5-fold difference from the HLM assay, i.e., 95% match (Table 1).

The higher IC_{50} values in the MTP assay at 45 to 50 min compared to 10 min for the six compounds suggest that the higher concentration of CYP2D6 and the longer incubation period in the MTP assay may be more rapidly inactivating the test compounds than in HLM. This is evident because the amount of CYP2D6 used in each assay and the duration of the incubation are different. In HLM, it is estimated that the concentration of CYP2D6 is 5 pmol/mg protein (Shimada et al., 1996). Under our assay conditions for HLM, i.e., 1 mg/ml microsomal protein and a 200 μl volume, there is 1 pmol enzyme present. In the MTP assay, 5 pmol enzyme per well (in 200 μl) are required due to the low turnover rate of the enzyme for the substrate (Crespi et al., 1997). The incubation times are 15 and 45 min for the HLM assay and the MTP assay, respectively. Therefore, in the MTP assay, there is 5 times more enzyme present and the length of the incubation is approximately 3 times longer than in the HLM assay; this may result in a more rapid metabolism in the MTP assay. This is consistent with our finding that for those six compounds that showed higher IC_{50} values in the MTP assay at 45 to 50 min, the IC_{50} at 10 min matched more closely with the IC_{50} in HLM. To confirm that the shift to higher IC_{50} values over time is an indicator of metabolism, known substrates of CYP2D6 were tested (Spatzenegger and Jaeger, 1995). When dextro-

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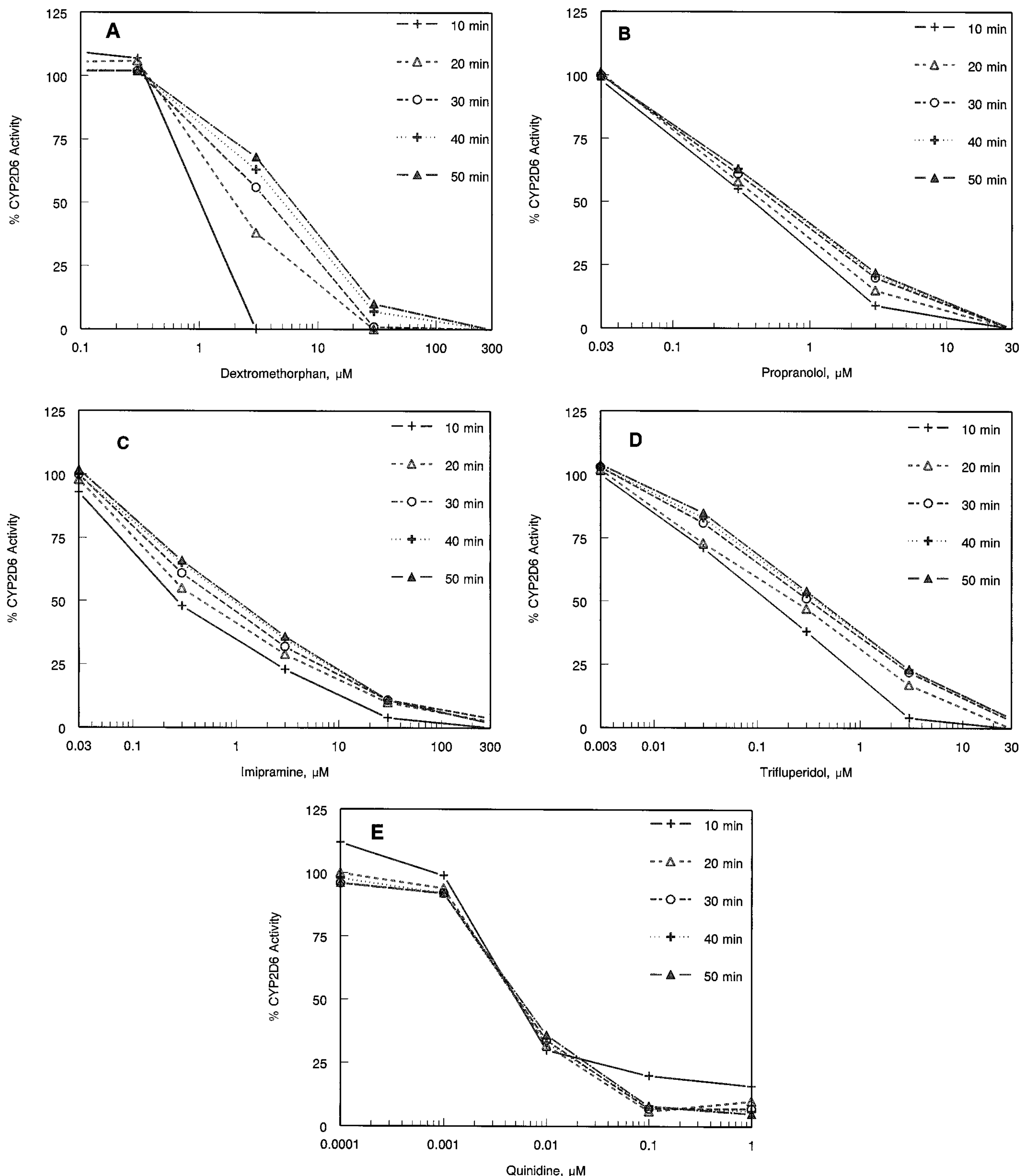

FIG. 1. *Inhibition curves for dextromethorphan (A), propranolol (B), imipramine (C), trifluperidol (D), and quinidine (E) at selected time points in the MTP assay.*

methorphan, imipramine, propranolol, and trifluperidol were evaluated in the MTP assay, the IC_{50} curves shifted to the right for all compounds, i.e., higher IC_{50} values over time (Fig. 1). In contrast, when quinidine was analyzed, the IC_{50} value did not change over time. This is expected because quinidine is a competitive inhibitor of CYP2D6 but not a substrate (Nielson et al., 1995). Nevertheless, this hypothesis needs to be further confirmed by monitoring the reaction mixture by liquid chromatography-mass spectrometry analysis. Using this generalization, a shift of inhibition curves to the *left* over time (i.e., lower IC_{50}) would suggest metabolism- or mechanism-based inhibition. Because there are no known specific metabolism-based inhibitors of CYP2D6 (Parkinson, 1996), preliminary experiments

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were carried out with the MTP assay using recombinant CYP3A4 from baculovirus-infected insect cells as described by Crespi et al. (1997; data not shown). The inhibition curves for erythromycin and troleandomycin, both of which are mechanism-based inhibitors of CYP3A4, shifted to the left, i.e., lower IC_{50} values over time, whereas the curve for ketoconazole, a nonmechanism-based inhibitor of CYP3A4, remained relatively stable over time. Therefore, these observations may have general application in rapidly screening NCEs for inhibition of cytochrome P-450 enzymes.

In comparing the results from the MTP assay to those obtained from CYP2D6 inhibition in HLM, it is important to point out that insect cell microsomes containing the expressed enzyme do not contain any other cytochrome P-450 isoforms. Thus, the potential for metabolism by any of the other cytochrome P-450 isoforms in liver microsomes does not exist in the Supersomes. If a compound is metabolized in HLM by one isoform to a less potent inhibitor for a second isoform, it is expected that its IC_{50} obtained with HLM would be higher than that obtained in the MTP assay. Therefore, it is reasonable to speculate that the three compounds with higher IC_{50} values in HLM might have been subjected to metabolism to less potent inhibitors by other cytochrome P-450 isoforms. However, it should be mentioned that using the recombinant CYP2D6 preparation results in a more intrinsically accurate estimate of the IC_{50} of a particular compound for this isoform, but may not reflect the apparent IC_{50} determined in HLM, which is probably more relevant in predicting drug-drug interactions. Furthermore, in the prediction of the in vivo inhibition potential for of a specific cytochrome P-450 and the consequent clinically relevant drug-drug interaction, plasma levels (better yet concentrations in the liver) of the compound and the potency of the inhibitor (better expressed as K_i) all must be taken into consideration.

Results of the current study indicate that the risk of overestimating the IC_{50} (false positive) for a test compound is minimized by using data acquired after shorter incubation time (10 min instead of 45 min). This results in increased predictability of the MTP from 85 to 95%. However, there is still a concern for the compounds that showed lower IC_{50} in the MTP assay (3 of 62, approximately 5%) because of the potential of discarding these three NCEs (false negatives). Nevertheless, in the final analysis, utilization of Supersomes coupled with the MTP technology has greatly helped us select appropriate NCEs quickly by enhancing throughput. This is a “balancing act” between discarding a small number of false negatives versus enhanced throughput, but the overall benefit derived by accelerating our discovery program outweighs the loss of approximately 5% of NCEs during the first supersomal screen.

In conclusion, the results of the present study demonstrate that the MTP assay using recombinant enzyme may be useful for screening large numbers of compounds for potential inhibition of CYP2D6. Furthermore, the IC_{50} values obtained at early time points in the MTP assay more closely predict the IC_{50} values obtained in HLM assay. However, the IC_{50} values of selected candidates should still be confirmed in HLM.

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References

Chen ZR, Somogyi AA and Bochner F (1990) Simultaneous determination of dextromethorphan and three metabolites in plasma and urine using high-performance liquid chromatography with application to their disposition in man. *Ther Drug Monit* **12:** 97–104.

Crespi CL, Miller VP and Penman PW (1997) Microtiter plate assays for inhibition of human drug-metabolizing cytochromes P450. *Anal Biochem* **248:**188–190.

Gonzalez F (1989) The molecular biology of cytochrome P450s. *Pharmacol Rev* **40:**243–288.

Guengerich FP (1995) Human cytochrome P450 enzymes, in *Cytochrome P450* (Ortiz-de Montellano PR ed.) pp 473–535, Plenum Press, New York.

Guengerich FP (1996) *In vitro* techniques for studying drug metabolism. *J Pharmacokinet Biopharm* **24:**521–533.

Halliday RC, Jones BC, Smith DA, Kitteringham NR and Park BK (1995) An investigation of the interaction between halofantrine, CYP2D6 and CYP3A4: Studies with human liver microsomes and heterologous enzyme expression systems. *Br J Clin Pharmacol* **40:**369–378.

Lewis DFV, Eddershaw PJ, Goldfarb PS and Tarbit MH (1997) Molecular modelling of cytochrome P4502D6 (CYP2D6) based on an alignment with CYP102: Structural studies on specific CYP2D6 substrate metabolism. *Xenobiotica* **27:**319–340.

Nielson F, Rosholm JU and Brøsen K (1995) Lack of relationship between quinidine pharmacokinetics and the sparteine oxidation polymorphism. *Eur J Clin Pharmacol* **48:**501–504.

Palamanda JR, Favreau LV, Lin CC and Nomeir AA (1998) Validation of a rapid microtiter plate assay to conduct cytochrome P4502D6 enzyme inhibition studies. *Drug Discovery Today* **3:**466–470.

Parkinson A (1996) An overview of current cytochrome P450 technology for assessing the safety and efficacy of new materials. *Toxicol Pathol* **24:**45–57.

Rodrigues AD and Roberts EM (1997) The *in vitro* interaction of dexmedetomidine with human liver microsomal cytochrome P4502D6 (CYP2D6). *Drug Metab Dispos* **25:**651–655.

Shimada T, Yamazaki H, Mimura M, Inui Y and Guengerich FP (1994) Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: Studies with liver microsomes from 30 Japanese and 30 Caucasians. *J Pharmacol Exp Ther* **270:**414–423.

Shimada T, Yamazaki H, Mimura M, Wakamiya N, Ueng YF, Guengerich FP and Inui Y (1996) Characterization of microsomal cytochrome P450 enzymes involved in the oxidation of xenobiotic chemicals in human fetal liver and adult lungs. *Drug Metab Dispos* **24:**515–522.

Spatzenegger M and Jaeger W (1995) Clinical importance of hepatic cytochrome P450s in drug metabolism. *Drug Metab Rev* **27:**397–417.

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