The human hepatic metabolism of simvastatin hydroxy acid is mediated primarily by CYP3A, and not CYP2D6

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Aims To identify the cytochrome P450 (CYP) isoforms responsible for the metabolism of simvastatin hydroxy acid (SVA), the most potent metabolite of simvastatin (SV).

Methods The metabolism of SVA was characterized *in vitro* using human liver microsomes and recombinant CYPs. The effects of selective chemical inhibitors and CYP antibodies on SVA metabolism were assessed in human liver microsomes.

Results In human liver microsomes, SVA underwent oxidative metabolism to three major oxidative products, with values for K_m and V_{max} ranging from about 50 to 80 μM and 0.6 to 1.9 $nmol\ min^{-1}\ mg^{-1}$ protein, respectively. Recombinant CYP3A4, CYP3A5 and CYP2C8 all catalysed the formation of the three SVA metabolites, but CYP3A4 was the most active. CYP2D6 as well as CYP2C19, CYP2C9, CYP2A6, CYP1A2 did not metabolize SVA. Whereas inhibitors that are selective for CYP2D6, CYP2C9 or CYP1A2 did not significantly inhibit the oxidative metabolism of SVA, the CYP3A4/5 inhibitor troleanomycin markedly (about 90%) inhibited SVA metabolism. Quercetin, a known inhibitor of CYP2C8, inhibited the microsomal formation of SVA metabolites by about 25–30%. Immunoinhibition studies revealed 80–95% inhibition by anti-CYP3A antibody, less than 20% inhibition by anti-CYP2C19 antibody, which cross-reacted with CYP2C8 and CYP2C9, and no inhibition by anti-CYP2D6 antibody.

Conclusions The metabolism of SVA in human liver microsomes is catalysed primarily ($\geq 80\%$) by CYP3A4/5, with a minor contribution ($\leq 20\%$) from CYP2C8. CYP2D6 and other major CYP isoforms are not involved in the hepatic metabolism of SVA.

Keywords: CYP2C8, CYP2D6, CYP3A4, CYP3A5, simvastatin, simvastatin hydroxy acid

Introduction

Simvastatin (SV) is used widely for the treatment of hypercholesterolaemia and hypertriglyceridaemia. In humans, it undergoes rapid metabolism to several oxidative metabolites, and a hydrolytic product, simvastatin hydroxy acid (SVA) [1, 2]. Among them, SVA is the most potent competitive inhibitor of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis [2]. Recently, the cholesterol-lowering effect of SV has been shown to be correlated negatively with the debrisoquine

metabolite ratio, an *in vivo* probe for CYP2D6 activity, in eight healthy volunteers [3]. Subsequently, the efficacy and primarily tolerability of SV in patients with hypercholesterolaemia has been shown to correlate with CYP2D6 genotype [4]. In the first study, all eight subjects exhibited a very narrow range of debrisoquine metabolite ratio (about threefold) and all were classified as extensive metabolizers [3]. In the second study, only five CYP2D6 alleles were analysed, and there was only one subject with multiple copies of CYP2D6 genes resulting in high CYP2D6 activity and low cholesterol reduction [4]. Despite these limitations, the authors suggested that CYP2D6 is involved in the metabolism of active metabolites of SV. In our previous study, CYP3A4/5, but not CYP2D6, was identified as the principal CYP isoform involved in the metabolism of SV [1]. To date, there have been only limited data on the

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Received 30 October 2002, accepted 13 December 2002.

enzymes catalysing the metabolism of SVA [5], and no reports for the other active metabolites of SV, presumably due to the unavailability of synthetic standards. Therefore, this study was conducted to investigate systematically the hepatic enzymes responsible for the metabolism of SVA, the only synthetic metabolite presently available.

Methods

Materials

^{14}C -SVA was synthesized at Merck Research Laboratories (Rahway, NJ, USA). Markers and chemical inhibitors of the cytochrome P450 family of enzymes were obtained commercially as previously described [1]. Monoclonal antibodies against human CYP2D6 were obtained from Gentest (Woburn, MA, USA), whereas monoclonal antibodies against human CYP3A and CYP2C19 were prepared at Merck Research Laboratories (West Point, PA, USA). The antibodies against CYP2D6 and CYP3A were specific to CYP2D6 and CYP3A4/5/7, respectively. Anti-CYP2C19 cross-reacted with CYP2C8 and CYP2C9, but not with CYP2D6 or CYP3A. Human recombinant P450s were purchased from Gentest (CYP3A4, CYP3A5, CYP2C19, CYP2C9, CYP2C8 supersomesTM, and CYP2A6 and CYP1A2 microsomes) or prepared at Merck Research Laboratories (CYP3A4 and CYP2D6). Human liver microsomes were obtained from various organizations (IIAM, Exton, PA, USA; Gentest; and Xenotech, Kansas City, KS, USA), which collect tissues in accordance with all pertinent regulations.

In vitro metabolism of SVA

Metabolism of SVA was studied following incubations of human liver microsomes (0.4 mg protein mL^{-1}) with ^{14}C -SVA (1–100 μM) and NADPH (1 mM) in 0.1 M sodium phosphate buffer pH 7.4 at 37 °C. The reaction was terminated after 12 min by the addition of 2 mL acetonitrile. The solvent extracts were evaporated and reconstituted for analysis by high-performance liquid chromatography (HPLC). A preliminary experiment showed that the rates of formation of all metabolites were linear with respect to the incubation time and protein concentration used. Because of its high liver to plasma partition ratio [2], the substrate concentration range used in this study covered clinically relevant concentrations of SVA [6].

The experiments with known chemical inhibitors of P450 were performed at a SVA concentration of 25 μM and inhibitor concentrations known to be selective for CYP2D6 (25 μM quinidine), CYP1A2 (50 μM furafylline), CYP2C9 (50 μM sulfaphenazole), CYP2C8 (20 μM quercetin) or CYP3A4/5 (50 μM troleandomycin) [7–9].

In experiments with furafylline and troleandomycin, the inhibitors were preincubated with liver microsomes and NADPH for 30 min at 37 °C before adding the substrate. All other inhibitors were coincubated with the substrate.

For immunoinhibition studies, microsomes were first incubated on ice for 20 min with antibodies or control sera, at the ratios of antibody to incubation mixture volume shown to have maximum inhibitory effects (50–100 μL IgG mg^{-1} microsomal protein or 20–30 μL IgG per 100 pmol P450). The reaction was then carried out as described above.

Incubations with human recombinant P450s were performed using 30 pmol P450 (CYP3A4, CYP3A5, CYP2C8, CYP2C9 and CYP2C19 supersomesTM), or 100 pmol P450 (CYP2A6, CYP2D6, and CYP1A2 microsomes), at 37 °C for up to 40 min. Kinetic studies of SVA metabolism by CYP3A4 and CYP2C8 also were conducted using 20 pmol CYP3A4 or 90 pmol CYP2C8 per incubation and 1–100 μM SVA incubated for 10 min at 37 °C.

Analytical procedures

An HPLC method previously described for SVA and its metabolites was used for quantification purposes [5]. This assay showed satisfactory linearity and precision (< 15% coefficient of variation). Identification of SVA metabolites was accomplished by using LC-MS techniques, with a Finnigan MAT LCQ ion trap mass spectrometer (Finnigan-MAT, San Jose, CA, USA), as described previously [5].

Assays for enzyme activities

Assays described previously for CYP3A (testosterone 6 β -hydroxylation), CYP2D6 (bufuralol 1'-hydroxylation), CYP2C9 (tolbutamide 3-methylhydroxylation), and CYP2C8 (paclitaxel 6 α -hydroxylation) activities [1, 10] were used for the present studies.

Data analysis

Apparent K_m and V_{max} values were estimated using a nonlinear regression program (Enfit; Biosoft, Ferguson, MO, USA). The intrinsic clearance (CL_{int}) was estimated by dividing V_{max} by K_m .

Results

Metabolism in human liver microsomes

In human liver microsomes, SVA underwent metabolism to at least three oxidative products (M1, M2 and M3). On the basis of UV and LC/MS/MS spectra, M1, M2

and M3 were identified as the 3',5'-dihydrodiol, 3'-hydroxy and 6'-exomethylene metabolites of SVA, respectively. Formation of these products in human liver microsomes was best described by single-enzyme Michaelis–Menten kinetics. The K_m and V_{max} values (Table 1) were higher and lower, respectively, than the corresponding values obtained previously for the metabolism of SV [1]. As a consequence, the CLint for SVA metabolism was much lower (more than fourfold) than that observed earlier for SV metabolism [1], suggesting that SVA was a much poorer substrate than SV, for CYP enzymes.

Metabolism by recombinant CYP isoforms

Of the cDNA-expressed CYP isoforms studied, CYP3A4, CYP3A5 and CYP2C8 metabolized SVA to its three products in a similar fashion to human liver microsomes. CYP3A4 metabolized SVA at the highest rate compared with the other isoforms. The CLint for all three routes was four- to ninefold higher for CYP3A4 than for CYP2C8 (Table 1). Both CYP3A4 and CYP2C8 catalysed the formation of SVA metabolites, with K_m values comparable (within threefold) to those obtained with human liver microsomes (Table 1). CYP2D6, CYP2A6, CYP2C9, CYP2C19, CYP1A2 and the control microsomes did not catalyse the oxidative metabolism of SVA to any appreciable extent (Table 1).

Chemical inhibition studies

Of the chemical CYP inhibitors tested, troleandomycin markedly (> 80%) inhibited the formation of the three metabolites in human liver microsomes pooled from 10 different subjects (Figure 1A). Quercetin, the known potent inhibitor of CYP2C8 (K_i 1 μM) [8], inhibited the formation of M1, M2 and M3 by about 30% (Figure 1A). Quercetin has also been reported to inhibit CYP3A4 activity, with an IC_{50} value of about 50 μM [9]. However, under our experimental conditions, quercetin (at 20 μM) only slightly ($\leq$ 10%) affected the formation of 3'*p*-hydroxypaclitaxel by CYP3A (data not shown). Quinidine, sulfaphenazole and furafylline did not affect the metabolism of SVA (Figure 1A).

Immunoinhibition studies

Anti-CYP3A antibody greatly inhibited (about 90%) the formation of the three major metabolites by human liver microsomes (Figure 1B). Anti-CYP2C antibody inhibited the metabolite formation by about 20%, whereas anti-CYP2D6 antibody showed minimal inhibitory effects (Figure 1B). Considering that CYP2C9 and CYP2C19 did not catalyse the metabolism of SVA, the

Table 1 Kinetic parameters for simvastatin hydroxy acid (SVA) metabolite formation by human liver microsomes and human recombinant CYPs.

	M1		M2		M3	
	K_m μM	V_{max} $nmol\ min^{-1}\ mg^{-1}$ or $nmol\ min^{-1}\ pmol^{-1}$ P450	CLint $mL\ min^{-1}\ mg^{-1}$ or $mL\ min^{-1}\ pmol^{-1}$ P450	K_m μM	V_{max} $nmol\ min^{-1}\ mg^{-1}$ or $nmol\ min^{-1}\ pmol^{-1}$ P450	CLint $mL\ min^{-1}\ mg^{-1}$ or $mL\ min^{-1}\ pmol^{-1}$ P450
Human liver microsomes	76 $\pm$ 35*	1.9 $\pm$ 1.8	0.020 $\pm$ 0.01	47 $\pm$ 12	0.86 $\pm$ 0.26	0.020 $\pm$ 0.01
CYP3A4	26	7.3 (2.6)†	0.28	29	4.6 (2.1)	0.16
CYP2C8	88	2.8 (0.27)	0.032	36	0.85 (0.17)	0.024
CYP3A5		(0.70)			(0.56)	
CYP2D6		(ND)‡			(ND)	
CYP2C9		(ND)			(ND)	
CYP2C19		(ND)			(ND)	
CYP1A2		(ND)			(ND)	
CYP2A6		(ND)			(ND)	

*Values are mean $\pm$ SD obtained from six individual subjects. V_{max} and CLint values are expressed per mg microsomal protein. †Values in parentheses were obtained using 100 μM SVA. V_{max} and CLint values are expressed per pmol P450. ‡ND, Not detectable ($<$ 0.005 $nmol\ min^{-1}\ pmol^{-1}$ P450).

Figure 1 Effects of chemical inhibitors of CYP (A) and anti-CYP antibodies (B) on the metabolism of simvastatin hydroxy acid (SVA) by human liver microsomes pooled from 10 subjects, and effects of anti-P450 antibodies on the formation of M3 in liver microsomes from individual subjects and in recombinant CYP3A4 and CYP2C8 (C). For figure 1C: Anti-2C (■); Anti-2D6 (▒); and Anti-3A (□). Results (mean ± SD) were based on triplicate determinations. All incubations were carried out in human liver microsomes in the absence or presence of inhibitors. Control activities (in the absence of inhibitors/antibodies) were 0.8, 0.4 and 0.3 nmol min⁻¹ mg⁻¹ protein for the formation of M1 (■), M2 (▒) and M3 (□), respectively. TAO, Troleandomycin; QC, quercetin; QD, quinidine; SZ, sulfaphenazole; FF, furafylline; HM1, HM2, HM3, HM4, HM5 and HM6, human liver microsomes from subjects 1, 2, 3, 4, 5 and 6, respectively; 3A4 and 2C8, CYP3A4 and CYP2C8, respectively.

inhibition of SVA metabolism by anti-CYP2C antibody could be attributed to the inhibition of CYP2C8 activity in liver microsomes.

Since the relative contributions of the metabolizing enzymes depend not only on their relative efficiency, but also on the amount of enzyme present in human livers, and considering the known intersubject variability of CYPs in humans, the relative contribution of CYP3A and CYP2C8 to human hepatic metabolism of SVA was assessed in the livers of five additional subjects. Shown in Figure 1C are the results obtained for M3 in human liver microsomes, compared with those observed with cDNA-expressed CYP3A4 and CYP2C8. Based on the extent of inhibition, the contribution of CYP3A4/5 ranged from 75% to 95%, whereas that of CYP2C8 was 5–20% in these livers (Figure 1C). Similar results also were obtained for M1 and M2 (data not shown).

Correlation studies

The formation rates of all three metabolites of SVA obtained from 12 human livers correlated well with each other ($r^2 > 0.98$), supporting the view that their formation was catalysed by the same enzymes. Only testosterone 6 β -hydroxylation and paclitaxel 6 α -hydroxylation showed a significant correlation ($r^2 = 0.54$ – 0.66 , $P < 0.005$) with the formation rates of M1, M2 and M3. Coincidentally, in this panel of microsomes, activities for CYP2C8 and CYP3A4/5 were also correlated to each other ($r^2 = 0.5$). No correlation ($r^2 < 0.10$) was found between SVA metabolism and CYP2D6 or CYP2C9 marker activities.

Discussion

The present investigation used four different *in vitro* approaches, namely metabolism by recombinant P450 isoforms, chemical inhibition, immunoinhibition, and correlation studies, to characterize the enzymes for the metabolism of SVA in human liver. The results of this study clearly indicate that CYP3A was the major ($\geq 80\%$) enzyme subfamily involved in SVA metabolism, as is the case for SV itself [1]. In addition, the present results rule out the possibility of significant CYP2D6 involvement in the metabolism of SVA, the most active metabolites of SV. Our results do not support the suggestion made by Nordin *et al.* [3] and Mulder *et al.* [4] that the apparent association between CYP2D6 polymorphism and the efficacy/tolerability of SV observed in patients was due to CYP2D6 involvement in the metabolism of SVA. In contrast, the present finding is in agreement with a more recent analysis by Geisel *et al.* [11] that the CYP2D6 polymorphism has no influence on the efficacy of SV in 41 patients with primary hypercholesterolaemia. Unlike the study of Mulder *et al.* [4], that of Geisel *et al.* [11] excluded patients with hypothyroidism, minimizing potential confounding effects from treatment with L-thyroxine, a known cholesterol-reducing agent. Geisel *et al.* [11] also used a more comprehensive genotyping procedure, which screened for 12 CYP2D6 alleles.

In addition, we demonstrated that CYP2C8 played a minor role ($\leq 20\%$) in the oxidative metabolism of SVA in human liver microsomes. The results also are consistent with our recent finding of a minimal effect of gemfibrozil, a potent CYP2C8 inhibitor, on human liver

microsomal metabolism of SVA [5, 10]. In contrast, gemfibrozil does inhibit the metabolism of cerivastatin [5, 10], whose oxidative metabolism is mediated significantly by CYP2C8 [12].

We thank Drs Donald E. Slaughter and Cuyue Tang for LC/MS/MS studies, Dr Magang Shou for providing CYP3A4 and CYP2D6, and Drs Conrad Raab, Allen Jones and Dennis Dean for synthesis and purification of ^{14}C -SVA.

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