Identification of Human Liver Cytochrome P450 Isoforms Involved in Autoinduced Metabolism of the Antiangiogenic Agent (Z)-5-[(1,2-Dihydro-2-oxo-3H-indol-3-ylidene)methyl]-2,4-dimethyl-1H-pyrrole-3-propanoic Acid (TSU-68)

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Abstract:

(Z)-5-[(1,2-Dihydro-2-oxo-3H-indol-3-ylidene)methyl]-2,4-dimethyl-1H-pyrrole-3-propanoic acid (TSU-68) is a new anticancer drug that inhibits angiogenic receptor tyrosine kinases, which play a crucial role in tumor-induced vascularization. TSU-68 undergoes hepatic oxidation and glucuronidation. Incubation of TSU-68 with human liver microsomes in the presence of NADPH resulted in the formation of three major metabolites: 5-, 6-, and 7-hydroxyindolinone derivatives. The 5-, 6-, and 7-hydroxylation followed simple Michaelis-Menten kinetics with \( \frac{V_{\text{max}}}{K_{\text{m}}} \) values (an indicator of intrinsic clearance) of 13, 25, and 6 \( \mu \)l/min/mg, respectively. Of the 10 cDNA-expressed human cytochrome P450 isoforms examined, only CYP1A1 and CYP1A2 exhibited appreciable TSU-68 hydroxylation activity. Inhibition studies with \( \alpha \)-naphthoflavone (a selective CYP1A2 inhibitor) and anti-CYP1A2 antibody also indicated the almost exclusive role of CYP1A2 in microsomal TSU-68 hydroxylation. Treatment of human hepatocytes with 10 \( \mu \)M TSU-68 resulted in a 28- to 140-fold increase in CYP1A1/2-mediated ethoxyresorufin O-deethylase activity. The protein levels of CYP1A2 were increased in TSU-68-treated hepatocytes, and those of CYP1A1, which were undetectable in control hepatocytes, were also increased to detectable levels in the TSU-68-treated hepatocytes. Thus, TSU-68 was shown to induce CYP1A1/2 expression, which was responsible for its hydroxylation. The observation that TSU-68 treatment resulted in a 10- to 45-fold increase in 5-, 6-, and 7-hydroxylation directly demonstrated the autoinduced hydroxylation of TSU-68. In conclusion, TSU-68 has the potential to cause induction of its own CYP1A1/2-mediated oxidative metabolism in humans. This autoinductive effect provides a clear explanation for the clinically observed decrease in TSU-68 plasma concentrations during repeated administration of the drug.

Vascular endothelial growth factor receptor, platelet-derived growth factor receptor, and fibroblast growth factor receptor are important for the growth and survival of endothelial cells during angiogenesis, a necessary step for tumor growth (Ferrara, 1999; Klint and Claesson-Welsh, 1999; Rosenkranz and Kazlauskas, 1999). TSU-68, developed as a novel tumor angiogenesis inhibitor, was shown to inhibit the tyrosine kinase activity of these receptors. Therefore, it has been expected that TSU-68 would induce hepatic P450 activity, which was responsible for its metabolism (autoinduction), thereby markedly decreasing plasma concentrations of the drug after repeated twice-daily administration for 1 month (Murakami et al., 2003; Xiong et al., 2004). Interestingly, this decrease occurred as early as the second dose of TSU-68 on day 1. In preclinical studies, TSU-68 administered to rats was shown to induce hepatic P450 activity, which was responsible for its metabolism (autoinduction), thereby markedly decreasing plasma concentrations of TSU-68 within a day (Kitamura et al., 2007). However, this finding provides no conclusive evidence that the clinically observed decrease is due to this autoinduction, because there are interspecies differences in P450 induction (Kern et al., 1997; Lu and Li, 2001; Standing its clinical pharmacokinetic properties and predicting the possibility of drug-drug interaction risks. It has been speculated on structural grounds that TSU-68 would be first metabolized by hydroxylation and acyl glucuronidation (Antonian et al., 2000). Little is known, however, about the chemical structure of TSU-68 hydroxylated metabolites and the cytochrome P450 isoforms responsible for this hydroxylation. Additionally, the clinical pharmacokinetics of TSU-68 have shown an approximately 50% decrease in the plasma concentrations of the drug after repeated twice-daily administration for 1 month (Murakami et al., 2003; Xiong et al., 2004). Interestingly, this decrease occurred as early as the second dose of TSU-68 on day 1. In preclinical studies, TSU-68 administered to rats was shown to induce hepatic P450 activity, which was responsible for its metabolism (autoinduction), thereby markedly decreasing plasma concentrations of TSU-68 within a day (Kitamura et al., 2007). However, this finding provides no conclusive evidence that the clinically observed decrease is due to this autoinduction, because there are interspecies differences in P450 induction (Kern et al., 1997; Lu and Li, 2001;...
Martignoni et al., 2006). It is therefore necessary to demonstrate in vitro that TSU-68 has the potential to induce its own metabolism in humans. The objectives of the present study were to investigate 1) the biotransformation and the enzyme kinetics of TSU-68 in human liver microsomes, 2) the human cytochrome P450 isoform(s) responsible for the hydroxylation of TSU-68, and 3) the capability of TSU-68 to induce the P450 isoforms in human cryopreserved hepatocytes.

Materials and Methods

Chemicals and Reagents.

TSU-68 was synthesized at SUGEN Inc. (South San Francisco, CA), and M1, M2, M3, and M5 (Fig. 1) were synthesized at Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan). Pooled and individual human liver microsomes were obtained from BD Gentest (Woburn, MA). Insect cell microsomes containing baculovirus-expressed human P450s (CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4) and UGTs (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17) were also obtained from BD Gentest. Rabbit anti-rat CYP1A2 serum (for immunoinhibition) and goat anti-rat CYP1A1 serum (for immunoblotting), reactive with both human CYP1A1 and CYP1A2, were purchased from Daiichi Pure Chemicals (Tokyo, Japan). Monoclonal mouse anti-human CYP2C8 antibody was obtained from BD Gentest. Rabbit anti-rat CYP1A2 serum (for immunoinhibition) and goat anti-rat CYP1A1 serum (for immunoblotting), reactive with both human CYP1A1 and CYP1A2, were purchased from Daiichi Pure Chemicals (Tokyo, Japan). Monoclonal mouse anti-human CYP2C8 antibody was obtained from BD Gentest. Cryopreserved human hepatocytes from donor HH59 were obtained from In Vitro Technologies (Baltimore, MD), and those from donors HH39 and HH426 were from XenoTech, LLC (Lenexa, KS). All other chemicals were of reagent grade or of the highest purity available commercially.

TSU-68 Hydroxylation in Microsomes. After a 5-min preincubation, at 37°C of a mixture containing 0.5 mg of protein/ml human liver or cDNA-expressed microsomes activated by 0.05 mg/ml alamethicin, 50 μM TSU-68, 8 mM MgCl₂, and 5 mM N-saccharic 1,4-lactone in 50 mM Tris buffer (pH 7.5), the reaction was initiated by the addition of 2 mM uridine 5′-diphosphoglucuronic acid (0.5 ml of the mixture) and terminated by the addition of acetonitrile (0.25 ml) 10 min later. DMSO (0.25 ml) was also added to the mixture. After centrifugation at 7000g for 10 min, the supernatant (30 μl) was applied to HPLC.

Hepatocyte Cultures. The cryopreserved human hepatocytes suspended in 10% fetal bovine serum-supplemented Williams’ E medium (Langford et al., 1989) were purified by Percoll gradient centrifugation (Hengstler et al., 2000). Cell viability before plating was determined by using the trypan blue exclusion method and ranged from 65 to 87%. The plates were plated onto collagen- precoated 24-well plates (1.5–2 × 10⁶ cells/well). After the cells had attached for 4 h, the medium was replaced with the serum-free supplemented medium. The cells were maintained in the cultures at 37°C in an atmosphere containing 5% CO₂ and 95% air. After 24 h in culture, the hepatocytes were exposed for 48 h to medium containing 10 μM TSU-68, 10 μM omeprazole, 25 μM rifampicin, or vehicle control (0.1% DMSO). At 24 h during this period, the medium was replaced with fresh medium containing the same concentrations of the chemicals being tested.

Enzyme Activity in Hepatocytes. At the end of the 48-h exposure period mentioned above, the cells were washed twice with 0.5 ml of Krebs-Henseleit buffer (containing 3 mM saline/lid for experiments on ethoxyresorufin O-deethylation and TSU-68 hydroxylation). The cells were then incubated for 2 h with 0.25 ml of 2 μM ethoxyresorufin with 10 μM dicumarol, 10 μM TSU-68 (for hydroxylation), or 125 μM testosterone or incubated for 15 min with 0.4 ml of 10 μM TSU-68 (for glucuronidation). Reactions were stopped by transferring the samples to cryovials, and 40 and 160 mM ascorbic acid was further added for experiments on TSU-68 hydroxylation and glucuronidation, respectively. After a TSU-68 analog and corticosterone were added as internal standards for the determination of TSU-68 metabolites and 6β-hydroxytestosterone, respectively, the samples were applied to LC/MS/MS. The samples for the determination of resorufin were directly applied to HPLC.

Immunoblotting Analysis of CYP1A1 and CYP1A2. Protein samples were extracted from cells by using M-PER cell lysis solution ( Pierce Chemical, Rockford, IL) and concentrated by centrifugation at 200,000g for 10 h. The samples were resolved by 10% NuPAGE Bis-Tris gel electrophoresis (Invitro-
gen, Carlsbad, CA) and electroblotted onto a polyvinylidene difluoride membrane in NuPAGE transfer buffer according to the manufacturer’s instructions. The membrane was blocked with 2% nonfat dried milk in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T) and probed with 1:6000 diluted anti-rat CYP1A1 for 1 h. The membrane was then rinsed with PBS-T and exposed for 1 h to 1:45,000 diluted secondary antibody labeled with horse-radish peroxidase. After having been rinsed with PBS-T, the membrane was examined for protein by using an enhanced chemiluminescence kit (ECL-Advance, Pierce Chemical). The band intensities were measured by densitometry using an LAS-3000 imaging analyzer (Fuji photo film; Fuji, Tokyo, Japan). CYP1A1 and CYP1A2 proteins were identified and quantified on the basis of comparison with the proteins produced from the corresponding baculovirus insect cell-expressed cDNA.

**HPLC Analysis.** TSU-68 metabolites in microsomal samples were analyzed by using a LC-10AD Shimadzu, Kyoto, Japan). Separation was achieved on a Capcell Pak C18 UG120 column (3 μm, 150 × 4.6 mm; Shiseido Fine Chemicals, Tokyo, Japan). A mobile phase consisting of 1% acetic acid and acetonitrile was delivered at a flow rate of 1.0 ml/min. Linear gradients of acetonitrile of 20 to 45% over 20 min and 30 to 45% over 12 min were performed for M1–M4 and M5, respectively. The analytes were monitored at a wavelength of 440 nm. Resorufin for CYP1A1/2 activity was monitored on the analytical column by using a mobile phase consisting of 20 mM phosphate buffer (pH 7.0)-acetonitrile (87:13, v/v) at a flow rate of 1 ml/min and was monitored with a fluorescence detector at an excitation wavelength of 575 nm and an emission wavelength of 595 nm.

**LC/MS/MS Analysis.** TSU-68 metabolites in hepatocyte samples were analyzed by LC/MS/MS using an API4000 LC/MS/MS system (Applied Biosystems/MD Sciex, Foster City, CA) coupled to an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA). Separation was achieved on a Capcell Pak C18 UG120 column (5 μm, 150 × 2.0 mm). A mobile phase consisting of 0.1% formic acid and acetonitrile was delivered as linear gradients of acetonitrile of 30 to 70% over 12 min and 70% for 8 min at a flow rate of 0.2 ml/min. The tandem mass spectrometer was operated at negative electrospay ionization with a 4.5 kV ionization potential and an ion source temperature of 300°C. Multiple reaction monitoring (MRM) was applied for the quantification. The MRM transition ions were m/z 325 → 281 for M1–M3, m/z 485 → 309 for M5, and m/z 349 → 172 for the internal standard. Mass spectrometric analysis was also performed by using this system.

Analysis of 6β-hydroxytestosterone was performed by using the LC/MS/MS system as described above. Separation was achieved on the analytical column with linear gradients from 30 to 70% acetonitrile in 0.1% formic acid over 5 min and 70% acetonitrile for 1 min at a flow rate of 0.2 ml/min. The tandem mass spectrometer was operated at positive electrospray ionization with a 5 kV ionization potential and an ion source temperature of 600°C. The MRM transition ions were m/z 305 → 269 for 6β-hydroxytestosterone and m/z 347 → 121 for corticosterone.

**Results**

**TSU-68 Hydroxylation in Human Liver Microsomes.** HPLC analysis of an incubation mixture containing TSU-68 and pooled human liver microsomes in the presence of NADPH indicated three major metabolite peaks (M1, M2, and M3) and one minor metabolite peak (M4) detected at 440 nm, which is a specific wavelength for TSU-68 and its metabolites. In the LC/MS/MS analysis, one of the peaks had a deprotonated molecular ion [M-H]- at m/z 325, which is 16 mass units higher than that of TSU-68. This gain in molecular weight is consistent with the hydroxylation of TSU-68. In addition, LC/MS/MS analysis indicated that m/z 325 of M1, M2, and M3 gave a common product ion at m/z 148, corresponding to a hydroxylated indolinone ring. The peaks of M1, M2, and M3 were assigned to 5-, 6-, and 7-hydroxylated indolinone derivatives (Fig. 1), respectively, by comparing their HPLC retention times with those of authentic standards. The product ions from m/z 325 of M4 were detected at m/z 132, corresponding to an intact indolinone ring, assuming the hydroxyl position of M4 to be relevant to either of the two methyl groups of the pyrrole ring (Fig. 1). No attempt was made to quantitatively examine M4 formation; because, aside from a lesser extent of its formation, M4 can be partially produced by nonenzymatic degradation of TSU-68.

**Identification of CYP Isoforms Responsible for TSU-68 Hydroxylation.** Of the cDNA-expressed human P450 isoforms examined, CYP1A1, CYP1A2, CYP2C8, CYP2D6, and CYP3A4 catalyzed TSU-68 hydroxylation, with by far the highest activities being observed for CYP1A1 and CYP1A2 (Table 1). No activity was detected for the other P450 isoforms examined (CYP2A6, CYP2B6, CYP2C9, CYP2C19, and CYP2E1). Inhibitory effects on TSU-68 hydroxylation in pooled human liver microsomes were examined by using the chemical inhibitors selective for the P450 isoforms that exhibited detectable catalytic activities. CYP1A1 expression is known to be extremely low compared with that of CYP1A2 in human liver microsomes (Murray et al., 1993). As shown in Table 2, marked inhibition of M1, M2, and M3 formation was achieved with α-naphthoflavone (CYP1A2-selective) and moderate inhibition with quercitin (CYP2C8-selective). Quinidine (CYP2D6-selective) and ketoconazole (CYP3A4-selective) failed to inhibit the formation. An immunoinhibition study was performed by using antibodies specific for CYP1A2 and CYP2C8 in an attempt to confirm the results of the chemical inhibition (Table 2). As expected, the anti-CYP1A2 antibody exhibited >95% inhibition of M1, M2, and M3 formation. In contrast, unlike quercitin, the anti-CYP2C8 antibody inhibited no inhibition of the formation; whereas 6α-hydroxylation of paclitaxel, a typical CYP2C8 substrate, was markedly inhibited by this antibody (data not shown). Therefore, the inhibition by quercitin was considered to result from its diverse inhibition selectivity. Overall, the results of these experiments demonstrated that CYP1A2 plays an almost exclusive role in TSU-68 hydroxylation in human liver microsomes. Similar trends were observed for M4 formation, although the quantitative evaluation could not be fully achieved for the reason mentioned above.

**Kinetics of TSU-68 Hydroxylation.** The kinetics of microsomal formation of M1, M2, and M3 was examined to characterize TSU-68 hydroxylation in human liver. For estimates of the K_m and V_max values were 6.7, 6.8, and 7.0 μM, and mean V_max, values, 81, 154, and 35 pmol/min/mg for M1, M2, and M3, respectively. There was an approximate 4-fold interindividual variation in the microsomal intrinsic clearance (V_max/K_m) of TSU-68 hydroxylation, based on variations

<table>
<thead>
<tr>
<th>cDNA-Expressed Human P450 isoform</th>
<th>Formation of TSU-68 Metabolite</th>
<th>pmol/min/mmol P450</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1</td>
<td></td>
<td>28.8</td>
</tr>
<tr>
<td>CYP1A2</td>
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<td>6.5</td>
</tr>
<tr>
<td>CYP2A6</td>
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<td>—</td>
</tr>
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<td>CYP2B6</td>
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<tr>
<td>CYP2C8</td>
<td></td>
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</tr>
<tr>
<td>CYP2C9</td>
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<td>—</td>
</tr>
<tr>
<td>CYP2D6</td>
<td></td>
<td>0.21</td>
</tr>
<tr>
<td>CYP2E1</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>CYP3A4</td>
<td></td>
<td>0.15</td>
</tr>
</tbody>
</table>

*—, not detected.

**TABLE 1**

TSU-68 hydroxylation by human cDNA-expressed P450 isoforms

TSU-68 (50 μM) was incubated with insect cell microsomes containing cDNA-expressed P450 isoforms in the presence of a NADPH-generating system.
in the $V_{\text{max}}$ values. The rank order of the $V_{\text{max}}/K_m$ for the hydroxylation of TSU-68 was consistently $M2 > M1 > M3$ for all four individuals.

Kinetic studies were also performed with cDNA-expressed human P450 isoforms (Table 3). CYP1A2 showed $K_m$ values of 10.4, 11.2, and 11.0 $\mu M$ for formation of M1, M2, and M3, respectively. These values were similar to the $K_m$ values obtained with the human liver microsomes. Relative $V_{\text{max}}$ values of M1, M2, and M3 formed by CYP1A2 were also similar to those obtained with the human liver microsomes. In contrast, CYP1A1 had a 13-fold lower $V_{\text{max}}$ value for M1 and M2 formation than CYP1A2. The $K_m$ for M3 formation was not determined because of its slight formation. Unlike CYP1A2, CYP1A1 had similar $V_{\text{max}}$ values for M1 and M2 formation and a very low $V_{\text{max}}$ value for M3 formation. These results suggest that CYP1A1 has higher catalytic activity toward the hydroxylation of TSU-68, especially M1 and M2 formation, than CYP1A2. CYP2C8 had a relative higher $K_m$ value besides a much lower $V_{\text{max}}$ value, suggesting its negligible contribution to hydroxylation of TSU-68.

**TSU-68 Glucuronidation in Human Liver Microsomes.** One chromatographic peak detected at 440 nm was observed after human liver microsomal incubation with TSU-68 in the presence of the UGT cofactor uridine 5’-diphosphoglucuronic acid. This peak had a deprotonated molecular ion [M-H] $^{-}$ at $m/z$ 485, corresponding to a glucuronide of TSU-68. The glucuronide (M5) was assigned to the acyl glucuronide (Fig. 1) by comparing the data with those obtained from an authentic standard. Of the cDNA-expressed human UGT isoforms examined, UGT1A1, UGT1A3, UGT2B4, and UGT2B7 catalyzed M5 formation. Thus, multiple UGT isoforms were involved in glucuronidation of TSU-68.

**P450 Induction by TSU-68 in Human Hepatocytes.** The inductive effect of TSU-68 on CYP1A1/2 and CYP3A4, both well known inducible P450 isoforms, was examined by using human hepatocytes. Human cryopreserved hepatocytes from three donors (HH59, HH939, and HH426) were treated with 10 $\mu M$ TSU-68 for 48 h. Ethoxyresorufin O-deethylase (EROD) activity (CYP1A1/2 activity) and testosterone 6β-hydroxylase activity (CYP3A4 activity) were compared between 0.1% DMSO (vehicle) and TSU-68-treated hepatocytes. A 28- to 140-fold increase in EROD activity was observed in TSU-68-treated hepatocytes (Fig. 3), which is similar to the results obtained by treatment with omeprazole, a known CYP1A1 inducer. In contrast, the testosterone 6β-hydroxylase activity was not affected by treatment with TSU-68, whereas treatment with rifampicin, a well known CYP3A4 inducer, caused the apparent induction of the activity (Fig. 4). These results suggest that TSU-68 has the potential to induce CYP1A1/2, but not CYP3A4 in humans.

In addition, immunoblot analysis of TSU-68-treated hepatocytes was performed to demonstrate the induction of CYP1A1 and CYP1A2 at the protein level and examine the relative amounts of these two isoforms. As shown in Fig. 5, treatment with TSU-68 caused an apparent increase in the level of CYP1A2 protein in hepatocytes from all three donors. Although CYP1A1 protein was not detected in vehicle-treated hepatocytes as previously reported (Pelkonen et al., 1998; Roos, 2002), TSU-68 treatment increased CYP1A1 protein to detectable levels. TSU-68-treated hepatocytes from donors HH59 and HH939 indicated that the protein levels of CYP1A1 were lower than those of CYP1A2, whereas those from donor HH426 indicated that the protein levels of CYP1A1 were similar to those of CYP1A2. Thus, interindividual differences in the relative amounts of CYP1A1 and CYP1A2 proteins were observed in TSU-68-treated hepatocytes.

**Autoinduction of TSU-68 Hydroxylation in Human Hepatocytes.** The autoinduction of TSU-68 hydroxylation via CYP1A1/2 can be indirectly explained by combining the above results. Moreover, in an attempt to provide direct evidence for this autoinduced hydroxylation, our group used TSU-68 as a substrate of CYP1A1/2 induced by TSU-68 in human hepatocytes. Before TSU-68 was added as a substrate, hepatocytes treated with TSU-68 for 48 h were washed twice with medium to minimize contamination by the metabolites produced from TSU-68 during the treatment. M1 and M2 were observed in control hepatocytes incubated for 2 h in the presence of 10 $\mu M$ TSU-68, as was the case for the microsomal incubation. M3 was also detectable, but it was below the limit of quantification. As shown in Fig. 6, treatment with TSU-68 resulted in 10- to 45-fold and 11- to 42-fold increases in M1 and M2 formation, respectively. M3 formation was also increased to the quantifiable level in the treated hepatocytes. This result demonstrates directly the fact that TSU-68 induced its own hydroxylation in human hepatocytes. Of TSU-68-treated hepatocytes from the three donors, the highest formation of M1, M2, and M3 was observed with donor HH939, which is consistent with the results on induced EROD activity. As expected, omeprazole also caused an apparent increase in this formation, supporting the

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**TABLE 2**

<table>
<thead>
<tr>
<th>Chemical inhibitor</th>
<th>Concentration or Volume</th>
<th>$V_{\text{max}}$</th>
<th>$K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Naphthoflavone (CYP1A2)</td>
<td>1 $\mu M$</td>
<td>21</td>
<td>26</td>
</tr>
<tr>
<td>Quercetin (CYP2C8)</td>
<td>10 $\mu M$</td>
<td>9</td>
<td>23</td>
</tr>
<tr>
<td>Quinine (CYP2D6)</td>
<td>30 $\mu M$</td>
<td>63</td>
<td>67</td>
</tr>
<tr>
<td>Ketoconazole (CYP3A4)</td>
<td>4 $\mu M$</td>
<td>98</td>
<td>97</td>
</tr>
</tbody>
</table>

**Note:** All values are expressed as mean of three experiments ± SD.
involvement of CYP1A1/2 in TSU-68 hydroxylation. In contrast, M5 formation in TSU-68-treated hepatocytes from the three donors was similar to that in vehicle-treated hepatocytes (81 ± 16% of the vehicle control); whereas increased M5 formation, although not significant, was observed in phenobarbital (an inducer of UGT)-treated hepatocytes. This result indicates that TSU-68 fails to induce its glucuronidation.

**Discussion**

The in vitro studies using human liver microsomes demonstrated that TSU-68 underwent hydroxylation and acyl glucuronidation and that the hydroxylation was catalyzed almost exclusively by CYP1A2. In addition, the induction studies using cryopreserved human hepatocytes indicated that treatment with TSU-68 caused a marked increase in CYP1A1/2-mediated EROD and in the protein expression of both CYP1A1 and CYP1A2. The combination of these results leads to the conclusion that TSU-68 has the potential to cause induction of CYP1A1/2 responsible for its own hydroxylation in humans. This autoinduction of TSU-68 hydroxylation was directly demonstrated in the induction study using TSU-68 as both an inducer and a substrate. A similar approach has been reported by using a labeled drug as a substrate (Pichard-Garcia et al., 2004).

In human liver microsomes, TSU-68 underwent predominant hydroxylation of the indolinone ring and, to a lesser extent, probably hydroxylation of the methyl group of the pyrrole ring. This oxidative pathway is essentially similar to that of SU5416, a tyrosine kinase inhibitor, which is structurally analogous to TSU-68 (Antonian et al., 2000). The metabolite profile of SU5416 assumes that hydroxyl groups of TSU-68 further undergo glucuronidation and/or sulfation.

**TABLE 3**

<table>
<thead>
<tr>
<th>Microsomes</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol/min/mg protein)</th>
<th>$V_{max}/K_m$ (μl/min/pmol P450)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human liver microsomes</td>
<td></td>
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<tr>
<td>HLM 31</td>
<td>3.9</td>
<td>70</td>
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<td>HLM 74</td>
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<td>HLM 81</td>
<td>7.3</td>
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<td>11.7</td>
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<tr>
<td>HLM 94</td>
<td>7.2</td>
<td>137</td>
<td>19.0</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>6.7 ± 2.0</td>
<td>81 ± 44</td>
<td>13.1 ± 7.0</td>
</tr>
</tbody>
</table>

| cDNA-expressed P450          |            |                                |                                  |
| CYP1A1                      | 0.76       | 28.0                           | 36.8                             |
| CYP1A2                      | 10.4       | 7.8                            | 0.75                             |
| CYP2C8                      | 57.8       | 0.42                           | 0.0073                           |

a — not calculated.

**Fig. 3.** Effect of TSU-68 on CYP1A1/2 activity in human hepatocytes. Human cryopreserved hepatocytes from three donors were treated for 48 h with 0.1% DMSO (vehicle), 10 μM TSU-68, or 10 μM omeprazole (positive control). CYP1A1/2 activity was determined as the formation of resorufin after incubation of 2 μM ethoxyresorufin with hepatocytes for 2 h. Each bar represents mean ± S.D. (n = 3 wells/treatment).

**Fig. 4.** Effect of TSU-68 on CYP3A activity in human hepatocytes. Human cryopreserved hepatocytes from three donors were treated for 48 h with 0.1% DMSO (vehicle), 10 μM TSU-68, or 25 μM rifampicin (positive control). CYP3A4 activity was determined as the formation of 6β-hydroxytestosterone after incubation of 125 μM testosterone with hepatocytes for 2 h. Each bar represents the mean ± S.D. (n = 3 wells/treatment).
Despite failure of the anti-CYP2C8 antibody to inhibit the formation, quercetin, a typical CYP2C8 inhibitor, was able to inhibit the formation. This was probably due to the cross-inhibitory effect of quercetin on CYP1A2 activity (Dierks et al., 2001).

TSU-68 is metabolized not only by hydroxylation but also via acyl glucuronidation to form M5. The intrinsic clearance for TSU-68 glucuronidation in human liver was approximately 5-fold higher than that in rat liver, whereas the total intrinsic clearance for TSU-68 hydroxylation was comparable between rats and humans (unpublished data). This finding implies that glucuronidation in humans makes a larger contribution to TSU-68 metabolism than that in rats. However, rat urinary and biliary excretion of M5 (2% of the dose) after oral administration of TSU-68 was much lower than the total excretion of the hydroxylated metabolites and their conjugates (>22% of the dose), suggesting that hydroxylation is the highly predominant metabolic route in rats (unpublished data). Therefore, even in humans, hydroxylation would be expected to be a primary route of TSU-68 elimination, especially under induction of CYP1A1/2. This expectation is consistent with the clinically observed 50% decrease in the TSU-68 plasma concentrations (Murakami et al., 2003; Xiong et al., 2004), based on the premise that this decrease is caused by only induction of TSU-68 hydroxylation.

In general, CYP1A1 and probably CYP1A2 are transcriptionally activated by the binding of ligand to the arylhydrocarbon receptor (AhR). The binding of TSU-68 to the AhR, although not experimentally confirmed, is supported by the published finding that indirubin, which shares a common partial chemical structure with TSU-68, shows marked AhR ligand-binding activity (Sugihara et al., 2004). CYP3A4, known to be inducible by many xenobiotics, was hardly affected by TSU-68 treatment. The CYP2B and CYP2C families, although not evaluated in the present study, are also unlikely to be induced by TSU-68 treatment; because these families have a similar transcriptional mechanism as CYP3A4 through a common ligand-activated nuclear receptor (Lin, 2006). In fact, to our knowledge, there are no xenobiotics selectively inducing these families without the induction of CYP3A4. It has been reported that UGT1A1, UGT1A6, and UGT1A9 are transcriptionally activated by AhR ligands in human cell lines (Yueh et al., 2003). However, there is disagreement among the literature as to the inductive effect of the AhR ligands on UGT1A1 activity in human hepatocytes (Li et al., 1999; Ritter et al., 1999). A comprehensive study using various prototypic UGT inducers also suggests that induction of UGT activities in human hepatocytes is ambiguous and exhibits a large interindividual variability, compared with that of P450 activities (Soars et al., 2004). This trend was also observed in induction of TSU-68 glucuronidation by phenobarbital, a known UGT inducer.

In human liver microsomes and untreated hepatocytes, CYP1A protein expression detected by immunoblot analysis is almost entirely due to CYP1A2, with CYP1A1 being rarely detected (Murray et al., 1993; Pelkonen et al., 1998; Roos, 2002). Treatment of hepatocytes with 2,3,7,8-tetrachlorodibenzo-p-dioxin and 3-methylcholanthrene preferably induced CYP1A2, but not CYP1A1 proteins, whereas treatment with β-naphthoflavone induced CYP1A1 and CYP1A2 proteins at comparable levels (Runge et al., 2000; Xu et al., 2000; Zhang et al., 2006). In the case of TSU-68, the relative amounts of induced CYP1A1 and CYP1A2 proteins were different among the three donors. Such interindividual differences have also been shown in the case of benzo[k]fluoranthene-treated hepatocytes (Liu et al., 2001). As reported previously (Santostefano et al., 1997), with less exposure to inducers there is a greater difference between the protein levels of CYP1A1 and CYP1A2, with CYP1A1 levels being extremely lower than CYP1A2 levels in untreated hepatocytes. For that reason, in the clinical settings in which hepatic exposure to TSU-68 is probably lower than in the in vitro exposure, CYP1A1 protein levels would be expected to be relatively low compared with those of CYP1A2. However, CYP1A1 has a much higher \( V_{\text{max}}/K_m \) for M1 and M2 formation than CYP1A2 does. From this point of view, CYP1A1, even at its lower protein levels, might play a significant role in hydroxylation of TSU-68.

TSU-68 treatment resulted in the large magnitude of fold induction of CYP1A1 compared with that of CYP1A2 (Fig. 5), as was the case for the other CYP1A inducers. This fold induction of CYP1A1 might be reflected in the large increases (>10-fold) in EROD and TSU-68 hydroxylation in the TSU-68-treated hepatocytes, because CYP1A1 has the higher catalytic activity toward EROD than CYP1A2 (Ueng et al., 2006), as well as TSU-68 hydroxylation. Additionally, compared with cDNA-expressed CYP1A2, the pattern of M1–M3 formation in cDNA-expressed CYP1A1 were similar to that in the TSU-68 treated...
hepatocytes, in which the formation rates of M1 and M2 were similar whereas those of M3 were much lower. This similarity could be due to a large contribution of CYP1A1.

Species differences are known to exist in inductive effects of some P450 inducers. For instance, omeprazole and rifampicin are effective inducers of CYP1A and CYP3A, respectively, in human hepatocytes; whereas these drugs cause little or negligible induction of the corresponding P450 isoforms in rat hepatocytes (Kern et al., 1997; Lu and Li, 2001). Besides the species differences, one case report has clinically documented the fact that omeprazole had little effect on CYP1A1 metabolism (Dilger et al., 1999).

In contrast, TSU-68 exhibited a similarity in autoinduction between rats and humans. We obtained evidence that in rat liver as well as in human hepatocytes the protein expression and catalytic activity of CYP1A1/2 were induced by TSU-68, and, moreover, that hydroxylation of TSU-68 was catalyzed mainly by CYP1A1/2. This similarity makes the extrapolation of in vivo results between rats and humans more reliable. Furthermore, our group’s previous work with rats demonstrated that decreased plasma concentrations of TSU-68 resulted from the autoinduction (Kitamura et al., 2001). Overall, therefore, the human in vitro results obtained in this study and the in vivo finding in rats strongly support the idea that the clinically observed decrease in the plasma concentrations of repeatedly administered TSU-68 is attributed to autoinduction via CYP1A1/2.

References
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Identification of P450 Isoforms Autoinduced by TSU-68