Identification of human liver cytochrome P450 isoforms involved in autoinduced metabolism of the anti-angiogenic agent TSU-68 ((*Z*)-5-[(1,2-dihydro-2-oxo-3*H*-indol-3-ylidene)methyl]-2,4-dimethyl-1*H*-pyrrole-3-propanoic acid).

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Running title: Identification of P450 isoforms autoinduced by TSU-68

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Number of Text pages: 33

Number of Tables: 3

Number of Figures: 6

Number of References: 32

Number of words in the Abstract: 245

Number of words in the Introduction: 378

Number of words in the Discussion: 1231

Abbreviations:

TSU-68 (SU6668),

(*Z*)-5-[(1,2-dihydro-2-oxo-3*H*-indol-3-ylidene)methyl]-2,4-dimethyl-1*H*-pyrrole-3-propano ic acid; HPLC, high-performance liquid chromatography; LC/MS/MS, liquid chromatography-tandem mass spectrometry; ESI, electrospray ionization; NADPH, reduced nicotinamide adenine dinucleotide phosphate; UGT, UDP-glucuronosyltransferase; UDPGA, uridine 5'-diphosphoglucuronic acid; EROD, ethoxyresorufin *O*-deethylation; AhR, arylhydrocarbon-receptor; DMSO, dimethyl sulfoxide.

[Abstract]

TSU-68 ((*Z*)-5-[(1,2-dihydro-2-oxo-3*H*-indol-3-ylidene)methyl]-2,4-dimethyl-1*H*-pyrrole-3-propanoic acid) is a new anti-cancer 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 V_{max}/K_m values (an indicator of intrinsic clearance) of 13, 25, and 6 μl/min/mg, respectively. Of the ten cDNA-expressed human cytochrome P450 isoforms examined, only CYP1A1 and CYP1A2 exhibited appreciable TSU-68 hydroxylation activity. Inhibition studies with α-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 μM 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.

[Introduction]

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 have an anti-tumor effect on many diverse solid tumors. Currently, TSU-68 is being studied in phase I/II clinical trials to investigate its clinical response, tolerability, and pharmacokinetics.

In humans, the unchanged drug excreted in urine accounts for a low percentage of the orally administered TSU-68, indicating that TSU-68 is eliminated predominantly through hepatic metabolism. Characterizing the metabolism of TSU-68 is of great importance for understanding 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 Day1. 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.

[Methods]

Chemicals and reagents

TSU-68, (*Z*)-5-[(1,2-dihydro-2-oxo-3*H*-indol-3-ylidene)methyl]-2,4-dimethyl-1*H*-pyrrole-3-propanoic acid, 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 GENTEST Corp. (Woburn, MA). Insect cell microsomes containing baculovirus-expressed human CYPs (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 GENTEST Corp. 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 from Gentest Corp. Cryopreserved human hepatocytes from donor HH59 were obtained from In Vitro Technologies Inc. (Baltimore, MD); and those from HH393 and HH426, 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 consisting of human liver or cDNA-expressed microsomes and TSU-68, the reaction was initiated by the addition of a

5-min preincubated NADPH-regenerating system. The final mixture (0.5 ml) contained 1 mg/ml microsomal protein, 0.2–500 μM TSU-68, 1.3 mM β-$NADP^+$, 3.3 mM glucose 6-phosphate, 0.4 units/ml glucose-6-phosphate dehydrogenase, 3.3 mM $MgCl_2$, and 0.1 M phosphate buffer (pH 7.4). The reaction was terminated 5 or 10 min after initiation by the addition of an equal volume of methanol. After centrifugation at 7000g for 10 min, the supernatant (50 μl) was applied to HPLC. Under these assay conditions, reactions were linear with respect to protein concentration and time of incubation. Kinetic data (K_m and V_{max} values) were calculated by fitting to the Michaelis-Menten equation using SigmaPlot (Systat Software, Inc., San Jose, CA) Inhibition studies were performed by using the mixture described above, except that the microsomes were preincubated with α-naphthoflavone (1 or 10 μM), quercetin (10 or 30 μM), quinidine (4 or 40 μM) or ketoconazole (1 or 2 μM) for 5 min or preincubated with anti-CYP1A2 antiserum or anti-CYP2C8 antibody (20 or 40 μl) for 30 min. The concentrations of these chemical inhibitors were chosen based on previously published data (Bu et al., 2001; Kim et al., 2005).

TSU-68 glucuronidation in microsomes

After a 5-min preincubation, at 37°C, of a mixture containing 0.5 mg protein/ml human liver or cDNA expressed microsomes activated by 0.05 mg/mL alamethicin, 50 μM TSU-68, 8 mM $MgCl_2$, and 5mM D-saccharic 1,4-lactone in 50 mM Tris buffer (pH 7.5), the reaction was initiated by the addition of 2 mM UDPGA (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 7000*g* 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 (Lanford et al., 1989) were purified by Percoll gradient centrifugation (Hengstler et al., 1994). Cell viability before plating was determined by using the trypan blue exclusion method and ranged from 65% to 87%. The cells were plated onto collagen-precoated 24-well plates ($1.5–2 \times 10^5$ 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% CO_2 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-hr exposure period mentioned above, the cells were washed twice with 0.5 ml of Krebs-Henseleit buffer (containing 3 mM salicylamide 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 analogue 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, Rockford, IL) and concentrated by centrifugation at 200,000*g* for 10 h. The samples were resolved by 10% NuPAGE Bis-Tris gel electrophoresis (Invitrogen, Carlsbad, CA) and electroblotted onto a PVDF 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 hr. The membrane was then rinsed with PBS-T and exposed for 1 h to 1:45,000 diluted secondary antibody labeled with horseradish peroxidase. After having been rinsed with PBS-T, the membrane was examined for protein by using an enhanced chemiluminescence kit (ECL-Advance from Pierce). The band intensities were measured by densitometry using an LAS-3000 imaging analyzer (Fuji photo film, Tokyo, Japan). CYP1A1 and CYP1A2 proteins were identified and quantified based on 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 Shimadzu

LC-10AD (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 ml/min. Linear gradients of acetonitrile of 20 to 45% over 20 min and 30 to 45% over 12 min were conducted for M1–M4 and M5, respectively. The analytes were monitored at a wavelength of 440 nm. Resorufin for CYP1A1/2 activity was separated on the analytical column by using a mobile phase consisting of 20mM 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/MDS Sciex, Foster City, CA) coupled to an Agilent 1100 (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 MS/MS was operated at negative ESI 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.

The 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 MS/MS was operated at positive ESI 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 analysis, all 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 non-enzymatic 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 quercetin (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 quercetin, the anti-CYP2C8 antibody exhibited 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 quercetin 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}, human liver microsomes from each of four donors were incubated with 1–100 μM TSU-68. The formation of M1, M2 and M3 followed Michaelis-Menten kinetics, and the corresponding Eadie-Hofstee plots revealed approximately monophasic profiles, suggesting a single enzyme-binding site (Fig. 2). As shown in Table 3, mean K_m 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 in the V_{max} values. The rank order of the V_{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 μM 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_{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 K_m 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_{max} values for M1 and M2 formation, and a very low V_{max} value for M3 formation. These results suggest that CYP1A1 has the 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_{max}, 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 following human liver microsomal incubation with TSU-68 in the presence of the UGT cofactor UDPGA. 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, HH393, and HH426) were treated with 10 μM 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 CYP1A 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.

Additionally, 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 HH393 indicated that the protein levels of CYP1A1 were lower than those of CYP1A2, while 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 adding TSU-68 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 μM 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 directly demonstrates 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 HH393, which is consistent with the results on induced EROD activity. As expected, omeprazole also caused an apparent increase in this formation, supporting the 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. Using the two complementary approaches of the metabolism studies with cDNA-expressed P450 isoforms and the inhibition studies with antibody inhibitors, we obtained evidence that CYP1A2 almost exclusively contributes to TSU-68 hydroxylation in human liver microsomes. This large contribution is in agreement with the kinetic result showing that K_m of human liver microsomes with little interindividual variation is close to that of the cDNA-expressed CYP1A2. 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 by not only 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 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 is consistent with 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 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. 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, as 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; while 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 previously reported (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 where 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_{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, as well as TSU-68 hydroxylation. Additionally, compared with cDNA-expressed CYP1A2, the pattern of M1–M3 formation in cDNA-expressed CYP1A2 were similar to that in the TSU-68 treated hepatocytes, where the formation rates of M1 and M2 were similar while 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 that omeprazole had little effect on CYP1A metabolism (Dilger et al., 1999). In contrast, TSU-68 exhibited a

similarity in the 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., 2007). 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

Antonian L, Zhang H, Yang C, Wagner G, Shawver LK, Shet M, Ogilvie B, Madan A, and Parkinson A (2000) Biotransformation of the anti-angiogenic compound SU5416. Drug Metab Dispos 28:1505–1512.

Bu HZ, Knuth K, Magis L, and Teitelbaum P (2001) High-throughput cytochrome P450 (CYP) inhibition screening via a cassette probe-dosing strategy. V. Validation of a direct injection/on-line guard cartridge extraction-tandem mass spectrometry method for CYP1A2 inhibition assessment. Eur J Pharm Sci 2:447–452.

Dierks EA, Stams KR, Lim HK, Cornelius G, Zhang H, and Ball SE (2001) A method for the simultaneous evaluation of the activities of seven major human drug-metabolizing cytochrome P450s using an *in vitro* cocktail of probe substrates and fast gradient liquid chromatography tandem mass spectrometry. Drug Metab Dispos 29:23–29.

Dilger K, Zheng Z, and Klotz U (1999) Lack of drug interaction between omeprazole, lansoprazole, pantoprazole and theophylline. Br J Clin Pharmacol. 48:438–444.

Ferrara N (1999) Molecular and biological properties of vascular endothelial growth factor. J Mol Med 77:527–543.

Hengstler JG, Ringel M, Biefang K, Hammel S, Milbert U, Gerl M, Klebach M, Diener B, Platt KL, Böttger T, Steinberg P, and Oesch F (2000) Cultures with cryopreserved hepatocytes: applicability for studies of enzyme induction. Chem Biol Interact. 125:51–73.

Kern A, Bader A, Pichlmayr R, and Sewing KF (1997) Drug metabolism in hepatocyte sandwich cultures of rats and humans. Biochem Pharmacol. 54:761–772.

Kim MJ, Kim H, Cha IJ, Park JS, Shon JH, Liu KH, and Shin JG (2005)

High-throughput screening of inhibitory potential of nine cytochrome P450 enzymes *in vitro* using liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 19:2651–2658.

Kitamura R, Yamamoto Y, Nagayama S, and Otagiri M (2007) Decrease in plasma concentrations of antiangiogenic agent TSU-68 ((Z)-5-[(1,2-dihydro-2-oxo-3H-indol-3-ylidene)methyl]-2,4-dimethyl-1H-pyrrole-3-propanoic acid) during oral administration twice a day to rats. Drug Metab Dispos 35:1611–1616.

Klint P and Claesson-Welsh L (1999) Signal transduction by fibroblast growth factor receptors. Front Biosci 4:165–177.

Lanford RE, Carey KD, Estlack LE, Smith GC, and Hay RV. (1989) Analysis of plasma protein and lipoprotein synthesis in long-term primary cultures of baboon hepatocytes maintained in serum-free medium. In Vitro Cell Dev Biol. 25:174–182.

Li AP, Hartman NR, Lu C, Collins JM, and Strong JM (1999) Effects of cytochrome P450 inducers on 17α-ethinyloestradiol (EE2) conjugation by primary human hepatocytes. Br J Clin Pharmacol 48:733–742.

Lin JH (2006) CYP induction-mediated drug interactions: *in vitro* assessment and clinical implications. Pharm Res 23:1089–1116.

Liu N, Zhang QY, Vakharia D, Dunbar D, and Kaminsky LS (2001) Induction of CYP1A by benzo[*k*]fluoranthene in human hepatocytes: CYP1A1 or CYP1A2? Arch Biochem Biophys 389:130–134

Lu C and Li AP (2001) Species comparison in P450 induction: effects of dexamethasone, omeprazole, and rifampin on P450 isoforms 1A and 3A in primary cultured

hepatocytes from man, Sprague-Dawley rat, minipig, and beagle dog. Chem Biol Interact 134:271–281.

Martignoni M, Groothuis GM, and de Kanter R (2006) Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. Expert Opin Drug Metab Toxicol 2:875–894.

Murakami H, Yamamoto N, Shimoyama T, Ueda Y, Yamada Y, and Tamura T (2003) Phase I, pharmacokinetic, and biological studies of TSU-68, the oral vascular endothelial growth factor receptor tyrosine kinase inhibitor, administered after meals in patients with advanced solid tumors. Proc Am Soc Clin Oncol 22:Abstract 870.

Murray BP, Edwards RJ, Murray S, Singleton AM, Davies DS, and Boobis AR (1993) Human hepatic CYP1A1 and CYP1A2 content, determined with specific anti-peptide antibodies, correlates with the mutagenic activation of PhIP. Carcinogenesis. 14:585–592.

Pelkonen O, Mäenpää J, Taavitsainen P, Rautio A, and Raunio H (1998) Inhibition and induction of human cytochrome P450 (CYP) enzymes. Xenobiotica 28:1203–1253.

Pichard-Garcia L, Weaver RJ, Eckett N, Scarfe G, Fabre JM, Lucas C, and Maurel P (2004) The olivacine derivative S 16020 (9-hydroxy-5,6-dimethyl-N-[2-(dimethylamino)ethyl)-6H-pyrido(4,3-B)-carbazole-1-carboxamide) induces CYP1A and its own metabolism in human hepatocytes in primary culture. Drug Metab Dispos 32:80–88.

Ritter JK, Kessler FK, Thompson MT, Grove AD, Auyeung DJ, and Fisher RA (1999) Expression and inducibility of the human bilirubin UDP-glucuronosyltransferase UGT1A1 in liver and cultured primary hepatocytes: evidence for both genetic and

environmental influences. Hepatology 30:476–484.

Roos PH (2002) Differential induction of CYP1A1 in duodenum, liver and kidney of rats after oral intake of soil containing polycyclic aromatic hydrocarbons. Arch Toxicol 76:75–82.

Rosenkranz S and Kazlauskas A (1999) Evidence for distinct signaling properties and biological responses induced by the PDGF receptor α and b subtypes. Growth Factors 16:201–216.

Runge D, Kohler C, Kostrubsky VE, Jager D, Lehmann T, Runge DM, May U, Stolz DB, Strom SC, Fleig WE, and Michalopoulos GK (2000) Induction of cytochrome P450 (CYP)1A1, CYP1A2, and CYP3A4 but not of CYP2C9, CYP2C19, multidrug resistance (MDR-1) and multidrug resistance associated protein (MRP-1) by prototypical inducers in human hepatocytes. Biochem Biophys Res Commun. 273:333–341.

Santostefano MJ, Ross DG, Savas U, Jefcoate CR, and Birnbaum LS (1997) Differential time-course and dose-response relationships of TCDD-induced CYP1B1, CYP1A1, and CYP1A2 proteins in rats. Biochem Biophys Res Commun 233:20–24.

Soars MG, Petullo DM, Eckstein JA, Kasper SC, and Wrighton SA (2004) An assessment of UDP-glucuronosyltransferase induction using primary human hepatocytes. Drug Metab Dispos. 32:140–148.

Sugihara K, Kitamura S, Yamada T, Okayama T, Ohta S, Yamashita K, Yasuda M, Fujii-Kuriyama Y, Saeki K, Matsui S, and Matsuda T (2004) Aryl hydrocarbon receptor-mediated induction of microsomal drug-metabolizing enzyme activity by indirubin and indigo. Biochem Biophys Res Commun 318:571–578.

Ueng YF, Don MJ, Jan WC, Wang SY, Ho LK, and Chen CF (2006) Oxidative metabolism of the alkaloid rutaecarpine by human cytochrome P450. Drug Metab Dispos 34: 821–827.

Xiong HQ, Herbst R, Faria SC, Scholz C, Davis D, Jackson EF, Madden T, McConkey D, Hicks M, Hess K, Charnsangavej CA, and Abbruzzese JL (2004) A phase I surrogate endpoint study of SU6668 in patients with solid tumors. Invest New Drugs 22:459–466.

Xu L, Li AP, Kaminski DL, and Ruh MF (2000) 2,3,7,8-Tetrachlorodibenzo-p-dioxin induction of cytochrome P4501A in cultured rat and human hepatocytes. Chem Biol Interact 124:173–189.

Yueh MF, Huang YH, Hiller A, Chen S, Nguyen N, and Tukey RH (2003) Involvement of the xenobiotic response element (XRE) in Ah receptor-mediated induction of human UDP-glucuronosyltransferase 1A1. J Biol Chem 278:15001–15006.

Zhang ZY, Pelletier RD, Wong YN, Sugawara M, Zhao N, and Littlefield BA (2006) Preferential inducibility of CYP1A1 and CYP1A2 by TCDD: differential regulation in primary human hepatocytes versus transformed human cells. Biochem Biophys Res Commun. 341:399–407.

Legend for figures

Figure 1. Proposed biotransformation pathways of TSU-68 in human liver microsomes.

Figure 2. Eadie-Hofstee plots for TSU-68 hydroxylation by human liver microsomes.

TSU-68 (1–100 μM) was incubated with human liver microsomes (1 mg protein/ml) in the presence of the NADPH-generating system. Incubations were conducted at 37ºC for 10 min. M1, M2, and M3 correspond to 5-, 6-, and 7-hydroxylated TSU-68, respectively. Each point represents the mean value in liver microsomes from four donors.

Figure 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 per treatment).

Figure 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-hydroxy testosterone after incubation of 125 μM

testosterone with hepatocytes for 2 h. Each bar represents the mean + S.D. (n = 3 wells per treatment).

Figure 5. Effect of TSU-68 on CYP1A1 and CYP1A2 protein expression.

Human cryopreserved hepatocytes from three donors were treated for 48 h with 0.1% DMSO (vehicle) or 10 μM TSU-68. Protein extracts were isolated from hepatocytes and analyzed by immunoblotting for CYP1A1 and CYP1A2. CYP1A1 and CYP1A2 protein expression was identified and quantified based on comparison with the protein produced from corresponding baculovirus insect cell-expressed cDNA. ND, not detected.

Figure 6. Effect of TSU-68 on its hydroxylation 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). Formation of M1, M2, and M3 was determined after incubation of 10 μM TSU-68 with the treated hepatocytes for 2 h. Each bar represents mean + S.D. (n = 3 wells per treatment).

Table 1 TSU-68 hydroxylation by human cDNA-expressed CYP isoforms.

cDNA-expressed human CYP isoform	Formation of TSU-68 metabolite (pmol/min/pmol P450)		
	M1	M2	M3
CYP1A1	28.8	29.1	0.57
CYP1A2	6.5	21.1	3.9
CYP2A6	–	–	–
CYP2B6	–	–	–
CYP2C8	0.18	0.04	0.03
CYP2C9	–	–	–
CYP2C19	–	–	–
CYP2D6	–	0.21	–
CYP2E1	–	–	–
CYP3A4	–	0.15	–

TSU-68 (50 μM) was incubated with insect cell microsomes containing cDNA-expressed CYP isoform in the presence of a NADPH-generating system.

– : Not detected.

Table 2 Effects of chemical and antibody inhibitors of cytochrome P450 isoforms on TSU-68 hydroxylation by pooled human liver microsomes.

Inhibitor	Concentration or volume	Residual activity (% of control)		
		M1	M2	M3
Chemical inhibitor				
α-Naphthoflavone (CYP1A2)	1 μM	21	19	26
	10 μM	9	21	23
Quercetin (CYP2C8)	10 μM	63	72	67
	30 μM	40	47	45
Quinidine (CYP2D6)	4 μM	98	115	97
	40 μM	88	146	91
Ketoconazole (CYP3A4)	1 μM	104	115	109
	2 μM	103	113	105
Antibody inhibitor				
Anti-CYP1A2	20 μl	8	4	7
	40 μl	3	2	4
Anti-CYP2C8	20 μl	96	102	96
	40 μl	94	101	95

The chemical and antibody inhibitors were preincubated with TSU-68 (50 μM) and human liver microsomes (1 mg protein/ml) at 37 ℃ for 5 and 30 min, respectively.

Table 3 Enzyme kinetic parameters for TSU-68 hydroxylation by human liver and cDNA-expressed CYP isoform microsomes.

Microsomes	K_m			V_{max}			V_{max}/K_m		
	M1	M2	M3	M1	M2	M3	M1	M2	M3
Human liver microsomes		(μM)			(pmol/min/mg protein)			(μl/min/mg protein)	
HLM 31	3.9	3.6	4.2	70	132	32	18.0	36.8	7.6
HLM 74	8.5	8.0	8.9	31	55	14	3.7	6.9	1.6
HLM 81	7.3	7.2	7.2	85	153	36	11.7	21.4	4.9
HLM 94	7.2	8.2	7.6	137	275	59	19.0	33.4	7.8
Mean±S.D.	6.7±2.0	6.8±2.2	7.0±2.0	81±44	154±91	35±18	13.1±7.0	24.6±13.5	5.5±2.9
cDNA-expressed CYP		(μM)			(pmol/min/pmol P450)			(μl/min/pmol P450)	
CYP1A1	0.76	0.86	–	28.0	30.5	0.8	36.8	35.5	–
CYP1A2	10.4	11.2	11.0	7.8	25.7	4.7	0.75	2.29	0.43
CYP2C8	57.8	66.0	44.7	0.42	0.09	0.06	0.0073	0.0014	0.0014

– : Not calculated.

Fig.1

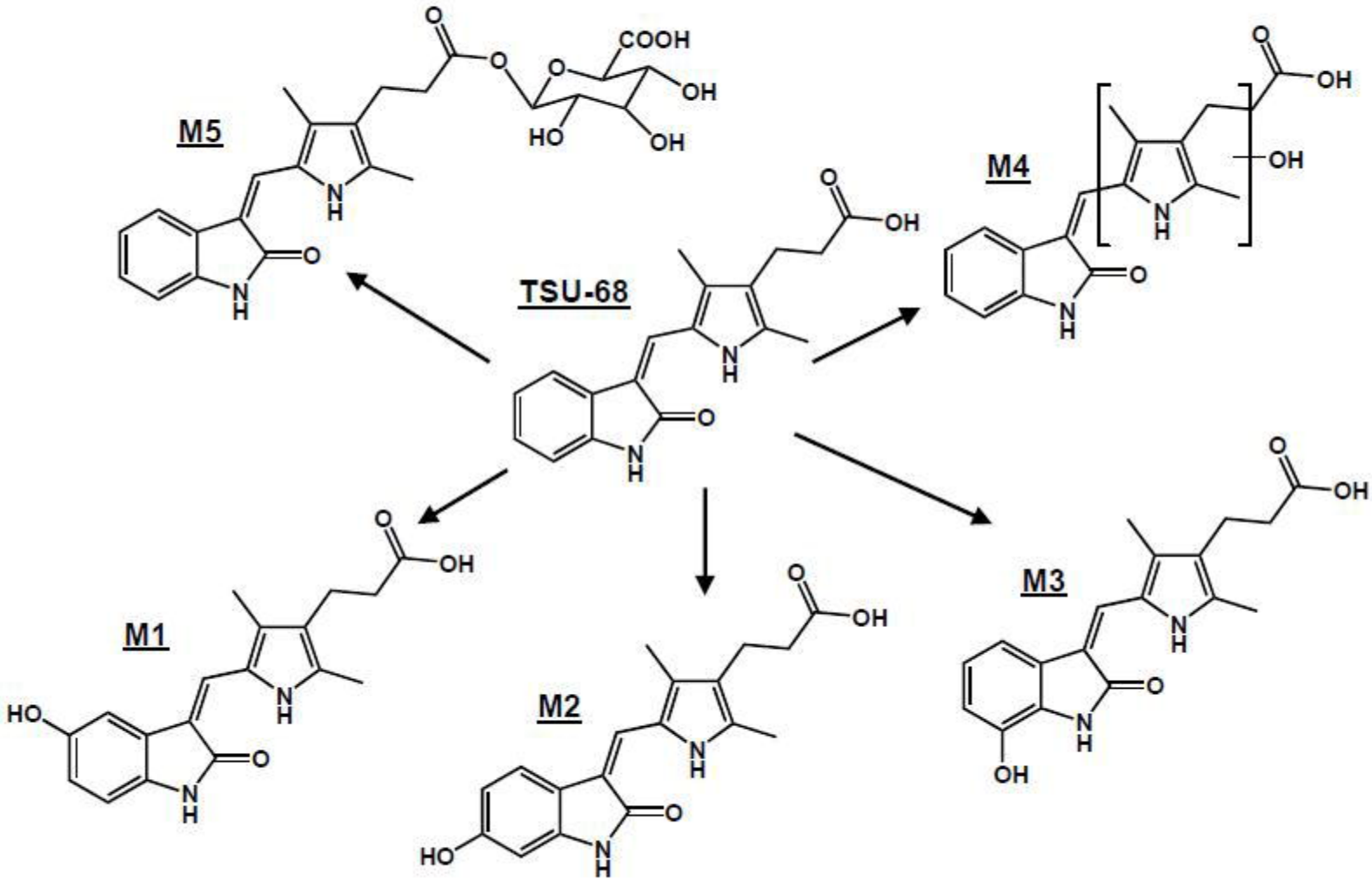


Fig.2

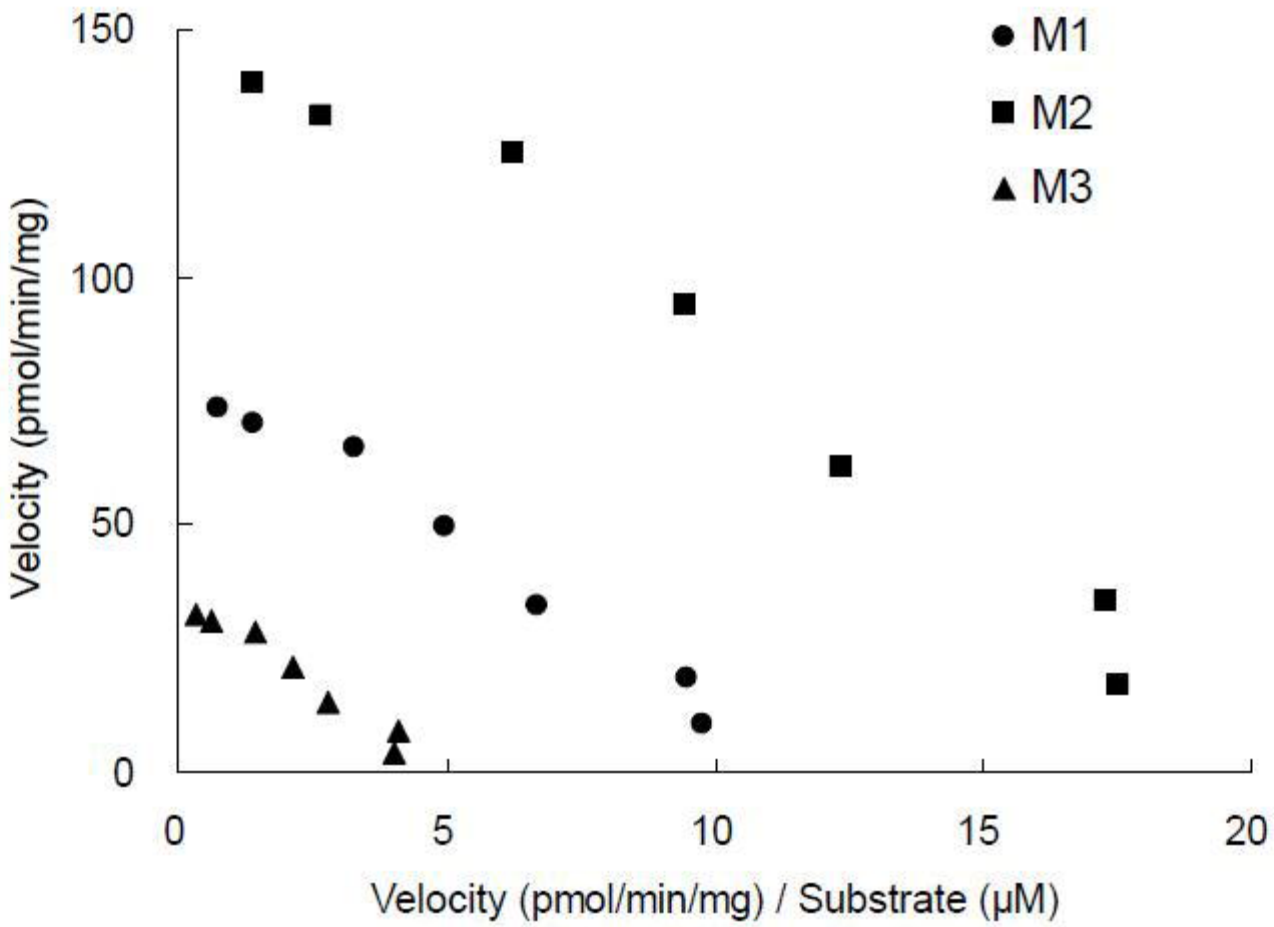

150
100
50
0
Velocity (pmol/min/mg)
M1
M2
M3
0
5
10
15
20
Velocity (pmol/min/mg) / Substrate (μM)

Fig.3

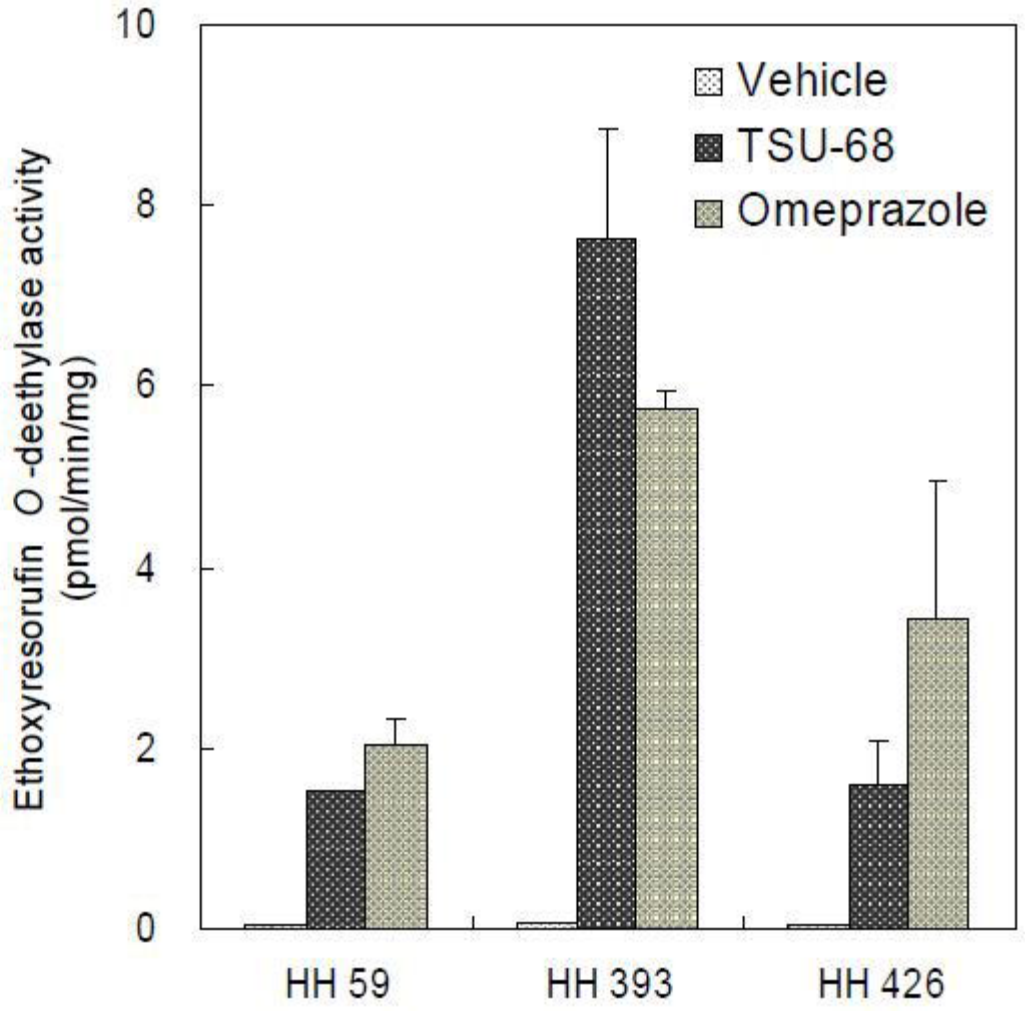

Ethoxyresorufin O-deethylase activity
(pmol/min/mg)
10
8
6
4
2
0
Vehicle
TSU-68
Omeprazole
HH 59
HH 393
HH 426

Fig.4

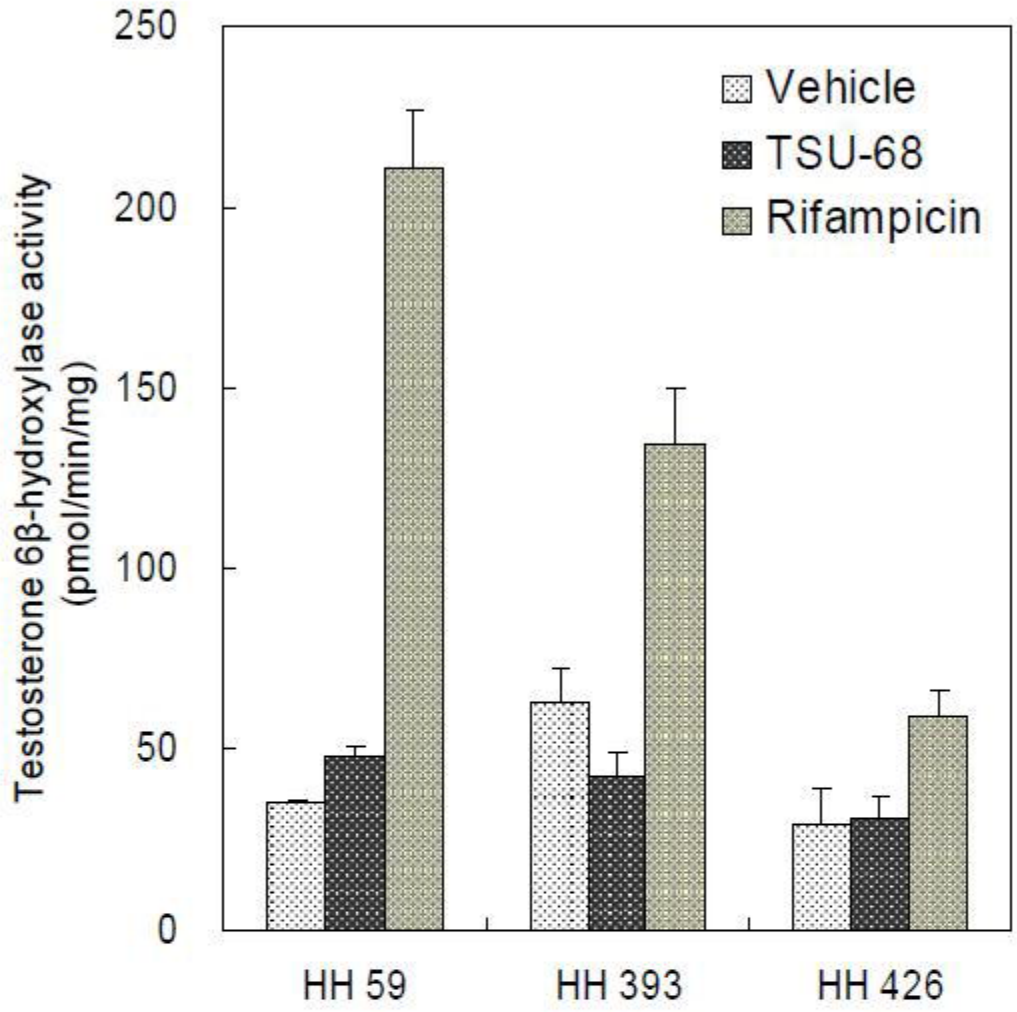

Testosterone 6β-hydroxylase activity
(pmol/min/mg)
250
200
150
100
50
0
Vehicle
TSU-68
Rifampicin
HH 59
HH 393
HH 426

Fig.5

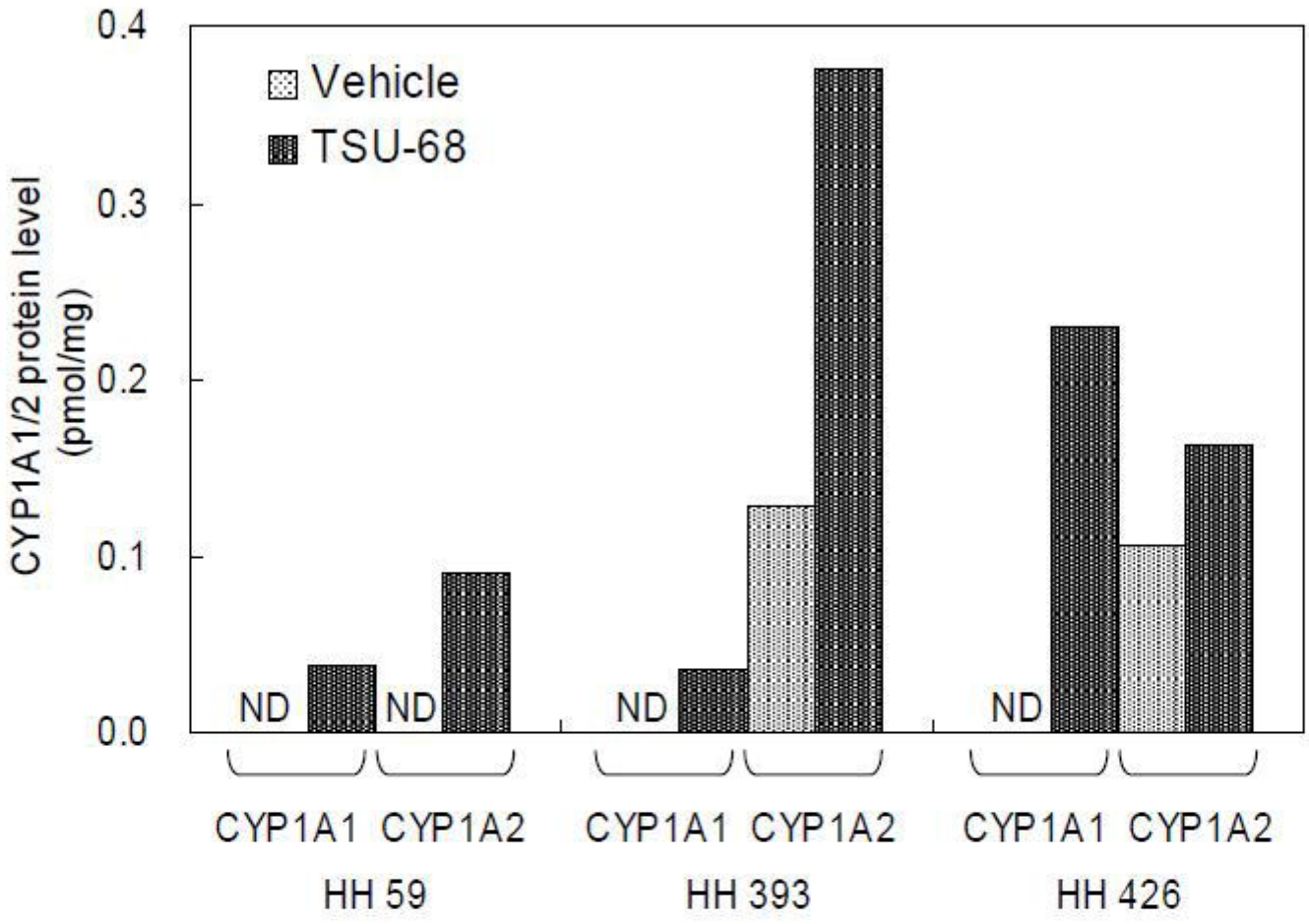
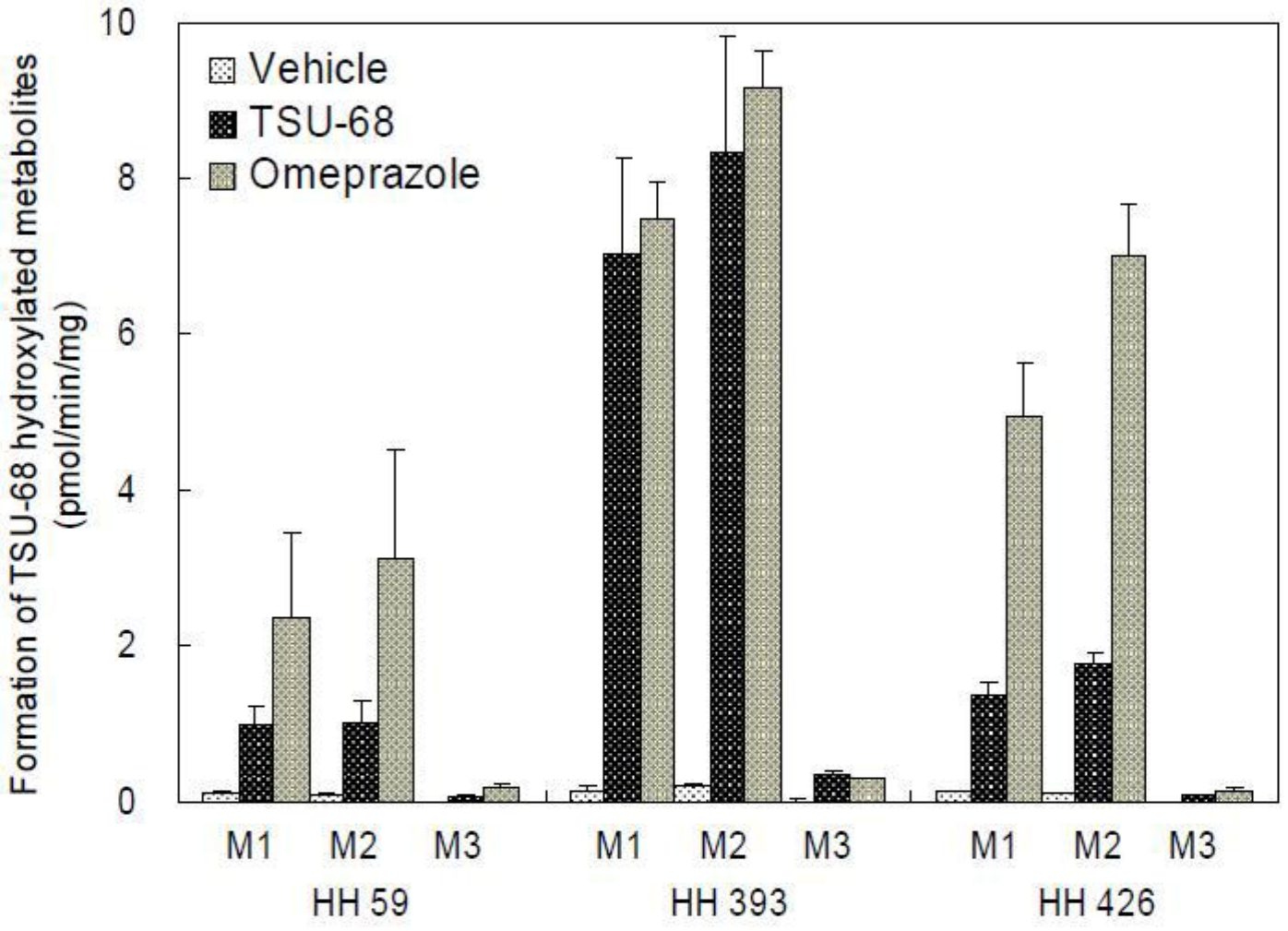

0.4
0.3
0.2
0.1
0.0
CYP1A1/2 protein level (pmol/mg)
Vehicle
TSU-68
ND
ND
ND
ND
CYP1A1
CYP1A2
CYP1A1
CYP1A2
CYP1A1
CYP1A2
HH 59
HH 393
HH 426

Fig.6


Formation of TSU-68 hydroxylated metabolites (pmol/min/mg)
10
8
6
4
2
0
Vehicle
TSU-68
Omeprazole
M1
M2
M3
HH 59
HH 393
HH 426