DMD Fast Forward. Published on September 10, 2008 as doi:10.1124/dmd.108.022830

CYP2C9-Catalyzed Metabolism of *S*-Warfarin to 7-Hydroxywarfarin In vivo and In vitro in Chimeric Mice with Humanized Liver

Tae Inoue, Kayoko Nitta, Kazumi Sugihara, Toru Horie,
Shigeru Ohta, and Shigeyuki Kitamura

Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, 734-8553, Japan (T.I., K.N., K.S., S.O., S.K.); PhoenixBio, Co., Ltd., Higashihiroshima, 739-0046, Japan (T.I., T.H.); Nihon Pharmaceutical University, Ina-machi, Saitama 362-0806, Japan (S.K.)

a) Running title: Metabolism of warfarin in human chimeric mice

b) Corresponding author : Dr. Shigeru Ohta

Graduate School of Biomedical Sciences, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8553, Japan

Fax number 81-82-257-5329

e-mail address sohta@hiroshima-u.ac.jp

c) The number of pages: 20

The total number of references: 25

The number of word: Abstract 193, Introduction 626, Discussion 825

The number of Figure: 4, The number of Table: 1

d) Abbreviations: HPLC, high-performance liquid chromatography; P450, cytochrome P450; AUC, area under the plasma concentration-time curve; hAlb, human albumin; RI, replacement index; uPA$^{(+/+)}$/SCID mice, urokinase-type plasminogen activator-transgenic severe combined immunodeficient mice.

ABSTRACT

Chimeric mice having humanized liver were constructed by transplantion of human hepatocytes. In this study, we investigated whether these mice have a similar capacity for drug metabolism to humans by examining hydroxylation of *S*-warfarin, which is predominantly metabolized to *S*-7-hydroxywarfarin, catalyzed by CYP2C9, in humans, but not mice. The 7-hydroxylating activity of chimeric mouse liver microsomes toward *S*-warfarin was about ten-fold higher than that of control (urokinase-type plasminogen activator-transgenic severe combined immunodeficient) mice. The 7-hydroxylase activity of chimeric mouse liver microsomes was markedly inhibited by sulfaphenazole, as was that of human liver microsomes, whereas the activity of control mice was unaffected. The CYP2C isoform in chimeric mouse liver was also confirmed to be the human isoform, CYP2C9, by immunoblot analysis. In the present in vivo study, the level of *S*-7-hydroxywarfarin in plasma of chimeric mice was about seven-fold higher than that in control mice, in agreement with the in vitro data. Thus, the CYP2C isoform in chimeric mice functions in vivo and in vitro as a human isoform, CYP2C9. These results suggest that chimeric mice with humanized liver could be useful to predict drug metabolism in humans, at least as regards CYP2C9-dependent metabolism.

Introduction

Chimeric mice have been constructed by transplantion of human hepatocytes into urokinase-type plasminogen activator-transgenic SCID mice (Dandri et al., 2001; Mercer et al., 2001). These chimeric mice were suggested to be useful as an in vivo model for studies on human liver diseases and hepatotropic viruses. Prolonged infections of human hepatitis B or C virus can be maintained in these mice (Dandri et al., 2001; Mercer et al., 2001). However, the chimeric mice used in those experiments did not have a high level of replacement with human hepatocytes. Recently, Tateno et al. (2004) prepared chimeric mice in which the liver was almost completely repopulated with human hepatocytes. Furthermore, they reported that cytochrome P450 subtypes in liver microsomes of chimeric mice in which more than 80 % of hepatocytes had been replaced with human hepatocytes were similar to those of the donor human liver (Tateno et al., 2004). Therefore, the chimeric mice constructed by Tateno et al. appear to be an excellent in vivo model for prediction of drug metabolism and drug-drug interactions due to drug induction and inhibition of drug-metabolising enzymes in humans. Katoh et al. (2004; 2005a) reported that cytochrome P450 (P450) isoforms and phase II enzymes in chimeric mice in which the human hepatocyte replacement rate was nearly 90 % were almost the same as those in human liver, based on quantitation of enzyme proteins. They also showed that the chimeric mice are a useful animal model to estimate the inductive effect on P450 in humans (Katoh et al., 2005b). We have shown that aldehyde oxidase, a cytosolic drug-metabolizing enzyme, in chimeric mice has functional characteristics almost identical with those of human aldehyde oxidase (Kitamura et al., 2008). Furthermore, Nishimura et al. (2005) reported that hepatocytes from chimeric mice with nearly completely humanized liver are a useful tool for screening the potency of new drugs to induce drug-metabolizing enzymes in human. Here, we investigated whether these mice have a similar capacity for CYP2C9-dependent metabolism of *S*-warfarin to that of humans.

Warfarin is widely used as an anticoagulant drug in humans, but it has a narrow therapeutic index and requires tight control of the dosage regimen. Racemic warfarin has been used clinically as an oral drug and as an environmental rodenticide, but the two isomers differ in their pharmacodynamics. *S*-Warfarin is pharmacologically active, while the *R*-enantiomer is essentially inactive in humans (O'Reilly, 1974). *R*- and *S*-Warfarin are metabolized via CYP1A1, 2B1, 2C6, 2C11 and 3A2 in rats (Kaminsky et al., 1983). In humans, *S*-warfarin is mainly metabolized to inactive 7-hydroxywarfarin and also to 6-hydroxywarfarin as a minor metabolite by CYP2C9 (Rettie et al., 1992; Kaminsky et al., 1993). CYP2C8, 2C18 and 2C19 expressed in yeast generated the 4'-hydroxyl metabolite from *S*-warfarin (Kaminsky et al., 1993). *R*-Warfarin is metabolized to 6-, 8- and 10-hydroxywarfarin via CYP1A2, 2C19 and 3A4 in humans (Rettie et al., 1989; Wienkers et al., 1996; Kaminsky and Zhang, 1997; Yamazaki and Shimada, 1997). *S*-Warfarin is metabolized more rapidly than the *R*-enantiomer, and in particular, 7-hydroxylation was 8-fold higher for *S*-warfarin than for *R*-warfarin (Park, 1988; Yamazaki and Shimada, 1997). The interaction of warfarin with various drugs, natural substances and foods via CYP2C9 is clinically significant (Toon et al., 1986; Rettie et al., 1992; Greenblatt and von Moltke, 2005).

In this study, we estimated the activity of CYP2C isozyme in the livers of control (urokinase-type plasminogen activator-transgenic severe combined immunodeficient: uPA$^{(+/+)}$/SCID) mice and chimeric mice with humanized liver by using *S*-warfarin as a substrate. The amounts of warfarin and its 7-hydroxyl derivative in the blood of chimeric mice dosed with warfarin were also measured by using high-performance liquid chromatography (HPLC). We demonstrated that oxidation of *S*-warfarin to *S*-7-hydroxywarfarin was catalyzed by CYP2C9 in the chimeric mice, both in vivo and in vitro.

Materials and Methods

Materials. *S*-Warfarin was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). *S*-7-Hydroxywarfarin and *S*-6-hydroxywarfarin were purchased from Ultrafine Chemicals Ltd. (Manchester, England). Pooled human liver microsomes and anti-CYP2C9 were purchased from BD Biosciences (San Jose, CA).

Animals. Chimeric mice with humanized liver were prepared according to the method of Tateno et al. (2004). Human hepatocytes were transplanted into uPA$^{(+/+)}$/SCID mice (20-30 days after birth), and progressively repopulated the mouse host liver. Hepatocytes from a male Caucasian (13 years old) were obtained from In Vitro Technologies (Catonsville, MD). In the case of Western blot analysis, hepatocytes from a male Caucasian (4 years old) and a male Caucasian (6 years old) were used as donors. To estimate the humanization of the chimeric mice, the replacement index (RI) of the mice with the human hepatocytes was determined by measuring human albumin (hAlb) in blood collected periodically from the tail vein, using a Human Albumin ELISA Quantitation kit (Bethyl Laboratories Inc., Montgomery, TX). The uPA$^{(+/+)}$/SCID mice were generated by crossing uPA mice (B6SJL-TgN(Alb1Plau)144Bri; The Jackson Laboratory, Bar Harbor, ME) with SCID mice (Fox Chase SCID C.B-17/Icr-*scid* Jcl; Clea Japan Inc., Tokyo, Japan). The mice were housed in cages on a 12-h light/dark cycle at 25^{o}C, with free access to tap water and diets. Chimeric mice were given CRF1 containing Vitamin C, sterilized by γ-ray irradiation (Oriental Yeast Co., Ltd., Tokyo, Japan) and control mice were given CRF1 (Oriental Yeast Co., Ltd.).

Administration of *S*-Warfarin to Mice. *S*-Warfarin dissolved in saline (100 mg/ml) was administered to mice intraperitoneally at a single dose of 30 mg/kg. Blood was collected at 0.5, 1, 3, 4 or 6 hr after treatment.

Liver Preparations. Livers were excised from control (uPA$^{(+/+)}$/SCID mice) and chimeric male mice (9-12 weeks age) and homogenized in four volumes of

1.15 % KCl. The microsomal fraction was obtained from the homogenate by successive centrifugation at 9,000 x *g* for 20 min and 105,000 x *g* for 60 min. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard protein.

Immunoblot Analysis of CYP2C Isoforms. The levels of CYP2C9 protein were determined by immunoblot analysis. Mouse microsomal proteins (5 μg) were separated by SDS-polyacrylamide gel electrophoresis (10% gel) and transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA) by electroblotting. Membranes were then incubated with 5% skimmed milk in 25 mM tris-buffered saline (pH 7.6) - 0.1% Tween 20 for 1 h and probed with an anti-rat CYP2C9 (1:1000) for 1 h. After washing, antibody binding was detected with horseradish peroxidase-conjugated rabbit anti-rat IgG, followed by development with ECL Plus (GE Healthcare, Buckinghamshire, England).

Assay for Warfarin Oxidase Activity. Warfarin oxidase activity was measured using a slight modification of the method of Lang and Bocker (1995). The amounts of *S*-7-hydroxywarfarin formed were measured by HPLC. Briefly, the incubation mixture consisted of 0.2 μmol of *S*-warfarin and liver microsomes equivalent to 50 - 100 mg of liver wet weight in a final volume of 0.5 ml of 0.1 M K, Na-phosphate buffer (pH 7.4). The incubation was performed at 37º for 20 min. Then 10 μg of 2-benzylphenol (an internal standard) and 1 ml of acetonitrile were added. After centrifugation, an aliquot of the supernatant was subjected to analysis by HPLC.

Measurement of Warfarin and its Metabolites in Plasma. *S*-Warfarin and *S*-7-hydroxywarfarin in blood of mice were determined according to the method of Zhu and Shin (2005). Ten μg of 2-benzylphenol as an internal standard and 0.6 g of KCl were added to the plasma and the mixture was extracted 3 times with 5 ml of diethyl ether. The amounts of *S*-warfarin and *S*-7-hydroxywarfarin in the extract were determined by using HPLC as follows.

HPLC. HPLC was performed using a Hitachi L-6000 chromatograph (Hitachi Co. Ltd., Tokyo, Japan) fitted with an Inertsil ODS-3 column (150 cm x 4.6 mm, GL Science Inc., Tokyo, Japan). The mobile phase was acetonitrile-0.5 % phosphoric acid (38 : 62 v/v). The chromatograph was operated at a flow rate of 1 ml/min, with a detection wavelength of 254 nm. The elution times of *S*-warfarin, *S*-7-hydroxywarfarin, *S*-6-hydroxywarfarin and IS were 28.2, 13.3, 10.2 and 42.3 min, respectively. Standard curves of *S*-warfarin and *S*-7-hydroxywarfarin were linear in the ranges of 50 - 0.1 ng and 15 - 0.05 ng, respectively. For *S*-warfarin, y=0.0911x with R^2=0.9800, while for *S*-7-hydroxywarfarin, y=0.0417x with R^2=0.9919. For quantification of incubated samples, peak-area ratios relative to that of the internal standard were used.

Statistical Analysis. Values are presented as means and standard deviations. Comparison of average AUC value between chimeric mice and control mice were made using the unpaired Student's *t* test with Excel software (Microsoft, Redmond, WA). A p value < 0.05 was considered statistically significant.

Results

7-Hydroxylase Activity of Liver Micosomes from Control and Chimeric Mice. 7-Hydroxylase activities of liver microsomes of control and chimeric mice toward *S*-warfarin were assayed. The calibration plot for *S*-7-hydroxywarfarin was linear in the range of 200 - 4 pmol/sample, and the detection limit was 0.1 pmol/min/mg protein. When *S*-warfarin was incubated with liver microsomes from control mice and from chimeric mice with a high level of hepatocyte replacement with human hepatocytes (>80 %) in the presence of NADPH, marked difference of their activities was observed. The HPLC peak of *S*-7-hydroxywarfarin formed by liver microsomes of chimeric mice was much higher than that in the case of control mice, but no other peaks due to metabolites of *S*-warfarin were detected (data not shown). The 7-hydroxylating activity of the chimeric mouse liver microsomes

toward *S*-warfarin was about ten-fold higher than that in the case of control mice. When the activities were compared among chimeric mice with different levels of replacement with human hepatocytes (40-50, 60-70 and 80-90 %), as judged from the hAlb concentration in the blood, clear differences were observed (Fig. 1). The 7-hydroxylating activity toward *S*-warfarin increased with increasing replacement ratio with human hepatocytes. Further, the 7-hydroxylase activity of liver microsomes of chimeric mice was markedly inhibited by sulfaphenazole, an inhibitor of CYP2C9 (Rettie et al., 1992; Yamazaki and Shimada, 1997), as is the case for human liver microsomes, but the activity of control mice was unaffected (Table 1). Further characterization of the CYP2C isoform in these liver microsomes was conducted by Western blot analysis using CYP2C9 antibody. The CYP2C isoform in chimeric mouse liver was confirmed to be the human isoform, CYP2C9, by immunoblot analysis (Fig. 2). The density of the band in the case of liver microsomes chimeric mice with a high level of displacement (>80 %) with human hepatocytes was close to that of pooled human liver microsomes. These results suggest that P450 in livers of chimeric mice functions as a human-type P450, i.e., CYP2C9.

(Fig. 1) (Fig. 2) (Table 1)

In Vivo Metabolism of *S*-Warfarin to the 7-Hydroxyl Derivative in Chimeric Mice. *S*-Warfarin and *S*-7-hydroxywarfarin in the plasma of control and chimeric mice after the administration of *S*-warfarin were assayed. The calibration plots for *S*-warfarin and *S*-7-hydroxywarfarin were linear in the ranges of 100 - 1 ng/ml of plasma and 30 - 0.5 ng/ml of plasma, respectively. The detection limits were 1 and 0.5 ng/ml of plasma, respectively. When *S*-warfarin was administered to control and chimeric mice, peaks of warfarin and *S*-7-hydroxywarfarin were found in HPLC chromatograms of the extract of the plasma of these mice. No peak of *S*-6-hydroxywarfarin was seen at 10.2 min, the

elution time of an authentic sample. The peak levels of *S*-warfarin in the plasma of control mice at various times after administration were approximately the same as those in chimeric mice. In contrast, the HPLC peaks of *S*-7-hydroxywarfarin in chimeric mice with high levels of human hepatocyte replacement were much higher than those in control mice (Fig. 3). The area under the plasma concentration-time curve (AUC) of *S*-7-hydroxywarfarin was greater in chimeric mice than that in control mice. Cmax in the plasma of control mice was also higher than that of chimeric mice (Fig. 4). Parameters of *S*-7-hydroxywarfarin in control (uPA $^{(+/+)}$/SCID and uPA $^{(wt/wt)}$/SCID) and chimeric mice were as follows: AUC 6.4, 4.4 and 36.4, Cmax 1.0, 2.6 and 8.5, Tmax 1.0, 3.1 and 2.8, respectively. Parameters of *S*-warfarin in control (uPA $^{(+/+)}$/SCID and uPA $^{(wt/wt)}$/SCID) and chimeric mice were as follows: AUC 170.6, 151.3 and 243.2, Cmax 56.5, 37.0 and 59.1, Tmax 1.58, 1.58 and 1.58, respectively. The AUC value of S-warfarin showed no significant difference between chimeric mice and control mice, whereas those of *S*-7-hydroxywarfarin differed significantly by Student's *t* test. This confirms that the in vivo metabolism of *S*-warfarin closely reflects the in vitro metabolism of *S*-warfarin in liver microsomes of chimeric mice.

(Fig. 3) (Fig. 4)

These results suggest that the chimeric mice could be useful to predict drug metabolism in humans, at least as regards CYP2C9-dependent metabolism.

Discussion

In this study, we demonstrated that a microsomal drug-metabolizing enzyme in chimeric mice with a high level of hepatocyte replacement with human hepatocytes functions as human-type P450, CYP2C9, both in vivo and in vitro. Further, the conversion of *S*-warfarin to the 7-hydroxyl metabolite in chimeric mice was similar to that in humans. The difference between the control mice and the chimeric mice reflects the differences of CYP2C isoform between mice and humans. *S*-Warfarin is known to be mainly oxidized to the 7-hydroxyl metabolite by CYP2C9. In mice, however, *S*-warfarin is not so actively oxidized to 7-hydroxywarfarin as it is in humans. *S*-Warfarin is therefore a good substrate to distinguish the P450 isoform in mice and humans. It appears to be possible to predict which new drugs would be metabolized by CYP2C9, by means of preclinical drug metabolic studies in these chimeric mice. We would not expect any marked difference between humanized chimeric mice and control mice as regards formation of *S*-4'-hydroxywarfarin and *S*-10-hydroxywarfarin by other CYPs, because although the CYP2C subfamily shows different substrate specificities between humans and mice, other CYPs do not.

We confirmed the usefulness of chimeric mice for studies of drug disposition. Until now, studies of drug metabolism in chimeric mice have been restricted to in vitro microsomal oxidase enzymes and phase II enzymes (Katoh et al., 2004; 2005b). In those reports, it was demonstrated that isoforms of P450 and phase II enzymes in chimeric mouse liver behaved similarly to those in human liver in in vitro experiments. Recently, we showed that aldehyde oxidase in chimeric mice functions as a human-type aldehyde oxidase in vivo and in vitro (Kitamura et al., 2008). In this study, we extended the studies in chimeric mice to in vivo and in vitro metabolism of *S*-warfarin by CYP2C9. This is the first report that a hepatic drug metabolizing enzyme, P450, in chimeric mice acts as a human enzyme at the in vivo level. A good in vivo-in vitro correlation of *S*-warfarin oxidase activity due to P450 in chimeric mice was demonstrated in this study. The amounts of *S*-7-hydroxywarfarin found in

plasma of chimeric mice were correlated with the in vitro warfarin oxidase activity of liver microsomes. Furthermore, the activity of chimeric mice varied in a hAlb concentration-dependent manner in both in vivo and in vitro studies. This fact suggests that the formation of the 7-hydroxyl derivative from *S*-warfarin in chimeric mice in vivo can be ascribed to human CYP2C9. This in vivo-in vitro correlation is important, because studies in chimeric mice would have various advantages for predicting drug interactions in humans. However, the role of enzyme activity in extrahepatic organs must also be considered. P450 is known to exist in organs other than liver. However, liver CYP2C9 is the major determinant of in vivo *S*-warfarin metabolism. In preclinical tests using chimeric mice, it would be important to confirm that the liver enzyme functions as the major metabolic enzyme at the in vivo level, as appears to be the case for CYP2C9-catalyzed warfarin metabolism in these chimeric mice.

Recently, transgenic mouse models containing individual human P450 isoforms, such as CYP2D6 and CYP3A4, have been generated (Corchero et al., 2001; Robertson et al., 2003; Zhang et al., 2003; Miksys et al., 2005). These mice could be useful to predict the toxicity and pharmacological effects of various chemicals. Usually, human hepatocytes are used in vitro for such studies, and it is the case that cultured human hepatocytes express most of the hepatic drug-metabolizing enzymes found in vivo. However, the activities of these enzymes tend to decrease during culture, due to a decrease of P450 gene transcription (Rodriguez-Antona et al., 2002). Chimeric mice can be regarded as a convenient source of fresh “human” hepatocytes.

The replacement index (RI) with human hepatocytes in the chimeric mice was evaluated from the hAlb concentration. In this study, we found that warfarin 7-hydroxylase activity and the level of CYP2C9 protein in mouse liver microsomes correlated reasonably well with the hAlb level in the blood of the chimeric mice. This indicates that P450 changed from murine form to human form in parallel with the replacement ratio with human hepatocytes. Katoh et al. (2004) also found that

the expression of human CYP2C9 mRNA was correlated with the concentration of hAlb in chimeric mice. Diclofenac 4'-hydroxylation is catalyzed by CYP2C9 (Nakajima et al., 2002), and Tateno et al. (2004) reported that diclofenac 4'-hydroxylase activity in chimeric mice having a high replacement ratio with human hepatocytes was much higher than that of control untreated mice.

Functional induction in addition to increased expression of CYP2C9 mRNA and protein (not murine cyp2c) in chimeric mice has been reported (Katoh et al., 2005b). Many drug interactions with warfarin are caused by inhibition or induction of CYP2C9 (Rettie et al., 1992; Yamazaki and Shimada, 1997). Therefore, the present chimeric mouse model with humanized liver should be useful for estimation and prediction of in vivo drug-drug interactions involving warfarin and CYP2C9-metabolized drugs in humans.

Footnotes

This study was partly supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Japanese Ministry of Education, Science, Sports and Culture, and the Japanese Ministry of the Environment.

References

Corchero J, Granvil CP, Akiyama TE, Hayhurst GP, Pimprale S, Feigenbaum L, Idle JR, and Gonzalez FJ (2001) The *CYP2D6* humanized mouse. Effect of the human *CYP2D6* transgene and *HNF4α* on the disposition of debrisoquine in the mouse. *Mol Pharmacol* **60:**1260-1267.

Dandri M, Burda MR, Torok E, Pollok JM, Iwanska A, Sommer G, Rogiers X, Rogler CE, Gupta S, Will H, Greten H, and Petersen J (2001) Repopulation of mouse liver with human hepatocytes and in vivo infection with hepatitis B virus. *Hepatology* **33:**981-988.

Greenblatt DJ and von Moltka LL (2005) Interaction of warfarin with drugs, natural substances and foods. *J Clin Pharmacol* **45:**127-132.

Kaminsky LS, de Morais SM, Faletto MB, Dunbar DA, and Goldstein JA (1993) Correlation of human cytochrome P4502C substrate specificities with primary structure: Warfarin as a probe. *Mol Pharmacol* **43:**234-239.

Kaminsky LS, Guengerich FP, Dannan GA, and Aust SD (1983) Comparisons of warfarin metabolism by liver microsomes of rats treated with a series of polybrominated biphenyl congeners and by the component-purified cytochrome P-450 isozymes. *Arch Biochem Biophys* **225:**398-404.

Kaminsky LS and Zhang Z-Y (1997) Human P450 metabolism of warfarin. *Pharmacol Ther* **73:**67-74.

Katoh M, Matsui T, Nakajima M, Tateno C, Kataoka M, Soeno Y, Horie T, Iwasaki K, Yoshizato K, and Yokoi T (2004) Expression of human cytochromes P450 in chimeric mice with humanized liver. *Drug Metab Dispos* **32:**1402-1410.

Katoh M, Matsui T, Okumura H, Nakajima M, Nishimura M, Naito S, Tateno C, Yoshizato K, and Yokoi T (2005a) Expression of human phase II enzymes in chimeric mice with humanized liver. *Drug Metab Dispos* **33:**1333-1340.

Katoh M, Matsui T, Nakajima M, Tateno C, Soeno Y, Horie T, Iwasaki K, Yoshizato K, and Yokoi T (2005b) In vivo induction of human cytochrome

P450 enzymes expressed in chimeric mice with humanized liver. *Drug Metab Dispos* **33:**754-763.

Kitamura S, Nitta K, Tayama Y, Tanoue C, Inoue T, Horie T, and Ohta S (2008) Aldehyde oxidase-catalyzed metabolism of N^1-methylnicotinamide in vivo and in vitro in chimeric mice with humanized liver. *Drug Metab Dispos* in press.

Lang D and Bocker R (1995) Highly sensitive and specific high-performance liquid chromatographic analysis of 7-hydroxywarfarin, a marker for human cytochrome P-4502C9 activity. *J Chromatogr B* **672:**305-309.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:**265-275.

Mercer DF, Schiller DE, Elliott JF, Douglas DN, Hao C, Rinfret A, Addison WR, Fischer KP, Churchill TA, Lakey JR, Tyrrell DL, and Knneteman NM (2001) Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* **7:**927-933.

Miksys SL, Cheung C, Gonzalez FJ, and Tyndale RF (2005) Human CYP2D6 and mouse CYP2DS: Organ distribution in a humanized mouse model. *Drug Metab Dispos* **33:**1495-1502.

Nakajima M, Tane K, Nakamura S, Shimada N, Yamazaki H, and Yokoi T (2002) Evaluation of approach to predict the contribution of multiple cytochrome P450s in drug metabolism using relative activity factor: effects of the differences in expression levels of NADP-cytochrome P450 reductase and cytochrome b5 in the expression system and the differences in the marker activities. *J Pharm Sci* **91:**952–963.

Nishimura M, Yokoi T, Tateno C, Kataoka M, Takahashi E, Horie T, Yoshizato K, and Naito S (2005) Induction of human CYP1A2 and CYP3A4 in primary culture of hepatocytes from chimeric mice with humanized liver. *Drug Metab Pharmacokinet* **20:**121-126.

O'Reilly RA (1974) Studies on the optical enantiomorphs of warfarin in man. *Clin Pharmacol Ther* **16:**348-354.

Park BK (1988) Warfarin: Metabolism and mode of action. *Biochem Pharmacol* **37:**19-27.

Rettie AE, Eddy AC, Heimark LD, Gibaldi M, and Trager WF (1989) Characteristics of warfarin hydroxylation catalyzed by human liver microsomes. *Drug Metab Dispos* **17:**265-270.

Rettie AE, Korzekwa KR, Kunze KL, Lawewnce RF, Eddy AC, Aoyama T, Gelboin HV, Gonzalez FJ, and Trager WF (1992) Hydroxylation of warfarin by human cDNA-expressed cytochrome P-450: A role for P-4502C9 in the etiology of (*S*)-warfarin-drug interactions. *Chem Res Toxicol* **5:**54-59.

Robertson GR, Field J, Goodwin B, Bierach S, Tran M, Lehnert A, and Liddle C (2003) Transgenic mouse models of human *CYP3A4* gene regulation. *Mol Pharmacol* **64:**42-50.

Rodriguez-Antona C, Donato MT, Boobis A, Edwards RJ, Watts PS, Castell JV, and Gomez-Lechon MJ (2002) Cytochrome P450 expression in human hepatocytes and hepatoma cell lines: molecular mechanisms that determine lower expression in cultured cells. *Xenobiotica* **32:**505-520.

Tateno C, Yoshizane Y, Saito N, Kataoka M, Utoh R, Yamasaki C, Tachibana A, Soeno Y, Asahina K, Hino H, Asahara T, Yokoi T, Furukawa T, and Yoshizato K (2004) Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* **165:**901-912.

Toon S, Low LK, Gibaldi M, Trager WF, O'Reilly, Motley CH, and Goulart DA (1986) The warfarin-sulfinpyrazone interaction: Stereochemical considerations. *Clin Pharmacol Ther* **39:**15-24.

Wienkers LC, Wurden CJ, Storch E, Kunze KL, Rettie AE, and Trager WF (1996) Formation of (R)-8-hydroxywarfarin in human liver microsomes: A new metabolic marker for the (S)-mephenytoin hydroxylase, P4502C19. *Drug Metab Dispos* **24:**610-614.

Yamazaki H and Shimada T (1997) human liver cytochrome P450 enzymes involved in the 7-hydroxylation of *R*- and *S*-warfarin enantiomers. *Biochem Pharmacol* **54:**1195-1203.

Zhang W, Purchio AF, Chen K, Wu J, Lu L, Coffee R, Contag PR, and West DB (2003) A transgenic mouse model with a luciferase reporter for studying in vivo transcriptional regulation of the human CYP3A4 gene. *Drug Metab Dispos* **31:**1o54-1064.

Zhu X and Shin WG (2005) Gender differences in pharmacokinetics of oral warfarin in rats. *Biopharm Drug Dispos* **26:**147-150.

Legends to Figures

Fig. 1. *S*-Warfarin oxidase activity of liver microsomes of chimeric mice.
Oxidase activity in liver of chimeric mice with various values of replacement index with human hepatocytes (RI) is shown. Each column represents the mean of triplicate determinations of an individual animal. The oxidase activity was assayed by measuring *S*-7-hydroxywarfarin formed by means of HPLC, as described under *Materials and Methods.*

Fig. 2. Western blots probed with anti-rat CYP2C9 antibody for mouse liver microsomes.

Fig. 3. HPLC chromatograms of the metabolites of *S*-warfarin in plasma of control (uPA$^{(+/+)}$/SCID) mice (A) and chimeric mice having a high level of replacement with human hepatocytes (B).

Fig. 4. Plasma concentration-time profiles of *S*-warfarin and the 7-hydroxylated metabolite after oral administration of *S*-warfarin to chimeric mice (A) and control (uPA$^{(+/+)}$/SCID and uPA$^{(wt/wt)}$/SCID) mice (B, C).
Each value represents the mean + S.D. of four determinations. *S*-Warfarin was orally administered at a dose of 30 mg/kg to male control and chimeric mice. Blood (0.05 ml) was collected at intervals. Amounts of *S*-warfarin and *S*-7-hydroxywarfarin in the plasma were determined using HPLC as described in Materials and Methods.

DMD Fast Forward. Published on September 10, 2008 as DOI: 10.1124/dmd.108.022830
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 1

Inhibitory effect of sulfaphenazole on S-warfarin 7-hydroxylase activity of liver microsomes of control and chimeric mice.

Liver microsomes	Alone	+ Sulfaphenazole	
	7-Hydroxylase activity (pmol/min/mg protein)		
			(% of control)
uPA$^{(wt/wt)}$/SCID mouse liver microsomes	0.39	0.30	(92 %)
Chimeric mouse liver microsomes	5.46	1.23	(23 %)
Pooled human liver microsomes	2.36	0.24	(10 %)

7-Hydroxylase activity toward *S*-warfarin of liver microsomes of control and chimeric mice, and pooled human liver microsomes was assayed in the presence and absence of sulfaphenazole (1×10^{-4} M) as described in Materials and Methods.

DMD Fast Forward. Published on September 10, 2008 as DOI: 10.1124/dmd.108.022830
This article has not been copyedited and formatted. The final version may differ from this version.

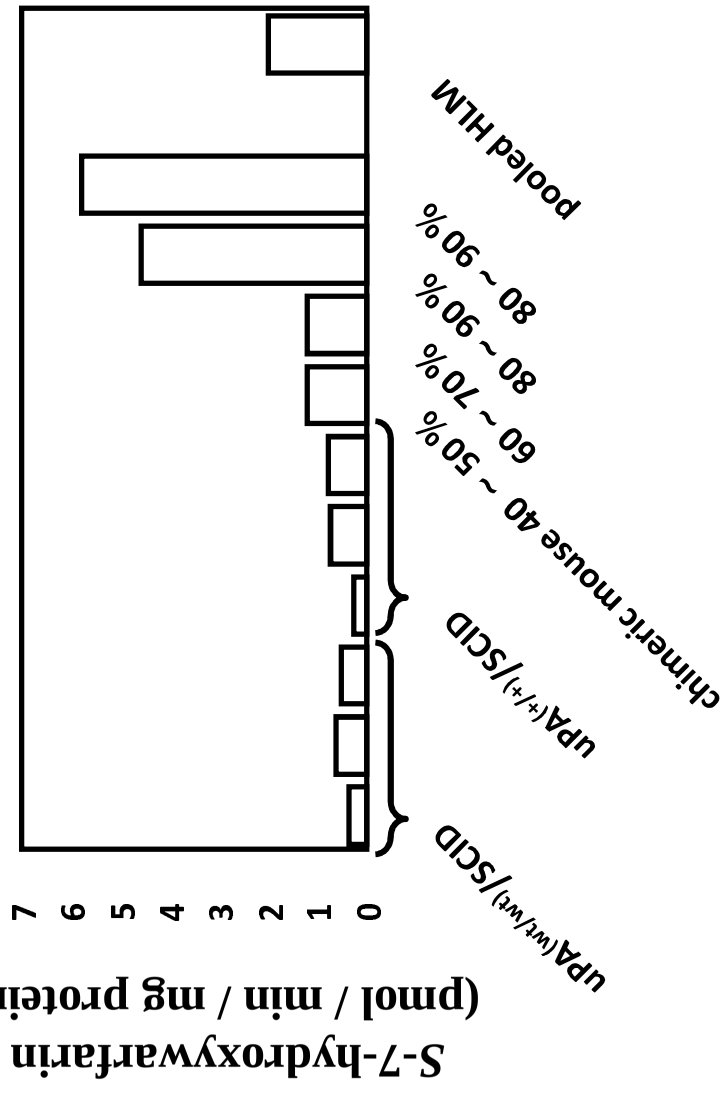


Fig.1

Fig.2

Standard

pooled HLM

uPA(wt/wt)/SCID

uPA(+/+)/SCID

Chimeric mouse 80 ~ 90%

80 ~ 90%

50 ~ 60%

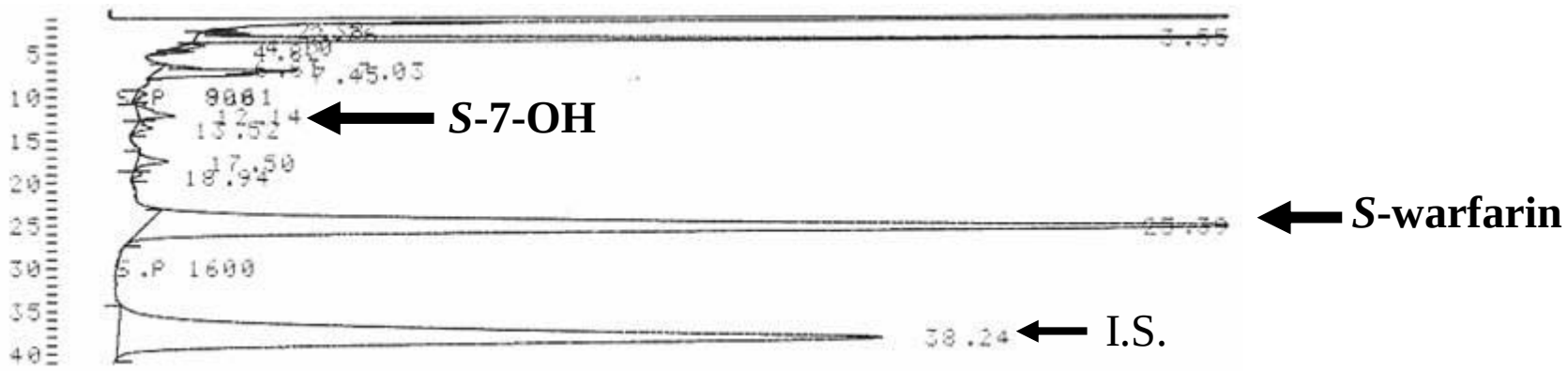
20 ~ 30%

Donor : Caucasian male, 4 years old

Donor : Caucasian female, 6 years old

Fig.3

(A) uPA $^{(+/+)}$/SCID mouse



(B) Chimeric mouse

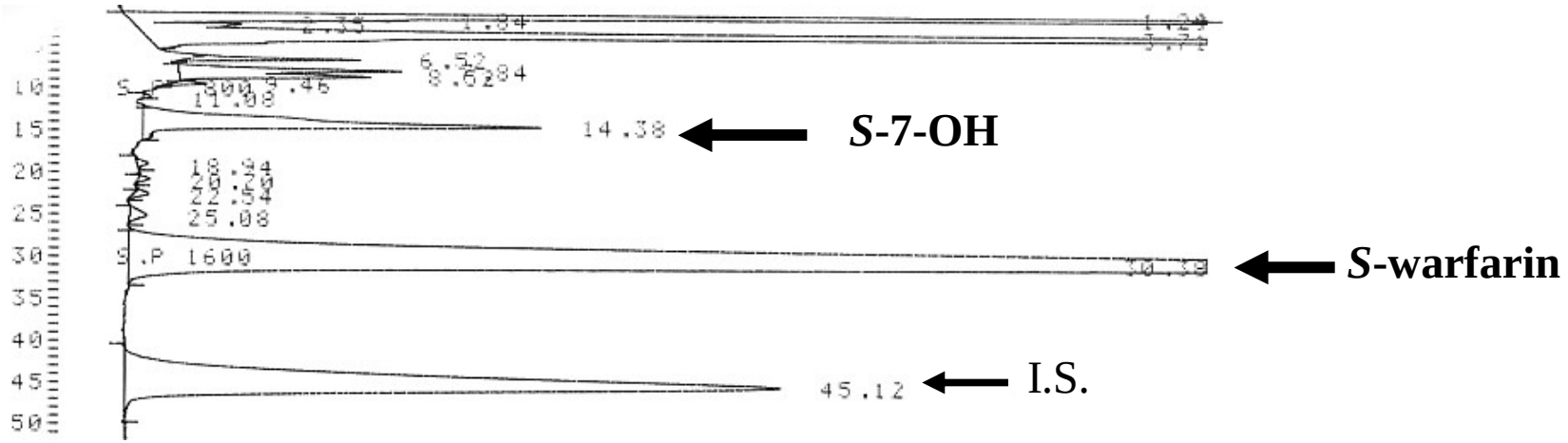


Fig.4