# Investigation of hepatic warfarin metabolism activity in rodenticide-resistant black rats (*Rattus rattus*) in Tokyo by *in situ* liver perfusion

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**Abstract**

Anti-blood coagulation rodenticides, such as warfarin, have been used all over the world. They inhibit vitamin K epoxide reductase (VKOR), which is necessary for producing several blood clotting factors. This inhibition by rodenticides results in lethal hemorrhage in rodents. However, heavy usage of these agents has led to the appearance of rodenticide-resistant rats. There are two major mechanisms underlying this resistance, i.e., mutation of the target enzyme of warfarin, VKOR, and enhanced metabolism of warfarin. However, there have been few studies regarding the hepatic metabolism of warfarin, which should be related to resistance. To investigate warfarin metabolism in resistant rats, *in situ* liver perfusion of warfarin was performed with resistant black rats (*Rattus rattus*) from Tokyo, Japan. Liver perfusion is an *in situ* methodology that can reveal hepatic function specifically with natural composition of the liver. The results indicated enhanced hepatic warfarin hydroxylation activity compared with sensitive black rats. On the other hand, in an *in vitro* microsomal warfarin metabolism assay to investigate kinetic parameters of cytochrome P450, which plays a major role in warfarin hydroxylation, the $V_{max}$ of resistant rats was slightly but significantly higher compared to the results obtained in the *in situ* study. These results indicated that another factor like electron donators may also contribute to the enhanced

metabolism in addition to high expression of cytochrome P450.

**Keywords**

warfarin, rodenticide resistance, *Rattus rattus*, liver perfusion, metabolism, cytochrome P450

## 1. Introduction

Rodents cause significant damage to human activities. Among these rodents, two species in the genus *Rattus* are dominant in urban areas all over the world, i.e., *Rattus norvegicus* (brown rats) and *Rattus rattus* (black rats). Brown rats are predominant in Europe and the USA, while black rats are more common in Japan [1]. These animals act as vectors of various zoonotic diseases, including plague, *Leptospira interrogans*, Lime disease caused by *Borrelia burgdorferi*, and hemorrhagic fever with renal syndrome caused by hantaviruses [2,3]. Rats also have economic effects as they chew through electrical wires and optical cables, which may result in fire or loss of data. They feed on agricultural products and stored products. In addition, they contaminate these products with their feces and urine, and therefore represent a serious hygiene problem [4]. Thus, control of rats using rodenticides is necessary.

Vitamin K antagonists, including warfarin, have been used as rodenticides all over the

55 world for more than 50 years. They target the endoplasmic enzyme vitamin K-epoxide
56 reductase (VKOR), which is necessary for producing blood clotting factors II, VII, IX,
57 and X. The loss of these factors results in lethal hemorrhage in rodents [5,6]. However,
58 heavy use of these rodenticides, especially in urban areas, has led to the acquisition of
59 resistance among rodents. The first resistant brown rats were observed in Scotland in
60 1958 [7]. Subsequently, rodenticide-resistant rodents were observed all over the world
61 (e.g., Korea, Thailand, European countries, Japan, and the USA) [1,8,9]. The
62 mechanism underlying resistance was unclear until the prime component of VKOR was
63 identified as vitamin K epoxide reductase complex subunit 1 (*Vkorc1*), and mutation in
64 *Vkorc1* was shown to cause significant reduction of sensitivity of VKOR to warfarin
65 [10]. Brown rats with mutations of Leu120Gln, Leu128Gln, and Tyr139Phe or
66 Tyr139Ser were found to show strong resistance to rodenticide [9]. These mutations
67 were found mainly in European countries, and also in Korea.
68 On the other hand, the situation in *Rattus rattus* seems to be different from that in *Rattus*
69 *norvegicus*, as resistance seems not only to involve VKOR mutation. Recently, Goulois
70 et al. reported that Tyr25Phe in black rats caused resistance to rodenticides [11]. Tanaka
71 et al. reported that Japanese warfarin-resistant black rats had different *Vkorc1* mutations
72 (Ala41Val, Ala41Thr, Arg61Trp, and Leu76Pro) but not Leu120Gln and Tyr139Phe.

73 Moreover, they showed resistance to warfarin *in vivo*, but their VKOR did not seem to
74 show resistance to warfarin in an *in vitro* DTT-driven microsomal VKOR assay [12].
75 Another possible mechanism was shown to involve metabolism. Warfarin is
76 metabolized to hydroxywarfarin by cytochrome P450 (CYPs or P450). In rats, warfarin
77 is hydroxylated to 4'-, 6-, 7-, 8-, and 10-hydroxywarfarin (OH-warfarin) by CYP1A1,
78 2B1, 2C6, 2C11, and 3A2, respectively [13] (Fig. 1). Ishizuka et al. reported that
79 warfarin-resistant black rats in Tokyo showed relatively high levels of CYP3A
80 expression [14]. We reported previously that black rats in Tokyo showed significantly
81 higher clearance of warfarin after warfarin administration [15]. In this study, resistant
82 black rats showed significantly higher concentrations of OH-warfarin in plasma,
83 indicating that rapid excretion of warfarin was due to enhanced metabolism. Based on
84 these results, it was deemed necessary to investigate hepatic metabolic activity of these
85 resistant rats. To evaluate biogenic hepatic function, *in situ* liver perfusion was
86 considered to be suitable [16].
87 Liver perfusion is an *in situ* method to investigate hepatic activity by injection of
88 perfusate from the portal vein and collection of perfusate from the caudal vena cava.
89 This *in situ* analysis is superior to *in vivo* assays and *in vitro* assays to investigate
90 hepatic activity. First, *in vivo* studies can observe biological natural reactions but it is

91 often difficult to eliminate the effects of other organs. *In vitro* studies are good for
92 qualitative and quantitative analyses of one phenomenon, but it is difficult to simulate
93 the *in vivo* conditions. By liver perfusion, we can observe natural biological reactions
94 with high specificity for the liver [17]. In this study, we investigated hepatic metabolic
95 activity of warfarin in warfarin-resistant black rats from Tokyo, Japan.
96

97 **2. Materials and Methods**

98 **2.1. Animals**

99 Warfarin-susceptible and warfarin-resistant black rats (*Rattus rattus*) were supplied by
100 Ikari Corporation (Tokyo, Japan). They have been maintained as closed colonies in the
101 laboratory after being caught in the wild. Susceptible rats (S-rats) were from Ogasawara
102 Islands in Japan, and resistant rats were from Shinjuku, one of the most urbanized areas
103 in Tokyo, Japan.
104 All rats used in this study were male (mean weight = 124 ± 24 g, mean age based on eye
105 lens [18] = 215.3 ± 96.0 days, $n$ = 14). Animals were kept under a 12/12-hour light/dark
106 cycle at 20°C – 23°C. Food (CE-2; CLEA, Tokyo, Japan) and water were available ad
107 libitum, and they were not fasted before the experimental procedures. All animal care

and experimental procedures were performed in accordance with the Guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and were approved by the Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (Approval number: 14-0142).

## 2.2. Chemicals

The following chemicals and reagents were obtained from the sources shown: warfarin metabolites 4'-, 6-, 7-, 8-, and 10-hydroxywarfarin (Ultrafine Chemicals, Manchester, UK); warfarin-sodium, methanol, diethyl ether, ammonium acetate, acetic acid, KCl, $MgCl_2$, Na $H_2PO_4$, $K_2HPO_4$, $KH_2PO_4$, $Na_2SO_4$, $CaCl_2$, $NaHCO_3$, $Na_2CO_3$, NaCl, NaOH, glucose, glucose-6-phosphate (G6P) oxazepam, phenol reagent (Folin–Ciocalteu reagent), nicotinamide adenine dinucleotide phosphate (NADPH) (Wako Pure Chemical Ind., Osaka, Japan); $CuSO_45H_2O$ was purchased from Kanto Chemicals (Tokyo, Japan). HEPES was purchased from Dojindo Laboratories (Kumamoto, Japan). Bovine serum albumin, β-glucuronidase, oxazepam glucuronide, phenyl-d5-7-hydroxywarfarin, and racemic warfarin were purchased from Sigma-Aldrich (St. Louis, MO). Goat anti-rat CYP1A1, 2B1, 2C6, 2C11, and CYP reductase antibodies and rabbit anti-rat CYP3A2 antibody were purchased from Daiichi Pure Chemicals (Tokyo, Japan). Mouse

anti-GAPDH antibody, donkey anti-goat IgG, goat anti-rabbit IgG, and goat anti-mouse IgG conjugated with horseradish peroxidase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Midazolam ("Midazolam SANDOZ") was obtained from SANDOZ (Tokyo Japan). Butorphanol tartrate (Vetorphale) was obtained from Meiji Seika (Tokyo, Japan). Medetomidine (Dorbene vet) was obtained from Kyoritsu Seiyaku (Tokyo, Japan). Isoflurane was obtained from DS Pharma Animal Health (Osaka, Japan).

### 2.3. *In situ* liver perfusion of warfarin

Liver perfusion was performed according to the method of Inoue and Sugano with small modifications [19,20]. First, rats ($n = 3$ in each group) were anesthetized by intraperitoneal injection of medetomidine (0.375 mg $kg^{-1}$), midazolam (2.0 mg $kg^{-1}$), and butorphanol (2.5 mg $kg^{-1}$). Anesthesia was maintained with 2.5% isoflurane during surgery.

After induction of anesthesia, laparotomy was performed to visualize the liver, portal vein, caudal vena cava, and bile duct. The bile duct was cannulated with PE-10 polyethylene tube (Becton Dickinson, Sparks MD), cannulation of the portal vein and caudal vena cava was performed with No. 4-6 polyethylene tubing (Hibiki, Tokyo,

144 Japan). After these surgical procedures, Krebs–Ringer HEPES buffer (115 mM NaCl,
145 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $NaH_2PO_4$, 1.2 mM $Na_2SO_4$, 2.5 mM $CaCl_2$, 25
146 mM $NaHCO_3$, 10 mM glucose) was pumped through the portal vein cannula with an
147 MP-2000 pump (EYELA, Tokyo, Japan).
148 Warfarin at a concentration of 0.5 mM in Krebs–Ringer HEPES buffer was pumped
149 through the portal vein cannula for 30 minutes after 15 minutes of pre-perfusion.
150 Following perfusion of warfarin, it was flushed out by perfusion of normal buffer for 15
151 minutes. The perfusate through the caudal vena cava cannula was collected every 1
152 minute for 45 minutes, and that through the bile duct cannula was collected every 5
153 minutes. Rats were euthanized by exsanguination during perfusion.
154

155 **2.4. Extraction of warfarin from the perfusate**

156 Warfarin and hydroxywarfarin were extracted from the perfusates with liquid–liquid
157 distribution [15]. Briefly, perfusate was mixed with 0.1 mM of sodium acetate buffer
158 (pH = 5.0), with 7-hydroxy-d5-warfarin as an internal standard. Liquid–liquid
159 distribution was performed with diethyl ether; the organic layer was acquired and
160 evaporated to dryness under a gentle steam of $N_2$ gas. The residue was dissolved in 1
161 mL of methanol. The solution was centrifuged at 15000 × *g* for 10 minutes and filtered

with 0.2 μm Chromatodisc Sample Syringe Filters (GL Science, Tokyo, Japan).

The concentrations of these compounds were determined by high-performance liquid chromatography (HPLC) (Shimadzu 20 series; Shimadzu, Kyoto, Japan) coupled with electrospray ionization triple quadrupole mass spectrometry (ESI/MS/MS, LC-8040; Shimadzu). The area under the curve of the time–concentration curve (Fig. 2) was calculated using GraphPad Prism7 (GraphPad Software, La Jolla, CA).

Spike and recovery test with Krebs–Ringer HEPES buffer was performed to investigate recovery rate. At first, liver perfusion without warfarin was performed with Sprague Dawley rat to collect perfusate for the test. 100 μL of mixture of 4', 6, 7, 8, and 10-OH warfarin, 7-hydroxy-d5-warfarin and warfarin were added to 1 mL of perfusate. After that, extraction was performed as described above. This procedure was repeated 4 times. The recovery rate of each compounds was described in Table 1. The limit of detection (LOD) and the limit of quantification (LOQ) calculated by standard curve ($n$ = 10) was also described in Table 1.11.39 nM. To calculate inter- and intraday precision (%RSD) and accuracy (%RE) low (10 nM), middle (50 nM) and high (200 nM) concentration standard mixture was analyzed ($n$ = 8). The results were in Table 1.

## 2.5. Preparation of liver microsomes

Liver microsomes were prepared by the method of Omura and Sato [21]. Briefly, liver samples used to obtain the microsome fraction were collected from S-rats and R-rats without liver perfusion ($n = 4$ each group). Aliquots of liver tissue were homogenized with about three volumes of potassium phosphate buffer (KPB, 0.1 M, pH 7.4) chilled on ice. The homogenates were centrifuged twice at 9000 × *g* at 4°C for 20 minutes. The supernatants were filtered with gauze and centrifuged twice at 105000 × *g* at 4°C for 60 minutes. The pellets were homogenized in 0.1 M KPB on ice. The microsomal homogenates were stored at −80°C until use. The protein concentration of microsomes was measured according to the method of Lowry et al. [22]. The concentrations of CYPs were determined by the method of Omura and Sato [21].

## 2.6. Warfarin metabolic activity *in vitro*

The hepatic CYP metabolic activity was investigated *in vitro* [23]. The reaction mixture included magnesium chloride (final concentration, 3 mM), G6P (final concentration, 5 mM), warfarin-sodium (final concentrations, 10, 25, 50, 100, 200, 400, and 800 μM) were mixed with microsomes diluted with 0.1 M KPB (final concentration, 1.0 mg protein $mL^{-1}$) on ice. The total volume of each reaction mixture was 250 μL. Samples

were preincubated for 5 minutes at 37°C. A mixture of G6PDH (final concentration, 2 IU/mL) and β-NADPH (final concentration, 0.5 mM) was added to each sample to start the reaction. After 10 minutes, 1 mL of methanol chilled on ice was added to stop the reaction. The concentration of warfarin metabolites was quantified by HPLC-ESI/MS/MS. Data were fitted to the Michaelis–Menten model using GraphPad Prism 7 (GraphPad Software) to calculate kinetic parameters ($K_m$ and $V_{max}$).

### 2.7. Immunoblotting of microsomal proteins

Western blotting of liver microsomal CYPs was performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [24]. Samples containing 10 μg of microsomal protein were electrophoresed on 10% polyacrylamide gels. The proteins were then transferred onto nitrocellulose membranes (Tokyo Roshi Kaisya Ltd., Tokyo, Japan). The membranes were incubated in 5% skim milk for 1 hour to block nonspecific binding. They were then exposed to antibodies at 4°C overnight. Detection was performed using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA).

### 2.8. Statistical analyses

Student's *t* test was performed using the JMP IN v. 5.1 package (SAS Institute, Cary, NC). In all analyses, $P = 0.05$ was taken to indicate statistical significance. Error bars indicate the standard error (SE).

## 3. Results

### 3.1. Liver perfusion

The hepatic metabolic activity of warfarin was investigated by liver perfusion. Figure 2 shows the concentration of hydroxywarfarin in perfusate obtained through vena cava cannulation. A single concentration of 0.5 mM warfarin was introduced for the first 15 minutes via portal vein cannulation, and then normal perfusate was used for washing for 30 minutes. Warfarin and 4', 6, 7, 8, and 10-hydroxywarfarin were detected in the perfusate of both S-rats and R-rats by HPLC-ESI-MS/MS. The AUC of each metabolite is shown in Table 2. All of five metabolites of R-rats showed significantly higher AUC than those of S-rats ($P = 0.05$), although there were no significant differences in AUC of warfarin (data not shown). It was difficult to quantify the concentration of hydroxywarfarin in perfusate obtained through the bile duct as the amount of perfusate

231 was too small.

232

233 **3.2. Metabolic activity *in vitro***

234 Kinetic parameters of CYPs related to warfarin metabolism were investigated *in vitro*
235 using the liver microsomal fraction. Figure 3 shows the Michaelis–Menten plots of each
236 hydroxywarfarin, and Table 3 shows the kinetic parameters, $K_m$, $V_{max}$, and $V_{max}/K_m$.
237 Several metabolite parameters were significantly higher in R-rats than in S-rats. With
238 the exception of 7-OH warfarin, the $V_{max}$ was significantly higher in R-rats than in
239 S-rats, and $V_{max}/K_m$ of 4'-, 7-, and 8-OH warfarin were also significantly higher in R-rats
240 than in S-rats. However, the $V_{max}/K_m$ of 6-OH warfarin in S-rats was significantly higher
241 than that in R-rats.

242

243 **3.3. Immunoblotting of CYPs**

244 Western blotting was performed to quantify the expression levels of CYPs, which play
245 roles in warfarin metabolism. Figure 4 shows the ratios of the expression levels of CYPs
246 in the microsomal fractions of R-rats and S-rats. With the exception of CYP2C6, R-rats
247 showed significantly higher levels of CYPs (by 1.2 – 1.3-fold) than S-rats.

## 4. Discussion

Warfarin is metabolized by several CYPs and warfarin metabolism often influences the efficacy of warfarin not only in resistant rats but also in human subjects. It has been used for treatment of human thrombosis, and known to have a narrow effective dosage range [25]. Genetically allele of CYP2C9 and CYP3A5 have influence on warfarin sensitivity [26]. CYPs can be induced by various agents, i.e., CYP1As are induced by TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) through aryl hydrocarbon receptors (AhRs), CYP2 and 3 are induced by various compounds, such as phenobarbital, through the constitutive androstane receptor (CAR) or pregnane X receptor (PXR) [27 – 29]. Some agents that activate these receptors can decrease the effect of warfarin. Therefore, metabolism should be examined to investigate the efficacy of warfarin.

In this study, hepatic warfarin metabolic activity of warfarin-resistant black rats from Tokyo, Japan, were investigated by *in situ* and *in vitro* analyses. R-rats from Tokyo showed enhanced warfarin metabolism in both analyses (Figs. 2, 3). However, the extents of both sets of results were slightly different. In the *in situ* analysis, the AUCs of all metabolites in R-rats were approximately 2.6 – 8.7-fold higher than those of S-rats (Table 2). However, the differences in the *in vitro* study were relatively modest with

R-rats showing approximately 1.2 – 3-fold higher $V_{max}$ than S-rats. Moreover, $V_{max}$ of 6-OH was lower in R-rats than S-rats (Table 3). Therefore, it is necessary to take into account the features of each method to determine the "*in vivo*" metabolic activity of R-rats.

The *in situ* analysis allows observation of hepatic metabolic activity under almost natural conditions (i.e., distribution of warfarin from blood vessels to organs, natural structure of tissue). This method seems to be able to represent the almost natural activity of the liver better than *in vitro* analysis or cell culture assay. The observations for OH-warfarin also indicated that the *in situ* results are essentially representative of the *in vivo* metabolic activity. The AUCs of OH-warfarins showed that 4'-OH-warfarin is dominant followed in decreasing order by 8-OH > 10-OH > 6-OH > 7-OH in both strains. However, in the *in vitro* study, the $V_{max}/K_m$ of 8-OH-warfarin was highest followed by 4'-OH > 10-OH > 6-OH > 7-OH in this order. In our previous study, 4'-OH-warfarin showed the highest concentration in plasma and the other metabolites showed the same tendency as the *in situ* results [15]. These discrepancies between *in vitro* and *in situ* analyses have been reported previously. Itoh et al. reported different reactions of α- and β-adrenergic receptors between hepatocytes in culture and liver perfusion [30]. Mine et al. also reported differences in sensitivity to glucagon [16]. This

was considered to be because the liver contains not only hepatic parenchymal cells. On the other hand, in our liver perfusion method, we injected 0.5 mM warfarin, which was a higher concentration than the level in blood after administration [15]. It may be also effective to use a lower concentration. The AUC of warfarin was not significantly different between S-rats and R-rats, and therefore the injection volume of warfarin seemed to be the same. Thus, a portion was metabolized so the difference in AUC of OH-warfarin resulted from differences in metabolic activities.

Bile excretion of chemicals plays an important role in their removal from the body. In this study, it was not possible to measure the concentration in bile perfusate due to the small yield. We found that a body weight of at least 250 g was required to obtain a sufficient amount of bile perfusate when using Sprague–Dawley rats (data not shown). Hydroxylation by CYPs is known as phase I reaction of metabolism, after which OH-warfarin is conjugated to glucuronic acid [31,32]. Generally, glucuronidated drugs are excreted to bile through cMOAT (canalicular multispecific organic anion transporter 1), a member of the ATP-binding cassette transporters expressed in the canalicular membrane of hepatocytes [33]. Further studies with collection of bile perfusate and detection of warfarin glucuronide are needed to gain a better understanding of warfarin metabolism.

In the *in vitro* study, we plotted the Michaelis–Menten curve to investigate the enzymatic activity of CYPs, i.e., $V_{max}/K_m$ represents the enzyme activity [34]. Although it is difficult to extrapolate these values to *in vivo* metabolic ability, these values in R-rats may be small therefore causing the enhanced metabolism observed in the *in situ* experiments. Moreover, the expression levels of CYPs determined by western blotting were also significantly higher in R-rats, although the extent was modest (1.1 – 1.3-fold) (Fig. 4). Thus, we cannot deny possibility that another factors contribute to enhanced metabolism observed in situ analysis. Of course, it is possible that these high expression level of CYPs are direct reason of it.

To examine *in vitro* activity, we extracted CYPs in the microsomal fraction and added various co-factors, such as magnesium chloride, G6P, and NADPH, for the reaction. Thus, if the cause of the enhanced metabolism *in vivo* is related to such factors, the difference in results obtained by *in vitro* analysis should be modest. Metabolomics and microarray analyses seem to be efficient means of verifying this possibility, although relatively little genetic information is available regarding *Rattus rattus*.

NADPH seems to influence enhanced metabolism as the activities of all CYPs require electron donation from cytochrome P450 reductase (CPR) and NADPH [35]. Previously, Ishizuka et al. reported that warfarin-resistant rats in Tokyo showed elevated

CPR activity, which may result in activation of CYPs [14]. In our study, the CPR expression level was also investigated by western blotting, but no significant differences were detected between S-rats and R-rats (data not shown). However, the amount of NADPH can influence on their activity. Indeed, overexpression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which plays a role in producing NADPH, can cause drug resistance in several human cancers, such as renal cell carcinoma, pancreatic carcinomas, and prostate cancer [36]. Although the underlying mechanisms are unclear, these cancers have been suggested to show increased malignancy through the provision of the necessary nutrients or interactions of GAPDH with Nm23-H1, a putative metastasis suppressor. Although there have been no reports showing the relationships between rodenticide resistance and these factors, further studies regarding this topic would be worthwhile.

## 5. Conclusions

In this study, we showed that warfarin-resistant rats from Tokyo had enhanced hepatic metabolic activity by liver perfusion analysis, which suggested the importance of *in vivo* and *in situ* studies to evaluate biogenic enzymatic activities.

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**Figure/Table Legends**

356 Fig. 1. Metabolic pathway of warfarin. Warfarin is hydroxylated by CYPs.

357

358 Fig. 2. Hydroxywarfarin concentrations in the perfusate after warfarin perfusion. The
359 x-axis shows time (minutes) and the y-axis shows concentration of hydroxywarfarin
360 (nM). Black triangles, R-rats; white circles, S-rats. Error bars represent SE.

361

362 Fig. 3. Michaelis–Menten plots of hydroxywarfarin. Black triangles, R-rats; white
363 circles, S-rats. Error bars represent SE. The x-axis shows concentration of substrate
364 (warfarin, μM), and the y-axis shows velocity of reaction (pmol mg protein$^{-1}$ min$^{-1}$). *
365 in the figure represents significant differences of $V_{max}/K_m$ ($P = 0.05$). Kinetic parameters
366 ($K_m$, $V_{max}$, and $V_{max}/K_m$) calculated using these plots are shown in Table 3.

367

368 Fig. 4. Immunoblotting of Cytochrome P450s. The y-axis indicates the ratio of
369 R-rats/S-rats. Detection was performed by HRP reaction by Pierce WB. Error bars
370 represent SE. *, Significant difference between S-rats and R-rats ($P = 0.05$).

371

372 Table 1. Method validation of quantification by HPLC-MS.

373 Table 2. AUC of hydroxywarfarin in perfusate. The AUC of each hydroxywarfarin was

374 calculated by GraphPad Prism 7. *, Significant difference between S-rats and R-rats ($P$

375 = 0.05).

376

377 Table 3. Kinetic parameters of hydroxywarfarin. $K_m$ and $V_{max}$ were calculated according

378 to the Michaelis–Menten plots in Fig. 3 by GraphPad Prism 7. *, Significant difference

379 between S-rats and R-rats ($P$ = 0.05).

380 Table 1

| | Intraday precision (%RSD) and accuracy (%RE) | | | | Innerday precision (%RSD) and accuracy (%RE) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | RSD | | | RE | RSD | | | RE | | | |
| | 10 nM | 50 nM | 200 nM | | 10 nM | 50 nM | 200 nM | | Recovery rate(%) | LOD (nM) | LOQ (nM) |
| 4'OH | 13.11 | 12.21 | 14.90 | 1.09 | 13.59 | 12.32 | 15.20 | 0.64 | 77.34±5.84 | 10.1 | 30.6 |
| 6OH | 12.61 | 11.37 | 13.14 | 2.69 | 8.62 | 6.51 | 10.34 | 0.63 | 78.88±8.29 | 7.89 | 23.91 |
| 7OH | 17.63 | 15.02 | 14.27 | 1.09 | 12.78 | 5.71 | 6.51 | 1.51 | 82.11±14.12 | 7.38 | 22.35 |
| 8OH | 15.49 | 11.06 | 14.04 | 1.37 | 12.69 | 9.63 | 6.21 | 2.69 | 84±5.91 | 7.89 | 23.91 |
| 10OH | 13.23 | 14.93 | 17.50 | 2.21 | 13.83 | 11.94 | 8.64 | 1.15 | 106.97±3.78 | 8.12 | 24.62 |
| Warfarin | 19.70 | 15.49 | 15.33 | 2.05 | 5.66 | 5.81 | 3.77 | 0.38 | 94.68±6.37 | 36.21 | 109.74 |
| d5-7OH | 13.32 | 11.92 | 13.35 | 0.51 | 11.18 | 6.10 | 6.34 | 1.37 | 83.76±8.65 | | |

381

382 Table 2

| AUC (nM $min^{-1}$) | 4'OH | 6OH | 7OH | 8OH | 10OH |
|---|---|---|---|---|---|
| R-rats | 9983±1753* | 1709±294* | 739±235* | 1211±241* | 2376±109* |

| | | | | | |
|---|---|---|---|---|---|
| S-rats | 1389±333 | 196±37 | 132±86 | 209±117 | 915±268 |

383

384 Table 3

| 4'OH | R-rats | S-rats |
|---|---|---|
| Vmax (pmol/mg protein /min) | 66.5±9.5* | 56.7±7.7 |
| Km (μM) | 199.8±28.4 | 270.6±44.6 |
| Vmax/Km | 0.33±0.05* | 0.25±0.06 |
| 6OH | | |
| Vmax (pmol/mg protein /min) | 2.63±0.45* | 2.29±0.37 |
| Km (μM) | 96.6±18.9 | 140.9±116.9 |
| Vmax/Km | 0.034±0.011* | 0.058±0.019 |

| | | |
|---|---|---|
| 7OH | | |
| Vmax (pmol/mg protein /min) | 19.5±3.8 | 10.9±1.6 |
| Km (μM) | 136.0±23.2 | 219.0±58.4 |
| Vmax/Km | 0.180±0.05* | 0.068±0.018 |
| 8OH | | |
| Vmax (pmol/mg protein /min) | 6.33±1.11* | 4.78±1.00 |
| Km (μM) | 11.0±1.8* | 10.8±1.4 |
| Vmax/Km | 0.60±0.14* | 0.45±0.10 |
| 10OH | | |
| Vmax (pmol/mg protein /min) | 50.1±12.0* | 46.7±9.2 |
| Km (μM) | 320.1±33.1 | 310.3±41.6 |

| | | |
|---|---|---|
| Vmax/Km | 0.15±0.04 | 0.16±0.04 |

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