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TITLE

Novel revelation of warfarin resistant mechanism in roof rats (*Rattus rattus*) using pharmacokinetic/ pharmacodynamic analysis.

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ABSTRACT

Roof rats (*Rattus rattus*) live mainly in human habitats. Heavy use of rodenticides, such as warfarin, has led to the development of drug resistance, making pest control difficult. There have been many reports regarding mutations of vitamin K epoxide reductase (VKOR), the target enzyme of warfarin, in resistant rats. However, it has been suggested there are other mechanisms of warfarin resistance. To confirm these possibilities, closed colonies of warfarin-susceptible roof rats (S) and resistant rats from Tokyo (R) were established, and the pharmacokinetics/pharmacodynamics of warfarin in rats from both colonies were investigated. R rats had low levels of warfarin in serum

and high clearance activity. These rats can rapidly metabolize warfarin by hydroxylation. The levels of accumulation in the organs were lower than those of S rats. R rats administered warfarin showed high expression levels of CYP2B, 2C, and 3A, which play roles in warfarin hydroxylation, and may explain the high clearance ability of R rats. The mechanism of warfarin resistance in roof rats from Tokyo involved not only mutation of VKOR but also high clearance ability due to high levels of CYP2B, 2C and 3A expression possibly induced by warfarin.

1 INTRODUCTION

Wild roof rats (*Rattus rattus*) mainly live in proximity to human habitats. Wild rats are harmful to humans as they carry various zoonotic diseases, including plague, *Leptospira interrogans*, and hemorrhagic fever with renal syndrome caused by species of hantaviruses [1,2] Therefore, extermination of these rats is necessary for public health. Rodenticides, such as warfarin, have been used since the 1950s. However, their continual use has resulted in the development of drug resistance in rodents, thus making rodent pest control difficult, especially in urbanized areas [3].

Warfarin has been used as a rodenticide worldwide due to its safety (sufficient for its use in clinical medicine as an anticoagulant). Warfarin inhibits blood coagulation by inhibiting the activity of vitamin K epoxide reductase (VKOR), which is the enzyme necessary for producing vitamin K-dependent clotting factors II, VII, IX, and X. Loss of these clotting factors leads to lethal hemorrhage [4,5].

Warfarin-resistant rats have been reported worldwide in urban areas, including Cambridge in the UK [6], Dorsten in Germany [7], California and Chicago in the USA [8], and Tokyo in Japan [9]. Rost et al. (2004) [10] reported that warfarin resistance of brown rats (*Rattus norvegicus*) is due to mutation of the vitamin K epoxide reductase

complex subunit 1 (*Vkorc1*), which encodes VKOR. Lasseur et al. (2005) [11] measured the kinetics of mutant VKOR and showed that Tyr139Pro mutant VKOR of brown rats has markedly lower warfarin K_i than wild-type, i.e., mutant VKOR has low sensitivity to warfarin.

However, it has been suggested that *Vkorc1* mutation is not the only mechanism of warfarin resistance [12]. Heiberg (2009) [13] reported warfarin-resistant rats without *Vkorc1* mutation. In addition, Japanese warfarin-resistant rats are known to have several mutations of *Vkorc1* (Ala41Val, Ala41Thr, Arg61Trp, and Leu76Pro) and most of them are roof rats [14]. However, there have been no reports of wild rats with Tyr139Pro mutation. Furthermore, the V_{max}/K_m of these four mutant VKOR, an indicator means enzyme efficiency, was approximately 0.3 – 0.5 times that of wild-type [14], while that of Tyr139Pro mutation was 1.25 times the wild-type. These findings also suggest the existence of another cause of warfarin resistance, especially in Japanese roof rats.

Another possible mechanism for warfarin resistance may involve enhanced metabolism of the drug by cytochrome P450 [15]. Cytochrome P450 (CYP or P450) constitutes the major enzyme family that plays a role in the oxidative transformation of xenobiotics capable of metabolizing 70% – 80% of all drugs in clinical use [16,17]. Warfarin is also metabolized by several forms of CYP [18]. In rats, warfarin is hydroxylated to 4'-, 6-, 7-, 8-, and 10-OH warfarin (Figure 1), which is mainly catalyzed by CYP1A, CYP2B, CYP2C, and CYP3A subfamilies in rats [19]. The five hydroxides (4'-, 6-, 7-, 8-, and 10-OH warfarin) are glucuronidated by UDP-glucuronosyltransferase (UGT) to increase water solubility and excreted mainly in the urine [20]. Ishizuka et al. (2007) [15] reported that warfarin-resistant roof rats in Tokyo, Japan, had high microsomal warfarin metabolic activity by P450, which seemed to increase warfarin clearance ability. The combined administration of warfarin and P450 inhibitor increase

the mortality rate of warfarin-resistant rat, compared to 0% lethality in warfarin or P450 inhibitor mono-treated animals.

For screening of warfarin resistance factors in roof rats, the pharmacokinetics of warfarin in resistant rats in *in vivo* level should be assessed.

Warfarin-resistant rats are present in the wild, and their wildness and individual differences make it difficult to perform *in vivo* experiments. Based on this reason, closed colonies of warfarin-susceptible and warfarin-resistant roof rats have been established. Wild warfarin-resistant roof rats in Tokyo, Japan, and warfarin-susceptible roof rats from Ogasawara Islands, Japan, were caught and have been kept and bred in the special conditioned rooms for roof rats for more than 10 years.

The present study was performed to investigate the pharmacokinetics/pharmacodynamics of warfarin in Japanese wild warfarin-resistant roof rats (*Rattus rattus*) using these closed colonies to determine their mechanism of warfarin resistance.

<Figure 1>

2 MATERIALS AND METHODS

2.1 Animals

Warfarin-susceptible and warfarin-resistant roof rats (*Rattus rattus*) were supplied by Ikari Corporation (Tokyo, Japan). These two strains were originally caught in the wild, and maintained as closed colonies in the laboratory of Ikari Corporation. Susceptible rats were originally from Ogasawara Islands, Japan, while the resistant rats were from Shinjuku, one of the most highly urbanized areas of Tokyo, Japan. Breeding of these roof rats was succeeded by keeping them in the three-dimensional room because it is difficult for roof rats to breed in the one-story room.

All rats used in this study were male (mean of weight was 112.9 ± 3.1 g and mean of age

based on eye lens[21] was 208.3 ± 27.9 days, $n = 26$). They were housed under a 12/12 hour light/dark cycle at $20 - 23^{\circ}\text{C}$. Food (CE-2, CLEA, Tokyo, Japan) and water were available ad libitum, and they were not fasted before/during the experiments.

All animal care and experimental procedures were performed in accordance with the Guidelines of the AAALAC, and were approved by the Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (Approved 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); warfarin-sodium, ethanol, methanol, diethyl ether, ammonium acetate, acetic acid, K_2HPO_4 , KH_2PO_4 , Na_2CO_3 , NaCl , NaOH , oxazepam, phenol reagent (Folin–Ciocalteu reagent) (Wako Pure Chemical Ind., Osaka, Japan); $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was purchased from Kanto Chemicals (Tokyo, Japan). Bovine serum albumin, β -glucuronidase, carbamazepine, oxazepam glucuronide, phenyl-d5-7-hydroxywarfarin and racemic warfarin were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Anti-rat CYP1A1, 2B1, 2C6, 2C11, and CYP reductase antibodies from goat and anti-rat CYP3A2 antibody from rabbit were purchased from Daiichi Pure Chemicals (Tokyo, Japan). Anti-GAPDH antibody from mouse anti-goat IgG from donkey, anti-rabbit IgG from goat, and anti-mouse IgG from goat conjugated with horseradish peroxidase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Heparin was purchased from Mochida Pharmaceutical CO., LTD (Tokyo, Japan). 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 Warfarin administration and blood collection

Before administration, rats were anesthetized with medetomidine (0.375 mg kg^{-1}), midazolam (2.0 mg kg^{-1}), and butorphanol (2.5 mg kg^{-1}) (i.p.), and anesthesia was maintained with 2.5% – 3% isoflurane. Under anesthesia, the cervical cutis was incised to visualize the jugular vein. Then, 10 mg kg^{-1} of warfarin dissolved in distilled water was administered orally ($n = 4$, male) or i.v. ($n = 4$, male) via the jugular vein. Rats were placed on a disposable heating pad to maintain their body temperature.

Samples of approximately blood ($100 \mu\text{l}$) were taken from the jugular vein through the sternocleidomastoid muscle at 5 minutes, and 1, 2, 4, 6, 10, 26, 33, and 51 hours after administration. Administration and blood collection were performed under 2.5% – 3% isoflurane anesthesia. Prothrombin time was measured at 5 minutes, and 6, 10, 26, 33, and 51 hours with CoaguCheck XS (Roche Diagnostics, Basel, Switzerland). After sampling at 51 hours, rats were euthanized with an overdose of isoflurane, and the liver, kidneys, spleen, heart, lungs, testis, and brain were collected.

2.4 Urine and feces collection after oral warfarin administration

For urine and feces collection, warfarin (10 mg kg^{-1}) was administered orally to susceptible/resistant rats (male, $n = 5$). Rats were anesthetized briefly with isoflurane, and warfarin (10 mg kg^{-1}) was administered orally. The rats were then kept in metabolic cages (Shinano Corporation, Tokyo, Japan), one per cage, for 48 hours with food and water available ad libitum. Urine and feces were collected at 24 and 48 hours.

2.5 Warfarin extraction from plasma, urine, feces, and tissues

Plasma was isolated from blood samples by centrifugation. All samples (plasma, organs, urine, and feces) were stored at -20°C . Aliquots of plasma (1 – 20 μl), urine (2 μl), and homogenized tissues (50 – 100 mg) were added to 15-ml centrifuge tubes with 0.1 M sodium acetate (2 ml), 1 μM glucuronidated oxazepam (100 μl , as an internal standard of warfarin and an indicator of deconjugation), 1 μM phenyl-d5-7-hydroxywarfarin (100 μl , as an internal standard of hydroxywarfarin), and 5000 unit β -glucuronidase (100 μl).

The mixtures were incubated overnight at 37°C . After incubation, diethyl ether (5ml $\times$ 2) was added to the tubes and vortexed for 2 minutes, followed by centrifugation at $3000 \times g$ for 10 minutes. The organic layer was obtained and evaporated to dryness under the gentle steam of N_2 gas. The residue was redissolved in MeOH (1ml) with 50 μM carbamazepine (10 μl , as the syringe spike). The solution was centrifuged at $15000 \times g$ for 10 minutes and filtered with 0.2 μm Chromatodisc Sample Syringe Filters (GL Science, Tokyo, Japan).

2.6 HPLC/MS conditions

Samples were analyzed by high-performance liquid chromatography (HPLC) coupled with electrospray ionization ion-trap tandem mass spectrometry (ESI/MS/MS, LTQ Orbitrap, LC-8030; Shimadzu, Kyoto, Japan) using a C18 column (Symmetry Shield, RP18 2.1×150 mm, 3.5 μm) (Waters, Milford, MA). The mobile phase was 10 mM ammonium acetate in 10% MeOH, pH 5.0 (A), and 100% MeOH (B). An injection volume of 10 μl , a flow rate of 0.3 ml/min, and a column temperature of 50°C were used throughout. In HPLC, the solvent gradient was as follows: 2% mobile phase B from 2 to 27 minutes followed by a linear gradient to 90%, 90% mobile phase B from

27 to 30 minutes, and then 0% mobile phase B from 30 to 35 minutes. The collision energies (CE) and other MS parameters were optimized and are shown in Table S-1.

2.7 Quality control and quality assurance

Spike and recovery test with liver sample was performed to investigate recovery rate. The recovery rates of 4'-, 6-, 7-, and 8-OH warfarin were $90.61\% \pm 25.02\%$ ($n = 4$), while that of 10-OH warfarin was $57.45\% \pm 17.00\%$ ($n = 4$). The recovery rate of warfarin was $108.22\% \pm 31.72\%$. The limit of detection (LOD) of OH warfarin was 3.76 nM, the limit of quantification (LOQ) was 11.39 nM. These values for warfarin were 87.57 nM and 265.36 nM, respectively.

2.8 Pharmacokinetic analysis of warfarin

Non-compartment models were fitted to the plasma concentration–time data of warfarin and its metabolites, and standard pharmacokinetic parameters were derived.[22] Curve fitting was performed with Phoenix WinNonLin (Certara, Princeton, NJ). The area under the curve (AUC) from 0 to 51 hours was calculated using the linear trapezoidal rule. Clearance was calculated from the equation (dose/AUC). Bioavailability was calculated from the equation $(AUC_{p.o.}/dose_{p.o.}) / (AUC_{i.v.}/dose_{i.v.})$.

2.9 *Vkorc1* sequence

The quality of our closed colony was validated by determining the *Vkorc1* sequence according to the method of Tanaka et al. (2012).[14] Briefly, genomic DNA was extracted with a Wizard Genomic DNA Purification Kit (Promega, Madison, WI). DNA was amplified by polymerase chain reaction (PCR) using primers specific for rat *Vkorc1* (Tanaka et al. 2012). PCR was performed using PrimeSTAR® Max DNA Polymerase

(Takara, Shiga, Japan). The PCR profile consisted of 10 s at 98°C, followed by 35 cycles of 10 s at 98°C, 5 s at 60°C, and 30 s at 72°C. The PCR products were directly sequenced using BigDye Terminator version 1.1 (Applied Biosystems, Foster City, CA). Ethanol precipitation was performed after the amplification reaction, and the nucleotide sequence was analyzed by an automated DNA sequencer (ABI Prism 310 Genetic Analyzer) following the manufacturer's instructions.

2.10 Preparation of liver microsomes

Liver microsomes were prepared according to the method of Omura and Sato (1964).[23] Briefly, samples of liver (2-3 g) tissue were homogenized with three volumes of potassium phosphate buffer (0.1 M, pH 7.4) on ice. The homogenates were centrifuged twice at $9000 \times g$ at 4°C for 20 minutes each time. The supernatants were decanted into ultracentrifuge tubes after gauze filtration and centrifuged twice at $105000 \times g$ at 4°C for 60 minutes. The pellets were homogenized in 0.1 M potassium phosphate buffer in 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. (1951). P450 concentration was measured by the method of Omura and Sato (1964).

2.11 Immunoblotting of microsomal proteins

Immunoblotting of liver microsomal CYPs was performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli et al. (1970).[25] Samples containing microsomal protein (10 µg) were electrophoresed on 10% polyacrylamide gels. After electrophoresis, proteins were transferred onto nitrocellulose membranes (Tokyo Roshii Kaisya Ltd., Tokyo, Japan). The membranes were incubated in 5% skim milk for 1 hour to block nonspecific

binding and then exposed to antibodies at 4°C overnight. Detection was performed using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, Massachusetts).

2.12 Statistical analyses

Student's *t* test or Dunnett's multiple comparison test was performed using the JMP IN v. 5.1 package (SAS Institute, Cary, North Carolina). Repeated measures ANOVA was performed using the GraphPad Prism 5 (GraphPad Software, La Jolla, San Diego, California). In all analyses, $p < 0.05$ was taken to indicate statistical significance. Error bars represent the standard error (SE). Significant differences of pharmacokinetics of warfarin and OH-warfarin were tested using repeated measuring ANOVA. If a significant interaction of strains and time was detected, student's *t* test was performed to compare the concentration of warfarin or OH-warfarin of each concentration.

3 RESULTS

3.1 Sequence of *Vkorc1*

A single missense mutation was found in *Vkorc1* of the resistant (R) rats (Leu76Pro). The rats with this mutation are known to survive for 30 days with a diet containing 0.025 % of warfarin [14]. This result confirmed maintenance of the *Vkorc1* mutation in the closed colony of warfarin-resistant (R) rats.

<Figure 2>

3.2 Pharmacodynamics of warfarin; prothrombin time after warfarin administration

Figure 2 shows prothrombin time (international normalized ratio; INR) after oral

administration of warfarin. Prothrombin time is an indicator of blood coagulation capacity; high INR means blood will not coagulate, and normal INR is approximately 1.0.

Warfarin-susceptible (S) rats showed high INR (≥ 8.0) from 26 to 51 hours and showed significant difference compared to the value at 0 hours (Dunnett's test $p = 0.05$). The upper limit that can be determined by CoaguCheck XS is 8.0, so it is possible that this value was higher than 8.0. In contrast, that of R rats did not change markedly and there was no significant difference compared to the value at 0 hours.

<Figure 3>

<Table 1>

3.3 Pharmacokinetics of warfarin after p.o. and i.v. administration

Figure 3 shows warfarin plasma concentration–time profiles after p.o. or i.v. administration at a dose of 10 mg kg^{-1} at time 0. Table 1 shows the pharmacokinetic parameters. The area under the curve (AUC) of R rats of both p.o and i.v. administration was significantly lower than that of S rats, and the clearance of R rats of both administration, which was calculated by dividing AUC by the dose was significantly higher than that of S rats. In contrast, Both S and R rats had bioavailability of approximately 70% (Table 1).

<Figure 4>

<Table 2>

3.4 Pharmacokinetics of hydroxywarfarin

The plasma concentration–time profiles of 4'-hydroxywarfarin, one of metabolites of warfarin are also shown in Figure 4. Pharmacokinetic parameters of 4'-hydroxywarfarin

are shown in Table 2. The C_{max} of i.v. administration of R rats was significantly higher than that of S rats. And the T_{max} of i.v. administration of R rats was significantly earlier than that of S rats. The results of other four metabolites (6, 7, 8, and 10-OH warfarin) are shown in Figure S1, S2 and Table S2, S3.

<Figure 5>

3.5 Excretion in urine and feces

The amounts of warfarin and its metabolites excreted in urine at 48 h are shown in Figure 5. At 24 and 48 hours after administration, there were no significant differences in total amounts of hydroxywarfarin and parent warfarin in urine between S rats ($441 \pm 127 \mu\text{g}$ at 48 h) and R rats ($629 \pm 63 \mu\text{g}$ at 48 h). The amounts of 4'-OH warfarin in urine of R rats at 24 and 48 hours ($227.5 \pm 37.5 \mu\text{g}$ at 48 h) were significantly higher than those of S rats ($78.7 \pm 21.5 \mu\text{g}$ at 48 h). Excrete amounts of other metabolites are shown in Table-S4. In contrast, there were no differences in levels of the five metabolites and parent warfarin examined in the feces of S and R rats (Figure S3).

<Figure 6>

3.6 Organ accumulation

The concentrations of warfarin and its metabolites in the liver and kidney were also determined 51 hours after administration. The concentrations of warfarin in the liver and kidney of S rats ($1378.4 \pm 509.6 \text{ ng mg}^{-1}$ in the liver and $487.5 \pm 300.9 \text{ ng mg}^{-1}$ in the kidney) were significantly higher than those of R rats ($61.3 \pm 16.2 \text{ ng mg}^{-1}$ in the liver and $29.3 \pm 28.2 \text{ ng mg}^{-1}$ in the kidney) (Figure 6). The concentrations of 4'-OH in the liver and 10-hydroxywarfarin in the liver and kidney were also significantly higher in S rats than R rats (shown in Figure S4 and Table S5).

<Figure 7>

3.7 Immunoblotting of CYP

The expression levels of CYPs, which play roles in metabolizing warfarin, were measured by western blotting (Figure 7). Microsomes prepared from S and R rats administered warfarin at a dose of 10 mg kg⁻¹ p.o. were used. Figure 7 shows the ratio of expression levels of CYPs to that of GAPDH. The expression levels of CYP protein which react with anti-CYP2B1, 2C, 2C11, and 3A2 antibodies in R rats were significantly higher than those of S rats.

4 DISCUSSIONS

Warfarin is known to have a lethal effect on rodents when it is taken continuously, and it takes around a week to reach lethal dose even for susceptible rats. *LD*₅₀ of warfarin single administration is 58 mg kg⁻¹ [26], however, that of repeated administration for five days is much lower; 17 mg kg⁻¹ (3.4 mg kg⁻¹ per day) [27,28]. The reason why it takes a week for warfarin to have lethal effect to rodents and repeated administration demands lower amount of warfarin for lethal effect compared to single administration is unclear, but may be due to accumulation of warfarin. This factor may have something to do with warfarin resistance. However pharmacokinetic of warfarin in warfarin-resistant rats is unclear.

In this study, we analyzed the pharmacokinetics of warfarin in warfarin-resistant (R) and warfarin-susceptible (S) male roof rats. R rats showed low AUC and high clearance activity of warfarin after both p.o. and i.v. administration (Table 1). However, there was no significant difference in BA between the two strains, which means absorption and

first pass effect in liver was not different between two strains.

Although there was no difference in BA of warfarin, R rats seemed to have the ability to metabolize warfarin rapidly. Pharmacokinetics of hydroxywarfarin of i.v. administrations showed the basis of rapid excretion. The concentrations of 4'-, 6-, 7-, and 8-OH warfarin at 1 hour after administration were significantly higher in R rats than S rats although administration volume (C_{max}) of warfarin was larger in S rats (shown Table 2, Figure S2, and Table S1). In addition, T_{max} of 4'-, 6- and 7-OH warfarin of R rats was significantly earlier than that of S rats. Thus, the metabolic ability of CYPs, which catalyze warfarin hydroxylation, is higher in R rats than S rats. The tendency of early T_{max} was also observed in p.o. administration but no significantly difference (Figure S1).

Clinically, polymorphism of *CYP2C9* is known to influence warfarin sensitivity in human [29–31]. Several genotypes of *CYP2C9* reduce the clearance rate of warfarin.

Joffe et al. [30] showed the relationships of *CYP2C9* polymorphism and efficacy of warfarin. They reported that rates of excessive (INR>6.0) anticoagulation (and bleeding) were 3 times higher in the homozygotes of *CYP2C9* mutants (14.7%) than in the wild types (4.5 %). Tabrizi et al. [31] also reported that patients with *CYP2C9* polymorphisms needed significantly lower warfarin doses (30.6 ± 2.5 mg) compared to patients with wild-type genotypes ($40.1 \pm$ mg). Conversely, the induction of CYPs by various drugs can enhance warfarin metabolism and result in a reduced effect [32]. The high clearance of warfarin in R rats seemed to due to high metabolic activity by CYPs.

Immunoblotting analysis revealed that the relatively high expression levels of CYP2B, 2C and 3A in R rats after warfarin administration (Figure 7). These results suggest that the high warfarin metabolic activity of R rats seems to be due to the high expression level of these CYPs.

CYP2B, 2C, and 3A are induced through several nuclear receptors, i.e., constitutively active receptor (CAR) [33] and pregnane X receptor (PXR) [34,35]. If warfarin can activate these nuclear receptors in R rats, it is likely that warfarin-resistant rats from Tokyo used in these experiments would have high induction of CYPs when exposed to rodenticides.

There have been some reports that some 2nd generation rodenticides can induce CYPs. The wild voles (*Microtus californicus*) living in highly rodenticide-contaminated areas showed high microsomal rodenticide metabolic activity [36]. In addition, Fujita et al. (2001) [37] reported upregulated CYP2B and 3A expression in wild voles living in areas with heavy pesticide use. Mutations in these nuclear receptors can enhance induction activity of CYPs. Heiberg and Markussen [38] performed bromadiolone exposure experiments and showed the overexpression of *Cyp2e1*, *2c13*, *3a2* and *3a3* in resistant Norway rats. Lamba et al. (2008) [39] reported that a missense mutation in the PXR promoter region increased the expression level of PXR and activity of CYP3A4 in human primary hepatocytes and liver. This polymorphism is known to reduce the concentrations of several drugs in serum [40]. These reports suggest the possibility of rodenticide-mediated CYP induction in rodenticide-resistant rats.

In the result of excretion of urine, however, amounts of OH-warfarin were not significant different except for 4'-OH warfarin. Seeing PK of warfarin, most of warfarin disappeared at 26 h from the body, so it is natural that total amount in urine at 24 h and 48 h showed no significantly difference. In addition, the plasma concentration of 4'-OH warfarin at 1 h was the highest of the five metabolites. These results imply that large amount of 4'-OH warfarin produced by CYP2C11 may be the main factor of warfarin resistance.

CYP2C11 of rats is known to be a male-specific enzyme [41,42]. CYP2C11 is

physiologically regulated by GH [43], and induced by xenobiotics via CAR, but many parts of the pathways remain unexplained. In addition to CYP2C11, other CYPs also show sex-related differences in expression. Therefore, it will be necessary to investigate the pharmacokinetic profiles of female roof rats.

The results of urine indicate another possibility of warfarin metabolism in R rats; R rats have not only high catalytic activity of 4'-OH hydroxylation by CYPs but also rapid excretion ability. After hydroxylation, hydroxywarfarin is conjugated by UGT and excreted in urine. In humans, 8-OH warfarin is mainly glucuronide conjugated by UGT1A1 and UGT1A10, 6- and 7-OH warfarin are also conjugated but relatively low rate, while 4'- and 10-OH warfarin are seldom conjugated [20]. Rats also convert hydroxywarfarins to glucuronides, although little is known regarding specific enzymes involved in these reactions [44,45]. In this results, 8-OH warfarin was dominant in urine of both strains, so it is possible the conjugation activity of hydroxywarfarin of rats is similar to that of humans. If rats have low 4'-OH warfarin glucuronidation activity similar to humans, this result suggests that R rats have high 4'-OH warfarin glucuronidation activity. This may also contribute to high clearance of warfarin and resistance of R rats because hydroxywarfarin also can inhibit VKOR [46]. Therefore, further studies to measure UGT activity in vitro are necessary.

Although even S rats excreted most of warfarin within 48 h after single administration, S rats tended to accumulate warfarin compared to R rats (Figure 6). Continuous feeding can cause warfarin accumulation and WHO (1981) defined warfarin resistance rats as rats which can survive with warfarin containing food for 28 days,[47] rapid excrete of warfarin may enable R rats to resist continuous warfarin exposure.

5 CONCLUSIONS

This study showed the high metabolic activity of warfarin in warfarin-resistant (R) roof rats (*Rattus rattus*) *in vivo* and high expression levels of several CYP subfamilies. The R rats also had *Vkorc1* mutation, and pharmacodynamic analysis showed that warfarin had no effect on R rats, at least with a single administration (Figure 2). These findings indicated that several mechanisms contribute to warfarin resistance of roof rats in Tokyo.

However, the contribution rate of each factor to resistance is unclear, and further studies are necessary to determine the relationships between pharmacokinetic features and *Vkorc1* mutation of R rats and resistance to warfarin. On the other hand, it is unclear how they achieve high levels of CYP expression, although it is possible that warfarin mediates CYP induction in R rats. This hypothesis should be evaluated in further studies. Our closed colony of warfarin-resistant rats enabled us to assess the effects of warfarin exposure on various biological responses, such as the expression level of CYPs, without the effects of other environmental xenobiotic chemicals.

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FIGURE LEGENDS

Figure 1. Metabolic pathways of warfarin (Ishizuka et al. 2007). Warfarin is hydroxylated by various members of the Cytochrome P450 superfamily (CYPs). There are five hydroxides of warfarin: 4'-, 6-, 7-, 8-, and 10-OH warfarin.

Figure 2. Prothrombin time was measured in blood samples obtained after warfarin administration at a dose of 10 mg kg⁻¹ to evaluate the extrinsic pathway of coagulation. High PT-INR indicates that a long time is required for blood to coagulate. Normal PT/INR is approximately 1.0, and the maximum value CoaguCheck XS can measure is 8. White circles mean value of susceptible rats (n = 4), black triangles mean that of

resistant rats ($n = 4$). * means significantly higher values compared to that of at 5 min (Dunnett's test, $p = 0.05$).

Figure 3. Time course of changes in plasma warfarin concentration after oral or intravascular administration of 10 mg kg^{-1} of warfarin. Blood collection was performed at 5 minutes, 1, 2, 4, 6, 10, 26, 33, 51 hours after administration. White circles mean concentration of susceptible rats ($n = 4$), black triangles mean that of resistant rats ($n = 4$).

Figure 4. Time course of changes in plasma 4'-hydroxywarfarin concentration after oral (A) or intravascular (B) administration of warfarin at a dose of 10 mg kg^{-1} . White circles mean concentration of susceptible rats ($n = 4$), Black triangles mean that of resistant rats ($n = 4$).

Figure 5. Excretion levels of warfarin and its metabolites in urine of susceptible rats ($n = 5$) and resistant rats ($n = 5$). Rats were kept in metabolic cages after oral administration of 10 mg kg^{-1} of warfarin for 48 h. Urine and feces were collected at the time of 24 h and 48 h. * means there is significantly difference between both strains (student's t test, $p = 0.05$).

Figure 6. Warfarin concentration in liver and kidney after 51 h from oral administration of 10 mg kg^{-1} of warfarin. Warfarin concentrations were measured in the livers of susceptible ($n = 6$) and resistant ($n = 6$) roof rats. * means there is significantly difference between both strains (student's t test, $p = 0.05$).

Figure 7. Western blotting of microsomal protein from rats administered warfarin at a dose of 10 mg kg^{-1} p.o. The livers were collected 51 hours after administration and microsomes were made from those livers. The expression levels of each CYPs and GAPDH were detected in same membrane by chemiluminescence of horseradish peroxidase. Y axis means the ratio of signal intensity of CYP to GAPDH.

warfarin-susceptible ($n = 7$) and -resistant ($n = 7$) males were measured. * means there is significant difference between two strains (student's t test, $p = 0.05$). The isoform name of CYPs were referred from *Rattus Norvegicus*.

Table 1 Mean $\pm$ SE pharmacokinetic parameters of warfarin

		C_{max} (ng/ μ l)	AUC (ng/ μ l) / h	Clearance (μ l/h)	Bio availability (%)
Susceptible	PO	72.50 $\pm$ 11.07	728.52 $\pm$ 161.49	1927.9 $\pm$ 308.1	69.53
	IV	232.61 $\pm$ 34.61	877.93 $\pm$ 142.87	137.23 $\pm$ 27.02	
Resistance	PO	40.29 $\pm$ 15.88*	182.71 $\pm$ 58.72*	8014.6 $\pm$ 2264.5*	72.34
	IV	133.09 $\pm$ 6.57*	252.88 $\pm$ 15.35*	409.08 $\pm$ 30.16*	

Table. 1 Mean $\pm$ standard error pharmacokinetic parameters of warfarin after p.o. and i.v. administration at a dose of 10 mg/kg to susceptible and resistant rats ($n = 4$, respectively). C_{max} ; the maximum value of warfarin concentration in plasma, AUC; area under curve of plasma-time profile. Clearance was calculated by dividing AUC by dose volume. * means there is significantly difference between both strains (student's t test, $p = 0.05$).

Table 2 Mean $\pm$ SE pharmacokinetic parameters of 4'-OH warfarin

		T_{max} (h)	C_{max} (ng/ μ l)	AUC (h $\times$ ng/ μ l)
Susceptible	PO	6.50 $\pm$ 1.26	3.42 $\pm$ 1.06	53.41 $\pm$ 15.40
	IV	3.50 $\pm$ 0.50	3.87 $\pm$ 0.62	35.82 $\pm$ 6.36
Resistance	PO	1.19 $\pm$ 0.08	2.52 $\pm$ 0.47	23.14 $\pm$ 2.75
	IV	1.25 $\pm$ 0.25*	7.54 $\pm$ 0.41*	24.42 $\pm$ 2.70

Table 2. Mean ($\pm$ standard error) pharmacokinetic parameters of hydroxywarfarin after oral or intravascular administration at a dose of 10 mg/kg to susceptible ($n = 4$) and resistant rats ($n = 4$). T_{max} means the time when concentration reach maximum value (C_{max}). AUC; area under curve of plasma-time profile of OH-warfarin. * means there is significantly difference between both strains (student's t test, $p = 0.05$).

SUPPLEMENTAL FIGURES

Figure S1. Time course of changes in plasma hydroxywarfarin concentration after oral administration of warfarin at a dose of 10 mg kg⁻¹. A: 4'-OH warfarin, B: 6-OH warfarin, C: 7-OH warfarin, D: 8-OH warfarin and E: 10-OH warfarin. White circles mean concentration of susceptible rats ($n = 4$), Black triangles mean that of resistant rats ($n = 4$).

Figure S2. Time course of changes in plasma hydroxywarfarin concentration after i.v. administration of warfarin at a dose of 10 mg kg⁻¹. A: 4'-OH warfarin, B: 6-OH warfarin, C: 7-OH warfarin, D: 8-OH warfarin and E: 10-OH warfarin.

Figure S3. Excretion levels of warfarin and its metabolites in feces of susceptible rats (S, $n = 5$) and resistant rats (R, $n = 5$). Rats were kept in metabolic cages after oral administration of 10 mg kg⁻¹ of warfarin for 48 h. Feces were collected at the time of 24 h and 48 h.

Figure S4. Hydroxywarfarin concentration in liver and kidney at 51 h after the oral administration of hydroxywarfarin. OH-warfarin concentrations in liver of susceptible ($n = 6$) and resistant ($n = 6$) roof rats were measured. * means there is significantly difference between both strains, S and R (student's t test, $p = 0.05$ shown in Table 6).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7