The genetic mechanisms of warfarin resistance in *Rattus rattus* found in the wild in Japan

Kazuyuki D. Tanaka[1)], Yusuke K. Kawai[1)], Yoshinori Ikenaka[1)], Tsunehito Harunari[2)], Tsutomu Tanikawa[2)], Shima Ando[1)], Hee won Min[1)], Fumie Okaijma[1)], Shoichi Fujita[1)], Mayumi Ishizuka[1)],

[1)]Laboratory of Toxicology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Kita-18 Nishi-9, Kita-ku, Sapporo 060-0818, Japan

[2)]Technical Research laboratory, Ikari Corporation, Chiba, 260-0844, Japan

Corresponding author: Dr. Mayumi Ishizuka,
Laboratory of Toxicology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Kita-18 Nishi-9, Kita-ku, Sapporo 060-0818, Japan,
Tel:81-11-706-6949 / Fax:81-11-706-5105
e-mail: ishizum@vetmed.hokudai.ac.jp

Abstract

Warfarin is commonly used worldwide as a rodenticide. It inhibits blood coagulation by inhibiting vitamin K 2,3-epoxide reductase (VKOR) activity leading to hemorrhage. However, it has been reported that repeated or long-term treatment with warfarin results in resistance emerging in wild rodents. Such resistance may explain why it is difficult to control rodents in many regions in Japan. In this report, we studied mutations in the *VKOR* gene (including the VKOR complex subunit 1 (*VKORC1*)), while also analyzing VKOR and clotting factor activity in black rats (*Rattus rattus*) in order to understand better the mechanism of warfarin resistance in this species.

We sequenced the *VKORC1* gene from 275 rats living in the wild in Japan. We found several types of novel base substitutions, some of which conferred warfarin resistance.

There was no difference in coagulation times between warfarin-sensitive and resistant rats measured under physiological conditions. However, after warfarin administration, no effect was noted in warfarin-resistant rats, although a prolonged coagulation time was noted in warfarin-sensitive rats.

We also determined the kinetic differences in hepatic microsomal VKOR-dependent activity between warfarin-resistant and sensitive rats. Warfarin-resistant rats showed 2-3-fold lower V_{max}/K_m values than did sensitive rats. In addition, we report that resistant rats found in the Tokyo area had a VKOR activity which was poorly inhibited by warfarin.

Finally, we conclude that reduced VKOR activity and warfarin resistance in the Japanese black rat might be due to mutations in the *VKORC1* gene. However, further study is needed to clarify how such rats can maintain adequate vitamin K-dependent clotting factor levels, while simultaneously exhibiting low VKOR activity and warfarin resistance.

Keywords: warfarin resistance, *Rattus rattus*, vitamin K epoxide reductase, VKOR activity, coagulation time

1. Introduction

The wild black rat *(Rattus rattus)* and brown rat (*Rattus norvegicus*) live in proximity to human habitats where they may not only cause physical damage to buildings, but also carry pathogens which cause human diseases such as the plague, *Angiostrongylus cantonensis* meningitis, hemorrhagic fever with renal syndrome (HFRS), and Weil's disease. Therefore, pest control is a critical factor in public health initiatives. Since the 1950s, rodenticides such as warfarin have been used worldwide to control rodents due to their convenience, safety, and minimal impact on the environment.

However, the continued use of rodenticides may result in the selection of drug resistance in rodents and lead to the failure of rat control. Rats that are resistant to warfarin were first described in Scotland in 1958 [1]. Since then, resistant rats have been reported all over the world, including Great Britain [1], Denmark [2], Germany [3], France [4], the USA [5], and Australia [6], but there is a paucity of information about resistant rats in Asia.

The pharmacological target of warfarin is vitamin K 2,3-epoxide reductase (VKOR). Through the inhibition of VKOR, warfarin blocks the vitamin K cycle and inhibits the γ-carboxylation of the vitamin K-dependent clotting factors II, VII, IX, and X. Loss of these clotting factors inevitably leads to lethal hemorrhage [7, 8]. In addition, it has been reported that warfarin-resistant rats have lower clotting factor activities and that they need more dietary vitamin K_1 than warfarin-sensitive rats to maintain normal blood clotting activity [9].

Several mechanisms of warfarin resistance in wild rats have been suggested. One possible mechanism may involve mutation of the *VKOR* gene. Rost *et al*. [10] reported that specific mutations in a gene, they named VKORC1, conferred resistance to warfarin in man, rats and mice. In addition, Pelz *et al*. [11] also concluded that the Tyr139Phe mutation in *VKORC1* forms the genetic basis of the anticoagulant resistance seen in brown rats in experiments using recombinant *VKORC1* mutants. Furthermore, Lasseur *et al*. [12] reported that warfarin-resistant brown rats with this same mutation showed VKOR activity with a low V_{max}. However, the very low K_m of this enzyme in resistant rats caused the enzymatic efficiency (V_{max}/K_m) to be very similar to, or even higher than that of sensitive brown rats.

In contrast to the predominance of brown rats in Europe, black rats are the dominant species in Asia and Africa. Rodenticide-resistant wild rats in these areas also pose serious pest control problems. In fact warfarin-resistant rats, sometimes nicknamed 'super rats', are also found in and around Tokyo [13, 14]. Furthermore, it has been claimed that 80% of wild rats in Tokyo develop warfarin resistance, highlighting the fact that there are many places where it is difficult to control rodents in Japan, in both urban and rural settings.

In a previous study, we reported that we did not find the VKOR 139 tyrosine substitution, a frequently seen mutation in European brown rats, in warfarin resistant Japanese black rats [15].

There is a possibility that the mutation profiles of *VKORC1* in the black rat may differ from those of the brown rat. In this study, we aimed to characterize *VKOR* genotypes and phenotypes in warfarin-resistant and sensitive black rats.

2. Materials

2.1. Chemicals

HEPES was purchased from Dojindo Laboratories (Kumamoto, Japan). $CuSO_4 \cdot 5H_2O$, vitamin E, and vitamin K were purchased from Kanto Chemicals (Tokyo, Japan). Bovine serum albumin and racemic warfarin were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Warfarin-sodium, DTT, dichloromethane, ethanol, methanol, H_2O_2, K_2HPO_4, KH_2PO_4, $MgCl_2$, Na_2CO_3, NaCl, NaOH, and phenol were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Animals

Wild rats were initially trapped from 19 prefectures in Japan where rodent control problems exist (Fig.1). Warfarin-sensitive and resistant black rats were supplied by the Ikari Corporation.

We identified *Rattus* species based on DNA Sanger Sequencing of cytochrome b5 and CO-I (cytochrome c oxidase subunit I) of mitochondrial DNA. Only rats verified as *R. rattus* were used further during this present study. We estimated the age of the rats (as described in the Methods section) and used sexually mature animals for our study. The warfarin-sensitive rats were originally trapped from the Ogasawara Islands [16]. Six rats (three males and three females) were caught on the islands in 1989, and a closed colony has since been maintained in the laboratory of the Ikari Corporation. Rats from the closed colony were used as warfarin-sensitive animals.

Rats that survived longer than four weeks on a warfarin diet were used as warfarin-resistant rats according to the World Health Organization (WHO) regulations. Four warfarin-resistant Tokyo rats (two males and two females) were bred for two years and the progeny used as warfarin-resistant animals [17].

All rats were housed, one per cage, under standard laboratory conditions (with a 12 h light and 12 h dark cycle) with food and water available *ad libitum*. Treatment of all animals was performed according to the policies of the Institutional Animal Care and Use Committee of Hokkaido University.

3. Methods

3.1. Sequencing analysis of *VKORC1*

Total RNA was extracted from warfarin-sensitive and resistant rat livers using TRI Reagent (Sigma-Aldrich). RNA concentrations were spectrophotometrically measured (λ = 260 nm). The 260/280 ratios were seen to lie between 1.8 and 2.

The cDNA was synthesized from total RNA. A mixture of total RNA, oligo(dT) (Toyobo, Osaka, Japan), and distilled deionized water was incubated at 70°C for 10 min and then cooled on ice for 1 min. A 5-fold concentrated buffer, 1 mM dNTP mixture, and reverse transcriptase (Rever Tra Ace Toyobo) were added to the mixture and then incubated at 42°C for 50 min and at 99°C for 5 min.

In the event that we could not extract total RNA from the liver samples, we extracted genome DNA from tail samples using a GenEluteTM Mammalian Genomic DNA Minprep Kit (Sigma-Aldrich).

The cDNA or genome DNA was amplified by polymerase chain reaction (PCR) using specific primers for rat *VKORC1*. The sequences of the sense and antisense primers were 5'-GTGTCTGCGCTGTACTGTCGACATC-3' and 5'-TAAGGCAAAGCAAGTCATGTCAGCCTGG-3', respectively. PCR was performed using Ex Taq (Takara, Tokyo, Japan). The PCR cycle program was on cycle of 90 sec at 94°C, 40 cycles of a 30 sec at 94°C, a 45 sec at 68°C, and 60 sec at 72°C, with one cycle of final extension for 5 min at 72°C. The PCR products were used for sequencing analysis directly.

The sequencing PCR reaction was performed using a BigDye Terminator version 1.1 (Applied Biosystems, Foster City, CA, USA). The sense and antisense primers for cDNA sequencing were 5'-TGTCGACATGGGCACCACCTGGAG-3' and 5'-ATGAGGTGGGACCTCAGGGCTTTTTG-3' and for genome DNA sequencing were 5'-TCTATTCTGCGGACACTG-3' and 5'-AAGTGTTCTCCTTGTGAGGA-3' and 5'-CCTGTCATTGGGGAGGTGTT-3'. 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.

3.2. Age determination of black rats

We estimated the ages of wild black rats by determining the paired eye lens weights [18]. Both eyes were removed and placed in 10% formalin immediately after sacrifice. The eyes were fixed in formalin at room temperature for four weeks. After fixation, the tissue around the lens was removed, and the lenses were washed with distilled water. The lenses were dried at 60°C for three days in an oven and weighed to estimate the rats' ages using Tanikawa's method [18].

3.3. Warfarin sensitivity test

Some rats were given a diet containing 0.025% (w/w) warfarin for four weeks. Rats that survived longer than four weeks on this diet were used as warfarin-resistant rats according to the WHO regulations.

3.4. Measurement of coagulation times

Warfarin at a dose of 3 mg kg^{-1} was administered to rats orally. Blood samples were collected from the cardiac or femur veins and anticoagulated with 3.8% sodium citrate (10%, v/v) at 0, 24, and 48 h after administration of warfarin. Plasma was separated by centrifugation at 2000 g for 10 min, and plasma samples were stored at -20°C prior to assay. Prothrombin time (PT), activated partial thromboplastin time (APTT), and heparin protamine titration (HPT) were measured using a CGO2 coagulation analyzer (ATWill, Kanagawa, Japan) with a dry-hemato system (ATWill). PT is a measurement of the extrinsic clotting pathway, which includes factor II (prothrombin), V, VII, X, and fibrinogen (factor I). APTT is a measurement of the intrinsic clotting pathway, which includes factors II, V, VIII, IX, X, XI, XII and fibrinogen. HPT reflects the vitamin K-dependent coagulation factors, II, VII, IX, and X.

3.5. Measurement of VKOR activity

3.5.1. Preparation of liver microsomes

Liver microsomes were prepared according to the method of Omura and Sato [19]. The livers were homogenized in potassium phosphate buffer (0.1 M, pH 7.4) added at a volume three times greater than that of the liver. The homogenates were centrifuged at 9000 g at 4°C for 20 min. The supernatant was decanted to an ultracentrifugation tube and centrifuged at 105000 g at 4°C for 60 min. The pellet was homogenized in potassium phosphate buffer (0.1 M, pH 7.4) on ice. The microsomal homogenates were transferred to 1.5 ml tubes and stored at -80°C prior to use.

3.5.2 Preparation of vitamin K epoxide

Vitamin K epoxide was prepared according to the method of Tishler *et al.* [20]. 750 μL of 30% H_2O_2 and 750 μL DDW containing 0.3 g Na_2CO_3 were added to a solution of 0.3 g vitamin K in 15

mL ethanol. The mixture was kept at 75°C and stirred vigorously for about 15 minutes until the yellow color of the solution changed to a pale pink. The mixture was cooled to room temperature for 5 min, diluted with 21 mL of DDW, and extracted with 180 ml diethyl ether. After centrifugation (1000 g, 10 min), the diethyl ether layer was removed and evaporated by a centrifugal evaporator (Eyela, Tokyo, Japan). The reaction product was refined by high-performance liquid chromatography (HPLC) using the following components: a PU-980 pump (Jasco, Tokyo, Japan); an Inertsil PREP-ODS column, 30.0 × 250 mm (GL Science Inc. Tokyo, Japan); a Mightysil, RP-18GP Aqua guard column (Kanto, Kanto Chemical Co. Inc. Tokyo, Japan); and a 4.6 × 5mm, 5 μm SPD-6AV detector (Shimadzu, Kyoto, Japan); the detection wavelength was 270 nm; the flow rate 10.0 ml min^{-1}; the mobile phase 3% DDW in methanol.

The concentration of vitamin K epoxide was determined by spectrophotometry using molar absorption coefficients of 30800 $M^{-1}cm^{-1}$ at 226 nm (spectrophotometer: U-3300 Hitachi) [21]. The vitamin K epoxide was kept at 4°C and shielded from light until use.

3.5.3. Enzymatic reaction

VKOR activity was measured using rat liver microsomes [15]. Microsomes from black rats were diluted in 0.1 M HEPES buffer (pH 7.4) to a final concentration of 4 mg ml^{-1} protein. The reaction mixture (500 μL) contained 0, 6.25, 12.5, 25, 50, 100, or 200 μM of vitamin K_1 epoxide. These samples were preincubated at 37°C for 5 minutes, and 10 μL of 100 mM DTT (for a final concentration of 2 mM) was added to the mixture to start the reaction. The reaction was maintained at 37°C for 5 minutes and stopped by the addition of 0.5 ml dichloromethane. A wash solution of 500 μL 1.5% NaCl was added, and then 3.7 mL dichloromethane containing vitamin E as the internal standard was added to the extract. After centrifugation (1000 g, 10 min), the aqueous layer was removed with an aspirator. Then 3 ml of the dichloromethane layer was removed and evaporated by a centrifugal evaporator. Vitamin K_1 was measured by HPLC using the following components: a LC-9A pump (Shimadzu); a SPD-6A detector (Shimadzu); a C-R6A printer (Shimadzu); a 234 autoinjector (Gilson); a Mightysil C18 GP Aqua column (Kanto Chemical Co. Inc., Tokyo, Japan); a 4.6 x 250 mm, 5 μm; a Mightysil C18 GP Aqua guard column (Kanto Chemical); a 4.6 x 5 mm, 5 μm; mobile phase composed of methanol and DW (ratio 100:3); a detection wavelength of 270 nm and a flow rate of 1.0 ml min^{-1} were also employed.

3.6. Warfarin inhibition assay of VKOR

The VKOR activity inhibition rate was measured using the same conditions as used for the measurement of VKOR activity, with 1 and 10 μM warfarin-sodium solutions at a VKO concentration of 20 μM.

VKOR activity data were fitted by nonlinear regression analysis to the Michaelis-Menten equation.

Estimates of the apparent K_m and V_{max} values were obtained by regression analysis of a hyperbola using Graph Pad Prism version 5 (GraphPad Software, Inc., San Diego, CA, USA).

3.7. Homology modeling and docking simulations

The Swiss Model program was used for modeling the three-dimensional (3-D) structure of warfarin-sensitive rat VKOR [22, 23, 24]. The final models were refined by energy minimization using the Swiss-Pdb Viewer Deep View version 4.0.1 [25, http://www.expasy.org/spdbv/]. Energy minimization was performed on the VKOR region at residues 33-78, which is considered to be located in the cytoplasm [26, 27], and includes the mutated residues. Warfarin-resistant rats' VKOR structures were predicted using a Swiss-Pdb Viewer. Using the Swiss-Pdb Viewer, energy minimization was performed wherever point mutations occurred.

The MolDock algorithm was employed using the Molegro Virtual Docker (MVD) platform (Molegro ApS, Aarhus, Denmark, http://www.molegro.com) to perform docking simulations [28]. Each VKOR structure obtained by homology modeling was taken as a macromolecule. The structure of the deprotonated warfarin molecule was modeled using the ChemSketch Freeware (http://acdlabs.eu/company/reference.php) and was used as a VKOR ligand. After importing the ligands and the proteins, all parameters were assigned using the automated preparation function, and cavity detection was performed in MVD. The cavity which was estimated to be located in the membrane was selected for the docking simulation. For each compound, the number of runs was set to 50. Other parameters were used in the default setting. Simulations were ranked in order according to the rerank score.

3.8. Statistical analyses

A Student's t-test or Tukey's honestly significantly different (HSD) test were performed using the JMP IN® v. 5.1 package. Statistical significance was considered to occur at p values < 0.05. Error bars in the bar charts represent the standard deviation (SD).

4. Results

4.1. Sequences of *VKORC1*

Table 1 shows the substitution of amino acid of the *VKORC1* gene in *Rattus rattus* （GenBank AB702679. Our study revealed many types of novel mutations in *VKORC1*. First, a base substitution of T227C led to a Leu76Pro amino acid mutation. Second, a base substitution of C181T led to an Arg61Trp amino acid mutation. These two mutations were found in rats caught in and around the Tokyo area (Tokyo, Chiba, Kanagawa). Some of these rats had two heterozygous mutations at these positions. Third, a base substitution of G121A led to an Ala41Thr amino acid mutation. This mutation was found in rats trapped in Osaka. Some rats from Niigata, Gunma, and Fukuoka had a base substitution of C122T which led to an Ala41Val amino acid mutation. Six other patterns of substitution in *VKORC1* were found.

Moreover, when the gene sequences were compared to those of *Rattus norvegicus*, four base substitutions of C36T, T268A, C321A, and C411T were noted. The substitution at position 268 led to an Ile90Leu amino acid mutation.

4.2. Warfarin survival test

Black rats with Arg35Pro, Arg40 Gly and Arg58Trp substitutions died within four weeks of being fed a warfarin-containing diet. These rats were deemed to be warfarin-sensitive. By contrast, rats with Ala41Val, Ala41Thr, Arg61Trp, and Leu76Pro substitutions were deemed to be warfarin-resistant.

4.3. Measurement of coagulation time

We measured the PT, APTT, and HPT coagulation times of warfarin-sensitive (Ogasawara) and resistant (Tokyo) rats, untreated with warfarin. There was no difference between warfarin-sensitive (PT: 13.6 ± 2.2; APTT: 26.2 ± 6.9; HPT: 29.9 ± 9.2 [sec]) and resistant (PT: 12.8 ± 2.2; APTT: 22.1 ± 6.6; HPT: 27.7 ± 10.4 [sec]) rats in any coagulation times under physiological conditions.

The PT, APTT, and HPT time course changes in warfarin-sensitive (Ogasawara) and resistant (Tokyo) rats after the administration of warfarin. At 24 h and 48 h, following administration, PT and HPT times of warfarin-sensitive rats were prolonged in comparison to sensitive rats tested at 0 h (PT 0 h: 13.6 ± 2.2; 24 h: 24.1 ± 4.2; 48 h: 48.6 ± 17.0 and HPT 0 h: 29.9 ± 9.2; 24 h: 87.0 ± 19.7; 48 h: 285.5 ± 123.3 [sec]). At 48 h following administration, APTT times of warfarin-sensitive rats were significantly prolonged in comparison to rats tested at 0 h, although there was no change compared to rats tested at 24 h after administration (APTT 0 h: 26.2 ± 6.9; 24 h: 26.7 ± 10.2; 48 h: 47.3 ± 19.3 [sec]). In warfarin-resistant rats, there was no change in the time course of coagulation times after the administration of warfarin (PT 0 h: 12.8 ± 2.2; 24 h: 15.2 ± 1.4; 48 h: 11.8 ± 1.2; APTT 0 h: 22.1

± 6.6; 24 h: 19.4 ± 2.1; 48 h: 19.3 ± 2.9; HPT 0 h: 27.7 ± 10.4; 24 h: 40.6 ± 9.9; 48 h: 22.5 ± 5.2 [sec]).

Table 2 shows the PT, APTT, and HPT times of warfarin-sensitive rats (Ogasawara) and warfarin-resistant rats 24 h after the administration of warfarin (table 2). The warfarin-sensitive rats had longer coagulation times in terms of PT and HPT than the resistant rats. However, there was no significant difference in APTT except in the case of Ogasawara rats and Niigata rats where we detected prolonged coagulation times in sensitive rats after warfarin administration (PT Ogasawara: 24.1 ± 4.2; Tokyo: 15.3 ± 1.7; Niigata: 13.0 ± 1.1; Osaka: 15.8 ± 4.5; APTT Ogasawara: 26.7 ± 10.2; Tokyo: 19.4 ± 2.5; Niigata: 17.5 ± 3.3; Osaka: 19.5 ± 2.3; HPT Ogasawara: 87.0 ± 19.7; Tokyo: 41.0 ± 12.1; Niigata: 29.8 ± 6.7; Osaka: 38.7 ± 24.8 [sec]).

4.4. Kinetic parameters of VKOR activity

The kinetic parameters of VKOR activity were estimated by the measurement of the reaction velocity. Fig. 2 shows the results of such data fitted by nonlinear regression analysis to the Michaelis-Menten equation. V_{max}, K_m, and V_{max}/K_m values, obtained from Lineweaver-Burk plots, are shown in Table 3. Niigata, Osaka, and Tokyo warfarin-resistant rats showed a three to six-fold lower V_{max} (Niigata: 160 ± 52; Osaka: 300 ± 160; Tokyo: 170 ± 63 [pmol/min/mg protein]) as compared to Ogasawara warfarin-sensitive rats (1000 ± 520 [nmol/min/mg protein]), although they showed almost the same K_m value. Thus warfarin-resistant rats showed a two to three-fold lower V_{max}/K_m (Niigata: 1.2 ± 0.35; Osaka: 0.89 ± 1.2; Tokyo: 1.1 ± 0.35) compared to sensitive rats (2.6 ± 0.60).

4.5. Warfarin inhibition assay

Fig. 3 shows the rates of inhibition of VKOR by warfarin in warfarin-sensitive rats (Ogasawara) and warfarin-resistant rats (Tokyo). There were significant differences between sensitive and resistant rats at a 10 μM warfarin concentration (sensitive rats at 0 μM: 100±49.6; 1 μM: 24.1±9.0; 10 μM: 17.9±1.7; resistant rats at 0 μM: 100±61.2; 1 μM: 62.7±37.3; 10 μM: 74.5±13.6).

4.6. Homology modeling and docking simulation

To characterize the structural differences in VKOR between sensitive and resistant rats, hypothetical three-dimensional structures of VKOR were made using homology modeling and energy minimization data. Docking simulations were performed by MVD. We used the predicted 3-D structures of rat VKOR as the macromolecule, while the molecular structure of warfarin, which was constructed on ChemSketch freeware, was modeled as the ligand. No differences were seen between the best rerank scores of warfarin-sensitive rat VKOR and resistant rat VKOR (TokyoA: Arg61Trp; TokyoB: Leu76Pro; TokyoC: Arg61Trp; Leu76Pro) (Table 4). However the substitution positions were divided into two types: those associated with sensitivity and those associated with

resistance (Table 4 and Fig. 4).

5. Discussion

As shown in Table 1, we sequenced the *VKORC1* gene in black rats from various locations in Japan. Altogether, we found nine types of mutations in our sample. Rost *et. al.* [29] and Diaz *et. al.* [30] reported many different mutations in *VKORC1* in brown and black rats from different parts of the world. In particular, warfarin-resistant rats had substitutions at positions 33, 35, 59, 120, 128, 139; however, no rats were reported to have the same *VKORC1* mutations as described in the present study except for the Ala21Thr and Arg35Pro substitutions. In China, Wang *et al.* [31] reported that the warfarin-resistant species *Rattus losea* had an Arg58Gly substitution in VKORC1. No Japanese wild rats were found to have this mutation.

Warfarin sensitivity tests were performed according to WHO guidelines. In these assays, we found novel mutations in warfarin-resistant black rats. Therefore, we suggest that substitutions of Ala41Val, Ala41Thr, Arg61Trp, Leu76Pro, or a heterozygous mutation at positions 61 and 76 may cause a conformational change in VKORC1 in these resistant rats. Interestingly, rats in China with an Arg58Gly substitution were resistant whereas rats with an Arg58Trp substitution were sensitive to warfarin.

Greaves *et al*. [9] reported that warfarin-resistant brown rats had lower clotting factor activity and that they needed more dietary vitamin K than warfarin-sensitive rats to maintain normal blood clotting capability. However, we found no difference in PT, APTT, or HPT coagulation times between warfarin-sensitive and resistant black rats.

After the administration of warfarin, there was no change in the coagulation time course in resistant rats, although there was a marked prolongation in the coagulation time course in sensitive rats at 24 and 48 h post-warfarin administration. In addition, as shown in Table 2, when comparing resistant rats (Arg61Trp and Leu76Pro, Ala41Val, Ala41Thr mutants) with sensitive rats (wild types), it was noted that 24 h after warfarin administration, the sensitive rats had prolonged coagulation times as compared to resistant rats when PT and HPT tests were performed. In the case of laboratory rats, Wistar rats had similar coagulation times to the warfarin-sensitive rats we used in the present study [32]. Indeed, it has been reported that after the administration of warfarin to Wistar rats, the PT and APTT measurements were both prolonged [33]. In the case of Sprague-Dawley rats, the PT was reported to be 14.5±1.4 sec while warfarin-resistant rats with the Tyr139Phe *VKORC1* mutation had a PT of 15.5±1.4 sec. After the administration of chlorophacinone, another type of anticoagulant, the PT times increased dramatically in sensitive rats but only slightly in resistant rats [34] (Table 2). Thus these resistant rats may produce vitamin K-dependent clotting factors at the same levels as sensitive rats irrespective of warfarin treatment regimen.

We also found differences in the kinetic parameters of VKOR in resistant rats compared to sensitive rats. As indicated in Table 3, resistant rats showed significantly lower V_{max}/K_m ratios than

sensitive rats under physiological conditions. Thus the VKOR of resistant rats showed low enzymatic efficiencies even at low (biological) substrate concentrations. This result is different from a previous study of warfarin-resistant brown rats in France [12]. That study reported that the V_{max}/K_m in warfarin-resistant rats was 1.25 times higher than that in sensitive rats; however, the V_{max}/K_m in resistant rats in the present study was lower (by a factor of between 0.34 and 0.46) than that of sensitive rats. Such low VKOR activity in these resistant rats was suggested to be due to mutations in *VKORC1*. In support of this hypothesis, Pelz *et al*. [11] demonstrated a low vitamin K_1 epoxide reduction activity in mutated recombinant VKORC1. These authors constructed variant VKORC1 products (with Tyr139Cys, Tyr139Ser, Tyr139Phe and Leu128Gln, Leu128Ser mutations) reflecting the sequences of warfarin-resistant brown rats that had been found in Europe. By recombinant expression of *VKORC1* constructs in HEK293 cells, they demonstrated that a mutation at Tyr139 or Leu128 markedly suppressed VKOR activity.

In Japan, there is particular concern at the Tokyo rat problem since 80% of the rat population has become warfarin-resistant. We focused attention on the substitutions at amino acid positions 61 and 76 in VKORC1. Initially, we performed docking simulations to model the interaction between VKOR and warfarin. We concluded that the molecular distance between VKOR and warfarin in warfarin-resistant rats is longer than that in sensitive rats (Fig. 4 and Table 4). This observation may explain the warfarin insensitivity of VKOR in warfarin-resistant rats; the electron-transfer rate between proteins would decrease exponentially as the separation between redox centers is increased. The calculated distance decay constant was $1.44\,Å^{-1}$ [35]. Moreover, in the work of two other groups, after determining the crystal structure of a VKOR fusion protein and a Trx-like domain derived from *Synechococcus* sp., an electron transfer pathway was proposed to explain how electron flow from reduced cysteines in newly synthesized proteins, passing through a Trx-like protein and VKOR, could reduce either vitamin K epoxide to quinine or quinine to hydroquinone [36, 37]. In mammalian VKOR, including humans, most mutations are mapped close to the quinine binding site. The striking clustering of mutations indicates that warfarin binds to the same site as quinine, or at least close to it. Amino acid positions 41, 61, and 76 in VKORC1, which are substituted in warfarin-resistant rats, are close to the quinine binding site.

As shown in Fig. 3, we found the activity of warfarin-resistant rats in Tokyo to be insensitive to warfarin. It is obviously advantageous for these rats to acquire warfarin-resistance, which we postulate might be caused by substitution-induced conformational changes. It was reported that Tyr139Phe mutated rats also showed resistance to the second generation warfarin, such as bromadiolone [38]. A chemical model for the mechanism of inhibition of VKOR by warfarin was proposed by Silverman [39]. Furthermore, the inhibition of VKOR by warfarin is non-covalent in nature, involving the binding of deprotonated warfarin to the active site of VKOR [40].

In conclusion, we determined the genotype of *VKORC1* in Japanese black rats and classified

nine types of mutations, four of which had critical effects on warfarin sensitivity. The mutation pattern of *VKORC1* in black rats is unique and therefore different from that of brown rats. In addition, resistant black rats have lower enzymatic efficiencies compared to warfarin-sensitive rats; these results differ from those obtained in warfarin-resistant European brown rats. However, further study is needed to clarify how these rats produce sufficient vitamin K-dependent clotting factors while simultaneously exhibiting reduced VKOR activity and warfarin resistance.

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Table 1. VKORC1 amino acid sequences in wild Japanese black rats.

VKORC1 amino acid position	wild type	mutant	sample number	warfarin sensitivity
21	Ala	Thr	3	no test
28	His	Gly	5	no test
35	Arg	Pro	16	sensitive
40	Arg	Gly	2	sensitive
41	Ala	Thr	16	resistant
41	Ala	Val	17	resistant
58	Arg	Trp	11	sensitive
61	Arg	Trp	38	resistant
76	Leu	Pro	44	resistant
139	Tyr	Phe	0	resistant

Japanese wild rats showed nine different VKORC1 mutations. Rats with Ala41Val, Ala41Thr, Arg61Trp, and Leu76Pro mutations exhibited warfarin resistance.

Table 2. Comparison of coagulation times in warfarin-sensitive and resistant rats 24 h after warfarin administration.

		PT [sec]	APTT [sec]	HPT [sec]	state	reference
Ogasawara (n=7)	(black rats)	24.1±4.2	26.7±10.2	87.0±19.7	24h after warfarin administration	this study
Tokyo (n=3)	(black rats)	15.3±1.7*	19.4±2.5	41.0±12.1*	24h after warfarin administration	this study
Niigata (n=11)	(black rats)	13.0±1.1*	17.5±3.3*	29.8±6.7*	24h after warfarin administration	this study
Osaka (n=3)	(black rats)	15.8±4.5*	19.5±2.3	38.7±24.8*	24h after warfarin administration	this study
Wistar rats	(brown rats)	13.9~21.1	15.2~33.9	-	basal level, control group	[32],[33]
SD rats	(brown rats)	14.5±1.4	-	-	basal level	[34]
Warfarin resistant rats	(brown rats)	15.5±1.4	-	-	basal level	[34]
					(black rats:*Rattus rattus, brown rats*:*Rattus norvegicus*)	

The PT and HTP of warfarin-resistant rats were shorter than those of warfarin-resistant rats 24 h after warfarin administration (*P < 0.05)

Table 3. Comparison of the kinetic parameters of VKOR in warfarin-sensitive and resistant rats.

derivation	the types of VKORC1	V_{max} [pmol/min/mg protein]	K_m [μM]	V_{max}/K_m
Ogasawara	wild type (n=3)	1000±520	400±240	2.6±0.60
Tokyo	Arg61Trp, Leu76Pro (n=3)	170±63*	150±41	1.1±0.35*
Niigata	Ala41val (n=3)	160±120*	140±100	1.2±0.35*
Osaka	Ala41Thr (n=2)	300, 160	340, 130	0.89, 1.2

***p < 0.05**

Rats with VKORC1 mutations, conferring warfarin resistance, showed a 3-6-fold lower V_{max} than did warfarin-sensitive rats, although the K_m value was nearly the same. Thus, warfarin-resistant rats showed a 2-3-fold lower enzymatic efficiency (V_{max}/K_m) than sensitive rats.

Table 4. Rerank scores and molecular distances (VKOR and warfarin)

	Rerank Score	Distance [Å]
sensitive	-92.0113	3.96408
Arg61Trp	-94.5704	6.22909
Leu76Pro	-94.1791	5.82709
Arg61Trp, Leu76Pro	-91.5158	5.81912

The best rerank score positions were chosen. The distances are measurements of the spaces between the sulfur atom of the 135-Cys and the carbon atom of the 2-carbon in the warfarin molecule.

Figure 1. Map showing the locations and number of wild warfarin-sensitive and resistant black rats that were collected for this study.

We trapped a total of 275 wild rats from 19 prefectures in Japan where rodent control problems exist.

Figure 2. VKOR activity of warfarin-sensitive and resistant rats.

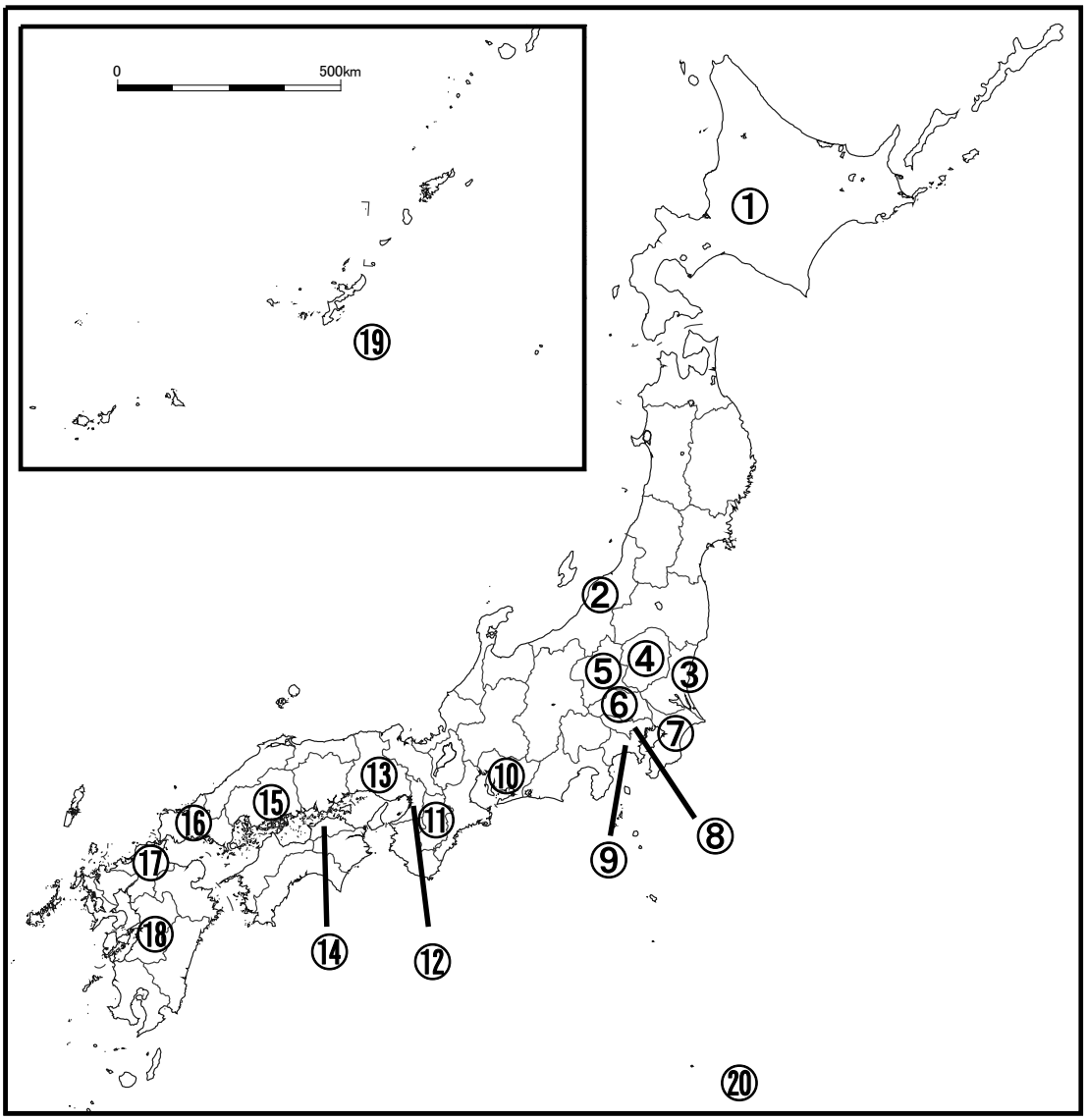
VKOR activity was measured using rat liver microsomes. The reaction was performed with substrate concentrations ranging between 0 and 200 μM. A) VKOR activity of Niigata rats (n=3) and Ogasawara rats (n=3). B) VKOR activity of Osaka rats (n=2) and Ogasawara rats (n=3). C) VKOR activity of Tokyo rats (n=3) and Ogasawara rats (n=3). The data were fitted by nonlinear regression analysis to the Michaelis-Menten equation. Error bars show SD. The kinetic parameters of K_m and V_{max} are shown in Table 3.

Figure 3. Warfarin inhibition rates in warfarin-sensitive and resistant rats.

Each activity score represents the ratio to enzyme activity under warfarin-free conditions. There are significant differences between warfarin-sensitive rats (n=4) and warfarin-resistant rats (n=4) in the presence of 10 μM warfarin. Error bars show SD. *P < 0.05

Figure 4. The states of the most stable positions.

We used predicted 3-D structures of rat VKOR as macromolecules and the warfarin molecule, the structure of which was constructed on ChemSketch freeware, as the ligand. The best rerank scores were not different between warfarin-sensitive rat VKOR and resistant rat VKOR (TokyoA: Arg61Trp, TokyoB: Leu76Pro, TokyoC: Arg61Trp, Leu76Pro). However the molecular distances between the VKOR and warfarin in all types of warfarin-resistant rats was longer than in sensitive rats.



	Place	sample number
①	Hokkaido	32
②	Niigata	15
③	Ibaraki	1
④	Tochigi	3
⑤	Gunma	1
⑥	Saitama	30
⑦	Chiba	19
⑧	Tokyo	56
⑨	Kanagawa	9
⑩	Aichi	15

	Place	sample number
⑪	Nara	3
⑫	Osaka	22
⑬	Hyogo	4
⑭	Kagawa	1
⑮	Hiroshima	11
⑯	Yamaguchi	1
⑰	Fukuoka	4
⑱	Kumamoto	2
⑲	Okinawa	7
⑳	Ogasawara	39

In total, 275 wild rats were trapped from 19 prefectures across Japan.

Figure 1

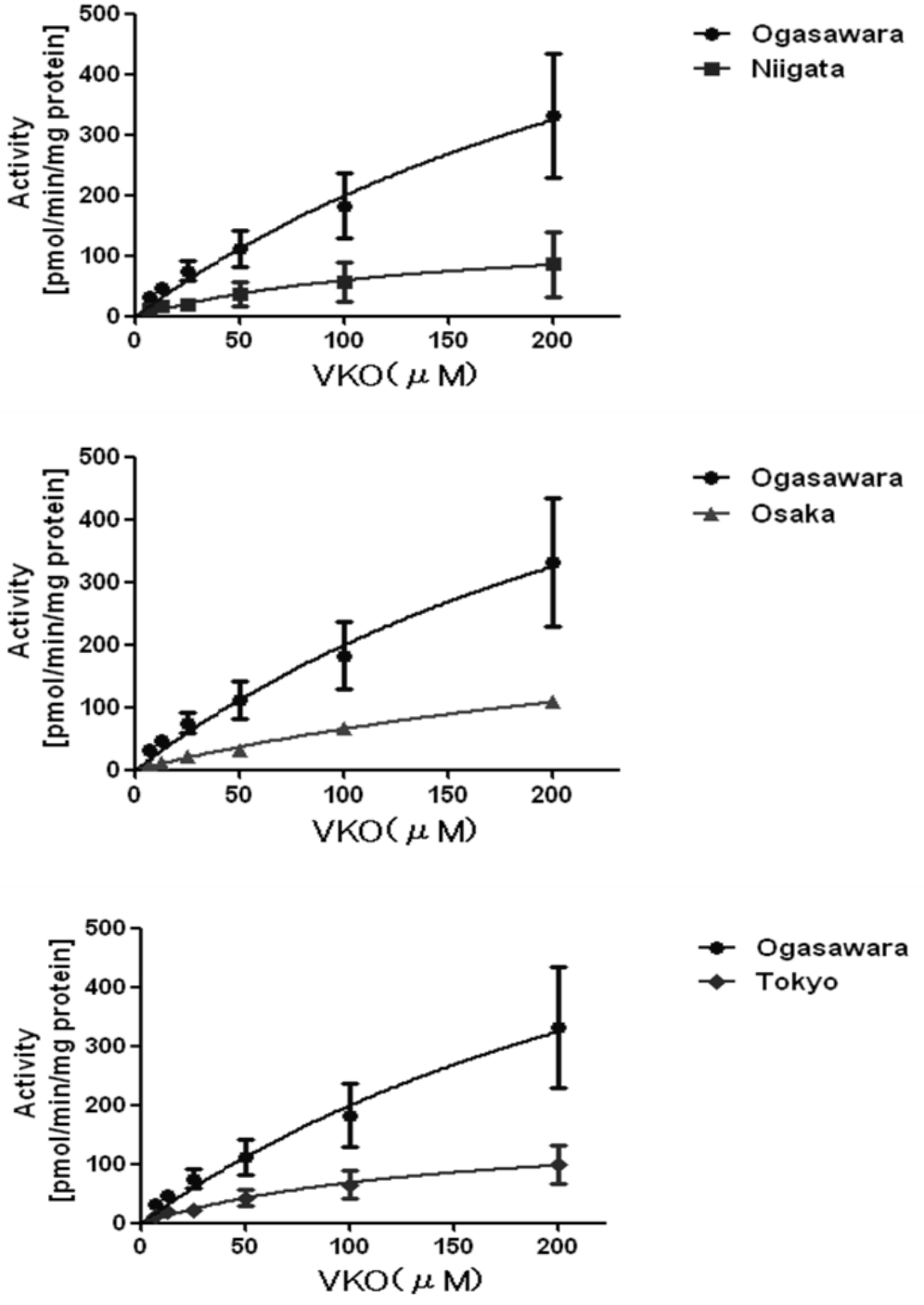

Activity
[pmol/min/mg protein]
500
400
300
200
100
0
0
50
100
150
200
VKO(μ M)
Ogasawara
Niigata
Activity
[pmol/min/mg protein]
500
400
300
200
100
0
0
50
100
150
200
VKO(μ M)
Ogasawara
Osaka
Activity
[pmol/min/mg protein]
500
400
300
200
100
0
0
50
100
150
200
VKO(μ M)
Ogasawara
Tokyo

Figure 2

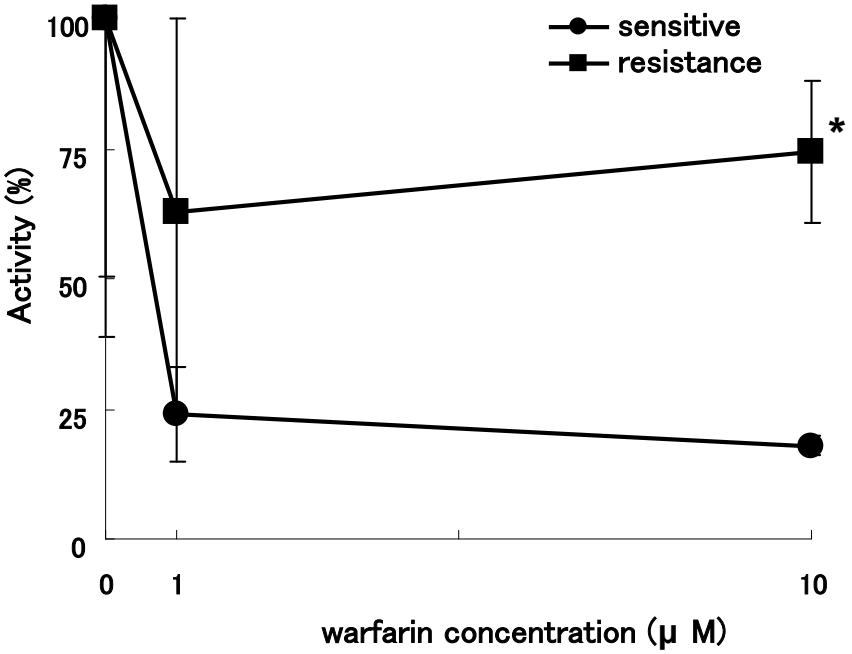

sensitive
resistance
100
75
50
25
0
Activity (%)
*
0
1
10
warfarin concentration (μ M)

Figure 3

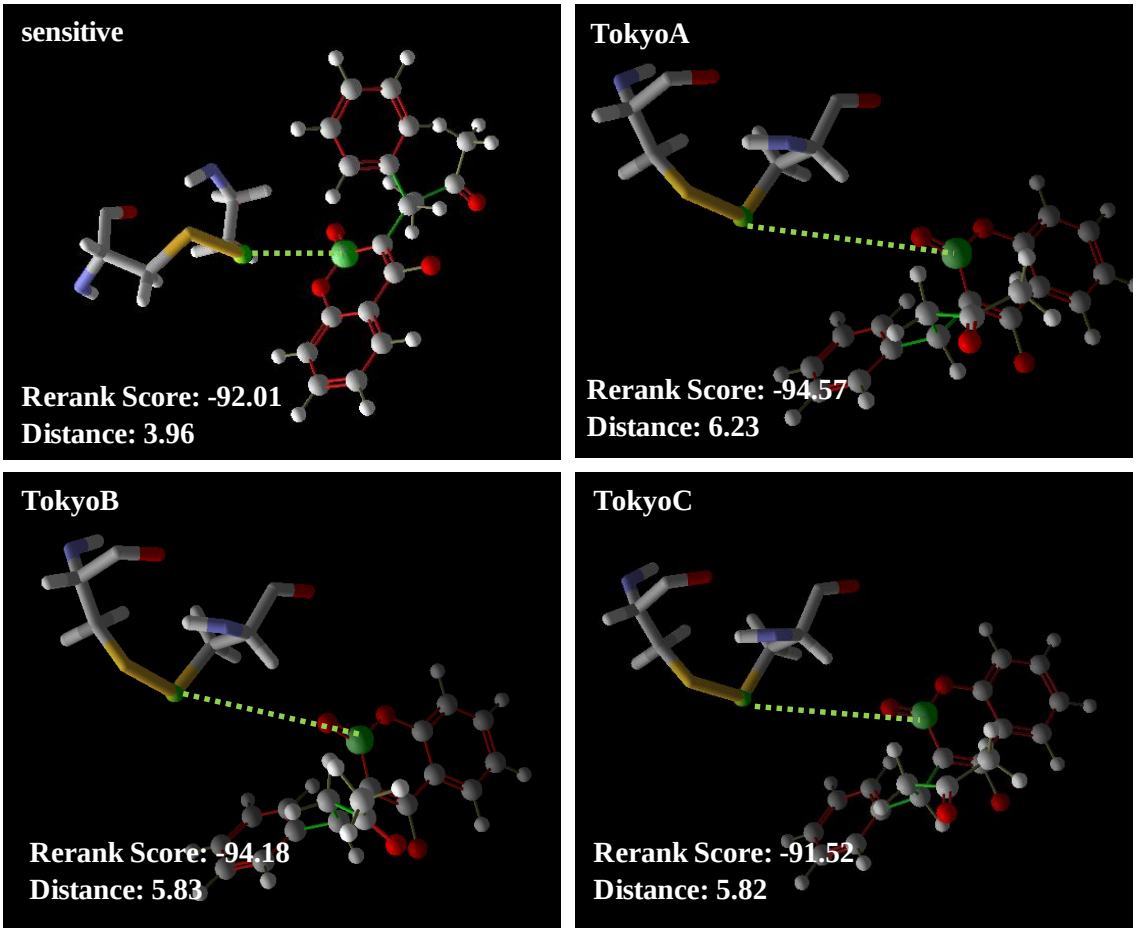

sensitive
Rerank Score: -92.01
Distance: 3.96
TokyoA
Rerank Score: -94.57
Distance: 6.23
TokyoB
Rerank Score: -94.18
Distance: 5.83
TokyoC
Rerank Score: -91.52
Distance: 5.82

Figure 4