The VKORC1 ER-luminal loop mutation (Leu76Pro) leads to a significant resistance to warfarin in black rats (*Rattus rattus*)

Kazuki Takeda[a], Yoshinori Ikenaka[a,b], Denis Fourches[c], Kazuyuki D. Tanaka[d], Shouta M.M. Nakayama[a], Dhoha Triki[c], Xinhao Li[c], Manabu Igarashi[e,f], Tsutomu Tanikawa[d], and Mayumi Ishizuka[a*]

[a]Laboratory of Toxicology, Department of Environmental Sciences, Faculty of Veterinary Medicine, Hokkaido University, Kita-18 Nishi-9, Kita-ku, Sapporo, 060-0818, Japan

[b]Water Research Group, Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa

[c]Department of Chemistry, Bioinformatics Research Center, North Carolina State University, Raleigh, NC, USA

[d]Technical Research Laboratory, IKARI SHODOKU CO., Ltd., Narashino, Chiba, Japan

[e]Division of Global Epidemiology, Research Center for Zoonosis Control, Hokkaido University, Sapporo, Japan

[f]Global Station for Zoonosis Control, Global Institution for Collaborative Research and Education, Hokkaido University, Sapporo, Japan

*Corresponding author. E-mail address: ishizum@vetmed.hokudai.ac.jp (M. Ishizuka)

Abbreviations: VKOR, vitamin K epoxide reductase; VKORC1, VKOR complex subunit 1; VKO, vitamin K epoxide; VK, vitamin K quinone; KH_2, vitamin K hydroquinone; THP, tris(3-hydroxypropyl)phosphine; S-rats, warfarin-susceptible rats; R-rats, warfarin-resistant rats; KPB, potassium phosphate buffer; MD, molecular dynamics; CDS, Coding sequences; LOD, Limit of detection; RMSF, Root Mean Square Fluctuation; SP, Standard precision; K_m, Michaelis constant; V_{max}, maximum velocity; IC_{50}, half maximal inhibitory concentration

Abstract

Well-known 4-hydroxycoumarin derivatives, such as warfarin, act as inhibitors of the vitamin K epoxide reductase (VKOR) and are used as anticoagulants. Mutations of the VKOR enzyme can lead to resistance to those compounds. This has been a problem in using them as medicine or rodenticide. Most of these mutations lie in the vicinity of potential warfarin-binding sites within the ER-luminal loop structure (Lys30, Phe55) and the transmembrane helix (Tyr138). However, a VKOR mutation found in Tokyo in warfarin-resistant rats does not follow that pattern (Leu76Pro), and its effect on VKOR function and structure remains unclear. We conducted both *in vitro* kinetic analyses and *in silico* docking studies to characterize the VKOR mutant. On the one hand, resistant rats (R-rats) showed a 37.5-fold increased IC_{50} value to warfarin when compared to susceptible rats (S-rats); on the other hand, R-rats showed a 16.5-fold lower basal VKOR activity (V_{max}/K_m). Docking calculations exhibited that the mutated VKOR of R-rats has a decreased affinity for warfarin. Molecular dynamics simulations further revealed that VKOR-associated warfarin was more exposed to solvents in R-rats and key interactions between Lys30, Phe55, and warfarin were less favored. This study concludes that a single mutation of VKOR at position 76 leads to a significant resistance to warfarin by modifying the types and numbers of intermolecular interactions between the two.

Keywords: vitamin K epoxide reductase, warfarin, rodenticide-resistance, molecular docking, molecular dynamics simulations

1 Introduction

Vitamin K was first discovered in 1929 as an essential nutrient for blood coagulation. However, cloning the vitamin K epoxide reductase complex 1 (*VKORC1*) gene was not successful until 2004. The gene encodes VKOR, which plays a role in the reactivation of vitamin K [1,2]. Production of vitamin K-dependent proteins, such as blood clotting factors, osteocalcin, and matrix gla protein is dependent on gamma-carboxylation [3]. Vitamin K hydroquinone (KH_2) can act as a cofactor of gamma-glutamyl carboxylase (GGCX), which plays a role in gamma-carboxylation of certain glutamic acid residues. KH_2 is oxidized to vitamin K 2,3-epoxide (VKO) through this reaction. VKO is regenerated to vitamin K quinone (VK) by VKOR and is subsequently converted to KH_2. This series of reactions constitute the vitamin K cycle (Fig. 1) [4]. Interestingly, 4-hydroxycoumarins derivatives, such as warfarin, work as inhibitors of VKOR and have been widely used as anticoagulants in the pharmaceutical industry, and rodenticides.

Various gene mutations have been reported in the *VKORC1* gene and are known to have a significant impact on warfarin sensitivity. In clinical applications, it is known that interindividual variables result in wide variations of therapeutic dosages of warfarin. One of the key factors is the *VKORC1* gene polymorphisms. To date, 26 polymorphisms have been found to impact warfarin resistance (dosage differences are between 2 and 7-fold [5]). For pest control, the appearance of rodenticide-resistant rats has been a serious problem. Since Rost *et al.* reported in 2004 that rodenticide-resistant rats had mutations in their *VKORC1* gene, resistant rats with various VKOR mutations have been found all around the world [2,6]. Therefore, for the proper use of anticoagulants, it is essential to understand the genetic polymorphisms of VKORC1 and their effect on structure and phenotype.

To date, the only X-ray crystal structure publicly available is from a bacterial (Synechococcus) VKOR homolog (PDB: 3KP9). Therefore, most studies with mammalian VKOR have been based on homology models based on that structure [7]. Several studies have suggested that human VKOR contains four transmembrane domains with four conserved cysteines, which are found either in an ER-luminal loop structure (Cys43 and Cys51) or are involved in the active center (Cys132 and Cys135) [8,9]. This active center is highly conserved

in animals and is called a CXXC motif. These cysteines form disulfide bonds that play a key role in electron shuffling during the reaction of the reduction of VKO. VKOR forms two structures depending on its disulfide bond status, one is Cys43-Cys51 and Cys-132-Cys135 (model 1), the other is Cys 51-Cys135 (model 2) (Fig. S1). The first bond of model 1 is reduced by a redox partner at the beginning of the reaction. Then, a new disulfide bond is formed (model 2), and the cysteine residues at position135 reduces VKO.

Several residues have been proposed as warfarin-binding sites. One is the TYA motif-containing Thr138 and Tyr139, located in the fourth helix [10]. Czogalla *et al*. suggested that phenylalanine in position 55 is the most important binding site, and VKOR and warfarin are believed to compete for this particular site [11]. Most of the warfarin-resistant mutations are close to Phe55 and Thr138, e.g., Trp59Arg, Tyr139Cys, Tyr139Phe, and Ala143Val [12–15].

Warfarin-resistant rats found in Tokyo, however, have been characterized to have a mutation in position 76, which is within the ER loop (Leu76Pro) and far from both the binding sites and active center [16]. In humans, mutations in this loop (positions 71–77) also have the potential to trigger resistance [17]. However, it was not possible to measure the IC_{50} value of those VKOR variants as their basal activity was too low for detection with the classical dithiothreitol (DTT)-driven VKOR assay. Czogalla succeeded in measuring IC_{50} using VKOR and factor IX in a co-expressed cell-based assay; however, this method is not suitable for kinetic analysis of VKOR [18]. Moreover, it is still unclear how these mutations cause a structural change that leads to resistance. The lack of analytical methods with DTT also did not allow us to obtain accurate inhibition values for VKOR with the Leu76Pro mutation. Thus, it is necessary to characterize the functional and structural effects of mutations in the ER loop.

In this study, we succeeded in measuring the activity of VKOR with the Leu76Pro mutation using liver microsomal fractioning with tris(3-hydroxypropyl)phosphine (THP) as a mild reductant of VKOR. HPLC-APCI-MS enabled us to measure vitamin K with high sensitivity. For structural analysis, 3D docking models were used to assess the differential binding potential. Molecular dynamics were analyzed to assess the interaction between amino acids of VKOR and its ligands.

2 Materials and Methods

2-1: Chemicals

We purchased vitamin K1 from Kanto Chemicals (Tokyo, Japan). Vitamin K1 epoxide, tris, and glycine were obtained from Sigma-Aldrich (St. Louis, MO, USA). Vitamin K1-d7 was obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA). From Wako Pure Chemical Industries (Osaka, Japan), we purchased acetic acid, diethyl ether, DTT, ethanol, HEPES, methanol, K_2HPO_4, KH_2PO_4, NaOH, NaCl, HCl, glycerol, bromophenol blue, sodium dodecyl sulfate, and warfarin–sodium. THP was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). PVDF membranes (0.2 μM) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Anti-VKORC1 rabbit monoclonal antibody (ab206656), anti-RPS6 rabbit polyclonal antibody (ab40820), and goat anti-rabbit IgG H&L (HRP) (ab205718) were purchased from Abcam (Cambridge, UK). Tween 20 was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

2-2: Animals

Closed colonies of warfarin-susceptible and warfarin-resistant black rats (*Rattus rattus*) were obtained from Ikari Shodoku Co. Ltd. (Tokyo, Japan). All rats used in this study were male (mean weight = 120.4 ± 10.5 g, mean age based on eye lens = 180.5 ± 40.2 days, n = 8) [19]. Rats were kept under a 12/12-hour light/dark cycle between 20°C and 23°C. Food (CE-2; CLEA, Tokyo, Japan) and water were available *ad libitum*. Rats were sacrificed with high concentrations of isoflurane. The liver was removed immediately and flash-frozen in liquid nitrogen. Samples were kept at −80°C until use. All animal treatments and experimental procedures were performed following 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 Faculty School of Veterinary Medicine, Hokkaido University (Approval number: 14-0142).

2-3: Sequence analysis

The complete coding sequences (CDS) of *VKORC1* of both susceptible (S-) and

resistant (R-) rats were analyzed. Genomic DNA was extracted from their liver samples using Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer's instructions. PCR was performed using PrimeSTAR Max DNA Polymerase (Takara Bio Inc, Shiga, Japan) with specific primers for rat *VKORC1* under the following conditions: 35 cycles of 94°C for 5 s, 60°C for 5 s, and 72°C for 15 s [16]. The sequence analysis of the VKORC1 PCR products was performed by FASMAC Co. Ltd. (Kanagawa, Japan). Sequences of the primers used in this study are detailed in Table S1.

2-4: Microsome preparation

Microsomal fractions were prepared from liver samples [20]. Approximately 3.0 g of liver samples were homogenized in 9.0 mL of potassium phosphate buffer (KPB, 0.1 M, pH 7.4). The homogenates were centrifuged at 9,000 ×g at 4°C for 20 min. The supernatants were filtered through gauze and centrifuged at 105,000 ×g at 4°C for 60 min. The pellets were dissolved in 0.1 M KPB. The microsomal fraction was stored at −80°C until analysis. The protein concentration of microsomes was measured using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

2-5: VKOR assay

In vitro kinetic assays of VKOR activity were performed as previously reported [21]. Briefly, reaction mixtures containing 1.0 mg/mL of microsomes, and 5, 10, 25, 50, 100, 300, and 750 μM of VKO were prepared in HEPES buffer (0.1 M, pH 7.4) to a total volume of 100 μL. Each sample was pre-incubated by gentle shaking in a water bath at 37°C for 5 min. Reactions were started by the addition of THP (final concentration, 1 mM) and incubated for an additional 20 min. Iced diethyl ether (1 mL) was added to terminate the reaction. For the inhibition tests, reaction mixtures containing 50 μM of VKO and 0, 0.01, 0.05, 0.1, 0.25, 0.5, and 1 μM of warfarin–sodium were prepared. Direct reduction of VKO by reductants was also measured using the reaction mixture without microsomes, and the value was subtracted from the samples, including microsome homogenates. Each of these experiments was performed in duplicate.

Purification of vitamin Ks and quantification by mass spectrometry were also performed according to the method of Takeda et al. [21]. VK and VKO were extracted from the reaction mixture using the liquid–liquid extraction method, and vitamin K1-d7 was added as an internal standard (final concentration, 100 nM). Liquid–liquid extraction was performed in 5 mL of diethyl ether, and the organic layer was aspirated and evaporated under a gentle N_2 gas stream. The solute was re-dissolved in 0.2 mL of methanol. Vitamin K and an internal standard were measured using HPLC (Shimadzu 20 series; Shimadzu, Kyoto, Japan) coupled with atmospheric pressure chemical ionization triple quadrupole mass spectrometry (APCI/MS/MS, LC-8040; Shimadzu) equipped with a C18 column (Inertsil ODS-3, 2.1 × 150 mm, 5.0 μm), from GL Science (Tokyo, Japan). Spike and recovery tests were performed to assess the recovery rate. Vitamin K1, vitamin K1 epoxide, and vitamin K1-d7 (100 nM) were added to 1 mL of HEPES buffer containing 1 mg/mL of microsome. After that, the extraction was performed as described above. This procedure was repeated four times. The recovery rates of vitamin K1, vitamin K1 epoxide, and vitamin K1-d7 were 83.89 ± 1.62%, 77.89 ± 1.49%, and 83.49 ± 1.64%, respectively. The limit of detection (LOD) was calculated using the standard curve (n = 8). The LODs of vitamin K1, vitamin K1 epoxide, and vitamin K1-d7 were 1.40, 5.21, and 3.04 nM, respectively.

2-6: Western blotting

Immunoblotting was performed for semi-quantification of the hepatic expression level of VKORC1. Microsomal fractions were separated using SDS-polyacrylamide gel electrophoresis (20 μg/lane) on 4–20% gradient gels (Bio-Rad Laboratories, Berkeley, CA, USA) under standard denaturing conditions. Proteins were transferred onto a 0.2-μm PVDF membrane using a semi-dry western blot. The membrane was incubated for 1 h in a protein-free blocking buffer (Takara Bio). Immunodetection analysis was performed on the blotted membrane with SNAP i.d. 2.0 (Merck Millipore, Burlington, MA, USA) using mouse polyclonal anti-VKORC1 antibody, anti-RPS-6 antibody, and HRP-conjugated anti-mouse IgG (Abcam). 5 mL of antibody solutions (diluted to 1:5000), and 30 mL of rinse buffer (0.1 M PBS supplemented with 0.05% Tween 20) are poured over the surface of the PVDF membrane

in the system. After 30 min incubation, each solution is forced through the membrane using a vacuum pump. HRP-specific signals were visualized using the Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific).

2-7: Molecular docking

3D homology models of each VKORC1 variant were obtained using BIOVIA Discovery Studio (Dassault Systèmes S.E., Paris, France). Model 1 (Cys43-Cys51, Cys132-Cys135) and Model 2 (C51-C135) VKOR of S- and R-rats were constructed and their energy minimization was performed using CHARMm forcefield [22].

Small molecule ligands (warfarin, VKO) were further preprocessed according to the following protocol. Explicit hydrogen atoms were added, and ionizable compounds were converted to their most probable charged forms at pH 7.0 ± 2.0 using the LigPrep software within the Schrödinger Small Molecule Discovery suite (Schrödinger, New York, NY, USA). The active site was defined by a 10 Å × 10 Å × 10 Å grid box. The box size was set according to the size of the native ligand. A scaling factor of 0.8 was applied to the van der Waals radii. The OPLS3 force field was used for grid generation [23]. Default settings were used for all remaining parameters.

Warfarin and VKO were docked into the binding pocket of the VKOR using GLIDE software (Schrödinger) with a standard precision scoring function [24]. We used the Glide Docking Score and Glide eModel to evaluate each docking pose. The docking score enables ligand ranking based on the estimation of the ligand's predicted binding affinity for the pocket, whereas the eModel score helps in evaluating the likelihood of the ligand conformation and the overall complex.

2-8: Molecular dynamics simulations

The dynamic non-covalent interactions between VKOR–VKO and VKOR–warfarin were simulated using the GPU-accelerated Desmond software (D.E. Shaw Research, New York, NY, USA) [25]. Each protein–ligand complex was built using the best Glide docking pose at the initial starting point, which was then explicitly solvated with water molecules. Full-atom

simulation ran for 50 ns with warfarin and 100 ns with VKO with the TIP3P solvation model and POPC membrane model, an OPLS3 force field energy minimization, and 10 Å orthorhombic volume. The calculation time step was fixed at 1.0 fs, with an integration output of 1.0 ps. The NPT ensemble was employed with a temperature fixed at 300 K and pressure at 1.01 bar. The integration time step was set at 2 fs. Default settings were used for all other parameters.

2-9: Statistics

Values obtained from the *in vitro* kinetic assay were fitted to nonlinear regression with the Michaelis–Menten equation or inhibition curve. Estimations of apparent V_{max}, K_m, V_{max}/K_m, and IC_{50} values were obtained using GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA). Data were analyzed using the Student *t*-test with a significance level of $p = 0.05$ with JMP 14 (SAS Institute, Cary, NC, USA).

3 Results

3-1: Sequence analysis of VKORC1

Fig. 2 shows the CDS of VKORC1 and the resulting amino acid sequences. A base substitution at position 227 (T→C) resulted in a mutation of leucine at position 76 to a proline. This substituted amino acid was detected in all R-rats (n = 4). The complete sequence is available in the Supplementary Materials.

3-2: Michaelis–Menten analysis of vitamin K1 epoxide reduction

The enzyme kinetics activities were examined by an *in vitro* study to evaluate VKOR activity in both S- and R-rats using VKO as a substrate. S-rats showed a good regression of VKO at a concentration ranging between 10 and 750 µM. However, R-rats showed lower basal activity and reached a plateau at 100 µM. R-rats also showed non-specific reduction at higher concentrations, possibly due to compensation through another enzyme (shown in Fig. S2). Consequently, we used 100 µM as a maximum concentration in the nonlinear regression of the Michaelis–Menten model for VKOR activity (Fig. 3). Table 1 shows the kinetic parameters

calculated with the equation. The maximum reaction rate (V_{max}), the Michaelis constant (K_m), and the hepatic intrinsic clearance (V_{max}/K_m) were determined to evaluate the VKOR function. Although there was no significant difference in K_m values, S-rats showed a 13.5-fold higher V_{max} compared to R-rats. Therefore, S-rats showed a 16.5-fold higher V_{max}/K_m value compared with R-rats.

Table 1: Kinetic parameters of VKOR

	S-rat	R-rat
V_{max} (pmol/mg protein /min)	2.57 ± 0.55	0.19 ± 0.06*
K_m (μM)	12.1 ± 1.9	11.9 ± 2.9
V_{max}/K_m	0.231 ± 0.056	0.014 ± 0.003*
IC_{50} (μM)	0.140 ± 0.046	5.250 ± 0.799*
Log IC_{50} (μM)	−0.966 ± 0.137	0.692 ± 0.074*

* Suggests significant differences (Student *t*-test, $p = 0.05$).

3-3: Inhibition assay by warfarin

An inhibition assay was performed to evaluate the sensitivity of VKOR to the rodenticide warfarin. Fig. 4 is the inhibition curve after normalization to its basal VKOR activity without warfarin. The resulting IC_{50} value calculated by the fitting is also shown in Table 1. S-rats showed a strong regression with a concentration of the inhibitor ranging from 0.01 to 5 μM. R-rats, on the contrary, showed regression only with a higher concentration range from 0.1 to 100 μM. As a result, R-rats have 37.5-fold increased IC_{50} value compared with S-rats.

3-4: Quantification of VKORC1

Western blotting with a microsomal fraction of the liver was performed to quantify VKORC1 protein expression in the liver. Ribosomal protein S6 (RPS6) was used as a loading control. Fig. 5 shows the western blot and average expression level. There was no significant difference in VKORC1 protein concentrations between S- and R-rats after normalization to RPS6. There were also no significant differences in VKORC1 or PRS6 expression levels

between S- and R-rats.

3-5: Molecular docking

A molecular docking study was performed to evaluate the potential of warfarin and VKO binding to wild-type and mutated VKOR. Homology models of VKOR in R- and S-rats (R-VKOR and S-VKOR, respectively) were constructed for both model 1 (Cys43-Cys51, Cys132-Cys135) and model 2 (Cys51-Cys132). Fig. 6 shows the optimized 3D structures of the resulting complexes, with both VKO and warfarin docked inside the binding pocket of VKOR. The Glide docking and eModel scores are captured in Table 2. All modeled complexes resulted in reasonable docking scores, when using a docking score of ≤−7.0 kcal/mol and a eModel score of ≤−50 kcal/mol as a standard threshold [26]. However, R-VKOR showed a less favorable docking score with warfarin compared with those of S-VKOR in both models 1 and 2. This computational result suggests that R-VKOR has a lower affinity to warfarin, which is in agreement with the experimental data obtained from R-rats. On the contrary, R-rats showed almost the same (model 1) or better (model 2) docking score for VKO when compared with S-rats.

Table 2. Docking scores and e-model scores

Ligand	Protein	Docking score (kcal/mol)	Glide eModel (kcal/mol)
Warfarin	S-VKOR (model 1)	−8.491	−80.551
	R-VKOR (model 1)	−8.013	−73.051
	S-VKOR (model 2)	−8.451	−77.177
	R-VKOR (model 2)	−7.684	−66.361
Vitamin K epoxide	S-VKOR (model 1)	−7.979	−77.886
	R-VKOR (model 1)	−8.913	−82.303
	S-VKOR (model 2)	−8.23	−80.723
	R-VKOR (model 2)	−8.118	−70.826

3-6: Molecular dynamics simulations

We also conducted molecular dynamics (MD) simulations to investigate the possible mechanisms causing the lower affinity of warfarin at R-VKOR. All previously shown VKOR–ligand complexes were simulated (see Methods for simulation parameters). The resulting trajectories are available as movies in the Supplementary Materials. Fig. 7 depicts the dynamic non-covalent interactions identified between the different small molecule ligands and the amino acid residues in VKOR (VKOR model 1 with warfarin, VKOR model 2 with VKO). S-VKOR showed a high affinity to warfarin (Fig. 7, both hydrophobic and polar interactions with warfarin shown as blue and green lines, respectively). When complexed with R-VKOR, warfarin was found to be partially or wholly exposed to solvent and showed weak interactions.

It should be emphasized that Phe55, a key residue for warfarin binding to VKOR, showed less possible interactions with R-VKOR (6% occurrence in total frames) compared with S-VKOR (30% occurrence) (Fig 7A, B). These values express how much the interaction occurs over the MD simulation time. With VKO, both S- and R-VKOR showed strong hydrophobic interactions (Fig. 7C, D). However, Cys135 showed a lower interaction with R-VKOR compared with S-VKOR. We calculated the distance between the aromatic ring of Phe55 and warfarin to assess their interaction (Fig. 8-A) and found S-VKOR to be generally closer (4–5 Å) than R-VKOR (5–7 Å), which can explain the higher interaction. The distance between the oxygen atom (o3) in an ethylene oxide of warfarin and the active cysteine residue in Cys135 was also measured (3–5 Å in S-VKOR and 3–4.5 Å in R-VKOR shown in Fig. 8-B). Root Mean Square Fluctuation (RMSF) as an indicator of flexibility within the protein structure for each VKOR residue is shown in Fig. S3.

4 Discussion

In this study, we performed *in vitro* kinetic assays with the microsomal VKOR fraction and *in silico* cheminformatics modeling. Warfarin-resistant rats with the Leu76Pro mutation showed an approximately 37.5-times higher IC_{50} value for warfarin than that found for susceptible rats (Fig. 4). Mutations around the binding sites and active site in VKOR known to cause resistance in rats showed a 40-fold higher IC_{50} (French-resistant rats having Thy139Phe)

[12]. Other mutations leading to resistance in humans showed 2.5- to 50-fold higher values [18]. Although the mutation in the rat VKOR is in the loop area, it has a considerably higher resistance than the other mutations. Docking simulations have predicted that R-VKOR could have a lower affinity for warfarin in both models 1 and 2 (Table 2). Importantly, these results are consistent with the high IC_{50} value observed *in vitro*.

MD simulations further showed higher hydrophobic and ionic interactions with warfarin in S-VKOR compared with R-VKOR. A possible explanation is that S-VKOR has stronger interactions between binding sites and warfarin. Interaction modeling between warfarin and Phe55 of S-VKOR yielded 27%, whereas that of R-VKOR was only 6%. Interactions between warfarin and the potential warfarin-binding residue Lys30 was 90% in S-VKOR but only 46% in R-VKOR (Fig. 7). The distance between Phe55 and warfarin is considerably less in S-VKOR than in R-VKOR (4–5 Å in S-VKOR and 5–7 Å in R-VKOR; Fig. 8-A). In the MD simulation, we also measured the RMSF, which is an indicator of protein plasticity. RMSF in the loop structure of R-VKOR showed a lower value than that of S-VKOR, which can explain lower interactions between binding sites in the loop structure and warfarin.

R-rats showed markedly lower VKOR activity (V_{max}, V_{max}/K_m) in an *in vitro* THP-driven assay (Fig. 3). V_{max}/K_m is an indicator of activity in low physiological substrate concentrations. A lower value means that R-rats seem to have lower VKOR activity even in this physiological situation. Interestingly, the results of the docking simulation showed that R-rats have almost the same score as S-rats in model 1, and an even higher score in model 2 (Table 2). MD simulations also suggested that they have almost the same affinity to VKO through tight hydrophobic interactions (Fig. 7). Considering that the K_m value is an index of the affinity between the enzyme and substrate and that there was no significant difference between the K_m value for the two groups, the cause of low VKOR activity in R-rats may be a result of VKOR functionality. MD can model the affinity between the active center and VKO. Cys135 of S-VKOR showed that interactions occur during 63% of the simulation time, whereas that of R-rats was only 49%, although the binding distance itself was not significantly different (approximately 3–5 Å in S-VKOR and 3–4.5 Å in R-VKOR). This may contribute to lower activity. In addition, RMSF in the loop part, more specifically in position 43, which is an active

cysteine, was low in the R-VKOR (Fig. S3). This low flexibility of active Cys43 in R-VKOR might further cause unfavorable energy to convert its disulfide bonds from model 1 (Cys43-Cys51 and Cys-132-Cys135) to model 2 (Cys51-Cys135) and vice versa.

Measuring VKOR activity has been a limitation in this study. So far, two ways of measurement have been described to assess VKOR activity. One is a DTT-driven assay using a microsomal fraction. It was the first reported VKOR activity assay in 1976 [27]. DTT is used as a reductant for VKOR because the physiological redox partner of VKOR is unknown. However, using DTT has been reported as problematic because it can reduce VKO directly and can pass the first disulfide bond [28]. A cell-based assay was developed to assess VKOR activity under more physiological conditions [29]. It uses HEK293 cells co-expressed with human factor IX and VKOR to measure the activity of carboxylated factor IX. It is also possible to use endogenous redox partners to measure the IC_{50} value, but it is not possible to calculate the enzyme kinetics of VKOR.

For the assessment of human VKOR mutations at positions 71 and 77, Hodroge *et al.* reported that the activity of these VKOR variants was too low for the DTT-driven assay. On the contrary, the activity of factor IX in those variants was almost the same as in the wild-type using the cell-based assay [17,18]. The mechanism behind these differences remains unclear, but one possibility is that the lower activity of VKOR is sufficient for the secretion of carboxylated factor IX within the 72-h incubation. We used THP as an alternative mild reductant to DTT because it was found to be a selective reductant for disulfide bonds and suitable for the *in vitro* VKOR assay [30]. Therefore, a lower direct reduction by the reductant was achieved (data not shown), and we succeeded in measuring the IC_{50} value with high sensitivity with detection by HPLC-APCI-MS. This cell-based assay should also be able to assess the activity of this mutation, but the physiological redox partner, a kind of disulfide isomerase, of VKOR remains unidentified. Further examination of VKOR using both the THP-driven assay and cell-based assay will be helpful in revealing the whole picture of the VKOR function.

Previously, we performed pharmacokinetic/pharmacodynamic analyses of warfarin using these S- and R-rats [31]. The basal PT value was not significantly different between them.

This result suggests that the low R-VKOR activity may be sufficient for physiological roles or that other enzymes compensate and contribute to vitamin K-dependent blood coagulation factor production. On the contrary, Jacob *et al.* reported that resistant rats with a Thr139Phe mutation showed an increased need for vitamin K in the diet [32]. Each VKOR variant seems to show the different physiological functions of VKOR. Therefore, the effect of VKOR mutations in physiological VK homeostasis still requires additional studies, especially with respect to evaluating the resistance to anticoagulants.

5 Conclusions

In conclusion, this study helps understand the potential effects of a mutation on the ER-luminal loop of VKOR (Leu76Pro) that causes significant resistance to warfarin *in vitro* by decreasing the affinity between binding sites.

Acknowledgments: The analyses were technically supported by Mr. Takahiro Ichise, and Ms. Nagisa Hirano. The English in this manuscript was proofread by Enago (Crimson Interactive Pvt. Ltd. Mumbai, Maharashtra, Republic of India).

Funding: This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan via awards made to M. Ishizuka [grant numbers 16H0177906, 18K1984708], Y. Ikenaka [grant numbers 17K2003807, 18H0413208], S.M.M. Nakayama [grant numbers 16K16197, 17KK0009], K. Takeda [grant numbers 17J0580808], as well as to the Environment Research and Technology Development Fund [grant numbers SII-1/3-2, 4RF-1802/18949907] of the Environmental Restoration and Conservation Agency of Japan. We also acknowledge financial support from The Soroptimist Japan Foundation, The Nakajima Foundation, The Sumitomo Foundation, The Nihon Seimei Foundation, and The Japan Prize Foundation. This research was also supported by JST/JICA, SATREPS (Science and Technology Research Partnership for Sustainable Development).

Declarations of Interest: none.

Figures Captions

Fig.1: Vitamin K cycle. Vitamin K hydroquinone (KH_2) acts as a cofactor of dependent carboxylation of glutamic acid to γ-carboxyglutamic acid by GGCX. Subsequently, KH_2 is oxidized to vitamin K epoxide (VKO). VKO is reduced to vitamin K quinone (VK) and KH_2 in the cycle. VKOR plays a role in the reduction of VKO to VK. VKOR, VKR, and NQO1 catalyze the conversion of VK to VKH_2. VKOR; Vitamin K epoxide reductase, VKR; Vitamin K reductase NQO1; NAD(P)H quinone oxidoreductase 1, KH_2; vitamin K hydroquinone, VKO; vitamin K epoxide, VK; vitamin K quinone.

Fig. 2: The coding sequence (CDS) of the *VKORC1* gene. Alignment of CDS of the *VKORC1* gene in warfarin-sensitive (S-) and resistant (R-) rats. The sequence at the vicinity of the mutation (Leu76Pro) in both strains is shown.

Fig. 3: Enzyme kinetics of VKOR activity. VKOR activity was measured using vitamin K1 epoxide as a substrate. The x-axis represents the VKO concentration, and the y-axis represents the velocity of the VKOR activity. Data were fitted to a nonlinear regression curve using the Michaelis−Menten equation. The kinetic data are shown in Table 1. A) Comparison of S- and R-rats. B) Expanded view of the R-rats. The plots show the average ± SE, n = 4 of each strain.

Fig. 4: Inhibition curve of VKOR activity by warfarin. VKOR activity was measured in the presence of various warfarin concentrations (0–2.0 μM). The activity was normalized to the activity without warfarin. The plots show the average ± SE, n = 4 of each strain.

Fig. 5: Immunoblotting of VKORC1. Western blotting was performed using a liver microsomal fraction. RPS6 was used as a loading control. The y-axis represents the ratio of VKORC1 and RPS6.

Fig. 6: Docking pose between the homology modeled rVKORC1 and warfarin or VKO. A

ribbon diagram of the hVKORC1 homology model with docked ligands depicted as sticks. The VKOR structure was described as a cartoon. Some of the important residues (active four cysteines; Cys43, 51, 132, 135, and binding sites found in following molecular dynamics simulation; Lys30, Phe55, Thr138) are pictured. The docking score is shown in Table 2.

Fig. 7: Schematic of detailed ligand atom interactions with the protein residues. Interactions that occur in more than 5.0% of the 100.00 ns simulation time are shown as arrows. Hydrophobic interaction is shown as a green line around ligands. The blue line represents an ionic interaction. It is possible to have interactions with >100% as some residues may have multiple interactions of a single type with the same ligand atom.

Fig. 8: (A) Distance between warfarin and Phe55 of rVKORC1. The frequency of each distance that occurs in the 50.00 ns simulation time is shown. The distance between the aromatic carbon (a7) of warfarin and aromatic carbon of the phenol residues in Phe55 was measured. (B) Distance between VKO and Cys135 of rVKORC1. The frequency of each distance that occurs in the 100.00 ns simulation time is shown. The distance between the oxygen atom (o3) in an ethylene oxide of warfarin and the active cysteine residue in Cys135 was measured.

References

[1] H. Dam, Hæmorrhages in Chicks Reared on Artificial Diets: a New Deficiency Disease, Nature. 133 (1934) 909–910. doi:10.1038/133909b0.

[2] S. Rost, A. Fregin, V. Ivaskevicius, E. Conzelmann, K. Hörtnagel, H.-J. Pelz, K. Lappegard, E. Seifried, I. Scharrer, E.G.D. Tuddenham, C.R. Müller, T.M. Strom, J. Oldenburg, Mutations in VKORC1 cause warfarin resistance and multiple coagulation factor deficiency type 2., Nature. 427 (2004) 537–41. doi:10.1038/nature02214.

[3] C. Vermeer, L.J. Schurgers, A COMPREHENSIVE REVIEW OF VITAMIN K AND VITAMIN K ANTAGONISTS, Hematol. Oncol. Clin. North Am. 14 (2000) 339–353. doi:10.1016/S0889-8588(05)70137-4.

[4] J. Oldenburg, M. Marinova, C. Müller-Reible, M. Watzka, The Vitamin K Cycle, Vitam. Horm. 78 (2008) 35–62. doi:10.1016/S0083-6729(07)00003-9.

[5] M. Watzka, C. Geisen, C.G. Bevans, K. Sittinger, G. Spohn, S. Rost, E. Seifried, C.R. Müller, J. Oldenburg, Thirteen novel VKORC1 mutations associated with oral anticoagulant resistance: Insights into improved patient diagnosis and treatment, J. Thromb. Haemost. 9 (2011) 109–118. doi:10.1111/j.1538-7836.2010.04095.x.

[6] S. Rost, H.-J. Pelz, S. Menzel, A.D. MacNicoll, V. León, K.-J. Song, T. Jäkel, J. Oldenburg, C.R. Müller, Novel mutations in the VKORC1 gene of wild rats and mice – a response to 50 years of selection pressure by warfarin?, BMC Genet. 10 (2009) 4. doi:10.1186/1471-2156-10-4.

[7] W. Li, S. Schulman, R.J. Dutton, D. Boyd, J. Beckwith, T.A. Rapoport, Structure of a

bacterial homologue of vitamin K epoxide reductase., Nature. 463 (2010) 507–12. doi:10.1038/nature08720.
[8] M.A. Rishavy, A. Usubalieva, K.W. Hallgren, K.L. Berkner, Novel Insight into the Mechanism of the Vitamin K Oxidoreductase (VKOR), J. Biol. Chem. 286 (2011) 7267–7278. doi:10.1074/jbc.M110.172213.
[9] W.D. Van Horn, Structural and functional insights into human vitamin K epoxide reductase and vitamin K epoxide reductase-like1, Crit. Rev. Biochem. Mol. Biol. 48 (2013) 357–372. doi:10.3109/10409238.2013.791659.
[10] S. Wu, X. Chen, D.-Y. Jin, D.W. Stafford, L.G. Pedersen, J.-K. Tie, Warfarin and vitamin K epoxide reductase: a molecular accounting for observed inhibition., Blood. 132 (2018) 647–657. doi:10.1182/blood-2018-01-830901.
[11] K.J. Czogalla, A. Biswas, K. Höning, V. Hornung, K. Liphardt, M. Watzka, J. Oldenburg, Warfarin and Vitamin K compete for binding to Phe55 in human VKOR, Nat. Struct. Mol. Biol. 24 (2017) 77–85. doi:10.1038/nsmb.3338.
[12] R. Lasseur, C. Longin-Sauvageon, B. Videmann, M. Billeret, P. Berny, E. Benoit, Warfarin resistance in a French strain of rats., J Biochem Mol Toxicol. 19 (2005) 379–85. doi:10.1002/jbt.20104.
[13] H.-J. Pelz, Spread of resistance to anticoagulant rodenticides in Germany, Int. J. Pest Manag. 53 (2007) 299–302. doi:10.1080/09670870701245223.
[14] E.B. Wilms, D.J. Touw, J.M.H. Conemans, R. Veldkamp, M. Hermans, A new VKORC1 allelic variant (p.Trp59Arg) in a patient with partial resistance to acenocoumarol and phenprocoumon, J. Thromb. Haemost. 6 (2008) 1224–1226. doi:10.1111/j.1538-7836.2008.02975.x.
[15] S. Rost, H.-J. Pelz, S. Menzel, A.D. MacNicoll, V. Leon, K.-J. Song, T. Jaekel, J. Oldenburg, C.R. Muller, R. Bell, P. Caldwell, A. Zimmermann, J. Matschiner, S. Rost, A. Fregin, V. Ivaskevicius, E. Conzelmann, K. Hortnagel, H. Pelz, K. Lappegard, E. Seifried, I. Scharrer, E. Tuddenham, C. Muller, T. Strom, J. Oldenburg, T. Li, C. Chang, D. Jin, P. Lin, A. Khvorova, D. Stafford, J. Sadowski, C. Esmon, J. Suttie, C. Vermeer, M. Knapen, K. Jie, D. Grobbee, E. Cranenburg, L. Schurgers, C. Vermeer, C. Boyle, R. Redfern, J. Gill, F. Rowe, C. Plant, A. Bradfield, M. Lund, R. Johnson, A. MacNicoll, J. Gill, H. Pelz, S. Rost, M. Hunerberg, A. Fregin, A. Heiberg, K. Baert, A. MacNicoll, C. Prescott, A. Walker, J. Oldenburg, C. Muller, M. Hermodson, J. Suttie, K. Link, J. Greaves, P. Ayres, H. Thijssen, C. Janssen, J. Mosterd, J. Bishop, D. Hartley, G. Partridge, D. Cain, S. Hutson, R. Wallin, A. MacNicoll, H. Thijssen, T. Misenheimer, J. Suttie, A. MacNicoll, R. Dean, J. Greaves, P. Ayres, S. Rost, A. Fregin, M. Hünerberg, C. Bevans, C. Muller, J. Oldenburg, J. Mach, D. Harrington, S. Underwood, C. Morse, M. Shearer, E. Tuddenham, A. Mumford, R. Lasseur, C. Longin-Sauvageon, B. Videmann, M. Billeret, P. Berny, E. Benoit, J. Tie, C. Nicchitta, G. von Heijne, D. Stafford, Novel mutations in the VKORC1 gene of wild rats and mice - a response to 50 years of selection pressure by warfarin?, BMC Genet. 10 (2009) 4. doi:10.1186/1471-2156-10-4.
[16] K.D. Tanaka, Y.K. Kawai, Y. Ikenaka, T. Harunari, T. Tanikawa, S. Ando, F. Okajima, S. Fujita, M. Ishizuka, The genetic mechanisms of warfarin resistance in Rattus rattus found in the wild in Japan, Pestic. Biochem. Physiol. 103 (2012) 144–151. doi:10.1016/j.pestbp.2012.04.011.
[17] A. Hodroge, B. Matagrin, C. Moreau, I. Fourel, A. Hammed, E. Benoit, V. Lattard, VKORC1 mutations detected in patients resistant to vitamin K antagonists are not all associated with a resistant VKOR activity, J. Thromb. Haemost. 10 (2012) 2535–2543. doi:10.1111/jth.12019.
[18] K.J. Czogalla, A. Biswas, A.C. Wendeln, P. Westhofen, C.R. Müller, M. Watzka, J. Oldenburg, Human VKORC1 mutations cause variable degrees of 4-hydroxycoumarin resistance and affect putative warfarin binding interfaces, Blood. 122 (2013) 2743–2750. doi:10.1182/blood-2013-05-501692.
[19] T. Tanikawa, An Eye-Lens Weight Curve for Determining Age in Black Rats, Rattus rattus, J. Mammal. Soc. Japan. 18 (1993) 49. doi:10.11238/jmammsocjapan.18.49.
[20] T. Omura, R. Sato, The Carbon Monoxide-binding pigment of Liver Microsomes I. Evidence for its Hemoprotein Nature, J. Biol. Chem. 239 (1964) 2370–2378. http://hero.epa.gov/index.cfm/reference/details/reference_id/12704 (accessed

November 9, 2015).
[21] K. Takeda, A. Morita, Y. Ikenaka, S.M.M. Nakayama, M. Ishizuka, Comparison of two reducing agents dithiothreitol and tris(3-hydroxypropyl)phosphine for in vitro kinetic assay of vitamin K epoxide reductase, Vet. Anim. Sci. (2020) 100095. doi:10.1016/j.vas.2020.100095.
[22] B.R. Brooks, R.E. Bruccoleri, B.D. Olafson, D.J. States, S. Swaminathan, M. Karplus, CHARMM: A program for macromolecular energy, minimization, and dynamics calculations, J. Comput. Chem. 4 (1983) 187–217. doi:10.1002/jcc.540040211.
[23] E. Harder, W. Damm, J. Maple, C. Wu, M. Reboul, J.Y. Xiang, L. Wang, D. Lupyan, M.K. Dahlgren, J.L. Knight, J.W. Kaus, D.S. Cerutti, G. Krilov, W.L. Jorgensen, R. Abel, R.A. Friesner, OPLS3: A Force Field Providing Broad Coverage of Drug-like Small Molecules and Proteins, J. Chem. Theory Comput. 12 (2016) 281–296. doi:10.1021/acs.jctc.5b00864.
[24] R.A. Friesner, J.L. Banks, R.B. Murphy, T.A. Halgren, J.J. Klicic, D.T. Mainz, M.P. Repasky, E.H. Knoll, M. Shelley, J.K. Perry, D.E. Shaw, P. Francis, P.S. Shenkin, Glide: A New Approach for Rapid, Accurate Docking and Scoring. 1. Method and Assessment of Docking Accuracy, J. Med. Chem. 47 (2004) 1739–1749. doi:10.1021/jm0306430.
[25] K.J. Bowers, D.E. Chow, H. Xu, R.O. Dror, M.P. Eastwood, B.A. Gregersen, J.L. Klepeis, I. Kolossvary, M.A. Moraes, F.D. Sacerdoti, J.K. Salmon, Y. Shan, D.E. Shaw, Scalable Algorithms for Molecular Dynamics Simulations on Commodity Clusters, in: ACM/IEEE SC 2006 Conf., IEEE, 2006: pp. 43–43. doi:10.1109/SC.2006.54.
[26] M.A. Kuenemann, P.A. Spears, P.E. Orndorff, D. Fourches, *In silico* Predicted Glucose-1-phosphate Uridylyltransferase (GalU) Inhibitors Block a Key Pathway Required for *Listeria* Virulence, Mol. Inform. 37 (2018) 1800004. doi:10.1002/minf.201800004.
[27] P.A. Friedman, M. Shia, Some characteristics of a vitamin K-dependent carboxylating system from rat liver microsomes, Biochem. Biophys. Res. Commun. 70 (1976) 647–654. doi:10.1016/0006-291X(76)91096-2.
[28] E.F. Hildebrandt, P.C. Preusch, J.L. Patterson, J.W. Suttie, Solubilization and characterization of vitamin K epoxide reductase from normal and warfarin-resistant rat liver microsomes, Arch. Biochem. Biophys. 228 (1984) 480–492. doi:10.1016/0003-9861(84)90014-6.
[29] A. Fregin, K.J. Czogalla, J. Gansler, S. Rost, M. Taverna, M. Watzka, C.G. Bevans, C.R. MüLler, J. Oldenburg, A new cell culture-based assay quantifies vitamin K 2,3-epoxide reductase complex subunit 1 function and reveals warfarin resistance phenotypes not shown by the dithiothreitol-driven VKOR assay, J. Thromb. Haemost. 11 (2013) 872–880. doi:10.1111/jth.12185.
[30] C. Krettler, C.G. Bevans, C. Reinhart, M. Watzka, J. Oldenburg, Tris(3-hydroxypropyl)phosphine is superior to dithiothreitol for in vitro assessment of vitamin K 2,3-epoxide reductase activity, Anal. Biochem. 474 (2015) 89–94. doi:10.1016/j.ab.2014.12.004.
[31] K. Takeda, Y. Ikenaka, T. Tanikawa, K.D. Tanaka, S.M.M. Nakayama, H. Mizukawa, M. Ishizuka, Novel revelation of warfarin resistant mechanism in roof rats (Rattus rattus) using pharmacokinetic/pharmacodynamic analysis, Pestic. Biochem. Physiol. 134 (2016) 1–7. doi:10.1016/j.pestbp.2016.04.004.
[32] J. Jacob, S. Endepols, H.-J. Pelz, E. Kampling, T.G. Cooper, C.H. Yeung, K. Redmann, S. Schlatt, Vitamin K requirement and reproduction in bromadiolone-resistant Norway rats, Pest Manag. Sci. 68 (2012) 378–385. doi:10.1002/ps.2273.