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

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20
21 **Abbreviations:** VKOR, vitamin K epoxide reductase; VKORC1, VKOR complex subunit 1; VKO, vitamin K
22 epoxide; VK, vitamin K quinone; KH₂, vitamin K hydroquinone; THP, tris(3-hydroxypropyl)phosphine; S-rats,
23 warfarin-susceptible rats; R-rats, warfarin-resistant rats; KPB, potassium phosphate buffer; MD, molecular
24 dynamics; CDS, Coding sequences; LOD, Limit of detection; RMSF, Root Mean Square Fluctuation; SP, Standard
25 precision; K_m, Michaelis constant; V_{max}, maximum velocity; IC₅₀, half maximal inhibitory concentration

26
27

28 **Abstract**

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

45

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

48

49

50 **1 Introduction**

51 Vitamin K was first discovered in 1929 as an essential nutrient for blood coagulation.
52 However, cloning the vitamin K epoxide reductase complex 1 (*VKORC1*) gene was not
53 successful until 2004. The gene encodes VKOR, which plays a role in the reactivation of
54 vitamin K [1,2]. Production of vitamin K-dependent proteins, such as blood clotting factors,
55 osteocalcin, and matrix gla protein is dependent on gamma-carboxylation [3]. Vitamin K
56 hydroquinone (KH₂) can act as a cofactor of gamma-glutamyl carboxylase (GGCX), which
57 plays a role in gamma-carboxylation of certain glutamic acid residues. KH₂ is oxidized to
58 vitamin K 2,3-epoxide (VKO) through this reaction. VKO is regenerated to vitamin K quinone
59 (VK) by VKOR and is subsequently converted to KH₂. This series of reactions constitute the
60 vitamin K cycle (Fig. 1) [4]. Interestingly, 4-hydroxycoumarins derivatives, such as warfarin,
61 work as inhibitors of VKOR and have been widely used as anticoagulants in the pharmaceutical
62 industry, and rodenticides.

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

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

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

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

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

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

107

108 **2 Materials and Methods**

109 *2-1: Chemicals*

110 We purchased vitamin K1 from Kanto Chemicals (Tokyo, Japan). Vitamin K1 epoxide,
111 tris, and glycine were obtained from Sigma-Aldrich (St. Louis, MO, USA). Vitamin K1-d7 was
112 obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA). From Wako Pure
113 Chemical Industries (Osaka, Japan), we purchased acetic acid, diethyl ether, DTT, ethanol,
114 HEPES, methanol, K₂HPO₄, KH₂PO₄, NaOH, NaCl, HCl, glycerol, bromophenol blue, sodium
115 dodecyl sulfate, and warfarin–sodium. THP was obtained from Santa Cruz Biotechnology
116 (Dallas, TX, USA). PVDF membranes (0.2 μM) were purchased from Thermo Fisher Scientific
117 (Waltham, MA, USA). Anti-VKORC1 rabbit monoclonal antibody (ab206656), anti-RPS6
118 rabbit polyclonal antibody (ab40820), and goat anti-rabbit IgG H&L (HRP) (ab205718) were
119 purchased from Abcam (Cambridge, UK). Tween 20 was obtained from Tokyo Chemical
120 Industry Co., Ltd. (Tokyo, Japan).

121

122 *2-2: Animals*

123 Closed colonies of warfarin-susceptible and warfarin-resistant black rats (*Rattus*
124 *rattus*) were obtained from Ikari Shodoku Co. Ltd. (Tokyo, Japan). All rats used in this study
125 were male (mean weight = 120.4 ± 10.5 g, mean age based on eye lens = 180.5 ± 40.2 days, n
126 = 8) [19]. Rats were kept under a 12/12-hour light/dark cycle between 20°C and 23°C. Food
127 (CE-2; CLEA, Tokyo, Japan) and water were available *ad libitum*. Rats were sacrificed with
128 high concentrations of isoflurane. The liver was removed immediately and flash-frozen in
129 liquid nitrogen. Samples were kept at –80°C until use. All animal treatments and experimental
130 procedures were performed following the Guidelines of the Association for Assessment and
131 Accreditation of Laboratory Animal Care International (AAALAC) and were approved by the
132 Animal Care and Use Committee of the Faculty School of Veterinary Medicine, Hokkaido
133 University (Approval number: 14-0142).

134

135 *2-3: Sequence analysis*

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

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

144

145 *2-4: Microsome preparation*

146 Microsomal fractions were prepared from liver samples [20]. Approximately 3.0 g of
147 liver samples were homogenized in 9.0 mL of potassium phosphate buffer (KPB, 0.1 M, pH
148 7.4). The homogenates were centrifuged at 9,000 ×g at 4°C for 20 min. The supernatants were
149 filtered through gauze and centrifuged at 105,000 ×g at 4°C for 60 min. The pellets were
150 dissolved in 0.1 M KPB. The microsomal fraction was stored at –80°C until analysis. The
151 protein concentration of microsomes was measured using Pierce BCA Protein Assay Kit
152 (Thermo Fisher Scientific).

153

154 *2-5: VKOR assay*

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

166 Purification of vitamin Ks and quantification by mass spectrometry were also
167 performed according to the method of Takeda et al. [21]. VK and VKO were extracted from
168 the reaction mixture using the liquid–liquid extraction method, and vitamin K1-d7 was added
169 as an internal standard (final concentration, 100 nM). Liquid–liquid extraction was performed
170 in 5 mL of diethyl ether, and the organic layer was aspirated and evaporated under a gentle N₂
171 gas stream. The solute was re-dissolved in 0.2 mL of methanol. Vitamin K and an internal
172 standard were measured using HPLC (Shimadzu 20 series; Shimadzu, Kyoto, Japan) coupled
173 with atmospheric pressure chemical ionization triple quadrupole mass spectrometry
174 (APCI/MS/MS, LC-8040; Shimadzu) equipped with a C18 column (Inertsil ODS-3, 2.1 × 150
175 mm, 5.0 μm), from GL Science (Tokyo, Japan). Spike and recovery tests were performed to
176 assess the recovery rate. Vitamin K1, vitamin K1 epoxide, and vitamin K1-d7 (100 nM) were
177 added to 1 mL of HEPES buffer containing 1 mg/mL of microsome. After that, the extraction
178 was performed as described above. This procedure was repeated four times. The recovery rates
179 of vitamin K1, vitamin K1 epoxide, and vitamin K1-d7 were 83.89 ± 1.62%, 77.89 ± 1.49%,
180 and 83.49 ± 1.64%, respectively. The limit of detection (LOD) was calculated using the
181 standard curve (n = 8). The LODs of vitamin K1, vitamin K1 epoxide, and vitamin K1-d7 were
182 1.40, 5.21, and 3.04 nM, respectively.

183

184 *2-6: Western blotting*

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

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

198

199 *2-7: Molecular docking*

200 3D homology models of each VKORC1 variant were obtained using BIOVIA
201 Discovery Studio (Dassault Systèmes S.E., Paris, France). Model 1 (Cys43-Cys51, Cys132-
202 Cys135) and Model 2 (C51-C135) VKOR of S- and R-rats were constructed and their energy
203 minimization was performed using CHARMM forcefield [22].

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

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

218

219 *2-8: Molecular dynamics simulations*

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

224 simulation ran for 50 ns with warfarin and 100 ns with VKO with the TIP3P solvation model
225 and POPC membrane model, an OPLS3 force field energy minimization, and 10 Å
226 orthorhombic volume. The calculation time step was fixed at 1.0 fs, with an integration output
227 of 1.0 ps. The NPT ensemble was employed with a temperature fixed at 300 K and pressure at
228 1.01 bar. The integration time step was set at 2 fs. Default settings were used for all other
229 parameters.

230

231 2-9: Statistics

232 Values obtained from the *in vitro* kinetic assay were fitted to nonlinear regression with
233 the Michaelis–Menten equation or inhibition curve. Estimations of apparent V_{max} , K_m , V_{max}/K_m ,
234 and IC_{50} values were obtained using GraphPad Prism 8 software (GraphPad Software Inc., San
235 Diego, CA, USA). Data were analyzed using the Student *t*-test with a significance level of $p =$
236 0.05 with JMP 14 (SAS Institute, Cary, NC, USA).

237

238 3 Results

239 3-1: Sequence analysis of VKORC1

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

244

245 3-2: Michaelis–Menten analysis of vitamin K1 epoxide reduction

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

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

258

259 Table 1: Kinetic parameters of VKOR

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

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

261

262 3-3: Inhibition assay by warfarin

263 An inhibition assay was performed to evaluate the sensitivity of VKOR to the
 264 rodenticide warfarin. Fig. 4 is the inhibition curve after normalization to its basal VKOR
 265 activity without warfarin. The resulting IC_{50} value calculated by the fitting is also shown in
 266 Table 1. S-rats showed a strong regression with a concentration of the inhibitor ranging from
 267 0.01 to 5 μM . R-rats, on the contrary, showed regression only with a higher concentration range
 268 from 0.1 to 100 μM . As a result, R-rats have 37.5-fold increased IC_{50} value compared with S-
 269 rats.

270

271 3-4: Quantification of VKORC1

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

277 between S- and R-rats.

278

279 3-5: Molecular docking

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

293

294 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

295

296 3-6: Molecular dynamics simulations

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

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

318

319 4 Discussion

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

325 [12]. Other mutations leading to resistance in humans showed 2.5- to 50-fold higher values
326 [18]. Although the mutation in the rat VKOR is in the loop area, it has a considerably higher
327 resistance than the other mutations. Docking simulations have predicted that R-VKOR could
328 have a lower affinity for warfarin in both models 1 and 2 (Table 2). Importantly, these results
329 are consistent with the high IC_{50} value observed *in vitro*.

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

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

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

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

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

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

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

390

391 **5 Conclusions**

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

395

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412

413 **Figures Captions**

414 Fig.1: Vitamin K cycle. Vitamin K hydroquinone (KH₂) acts as a cofactor of dependent
415 carboxylation of glutamic acid to γ -carboxyglutamic acid by GGCX. Subsequently, KH₂ is
416 oxidized to vitamin K epoxide (VKO). VKO is reduced to vitamin K quinone (VK) and KH₂
417 in the cycle. VKOR plays a role in the reduction of VKO to VK. VKOR, VKR, and NQO1
418 catalyze the conversion of VK to VKH₂. VKOR; Vitamin K epoxide reductase, VKR; Vitamin
419 K reductase NQO1; NAD(P)H quinone oxidoreductase 1, KH₂; vitamin K hydroquinone,
420 VKO; vitamin K epoxide, VK; vitamin K quinone.

421

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

425

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

431

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

435

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

439

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

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

445

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

451

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

458

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