Heterogeneity of the Coumarin Anticoagulant Targeted Vitamin K Epoxide Reduction System. Study of Kinetic Parameters in Susceptible and Resistant Mice (Mus musculus domesticus)

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ABSTRACT: Vitamin K epoxide reductase (VKOR) activity in liver microsomes from a susceptible and a genetically warfarin-resistant strain of mice (Mus Musculus domesticus) was analyzed to determine the mechanism of resistance to this 4-hydroxycoumarin derivative. Kinetic parameters for VKOR were calculated for each strain by incubating liver microsomes with vitamin K epoxide ± warfarin. In susceptible mice, an Eadie–Hofstee plot of the data was not linear and suggested the involvement of at least two different components. Apparent kinetic parameters were obtained by nonlinear regression using a Michaelis–Menten model, which takes into account two enzymatic components. Component A presents a high $K_m$ and a high $V_m$, and as a consequence only an enzymatic efficiency $V_m/K_m$ was obtained (0.0024 mL/min/mg). Estimated warfarin $K_i$ was 0.17 $\mu\text{M}$. Component B presented an apparent $K_m$ of 12.73 $\mu\text{M}$, an apparent $V_m$ of 0.32 nmol/min/mg, and an apparent $K_i$ for warfarin of 6.0 $\mu\text{M}$. In resistant mice, the enzymatic efficiency corresponding to component A was highly decreased (0.0003–0.00066 mL/min/mg) while the $K_i$ for warfarin was not modified. The apparent $V_m$ of component B was poorly modified between susceptible and resistant mice. The apparent $K_m$ of component B observed in resistant mice was similar to the $K_m$ observed in susceptible mice. These modifications of the catalytic properties are associated with a single nucleotide polymorphism (T175G) in the VKOR-C1 gene, which corresponds to a Trp59Gly mutation in the protein.

INTRODUCTION

Prevention of thromboembolic disease is based on the use of 4-hydroxy-coumarin derivatives such as warfarin. Warfarin response varies markedly between individuals, requiring that its anticoagulant activity must be monitored by frequent blood testing. Single nucleotide polymorphisms in CYP 2C9, an enzyme important for its metabolism, and vitamin K epoxide reductase (VKOR), its enzyme target, are believed to contribute equally to this variation in human response (about 30% each, [1–5]).

Warfarin is also widely used in the control of feral rodent proliferation and particularly in mice. The continual use of rodenticides for 50 years, such as warfarin, has resulted in the selection and development of resistant rat strains [6–9] and resistant mouse strains [10–13] in a number of geographic locations. We postulate that the description of resistance mechanisms selected in wild animals can be an interesting way to (1) understand the resistance mechanisms observed in human beings and (2) to adapt rodent control strategy.

Warfarin blocks the vitamin K cycle through the inhibition of the liver microsomal vitamin K 2,3-epoxide reductase (VKOR). Compounds that inhibit the reduction of vitamin K epoxide, such as warfarin, prevent
the biological activation of blood-clotting factors and cause internal bleeding [14].

In brown rats (Rattus norvegicus), different mechanisms are proposed to be the origin of different resistant strains [15,16]. The overexpression of calumenin, a chaperone protein, which acts in protecting the VKOR against the effects of warfarin, seems to be a mechanism involved in a strain of rats from Chicago [17]. In Europe, an important single nucleotide polymorphism (SNP) in the vitamin K epoxide reductase subunit 1 (VKORC1) (Y139F) was described in wild rats [18], and the catalytic consequences of this mutation have been previously described by our group [19].

Contrary to brown rats, anticoagulant resistance and kinetic parameters of VKOR have been poorly studied in house mice (Mus musculus domesticus) [20]. A study showed that VKOR activity in some wild warfarin-resistant house mice trapped in Denmark had reduced sensitivity to warfarin inhibition [13]. Other warfarin-resistant wild mice in the same study were found to have VKOR activity that was equally sensitive to warfarin inhibition as the VKOR from warfarin-susceptible mice. These results show that resistant mice do not necessarily have an impaired VKOR activity [13,21].

In this paper, we compare the catalytic properties of the VKOR of liver microsomes from a susceptible or a resistant mouse strain in order to understand the resistance mechanism.

**MATERIALS AND METHODS**

**Animals**

Warfarin-susceptible and warfarin-resistant mice, bred in the Lyon College of Veterinary Medicine (ENVL), were initially trapped from French farms in the 1980s. Each strain received the same feed and water ad libitum. Animals were not supplemented in vitamin K and received a standard feed (Scientific Animal Food and Engineering, reference A04 for adult rodent). The warfarin-resistant mice survived a 6-day no choice feeding of 0.025% warfarin-containing oats [22]. Susceptibility was estimated at 100% for the susceptible mouse strain. All experimental protocols were approved by the ethics committee of the Lyon College of Veterinary Medicine and followed the guidelines described in the National Health Institute for the Care and Use of Laboratory Animals. Mice were euthanized by decapitation under isoflurane anesthesia, 3–4 months after the termination of warfarin exposure. Experiments were also conducted on laboratory mice (Charles River Laboratories, St Germain sur l’Arbresle, France). The liver from 10 wild warfarin-resistant, 10 wild warfarin-susceptible mice, and 10 laboratory mice were removed and stored in liquid nitrogen until assay. Microsomes were prepared with pooled livers from five warfarin-susceptible or five warfarin-resistant mice. Microsomes from individual livers were also prepared. Others five livers from warfarin-susceptible or warfarin-resistant mice were used to obtain individual microsomes from each mouse previously genotyped.

**Tissue Preparation**

Microsomes were prepared from thawed livers by differential centrifugation as described by Moroni et al. [23]. Protein concentrations were determined by the method of Bradford [24] using bovine serum albumin as a standard. Microsomes were frozen at −80°C until analysis.

**VKORC1 Genotyping**

Genomic DNA was extracted from susceptible and resistant mice liver samples using the DNA genomic isolation system. DNA concentrations were evaluated spectrophotometrically at 260 nm. The 260/280 ratios were between 1.8 and 2. PCR was performed with 1 µg of extracted genomic DNA using specific primers of mice VKORC1 (VKORC1 GenBank accession # NC-000073). The sequence of the sense primer VKORC1-S and the antisense primer VKORC1-AS was 5′-AGCCTCTCCAATCACAATCCC-3′ and 5′-CCATGTAGTGACTTCCAGGG-3′, respectively. Amplification was performed at 94°C for 90 s, 29 cycles of 94°C for 30 s, 62°C for 45 s, 72°C for 3 min, followed by a final extension at 72°C for 5 min. The size of the amplified fragment was 1650 base pairs (bp). Sequencing of PCR products was systematically done (GenomExpress, Meylan, France).

**Kinetic Studies on Vitamin K Epoxide Reductase**

Microsomal VKOR activity was assayed as described earlier [25–27], except that the reaction solution contained 2 mM dithiothreitol (DTT) and buffer solution was 200 mM Tris-HCl/0.15 M KCl buffer (pH 7.4); therefore, 1.5 mg of protein was added. VKOR activity was assayed at 30°C. Warfarin was added before DTT according to [25–27]. The reaction was started by the addition of vitamin K epoxide (vitamin KO) solution in 1% Triton X-100. All incubation mixtures contained the same detergent concentration. The incubation time was 9 min, and the reaction was stopped by addition of 4 mL of iced 1:1 isopropanol/hexane solution. After
centrifugation (5000 × g, 5 min), the hexane layer was removed and dried in nitrogen. The dry residue was immediately dissolved in 0.2 mL of isopropanol. Vitamin K was measured by high pressure liquid chromatography (HPLC) (D7000 HS Hitachi AS 2000 automatic injector, injection volume of 50 μL, L 7100 pump, L 4000 UV detector). Separation was achieved on a LiChrospher 100 RP18e, 250 × 4 mm, 5 μm analytical column run at 2 mL/min in 100% methanol HPLC grade. External standards of vitamin K and vitamin KO were analyzed and quantified at 240 nm. Kinetic parameters of VKOR activity (apparent K_m and reaction velocity (V_m) values) were studied using pooled microsomes, and incubations were performed in triplicates. The substrate vitamin KO concentrations ranged from 12.5 to 400 μM. Inhibition parameters were determined by incubating vitamin KO in the presence of 0, 0.5, 1, 2, or 5 μM warfarin with pooled hepatic microsomes from susceptible and resistant mice. Apparent K_i values were calculated. A nonlinear model using the Rfit program developed by Ihaka and Gentleman was fitted to the data.

Individual liver microsomes from five susceptible or resistant mice were used to assay VKOR activity at 400 μM vitamin KO ± 1 μM warfarin.

**Chemicals**

Vitamin K (Phylloquinone) was converted to vitamin KO according to Tishler et al. [28]. Purity was estimated by UV absorption at 254 nm and higher than 99%. Vitamin K, DTT, Tris buffer, isopropanol, hexane, and methanol were purchased from Sigma-Aldrich (France). Sodium warfarin was a gift from Liphatech (Pont du Casse, France). Primers for VKORC1 genotyping were prepared by Proligo (Paris, France). DNA genomic isolation system was from Promega (Carbonnières les Bains, France).

**RESULTS**

**Genotyping the VKORC1 Gene**

VKORC1 gene, from 400 bp upstream exon 1 to 100 bp downstream the last exon (exon 3), was sequenced in 10 warfarin-susceptible and warfarin-resistant mice. The coding sequence was identical with our susceptible mice to the published sequence (GenBank accession # NC-000073). In resistant mice, a unique SNP (T175G) was systematically observed and corresponded to a Trp59Gly mutation (Figure 1), not previously described. This mutation is located in exon 2. For the catalytic studies, only hepatic microsomes from homozygotic mice were used.

**Kinetic Parameters of VKOR Activity**

Preliminary investigations showed that the enzymatic activity was linear with respect to protein content and duration of incubation with different substrate concentrations from 12.5 to 400 μM. All data obtained from laboratory mice were comparable to data from wild warfarin-susceptible mice concerning kinetic parameters and warfarin inhibition. As a consequence, we present only results obtained from wild mice. In Figure 2A, the effect of vitamin KO concentration on the velocity of the enzyme reaction catalyzed by susceptible mice pooled liver microsomes is presented. Within the range of concentrations tested (12.5–400 μM), the velocity did not reach a plateau. The Eadie–Hofstee plot of the data (V/S as a function of V) was not linear (Figure 2B).

The effect of warfarin on VKOR activity was studied with warfarin concentrations ranging from 0.5 to 5 μM. The effect of warfarin at 1 μM on VKOR activity is presented in Figure 3A. The Eadie–Hofstee plot of VKOR activity in the presence of 1 and 5 μM warfarin is presented in Figure 3B.

When warfarin concentration was 0.5 μM, the inhibiting effect was negligible when KO concentrations were low (12.5 and 25 μM). This inhibiting effect dramatically increased when concentration of KO (the substrate) increased. Furthermore, inhibiting effect of warfarin was noticeable at low KO concentrations (12.5 and 25 μM) when warfarin was 2 or 5 μM only.

VKOR activity of antivitamin K resistant mice has also been studied. In Figure 4A, the velocity of the reaction is plotted as a function of the vitamin KO concentration. As described for susceptible mice, the velocity did not reach a plateau. The VKOR activity was lower in comparison to the VKOR activity of pooled liver microsomes from susceptible mice, particularly when KO concentrations increased (1.28 nmol/min/mg in susceptible mice and...
FIGURE 2. (A) Effect of vitamin K epoxide (vitamin KO) concentration on the velocity of reaction of vitamin K epoxide reductase in pooled liver microsomes from mice susceptible to anticoagulant (12.5–400 μM vitamin KO). Each experimental data is the result of a triplicate. Data are presented as mean ± 2SD (SD bars correspond to the repeatability of the experiment on the same liver pooled microsomes). (B) Effect of vitamin KO on VKOR activity, in pooled liver microsomes from susceptible mice, represented by an Eadie–Hofstee diagram.

0.48 nmol/min/mg in resistant mice with 400 μM vitamin KO). Indeed, the increased rate of the activity as a function of the substrate concentration between 100 and 400 μM appeared to be highly limited. Effects of warfarin were also studied. Figure 4B presents the VKOR activity as a function of the KO concentration in the presence of 1 μM warfarin. Again, warfarin inhibited the VKOR activity when KO concentration was high (≥100 μM) only. All kinetic data are presented in Table 1.
FIGURE 3. (A) Effect of the concentration of vitamin K epoxide (vitamin KO) on the activity of the vitamin K epoxide reductase (VKOR, in nmol min\(^{-1}\) · mg prot\(^{-1}\)) in the presence of 1 µM warfarin in pooled liver microsomes from susceptible mice. A model was fitted to the data by nonlinear regression. VKOR activities of both components A and B are presented. Each experimental data is the result of a triplicate. Data are presented as mean ± 2SD (SD bars correspond to the repeatability of the experiment on the same liver pooled microsomes). (B) Effect of vitamin KO, in the presence of 1 and 5 µM warfarin, on VKOR activity in pooled liver microsomes from susceptible mice, represented by the Eadie–Hofstee diagram.

The VKOR activity of individual liver microsomes from susceptible or resistant mice was studied. The results showed that VKOR activities for liver microsomes from susceptible mice were 1.17 nmol/min/mg (±0.11) and 0.46 nmol/min/mg (±0.07) in the absence of warfarin or in the presence of 1 µM of warfarin, respectively. For microsomes from resistant mice, the results were 0.47 nmol/min/mg (±0.06) and 0.29 nmol/min/mg (±0.05).

**DISCUSSION**

Rat resistance to anticoagulants is primarily associated with mutations in the VKORC1 gene localized on chromosome 1. Different mutations, such as Y139F, are responsible for resistance. The involvement of this mutation in catalytic properties of the VKORC1 protein is of great importance [19,29] as shown for the recombinant VKORC1 protein expressed in HEK 293 cell line [18]. We observed, using a Y139F strain of wild rats, major modifications of the catalytic constants of the liver VKOR activity (decreased $V_m$, decreased $K_m$, increased warfarin $K_i$ by 42-fold, [19]). On the other hand, some other mutations do not seem to provoke noticeable changes in the catalytic properties of VKOR [18].

In mice, resistance is supposed to be related to both metabolic modifications and mutations in the VKORC1 gene (on chromosome 7). The resistance of our strain
is checked by in vivo testing (feeding tests). Resistance level is about 100% in this strain. The genotyping of the VKORC1 gene demonstrates in our strain that resistance is associated with a mutation T175G (Trp59Gly). Nevertheless, in the absence of recombinant expression of this mutated protein, the enzymatic consequences of such an SNP need to be ascertained.

The Eadie–Hofstee plot of the velocity of the VKOR as a function of KO concentration indicates that a monoenzymatic system is not sufficient in order to describe the kinetics in pooled liver from susceptible mice. A Michaelis–Menten [\( V = \frac{V_m S}{K_m + S} \)] model cannot be used to fit the data. The velocity of VKOR when concentrations of the substrate are high (KO \( \geq \) 100 \( \mu \)M) appears to be proportional to the substrate concentration (Figure 2A). As a consequence, we tested a model composed of both a proportional component \( \left(\frac{V_m}{K_m}\right)_A S \) and the

FIGURE 4. (A) Effect of the concentration of vitamin K epoxide (vitamin KO) on the activity of the vitamin K epoxide reductase (VKOR, in nmol min\(^{-1}\) mg prot\(^{-1}\)) in pooled liver microsomes from the resistant mice. A model was fitted to the data by nonlinear regression. VKOR activities of both components A and B are presented. Each experimental data is the result of a triplicate. Data are presented as mean ± 2SD (SD bars correspond to the repeatability of the experiment on the same liver-pooled microsomes). (B) Effect of 1 \( \mu \)M warfarin on VKOR activity in pooled liver microsomes from resistant mice. VKOR activities of both components A and B are presented.
Michaelis–Menten component. A correct fit was obtained by nonlinear regression with the following equation \( V = (V_m / K_m) \times S + (V_{\text{mB}} S) / (K_{\text{mB}} + S) \). The deduced activity of components A and B and the observed VKOR activity, in susceptible mice, are presented in Figures 2A and 2B, respectively. Component A, the proportional component, is characterized by its enzymatic efficiency \( V_m / K_m \) only. The parameters \( K_m \) and \( V_m \) cannot be delineated. Such a hypothesis corresponds to a \( K_m \) being high with respect to the substrate concentrations tested. The enzymatic efficiency \( V_m / K_m \) of component A can be estimated at 0.0024. Component B is characterized by a \( K_m \) of 12.73 ± 0.93 \( \mu \)M and a \( V_m \) of 0.32 ± 0.04 nmol/min/mg, i.e., \( V_m / K_m = 0.0255 \) mL/min/mg.

The use of this proportional component (A) is very interesting when data corresponding to the inhibition by warfarin were analyzed. Indeed, the inhibiting effects of low concentrations of warfarin increased with the concentration of the substrate (Figure 3A), i.e., when the activity of component A is effective only. With 1 \( \mu \)M of warfarin, the Eadie–Hofstee plot of the data becomes quite linear and seems to be aligned with the representation of the unmodified component B (Figure 3B). As a consequence, the use of a monoenzymatic model, i.e., component B, is sufficient to fit correctly the data. Nevertheless, using the two-components model, data were fitted with component A whose enzymatic efficiency is dramatically reduced (0.0003 mL/mi/mg), while component B is not altered when 1 \( \mu \)M warfarin is used. These results suggest that component A presents a high sensitivity to the inhibition by warfarin, while component B seems to be relatively resistant. When 5 \( \mu \)M warfarin is used, the Eadie–Hofstee plot of the data (Figure 3B) is parallel to the data corresponding to 1 \( \mu \)M warfarin and to the Eadie–Hofstee plot of component B (in the absence of warfarin). This argues for warfarin being a noncompetitive inhibitor as described by Thijssen et al. [30]. The model equation becomes \( V = [(V_m / K_m) \times (1 / K_{\text{iA}})] S + [(V_{\text{mB}} (1 + 1 / K_{\text{iB}})] S) / (K_{\text{mB}} + S) \), in which \( S \) and \( I \) are controlled parameters while \( K_{\text{iA}} \) and \( K_{\text{iB}} \) were determined by nonlinear regression of the data. The calculated warfarin \( K_{\text{i}} \) for component A is 0.17 \( \mu \)M. For component B, \( K_{\text{i}} \) can be estimated at 6.0 ± 0.4 \( \mu \)M.

In resistant mice (Figure 4A), adjustment with a two-component model A and B gives component A with an enzymatic efficiency of 0.0006 mL/min/mg and component B with a similar apparent \( K_m \) (15.31 ± 4.92 \( \mu \)M) to susceptible mice but a \( V_m \) of 0.24 ± 0.01 nmol/min/mg. The catalytic properties are in this case similar to those of the susceptible mice in the presence of warfarin.

Warfarin (1 \( \mu \)M) inhibition is presented in Figure 4B. For component A, \( K_i \) was estimated at 0.19 ± 0.02 \( \mu \)M. For component B, \( K_i \) was estimated at 3.5 ± 0.3 \( \mu \)M. Kinetic parameters of VKOR of susceptible and resistant mice are presented in Table 1.

A detailed approach of kinetic parameters of VKOR activity cannot be achieved with microsomes from only one mouse. Pooled microsomes could be at the origin of a mixture of different enzymatic systems from different animals without coexisting in the same animal. Consequently, we confirmed these results mouse by mouse. Results from individual microsomes are very close to results obtained from pooled microsomes, showing that the microsome mixture is not the explanation of the observed phenomenon. Using affinity and inhibition values \( K_{\text{mB}}, K_{\text{iA}}, \) and \( K_{\text{iB}} \), respectively, in the two-component model, we calculated \( V_m / K_m \) and \( V_{\text{mB}} \) values. Values of \( V_m / K_m \) (0.0021 ± 0.0003 mL/min/mg) and \( V_{\text{mB}} \) (0.36 ± 0.04 nmol/min/mg) in susceptible mice are in conformity with values obtained from pooled microsomes. In individual microsomes from resistant mice, \( V_{\text{mB}} \) (0.36 ± 0.08 nmol/min/mg) is similar to values obtained in individual microsomes from susceptible mice and higher than values from pooled microsomes. \( V_m / K_m \) (0.0003 mL/min/mg) in individual microsomes from resistant mice is even lower than values obtained from pooled microsomes. The use of individual microsomes allows us to consolidate the selected model.

The enzymatic model is composed of two components. The first one corresponds to a low affinity but high capacity (component A), and the second one corresponds to a high affinity low capacity (component B). Furthermore, component A is very sensitive to warfarin (\( K_i \) 0.17–0.19 \( \mu \)M), while component B appears to be more resistant (\( K_i \) about 3.5–6.0 \( \mu \)M). Finally, the proportion between components A and B is dependent

### Table 1. Kinetic Parameters of Vitamin K Epoxide Reductase Activity in Pooled Liver Microsomes from Anticoagulant Susceptible Mice and in Liver Microsomes of Anticoagulant-Resistant Mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>VKOR</th>
<th>Component</th>
<th>( K_m ) (( \mu )M)</th>
<th>( V_{\text{mmax}} ) (nmol/min/mg)</th>
<th>( V_{\text{m}} / K_m ) (mL/min/mg)</th>
<th>( K_i ) (( \mu )M Warfarin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible</td>
<td>Liver</td>
<td>Component A</td>
<td>High</td>
<td>High</td>
<td>0.0024</td>
<td>0.17 (0.03)</td>
</tr>
<tr>
<td>mouse</td>
<td>VKOR</td>
<td>Component B</td>
<td>12.73 (0.93)</td>
<td>0.32 (0.04)</td>
<td>0.0255</td>
<td>5.97 (0.38)</td>
</tr>
<tr>
<td>Resistant</td>
<td>Liver</td>
<td>Component A</td>
<td>High</td>
<td>High</td>
<td>0.0006</td>
<td>0.19 (0.02)</td>
</tr>
<tr>
<td>mouse</td>
<td>VKOR</td>
<td>Component B</td>
<td>15.31 (4.92)</td>
<td>0.24 (0.01)</td>
<td>0.0156</td>
<td>3.5 (0.27)</td>
</tr>
</tbody>
</table>
on the strain (component A is highly decreased in resistant strain; component B is poorly modified), and these modifications are associated with the VKOR-C1 genotype.

In resistant mice, global VKOR activity is low with respect to susceptible mice. The respective proportion of the two components is strongly modified with component A (high $K_m$, low $K_i$) being considerably decreased while component B being similar (or poorly modified) in comparison to susceptible mice. As a consequence, the global VKOR activity corresponds to the activity of component B (low $K_m$, high $K_i$) only. The resulting global enzymatic system, in resistant animals, is characterized by a lower $K_m$ for vitamin KO and a higher $K_i$ for warfarin as compared with susceptible mice. Such consequences are similar to those described in rats [27,30,31], but by the use of a different mechanism [19].

The high $K_i$ for component B ($3.50–6.0 \mu M$) is responsible for a spontaneous level of resistance in susceptible mice. Indeed, this $K_i$ is clearly higher than the $K_i$ determined in susceptible rats ($0.5 \mu M$ according to [19]).

The involvement of a dual enzymatic system corresponding to the VKOR activity has never been described. Such two component systems may be caused by several mechanisms but does not necessarily mean that two different genes exist. Indeed, technical artifacts may lead to the suspicion of two coexisting systems. For instance, nonionic detergents such as Triton X-100, which are present in the incubation medium, enable partial solubilization of microsomal proteins and may suggest the presence of diverse subpopulations according to enzyme solubility (membrane-linked enzyme, solubilized enzyme, micellized enzyme). Such artifacts would give poorly repeatable results and would definitely be independent of the susceptible or resistant genotype of mice. On the contrary, the distribution of components A and B is strongly associated with this genotype. Furthermore, this distribution was not observed in rats, though the technical analysis was similar. These data suggest that our results are not due to artifactual observations.

The presence of two components may also be related to a posttranslational modification of VKOR, or it may be due to the existence of two isoforms of VKOR-C1. To date, we have no data to support either hypothesis; therefore, we use the generic A and B terms to describe these components.

The analysis of the catalytic constants corresponding to the VKOR in susceptible and resistant mice did not reveal clear modification of the kinetic constants except for the lowered expression of component A. Therefore, there is no evidence for catalytic modifications associated with the SNP detected. Only heterologous expression in cell line could provide reliable information on the catalytic consequences of this mutation. It is also possible that, similarly to the situation observed in human beings, this SNP is strongly associated with another that is present in the promoter of the VKORC1 gene [4].

Finally if we consider that the catalytic center is localized in the two transmembrane helix alpha (amino acid 100–120 and AA 128–148, [32,33]), the importance of AA59 (the mutation observed in our resistant strain is Trp59Gly), which is in a cytoplasmic loop of the protein, should be more in the interaction with the other proteins of the VKOR complex [34] or in the protein conformation. In such an hypothesis, the VKOR-C1 protein should be in equilibrium within two conformations (high affinity, low capacity, high $K_i$ corresponding to component B and low affinity, high capacity and low $K_i$, corresponding to component A) and the mutation should allow the protein to be in the first conformation only.

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**REFERENCES**


