Human VKORC1 mutations cause variable degrees of 4-hydroxycoumarin resistance and affect putative warfarin binding interfaces

Katrin J. Czogalla, Arijit Biswas, Ann-Christin Wendeln, Philipp Westhofen, Clemens R. Müller, Matthias Watzka, and Johannes Oldenburg

Institute of Experimental Haematology and Transfusion Medicine, University Clinic Bonn, Bonn, Germany

Key Points

- In vitro analysis of VKORC1 mutations perfectly reflects patients’ warfarin resistance phenotypes.
- In silico docking of warfarin on a VKORC1 model reveals a putative docking site in agreement with the locations of OACR-associated mutations.

Introduction

Initially introduced as a rodenticide, warfarin became the first synthetic oral anticoagulant (OAC) for human use and eventually one of the most prescribed drugs world-wide even though its mechanism of action was only recently clarified. In the 1970s, vitamin K 2,3-epoxide (K\textsubscript{2,3}) was discovered as a by-product of vitamin K metabolism. Since the discovery of warfarin-sensitive vitamin K 2,3-epoxide reductase complex subunit 1 (VKORC1), 26 human VKORC1 (hVKORC1) missense mutations have been associated with oral anticoagulant resistance (OACR). Since 2004, 26 different VKORC1-associated OACR mutations have been associated with OAC resistance (OACR), clinically defined as greatly elevated coumarin dosage requirements, or even complete resistance. Since 2004, 26 different OACR-associated missense mutations in human VKORC1 (hVKORC1) have been reported. However, the molecular mechanism of OAC inhibition of VKORC1 and how mutations in this enzyme lead to OAC resistance are still unclear. Historically, investigations of wild-type and mutant hVKORC1 have relied on the “classical” dithiothreitol-driven vitamin K 2,3-epoxide reductase (VKOR) assay that significantly impacts effective OAC dosage. Furthermore, pharmacokinetics of warfarin is influenced by several polymorphisms of cytochrome P450 isofom 2C9, the primary enzyme responsible for metabolizing 4-hydroxycoumarins. This anticoagulant effect of 4-hydroxycoumarins is widely used in prevention and therapy of arterial or venous thromboses. However, there are wide variations in therapeutic dosage due to interindividual variables such as age, gender, weight, and co-medications as well as pharmacogenetic and pharmacokinetic determinants. A major pharmacogenetic factor is a polymorphism in the promoter region of VKORC1 (VKORC1:c.-1639 G\textsubscript{A}) that significantly impacts effective OAC dosage. Furthermore, pharmacokinetics of warfarin is influenced by several polymorphisms of cytochrome P450 isofom 2C9, the primary enzyme responsible for metabolizing 4-hydroxycoumarins. Since the discovery of warfarin-sensitive vitamin K 2,3-epoxide reductase complex subunit 1 (VKORC1), 26 human VKORC1 (hVKORC1) missense mutations have been associated with oral anticoagulant resistance (OACR). Assessment of warfarin resistance using the “classical” dithiothreitol-driven vitamin K 2,3-epoxide reductase (VKOR) assay has not reflected clinical resistance phenotypes for most mutations. Here, we present half maximal inhibitory concentrations (IC\textsubscript{50}) results for 21 further hVKORC1 mutations obtained using a recently validated cell-based assay (J Thromb Haemost 11(5):872). In contrast to results from the dithiothreitol-driven VKOR assay, all mutations exhibited basal VKOR activity and warfarin IC\textsubscript{50} values that correspond well to patient OACR phenotypes. Thus, the present assay is useful for functional investigations of VKORC1 and oral anticoagulant inhibition of the vitamin K cycle. Additionally, we modeled hVKORC1 on the previously solved structure of a homologous bacterial enzyme and performed in silico docking of warfarin on this model. We identified one binding site delineated by 3 putative binding interfaces. These interfaces comprise linear sequences of the endoplasmic reticulum–lumenal loop (Ser52-Phe55) and the first (Leu22-Lys30) and fourth (Phe131-Thr137) transmembrane helices. All known OACR-associated hVKORC1 mutations are located in or around these putative interfaces, supporting our model. (Blood. 2013;122(15):2743-2750)
Table 1. Warfarin IC50 for hVKORC1 variants determined by cell culture-based and dithiothreitol-driven in vitro assays and compared with patient dosage phenotypes

<table>
<thead>
<tr>
<th>hVKORC1 variant</th>
<th>Warfarin IC50 ± SEM (nM)*</th>
<th>Variant IC50/wild-type IC50 ratio*</th>
<th>Mean patient dosage in HDT multiples [drug]**</th>
<th>Warfarin IC50 by dithiothreitol-driven VKOR assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>24.7 ± 3.6</td>
<td>1.0 ± 0.2</td>
<td>1.0 [W, P] (n = 77)</td>
<td>Warfarin-sensitive (by definition)</td>
</tr>
<tr>
<td>Ala26Thr</td>
<td>1224 ± 5.2</td>
<td>49.6 ± 0.1</td>
<td>&gt;3.0 [W] (n = 1)</td>
<td>11.2-Fold increased Ki (IC50)‡</td>
</tr>
<tr>
<td>Ala26Pro</td>
<td>74.1 ± 7.5</td>
<td>3.0 ± 0.2</td>
<td>&gt;2.0 [P] (n = 1)</td>
<td>Sensitive†</td>
</tr>
<tr>
<td>Leu27Val</td>
<td>62.0 ± 10.1</td>
<td>2.5 ± 0.2</td>
<td>&gt;3.0 [F], 1.0 [W] (n = 1)</td>
<td>Sensitive‡</td>
</tr>
<tr>
<td>His28Gln</td>
<td>72.2 ± 8.2</td>
<td>2.9 ± 0.2</td>
<td>3.5 [P] (n = 1)</td>
<td>Sensitive‡</td>
</tr>
<tr>
<td>Val29Leu</td>
<td>136.1 ± 3.3</td>
<td>5.5 ± 0.1</td>
<td>2.0 [W] (n = 1)</td>
<td>Absence of expression‡/sensitive§</td>
</tr>
<tr>
<td>Asp36Gly</td>
<td>78.5 ± 6.6</td>
<td>3.2 ± 0.2</td>
<td>3.0 [W] (n = 1)</td>
<td>Sensitive‡</td>
</tr>
<tr>
<td>Asp36Ty</td>
<td>93.9 ± 19.4</td>
<td>3.8 ± 0.3</td>
<td>1.5-3.5 [W] (n = 10)</td>
<td>Sensitive‡</td>
</tr>
<tr>
<td>Val45Ala</td>
<td>152.4 ± 4.4</td>
<td>6.2 ± 0.1</td>
<td>&gt;2.0 [W] (n = 1)</td>
<td>Sensitive‡/sensitive§</td>
</tr>
<tr>
<td>Ser52Leu</td>
<td>182.3 ± 8.9</td>
<td>7.4 ± 0.2</td>
<td>&gt;3.0 [P] (n = 1)</td>
<td>Low VKOR activity, Ki (IC50) determination not possible‡</td>
</tr>
<tr>
<td>Ser52Trp</td>
<td>140.9 ± 33.0</td>
<td>5.7 ± 0.2</td>
<td>3.5 [P] (n = 1)</td>
<td>Low VKOR activity, Ki (IC50) determination not possible‡</td>
</tr>
<tr>
<td>Val54Leu</td>
<td>112.6 ± 2.8</td>
<td>4.5 ± 0.1</td>
<td>1.5-5.5 [W] (n = 2)</td>
<td>4.6-Fold increased Ki (IC50)‡</td>
</tr>
<tr>
<td>Ser56Phe</td>
<td>167.1 ± 11.9</td>
<td>6.8 ± 0.2</td>
<td>&gt;5.0 [P] (n = 1)</td>
<td>Sensitive‡</td>
</tr>
<tr>
<td>Arg56Gly</td>
<td>85.7 ± 7.8</td>
<td>3.4 ± 0.2</td>
<td>5.0 [W] (n = 1)</td>
<td>Sensitive‡/sensitive§</td>
</tr>
<tr>
<td>Trp59Arg</td>
<td>433.5 ± 5.0</td>
<td>17.5 ± 0.1</td>
<td>7.0 [P] (n = 1)</td>
<td>Low VKOR activity, Ki (IC50) determination not possible‡</td>
</tr>
<tr>
<td>Trp59Cys</td>
<td>188.6 ± 8.4</td>
<td>7.6 ± 0.2</td>
<td>&gt;3.5 [P] (n = 1)</td>
<td>Sensitive†</td>
</tr>
<tr>
<td>Val65Leu</td>
<td>1858.4 ± 4.8</td>
<td>75.2 ± 0.1</td>
<td>&gt;5.0 [P] (n = 1)</td>
<td>Low VKOR activity, Ki (IC50) determination not possible‡</td>
</tr>
<tr>
<td>Val66Gly</td>
<td>69.4 ± 5.7</td>
<td>2.8 ± 0.2</td>
<td>2.5 [P] (n = 1)</td>
<td>Low VKOR activity, Ki (IC50) determination not possible‡</td>
</tr>
<tr>
<td>Val66Met</td>
<td>134.7 ± 7.3</td>
<td>5.4 ± 0.2</td>
<td>3.0-6.0 [W] (n = 7)</td>
<td>Low VKOR activity, Ki (IC50) determination not possible‡</td>
</tr>
<tr>
<td>Gly71Ala</td>
<td>127.4 ± 6.3</td>
<td>5.1 ± 0.2</td>
<td>&gt;2.0 [P] (n = 1)</td>
<td>Low VKOR activity, Ki (IC50) determination not possible‡</td>
</tr>
<tr>
<td>Asn77Ser</td>
<td>131.1 ± 15.5</td>
<td>5.3 ± 0.2</td>
<td>&gt;3.0 [P] (n = 1)</td>
<td>Low VKOR activity, Ki (IC50) determination not possible‡</td>
</tr>
<tr>
<td>Asn77Trp</td>
<td>96.3 ± 5.7</td>
<td>3.9 ± 0.2</td>
<td>3.5 [W] (n = 1)</td>
<td>Low VKOR activity, Ki (IC50) determination not possible‡</td>
</tr>
<tr>
<td>Ile123Asn</td>
<td>209.3 ± 3.7</td>
<td>8.5 ± 0.1</td>
<td>&gt;7.0 [P] (n = 1)</td>
<td>2.4-Fold increased Ki (IC50)‡</td>
</tr>
<tr>
<td>Leu128Arg</td>
<td>1226.8 ± 8.4</td>
<td>49.7 ± 0.1</td>
<td>&gt;4.0-7.0 [W] (n = 5)</td>
<td>Low VKOR activity, Ki (IC50) determination not possible‡/sensitive§</td>
</tr>
<tr>
<td>Tyr133His</td>
<td>113.1 ± 5.2</td>
<td>4.6 ± 0.2</td>
<td>&gt;3.0 [W] (n = 1)</td>
<td>3.6-Fold increased Ki (IC50)‡</td>
</tr>
<tr>
<td>TYA → Leu-Ile-Val</td>
<td>361.1 ± 11.1</td>
<td>14.6 ± 0.1</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*IC50 values determined by the cell-based assay method as cited in Fregin et al18 used for the present study.
†Data from Figures 2 and 3 and supplemental Table 3 of Watzka et al. HDT is defined as the high-dosage threshold and is equivalent to the mean patient population dosage divided by the control group (homozygous wild-type VKORC1 alleles with VKORC1.c-1698G>G haplotype) population mean OAC dosage for each drug, where the patient achieved stable anticoagulation with an international normalized ratio within the range 2.0-3.0. n, number of reported patients with mutation.13
‡Results from Hodroge et al, the study assumes that K_i = IC50.17
§Results calculated from data in Rost et al11 by methods detailed in Fregin et al.18 Itapatient had cytochrome P450 isoform 2C9*2*3 haplotype, which results in a reduced warfarin dosage requirement to achieve a stable, therapeutically normalized ratio compared with patients with wild-type cytochrome P450 isoform 2C9*1*1 haplotype.

For personal use only.on July 15, 2017. by guest.
In fact, average errors for our data were considerably less than this value (see error bars for each dose-response curve in Figure 1).

**Assessment of VKORC1 protein expression**

Relative VKORC1 variant expression levels in HEK 293T cells were determined by cloning the variant cDNAs into pcDNA3.1/myc-His expression vector (Life Technologies, Darmstadt, Germany). Cells were lysed after 72-hour expression and VKORC1 variants quantitated by densitometry of immunostained western blots (see supplemental Information for detailed methods). VKORC1 variants were detected using a polyclonal anti-C-Myc antibody (Sigma-Aldrich Chemie GmbH, Munich, Germany) and were normalized to constitutively expressed ERGIC-53 that was detected by

---

**Figure 1. Dose-response curves for hVKORC1 variants measured by the cell culture-based assay.** (A-F) Secreted FIX activity data for HEK 293T cells coexpressing FIX together with human wild-type or OACR-associated VKORC1 variants in the presence of various warfarin concentrations (0.00-1.0 μM). Data are mean values for n = 6 measurements and error bars show ± SEM. Data points and fitted curves for hVKORC1 wild-type and missense variants are color-coded according to the legend key in each panel.
stripping western blots after hVKORC1 immunostaining and reprobing using an anti-ERGIC-53 antibody (ER-Golgi intermediate compartment; Santa Cruz Biotechnology, Inc., Heidelberg, Germany).22

**Molecular modeling of hVKORC1 and in silico docking of warfarin on hVKORC1 model**

A model of VKORC1 was constructed by comparative methods based on the X-ray crystal structure of the bacterial homolog (Synechococcus sp.) of VKORC1 (PDB ID: 3KP9) (http://www.pdb.org/pdb/home/home.do accessed on 12.09.2012) at 3.6 Å resolution using the YASARA (version 12.9.27, 1993-2011 by Elmar Krieger) homology modeling program.23 Because VKORC1 is a transmembrane protein, membrane-embedded simulations were calculated for 10 nanoseconds in a phosphatidylcholine bilayer using YASARA. Docking of the anionic open-chain warfarin structure on the resulting hVKORC1 structure was performed using the AutoDock (1989-2013 by The Scripps Research Institute) implementation included in YASARA. A rigid, global rolling ball search was performed using stationary VKORC1 and translating/rotating the warfarin structure. The generated interactions from the global search were analyzed using constraints of 2 critical disulfide bonds (Cys43-Cys51 and Cys132-Cys135). The best model was chosen on the basis of pseudo-energy scoring. A detailed description of the modeling and docking methods is presented in the supplemental Information.

**Results**

All hVKORC1 missense variants investigated show in vitro warfarin resistance by the cell culture-based assay

In the present study, we investigated 21 OACR-associated VKORC1 missense mutations according to the cell culture-based assay conditions recently published by our group. Measured protein expression levels for all VKORC1 variants studied here range from one- to fivefold of the wild-type expression level (supplemental Figure 1; supplemental Information).22

We further determined in vitro cell culture-based warfarin IC50 values for the 21 OACR-associated missense hVKORC1 variants. All mutations investigated by our assay exhibit warfarin dose-response data shifted to greater values than those for wild-type hVKORC1 at warfarin concentrations of 0 to 1 μM (Figure 1). These results confirm that the present assay accurately reports data that are in agreement with the clinical human warfarin-resistance phenotypes (Table 1). To summarize the dose-response data, we calculated the IC50 of warfarin for each hVKORC1 variant by standard curve-fitting methods (Table 1). These data allow us to classify the OACR mutations into mild, moderate, severe, or complete in vitro resistance phenotypes (mild: IC50 <100 nM; moderate: IC50 100-200 nM; severe: IC50 200-350 nM; complete: IC50 >350 nM). We further calculated relative increases in measured in vitro warfarin IC50 values by normalizing to the wild-type value of 24.7 nM (Table 1, column 3). All mutations assessed by our assay exhibit warfarin dose-response data shifted to greater values than those for wild-type hVKORC1 at warfarin concentrations of 0 to 1 μM (Figure 1).

**Mutagenesis of Thr138-Tyr139-Ala140 causes complete warfarin resistance in vitro**

To investigate the role of the TYA motif (hVKORC1 residues 138-140) as a putative warfarin binding site in hVKORC1, we mutated the tripeptide to Leu-Ile-Val.5,29-31 In our in vitro assay, this VKORC1 variant exhibits a complete warfarin resistance, suggesting that the TYA motif forms a structural requirement for warfarin inhibition (Table 1, 14.6-fold greater IC50 than wild-type; Figure 1E, residual FIX activity of 50% at 1 μM warfarin).

**The hVKORC1 structural model characteristics**

In accordance with the template structure, our homology-based hVKORC1 model comprises of similar secondary structural elements and overall protein fold.23 Our hVKORC1 model is a 4 transmembrane α-helix (TM1-4) bundle structure (supplemental Table 1) with a long periplasmatic surface loop between TM1 and TM2 (Ala32-Ser79; for detailed validation, see supplemental Information). Like the bacterial homolog, our model consists of a highly conserved serine-rich helical segment (Ser52-Ser57) in hVKORC1 (Figure 2). The threonine, tyrosine, alanine (TYA) motif implicated in warfarin binding in previous studies forms part of the fourth trans-membrane α-helix and is deep within the membrane bilayer. The conserved cysteine pairs, Cys43-Cys51 and Cys132-Cys135, are located in the cytoplasmic loop and the fourth trans-membrane α-helix (Figure 2).

**Docking of warfarin to the homology model identifies putative binding surfaces**

We used the molecular model of hVKORC1 to investigate possible warfarin binding sites by in silico molecular docking. Results from a previous in vitro structure-activity study revealed that the open side-chain conformation of anionic racemic warfarin is the active inhibitory isomer.32 An initial coarse-grained rolling ball docking search using the anionic (deprotonated) open side-chain form of R-warfarin yielded 12 interaction geometries (supplemental Figure 5; supplemental Information). Visual inspection of the docked structures revealed 3 distinct regions of docked warfarin: the cytoplasmic...
aqueous/endoplasmic reticulum (ER) membrane interface (supplemental Figure 5A), the protein/lipid bilayer interface (supplemental Figure 5B), and the ER lumenal loop (supplemental Figure 5C). We chose to further investigate one docking position with the highest ranked pseudo-energy score (supplemental Figure 5; warfarin molecule colored red; Figure 3). This position is proximal to the loop cysteines and the CXXC motif. Based on this position, we could define 3 molecular surfaces on hVKORC1 that represent putative interaction interfaces for warfarin: (1) the ER end of the first transmembrane helix (Leu22-Lys30); (2) a face of the serine-rich helix (Ser52-Phe55); and (3) a segment of the fourth transmembrane helix continuing into the large ER loop (Phe131-Thr137) (Figures 3 and 4; Table 2). The TYA motif is in proximity to the third predicted interface (Phe131-Thr137) and therefore may be critical to the structural integrity of the binding pocket. Interestingly, all known 4-hydroxycoumarin resistant mutations are found clustered around these 3 putative interfaces. Except for Tyr139, all mutations affect residues predicted to be in the polar lipid head-group region of the ER membrane leaflet or on the ER lumen-exposed loop.

**Discussion**

In our present study, we demonstrate for the first time in vitro expression and IC_{50} data for 21 OACR-associated hVKORC1
mutations in addition to the 3 previously reported variants in Fregin et al.\textsuperscript{5,22} that are in excellent agreement with the reported phenotypes for patients harboring the respective mutations. These data allow us to rank the severity of the mutations as moderate to severe or complete warfarin resistance. Furthermore, we simulated warfarin binding on a structural model of hVKORC1 and identified putative warfarin binding interfaces consistent with locations of known OACR-associated VKORC1 mutations.

**Warfarin interaction with hVKORC1**

Evolutionarily, VKORC1 is an ancient enzyme that is also found in bacterial and archaeal genomes. The crystal structure of a bacterial homolog of VKORC1 (Synechococcus sp.) shows that it comprises 5 TMs (PDB ID:3KP9).\textsuperscript{23} The first 4 α-helices form the VKOR protein core domain (Pfrom family VKOR PF07884). The fifth TM is a prokaryotic-specific extension that results in placement of a fused thioredoxin (Trx)-like on the periplasmic side of the membrane and is lacking in mammalian VKOR homologs. Our hVKORC1 homology model is based on the available crystal structure of Synechococcus VKOR on which application of a direct primary sequence alignment provides support for the 4TM topology choice.\textsuperscript{3,34,35} Accordingly, for the 4TM model, the loop between the first and second TM α-helices is predicted to be located in the ER lumen. This topology is also supported by previously published experimental results for electron transfer from the conserved loop cysteines Cys43 and Cys51 to the CXXC motif in the fourth TM α-helix.\textsuperscript{20,23,33,34}

This electron transfer is essential for in vivo VKOR activity. Further experimental support for the 4TM topology model is provided by studies demonstrating that various ER luminal oxidoreductase proteins interact with the loop cysteines of hVKORC1.\textsuperscript{35,36} However, Tie et al.\textsuperscript{37} recently demonstrated in a VKORC1 and VKORCL1 double-knock-out cell line with their cell-based assay that Cys51, as well as in combination with Cys43, is not required for VKOR activity. Only the Cys43 is necessary to maintain VKOR activity. These data are seen as further support for the 3TM topology model in which the loop cysteines are located in the cytoplasm and are not required for electron transfer for active site regeneration after each cycle of oxidation. Thus, the membrane topology of hVKORC1 is still unclear, and currently there is a debate existing whether hVKORC1 comprises 3 or 4 joined transmembrane α-helices.\textsuperscript{15,20,23,35,36,38} A recent review by Van Horn\textsuperscript{39} comprehensively discusses the current experimental evidence for both models.

The clustering of OACR-associated mutations chiefly in the ER luminal loop and the adjacent ends of the transmembrane helices suggests that warfarin may bind in this region of hVKORC1.\textsuperscript{20,23,33} Our in silico warfarin docking results support this hypothesis, because the most favorable docking pseudo-energy scores were associated with this region. We have identified 3 discontinuous putative warfarin binding interfaces that are located in the ER luminal loop and adjacent regions of TM1 and TM4 (Figure 3 and 4; Table 2). Accordingly, all human OACR-associated VKORC1 mutations are located in or near to these interfaces, suggesting they influence the interaction of warfarin with hVKORC1. Warfarin binding might inhibit hVKORC1 activity in 3 possible ways: (1) binding of warfarin may sterically hinder the reduction of the disulphide bridge between Cys43 and Cys51 in the ER luminal loop by physiological redox partners (eg, protein disulfide isomerase or other Trx-domain oxidoreductases); (2) warfarin binding might disturb the electron transfer to the CXXC motif, the catalytically active center of VKORC1; or (3) warfarin might physically block access of K>O to the substrate binding pocket adjacent to the CXXC motif.

**Mutations located at the first putative warfarin binding interface**

The residues Leu22-Lys30 comprise the first putative warfarin binding interface. In total, there are 5 human OACR-associated VKORC1 mutations within this interface (Ala26Thr, Ala26Pro, Leu27Val, His28Gln, and Val29Leu) (Figure 4A; Table 2). The most severe warfarin resistance phenotype among these residues is associated with the Ala26Pro substitution (IC\textsubscript{50} of 1224.0 nM) (Table 1; Figure 1A). The substitution of alanine to a proline residue is predicted to break the natural helical conformation in this region and might induce major conformational changes to the putative warfarin binding site. However, the substitution of alanine to threonine resulted in only a mild resistance phenotype. Unlike alanine, threonine is a hydrogen bond donor and may have an influence on warfarin binding. The 3 other OACR-associated mutations in this region (His28Gln, Leu27Val, and Val29Leu) also result in mild to moderate warfarin resistance (Table 1; Figure 1A). Of these substitutions, the first involves the exchange of a charged for a neutral side chain, although both can form hydrogen bonds. The second and third variants are conservative substitutions involving aliphatic, hydrophobic side chains.

**Mutations at the second putative warfarin binding interface**

The second putative warfarin binding interface is located between Ser52 and Phe55. There are 3 mutations located within this interface (Ser52Leu, Ser52Trp, Val54Leu) and 8 more mutations that are in close proximity (Figure 4A; Table 2). This interface is part of the amphipathic highly conserved, serine-rich helix, which seems to be a common feature of VKOR in different species.\textsuperscript{23} Mutations causing 4-hydroxycoumarin resistance located at this interface generally exhibit a moderate warfarin resistance phenotype. Interestingly, 3 of 4 mutations affect serine residues (Ser52Leu, Ser52Trp, Ser56Phe). These substitutions reflect physicochemically nonconservative changes. Furthermore, these substitutions would disrupt any potential polar interactions with warfarin. Two further mutations are in close proximity to this interface. The Arg58Gly substitution might alter electrostatic potential and surface accessibility affecting warfarin binding. Mutations of Trp59 result in an ~8- to 75-fold increase of in vitro measured IC\textsubscript{50} corresponding to moderate to complete in vivo warfarin resistance depending on the specific

---

<table>
<thead>
<tr>
<th>Table 2. Warfarin binding interfaces on hVKORC1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Warfarin binding interface</strong></td>
</tr>
<tr>
<td>I</td>
</tr>
<tr>
<td>II</td>
</tr>
<tr>
<td>III</td>
</tr>
</tbody>
</table>

Membrane embedded loop segment: Gly62-Ser79 Val66Gly, Val66Met, Gly71Ala, Asn77Ser, Asn77Tyr.
Mutations distant from all 3 putative warfarin binding interfaces

There are 5 mutations associated with in vivo warfarin resistance in human patients that are not in proximity to the putative warfarin binding interfaces (Val66Gly, Val66Met, Gly71Ala, Asn77Ser, and Asn77Tyr) (Figure 4B; Table 2). These mutations are located within a segment of the ER luminal loop between TM1 and TM2 that potentially interacts with the surrounding lipid bilayer. Except for Val66Gly and Asn77Tyr that result in a mild resistance, all other mutations exhibit moderate resistance phenotypes. These mutations reflect semiconservative physiochemical changes with respect to the respective wild-type residues. Therefore, these mutations might induce minor changes in the conformation of the embedded portion of the loop and possibly affect the second putative warfarin binding interface.

In conclusion, our combined in vitro and in silico investigations of 21 OACR-associated hVKORC1 missense mutations reveal their respective mechanisms of action. In contrast to previously published in vitro data, the expression data for our study indicate wild-type or greater than wild-type expression levels for all variants expressed in HEK 293T cells. In vitro measurements of warfarin inhibition of hVKORC1 variants allow us to classify the mutations as mild, moderate, severe, or complete in vitro resistance phenotypes. Additionally, the calculated mutant/wild-type \( IC_{50} \) ratios are good approximations of the increased therapeutic 4-hydroxycoumarin dosage requirements for patients with the respective OACR mutations. The clustering of the OACR-associated mutations in the ER luminal loop as well as in adjacent transmembrane \( \alpha \)-helices suggests that these regions are important for warfarin binding. Accordingly, in silico docking studies revealed 3 putative warfarin binding interfaces for hVKORC1 harboring almost all of the 26 known OACR-associated hVKORC1 missense mutations.

Given the overall success of the current assay in revealing in vitro warfarin resistance phenotypes for the hVKORC1 missense mutations, this assay might represent a helpful tool for guidance of dosages in patients with these and still unknown mutations in hVKORC1. Furthermore, the assay can be used to probe the influence of any intracellular modulator of hVKORC1 enzymatic activity and thus represents an important tool for future investigations of VKORC1 function.

Acknowledgments

This work was supported, in part, by funding from Baxter Germany GmbH (to J.O.) and from the Deutsche Forschungsgemeinschaft grant O1100 5-1 (to M.W. and J.O.).

Authorship

Contribution: K.J.C., P.W., C.R.M., M.W., and J.O. designed the experiments; K.J.C. and A.-C.W. collected the data; K.J.C., A.B., M.W., and J.O. analyzed the data and produced the figures; A.B. was responsible for protein modeling and warfarin docking studies; and K.J.C., C.R.M., M.W., A.B., and J.O. drafted and edited the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Johannes Oldenburg, Institute of Experimental Haematology and Transfusion Medicine, University Clinic Bonn, Sigmund-Freud Str 25, 53105 Bonn, Germany; e-mail: johannes.oldenburg@ukb.uni-bonn.de.
References


31. Ji Y, Dey JK, Takekawa SW. The correlation of vitamin K epoxide to vitamin K quinine and vitamin K quinine to vitamin K hydroquinone uses the same active site cysteines. Biochemistry. 2007;46(24):7279-7283.


Human VKORC1 mutations cause variable degrees of 4-hydroxycoumarin resistance and affect putative warfarin binding interfaces

Katrin J. Czogalla, Arijit Biswas, Ann-Christin Wendeln, Philipp Westhofen, Clemens R. Müller, Matthias Watzka and Johannes Oldenburg

Updated information and services can be found at: http://www.bloodjournal.org/content/122/15/2743.full.html

Articles on similar topics can be found in the following Blood collections
  * Thrombosis and Hemostasis (1083 articles)

Information about reproducing this article in parts or in its entirety may be found online at: http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at: http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at: http://www.bloodjournal.org/site/subscriptions/index.xhtml