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## A novel vitamin K derived anticoagulant tolerant to genetic variations of vitamin K epoxide reductase

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### Abstract

**Background:** Vitamin K antagonists (VKAs), such as warfarin, have remained the cornerstone of oral anticoagulation therapy in the prevention and treatment of thromboembolism for over half a century. They function by impairing the biosynthesis of vitamin K-dependent (VKD) clotting factors through the inhibition of vitamin K epoxide reductase (VKOR). The challenge of VKAs therapy is their narrow therapeutic index and highly variable dosing requirements, which are partially due to the genetic variations of VKOR.

**Objectives:** The goal of this study was to search for an improved VKA that is tolerant to the genetic variations of its target enzyme.

**Methods:** A series of vitamin K derivatives with benzyl and related side-chain substitutions at the 3-position of 1,4-naphthoquinone were synthesized. The role of these compounds in VKD carboxylation was evaluated by mammalian cell-based assays and conventional *in vitro* activity assays.

**Results:** Our results showed that replacing the phetyl side-chain with a methylene cyclooctatetraene (COT) moiety at the 3-position of vitamin K<sub>1</sub> converted it from a substrate to an inhibitor for VKD carboxylation. Strikingly, this COT-vitamin K derivative displayed a similar inhibition potency in warfarin-resistant VKOR mutations whose warfarin resistance varied over

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400-fold. Further characterization of COT-vitamin K for the inhibition of VKD carboxylation suggested that this compound targets multiple enzymes in the vitamin K redox cycle. Importantly, the anticoagulation effect of COT-vitamin K can be rescued with high doses of vitamin K<sub>1</sub>.

**Conclusion:** We discovered a vitamin K analogue that functions as a VKA, and is tolerant to genetic variations in the target enzyme.

### Keywords

Coagulation factors carboxylation; Cyclooctatetraene; Oral anticoagulant; Vitamin K antagonist; Vitamin K epoxide reductase

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## Introduction

Thromboembolic events, resulting from atrial fibrillation (AF) and venous thromboembolism (VTE), are common complications that have high mortality and morbidity[1]. It is estimated that AF currently affects over 33 million individuals worldwide[2]. According to the American Heart Association, VTE (including deep vein thrombosis and pulmonary embolism) is the third leading vascular diagnosis after heart attacks and strokes, affecting between 300,000 to 600,000 Americans each year. Additionally, patients with active cancer are at an increased risk of arterial and venous thromboembolism[3]. Anticoagulant therapy has proven to be an effective strategy for the prevention and treatment of thromboembolism[4]. For over half a century, vitamin K antagonists (VKAs), such as warfarin, have remained the cornerstone of oral anticoagulation therapy[5].

Warfarin, approved by the US Food and Drug Administration (FDA) in 1954, is the most commonly prescribed oral anticoagulant worldwide[6, 7]. In 2017, more than 15 million prescriptions for warfarin were written in the United States alone[8]. Warfarin functions by impairing the biosynthesis of active vitamin K-dependent (VKD) clotting factors through the inhibition of vitamin K epoxide reductase (VKOR), a key enzyme for the redox cycling of vitamin K to support posttranslational carboxylation of clotting factors. Since the discovery of the gene encoding VKOR[9, 10], the mechanism of action and pharmacogenomics of warfarin have been extensively studied. Single-nucleotide polymorphisms in the VKOR gene have been identified as one of the key factors that are strongly associated with large warfarin dose variation phenotypes in humans[11, 12]. A cell-based functional study suggested that warfarin resistance among naturally occurring VKOR mutations varied over 100-fold[13]. Clinical observations indicate, that to achieve the desired anticoagulation efficacy, the interpatient variability of warfarin therapeutic dosages can have a 10- to 20-fold difference[14]. To assist physicians in estimating the appropriate warfarin dosage in anticoagulation therapy, several pharmacogenetic-based dosing algorithms using VKOR genotypes have been proposed[11, 15, 16]. It has been shown that approximately 30% of patients receiving warfarin would benefit from VKOR pharmacogenetics at the beginning of their warfarin therapy[17]. VKOR pharmacogenetics is thought to be so clinically useful that the US FDA revised its warfarin product labeling to reflect the contribution of VKOR genotypes in warfarin dose selection[18].

Despite the effectiveness, widespread use, and genotype-guided dosing, warfarin therapy requires routine monitoring of the International Normalized Ratio (INR) to ensure an optimal warfarin efficacy while limiting the risk of bleeding[6, 19]. This has been the main cost and obstacle in warfarin therapy. Over the past decade, several novel (or non-VKA) oral anticoagulants (NOACs) that directly targeting thrombin (dabigatran) or factor Xa (apixaban, edoxaban, and rivaroxaban) have been advocated as warfarin replacements. These NOACs do not require laboratory monitoring and offer a number of additional attractive advantages over warfarin, including: fixed dosing, predictable pharmacokinetics, and fewer food–drug interactions[20, 21]. Nevertheless, compared to warfarin, these NOACs are expensive, have limited antidotes available[22], have higher medication risks[23, 24], and have poorer patient adherence due to lack of monitoring[25]. Additionally, the NOACs often require an assessment of kidney functions and their efficacies have not been extensively tested in patients with severe renal dysfunction[23, 26]. These situations lead physicians to choose VKAs for anticoagulation control[27].

In addition to warfarin, acenocoumarol and phenprocoumon (4-hydroxycoumarin derivatives like warfarin) and fluindione (a derivative of 1,3-indandione, Figure 1A) are also widely prescribed VKAs for anticoagulation therapy[27, 28]. Warfarin is the most commonly prescribed anticoagulant worldwide, whereas other VKAs are commonly used in many European countries[29]. In general, the use of these VKAs is challenging because of their narrow therapeutic index and high variability in dosage requirements that are effected by many factors, including genetic variations of VKOR. In this study, we explored the possibility of converting vitamin K from a substrate to an inhibitor for VKD carboxylation. Vitamin K is a family of 2-methyl-1,4-naphthoquinone derivatives, which include the naturally occurring phyloquinone (vitamin K<sub>1</sub>) and menaquinones (vitamin K<sub>2</sub>), and the synthetic menadione (vitamin K<sub>3</sub>)[30]. Menaquinones differ from phyloquinone in that the side chain at the 3-position comprises a number of repeating prenyl units rather than the semi-saturated phytyl chain. The menaquinones are named according to the number of prenyl units contained within the side chain (i.e. MK-n). Since the core structure of vitamin K is distinct from that of the current clinically used VKAs, we reasoned that, as an inhibitor, vitamin K analogues should have different inhibition and binding characteristics to the target enzyme. Our results show that substituting the phytyl side-chain of vitamin K<sub>1</sub> with a methylene cyclooctatetraene (COT) moiety resulted in a vitamin K analogue that functions as an inhibitor for VKD carboxylation. Importantly, the inhibition potency of the COT-vitamin K derivative was tolerant to genetic variations of VKOR.

## Materials and Methods

### Reagents and cell lines –

Warfarin, vitamin K<sub>1</sub>, vitamin K<sub>1</sub> epoxide (KO), menadione, menaquinone-4 (MK-4), and MK-4 epoxide were obtained from Sigma-Aldrich (St. Louis, MO). Vitamin K derivatives (novel and known) were synthesized based on previously described methods[31–36]. Synthetic procedures, full characterization and copies of <sup>1</sup>H and <sup>13</sup>C NMR spectra can be found in Supporting Information. Xfect transfection reagent was from Clontech Laboratories, Inc. (Mountain View, CA). Mouse anti-carboxylated factor IX gla domain

(FIXgla) monoclonal antibody was from Green Mountain Antibodies (Burlington, VT). Horseradish peroxidase conjugated sheep anti-human protein C (PC) was from Affinity Biologicals, Inc. (Ancaster, ON, Canada). Cell counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Rockville, MD). ABTS (2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) peroxidase substrate kit for enzyme-linked immunosorbent assay (ELISA) was from KPL Inc. (Gaithersburg, MD).

Human embryonic kidney 293 (HEK293) cells were from ATCC (Manassas, VA). HEK293 cells stably expressing the reporter-protein FIXgla-PC (protein C with its Gla domain exchanged with that of factor IX) (FIXgla-PC/HEK293) were obtained, as previously described[37]. FIXgla-PC/HEK293 cells with their endogenous VKOR and VKOR-like enzyme (VKORL) knocked out (double gene knockout, DGKO) were obtained by TALENs-mediated genome editing[38]. FIXgla-PC/HEK293 cells with their endogenous gamma-glutamyl carboxylase (GGCX) or UbiA prenyltransferase domain-containing protein 1 (UBIAD1) gene knockout were obtained by CRISPR-Cas9-mediated genome editing[39].

#### **DNA Manipulations and plasmids construction –**

The cDNA encoding *Metridia* luciferase (used as an internal control for transfection efficiency) was cloned into one of the multi-cloning sites of the mammalian dual expression vector pBudCE4.1 (Life Technologies Corp., San Diego, CA). The resulting vector pBudCE4.1-*Met*.Luc was used as the cloning and expression vector for expressing VKOR and its variants. Naturally occurring VKOR mutations were created by QuickChange site-directed mutagenesis. The nucleotide sequences of all the constructs were verified by DNA sequencing at Eton Bioscience Inc. (Research Triangle Park, NC).

#### **Evaluation of vitamin K derivatives for VKD carboxylation in HEK293 cells –**

A cell-based activity assay was used to evaluate the vitamin K derivatives as a substrate or inhibitor for VKD carboxylation in FIXgla-PC/HEK293 cells, as previously described[37]. To assess the ability of the vitamin K derivatives as a substrate for VKD carboxylation, FIXgla-PC/HEK293 cells were cultured with a fixed concentration or with increasing concentrations of the vitamin K derivative in cell culture medium. To evaluate the efficiency of vitamin K derivatives as an inhibitor for VKD carboxylation, we cultured FIXgla-PC/HEK293 cells with increasing concentrations of the vitamin K derivative or warfarin (positive control) in cell culture medium containing 5  $\mu$ M KO. After 24 hours incubation, the carboxylation efficiency of the reporter-protein FIXgla-PC was determined directly from the cell culture medium by ELISA[37].

#### **Vitamin K reductase or KO reductase activity assay –**

The effect of vitamin K derivatives as inhibitors of vitamin K reductase or KO reductase activity was evaluated in FIXgla-PC/HEK293 reporter cells with either the endogenous VKOR/VKORL (DGKO) or GGCX knocked out, as previously described[40]. Enzymatic activity was evaluated using ELISA-based reporter-protein carboxylation and conventional HPLC-based vitamin K conversion assays. For the ELISA-based KO reductase activity assay, FIXgla-PC/HEK293 cells were cultured with a fixed concentration or with increasing concentrations of the vitamin K derivative in cell culture medium containing 5  $\mu$ M KO.

After 24 hours incubation, the efficiency of reporter-protein carboxylation was determined in the cell culture medium by ELISA, as previously described[37]. ELISA-based vitamin K reductase activity was performed using DGKO reporter cells with vitamin K<sub>1</sub> as the substrate. For the conventional HPLC-based KO reductase activity assay, GGCX-knockout FIXgla-PC/HEK293 cells were cultured with a fixed concentration or with increasing concentrations of the vitamin K derivative in cell culture medium containing 5 μM KO. K vitamins were extracted from the cells and the conversion of KO to vitamin K<sub>1</sub> was determined by reverse-phase HPLC[40]. Vitamin K reductase activity was evaluated using DGKO reporter cells with vitamin K<sub>1</sub> as the substrate. Since reduced vitamin K<sub>1</sub> (KH<sub>2</sub>), an intermediate product in VKD carboxylation, is unstable and difficult to accurately quantify from cells directly[41], we coupled the vitamin K reduction and epoxidation reactions to quantitate the final stable product, KO. Details of differentially determining the vitamin K cycle enzyme activity were summarized in Supplemental Table 1.

#### **Vitamin K epoxidation activity assay –**

The *in vitro* vitamin K epoxidation activity of GGCX was determined by the quantitation of KO formed during the carboxylation of FLEEL in the presence of FIX's propeptide, as previously described[42]. GGCX enriched microsomes from insect cells (Sf9) were used in this assay. K vitamins were extracted from the reaction mixture and quantitated by an HPLC assay.

#### **Effect of VKOR genetic variations on the inhibition potency of VKAs –**

The effect of VKOR mutations on the inhibition potency of VKAs were performed in DGKO cells, as previously described[38]. Briefly, the plasmid DNA of pBudCE4.1-*Met.Luc* containing the cDNA of wild-type or mutant VKOR was transiently expressed in the DGKO reporter cells. Transfected cells were cultured with complete medium containing 5 μM KO and increasing concentrations of VKAs. Reporter-protein carboxylation was determined by ELISA after 24 hours incubation. The VKAs inhibition potency was evaluated by determining the half-maximal inhibition concentration (IC<sub>50</sub>) of the VKAs using GraphPad Prism 8 software.

#### **UBIAD1-dependent carboxylation activity of vitamin K derivatives –**

To determine the UBIAD1-dependent carboxylation activity of the vitamin K derivatives, UBIAD1-knockout FIXgla-PC/HEK293 cells were cultured with cell culture medium containing increasing concentrations of vitamin K derivatives for 24 hours. The efficiency of reporter-protein carboxylation was determined by ELISA. To confirm that the vitamin K derivatives unable to support VKD carboxylation in the UBIAD1-knockout reporter-cells was due to the absence of the UBIAD1 gene, the UBIAD1 cDNA was re-introduced back into these reporter-cells by transient expression and the reporter-protein carboxylation in the transfected cells was determined. To confirm that the vitamin K analogue supported VKD carboxylation as a result of the conversion of the vitamin K derivatives to MK-4 by UBIAD1, HEK293 or UBIAD1-knockout, HEK293 reporter cells were cultured with the vitamin K derivatives and the production of MK-4 within these cells were determined using an HPLC assay[40].

## Results

### The 3-position side-chain of vitamin K plays an essential role in VKD carboxylation

Current clinically used VKAs are derivatives of 4-hydroxycoumarin and 1,3-indandione[43] (Figure 1A). The inhibition potency of these drugs to their target enzyme VKOR varies 10- to 200-fold depending on the genetic variations of VKOR[13, 44, 45]. To search for improved VKAs with less inhibition variabilities, we synthesized a series of vitamin K analogues (2-methyl-1,4-naphthoquinone derivatives) with different side-chains at the 3-position (Figure 1B). Modification of the 3-position side-chain has been shown to significantly affect vitamin K activity in supporting VKD carboxylation[46], and has successfully converted vitamin K to drugs with varying pharmacological functions[32, 47, 48]. In this study, we explored the possibility of using these types of vitamin K analogues as inhibitors for VKD carboxylation. Since the newly designed vitamin K analogues were different from the 4-hydroxycoumarin and 1,3-indandione in terms of containing a different core structure (Figure 1A), we expected that, as an inhibitor, the vitamin K analogues might also have a different mechanism of action from the current clinically used VKAs.

First, we determined the capability of these compounds as a substrate to support VKD carboxylation in HEK293 cells using our cell-based assay. Results in Figure 1C showed that side-chain substitutions at the 3-position of vitamin K differentially affected VKD carboxylation activity. For example, introduction of a phenyl group to the  $\omega$ -position of the 3-position side-chain (VK-2I-Ben) only had a minor effect on carboxylation activity. However, removal of the isoprenyl unit from VK-2I-Ben (VK-M-Ben) abolished carboxylation activity. On the other hand, introducing a fluorine atom at the 4-position of the phenyl group (VK-M-FB) recovered ~20% carboxylation activity. Strikingly, the methylene COT derived vitamin K (VK-M-COT) derivative displayed activity lower than the background activity (DMSO) (Figure 1C). We have shown previously that this background activity is a result of residual K vitamins in the fetal bovine serum supplemented in the cell culture medium[40]. Therefore, these results indicate that VK-M-COT could be an inhibitor for VKD carboxylation.

To better understand the effect of these vitamin K derivatives on VKD carboxylation, we performed a serial-concentration titration by incubating FIXgla-PC/HEK293 reporter cells with increasing concentrations of different vitamin K analogues in cell culture medium. Results in Figure 1D show that, consistent with the above results, VK-2I-Ben has a similar dose-response curve in the stimulation of VKD carboxylation as vitamin K<sub>1</sub>. However, VK-M-COT displays an inhibitory dose-response curve, suggesting that VKD carboxylation activity decreases when the concentration of VK-M-COT increases (Figure 1D). Together, these results suggest that VK-M-COT functions as an inhibitor for VKD carboxylation.

### The COT-vitamin K analogue targets multiple enzymes in the vitamin K cycle

To further confirm that COT-vitamin K was indeed an inhibitor for VKD carboxylation, we determined the inhibition potency of VK-M-COT on VKD carboxylation in HEK293 cells. We cultured FIXgla-PC/HEK293 cells with 5  $\mu$ M KO and with increasing concentrations of VK-M-COT or warfarin (positive control), and determined the carboxylation efficiency of

reporter-protein FIXgla-PC. Our results showed that VK-M-COT had a dose-response inhibition curve similar to warfarin for FIXgla-PC carboxylation (Figure 2A), although the inhibition potency of VK-M-COT was ~20-fold lower than warfarin (IC<sub>50</sub>, 249 nM vs. 12.5 nM) (Figure 2B). This result supported the above observation that VK-M-COT functions as an inhibitor in VKD carboxylation.

In the vitamin K cycle, when the VKD protein is carboxylated by GGCX, the reduced form of vitamin K (KH<sub>2</sub>) is oxidized to KO, which must be reduced back to vitamin K and then to KH<sub>2</sub> by a two-step reduction process (Figure 2C). To clarify whether VK-M-COT inhibits the reduction of KO to vitamin K, vitamin K to KH<sub>2</sub> or the epoxidation of KH<sub>2</sub> to KO, we used our recently established cell-based activity assays[40] and the conventional *in vitro* activity assay[42] (Supplemental Table 1). First, we examined the inhibition of reporter-protein carboxylation by VK-M-COT in FIXgla-PC/HEK293 cells using KO as the substrate and in DGKO cells using vitamin K<sub>1</sub> as the substrate. To exclude the effect of cytotoxicity in our cell-based assay, we also determined cell viability in the corresponding concentrations of VK-M-COT. Results in Figure 2D show that VK-M-COT inhibited reporter-protein carboxylation when either KO or vitamin K<sub>1</sub> were used as substrate, and these inhibitions were not due to the cytotoxicity of VK-M-COT.

Next, we determined KO and vitamin K reductase activities in HEK293 cells by directly measuring K vitamins within the cells using a conventional reversed-phase HPLC assay[40]. GGCX-knockout or DGKO reporter cells were cultured with VK-M-COT together with KO or vitamin K<sub>1</sub> as described above, and K vitamins were extracted from the treated cells for the HPLC assay. Since reduced vitamin K (KH<sub>2</sub>) is unstable and difficult to accurately quantify from cells directly[41], we coupled the vitamin K reduction and epoxidation reactions to measure the final stable product KO (Figure 2C). Our results show that VK-M-COT significantly decreased both the reduction of KO (Figure 3A) and vitamin K (Figure 3B), which are consistent with the results from our cell-based carboxylation activity assays (Figure 2D).

It should be noted that the above vitamin K reductase activity is a coupled activity of vitamin K reduction and epoxidation. Therefore, the inhibition of either vitamin K reductase, GGCX, or both of the enzymes will affect the coupled enzymatic activity assay. Since the identity of vitamin K reductase is unknown and the product (KH<sub>2</sub>) of the enzyme is unstable[41], we determined the effect of VK-M-COT on GGCX activity using a conventional *in vitro* activity assay[42]. Our result showed that VK-M-COT inhibits GGCX activity for vitamin K epoxidation (Figure 3C). Together, these results suggested that, unlike warfarin that only targets VKOR[37], VK-M-COT targets multiple enzymes in the vitamin K cycle.

### The anticoagulation effect of COT-vitamin K is tolerant to VKOR's genetic variations

Our previous study shows that all current clinically used VKAs are susceptible to genetic variations of VKOR[13]. As VKOR is also the target of VK-M-COT (Figure 2D and 3A) and the core structure of VK-M-COT (2-methyl-1,4-naphthoquinone) is different from that of clinically used VKAs (Figure 1A), we assumed that VK-M-COT could have a different binding site in the target enzyme, and thus, have different sensitivities to genetic variations

of VKOR. To test this hypothesis, we examined the inhibition potency of VK-M-COT among the most warfarin-resistant VKOR mutations (A26P, F55A, W59R, L128R, and Y139F) located at different regions of the VKOR molecule. Residue A26 is located within the first transmembrane domain (TMD1). Residues F55 and W59 are located in the loop region between TMD1 and TMD2, while residues L128 and Y139 are located within the last TMD near VKOR's active site. Consistent with previous studies[13, 49, 50], our results showed that compared to wild-type VKOR, the inhibition curves of these VKOR mutations by warfarin dramatically shifted toward higher warfarin concentrations, suggesting that these mutations are resistant to warfarin inhibition (Figure 4A). The  $IC_{50}$  values of warfarin to these mutations increased 60-fold (A26P) to 417-fold (F55A) (Table I). However, the inhibition potency of VK-M-COT to these warfarin-resistant VKOR mutations was similar to that of wild-type VKOR ( $IC_{50}$  variation is less than 3-fold) (Figure 4B and Table I). These results suggested that the anticoagulation efficiency of VK-M-COT is tolerant to VKOR genetic variations.

### **A ketone group at the 3-position side-chain of the vitamin K analogues recovered their VKD carboxylation activity**

It has been shown that a benzoyl substitution at the 3-position of menadione markedly influenced its redox properties through increased delocalization of  $\pi$ -electrons induced by the ketone group[31]. To explore this effect on VKD carboxylation, we introduced keto functionality into the core of the vitamin K derivatives, which either significantly decreased VKD carboxylation activity (VK-M-Ben and VK-M-FB) or functioned as an inhibitor for VKD carboxylation (VK-M-COT) (Figure 5A). We then examined the ability of these compounds as a substrate to support VKD carboxylation in HEK293 cells. Our results showed that, compared with the parent compounds (Figure 1), introducing a ketone group at the 3-position significantly increased VKD carboxylation activity (Figure 5B and 5C). For example, the ketone group increased the carboxylation activity of VK-M-Ben and VK-M-FB from 3% and 11% (Figure 1C) to 41% and 69% (Figure 5B), respectively. Interestingly, the introduction of a ketone group converted the COT-vitamin K derivative from an inhibitor (VK-M-COT) (Figure 2) to a substrate (VK-K-COT) for VKD carboxylation.

### **VKD carboxylation activity of VK-K-COT is dependent on UBIAD1**

Previous studies showed that in tissues, dietary phylloquinone is converted to menadione by the cleavage of the 3-position side-chain; then, menadione is converted to MK-4 by the prenyltransferase UBIAD1[51–53] (Figure 6A). *In vitro* studies showed that both vitamin  $K_1$  and MK-4, but not menadione, can efficiently support VKD carboxylation[54, 55]. To clarify whether VK-K-COT is directly involved in VKD carboxylation or is converted to MK-4 by UBIAD1 to support VKD carboxylation, we determined the VKD carboxylation activity of VK-K-COT in UBIAD1-knockout FIXgla-PC/HEK293 reporter cells. Results in Figure 6B show that both vitamin  $K_1$  and MK-4 can efficiently support VKD carboxylation in UBIAD1-knockout cells. However, menadione was unable to support reporter-protein carboxylation in these cells. This result suggests that vitamin  $K_1$  and MK-4, but not menadione, can directly function as cofactors for VKD carboxylation, which is consistent with previous *in vitro* and cell-based studies[39, 54, 55]. It is noteworthy that, unlike in HEK293 cells (Figure 5C), VK-K-COT was unable to support VKD carboxylation in



UBIAD1-knockout reporter cells (Figure 6B), suggesting that the VKD carboxylation activity of VK-K-COT is dependent on UBIAD1.

To further confirm the role of UBIAD1 in VK-K-COT supported VKD carboxylation, we introduced UBIAD1 back into the UBIAD1-knockout cells by transient expression. Our results showed that in the presence of UBIAD1, both menadione and VK-K-COT regained VKD carboxylation activity (Figure 6C), supporting the notion that the VKD carboxylation activity of VK-K-COT is dependent upon UBIAD1. We assumed that this UBIAD1-dependent carboxylation activity of VK-K-COT results from the conversion of VK-K-COT to MK-4. To test this hypothesis, we determined the production of MK-4 in wild-type and UBIAD1-knockout FIXgla-PC/HEK293 cells when these cells were cultured with VK-K-COT. Our results showed that, a significant amount of MK-4 and small amounts of MK-4 epoxide were observed in wild-type HEK293 cells where UBIAD1 was present (Figure 6D). Additionally, MK-4 epoxide accumulated in cells when VKOR was inactivated by warfarin. This result is consistent with the observation that KO accumulated in plasma when rats were treated with warfarin or in patients with VKA anticoagulation therapy[56, 57]. However, neither MK-4 nor MK-4 epoxide was observed in UBIAD1-deficient cells under the same conditions (Figure 6E). Together, these results support the hypothesis that VK-K-COT cannot directly function as a cofactor for VKD carboxylation, rather it needs to be converted to MK-4 by UBIAD1 in order to be active in the vitamin K cycle.

It is worth noting that no MK-4 production was observed from VK-M-COT, the counterpart of VK-K-COT which functions as an inhibitor of VKD carboxylation, by the UBIAD1-dependent metabolic pathway (Figure 6F and 6G). This suggests that the ketone group, at the 3-position side-chain, makes the side-chain of the vitamin K analogues more susceptible for cleavage and conversion to MK-4 as a substrate for VKD carboxylation.

### **The anticoagulation effect of VK-K-COT can be reversed by vitamin K<sub>1</sub>**

One of the challenges of anticoagulation therapy is reversing the anticoagulation effect to reduce bleeding risks[58]. Vitamin K<sub>1</sub> administration is an effective strategy for reversing excessive anticoagulation in patients receiving VKA therapy[59–61]. It is believed that the antidotal effect of vitamin K<sub>1</sub> is due to the existence of warfarin-resistant vitamin K reductase that directly reduces vitamin K<sub>1</sub> to KH<sub>2</sub> to support VKD carboxylation in the presence of warfarin[37, 62, 63]. Since VK-M-COT targets multiple enzymes in the VKD carboxylation process, we explored whether the anticoagulation effect of VK-M-COT could be reversed using high concentrations of vitamin K<sub>1</sub>. We cultured FIXgla-PC/HEK293 reporter-cells with a fixed concentration of warfarin or VK-M-COT to inactivate VKD carboxylation, and then using increasing concentrations of vitamin K<sub>1</sub> to reverse their anticoagulation effect. Results in Figure 7 show that the inhibition of VKD carboxylation by both warfarin and VK-M-COT can be reversed equally with higher concentrations of vitamin K<sub>1</sub>.

## **Discussion**

Vitamin K antagonists have been the mainstay of anticoagulation therapy for the past 60 years. Since the discovery of the first anticoagulant, dicoumarol, from spoiled sweet

clover[64], numerous coumarin derivatives have been chemically synthesized or isolated from natural sources for anticoagulation tests[27, 65, 66]. Currently, three coumarin derivatives (warfarin, acenocoumarol, and phenprocoumon) have been clinically used for anticoagulation therapy. These compounds have different half-lives, with long-acting coumarins appearing to provide a more stable anticoagulation effect[5]. Nevertheless, coumarin-derived VKAs have a narrow therapeutic range and require regular coagulation monitoring and dose adjustments to keep the anticoagulation intensity within the therapeutic range. This is partially due to the genetic variations of their target enzyme VKOR.

One of our long-term goals has been to search for an improved VKA that is not susceptible to the genetic variations of VKOR with the hope that a fixed dose of anticoagulant would achieve the desired anticoagulation effect, despite the genetic backgrounds of patients. Recently, we have shown that replacing the phenyl group of warfarin with COT significantly decreased the variation of warfarin resistance among naturally occurring VKOR mutations[34]. However, the resistance of VKOR mutations to COT-warfarin still had an over 25-fold difference. Additionally, COT-warfarin and warfarin displayed a similar pattern of resistance to all naturally occurring VKOR mutations. This could be due to the fact that their core structure, 4-hydroxycoumarin (Figure 1A), binds to the same site in VKOR. Therefore, it seemed logical that compounds with different core structures (i.e. other than coumarin) may be associated with a different binding site in the target enzyme, and thus have a different mechanism of action.

As a continuation to our previous work, we explored the possibility of derivatizing vitamin K as an inhibitor for VKD carboxylation. Vitamin K comprises a group of structurally similar compounds that share a 2-methyl-1,4-naphthoquinone core structure, and are distinctly different from the coumarins (Figure 1A), but all differ in the side-chain at the 3-position[30, 46]. Lowenthal *et al* have shown that replacing the 2-methyl group of phylloquinone with a chlorine atom resulted in an analogue (Chloro-K) that was a potent antagonist of vitamin K[67]. Because of its distinct structure and alternative mechanism of action from the coumarin anticoagulants[68], Chloro-K appears to be an effective anticoagulant in warfarin-resistant rats[69].

In this study, we focused on derivatization of the 3-position side-chain of vitamin K. Our results showed that substitution of the 3-position side-chain significantly affected its activity as a substrate for VKD carboxylation (Figure 1C). Interestingly, replacement of the phytyl side-chain with methylene COT converted the vitamin K core from a substrate to an inhibitor for VKD carboxylation (Figure 2). Characterization of methylene COT-vitamin K with the most warfarin-resistant VKOR mutations showed that methylene COT-vitamin K displayed a similar inhibition potency to wild-type VKOR and all the warfarin-resistant VKOR mutations, although these mutations affected the inhibition potency of warfarin for VKD carboxylation up to 400-fold (Table 1). These results suggested that, different from warfarin, a fixed dose of methylene COT-vitamin K should achieve a similar anticoagulation effect, despite genetic variations of VKOR.

Bleeding complications are the most concerning adverse event with the use of any anticoagulant. Therefore, a specific reversal agent is desired when anticoagulant therapy

needs to be reversed or neutralized[70]. Unlike warfarin, methylene COT-vitamin K appears to target multiple enzymes in the VKD carboxylation pathway (Figure 2D and Figure 3). We reasoned that a COT-vitamin K analogue that possesses vitamin K activity could be an ideal specific reversal agent for methylene COT-vitamin K. As mentioned, a previous study suggested that a ketone group at 3-position can alternate redox properties of the vitamin K core[31]. Therefore, we introduced a ketone group to the vitamin K derivatives used in this study. Our results showed that the ketone addition converted the COT-vitamin K derivative from an inhibitor (VK-M-COT) to a substrate (VK-K-COT) for VKD carboxylation (Figure 2 and 5). Further characterization of VK-K-COT supported VKD carboxylation, suggesting that VK-K-COT is not directly involved in vitamin K redox cycling, rather, its VKD carboxylation activity resulted from the conversion of VK-K-COT to MK-4 mediated by UBIAD1 (Figure 6). Nevertheless, our results showed that vitamin K<sub>1</sub> can efficiently reverse the anticoagulation effect of COT-vitamin K as in warfarin (Figure 7).

It should be noted that, as an analogue of vitamin K, VK-M-COT appears to target multiple enzymes in the vitamin K cycle (Figure 3). Therefore, genetic variations of other enzymes in the cycle, like GGCX, may also affect the anticoagulation efficacy of VK-M-COT. Future studies will explore how naturally occurring GGCX mutations affect the inhibition potency of VK-M-COT for VKD carboxylation. GGCX mutations mainly cause the combined vitamin K-dependent clotting factors deficiency (VKCFD)[71], and this symptom can be ameliorated by the administration of vitamin K<sub>1</sub>. As GGCX mutations do not appear to affect the efficiency of vitamin K therapy, we do not expect GGCX mutations to have a significant effect on the inhibition potency of the vitamin K analogue, VK-M-COT, on VKD carboxylation. Additionally, since the inhibition potency of VK-M-COT to VKD carboxylation was ~20-fold less than that of warfarin (Figure 2A and 2B), the other objective is to improve the binding affinity of VK-M-COT to the target enzymes by undertaking a structure activity relationship study, without compromising the resistance tolerance to genetic variations in vitamin K cycle enzymes.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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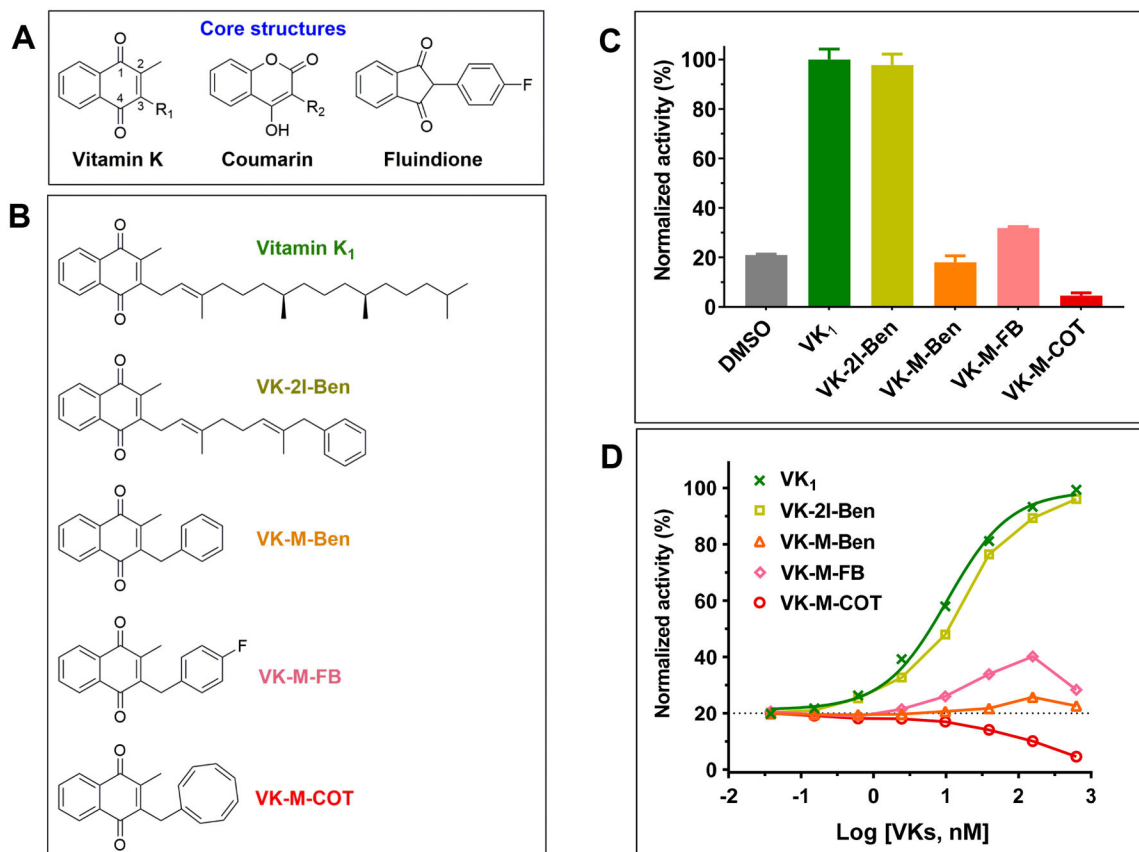
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### Essentials

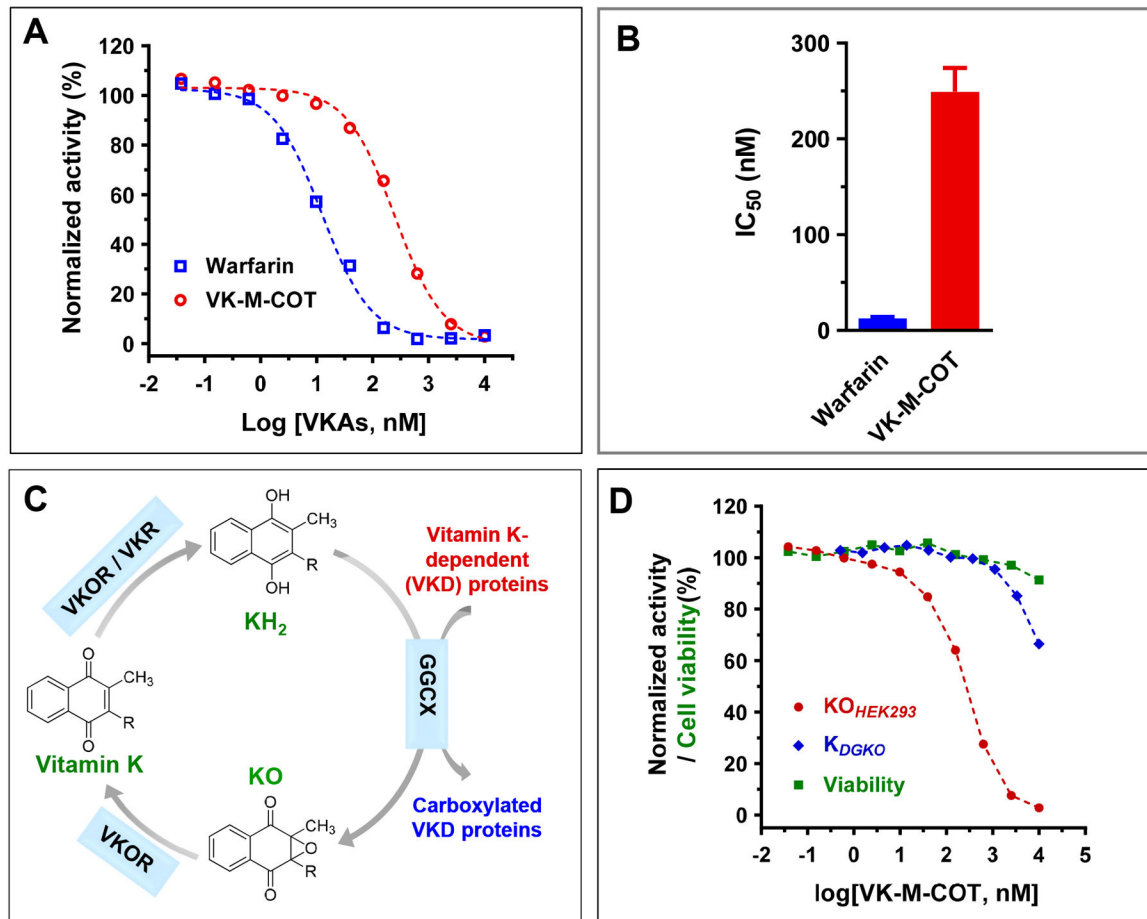
- Warfarin therapy has been the mainstay of treatment for thromboembolism
- The efficacy of warfarin therapy relies on the genetic variation of its target VKOR
- A cyclooctatetraene (COT) derived vitamin K analogue can function as an anticoagulant
- The anticoagulation efficacy of COT-vitamin K is independent on VKOR's genetic variation





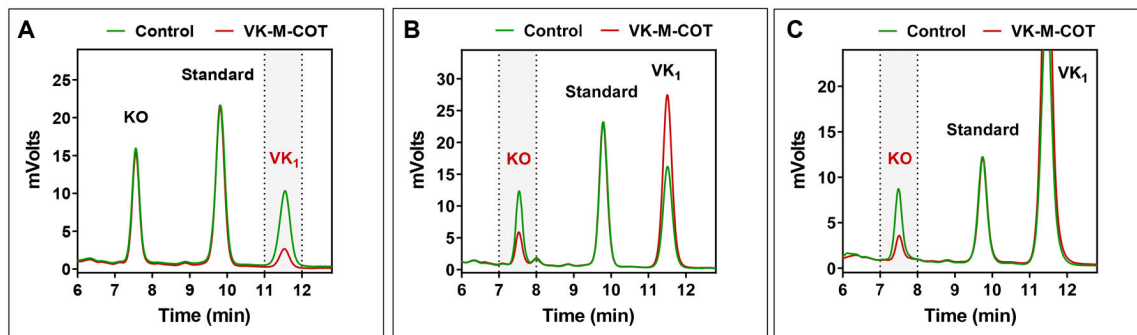
**Figure 1. Evaluation of vitamin K derivatives for supporting VKD carboxylation in HEK293 cells**

(A) Core structures of vitamin K and clinically used vitamin K antagonists. (B) Chemical structures of vitamin K derivatives used in this study. (C) Cell-based VKD carboxylation activity of different vitamin K derivatives in FIXgla-PC/HEK293 reporter cells. The reporter cells were cultured in cell culture medium containing 1  $\mu$ M vitamin K derivative as the substrate for 24 hours. Carboxylation activity was evaluated by determining the carboxylation efficiency of the reporter-protein FIXgla-PC using ELISA. The carboxylation activity of vitamin K<sub>1</sub> (VK<sub>1</sub>) as the substrate was normalized to 100%. (D) Concentration titration of vitamin K derivatives in FIXgla-PC/HEK293 reporter cells. The reporter cells were cultured with increasing concentrations of vitamin K derivatives for 24 hours. Reporter-protein FIXgla-PC carboxylation was determined and normalized as described in the legend of Figure 1C.



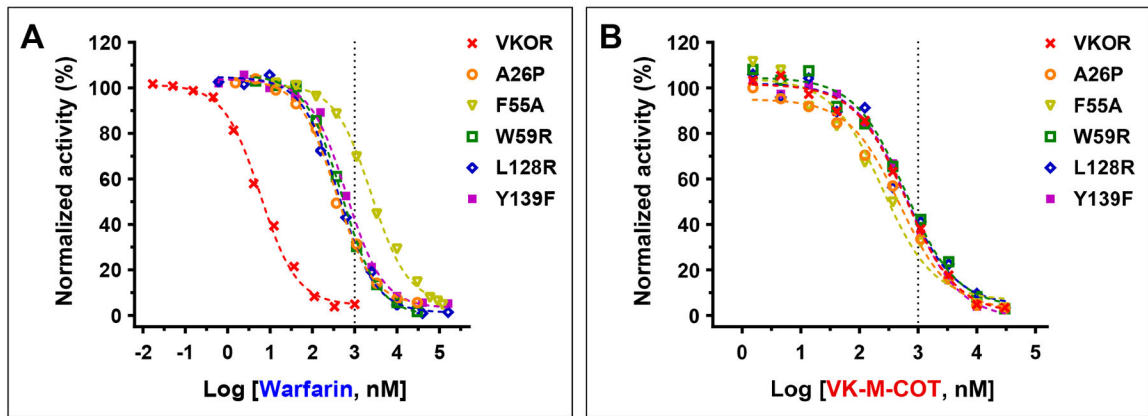
**Figure 2. Characterization of VK-M-COT as an inhibitor for VKD carboxylation in HEK293 reporter cells**

(A) Inhibition of VKD carboxylation by VK-M-COT (○) and warfarin (□) in HEK293 cells. FIXgla-PC/HEK293 cells were cultured with increasing concentrations of VK-M-COT or warfarin in cell culture medium containing 5 μM KO for the cell-based activity assay, as described in the legend of Figure 1C. (B) Inhibition potency of warfarin and VK-M-COT for VKD carboxylation. Half-maximal inhibition concentrations (IC<sub>50</sub>) of VK-M-COT and warfarin on VKD carboxylation were determined from Figure 2A using GraphPad Prism 8. (C) The vitamin K redox cycle. When VKD proteins are carboxylated by GGCX, the cofactor reduced vitamin K (KH<sub>2</sub>) is oxidized to vitamin K epoxide (KO), which needs to be reduced back to KH<sub>2</sub> by a two-step reduction of KO to vitamin K and vitamin K to KH<sub>2</sub>. (D) Inhibition of VKD carboxylation by VK-M-COT in HEK293 and DGKO reporter cells. FIXgla-PC/HEK293 or DGKO reporter cells were cultured with increasing concentrations of VK-M-COT in cell culture medium containing 5 μM KO (• KO<sub>HEK293</sub>) or vitamin K<sub>1</sub> (♦ K<sub>DGKO</sub>), respectively, for the cell-based activity assay and cell viability assays (■ viability).

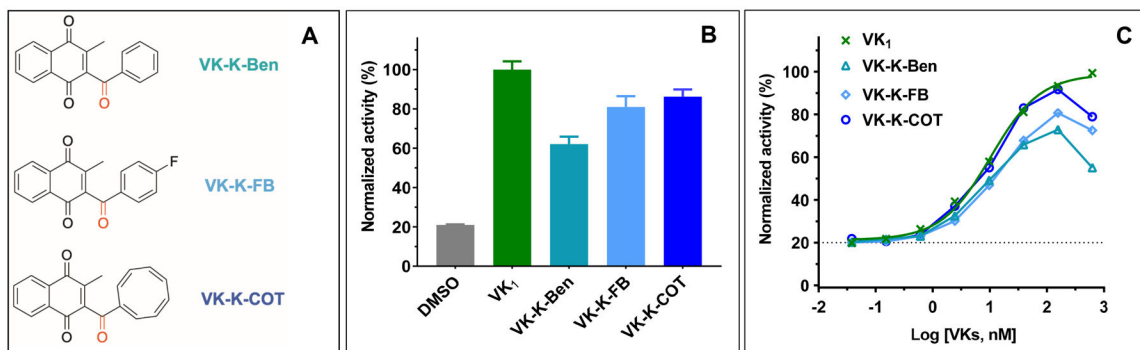


**Figure 3. Characterization of VK-M-COT as an inhibitor in the vitamin K redox cycle**

(A) Inhibition of KO to vitamin K<sub>1</sub> reduction by VK-M-COT in HEK293 cells. GGCX-knockout HEK293 cells were cultured with cell culture medium containing 10  $\mu$ M KO in the absence or presence of 10  $\mu$ M VK-M-COT for 6 hours. DMSO (solvent used for dissolving VK-M-COT) treated cells were used as the control. K vitamins were extracted from the treated cells for the conventional HPLC assay. KO reduction activity is evaluated by the production of vitamin K<sub>1</sub> (VK<sub>1</sub>) (labeled in red and marked with vertical dash lines) in the chromatogram. Standard: vitamin E acetate. (B) Inhibition of vitamin K reduction by VK-M-COT in HEK293 cells. DGKO cells were cultured with 10  $\mu$ M vitamin K<sub>1</sub> in the absence or presence of 10  $\mu$ M VK-M-COT for 6 hours. DMSO treated cells were used as the control. K vitamins were extracted from the treated cells for the conventional HPLC assay. Vitamin K reduction activity was evaluated by the production of KO (labeled in red and marked with vertical dash lines) in the chromatogram. (C) Inhibition of vitamin K epoxidation by VK-M-COT. GGCX enriched microsomes were cultured with FLEEL, FIX-propeptide, and fresh prepared KH<sub>2</sub> in the absence or presence of 100  $\mu$ M VK-M-COT. K vitamins were extracted from the reaction mixture for the conventional HPLC assay. Vitamin K epoxidation activity was evaluated by the production of KO (labeled in red and marked with vertical dash lines) in the chromatogram.

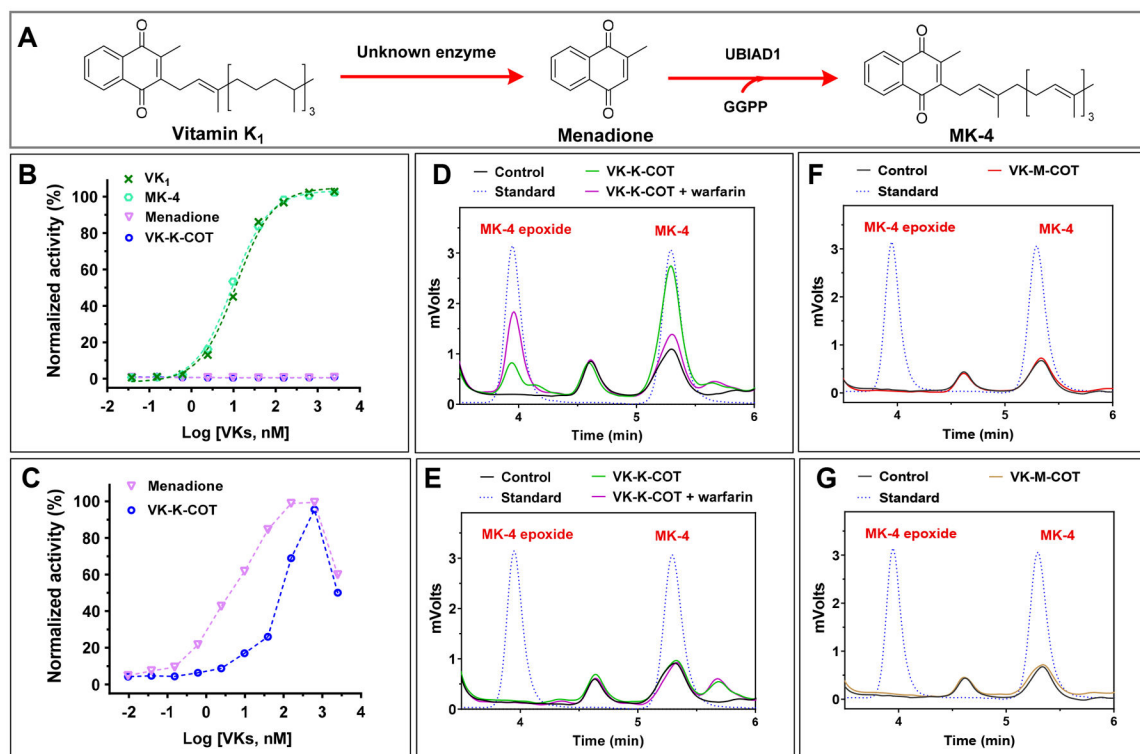


**Figure 4. Effect of VKOR mutations on the inhibition potency of warfarin and VK-M-COT**  
 VKOR mutations were transiently expressed in DGKO reporter cells. Transfected cells were cultured with 5  $\mu$ M KO with increasing concentrations of warfarin (**A**) or VK-M-COT (**B**) for 24 hours. The efficiency of reporter-protein FIXgla-PC carboxylation was determined as described in the legend of Figure 1C.



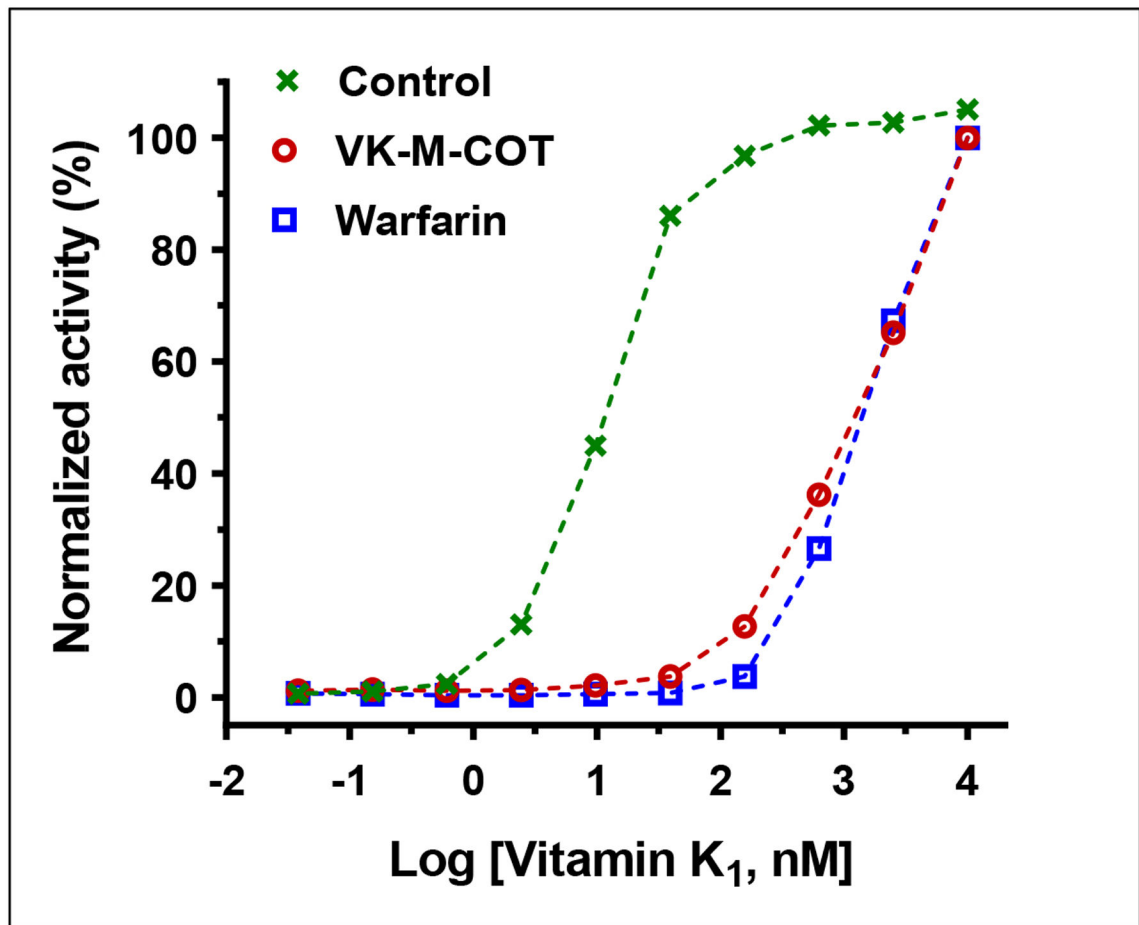
**Figure 5. Effect of a ketone group at the 3-position side-chain of the vitamin K derivatives on VKD carboxylation**

**(A)** Chemical structures of the ketone derived vitamin K analogues. **(B)** Cell-based VKD carboxylation activity of the ketone derived vitamin K analogues in FIXgla-PC/HEK293 reporter cells. Carboxylation activity was determined as described in the legend of Figure 1C. **(C)** Concentration titration of the ketone derived vitamin K analogues in FIXgla-PC/HEK293 reporter cells as described in the legend of Figure 1D.



**Figure 6. Characterization of UBIAD1-dependent VKD carboxylation of COT derived vitamin K analogue in HEK293 cells**

(A) Schematic diagram of the conversion of vitamin K<sub>1</sub> to MK-4 mediated by UBIAD1. (B) VKD carboxylation in UBIAD1-knockout HEK293 reporter cells using vitamin K<sub>1</sub> (VK), MK-4, menadione, or VK-K-COT as the substrate. UBIAD1-knockout reporter cells were cultured with increasing concentrations of the vitamin K analogues, and the efficiency of the reporter-protein FIXgla-PC carboxylation was determined as described in the legend of Figure 1D (C) Contribution of UBIAD1 to VKD carboxylation using menadione and VK-K-COT as the substrate. UBIAD1 was transiently expressed in UBIAD1-knockout reporter cells and the transfected cells were cultured with increasing concentrations of menadione or VK-K-COT. Carboxylation efficiency of reporter-protein FIXgla-PC was determined as described in the legend of Figure 1C. (D) (E) Conversion of VK-K-COT to MK-4 in HEK293 (D) and UBIAD1-deficient cells (E). FIXgla-PC/HEK293 cells (D) or UBIAD1-deficient HEK293 reporter cells (E) were cultured with 10 μM VK-K-COT in the absence or presence of 5 μM warfarin for 6 hours. DMSO treated cells were used as the control. K vitamins were extracted from the treated cells for the conventional HPLC assay. Pure MK-4 and MK-4 epoxide were used as standards to define the retention of these compounds in the chromatogram. (F) (G) Determination of the conversion of VK-M-COT to MK-4 in HEK293 (F) and UBIAD1-deficient cells (G) as described in the legend of Figure 6 D and E.



**Figure 7. Reversal of the anticoagulation effect of VK-M-COT and warfarin by vitamin K<sub>1</sub>**  
FIXgla-PC/HEK293 reporter cells were cultured in cell culture medium containing 3  $\mu$ M VK-M-COT, 3  $\mu$ M warfarin or DMSO (control) and increasing concentrations of vitamin K<sub>1</sub> for 24 hours. The carboxylation efficiency of the reporter-protein FIXgla-PC was determined as described in the legend of Figure 1D.

**Table I**

Inhibition potency of warfarin and VK-M-COT for VKOR mutations

VKOR	IC <sub>50</sub> ± standard deviation (nM)	
	VK-M-COT	Warfarin
Wild type	616 ± 74	5.9 ± 0.6
A26P	487 ± 75	360 ± 33
F55A	249 ± 66	2460 ± 235
W59R	615 ± 101	498 ± 37
L128R	575 ± 132	416 ± 49
Y139F	658 ± 54	633 ± 67

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