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New adjuvants to enhance anticoagulant activity of Warfarin

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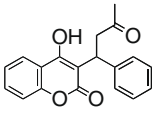
ABSTRACT

New adjuvants of warfarin anticoagulant activity have been developed. These compounds, which are 1,4-methano-1,2,3,4-tetrahydroanthracene-9,10-diol derivatives, act synergistically with warfarin to potentiate its anticoagulant effect. None of the compounds tested is an effective oral anticoagulant in the absence of warfarin.

Warfarin (Coumadin® **1**) has been in extensive clinical use for almost six decades now, having been approved for use in humans on June 8, 1954,[1] and it remains the only effective FDA-approved oral anticoagulant currently available for long-term prevention and treatment of thrombo-embolic problems. Its unique status among oral anticoagulants for long-term use leads to warfarin often being referred to as the 'gold standard' for oral anticoagulants.



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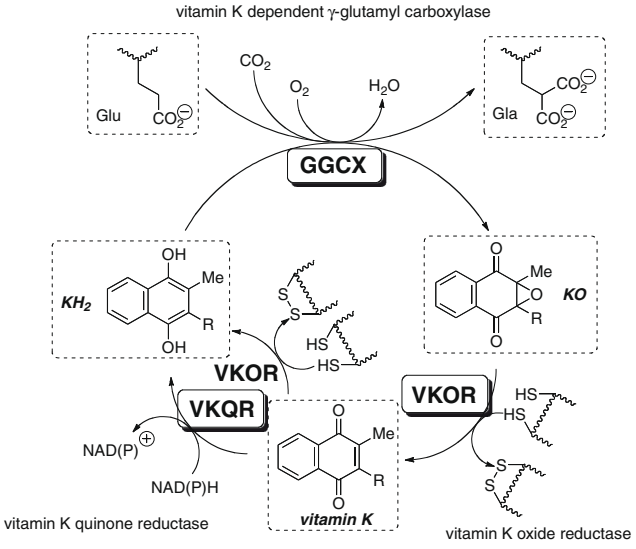
The blood clotting process involves vitamin K-mediated γ-carboxylation of glutamyl residues in precursor molecules for active clotting factors II, VII, IX, X, and naturally occurring anticoagulant proteins C, S and Z. This carboxylation requires the coordinated action of two primary enzymes: the vitamin K dependent γ-glutamyl carboxylase (GGCX), and the vitamin K 2,3-epoxide reductase (VKOR).

The function of vitamin K is generally discussed in terms of the vitamin K cycle[2] shown schematically in Scheme 1. The process involves GGCX, which couples the oxidation of the hydroquinone (reduced) form of vitamin K (KH_2) by molecular oxygen to the γ-carboxylation of the side chain of glutamate (Glu) residues by carbon dioxide. In the process, vitamin K is oxidized to the 2,3-epoxy-1,4-naphthoquinone (vitamin K epoxide, *KO*), and the Glu residues are converted to γ-carboxyglutamates (Gla). The cycle is then closed by reduction of *KO* to KH_2, a transformation carried out by VKOR, specifically subunit 1 (VKORC1), which regenerates the KH_2 in a two-step reduction using exogenous sulfhydryl cofactors.[3]



Scheme 1. The vitamin K cycle.

Much anticoagulant research has been focused on the interaction of warfarin with VKORC1, and some idea of the importance

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of this enzyme may be gauged from the fact that no fewer than four chapters in a recent volume of *Vitamins and Hormones* are dedicated to VKORC1.[4]

VKORC1 is a 163-residue protein with a mass of 18 kDa, which was discovered in 1970,[5] and whose gene was discovered in 2004.[6] This protein is the only one discovered to date that is capable of reducing vitamin K 2,3-epoxide to vitamin K. VKORC1 contains a CXXC sequence (CIVC, residues 132–135 on the third trans-membrane helix[7]) that is conserved across all species studied,[4a,8] and this appears to be the critical reducing center of the enzyme.

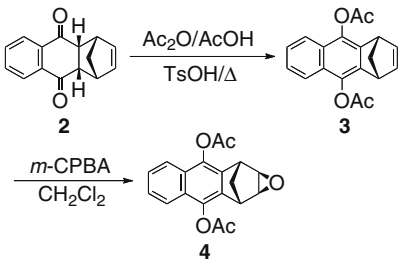
We have discovered a new class of compounds that exhibit synergistic enhancement of warfarin activity. They enhance the anticoagulant activity of the coumarin up to fourfold when co-administered orally, but exhibit no significant anticoagulant activity when administered alone. The compounds are *exo*-2,3-epoxy-1,2,3,4-tetrahydro-1,4-methano-9,10-anthracene-diol esters, and they appear to react with oxygen after hydrolysis in a manner similar to vitamin K to generate an analogue of vitamin K epoxide, which then acts as the potentiator of the anticoagulant activity of warfarin in rats.

The title compounds are prepared from the Diels–Alder adduct of 1,4-naphthoquinone and cyclopentadiene (**2**) by aromatization of the dione ring by boiling with acetic anhydride and acetic acid in the presence of *p*-toluenesulfonic acid as the catalyst to give the diacetate **3**. This diacetate was oxidized with *m*-chloroperoxy-benzoic acid in dichloromethane to give the corresponding *exo* epoxide, **4**.[9] These reactions are summarized in Scheme 2.[10]

Male, albino, Sprague–Dawley rats weighing 150–175 g were fed a rat chow composed of rolled oats, sugar and peanut oil (92:4:4) (w:w:w) plus a standard vitamin/mineral mix, and were provided with water ad libitum. The administration of anticoagulant was accomplished by uniformly dispersing the compound in the peanut oil used in the chow. The diets for co-administration were prepared by adding the adjuvant to previously prepared diet containing warfarin. The anticoagulant and adjuvant were administered daily throughout the course of the experiment. This diet has been used for feeding warfarin and other coumarin type compounds in the past, and we found that there was indeed excellent diet consumption with this formula for all compounds evaluated in these experiments.[11]

At the appropriate time (4 days or 10 days), the rats were anesthetized and sacrificed by cardiac puncture. Selected data from the feeding studies relevant to blood clotting are the prothrombin time (PT, in seconds, a measure of the extrinsic coagulation pathway), Factor VII content, and Factor X content in the blood (values expressed relative to the human scale in%). These are gathered in Table 1 (4-day study) and Table 2 (10-day), and graphically in Figure 1 (4-day) and Figure 2 (10-day); SEM refers to the standard error of the mean.

It is clear that at 4 days of administration, the first six sets of results do not differ significantly from control, which is evidence that neither compound **3** nor compound **4** (nor any of their metabolites) affects PT or the levels of the clotting factors VII and X; thus, used alone neither of these compounds effectively inhibits VKORC1. This plot also nicely illustrates how compound **3** marginally potentiates the activity of warfarin at 4 days of co-administration, while, in dramatic contrast to this, compound **4** almost completely reverses the effects of warfarin at 4 days of co-administration: the prothrombin time falls, and the levels of both Factor VII and Factor X are maintained, although the level of Factor VII is less that the level of control.



Scheme 2. Synthesis of the warfarin adjuvants.

Table 1
Prothrombin coagulation time (PT); Factor VII and Factor X levels at 4 days

Compds	Dosage (mg/kg)	PT (SEM) (s)	VII% (SEM)	X% (SEM)
Control	0	12.8 (0.5)	400.0 (33.2)	60.6 (3.0)
3	0.025	11.2 (0.2)	346.8 (33.6)	74.5 (8.0)
3	0.25	11.6 (0.06)	351.3 (11.2)	65.3 (2.6)
3	2.5	11.8 (0.3)	370.8 (7.4)	64.3 (2.4)
4	0.025	11.4 (0.2)	366.3 (7.8)	73.3 (4.0)
4	0.25	11.6 (0.09)	334.3 (26.3)	61.0 (5.6)
4	2.5	12.7 (0.7)	395.5 (18.0)	61.0 (3.7)
1	0.25	37.0 (5.9)	29.5 (4.1)	10.0 (0.0)
1 + **3**	0.25 + 2.5	40.5 (6.0)	20.3 (3.6)	4.8 (1.5)
1 + **4**	0.25 + 2.5	12.0 (0.2)	264.0 (27.5)	58.8 (3.6)

Table 2
Prothrombin coagulation time (PT); Factor VII and Factor X levels at 10 days

Compds	Dosage (mg/kg)	PT (SEM) (sec)	VII% (SEM)	X% (SEM)
Control		12.9 (0.7)	321.3 (14.7)	58.1 (1.8)
3	0.025	11.4 (0.06)	364.0 (21.0)	68.0 (0.6)
3	0.25	11.7 (0.08)	304.7 (15.4)	61.2 (2.1)
3	2.5	11.6 (0.08)	286.8 (10.0)	61.0 (1.8)
4	0.025	11.6 (0.06)	350.3 (11.3)	64.2 (1.5)
4	0.25	11.6 (0.09)	295.2 (8.7)	59.8 (2.7)
4	2.5	11.6 (0.08)	285.8 (10.0)	61.0 (1.8)
1	0.25	24.4 (3.6)	56.2 (5.6)	14.9 (1.8)
1 + **3**	0.25 + 2.5	35.4 (4.3)	35.0 (5.0)	7.7 (1.2)
1 + **4**	0.25 + 2.5	108.4 (38.4)	18.4 (8.6)	3.6 (1.2)

In similar fashion, the first six sets of results at 10 days do not differ significantly from control, which reinforces the conclusion that neither compound **3** nor compound **4** (nor any metabolite of either compound) is *per se* an inhibitor of VKORC1. This plot does, however, show how compound **3**, and, *much more effectively*, compound **4** both significantly enhance the anticoagulant effect of warfarin at 10 days.

ANOVA results for these observations show that the administration of **3** and **4** alone give results for PT, VII% and X% that are not significantly different from the control (p >0.1) at either 4 or 10 days of administration. The effects of added **3** on warfarin anticoagulation are not significant at 4 or 10 days of co-administration, although some marginal effects are noted at 10 days on the levels of factor VII (p = 0.025) and factor X (p = 0.014). Compound **4**, when co-administered with warfarin, has a significant effect on all measured anticoagulation markers compared to the administration of warfarin alone, as summarized in Table 3.

Clearly, the most striking of our results is the behavior of compound **4** over the time period of the experiment. After 4 days of co-administration, it is clearly a warfarin antagonist: with the exception of the Factor VII levels, which do not quite rise again to the levels of control, the results are not significantly different from control. At 10 days of co-administration, on the other hand, compound **4** acts as a warfarin *agonist*: PT is increased by almost an order of magnitude compared to control, and by a factor of over four relative to warfarin alone; the levels of Factor VII are reduced by a factor of close to 20 compared to control, and by a factor of three relative to warfarin alone; the levels of Factor X are reduced by a factor of close to 20 compared to control, and by a factor of

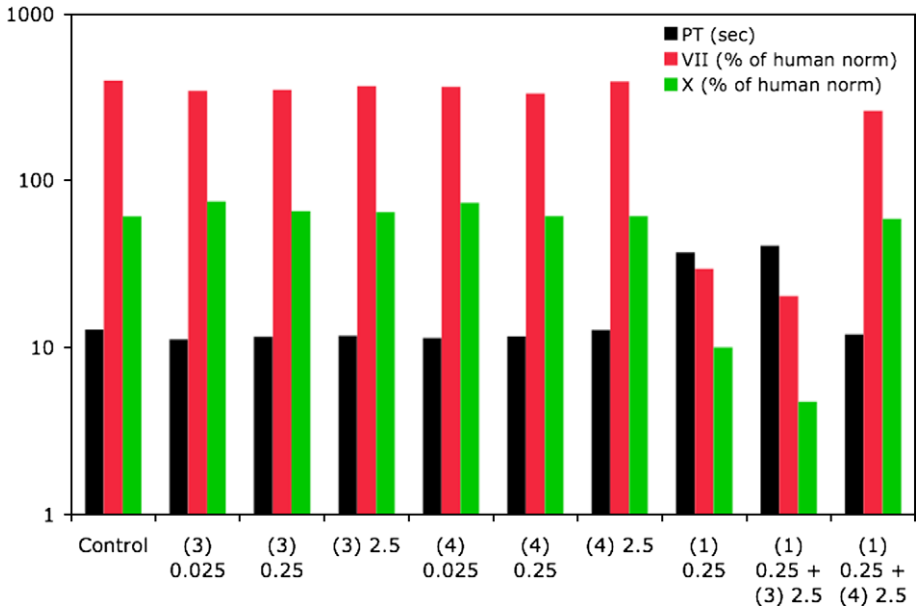


Figure 1. Results of administration of compounds **3** and **4** after 4 days.

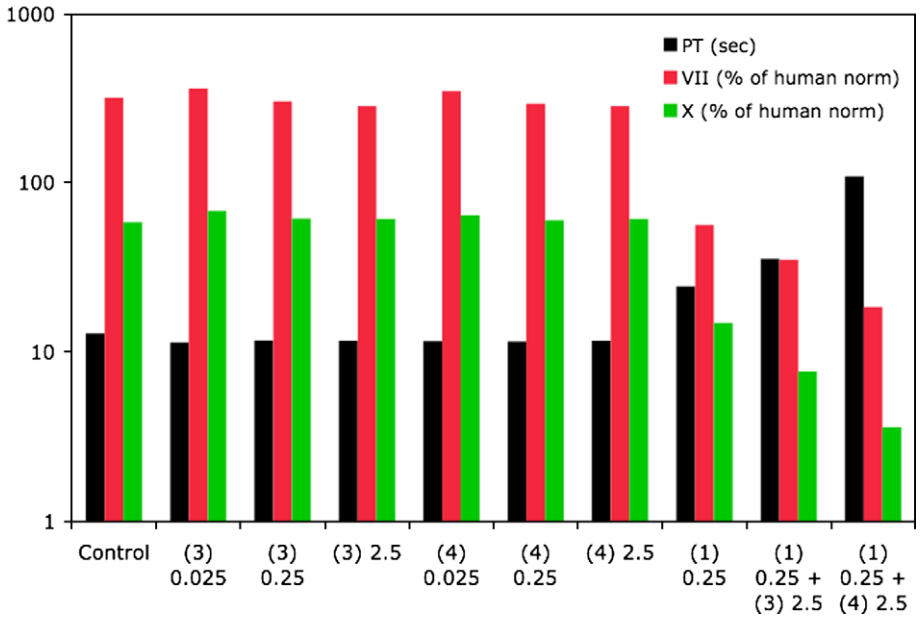


Figure 2. Results of administration of compounds **3** and **4** after 10 days.

Table 3
ANOVA *p* values for anticoagulation by co-administration of **1** and **4** compared to the administration of **1** alone

	4 days ($n = 4$)	10 days ($n = 6$)
PT	0.01	0.005
VII	2.7×10^{-7}	$<10^{-3}$
X	1.03×10^{-9}	0.0013

four relative to warfarin alone. After 10 days of co-administration, compound **4** effectively quadruples the anticoagulant activity of warfarin.

It is also important to note that during this study, the levels of alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatinine were never significantly different from the control. These data suggest that neither of the compounds exhibited hepatotoxicity or nephrotoxicity, whether administered alone to the rats or by co-administration with warfarin. This is consistent with the view that these compounds may well provide a useful new paradigm for oral anticoagulant development.

The results obtained using compound **4**, especially, were simultaneously striking and puzzling. Four days after the start of co-administration of **4** and warfarin, there is a marked *decrease* in the effectiveness of warfarin as an anticoagulant, while after 10 days of co-administration with warfarin, compound **4** obviously strongly *potentiates* the anticoagulant effects of warfarin. To our knowledge, this complete reversal of anticoagulant activity of a compound over time is unprecedented.

The observed results can be interpreted in terms of the binding of the two substrates to VKORC1. It is now accepted that warfarin binds to the oxidized (disulfide, inactive) form of VKORC1 in the vitamin K epoxide binding site.[12] We suggest that after 10 days the diester **3** has been largely converted to the epoxide **6**, which ought to be a structural analogue of vitamin K 2,3-epoxide. The oxidation, which may or may not be mediated by GGCX (the slowness of the oxidation likely argues against the involvement of the enzyme), is summarized in Scheme 3.

While one can make similar arguments for the action of compound **4** in the feeding test, it is clear that the presence of the epoxide ring has a profound effect on its activity compared to the analogues lacking this ring, and we do not believe that any of the arguments above would lead one to predict an order of magnitude difference in their activities due to this one simple change in the structure of the compound.

The problem posed by compound **4** is to simultaneously rationalize its suppression of warfarin activity after 4 days of co-administration, and its enhancement of warfarin activity after 10 days of co-administration. We suggest that the effects of compound **4** (or its hydrolysis product, **7**) on the anticoagulant activity of warfarin after 4 days may reflect the *removal* of warfarin by ether formation to give a conjugate such as **8**, which can subsequently be oxidized to **9** (Scheme 4).

We propose that the reaction between the epoxide **7** and warfarin may occur in the feed itself prior to consumption by the rat, or in plasma after consumption of the feed, and that it does not require the action of an enzyme. The reaction is expected to occur in the normal manner, with attack of the warfarin from the *endo* direction to give *trans* ring opening of the epoxide, yielding the ether **8**. Possible model reactions for the formation of ethers of warfarin under a variety of conditions are being carried out in our laboratories at this time.

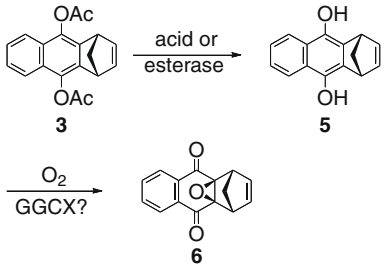
The conjugate **8**, we believe, does not exhibit warfarin activity, so the observed effects of the administration of **4** in the short term should be a loss of warfarin anticoagulant activity due to removal of warfarin from the blood stream. Lacking the reactive epoxide of **7**, compound **5** would be unable to form a similar conjugate, and would, therefore, be unable to exhibit the same anti-warfarin activity. The slow oxidation of **8** to the conjugate **9** gives an inhibitor capable of binding to two close, but separate binding sites of VKORC1.

It could be conjectured that the metabolism and clearance rates would very likely be such that steady state concentrations of warfarin and the adjuvant would be established early on, rendering unlikely the arguments about depletion of various species up to day 4 and a subsequent turn of events thereafter. However, it could also be countered that if the reaction between warfarin and the epoxide were rapid enough, the warfarin concentration available to the rat may be below the level required for effective anticoagulation. This would then mean that the major species present in the animal is, in fact, the conjugate **8**, and not warfarin itself. Answering this question clearly requires more experimentation. The anticoagulant activity observed at 10 days of continuous co-administration of warfarin and **4** would then be attributed to the slow oxidation of **8** to the active anticoagulant **9**, and since **8** is more lipophilic than warfarin, its half-life in the animal may be significantly longer.

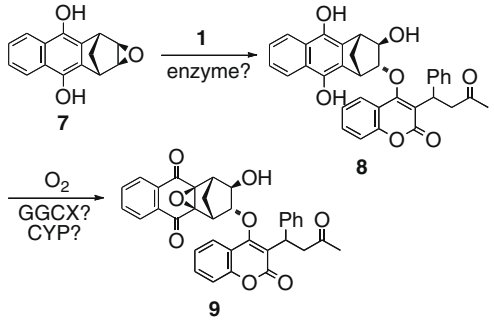
In this discussion it is important to remember that although the warfarin concentration may have reached a steady state by 4 days the ultimate anticoagulant effect as measured by prothrombin time is delayed due to the necessary degradation of vitamin K dependent clotting factors II, VII, IX and X with half-lives of 60, 4–6, 24 and 48 h, respectively.

The removal of warfarin itself from circulation is mainly by metabolism through cytochrome P450 oxidation.[13] Specifically, CYP2C9 has been invoked in the oxidation of warfarin to 7-hydroxywarfarin, and polymorphism in this enzyme is responsible for significant variation in clearance times of warfarin from individuals.[14] The average half-life of warfarin is 20–60 h, with mean value of approximately 40 h. If the removal of conjugate **8** has to occur by a similar oxidative pathway, this suggests that conjugate **8** is not readily oxidized to an analogue of 7-hydroxywarfarin by CYP2C9, and may provide a lead for developing new oral anticoagulants that are resistant to inactivation by CYP2C9.

Our work to elucidate the mechanism for enhancement of the anticoagulant action of warfarin by these compounds, especially compound **4**, is continuing.



Scheme 3. Conversion of **3** to the vitamin K 2,3-epoxide analogue **6**.



Scheme 4. Possible reactions of **3** affecting warfarin concentrations in blood.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.01.123.

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