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Warfarin glycosylation invokes a switch from anticoagulant to anticancer activity

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Warfarin **1**, a vitamin K antagonist (VKA), is a potent inhibitor of vitamin K epoxide reductase (VKOR) and acts by inhibiting the regeneration of reduced vitamin K, an essential cofactor for the clotting cascade.^[1] Warfarin, and other members of the 4-hydroxy coumarin family, were first used as a rodenticides before evolving into the most widely prescribed oral drug class to treat thromboembolic events, including atrial fibrillation and deep-vein thrombosis.^[2] While recent preclinical and clinical studies have implicated anticoagulants such as heparins in the reduction of tumor burden, metastatic potential and increasing overall patient survival,^[3] the utility of VKAs in the context of anticancer therapy has been restricted to the treatment of cancer-associated venous thromboembolism.^[4] Many 4-hydroxycoumarin analogs have been synthesized, culminating in derivatives with improved anti-VKOR potency and/or ADME properties.^[5] Despite both the natural abundance of 4-hydroxy coumarin analogs and extensive synthetic and semi-synthetic structural diversity, to the best of our knowledge the only glycosylated metabolites of warfarin described in the literature are four (C6-, C8-, C4- and C4'-) glucuronides as standards for warfarin phase II metabolism studies.^[6]

Given the precedent of glycosylation to modulate drug potency, mechanism, solubility, and ADME properties,^[7] we therefore set out to assess the potential impact of glycosylation on **1** activity. Herein we describe the synthesis of a library of 38 differentially-glycosylated warfarin neoglycosides. While this study revealed glycosylation to generally eliminate the native anti-VKOR activity of the parent, certain warfarin neoglycosides displayed surprising increases in anticancer cytotoxicity activity (nearly 2 orders of magnitude over the parent). Cumulatively, this study highlights the ability of glycosylation to fundamentally change the mechanism of a well-known pharmacophore and also provides a new practical method to prepare (*R*) and (*S*)-warfarin by the derivatization of racemic warfarin with a chiral auxiliary.

Neoglycorandomization takes advantage of the reactivity of unprotected free reducing sugars toward a methoxyamine-appended aglycon and enables rapid access to differentially

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glycosylated variants of targeted small molecules or proteins.^{[8] [9]} For rapid warfarin neoglycosylation, aglycon **3** was prepared according to Scheme 1 in two simple steps. Specifically, reaction of warfarin **1** with methoxyamine hydrochloride in pyridine resulted in the formation of **2** in a quantitative yield. The following reduction of the imine **2** using *tert*-butylamine.borane complex under acidic conditions resulted in the formation of the desired methoxyamine-appended aglycon **3**. The chemoselective neoglycosylation reaction of **3** with diverse free reducing sugars in DMF/AcOH (3:1) at 50°C provided the corresponding warfarin neoglycosides **4–41** with yields ranging from 2 to 70% (average overall 41%).^[10] All library members were purified by standard chromatography and LC-MS was subsequently used to assess purity and confirm identity.^[10]

As a first pass assessment, the cytotoxicity of the library members was evaluated on eight human cancer cell lines representing a panel of carcinomas including lung, colorectal, breast, CNS and ovarian. In this study, the neoglycosides **4–41** were compared to warfarin **1**, warfarin oxime **2** and warfarin neoaglycon **3** (Figure 1). From this assessment, 23 of the 38 library members displayed a better activity than warfarin **1** in at least one cancer cell line. The human colorectal HCT-15 cell line was most sensitive to warfarin neoglycosides with nine library members displaying an IC₅₀ below 20 μM (compared to an IC₅₀ of 543 μM for **1**), whereas NCI-H460 (human lung adenocarcinoma) was highly resistant. Interestingly, pentosylation (**32–41**, with the exception of the *D*-arabinoside **33**) led to drastic increases in anticancer activity and, in some cases cell line specificity, compared to the parent warfarin **1**. Specifically, the neoglycosides **38–41** presented an elevated potency from 8–70 fold across 7 cell lines while the effectiveness of members **22–24**, **34** and **35** was generally limited to HCT-15.

Although warfarin is prescribed as a racemate, (*S*)-warfarin exhibits 2–5 times more anticoagulant activity than the (*R*) enantiomer.^[11] To determine the impact of the C9 stereocenter upon the novel anticancer activities of respective neoglycosides, we developed a highly efficient three step process to prepare (*R*) and (*S*)-warfarin **1**. Several approaches for the synthesis of optically pure warfarin **1** have been reported in the literature and while asymmetric synthesis is among the most attractive strategies to date,^[12] limited availability of organocatalysts and a dependence upon chiral chromatography continue to limit preparative scale approaches to either warfarin enantiomer. Our approach to prepare (*R*) and (*S*)-warfarin in enantiopure form was achieved by derivatization of the racemate **42** to diastereomeric ketals (*S*)-**43** and (*R*)-**43** (Scheme 2), which, as anticipated, were chromatographically separable. The reaction of warfarin **1** with (2*R*,3*R*)-2,3-butanediol was unsuccessful due to a facile intramolecular cyclization to give the corresponding mixed ketal.^[12a, 13] Alternatively, methylation of warfarin **1** with trimethylsilyldiazomethane readily afforded **42** followed by the protection of the ketone to give the diastereoisomers **43**. Subsequent demethylation and the deprotection of the ketone of (*S*)-**43** and (*R*)-**43**, accomplished in a single step by a treatment with a mixture of TFA/H₂O, provided the desired (*S*)-warfarin and (*R*)-warfarin, respectively in 65% overall yield.^[14] Starting with pure (*S*)-warfarin and (*R*)-warfarin, the corresponding *D*-xylose and *D*-lyxose neoglycosides (based upon the most active carbohydrates elucidated in the racemic screen, Figure 1) were subsequently synthesized according to the Scheme 1. To our surprise, the activities of the (*R*)- or (*S*)-warfarin derived neoglycosides were indistinguishable from **39** or **41**, respectively.

The 4-hydroxy coumarins, including warfarin, inhibit vitamin K epoxide reductase complex 1 (VKORC1), which is involved in the vitamin K cycle. As a consequence, the suppression of VKORC1 activity compromises the γ-carboxylation of vitamin K dependant clotting factors II, VII, IX and X.^{[15][1]} To subsequently evaluate how the structural modification of warfarin influences the anticoagulant potential of library members, a subset of representative

neoglycosides were tested in a standard single dose VKORC1 assay (Figure 2).^[1a] At a final concentration of 1 μM of warfarin **1**, the remaining VKOR activity was 44% of the control (vehicle alone). While neoaglycon **3** displayed a similar anti-VKOR profile (albeit ~2-fold less potent), a 100-fold excess of each neoglycoside tested was required to approach the anti-VKOR activity of warfarin. Thus, the glycosylation of warfarin prohibits interaction with VKOR in a manner reminiscent to other biomodifications (e.g. P450-catalyzed hydroxylation in phase I metabolism),^[2a] where an increase in hydrophilicity is anticipated to prohibit anticoagulant activity in vivo.^[1a]

In conclusion, neoglycosylation has enabled the study of the impact of glycodiversification upon the activity of warfarin. In this study, we demonstrate that the glycosylation of warfarin can dramatically modulate both cancer cell line cytotoxicity as well as the VKOR inhibition of warfarin culminating in specific analogs which display a 70-fold increase in anticancer activity and a corresponding 100-fold lower anti-VKOR activity. Whether this remarkably 7,000-fold change in ‘specificity’ reflects the potential to achieve a useful therapeutic window in vivo remains to be determined. An efficient derivatization of warfarin, having potential for gram scale synthesis of pure (*R*)- and (*S*)-warfarin, has also been developed as part of this study and revealed the warfarin C9 stereochemistry to have no influence upon the novel activities of corresponding neoglycosides. While this study clearly illustrates the appended sugar to be critical for altering both the mechanism of action and potency of the parent drug, determination of the precise anticancer mechanism of action of warfarin neoglycosides remains to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Summary of IC_{50} data from the high-throughput cytotoxicity assay of **1–41** (reciprocal values displayed). Comparisons were performed against warfarin (**1**), warfarin oxime (**2**) and warfarin aglycon (**3**). A complete table of IC_{50} data and corresponding error values is provided in the Supporting Information. Cell lines used for the screening are HCT-15 (human colorectal adenocarcinoma), MCF7 (human breast adenocarcinoma), H1299 (human lung adenocarcinoma), SKOV-3 (human ovarian adenocarcinoma), SF-268 (human CNS adenocarcinoma), HT-29 (human colorectal adenocarcinoma), A549 (human lung adenocarcinoma), NCI-H460 (human lung adenocarcinoma).

Figure 2.
Vitamin K epoxide reductase activity as a function of warfarin analog concentration (μM).

Scheme 1.

Synthesis of neoglycosides library. a) MeONH_2 , pyridine, MeOH, 99%; b) $t\text{-Bu-NH}_2\cdot\text{BH}_3$, 10% aq. HCl, ethanol/dioxane (1:3), 86%; c) reducing sugar, DMF/AcOH (3:1).

Scheme 2.

Synthesis of optically pure warfarin neoglycosides. a) Me₃SiCHN₂, CH₂Cl₂, 76%; b) (2*R*, 3*R*)-2,3-butanediol, *p*-TSA, HC(OEt)₃, CH₂Cl₂, 86%; c) TFA/H₂O (4:1), 100%. d) MeONH₂, pyridine, MeOH, 99%; e) *t*-Bu-NH₂.BH₃, 10% aq. HCl, ethanol/dioxane (1:3), 86%; f) reducing sugar, DMF/AcOH (3:1), 30–45%. Formation of the β-anomer was typically observed (e.g., **39**: α/β 1:9; **41**: α/β 1:4) and sugar conjugation was found to improve aqueous solubility of warfarin (17 μg mL⁻¹) ~5-fold.