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RESEARCH PAPER

Effect of tecarfarin, a novel vitamin K epoxide reductase inhibitor, on coagulation in beagle dogs

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Background and purpose: Tecarfarin (ATI-5923) is a novel vitamin K epoxide reductase inhibitor that is metabolized by esterase (mainly human carboxylesterase 2) to a single major metabolite, ATI-5900, in rats, dogs and humans. Tecarfarin is not significantly metabolized by CYP450 enzymes. The objective of this study was to test and compare the efficacy of tecarfarin with that of warfarin, when administered either intravenously or once a day orally, to produce stable anticoagulation in beagle dogs.
Experimental approach: Effects on coagulation were assessed by measuring the activity levels of Factor VII and Factor X and thromboplastin-induced coagulation times, reported as prothrombin time (PT).
Key results: Continuous intravenous infusions and oral administration of tecarfarin and warfarin caused a dose-dependent decrease in activity of Factor VII and Factor X, and associated increase in PT. Intravenous fresh frozen canine plasma or subcutaneous vitamin K_1 treatment reversed the anticoagulant effects of orally administered tecarfarin. Consistent with the inhibitory effects of amiodarone on CYP2C9, co-administration of amiodarone significantly increased the anticoagulation effect of warfarin and plasma warfarin concentrations. In contrast, amiodarone had no effect on the anticoagulation induced by tecarfarin or tecarfarin plasma concentrations in this model.
Conclusions and implications: Overall, the data presented herein indicate that tecarfarin, via a vitamin K-dependent mechanism, causes changes in key parameters of haemostasis in beagle dogs that are consistent with effective anticoagulation. Compared to warfarin it has a decreased potential to interact metabolically with drugs that inhibit CYP450 enzymes and, therefore, may offer an improved safety profile for patients.
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Keywords: coagulation factors; warfarin; amiodarone; tecarfarin; beagle dogs

Abbreviations: DDI, drug-drug interaction; FFP, fresh frozen plasma; hCE, human carboxylesterase; HLM, human liver microsomes; HPBCD, hydroxypropyl-β-cyclodextrin; INR, international normalized ratio; PT, prothrombin time; QD, once daily; $VitK_1$, vitamin K_1; $VitK_2$, vitamin K_2; $VitK_1O$, vitamin K_1 epoxide; VKOR, vitamin K epoxide reductase; VKORC1, VKOR complex, subunit 1

Introduction

Warfarin (Figure 1A) is a vitamin K epoxide reductase (VKOR) inhibitor that inhibits the synthesis of functional clotting pro-factors II, VII, IX and X, as well as the naturally occurring endogenous anticoagulant proteins C and S. Warfarin is used to prevent thromboembolic events associated with many medical conditions such as atrial fibrillation, valvular heart disease and deep venous thrombosis (Hirsh *et al.*, 2001). The goal of anticoagulant therapy is to administer the proper dose to prevent clot formation or expansion without serious bleeding complications. Thus, to provide antithrombotic efficacy in the healthcare setting, steady-state plasma levels of warfarin must be maintained within a range that increases the blood clotting times to predetermined international normalized ratio (INR) values specific for particular clinical indications (Odén and Fahlén, 2002). Warfarin is an enantiomeric mixture, and most of the pharmacological activity of warfarin resides in the S-enantiomer. (S)-Warfarin is metabolized by CYP2C9 and CYP3A4, and is therefore subject to interactions with drugs that either inhibit or induce these CYP450 isoforms, thus leading to either excessive or suboptimal anticoagulation respectively (Holbrook *et al.*, 2005).

Tecarfarin (ATI-5923) [4-(4-hydroxy-2-oxo-2*H*-chromen-3-ylmethyl)-benzoic acid 2,2,2-trifluoro-1-methyl-1-trifluoromethyl-ethyl ester] (Figure 1B), a drug candidate

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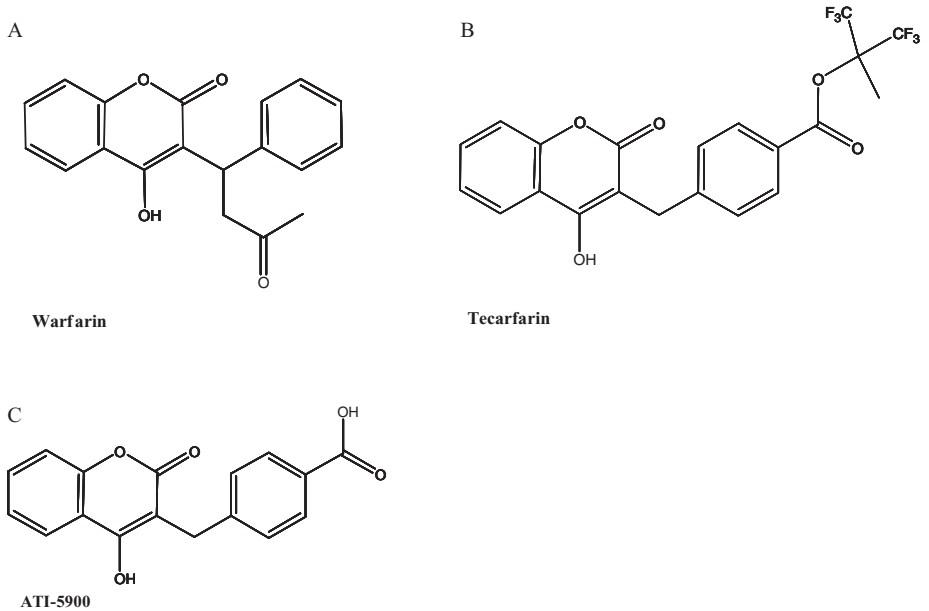


Figure 1 Chemical structures of (A) warfarin, (B) tecarfarin and (C) its metabolite ATI-5900.

currently in phase II/III clinical trials (see http://clinicaltrials.gov; Usman *et al*., 2008), is a VKOR inhibitor that was specifically designed to be non-oxidatively metabolized by human carboxylesterases (hCE) in order to yield more predictable metabolism and to decrease the likelihood of drug–drug interactions, especially with CYP450 inhibitors or inducers (Irwin *et al*., 2006). The metabolism of tecarfarin has been studied in rat, canine and human liver microsomes (HLM) *in vitro*. Tecarfarin is converted by esterases to ATI-5900 and this is quantifiable. The biotransformation of tecarfarin is completely independent of nicotinamide adenine dinucleotide phosphate (NADPH), the necessary cofactor for CYP450. The addition of NADPH does not result in additional metabolism, indicating that this biotransformation is independent of CYP450 (Irwin *et al*., 2006). The primary metabolite of tecarfarin in rats, dogs and humans is ATI-5900 [4-(4-hydroxy-2-oxo-2H-chromen-3-ylmethyl)-benzoic acid] (Figure 1C). Of the four vitamin K (VitK)-dependent clotting factors influenced by VKOR, Factor VII has the shortest half-life in dogs as well as in humans. Therefore, a reduction in Factor VII levels is an early indicator of anticoagulation. If VKOR inhibition persists, a reduction in the levels of other factors that have longer half-lives (e.g. Factor II with a half-life of 65 h and Factor X with a half-life of 40 h) also occurs (Hirsh *et al*., 2001).

In order to evaluate the pharmacokinetic (PK) and pharmacodynamic properties of tecarfarin and compare them to those of warfarin, beagle dogs were administered tecarfarin or warfarin by either continuous i.v. infusion or once daily (QD) oral dosing at doses sufficient to produce steady-state plasma concentrations consistent with stable anticoagulation. This study also provided insight into the degree of VKOR inhibition produced by tecarfarin and warfarin administration, as measured by prothrombin time (PT) or expressed as INR, and plasma activity levels of Factor VII and Factor X. Reversal of tecarfarin-induced anticoagulation was also assessed in adult beagle dogs by using either canine fresh frozen plasma (FFP) or vitamin K_1 ($VitK_1$), the recommended antidotes for excessive warfarin-induced anticoagulation. Finally, the potential of tecarfarin to interact with drugs that inhibit CYP450 was assessed by determining whether co-administration of amiodarone modified its anticoagulant effects in a similar manner to those of warfarin in beagle dogs. Amiodarone, and more specifically its metabolite, N-desethylamiodarone, are known to inhibit CYP2C9, which metabolizes (S)-warfarin to inactive hydroxylated metabolites (Almog *et al*., 1985).

Methods

Chemicals

The test drugs (tecarfarin, ATI-5900 and warfarin, sodium salts) were synthesized by ARYx Therapeutics (Fremont, CA, USA).

In vitro *experiments*. Pooled human microsomal suspension (20 $mg \cdot mL^{-1}$) was obtained from BD Biosciences (San Jose, CA, USA). $VitK_1$ was obtained from Aldrich (Saint Louis, MD, USA). Vitamin K_2 ($VitK_2$), potassium phosphate (dibasic), 3-[(3-cholamidopropyl) dimethulammonio]-2-hydroxy-1 -propanesulfonate (CHAPS) and dimethyl sulphoxide were obtained from Sigma (Saint Louis, MO, USA). Glacial acetic acid and isopropyl alcohol [high performance liquid chromotography (HPLC) grade] were obtained from Fischer (Pittsburgh, PA, USA). Potassium phosphate, (monobasic), potassium chloride, glycerol, zinc sulphate heptahydrate, zinc chloride, sodium acetate trihydrate and hexane (HPLC grade) were obtained from J.T. Baker (Phillipsburg, NJ, USA). Ethanol was obtained from Asper Alcohol and Chemical Co. Vitamin K_1-2,3-epoxide was synthesized according to Dowd *et al*. (1992).

In vivo *experiments*. Hydroxypropyl-β-cyclodextrin (HPBCD) was obtained from Cyclodextrin Technologies Development,

Inc. (High Springs, FL, USA). Paraoxon was obtained from Sigma. Amiodarone hydrochloride was obtained from Spectrum (Gardena, CA, USA). $VitK_1$ (phytonadione 10 mg·mL^{-1}; K-ject) was manufactured by Phoenix Scientific Inc. (St. Joseph, MO, USA). $VitK_1$ diluent was prepared using castor oil (70 mg·mL^{-1}; Sigma), dextrose monohydrate (37.4 mg·mL^{-1}; Sigma), water for injection (q.s.; B. Braun Medical Inc., Bethlehem, PA, USA) and benzyl alcohol (0.9% w/v; Sigma). Fresh frozen canine plasma was obtained from Hemopet (Garden Grove, CA, USA).

$VitK_1$ epoxide reductase inhibition in HLM

In order to assess the relative potencies of warfarin, tecarfarin and ATI-5900, pooled HLM (12.5 μL) were incubated in buffer (250 mM potassium phosphate, 500 mM potassium chloride, 20% glycerol and 0.75% CHAPS at pH 7.85) with vitamin K_1 epoxide ($VitK_1O$) and increasing concentrations of each of the test compounds (final concentrations: 0.01–100 μM for tecarfarin and warfarin and 0.01–1500 μM for ATI-5900) at 25°C, 70 rpm for 3 min. Reactions were initiated by the addition of dithiothreitol as a reducing agent (2 mM final concentration). After initiation, the samples were incubated at 25°C, 70 rpm for exactly 20 min and were the reaction quenched by the addition of a 15% w/v solution of aqueous ZnSO4 to precipitate proteins. $VitK_2$ (100 pmol) was then added as an internal standard to each incubation tube, followed by the addition of a 7:2 v/v mixture of hexane/isopropyl alcohol. The tubes were vortexed for 1 min and then centrifuged in order to break the emulsion. The hexane layers from these extractions were next transferred to test tubes and the solvent was evaporated under a stream of nitrogen. The residue from each tube was diluted in 100 μL of isopropyl alcohol and transferred to a vial for HPLC analysis. Aliquots were tested for formation of $VitK_1$ by HPLC with fluorescence detection. $VitK_1$, vitamin K_l epoxide ($VitK_1O$), and the internal standard $VitK_2$ were separated over a 20-min run time on an Agilent Hypersil ODS 5 μm (4.0 × 125 mm) HPLC column (Agilent Technologies, Santa Clara, MD, USA) with an isocratic mobile phase of 95% methanol containing 11 mM $ZnC1_2$, 5.5 mM NaOAc and 5.5 mM glacial acetic acid. With a flow rate of 1 mL·min^{-1}, retention times for $VitK_2$, $VitK_1O$ and $VitK_1$ were 6.9, 9.0 and 12.5 min respectively. Before entering the fluorescence detector, the mobile phase passed directly through a short in-line guard column packed with zinc powder in order to catalyse the reduction of the essentially non-fluorescent $VitK_1$ and $VitK_2$ quinones to their highly fluorescent dihydroquinone forms. Under these conditions, the $VitK_1O$ substrate also undergoes reduction to generate a fluorescent product. In-line filters were placed between the injector port and the ODS column as well as between the zinc column and the fluorescence detector. The fluorescence detector was optimized for $VitK_1$ detection with an excitation wavelength of 244 nm and emission detection at 430 nm. Data were analysed using Shimadzu EZSTART chromatography quantification software v 7.2.1 SP1 (Columbia, MD, USA). Quantification was achieved by comparison of peak areas to the internal standard $VitK_2$. Activities were reported relative to a negative control reaction (no inhibitor, VKOR activity = 100%). The half maximal inhibitory concentration (IC_{50}) for each test compound was used to determine the concentration range for use in subsequent kinetic studies.

Five sets of kinetic experiments were performed to determine the *K*i for each test compound in HLM. Each individual data set tested VKOR complex, subunit 1 (VKORC1) activity at substrate concentrations of 0.5, 1.0, 2.0, 5.0, 10, 20 and 100 μM (all reactions run in triplicate) at a single concentration of test compound. Inhibitory concentrations tested were 0.20, 0.65, 1.0 and 2.0 μM for tecarfarin (*K*i study), 100, 300, 500 and 1000 μM for ATI-5900, and 0.35, 0.70, 1.2 and 1.7 μM for warfarin. Each set included a control (no inhibitor present) to determine the maximal (100%) level of enzyme activity. Reaction velocities at each concentration of inhibitor were calculated and *K*i and K_m values were obtained by replotting data from Lineweaver–Burke analysis.

Pharmacokinetic studies

A total of 12 beagle dogs weighing between 7 and 10 kg (Marshall Farms, North Rose, NY, USA) were divided into four groups consisting of 1 male and 2 females in each group. Animals were given 0.3 mg·kg^{-1} of either tecarfarin or warfarin by either the i.v. or p.o. (gelatine capsule) route of administration. Drugs for i.v. administration were formulated in ethanol/5% dextrose 10:90 v/v, and were administered in a volume of 0.5 mL·kg^{-1}. Blood for plasma (2 mL per sample) was collected at specified time intervals (5, 10, 15, 30, 60, 120, 180, 240, 360, 480, 720 and 1440 min post-i.v. dosing or 30, 60, 120, 240, 360, 480, 720, 960 and 1440 min post-p.o. dosing) via the brachiocephalic artery in labelled Vacutainers (Becton Dickinson, San Jose, CA, USA) containing sodium heparin and paraoxon (0.04 mL of 0.4% in ethanol) in order to inhibit further metabolism of the drug by blood esterases. Plasma samples were analysed to measure the concentrations of tecarfarin and ATI-5900 by liquid chromatography/tandem mass spectrometry (LC/MS/MS) method. Non-compartmental modelling was performed using WinNonlin (Pharsight Corp., Mountain View, CA, USA) and the following PK parameters were determined: maximum peak plasma concentration (C_{max}), time to C_{max} (t_{max}), terminal half-life ($t_{1/2}$), area under the curve (AUC) and oral bioavailability (F).

In vivo *i.v. efficacy studies*

Nine female beagle dogs weighing between 7 and 10 kg (Marshall Farms) were each surgically implanted with two central venous catheters by DaVinci Biomedical Research Products, Inc. (South Lancaster, MA, USA). The procedures used were as humane as possible and complied with the U.S. guidelines for animal care.

The dogs were divided into three groups of 3 animals and received: vehicle [5% HPBCD in 12.5% distilled water and 87.5% normal saline (Group 1)]; escalating doses of tecarfarin [0.1 mg·kg^{-1}·day^{-1} for 9 consecutive days and 0.2 mg·kg^{-1}·day^{-1} for the next 9 days (Group 2)]; or warfarin [0.05 mg·kg^{-1}·day^{-1} for 3 days, 0.1 mg·kg^{-1}·day^{-1} for 5 days and 0.2 mg·kg^{-1}·day^{-1} for 9 days (Group 3)]. Test and control compounds were administered by continuous i.v. infusion (1.2 mL·h^{-1}) using AutoMed 3400 infusion pumps (Algos LC, Salt Lake City, UT, USA) placed in a jacket worn by each dog.

Fresh sterile stock solutions of the test compounds were prepared in 100 mL sterile bags and changed every 3 days. INR, drug levels and Factors VII and X activity levels were monitored.

In vivo *oral efficacy studies*

Three groups of 5 beagle dogs (2 males, 3 females) weighing between 7 and 12 kg (Marshall Farms) were used. Tecarfarin and warfarin were administered orally using gelatine capsules (Torpac Inc., Fairfield, NJ, USA) containing the test compounds as a powder. The doses were adjusted for the weight of each dog and prepared daily.

In the first set of experiments, tecarfarin (0.05 $mg \cdot kg^{-1}$) was administered p.o. twice daily for 21 days. In the second set of experiments, after a 2-week washout period, the same dogs received QD escalating oral doses of either tecarfarin (0.2 $mg \cdot kg^{-1}$ for 11 days, 0.25 $mg \cdot kg^{-1}$ for 10 days, and 0.3 $mg \cdot kg^{-1}$ for 14 days) or warfarin (0.2 $mg \cdot kg^{-1}$ for 5 days, 0.25 $mg \cdot kg^{-1}$ for 10 days, and 0.3 $mg \cdot kg^{-1}$ for 14 days). INR, drug levels and Factors VII and X activity were monitored daily.

In vivo *reversal study*

This study assessed the ability of i.v. FFP or s.c. $VitK_1$ treatment to reverse anticoagulation produced in adult beagle dogs by treatment with tecarfarin. Four groups of 3 dogs (1 male and 2 females per group) were used. All dogs received QD oral doses of tecarfarin (0.5 $mg \cdot kg^{-1}$) for 4 days. Twenty-four hours after the last dose, the dogs received either an i.v. infusion of canine FFP (9 $mL \cdot kg^{-1}$; group 1) or a s.c. injection of $VitK_1$ (2.5 $mg \cdot kg^{-1}$; group 2). Control animals received either i.v. or s.c. injections of 0.9% saline (9 $mL \cdot kg^{-1}$; group 3) or $VitK_1$ diluent (250 $\mu L \cdot kg^{-1}$; group 4). Note that $VitK_1$ was not injected i.v. due to a well-know risk of anaphylaxis with this compound (Riegert-Johnson and Volcheck, 2002). PT and Factor VII and Factor X activities were measured in plasma samples taken immediately prior to tecarfarin dosing each day (trough levels) and before and after administration of FFP or $VitK_1$.

In vivo *drug–drug interaction (DDI) study*

Two groups of 6 dogs (2 males and 4 females per group) weighing between 8 and 14 kg (Marshall Farms) were treated with tecarfarin (0.3 $mg \cdot kg^{-1}$ or adjusted dose) or warfarin (0.25 $mg \cdot kg^{-1}$ or adjusted dose) p.o., QD, for 11 days to produce a stable increase in PT. After 11 days of treatment, the dogs received, in addition to either tecarfarin or warfarin, 40 $mg \cdot kg^{-1}$ of amiodarone for 2 days, followed by 20 $mg \cdot kg^{-1}$ of amiodarone for 6 days. All treatments were stopped on day 20, and the recovery was monitored for 3 days. Tecarfarin, warfarin and amiodarone were administered p.o. in solid form using gelatine capsules (Torpac Inc.). The PT as well as Factors VII and X activities was determined, and plasma drug levels (tecarfarin, warfarin and amiodarone) were measured.

Blood sampling

Each morning, INR was measured in drops of blood freshly collected from the dog brachiocephalic vein using a point-of-care coagulation monitor (INRatio Meter, HemoSense Inc., San Jose, CA, USA). Blood samples (2 mL) were also obtained from the brachiocephalic vein at 24-h intervals during the treatment. Samples were collected into lithium heparin (Vacutainer tubes; Becton Dickinson) containing 0.4% paraoxon (Sigma) in ethyl alcohol. Following centrifugation, at least 1.0 mL of plasma was transferred to Eppendorf cryotubes and frozen at –70°C until analysed for Factor VII and X activity, and drug levels.

Coagulation factor assessments

Factor VII and X activities were measured using the chromogenic assay systems, Coaset F VII (manufactured by Chromogenix Instrumentation Laboratory SpA, Milan, Italy and distributed by DiaPharma Group Inc., West Chester, OH, USA) and DiaPharma Factor X (manufactured by R2 Diagnostics, South Bend IN, USA) respectively. Heparin did not interfere with the measurement of Factors VII and X (data not shown). The effects of test compounds on the activities of Factor VII or Factor X are expressed as a percentage of control baseline values.

Determination of PT

Samples of blood (2 mL) were collected into 3.2% citrate (MonoJect, Tyco Healthcare Group, Mansfield, MA, USA) from the brachiocephalic vein at 24-h intervals during treatment for the measurement of PT. PTs were measured by Quality Clinical Labs (Mountain View, CA, USA) using nephelometry (ACL 100 Coagulation System; Coulter/Instrumentation Laboratory, Fullerton, CA, USA).

Determination of drug concentrations

Plasma concentrations of tecarfarin and ATI-5900 were measured using LC/MS/MS methodology following protein precipitation with acetonitrile. The HPLC column was a Zorbax Eclipse XDB C18 (2.1 × 100 mm, 3.5 μm) from Agilent. The mobile phase consisted of 0.1% formic acid in acetonitrile and 0.1% formic acid in water. The flow rate was 0.200 $mL \cdot min^{-1}$ and the injection volume was 10 μL. The HPLC system (SHIMADZU LC-10AD vp, Shimadzu, Columbia, MD, USA) was coupled to the mass spectrometer (SCIEX API-3000 LC/MS/MS systems, AME Biosciences, Toroed, Norway) using a TurboIonSprayTM atmospheric pressure ionization inlet (AME Biosciences). The analysis was by negative ionization MS/MS. High purity nitrogen gas from a high-pressure liquid nitrogen Dewar flask was used for the nebulizer, TurboIonSpray, curtain and collision gases.

Plasma levels of tecarfarin, ATI-5900, warfarin and amiodarone were also measured in the drug–drug interaction study.

The lower limit of quantification for tecarfarin was 1.0 $ng \cdot mL^{-1}$ and the linear dynamic range was 1–1000 $ng \cdot mL^{-1}$. Accuracy was 85–105% over the linear dynamic range and precision was 4.2–13.4%.

Statistical analysis

Data are expressed as mean ± SEM. GraphPad Prism software (La Jolla, CA, USA) was used to determine IC_{50}. Differences in

PT values, Factor X levels and drug plasma concentrations were analysed using the Dunnett's multiple comparison test (reversal study) and the Wilcoxon signed rank test (DDI study) after analysis of variance.

Results

$VitK_1$ epoxide reductase inhibition in HLM

The objective of this experiment was to determine the inhibition of human liver microsomal VKORC1 activity by tecarfarin and its putative main metabolite, ATI-5900, in comparison to warfarin. All experimental incubations to determine IC_{50} values were run in duplicate at a $VitK_1$ epoxide concentration of 3.25 μM. The results (Figure 2) suggest that tecarfarin is equipotent with warfarin (IC_{50} = 0.67 μM and 0.84 μM for tecarfarin and warfarin, respectively) at inhibiting VKORC1 in HLM. By contrast, ATI-5900 appears to be a very poor VKORC1 inhibitor (IC_{50} = 270 μM). All three test compounds, tecarfarin, warfarin and ATI-5900, were determined to be non-competitive inhibitors of VKORC1 epoxide reductase activity in HLM. The *K*i values determined from either the slopes of the respective Lineweaver–Burke plots (Figure 3), or from their *y*-intercept values were, respectively, 0.49 and 0.63 μM for tecarfarin, 0.69 and 0.53 μM for warfarin, and 423 and 273 μM for ATI-5900.

Pharmacokinetic studies

Time-concentration profiles of tecarfarin and warfarin in dogs are given in Figure 4 and PK parameters are given in Table 1. After being administered tecarfarin, beagle dogs metabolized the compound into its main putative metabolite ATI-5900. After i.v. injection, the C_{max} for tecarfarin was about 25 times greater than the C_{max} observed with ATI-5900 (1155 ng·mL^{-1} vs. 46.2 ng·mL^{-1}, respectively). The total area under the curve (AUC_{ALL}) for tecarfarin was about 9 times the AUC_{ALL} observed with ATI-5900 (4618 ng·h^{-1}·mL^{-1} vs. 520 ng·h^{-1}·mL^{-1}, respectively). Ester hydrolysis of tecarfarin appeared to be slow, t_{max} for ATI-5900 being 1.7 h, compared to 0.14 h after i.v. bolus administration. The volume of distribution of tecarfarin at steady state (V_{ss}) was only 0.49 L·kg^{-1}, indicating that tecarfarin resides principally in the blood compartment. I.v. treatment was associated with an apparent $t_{1/2}$ of 6 h for both tecarfarin and ATI-5900.

Both tecarfarin and warfarin had high bioavailability (77% vs. 87% respectively). The p.o. data indicated a more rapid absorption for warfarin than for tecarfarin (t_{max} = 2.17 h vs. 4.67 h respectively).

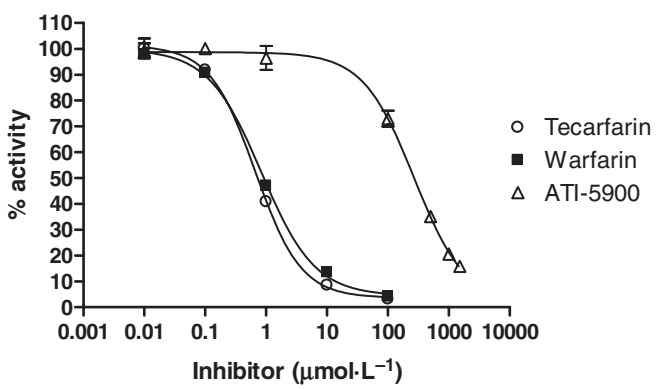


Figure 2 Inhibition of human liver microsomal VKORC1 activity by tecarfarin and its putative main metabolite, ATI-5900, in comparison to warfarin. Half maximal inhibitory concentration (IC_{50}) values calculated from the curves were tecarfarin 0.67 μM, warfarin 0.84 μM and ATI-5900 270 μM.

In vivo *efficacy: i.v. infusion study*

The i.v. infusion study was designed to determine the steady-state plasma concentrations that cause biochemical changes

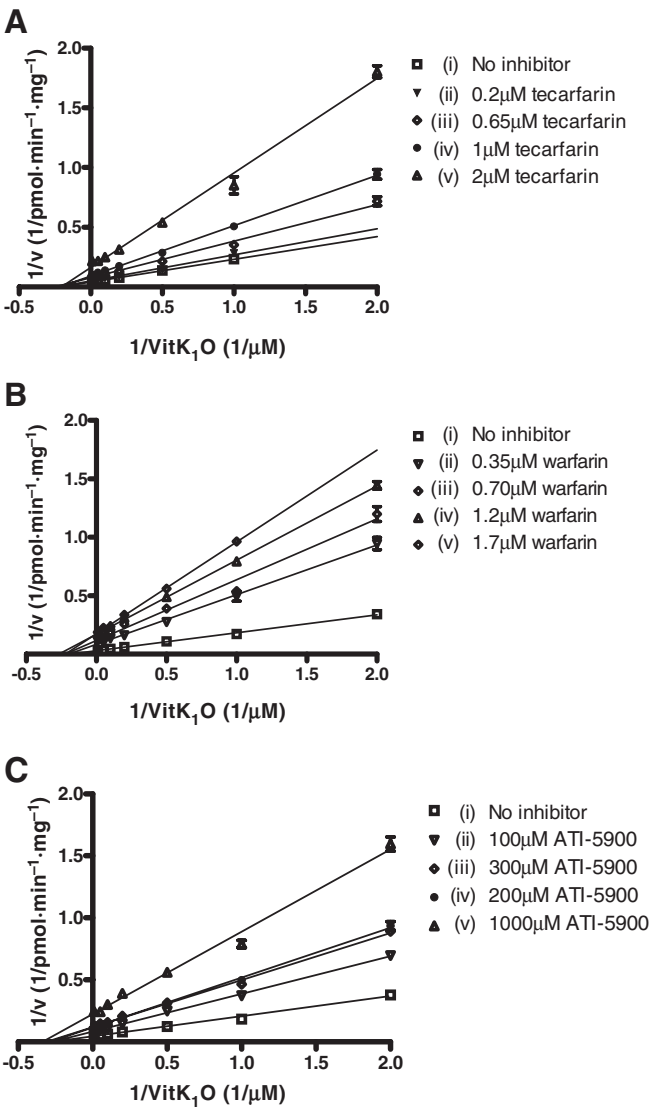


Figure 3 Lineweaver–Burke plots describing VKORC1 kinetics at various inhibitory concentrations of the test compounds in human liver microsomes. (A) For tecarfarin the respective K_m values for $VitK_1O$ determined from each reciprocal plot are: (i) 4.63 μM; (ii) 4.39 μM; (iii) 3.97 μM; (iv) 4.60 μM; and (v) 4.84 μM. (B) For warfarin the respective K_m values for $VitK_1O$ determined from each reciprocal plot are: (i) 5.22 μM; (ii) 5.05 μM; (iii) 4.44 μM; (iv) 3.70 μM; and (v) 4.48 μM. (C) For ATI-5900 the respective K_m values for $VitK_1O$ determined from each reciprocal plot are: (i) 3.62 μM; (ii) 3.71 μM; (iii) 3.21 μM; (iv) 3.47 μM; and (v) 2.97 μM.

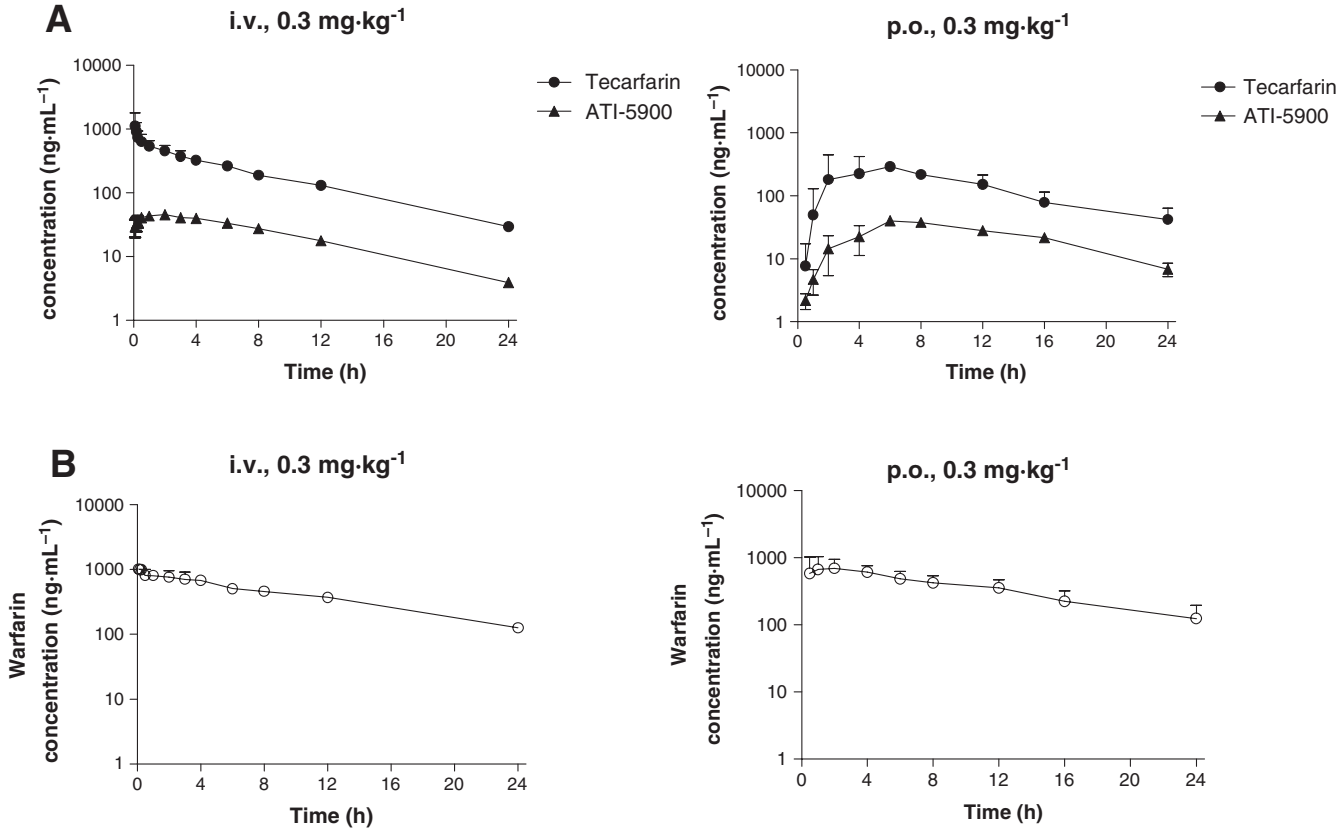


Figure 4 Kinetic profile of (A) tecarfarin and its main metabolite ATI-5900 and (B) warfarin in beagle dogs. Dogs were given a single 0.3 mg·kg^{-1} dose, i.v. (left panels) and p.o. (right panels), of either tecarfarin or warfarin, and plasma concentrations of test compounds were determined over a 24-h period. Data represent the mean ± SEM.

Table 1 Plasma pharmacokinetic parameters for tecarfarin and warfarin in dogs

	Tecarfarin		*Warfarin*	
	i.v., 0.3 mg·kg^{-1}	*p.o., 0.3 mg·kg^{-1}*	*i.v., 0.3 mg·kg^{-1}*	*p.o., 0.3 mg·kg^{-1}*
AUC_{0-24} (ng·h^{-1}·mL^{-1})	4618 ± 767	3257 ± 1430	9889 ± 1237	8565 ± 2932
C_{max} (ng·mL^{-1})	1155 ± 600	360 ± 123	1070 ± 181	753 ± 278
t_{max} (h)	0.14 ± 0.10	4.67 ± 2.31	0.17 ± 0.08	2.17 ± 1.76
$t_{1/2}$ (h)	5.84 ± 0.44	6.20 ± 1.40	8.69 ± 1.00	8.34 ± 2.15
CL (mL·kg^{-1}·h^{-1})	62.8 ± 11.0	NC	26.3 ± 2.6	NC
V_{ss} (L·kg^{-1})	0.49 ± 0.13	NC	0.32 ± 0.07	NC
F (%)	NC	77	NC	87

Data are expressed as mean ± s.d. (n = 3 dogs per group). NC, not calculated and/or not applicable.
AUC, area under the curve; C_{max}, maximum peak plasma concentration; t_{max}, time to C_{max}; $t_{1/2}$, terminal half-life; CL, clearance; V_{ss}, volume of distribution at steady state; F, bioavailability.

consistent with stable anticoagulation, and to provide insight into the degree and kinetics of VKOR inhibition following tecarfarin administration. For comparative purposes, warfarin was also included in the study. Tecarfarin was given at a dose of 0.1 and 0.2 mg·kg^{-1}·day^{-1} via a slow, constant infusion (1.2 mL·h^{-1}). Warfarin was given at doses of 0.05, 0.1 and 0.2 mg·kg^{-1}·day^{-1}. Doses were escalated when it was determined that plasma levels of drugs had reached steady state. Plasma concentrations of tecarfarin and warfarin at steady state seemed to be dose-dependent (Figure 5). Continuous i.v. infusion of tecarfarin and warfarin caused dose-dependent changes in the activity levels of Factor X and Factor VII and INR values (Figure 5). Infusion of vehicle alone had no effect on INR (data not shown). It was noted that there were differences in the abilities of the test compounds to cause changes in the measured haemostatic parameters. Warfarin and tecarfarin both increased the INR in a dose-dependent manner but tecarfarin was a more potent anticoagulant, on a dose basis, than warfarin.

In vivo *efficacy: oral study*

The relationships between i.v. dose and INR and between i.v. dose and plasma levels of drug, coupled with the findings of

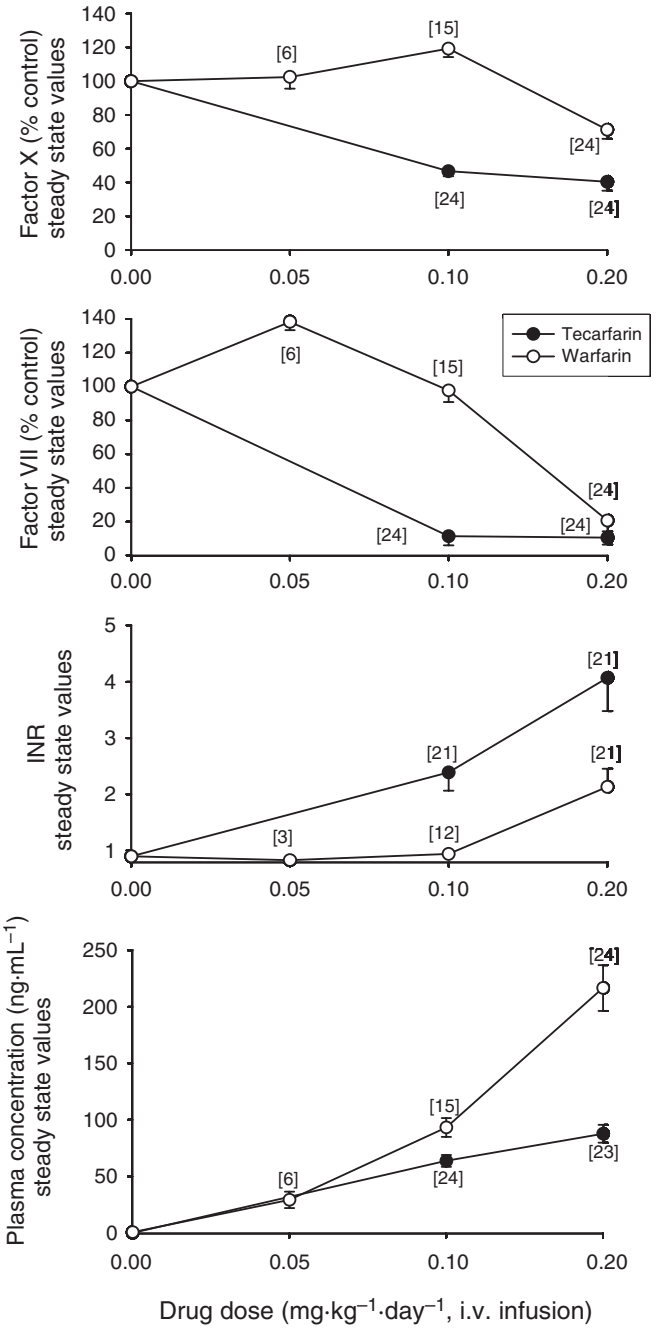


Figure 5 Relationship between i.v. infusion dose of tecarfarin, steady state anticoagulation effects and plasma concentration in beagle dogs compared to warfarin. Data shown represent the dose-dependent effect of tecarfarin on Factor X (top panel), Factor VII (top middle panel), INR (bottom middle panel) and plasma concentration (bottom panel) compared to warfarin. Values included in the analysis (*n*, the number of observations, is shown in parentheses) were those considered at steady state, that is, after more than 2 days of continuous drug infusion in dogs. Doses were escalated when it was determined that steady state had been achieved. Each data point represents the mean ± SEM (3 dogs per group).

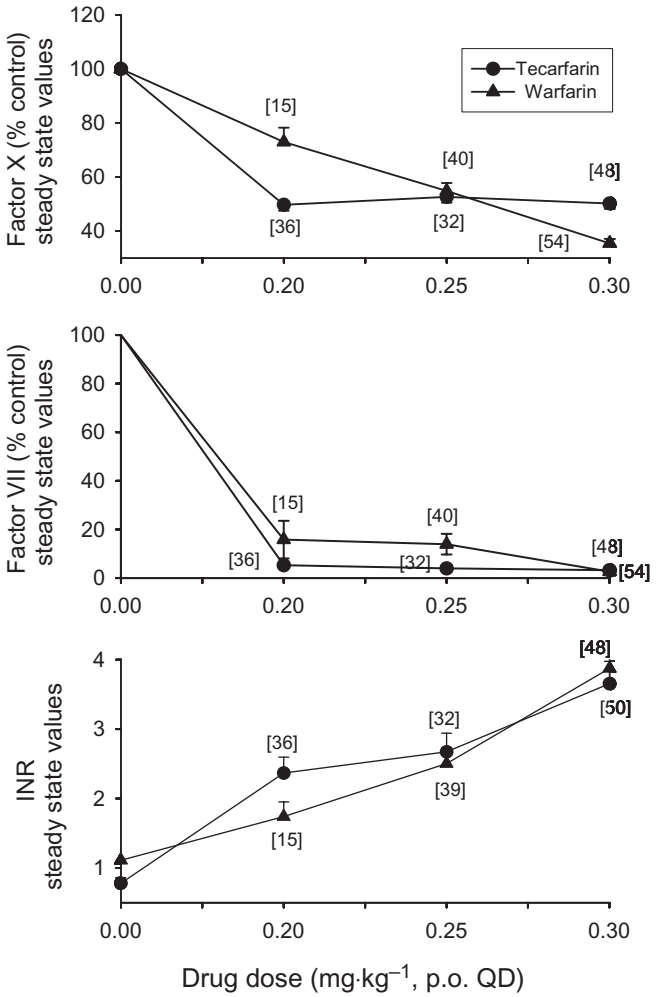


Figure 6 Relationship between orally administered doses of tecarfarin and warfarin given QD and steady state anticoagulation effects in beagle dogs. Data represent the dose-dependent effect of tecarfarin ($n = 4$) and warfarin ($n = 5$) on Factor X (top panel) and Factor VII (middle panel) and INR (bottom panel). Values included in the analysis (*n*, the number of observations is shown in parentheses) were those considered at steady state, that is, after more than 2 days of oral dosing in dogs. Doses were escalated when it was determined that steady state had been achieved. Each data point represents the mean ± SEM.

the PK experiment, were good predictors of the oral dose needed to achieve a reasonable 'therapeutic' INR in beagle dogs. This 'therapeutic' INR was chosen as a range of between 2 and 4, as is standard practice in humans. In this oral study the anticoagulant effects of tecarfarin, administered QD, were assessed in beagle dogs. Both tecarfarin and warfarin were administered at 0.2, 0.25 and 0.3 mg·kg^{-1} QD. The first observation was that the INR was highly dependent on the activity levels of the coagulation factors, similarly for tecarfarin and warfarin, both in this oral study and in the previous i.v. experiments (Figure 6). Figure 7 shows this correlation, where individual data points were plotted (i.v. data points). It is interesting to note that Factor VII activity was reduced by more than 80% before increases in INR were observed, whereas Factor X activity was only reduced by 40–50% before INR began to increase. Unlike in the i.v. experiments, there was no evidence that tecarfarin is more potent, on a dose basis, than warfarin, in depleting coagulation factors or in increasing INR.

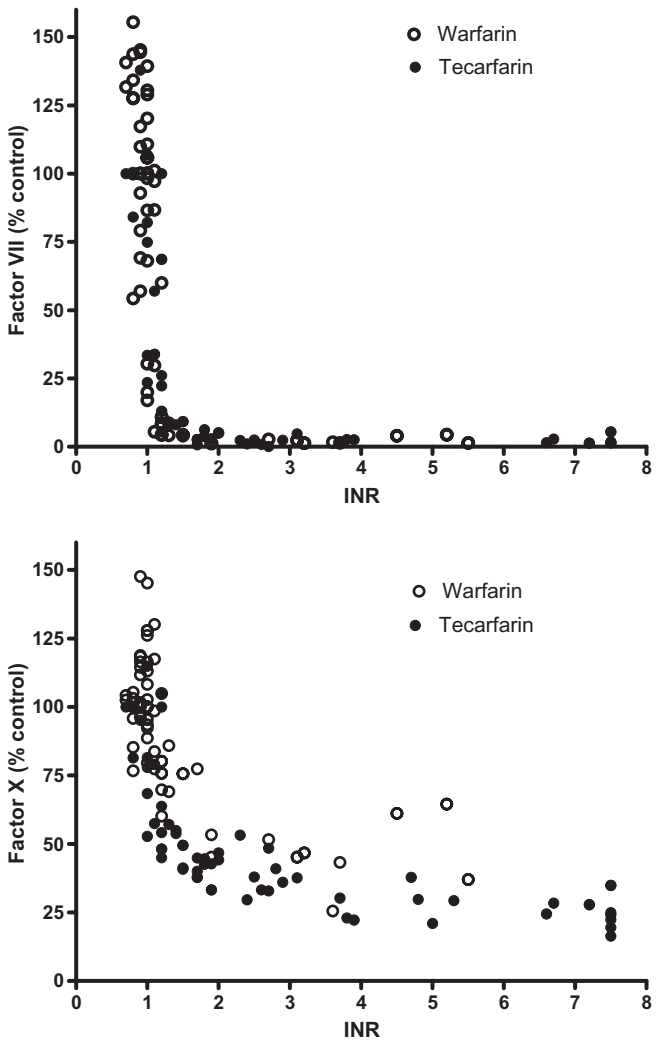


Figure 7 Effect of an i.v. infusion of warfarin (0.05, 0.1 and 0.2 mg·kg^{-1}·day^{-1}) and tecarfarin (0.1 and 0.2 mg·kg^{-1}·day^{-1}) on Factors VII and X levels. Data shown are the Factor VII and X values expressed as % of control (baseline) for n = 3 dogs per group. INR, international normalized ratio.

Reversal study

Once per day oral administration of tecarfarin (0.5 mg·kg^{-1}) increased PT in dogs to between 15 and 20 s (Figure 8) and decreased activity levels of Factor VII and Factor X to values compatible with an INR of 3 as seen in the i.v. and p.o. experiments (data not shown). Treatment with FFP (9 mL·kg^{-1}, i.v.) reversed the anticoagulant effects of tecarfarin. PTs returned to treatment baseline values within 15 min after FFP treatment (Figure 8), and within 4 h after treatment with VitK (Figure 8).

Drug–drug interaction study

The effects of co-administration of amiodarone on tecarfarin- or warfarin-induced anticoagulation were assessed in adult beagle dogs. PTs, Factor VII and Factor X activities and drug levels were measured in plasma samples taken immediately prior to drug dosing each day.

Animals (n = 5–6 per group) were dosed orally, QD for 11 days with either tecarfarin or warfarin at concentrations that induced a PT between 15 and 30 s. At the end of this 11-day treatment period, PT values were 26.3 ± 4.5 s and 18.9 ± 2.8 s for tecarfarin and warfarin-dosed animals respectively (Figure 9). Furthermore, Factor VII levels were reduced by 100% in both groups (data not shown) and Factor X activities were 31 ± 5 and 41 ± 3% of baseline for tecarfarin and warfarin, respectively (Figure 10).

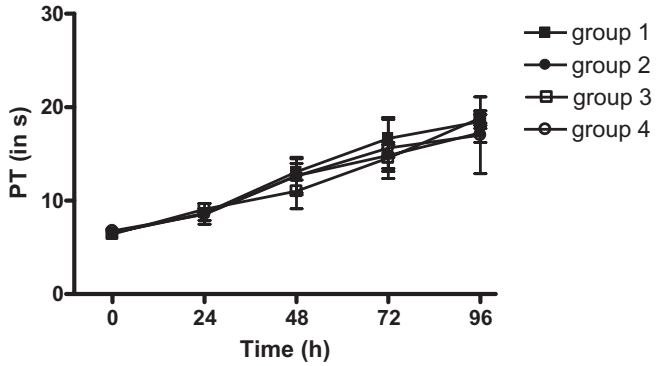
Starting at day 12 of warfarin treatment, co-administration of amiodarone for 8 days resulted in significant (P = 0.0313) increases in PT values (Figure 9) and further reductions in Factor X levels (P = 0.0313) (Figure 10) within 2 days. Neither PT nor Factor X levels were affected by concomitant tecarfarin and amiodarone treatment.

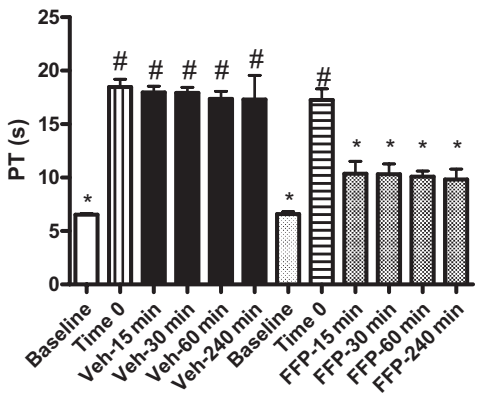
After 11 days of oral treatment with warfarin or tecarfarin, drug levels were 201 ± 39 ng·mL^{-1} and 57 ± 6 ng·mL^{-1}, respectively (Figure 11). Warfarin plasma levels were significantly (P = 0.0313) elevated during amiodarone co-administration, but tecarfarin concentrations were unaffected (Figure 11).

Discussion

Warfarin has been used for many years to prevent and treat thromboembolic complications associated with medical conditions such as atrial fibrillation, native and prosthetic valvular heart disease and deep venous thrombosis (Hirsh *et al.*, 2001). Warfarin is an inhibitor of VKOR, a hepatic microsomal enzyme that catalyses the post-translational conversion of specific glutamyl residues to γ-carboxyglutamyl (Gla) residues in serine proteases and cofactors of the coagulation cascade. These include clotting Factors II, VII, IX and X, as well as the endogenous anticoagulant proteins C, S and Z. Of the clotting factors, Factor VII has the shortest half-life (4–6 h in both dogs and humans). Therefore, a reduction in Factor VII activity serves as an early marker of warfarin-induced anticoagulation. Subsequently, reductions in the functional levels, and hence activities, of both Factor II and Factor X, which have longer half-lives (65 and 40 h in humans, 72 h in dogs, respectively; Monnet and Morgan, 2000), also occur if VKOR inhibition persists.

Thus, it should not be surprising that there is a time lag associated with drug administration, effective VKOR inhibition within hepatocytes (which is dependent upon dose and hepatocyte drug levels), and clinically effective anticoagulation. Determination of PT and its derivative measurement, the INR, are the most common ways used to monitor oral coagulation therapy (Quick, 1935). INR incorporates parameters to account for differences in instrumentation and reagents. PT is sensitive to defects in the extrinsic pathway Factors X, VII, V, II and I (fibrinogen). During the first few days of warfarin therapy, prolongation of PT mainly reflects a reduction of Factor VII, and subsequently a reduction of Factors X and II. The INR integrates these changes into a value predicting antithrombotic efficacy. Of note, the clinical antithrombotic efficacy of warfarin is more closely linked to reductions in Factor II and Factor X activity than to reductions in Factor VII (Hirsh *et al.*, 2001).





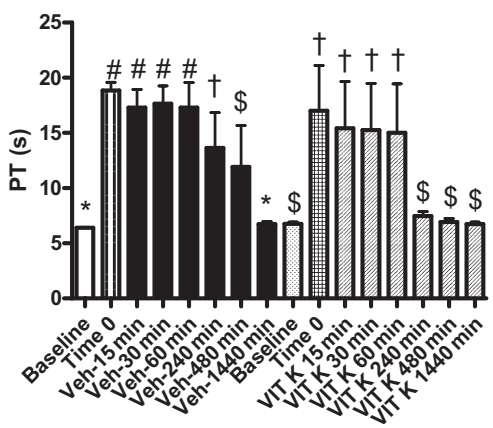


Figure 8 Effect of tecarfarin (0.5 mg·kg^{-1}) on prothrombin time (PT) followed by reversal of anticoagulation by i.v. fresh frozen canine plasma (FFP) or by s.c. VitK_1. Top panel: tecarfarin (0.5 mg·kg^{-1}) was administered to dogs orally, QD, for 4 days. Venous blood was sampled each day at drug trough level, just before the next dose, and PT was measured. Data represent the mean ± SEM of PT per group of dogs (4 groups of 3 dogs), which were subsequently used in the reversal study: Dogs from group 1 subsequently received vehicle i.v. (saline), dogs from group 2 subsequently received vehicle subcutaneously (vitamin K [VitK] diluent, s.c.), dogs from group 3 subsequently received FFP (9 mL·kg^{-1}, i.v.) and dogs from group 4 subsequently received VitK (2.5 mg·kg^{-1}, s.c.). Middle panel: 24 h following the last dose of tecarfarin, also taken as time 0, the animals received FFP (FFP, 9 mL·kg^{-1}; group 3, $n = 3$) or vehicle (Veh, saline; group 1, $n = 3$) i.v. and PT were measured at indicated intervals. After 4 days of oral treatment with tecarfarin, baseline PT was 17.3 ± 1.0 s and 18.5 ± 0.7 s, for the FFP and vehicle group respectively. Data represent the mean values for the first 240 min ± SEM. Bottom panel: 24 h following the last dose of tecarfarin, also taken as time 0, the animals received VitK (2.5 mg·kg^{-1}; group 4, $n = 3$) or vehicle (Veh, VitK diluent; group 2, $n = 3$) subcutaneously and PT were measured at indicated intervals. After 4 days of oral treatment with tecarfarin, baseline PT was 17.3 ± 4.1 s and 18.8 ± 0.8 s, for the VitK and vehicle group respectively. Data represent the mean values ± SEM. Time 0 values represent mean PT for all tecarfarin-treated animals ($n = 12$) prior to initiation of rescue treatment. Baseline values represent mean PT values prior to any treatment. The PT values obtained at indicated intervals following the initiation of FFP or VitK 'rescue' were compared with both mean baseline and to PT prior to initiation of rescue. Data were analysed using Dunnett's multiple comparison test, *$P < 0.01$ compared to time 0 values; \$$P < 0.05$ compared to time 0 values; #$P < 0.01$ compared to baseline values; and †$P < 0.05$ compared to baseline values.

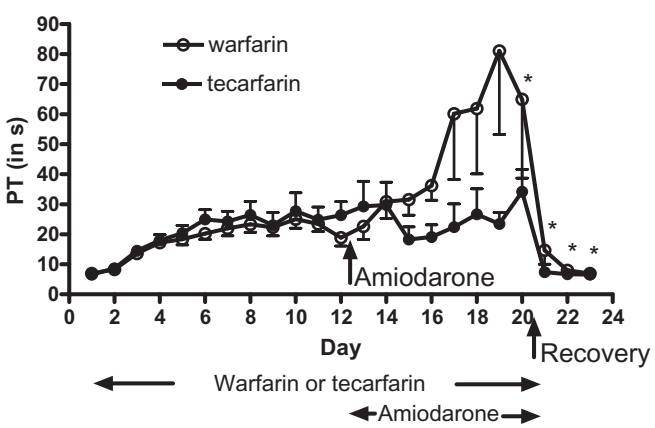


Figure 9 Effect of amiodarone on the coagulation of warfarin- or tecarfarin-treated beagle dogs. Groups of dogs were treated with tecarfarin (0.3 mg·kg^{-1} or adjusted dose; $n = 5$) or warfarin (0.25 mg·kg^{-1} or adjusted dose; $n = 6$) orally, QD, for 11 days to increase PT between 15 and 30 s. Amiodarone was then co-administered orally, QD, at 40 mg·kg^{-1} for 2 days and 20 mg·kg^{-1} for 6 days. PT values were measured daily. Data represent the mean PT values (in s) ± SEM. *$n = 5$: one dog had to be rescued with vitamin K (PT > 169 s).

In pre-clinical testing of new anticoagulants, drug development is complicated by two issues: (i) the utility of INR as a measure of anticoagulation has not been validated in animal models; and (ii) no 'gold standard' animal model exists to definitively predict the effectiveness of anticoagulant drugs in humans. In the light of these observations, when using animal models, drug effects on important VitK-dependent clotting factors (e.g. Factor VII and Factor X) should be used to complement measurements of INR as evidence of anticoagulation and potential antithrombotic efficacy. The recent approval by the FDA of the use of levels of Factor X as a surrogate to assess the degree of anticoagulation caused by warfarin in humans reaffirms the value of using Factor X in this manner (DiaPharma, 2004).

In the healthcare setting, to provide antithrombotic efficacy for major clinical indications such as atrial fibrillation, the steady-state drug levels of VKOR inhibitors need to be adjusted in a manner that reduces the functional (carboxylated form) levels of VitK-dependent clotting factors in the plasma to a degree that increases the values of INR to predetermined values based on clinical studies and outcomes.

The utilization of warfarin clinically is complicated by a number of factors. Recent pharmacogenomics studies

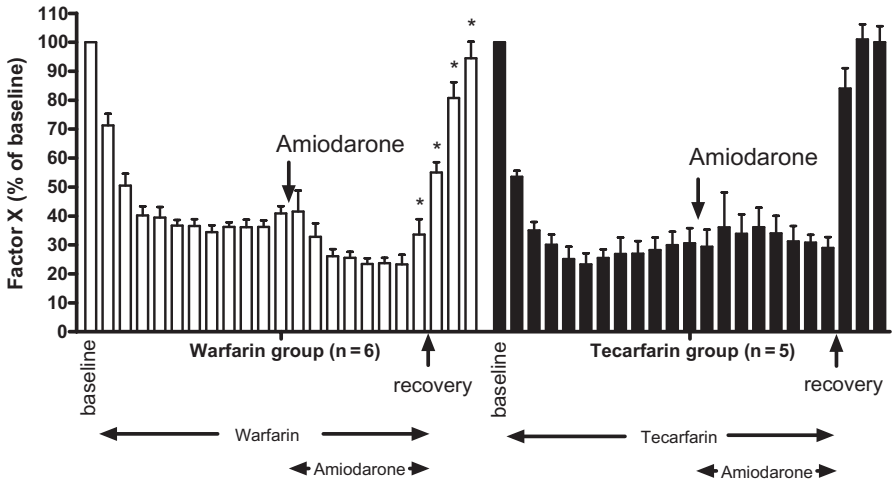


Figure 10 Effect of amiodarone on Factor X levels in warfarin- or tecarfarin-treated beagle dogs. Groups of dogs were treated with tecarfarin (0.3 mg·kg^{-1} or adjusted dose; $n = 5$) or warfarin (0.25 mg·kg^{-1} or adjusted dose; $n = 6$) orally, QD, for 11 days to increase prothrombin time (PT) between 15 and 30 s. Amiodarone was then co-administered orally, QD, at 40 mg·kg^{-1} for 2 days and 20 mg·kg^{-1} for 6 days. Factor levels were measured daily. Data represent the mean Factor X levels (in % from baseline) ± SEM. Data were analysed using the Wilcoxon signed rank test. The factor levels (warfarin and tecarfarin groups) obtained on days 10, 11 and 12 were averaged and compared to the average of days 15 through 19. The levels for the tecarfarin group were not statistically different but the *P*-value was 0.0313 for the warfarin group. *$n = 5$: one dog had to be rescued with vitamin K.

have identified genetic factors that influence warfarin-induced anticoagulation. Single nucleotide polymorphism in non-coding regions of the VKOR gene appear to determine the variability in warfarin response at the transcriptional level and approximately 25% of the variability in the required warfarin dose can be accounted for by genetic variations in VKOR (Rieder *et al.*, 2005). More importantly, patients with the common, functionally defective *2 and *3 allelic variants of the CYP2C9 enzyme require significantly lower maintenance doses of warfarin, and these CYP2C9 polymorphisms are associated with increased risk of bleeding (Higashi *et al.*, 2002). (S)-warfarin, the most potent enantiomer of commercial warfarin, is metabolized by CYP2C9 (Lindh *et al.*, 2005; Rieder *et al.*, 2005; Veenstra *et al.*, 2005). In addition, the relatively low therapeutic index of warfarin requires frequent visits to outpatient clinics to monitor the level of anticoagulation and drug–drug interactions, which affect the absorption or metabolic clearance of warfarin and therefore commonly modulate the level of anticoagulation (Serlin and Breckenridge, 1983). Despite the fact that oral warfarin for antithrombotic applications has a very favourable benefit–risk ratio, a VKOR inhibitor with an improved therapeutic index (e.g. CYP-independent metabolism, fewer adverse drug reactions) and requiring fewer visits to INR-monitoring clinics would be a valuable addition to the medical anticoagulant armamentarium. Towards this end, tecarfarin was found to be as potent a VitK antagonist as warfarin and its putative primary metabolite, ATI-5900, is essentially inactive with respect to human liver microsomal VKORC1 inhibition (Figure 2). This novel VKOR inhibitor has been designed to be hydrolysed by hCE in order to reduce the likelihood of drug–drug interactions with CYP450 inhibitors or inducers.

The primary objective of our studies was to determine the i.v. and oral doses of tecarfarin required to produce a steady state concentration consistent with anticoagulation and therapeutically effective antithrombotic actions and, also, to provide insight into the degree of VKOR inhibition produced after tecarfarin administration in beagle dogs compared to warfarin. Previous studies have shown that tecarfarin is hydrolysed via carboxylesterase to ATI-5900, and that this process is independent of CYP450. Studies using radioactively labelled tecarfarin in rats showed that approximately 60% of the radioactivity is excreted in the faeces and 30% excreted in the urine (data not shown). Continuous i.v. infusions of warfarin and tecarfarin caused a dose-dependent decrease in the levels of Factor VII and Factor X, and an increase in the values of INR (Figure 5). In the i.v. experiment, tecarfarin was also shown to be more potent than warfarin in inducing anticoagulation. These results suggest that tecarfarin causes changes in key parameters of haemostasis in dogs that are consistent with clinically effective anticoagulation. Based upon the doses used intravenously, oral doses (0.2–0.3 mg·kg^{-1} QD) of warfarin and tecarfarin were also found to dose-dependently increase the INR and decrease the levels of Factors VII and X. Unlike in the i.v. study, in this oral study tecarfarin did not appear to be more potent than warfarin (Figure 6). This may be due, among other possibilities, to either a lower bioavailability, or to a slower intestinal absorption of tecarfarin compared to warfarin, as suggested by the initial PK results.

To provide options to reverse possible excessive anticoagulation induced with the test compound, we assessed whether administration of FFP or VitK reversed tecarfarin-induced anticoagulation. In current guidelines, VitK (Crowther *et al.*, 2000) or FFP (see guideline for the use of fresh-frozen plasma, Medical Directors Advisory Committee, National Blood Transfusion Council, 1998) is the recommended treatment for excessive anticoagulation with warfarin (Yiu *et al.*, 2006). Both FFP and VitK treatment were effective at reversing the decrease in Factor VII and Factor X levels and the increase in PT values caused by 4 days of oral treatment with tecarfarin in

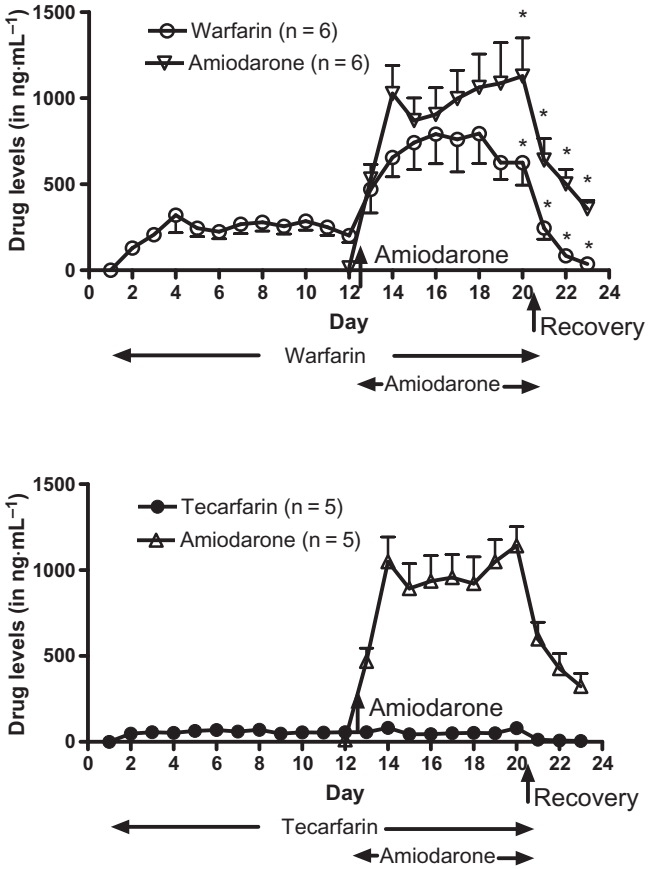


Figure 11 Effect of oral administration of amiodarone when co-administered with either warfarin or tecarfarin on drug (warfarin or tecarfarin) plasma levels in dogs. Groups of dogs were treated with tecarfarin (0.3 mg·kg^{-1} or adjusted dose; n = 5) or warfarin (0.25 mg·kg^{-1} or adjusted dose; n = 6) orally, QD, for 11 days to increase PT. Animals then received amiodarone (40 mg·kg^{-1} for 2 days then 20 mg·kg^{-1} for 6 days) orally, QD. Drug plasma levels were measured daily. Data represent the mean plasma level values ± SEM: warfarin group (top panel) and tecarfarin group (bottom panel). Data were analysed using the Wilcoxon signed rank test. The drug plasma levels (warfarin and tecarfarin) obtained on days 10, 11 and 12 were averaged and compared to the average of days 15 through 19. The tecarfarin levels were not statistically different but the P-value was 0.0313 for the warfarin group. *n = 5: one dog had to be rescued with vitamin K.

adult beagle dogs (Figure 8). This indicates they would be safe and efficient antidotes to possible excessive anticoagulation with this new compound. As suggested by the initial PK data in beagle dogs, the half-life of tecarfarin is shorter than the half-life of warfarin. As a result, s.c. VitK did not, initially, appear to reverse the anticoagulation effect of tecarfarin when compared to vehicle. This was especially noticeable because VitK was administered 24 h following the last dose of tecarfarin. By contrast, as expected, FFP had an immediate effect on reversing INR back to a physiological value. Nonetheless, despite the fact that Factors II and IX activities were not measured, the results of the reversal experiment, coupled with the finding that the INR induced by tecarfarin, similar to warfarin, is dependent on the depletion of coagulation Factors VII and X (Figure 7), and results of *in vitro* experiments in HLM, are strong evidence that the main mechanism of action of tecarfarin is, like warfarin, via inhibition of VitKO.

Finally, to assess the potential of tecarfarin to interact with other drugs, amiodarone was chosen as a typical CYP2C9 inhibitor that affects warfarin metabolism. Co-administration of amiodarone had no measurable effect on the blood levels or on the anticoagulation induced by tecarfarin in adult beagle dogs (Figure 11). In contrast, co-administration of amiodarone significantly increased warfarin levels and the resulting anticoagulation induced by warfarin.

In summary, the *in vitro* and *in vivo* pharmacological profile of tecarfarin is similar to that of warfarin. Effects on coagulation factors VII and X and reversal of anticoagulation by $VitK_1$, together with VKOR inhibition experiments in HLM, are strong evidence that the primary mechanism of action of tecarfarin, like warfarin, is VKOR inhibition. However, unlike warfarin, tecarfarin in beagle dogs is not metabolized through the CYP450 system and does not interact with amiodarone, a drug frequently co-administered with warfarin in the treatment of atrial fibrillation. These data suggest that tecarfarin may have a lower potential for adverse reactions resulting from interactions with other drugs or food that inhibit or induce CYP450 and with CYP450 genetic polymorphisms, and, therefore, may offer an improved safety profile to patients.

Conflict of interest

AER has no affiliation with ARYx Therapeutics; his laboratory performed the vitamin K epoxide redcutase assay in human liver microsomes. DMD is a Professor at University of Florida and is affiliated with ARYx. He is a shareholder and receives financial support from ARYx. All the other authors are full-time employees of ARYx and shareholders.

References

Almog S, Shafran N, Halkin H, Weiss P, Farfel Z, Martinowitz U *et al*. (1985). Mechanism of warfarin potentiation by amiodarone: dose- and concentration-dependent inhibition of warfarin elimination. *Eur J Clin Pharmacol* **28**: 257–261.

Crowther MA, Julian J, McCarty D, Douketis J, Kovacs M, Biagoni L *et al*. (2000). Treatment of warfarin-associated coagulopathy with oral vitamin K: a randomised controlled trial. *Lancet* **356**: 1551–1553.

DiaPharma (2004). *Factor X assay receives FDA approval for monitoring anticoagulation*. Available at: http://www.diapharma.com/company/news_archive/diapharma_factor_x_kit_cleared (accessed 2009).

Dowd P, Ham SW, Marchand AP, Rajapasksa D (1992). Mechanism of epoxidation of vitamin K with basic hydrogen peroxide. *J Org Chem* **57**: 3514–3516.

Higashi MK, Veenstra DL, Kondo LM, Wittkowsky AK, Srinouanprachanh SL, Farin FM *et al*. (2002). Association between CYP2C9 genetic variants and anticoagulation-related outcomes during warfarin therapy. *JAMA* **287**: 1690–1698.

Hirsh J, Dalen J, Anderson DR, Poller L, Bussey H, Deykin D (2001). Oral anticoagulants: mechanism of action, clinical effectiveness, and optimal therapeutic range. *Chest* **119**: 8S–21S.

Holbrook AM, Pereira JA, Labiris R, McDonald H, Douketis JD, Crowther M *et al*. (2005). Systematic overview of warfarin and its drug and food interactions. *Arch Intern Med* **165**: 1095–1106.

Irwin I, Davies A, Shao L, Kumaraswamy P, Lightning L, Choppin A *et al*. (2006). Pharmacokinetics and pharmacodynamics of ATI-5923, a novel VKOR inhibitor, in the dog. *Drug Metab Rev* **38**: 93.

Lindh JD, Lundgren S, Holm L, Alfredsson L, Rane A (2005). Several-fold increase in risk of overanticoagulation by CYP2C9 mutations. *Clin Pharmacol Ther* **78**: 540–550.

Medical Directors Advisory Committee, National Blood Transfusion Council (1998). Guideline for the use of fresh-frozen plasma. *S Afr Med J* **88**: 1344–1347.

Monnet E, Morgan M (2000). Effect of three loading doses of warfarin on the international normalized ratio for dogs. *Am J Vet Res* **61**: 48–50.

Odén A, Fahlén M (2002). Oral anticoagulation and risk of death: a medical record linkage study. *BMJ* **325**: 1073–1075.

Quick AJ (1935). The prothrombin time in hemophilia and in obstructive jaundice. *J Biol Chem* **109**: 73–74.

Rieder MJ, Reiner AP, Gage BF, Nickerson DA, Eby CS, McLeod HL *et al*. (2005). Effect of VKORC1 haplotypes on transcriptional regulation and warfarin dose. *N Engl J Med* **352**: 2285–2293.

Riegert-Johnson DL, Volcheck GW (2002). The incidence of anaphylaxis following intravenous phytonadione (vitamin K1): a 5-year retrospective review. *Ann Allergy Asthma Immunol* **89**: 400–406.

Serlin MJ, Breckenridge AM (1983). Drug interactions with warfarin. *Drugs* **25**: 610–620.

Usman MH, Notaro LA, Patel H, Ezekowitz MD (2008). New developments in anticoagulation for atrial fibrillation. *Curr Treat Options Cardiovasc Med* **10**: 388–397.

Veenstra DL, Blough DK, Higashi MK, Farin FM, Srinouanprachan S, Rieder MJ *et al*. (2005). CYP2C9 haplotype structure in European American warfarin patients and association with clinical outcomes. *Clin Pharmacol Ther* **77**: 353–364.

Yiu KH, Siu CW, Jim MH, Tse HF, Fan K, Chau MC *et al*. (2006). Comparison of the efficacy and safety profiles of intravenous vitamin K and fresh frozen plasma as treatment of warfarin-related over-anticoagulation in patients with mechanical heart valves. *Am J Cardiol* **97**: 409–411.