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RESEARCH PAPER

Effects of etravirine on the pharmacokinetics and pharmacodynamics of warfarin in rats

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BACKGROUND AND PURPOSE

Warfarin is often used with etravirine (ETV) to prevent HIV-related thromboembolic events. As both warfarin and ETV bind to plasma proteins and are metabolized by hepatic cytochrome P450s, they are likely to interact. Hence, we evaluated the effect of ETV on the pharmacokinetics and blood clotting time of racemic warfarin in rats.

EXPERIMENTAL APPROACH

Two groups of male Sprague-Dawley rats, in which the jugular vein had been cannulated, were studied. The control group (n = 10) received 1 mg·kg^{-1} racemic warfarin i.v., and the test group (n = 13) 1 mg·kg^{-1} of racemic warfarin followed by 25 mg·kg^{-1} ETV i.v. Serial blood samples were collected for up to 144 h and the blood clotting time (calculated as international normalized ratio [INR]) measured in blood plasma at each sample point. Plasma concentrations of R-warfarin, S-warfarin, R-7-hydroxywarfarin and S-7-hydroxywarfarin were measured by a LC/MS/MS method using a chiral lux cellulose-1 column. Pharmacokinetic parameters were analysed using non-compartmental methods.

KEY RESULTS

ETV significantly increased, by threefold, the systemic clearance and volume of distribution of S-warfarin, but not those of R-warfarin. ETV decreased the total AUC of warfarin, but had no effect on its elimination half-life. ETV also increased the systemic clearance of both R-7-hydroxywarfarin and S-7-hydroxywarfarin but only increased the volume of distribution of R-7-hydroxywarfarin. Interestingly, the effect of warfarin on blood clotting time (INR) was significantly increased in the presence of etravirine.

CONCLUSION AND IMPLICATIONS

Our data suggest that etravirine may potentiate the anticoagulant effect of warfarin and this could have clinical significance.

Abbreviations

Cl, clearance; ETV, etravirine; INR, international normalized ratio; MRT, mean residence time; NNRTI, non-nucleoside reverse transcriptase inhibitors; PD, pharmacodynamics; PK, pharmacokinetics; Q, blood flow; $t_{1/2}$, terminal half-life; Vd, volume of distribution; VKORC1, vitamin K epoxide reductase complex 1

Introduction

Warfarin is the most commonly prescribed anticoagulant in the world (Eriksson and Wadelius, 2012) and is used for the prevention of venous thromboembolism (Liu *et al.*, 2012), for the treatment of atrial fibrillation and deep vein thrombosis (Jones *et al.*, 2011). Warfarin extensively (99.83%) binds to the plasma protein albumin (Yacobi and Levy, 1975; Jensen

et al., 2012) and is metabolized by a pathway that involves cytochrome P450 (CYP450) (Kaminsky and Zhang, 1997). These factors indicate a potential for drug–drug interactions when it is co-administered with a wide range of drugs (Chu *et al.*, 2011). The warfarin molecule exists as enantiomers, which have identical chemical and physical properties, but exhibit different metabolic behaviour inside the body. Warfarin is given to patients as a racemic mixture consisting of equal amounts of the R- and S-enantiomers. The S-enantiomer is more potent (three to seven times) than the R-enantiomer in both humans and rats (Yacobi *et al.*, 1974; Jensen *et al.*, 2012). Warfarin is highly metabolized in the body by a stereo-specific pathway catalysed by cytochrome P450; R-warfarin is metabolized primarily by CYP3A4 to 10-hydroxywarfarin and CYP1A2 to 6- and 8-hydroxywarfarin, while S-warfarin is metabolized primarily by CYP2C9 to S-7-hydroxywarfarin (Kaminsky and Zhang, 1997). The most abundant metabolite of warfarin in humans is S-7-hydroxywarfarin (Jones *et al.*, 2010). The elimination half-life of warfarin is relatively long, that is 10–16 h in animals and 40–46 h in humans, which may potentially cause a significant change in its anticoagulant effect when it is administered concomitantly with other drugs (Panossian *et al.*, 2009).

The anticoagulant effect of warfarin occurs through a mechanism that involves inhibition of vitamin K epoxide reductase complex 1 (VKORC1) and vitamin K reductase. This prevents the formation of reduced vitamin K, which is essential for the hepatic synthesis of vitamin K-dependent coagulation factors II, VII, IX and X (Zhou and Chan, 2003). Clinically, one of the major challenges in warfarin therapy has arisen from the interindividual variation in response to the drug and hence deciding the correct therapeutic dose for each patient. Warfarin anticoagulant therapy is monitored by measuring the international normalized ratio (INR), a measure of three of the four vitamin K-dependent coagulation factors (II, VII and X). Normal coagulation time is indicated by an INR of 1 and when the INR is 2 the clotting time is doubled. If the INR is too high, the risk of internal bleeding is increased. Usually, a dose of 10–100 mg warfarin per week is sufficient to attain an optimal balance between adverse effect and efficacy. Hence, warfarin is considered to be a narrow therapeutic index drug (the therapeutic window range is an INR value of 2–3, Eriksson and Wadelius, 2012), and in humans, a precise control of the dosage regimen is essential.

Etravirine (ETV) is the first drug in the second generation of non-nucleoside reverse transcriptase inhibitors (NNRTIs) to be marketed for the treatment of HIV/AIDS. ETV is more effective than the first-generation NNRTIs as it is active against NNRTI-resistant HIV-1. It has properties that make it a suitable drug to be combined with other antiretrovirals for the chronic treatment of patients with HIV, who require therapy for prolonged periods of time (Boffito *et al.*, 2009). ETV is available in tablet form and the therapeutic dose is 200 mg p.o. twice a day. Most of the ETV (99.9%) administered binds to plasma proteins (Papendorp and Berk, 2009). The enzymes involved in the metabolism of ETV are CYP3A4, CYP2C9 and CYP 2C19 (Brown *et al.*, 2009). Interestingly, ETV, as well as being a substrate of these CYPs, also induces CYP3A4 but inhibits CYP2C9 and CYP2C19 (Kakuda *et al.*, 2012).

The rat model is commonly used as an animal model for the study of the effect of drug–drug and drug–herbal interactions on the pharmacokinetics and pharmacodynamics of drugs like warfarin (Slattery *et al.*, 1977; Yacobi *et al.*, 1980; Chan *et al.*, 2009; Chu *et al.*, 2011). It is probable that warfarin and ETV will interact when administered together as both are highly bound to protein and are metabolized by similar metabolic pathways. For most antiretrovirals, it is critical that their concentrations are maintained above the suggested minimum effective concentration throughout the dosing interval. However, long term use of NNRTIs can cause HIV-related thromboembolic events (Jacobson *et al.*, 2004; Ortiz *et al.*, 2007; Matta *et al.*, 2008), which require the administration of anticoagulants, such as warfarin. When ETV and warfarin were co-adminstered to humans, clinically, their effects suggest that a significant ETV-warfarin interaction occurs (Liedtke and Rathbun, 2009; Kakuda *et al.*, 2011). Also, an increase in INR value upon co- administration of warfarin and etravirne in humans has been reported (Nutescu *et al.*, 2011). However, information on the pharmacokinetic interaction between warfarin and ETV is limited. Therefore, the aim of the present study was to investigate the effect of ETV on the pharmacokinetics (PK) and pharmacodynamics (PD) of warfarin.

Methods

Chemicals and reagents

Racemic warfarin, racemic 7-hydroxywarfarin, acetaminophen (Internal Standard, IS), DMSO, HPLC-grade water and acetic acid was purchased from Sigma Aldrich, St. Louis (MO, USA). ETV as the standard powder (98% pure) was from Toronto research chemicals, Inc., North York (ON, Canada). Acetonitrile HPLC grade was from VWR International, Atlanta (GA, USA).

Pharmacokinetic studies in the rat

The animal experiment and protocol were reviewed and approved by the Institutional Animal Care and Use Committee at Texas Southern University. All experimental procedures were performed in accordance with the National Institutes of Health 'Guide for the Care and Use of Laboratory Animals, 8th Edition' (NIH Publication 2011).

Animals were housed separately in metabolic cages and kept in a 12 h light–dark cycle for a minimum of 7 days before being used in the experiments. The jugular veins of male adult Sprague-Dawley rats (Harlan Inc., Indianapolis, IN, USA) weighing 250–300 g were cannulated under anaesthesia the day before the study. The rats were anaesthetized with an i.m. injection of 1 mL kg^{-1} bodyweight using a cocktail containing ketamine (50mg mL^{-1}), xylazine (3.3mg mL^{-1}) and acetopromazine (3.3mg mL^{-1}) in sterile water for injection. Dosing solutions of racemic warfarin and ETV were prepared in normal saline and DMSO respectively. The rats were randomly divided into two groups. The treated group (n = 13) received 1 mg·kg^{-1} of racemic warfarin immediately followed by ETV (25 mg·kg^{-1}) i.v., whereas the control group (n = 10), received 1 mg·kg^{-1} of racemic warfarin and an equal volume of DMSO (without ETV) i.v. Blood samples (approxi-

mately 250 µL) were collected from the jugular vein cannula for up to 144 h. The samples were centrifuged at 17,383 *g* for 10 min, the plasma samples were collected, immediately stored at –80°C and analysed by an LC/MS/MS assay. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

LC/MS/MS analysis of warfarin in rat plasma

Plasma concentrations of R-warfarin, S-warfarin, R-7-hydroxywarfarin and S-7-hydroxywarfarin were determined by a LC/MS/MS assay method, which was previously validated (John *et al.*, 2012). Briefly, rat plasma sample (100 µL) was extracted and deproteinized by mixing it with 100 µL of an internal standard (IS) solution containing 500 ng of acetaminophen mL^{-1} of acetonitrile. The mixture was briefly vortexed for 30 s and centrifuged at 17,383 *g* for 5 min. Ten-microlitres of the supernatant was injected into the HPLC column. The chromatographic separation of warfarin enantiomers (R/S) and 7-hydroxywarfarin enantiomers (R/S) was achieved using a commercially available chiral column, Lux cellulose –1 with a dimension of 250 × 4.6 mm i.d. packed with 5-µm particles (Phenomenex, Torrance, CA, USA) in conjunction with a Lux Cellulose-1 guard column with a dimension of 4 × 3.0 mm (Phenomenex). A gradient elution was used, consisting of 0.1% acetic acid in water (mobile phase A) and 100% acetonitrile (mobile phase B). The injection volume was 10 µL and the total run time was 11 min. The LC/MS/MS analysis was done by using a 3200 QTRAP® (AB Sciex, Foster City, CA, USA) in the negative multiple reaction monitoring mode by monitoring the *m/z* transitions 323.0/176.9 for 7-hydroxywarfarin, *m/z* 307 → 161 for racemic warfarin, *m/z* 432.9 → 141.6 for ETV and *m/z* 149 → 107 for acetaminophen (I.S).

Pharmacodynamic studies in the rat

The degree of anticoagulation was assessed by means of the INR value measured by conducting a one stage INR test on the plasma sample, using a fully automated INR meter Roche CoaguChek® *XS* System (ThermoFisher Scientific, Houston, TX, USA). The INR measurements were performed on the same plasma samples collected for pharmacokinetic analysis. Approximately 8 µL of rat plasma was placed directly on the CoaguCheck *XS* PT Test test strips (ThermoFisher Scientific). The INR value was displayed in less than a minute and recorded. The *Roche CoaguChek XS* System provides test results if the INR value is between 0.8 and 8.0. If the results fall outside of this range, the meter displays <0.8 or >8.0. The *Roche CoaguChek XS* System has quality control functions integrated into the meter and the test strips. Hence the meter automatically runs its own quality control test as part of every test. *Roche CoaguChek XS* System is sensitive to various clotting factors including Factors II, V, VII, and X. The assay is also specific with no significant effect on test results in the presence of bilirubin up to 0.3 $mg{\cdot}mL^{-1}$; lipaemic samples containing up to 5 $mg{\cdot}mL^{-1}$ of triglcerides; haemolysis up to 10 $mg{\cdot}mL^{-1}$; heparin concentrations up to 0.8 $U{\cdot}mL^{-1}$; low molecular weight heparins up to 2 IU anti-factor Xa $activity{\cdot}mL^{-1}$; clopidogrel up to 0.2 $mg{\cdot}mL^{-1}$; and fondaparinux up to 5 $mg{\cdot}mL^{-1}$.

In vitro *effect of ETV on serum protein binding of warfarin enantiomers*

Plasma protein binding studies were conducted using an ultra filtration technique (Yu *et al.*, 2012). Briefly, freshly collected rat blank plasma samples were spiked with 20 $\mu g{\cdot}mL^{-1}$ of racemic warfarin with and without ETV (25 $\mu g{\cdot}mL^{-1}$). The plasma samples were equilibrated at 37°C for 30 min, and then transferred to ultra filtration centrifugal devices containing Amicon filter membranes (MW cut off = 30 000) and centrifuged for 10 min at 17,383 *g*. Aliquots of the ultra filtrate and the top non-filtrated plasma portion were analysed by the LC/MS/MS method. Each test was performed in triplicate.

Data analysis

Statistical interpretations of the data were conducted using SYSTAT 11 (SYSTAT Inc., Evanston, IL, USA). Sample data were expressed as means ± SD. Prior to the application of any statistical test, the Levene's Test for Equality of Variances was run on the variances of the observations in the individual groups. If the variances associated with any two mean values were statistically found to be homogeneous, then Student's *t*-test or Mann–Whitney *U*-test were used to determine the statistical significance between the control and the ETV treated groups. A *P*-value of less than 0.05 was considered as statistically significant. The i.v. plasma concentration-time data were pharmacokinetically analysed separately for each rat by classical non-compartmental techniques using the microcomputer-based nonlinear regression program, WinNonlin 2.1 (Pharsight Corporation, Mountain View, CA, USA). The noncompartmental pharmacokinetic parameters thus derived were the systemic plasma clearance (Cl), the apparent volume of distribution (Vd), the total area under the plasma drug concentration-time curve extrapolated to time infinity (AUC) and the mean residence time (MRT).

Results

Pharmacokinetics of R-warfarin and S-warfarin when co-administered with ETV

Figure 1A shows the mean plasma concentration versus time curve of R-warfarin with and without ETV. Co-administration of ETV lowered the plasma concentration of R-warfarin. The R-enantiomer in the control group was below the detection limit after 96 h whereas in the treated group, it was detectable up to 120 h. Figure 1B illustrates the mean plasma concentration versus time profile for S-warfarin. Similarly, the presence of ETV significantly lowered the plasma concentration of S-warfarin. S-warfarin was detectable up to 144 h after warfarin administration. In comparison, R-warfarin appeared to be eliminated more quickly than S-warfarin.

Table 1 summarizes the pharmacokinetics of R-warfarin and S-warfarin. For S-warfarin, a threefold increase was observed in the systemic Cl (4.13 $mL{\cdot}h^{-1}{\cdot}kg^{-1}$ without ETV vs. 12.99 $mL{\cdot}h^{-1}{\cdot}kg^{-1}$ with ETV) as well as the Vd (115.0 $mL{\cdot}kg^{-1}$ without ETV vs. 394.2 $mL{\cdot}kg^{-1}$ with ETV). R-warfarin also showed an increase in Cl and Vd, but these values were not statistically significant. ETV decreased the mean AUC_{∞} of both S-warfarin (188.7 $\mu g{\cdot}h{\cdot}mL^{-1}$ without ETV vs.

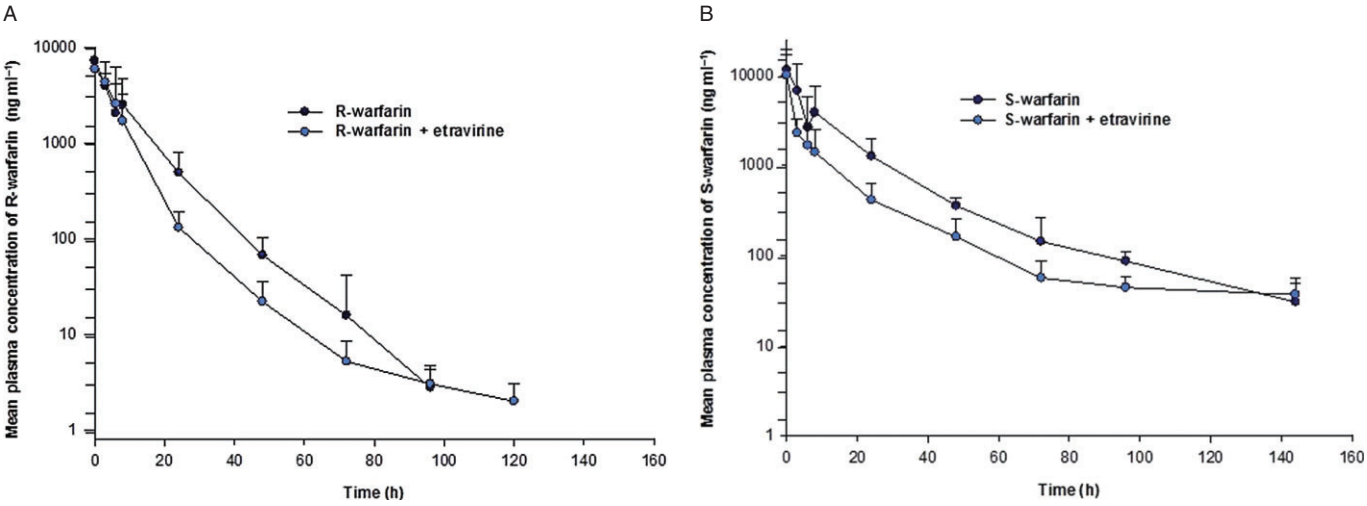

Figure 1

(A) Mean ± SD plasma concentration – time profiles of R-warfarin after i.v. administration of 1 mg·kg^{-1} racemic warfarin alone (n = 10) and after co-administration of 1 mg·kg^{-1} racemic warfarin and 25 mg·kg^{-1} etravirine i.v. (n = 13) in male Sprague-Dawley rats. (B) Mean ± SD plasma concentration – time profiles of S-warfarin after i.v. administration of 1 mg·kg^{-1} racemic warfarin alone (n = 10) and after co-administration of 1 mg·kg^{-1} racemic warfarin and 25 mg·kg^{-1} etravirine i.v. (n = 13) in male Sprague-Dawley rats.

Table 1

Comparative mean (±SD) pharmacokinetic parameters for R-warfarin and S-warfarin in male Sprague-Dawley rats after concomitant administration of racemic warfarin and etravirine i.v.[a]

Parameter	Warfarin alone	Warfarin + etravirine
n	*10*	13
	R-Warfarin	
AUC_{∞} (μg·h·mL^{-1})	74.34 ± 45.4	24.65 ± 7.86**
$t_{1/2}$ (h)	9.42 ± 2.0	13.4 ± 6.4
Vd (mL·kg^{-1})	248.5 ± 243	463.8 ± 332
Cl (mL·h^{-1}·kg^{-1})	16.8 ± 14	22.69 ± 8.90
MRT (h)	9.63 ± 3.5	8.89 ± 3.0
	S-warfarin	
AUC_{∞} (μg·h·mL^{-1})	188.7 ± 87.5	36.82 ± 13.0***
$t_{1/2}$ (h)	21.50 ± 11.2	21.01 ± 6.32
Vd (mL·kg^{-1})	115.0 ± 49.1	394.2 ± 149***
Cl (mL·h^{-1}·kg^{-1})	4.13 ± 1.7	12.99 ± 2.39***
MRT (h)	13.5 ± 4.5	18.83 ± 5.58*

Dat shown are means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.
[a]The rats were concomitantly administered 1mg·kg^{-1} racemic warfarin and 25mg·kg^{-1} etravirine i.v.
n, number of rats employed; AUC_{∞}, total area under the plasma concentration versus time curve; $t_{1/2}$, terminal half-life; Vd, apparent volume of distribution; Cl, systemic plasma clearance; MRT, mean residence time.

36.82 μg·h·mL^{-1} with ETV) and R-warfarin (74.3 μg·h·mL^{-1} without ETV vs. 24.65 μg·h·mL^{-1} with ETV). Half-life, both R- and S-warfarin did not show any statistically significant difference between warfarin alone or in combination with ETV group. For controls, S-warfarin demonstrated a higher AUC and half-life, but a lower Vd and Cl compared with its R-enantiomer.

Table 2 shows the pharmacokinetic parameters of the 7-hydroxywarfarin enantiomers in rats. Co-administration of ETV resulted in a significant increase in the Cl for R-7-hydroxywarfarin and S-7-hydroxywarfarin. Even though there was a significant, threefold, increase in the Vd for R-7-hydroxywarfarin in the presence of ETV, the S-7-hydroxy metabolite's increase in Vd was not significant. The half-life and AUC were decreased significantly in both the enantiomeric metabolites in the presence of ETV.

Pharmacodynamics of warfarin when co-administered with ETV

Figure 2 shows the comparison of the INR values between the warfarin only group and the warfarin plus ETV group. As expected, the mean INR value of the ETV-treated group was increased compared with the control group. Racemic warfarin administration with or without ETV led to a wide range of INR values, as measured by the device. The blank plasma prior to warfarin administration showed a mean INR value of 1.5 ± 0.1. The INR values measured throughout the experiments were in the range 2.43–8. The INR value was maximal at 24 h in both groups of rats and did not show any SD. When the INR values at each time point were compared between the control and treated groups, the INR values were significantly increased at 3 h (P = 0.0301) and 6 h (P = 0.0047) in the presence of ETV compared with the control group. The INR value did not return to the baseline value even after 6 days of warfarin administration (INR 4.9 control vs. INR 6.5 treated).

In vitro *effect of ETV on plasma protein binding of warfarin enantiomers*

In the presence of ETV, the amount of warfarin observed bound to protein was significantly decreased (99.34% ± 0.06

Table 2

Comparative pharmacokinetic parameters for racemic 7-hydroxywarfarin in male Sprague-Dawley rats after concomitant administration of racemic warfarin and etravirine[a]

Parameter	Warfarin alone	Warfarin + etravirine
n	6	6
	R-7OH warfarin	
AUC_{∞} (μg.h·mL^{-1})	1.90 ± 0.3	0.813 ± 0.34**
$t_{1/2}$ (h)	16.1 ± 3.0	15.25 ± 1.90*
Vd (L·kg^{-1})	5.98 ± 1.6	14.49 ± 2.90***
Cl (mL·h^{-1}·kg^{-1})	256.1 ± 36.5	675.9 ± 178***
MRT (h)	28.7 ± 4.4	15.89 ± 3.75***
	S-7OH warfarin	
AUC_{∞} (μg.h·mL^{-1})	1.410 ± 0.19	0.466 ± 0.04***
$t_{1/2}$ (h)	19.45 ± 2.80	13.2 ± 5.8*
Vd (L·kg^{-1})	9.34 ± 1.6	19.00 ± 10.0
Cl (mL·h^{-1}·kg^{-1})	335.4 ± 44.3	1104.7 ± 208.0***
MRT (h)	32.81 ± 4.60	15.50 ± 3.66***

Data shown are mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.
[a]The rats were concomitantly administered1mg·kg^{-1} racemic warfarin and 25mg·kg^{-1} etravirine i.v.
n, number of rats employed; AUC_{∞}, total area under the plasma concentration versus time curve; $t_{1/2}$, terminal half-life; V_d, apparent volume of distribution; Cl, systemic clearance; MRT, mean residence time.

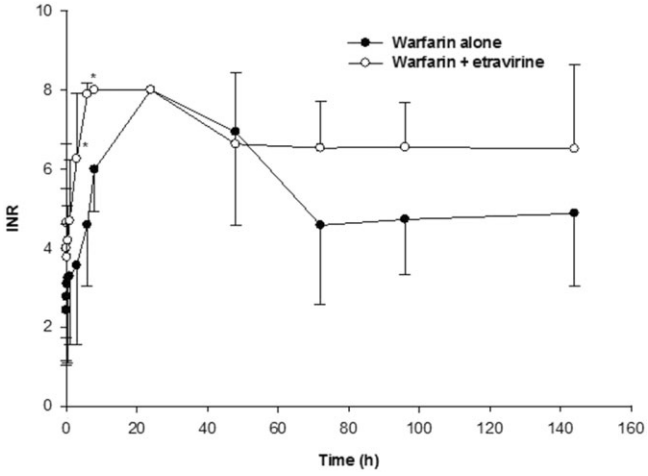

Figure 2

Mean international normalized ratio (INR) values – time profile from 0.03–144 h following i.v. administration of racemic warfarin (1 mg·kg^{-1}) (control group, n = 6) and after co-administration of 1 mg·kg^{-1} racemic warfarin with 25 mg·kg^{-1} etravirine i.v. Data are shown as means ± SD.*P < 0.05 versus control for 3 h and 6 h.

vs. 92.38% ± 0.04 (+ETV), P < 0.01 for R-warfarin and 99.26% ± 0.08 vs. 91.79% ± 0.09 (+ETV), P < 0.01 for S-warfarin).

Discussion and conclusions

The purpose of this study was to evaluate the effect of ETV on the pharmacokinetics and blood clotting time of racemic warfarin. The warfarin dose (1 mg·kg^{-1}) was chosen based on the multiple studies reported in the literature (Yacobi *et al.*, 1980; Levi *et al.*, 2003), whereas ETV dose was chosen based on the studies in our lab. All the rats were chosen randomly based on their body weight. Even though we started with an equal number of animals in both the control and treated groups, some rats were not used due to abnormal blood flow. We found that ETV significantly increased the systemic clearance of S-warfarin, its Vd and INR value in rats, but not the terminal half-life. The increase in S-warfarin systemic Cl in the presence of ETV most likely involves warfarin displacement from its protein binding sites by ETV. An *in vitro* plasma protein binding study conducted in pooled rat plasma further confirmed this displacement (unpublished data). Both warfarin and ETV are extensively bound to the plasma protein, albumin. Hence, ETV might have competitively displaced warfarin from albumin, a major binding site for warfarin. According to Bird and Carmona (2008), drugs with an extensive protein binding nature displace other drugs with the same characteristics when added as a cotreatment. Because warfarin has a narrow therapeutic index, this type of interaction, which increases its unbound plasma concentration, has prominent clinical significance. The pharmacological action of warfarin is highly dependent on the unbound warfarin concentration. Based on currently available information, it is also possible that increased Cl observed for S-warfarin is due to competition for the metabolizing enzyme between S-warfarin and ETV. S-warfarin is metabolized primarily by CYP2C9. The predominant metabolizing enzymes for ETV are CYP3A4, CYP2C9 and CYP 2C19. Interestingly, ETV is a substrate and inhibitor of CYP2C9 and CYP2C19. CYP2C9 plays an important role in the hepatic metabolism of both S-warfarin and ETV, therefore, it is possible that these two drugs compete with each other for their metabolizing enzyme and thereby ETV inhibits the metabolism of S-warfarin. This would decrease systemic Cl of S-warfarin which was not the case in our study.

Previous studies have demonstrated that these types of interactions may result in changes in binding characteristics of either drug (Sands *et al.*, 2002). The findings of warfarin–ibuprofen interaction studies in rats (Slattery *et al.*, 1977) are consistent with the notions that a highly protein-bound acidic drug like ETV will displace warfarin from the plasma proteins. Ibuprofen is extensively bound to protein in human plasma (Melillo *et al.*, 2004) (99% versus the 99.9% for ETV) and it has a pKa of 4.4 (Yazdanian *et al.*, 2004), which compares to 3.75 for ETV (Abobo *et al.*, 2010). The warfarin–ibuprofen interaction study confirmed that highly protein-bound acidic drugs increase the total Cl of warfarin, as observed in the present study. Moreover, an interaction study between warfarin and phenylbutazone in rats (Yacobi *et al.*, 1980) demonstrated an increased systemic clearance of warfarin on concomitant administration of the two drugs.

Phenylbutazone has extensive, 98% protein-binding characteristics (Sulkowska *et al*., 2008) similar to ETV (Papendorp and Berk, 2009).

In addition, theoretical considerations indicate that an essentially linear relationship exists between the systemic plasma clearance and unbound fraction of warfarin (Yacobi *et al*., 1980). This can be explained by following equations (see Sands *et al*., 2002).

$$Cl = Q[f_u(Cl_{int})]/\{Q + [f_u(Cl_{int})]\} \quad (1)$$

Where, Q = blood flow; f_u = unbound fraction; and Cl_{int} = intrinsic clearance.

For low hepatic clearance drugs, like warfarin, where Cl_{int} is small (extraction ratio, E = 0.003), Q is much larger than $[f_u\ (Cl_{int})]$. Thus, the denominator of the clearance equation becomes almost equal to Q, that is, $Q + [f_u\ (Cl_{int})] \sim Q$. In other words,

$$Cl \sim f_u(Cl_{int}) \quad (2)$$

Hence, an increase in systemic clearance could be due to an increase in unbound warfarin in blood. Patients with low warfarin Cl_{int} (e.g. cardiac and hepatic function-impaired patients) might be at greatest risk of adverse effects from this potential interaction. Even though systemic clearance of R-warfarin was increased in our study, it was not statistically significant. These may be attributed to a difference in the metabolic pathways of R-warfarin and ETV.

ETV co-administration significantly increased the Vd of S-warfarin in rats. This effect might be attributed to the fact that ETV administration increased the unbound warfarin concentration in plasma due to displacement of warfarin from its protein binding sites. A similar linear relationship between unbound warfarin and apparent Vd has been reported by Yacobi and Levi (1977) and Yacobi *et al*. (1980), explaining the protein binding of warfarin in rats. This can be explained further by the following equation (Eqn. 3) (Rowland and Tozer, 1995, third edition). As the unbound fraction of warfarin increases in plasma, there is an increase in the Vd. Although the Vd of R-warfarin was increased, this was not statistically significant.

$$V = V_P + V_{TW} \cdot f_U / f_{UT} \quad (3)$$

Interestingly, the increased effect of ETV observed on the Cl and Vd of the more potent S-warfarin was also associated with a significant effect on warfarin pharmacodynamics. In the present study, the pharmacodynamic results obtained in the presence of ETV are in agreement with the pharmocokinetic results. The significantly increased INR values for warfarin in the presence of ETV suggest that ETV potentiates the anticoagulant action of warfarin. These increased INR values could be due to an increased free fraction of S-warfarin in blood resulting from competitive protein binding displacement by ETV. It should be noted that the INR values did not return to the baseline even after 6 days (144 h) of warfarin administration (INR 4.9 control vs. INR 6.5 treated). The prolonged INR value is apparently related to the higher concentration of unbound warfarin in plasma. Similar results were observed in previous warfarin–drug interaction cases reported in patients (Liedtke and Rathbun, 2009). Furthermore, Nutescu *et al*. (2011) reported an increase in INR value upon co-administration of warfarin and ETV in humans. It is noteworthy to point out that the terminal half-life of both R-warfarin and S-warfarin was unaffected by the presence of ETV.

ETV was recently marketed as a treatment of HIV infection (Johnson and Saravolatz, 2009). A study (Kakuda *et al*., 2011) on the pharmacokinetic interactions between warfarin and non-antiretroviral drugs reported that the INR should be monitored when combining ETV with warfarin. Moreover, Liedtke and Rathbun (2009) reported that a potential warfarin–ETV interaction is anticipated upon their concomitant administration. So far, four cases of warfarin–NNRTIs interaction have been reported by Liedtke *et al*. Interestingly, the warfarin–ETV (NNRTI) interaction reported by Liedtke *et al*. is very similar to that of the present study in that ETV potentiated the anticoagulant effect of warfarin. Similar to the results of our study, the INR value was increased to 7 upon concomitant administration of ETV with warfarin. However, no studies have, as yet, been performed in humans or in animal models to elucidate the interaction between warfarin and ETV.

The simultaneous enantioseparation of racemic warfarin and 7-hydroxywarfarin in rat plasma was made possible by means of a chiral column (Lux cellulose –1) with superior specificity offered by LC/MS/MS. This enabled us to simultaneously characterize R-warfarin, S-warfarin, R-7-hydroxywarfarin and S-7-hydroxywarfarin in our rat samples. Warfarin metabolism follows a highly stereo-specific pathway (Zhou and Chan, 2002). S-warfarin is metabolized by CYP2C9 to form the primary metabolite 7-hydroxywarfarin and the minor metabolite 6-hydroxywarfarin, whereas R-warfarin is principally eliminated by CYP3A4 metabolism to form 10-hydroxywarfarin (Kaminsky and Zhang, 1997). Because S-warfarin is the pharmacologically active form, we studied its major metabolite, S-7-hydroxywarfarin, in the present study.

Table 2 shows the effect of ETV on the pharmacokinetics of 7-hydroxywarfarin. The systemic Cl and Vd for both R- and S-7-hydroxywarfarin was increased similar to their parent enantiomers. This effect could be attributed to a linear relationship between intrinsic Cl and unbound free fraction of 7-hydroxy metabolite as seen in Eqn. 2 and 3. The AUC, half-life and MRT were decreased for both the R- and S-warfarin metabolites, probably due to the distribution of the unbound 7-hydroxy warfarin metabolite to tissues and its elimination through different pathways.

Highly stereo-selective pharmacokinetics and pharmacodynamics makes warfarin susceptible to numerous drug–drug interactions. S-warfarin is metabolized by the polymorphic CYP2C9 enzyme and hence is the substrate selected to investigate the drug–drug interactions for CYP2C9 by the US Food and Drug Administration (FDA), while R-warfarin is metabolized by a combination of CYP isoforms including CYP1A2 and CYP 3A4. It has also been reported that the anticoagulant action of racemic warfarin is mostly due to the S-warfarin and to predict any drug–drug interaction requires only the monitoring of S-warfarin (Klein *et al*., 2012).

In the present study we showed that the concomitant administration of ETV with warfarin significantly increased the Vd and Cl of S-warfarin. These effects may be attributed to the displacement of warfarin from protein binding sites and a competitive inhibition of S-warfarin metabolizing enzymes

by ETV. The pharmacodynamic results further confirmed this drug–drug interaction. Further clinical studies are warranted to evaluate such drug interactions in humans.

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Conflict of interest

None.

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