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Research Article

# Protein Binding Drug-Drug Interaction between Warfarin and Tizoxanide in Human Plasma

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## Abstract

The goal of the in vitro research was to evaluate the potential for inhibition of warfarin protein binding by tizoxanide. Warfarin was of particular interest for the present investigation because it has been shown to be highly bound to plasma proteins and is a narrow therapeutic index drug. Tizoxanide is an active metabolite of an anti-infective prodrug nitazoxanide and also highly protein-bound medication. Both drugs are expected to be co-administered clinically. Protein binding of warfarin was investigated using a centrifugal ultrafiltration technique. Co-administration of tizoxanide significantly inhibited protein binding of warfarin for all concentrations tested. Tizoxanide increased free fraction ( $f_u$ ) of warfarin 34, 28, and 20-fold for concentration of 50, 75, and 100  $\mu\text{g/ml}$ , respectively. The interaction could potentially result in increasing the toxicity of warfarin therapy and the risk of bleeding.

**Keywords:** Protein binding; Interaction; Warfarin; Tizoxinide

## Introduction

Many drugs are bound to proteins within the body. There is marked variability in the extent to which the drugs bind to the proteins. The degree of drug-protein binding might affect a drug's efficiency. Drugs can be bound to plasma proteins as well as tissue proteins. Plasma protein binding can have multiple effects on the pharmacokinetics (PK) of a drug. The binding of drug to plasma proteins is a major determinant of drug disposition. When a drug is bound to the plasma proteins, it is not actively distributed to the site of action to interact with the target tissues. Moreover, the bound drug is kept in the blood, while the unbound (free) fraction may be metabolized or excreted. The binding has a very important effect also on drug pharmacodynamics (PD). Only the free drug interacts with receptors, therefore only the free drug can produce therapeutic effect. Common blood proteins that bind drugs are human serum albumins, lipoproteins, glycoproteins, and globulins. Albumin is the most abundant protein in the blood plasma. Many medications extensively bind to the albumins.

Protein binding interactions are displacement reactions, which have been implicated as the causative mechanisms in many drug-drug interactions. Competitive displacement interactions are predominant ones among protein binding reactions. The competitive protein binding drug-drug interactions result in increased the free plasma concentrations of the displaced medications. Clinically displacement reactions acquire importance when the displaced drug is highly bound to plasma proteins. In this case the displacer drug produces a very rapid and significant increase in the plasma concentrations of the displaced medication. The result of this interaction can be dramatic if the second drug is a drug with narrow therapeutic index. Such type of interactions can lead to enhanced pharmacological effects and, possibly, toxicity of the displaced medication.

A simple, generalized guideline for the evaluation of the clinical significance of protein binding interactions frequently cannot be applied. Instead, a careful analysis of protein binding effects must be

made on a drug-by-drug basis. Free, unbound concentrations should always be measured rather than estimated, and used as the primary input for PK/PD correlations, as well as for statements on the safety and efficacy of drugs [1].

Warfarin (Coumadin<sup>TM</sup>) is a widely used anticoagulant. Warfarin is indicated for treatment of venous thromboembolism, atrial fibrillation, mechanical and bioprosthetic heart valves, post-myocardial infarction, and recurrent systemic embolism. Warfarin consists of a racemic mixture of two active enantiomers: *R*- and *S*-forms [2,3]. Warfarin is a narrow therapeutic range drug and can cause major or fatal bleeding. Approximately 99% of the drug is bound to human plasma albumins [3]. One of the pharmacokinetic mechanisms for drug interactions with warfarin is protein binding interaction. This interaction can result in displacement of warfarin from the protein binding site, increase warfarin free plasma concentration, and increase the risk of warfarin toxicity. Free drug plasma concentrations must be carefully monitored during warfarin therapy.

Nitazoxanide (Alinia<sup>TM</sup>) is an anti-infective prodrug, which converts to an active metabolite tizoxanide very fast. The parent nitazoxanide is not detected in plasma. Tizoxanide is active against anaerobic bacteria, protozoan parasites, and viruses [4,5]. Tizoxanide is highly bound to plasma proteins (>99 %) [4,6]. Caution should be used when administering nitazoxanide concurrently with other highly plasma protein-bound drugs, especially drugs with narrow therapeutic indices, as competition for binding sites may occur.

Tizoxanide is compound that is expected to be co-administered with warfarin clinically and have the potential to alter warfarin protein binding. No drug-drug protein binding interaction studies have been reported for warfarin and tizoxanide.

The overall goal of the research was to evaluate the potential for inhibition of protein binding of warfarin by tizoxanide, which can be expected to be co-administered with warfarin in the patients.

## Materials and Methods

### Materials

Warfarin, DMSO, acetonitrile and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Tizoxanide was purchased from Cayman Chemical (Tallinn, Estonia). HPLC nylon filter (0.45 micron, 47 mm) was purchased from Pall Life Science (Port Washington, NY). Human plasma was purchased from Valley Biomedical (Winchester, VA). Amicon Centrifree YM-30 (molecular weight cut off 30K) centrifugal filter devices were obtained from Millipore Corporation (Billerica, MA, USA).

### Study groups

The studies were divided into two phases. Four experiments were performed for each study group. Phase I studies generated baseline protein binding values for warfarin. The compound was dosed alone. Phase II studies were conducted to assess the effect of tizoxanide on warfarin protein binding. Warfarin and tizoxanide were dosed together. Tizoxanide was selected based on its potential to be co-administered with warfarin in the clinical setting as well as its protein binding profile. Warfarin protein binding data of Phase I studies were compared to the warfarin binding values of Phase II studies. Warfarin concentrations were determined by HPLC.

### Analytical method for warfarin

Warfarin was measured in all samples by validated HPLC method. Separation was accomplished using a C18 column (100 mm X 4.6 mm, 2.6 $\mu$ , and 100A<sup>o</sup>) with a pre-column filter. The C18 column was temperature controlled at 30°C. Measurements were performed with ultraviolet detection at a wavelength of 220 nm. Mobile phase consisted of 35% acetonitrile and 65% 10 mM phosphate buffer (KH<sub>2</sub>PO<sub>4</sub>, pH 6.5). Analysis was conducted at ambient temperature at a constant flow rate of 1.0 mL/min using isocratic elution. Six microliters of the resultant ultrafiltrate was injected into the HPLC.

### Binding assessments

**Nonspecific binding to ultra filtration device:** A preliminary study was performed to identify warfarin nonspecific binding to the ultrafiltration device. Aqueous solution of warfarin was added to an ultrafiltration device (Amicon Centrifree Micropartition System<sup>®</sup>). The system was centrifuged for 15 minutes. The ultrafiltrate was assayed to determine warfarin binding to the ultrafiltration system. Samples were analyzed by HPLC. The studies were performed in triplicate.

**Protein binding determination:** Plasma protein binding of warfarin was evaluated using ultrafiltration. Studies were carried out at physiologic temperature and pH. Aliquots of human plasma were placed in test tubes and spiked with stock solutions of warfarin to generate samples of varying concentrations (e.g., 50, 75, and 100  $\mu$ g/ml). **Tizoxanide (500  $\mu$ g/mL)** was added only for interaction studies (Phase II). Samples were allowed to equilibrate in shaker bath (37OC) for 30 minutes. An aliquot (1 ml) of warfarin human plasma was added to an Amicon Centrifree Micropartition System<sup>®</sup> (MW cutoff 30,000). The system was centrifuged for 15 minutes @ 1500 g. The ultrafiltrate was assayed for determination of free warfarin concentrations. Samples were analyzed by HPLC. All experiments were performed in quadruplicate.

### Data analysis

Unbound fraction ( $f_u$ ) of warfarin was calculated as a ratio of unbound (ultrafiltrate) concentration and total plasma concentration.

$$f_u = \frac{C_u}{C_t}$$

Where:  $f_u$  is unbound (free) fraction

$C_u$  is unbound (free) concentration of warfarin

$C_t$  is total concentration of warfarin

### Statistical analysis

Mean estimates of protein binding parameters were compared using ANOVA. In this manner, differences in protein binding of warfarin were detected between study groups. Statistical significance was taken as a p-value <0.05.

## Results

Protein binding of warfarin was investigated using a centrifugal ultrafiltration technique. Experiments were conducted over a range of warfarin concentrations from 50 to 100 $\mu$ g/mL. Preliminary studies (i.e., fraction unbound  $\sim$  1) found negligible binding of warfarin to the ultrafiltration device (Amicon Centrifree<sup>™</sup> YM-30 micropartition devices). Warfarin protein binding data presented in Table 1.

Phase I studies generated baseline protein binding values for warfarin. Mean fraction unbound ( $f_u$ ) of warfarin was 0.012 in Phase I studies. No significant differences were found between concentrations studied.

Phase II studies were conducted to evaluate protein binding of warfarin in the presence of tizoxanide. Mean fraction unbound of warfarin in Phase II studies was 0.31. Tizoxanide increased free fraction ( $f_u$ ) of warfarin 34, 28, and 20-fold for concentration of 50, 75, and 100  $\mu$ g/ml, respectively. The results revealed that protein binding values were not significantly different within both study groups.

## Discussion

This research was conducted to characterize the effect of tizoxanide on protein binding of warfarin. Warfarin was of particular interest for the present investigation because it has been shown to be highly bound to plasma proteins and is a narrow therapeutic index drug. Tizoxanide, an active metabolite of an anti-infective prodrug nitazoxanide, is highly bound to plasma proteins and expected to be co-administered with warfarin clinically. There is a possibility for protein binding drug-drug interaction between warfarin and tizoxanide.

**Table 1:** Protein Binding of Warfarin.

Study Groups	Warfarin Concentrations ( $\mu$ g/mL)	Fraction Unbound of Warfarin*
Phase I: Warfarin alone	50	0.010 (0.003)
	75	0.011 (0.002)
	100	0.014 (0.001)
Phase II: Warfarin + Tizoxanide	50	0.339 (0.024)
	75	0.313 (0.008)
	100	0.277 (0.061)

\*Data reported as mean (SD)

The study evaluated the potential for statistically significant drug–drug interaction between warfarin and tizoxanide. Co-administration of tizoxanide significantly inhibited protein binding of warfarin for all concentrations tested. Tizoxanide amplified free concentrations of warfarin by displacing warfarin from the protein binding site.

The studies on supermolecular interaction of warfarin with human albumins revealed that warfarin predominantly bound to albumins [7]. Protein binding of tizoxanide is not well investigated. Some studies were conducted in goat plasma and in the solutions of albumin and alpha-1-acid-glycoprotein. The investigation showed that tizoxanide in plasma and in the albumin solution achieved a protein binding percentage of more than 95%, while in the solution of alpha-1-acid-glycoprotein; the percentage was only about 49%. The result suggested that tizoxanide has much more potent binding ability with albumin than with alpha-1-acid-glycoprotein, resulting from its acidic property [6].

The both compounds warfarin and tizoxanide bind predominantly to albumins and, most probably, competed for the same binding site of the protein. Albumins have two main drug binding sites characterized as Sudlow site I and Sudlow site II [8]. These sites bind drugs selectively. Warfarin primarily binds to the site I [9,10]. Because tizoxanide displaced warfarin from its binding site we can suggest that tizoxanide also has high affinity to the albumin binding site I and the both drugs have competed for the shared binding Sudlow site I of albumins. This assumption requires further investigation.

Displacement of one drug by another from blood proteins can modify the pharmacokinetics of the displaced drug. For some drugs, displacement from blood proteins causes only mild changes in free plasma drug concentrations and the altered plasma binding would not be expected to influence the pharmacological response. In this case dose adjustments are not required.

However, plasma binding displacement interactions become clinically important when the displaced medication is a narrow therapeutic index drug. There has been concern that inhibition of warfarin protein binding could affect the anticoagulant activity of the drug. By decreasing protein binding of warfarin, tizoxanide increased the plasma concentrations of free warfarin, although total drug concentrations in blood were unchanged. This could result in enhanced pharmacological response and increased toxicity of warfarin. It is very dangerous for the patient stabilized on warfarin. Such interaction can increase the risk of bleeding and may require a dose adjustment (reduction) of warfarin.

Furthermore, tizoxanide may displace other highly albumin-bound drugs from their binding sites. Caution should be used when administering nitazoxanide concurrently with highly plasma protein-bound drugs, especially drugs with narrow therapeutic

indices, as competition for binding sites may occur. Future studies are required to monitor and evaluate the effects of these potential drug–drug interactions. The studies will include in vitro experiments to assess valuable kinetic parameters of the interaction followed by experiments to evaluate the magnitude of the interaction in vivo.

## Conclusion

The studies provided meaningful insights regarding the inhibition of warfarin protein binding. Protein-binding displacement interactions were observed in the in vitro experiments with warfarin and tizoxanide. It was concluded that tizoxanide has the potential to alter protein binding of warfarin and produce variations in unbound warfarin plasma concentrations. Even slight variations in warfarin plasma exposure may result in large variations in the pharmacological response. The interaction could potentially result in increasing the toxicity of warfarin therapy and increase the risk of bleeding. However, further studies are needed to evaluate clinical significance of the finding. If the magnitude of the clinical interaction is significant, dose adjustments of warfarin may be necessary when co-administered with nitazoxanide.

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