DMD Fast Forward. Published on October 6, 2008 as doi:10.1124/dmd.108.023358
This article has not been copyedited and formatted. The final version may differ from this version.

DMD #23358

Mechanism-Based Inactivation of Cytochrome P450 2C9 by Tienilic Acid and (±)-Suprofen: A Comparison of Kinetics and Probe Substrate Selection

J. Matthew Hutzler*, Larissa M. Balogh, Michael Zientek, Vikas Kumar, and Timothy S. Tracy

Pharmacokinetics, Dynamics and Metabolism (PDM), Pfizer Global Research and Development, St. Louis Labs (J.M.H., V.K.), La Jolla Labs (M.Z.)

University of Washington, Department of Medicinal Chemistry, Seattle, WA 98195 (L.M.B.)

University of Minnesota, Department of Experimental and Clinical Pharmacology, Minneapolis, MN 55455 (T.S.T.)

DMD Fast Forward. Published on October 6, 2008 as DOI: 10.1124/dmd.108.023358
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: Inactivation of P450 2C9 and Probe Substrate Selection

*Address Correspondence to:

Dr. J. Matthew Hutzler

Pharmacokinetics, Dynamics and Metabolism (PDM)

Pfizer Global Research and Development,

700 Chesterfield Parkway West, T3A, Lab 319E

Chesterfield, MO 63017

Tel: 314-274-0261

Fax: 314-274-4426

Email: J.Matt.Hutzler@Pfizer.com

Text Pages: 29

Tables: 1

Figures: 6

References: 34

Abstract: 245

Introduction: 675

Discussion: 1768

Abbreviations:

SAR, structure-activity relationship; LC/MS/MS, liquid chromatography/tandem mass spectrometry; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; DDI, drug-drug interaction; NCE, new chemical entity

DMD Fast Forward. Published on October 6, 2008 as DOI: 10.1124/dmd.108.023358
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

In vitro experiments were conducted to compare k_{inact}, K_I and inactivation efficiency (k_{inact}/K_I) of P450 2C9 by tienilic acid and (±)-suprofen using (*S*)-flurbiprofen, diclofenac, and (*S*)-warfarin as reporter substrates. While the inactivation of P450 2C9 by tienilic acid when using (*S*)-flurbiprofen and diclofenac as substrates was similar (efficiency of ~9 ml/min/μmol), the inactivation kinetics were characterized by a sigmoidal profile. (±)-Suprofen inactivation of (*S*)-flurbiprofen and diclofenac hydroxylation was also described by a sigmoidal profile, although inactivation was markedly less efficient (~1 ml/min/μmol). In contrast, inactivation of P450 2C9 mediated (*S*)-warfarin 7-hydroxylation by tienilic acid and (±)-suprofen was best fit to a hyperbolic equation, where inactivation efficiency was moderately higher (10 ml/min/μmol), and ~3-fold higher (3 ml/min/μmol), respectively, relative to the other probe substrates, which argues for careful consideration of reporter substrate when assessing mechanism-based inactivation of P450 2C9 *in vitro*. Further investigations into the observed increased inactivation by tienilic acid relative to (±)-suprofen revealed that it is a higher affinity substrate, with a spectral binding affinity constant (K_s) of 2 μM and an *in vitro* half-life of 5 min, compared to a K_s of 21 μM and a 50 min *in vitro* half-life for (±)-suprofen. Lastly, a close analog of tienilic acid with the carboxylate functionality replaced by an oxirane ring, was devoid of inactivation properties, which suggests that an ionic binding interaction with a positively-charged residue in the P450 2C9 active site is critical for recognition and mechanism-based inactivation by these close structural analogs.

Cytochrome P450 enzymes are a superfamily of oxidative enzymes involved in the metabolism and clearance of a variety of structurally diverse therapeutic drugs. Within this family, P450 2C9 represents approximately 20% of the total hepatic P450 (Guengerich, 2002), and has recently been found to also represent about 15% of the total intestinal P450 (Paine et al., 2006). P450 2C9 is known to be involved in the metabolism of numerous acidic drugs, including non-steroidal anti-inflammatory drugs (NSAIDs) such as flurbiprofen, naproxen and diclofenac (Tracy et al., 1996; Tracy et al., 1997; Klose et al., 1998), as well as the low therapeutic index drugs warfarin and phenytoin (Rettie et al., 1992; Giancarlo et al., 2001). The significant role that P450 2C9 plays in the metabolism of drugs (roughly 20% of drugs on the market), in addition to its polymorphic expression, makes this enzyme of profound interest from a drug-drug interaction perspective.

Cytochrome P450 enzymes are often implicated in drug-drug interactions (DDIs) due to the inhibition of metabolic clearance, ultimately leading to increased exposure and potentially serious adverse events in the clinic. The prediction of *in vivo* DDIs with new chemical entities (NCEs) has become an increasingly important component of drug metabolism research within drug discovery, and continuous improvements have been made in our ability to predict this risk in the clinic (Brown et al., 2006; Obach et al., 2006; Lu et al., 2007). However, *in vitro-in vivo* extrapolations of DDIs have been complicated by multiple binding regions within the enzyme active site that produce substrate-dependent inhibition, a characteristic initially observed with P450 3A4 (Stresser et al., 2000; Wang et al., 2000). Information from crystal structures of P450 2C9 (Williams et al., 2003; Wester et al., 2004) suggests that the active site is quite

voluminous, which has been hypothesized to at least partially explain the numerous reports of complex kinetics that this enzyme displays (Korzekwa et al., 1998; Hutzler and Tracy, 2002; Hutzler et al., 2003). Kumar et al. have recently provided direct evidence of the potential complexities of P450 2C9 by testing a large set of competitive inhibitors against P450 2C9 using various probe substrates, indicating that inhibition potency was probe substrate-dependent (Kumar et al., 2006). As a result, it was suggested that to fully evaluate inhibition of P450 2C9 for predictive purposes, multiple probe substrates should be used, a practice that has become common when evaluating inhibition of P450 3A4.

Of equal importance to the identification of competitive inhibitors is the characterization of mechanism-based inactivation of cytochrome P450. Not evaluating time-dependence of P450 inhibition may lead to under-prediction of DDI risk. This concern is indicated by the numerous methodologies reported for predicting clinical DDIs using *in vitro* inactivation data (Obach et al., 2007; Riley et al., 2007; Venkatakrishnan et al., 2007). One of the first examples of mechanism-based inactivation of P450 2C9 involved the thiophene derivative tienilic acid (Lopez-Garcia et al., 1994), which was withdrawn from the market due to hepatotoxicity, the apparent result of an immune response to alkylation by the P450 2C9 protein (Lecoeur et al., 1994). More recently, the structural analog (±)-suprofen was shown to also be a mechanism-based inactivator of P450 2C9 (O'Donnell et al., 2003). Tienilic acid and (±)-suprofen are reported to cause mechanism-based inactivation by a similar mechanism, leading to covalent modification of the P450 2C9 apoprotein within the active site (Koenigs et al., 1999; O'Donnell et al., 2003), making these inactivators ideal for evaluating the substrate dependence of inactivation kinetics. In the studies described herein, tienilic acid and (±)-suprofen

(Figure 1) were used to investigate the following: whether the *in vitro* inactivation kinetics of P450 2C9 by tienilic acid and (±)-suprofen are dependent upon the reporter substrate used for measuring residual activity; and the mechanism behind the superior P450 2C9 inactivation kinetics observed with tienilic acid relative to (±)-suprofen. It is anticipated that this work may improve our rationale regarding probe substrate selection for measuring P450 2C9 inactivation rates *in vitro*, while concurrently provide additional insight into the structure-activity relationship (SAR) for inactivation of P450 2C9 by carboxylate-containing molecules.

DMD Fast Forward. Published on October 6, 2008 as DOI: 10.1124/dmd.108.023358
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

Chemicals. Potassium phosphate buffer, NADPH, $NADP^+$, isocitric acid, isocitrate dehydrogenase, magnesium chloride, tolbutamide, diclofenac, and 4-hydroxydiclofenac were purchased from Sigma-Aldrich (St. Louis, MO). Tienilic acid, analog 1 ((2,3-dichloro-4-(oxiran-2-ylmethoxy)phenyl)(thiophen-2-yl)methanone), (±)-suprofen, (*S*)-flurbiprofen, 4`-hydroxyflurbiprofen, (*S*)-warfarin, and 7-hydroxywarfarin were obtained from Pfizer internal compound library (St. Louis, MO). Cytochrome P450 2C9 Baculosomes® were purchased from Invitrogen (Carlsbad, CA). All other chemicals were obtained from commercial sources and were of the highest purity available.

P450 2C9 Inactivation Assays. Human recombinant P450 2C9 was assayed for residual activity with probe substrate ((*S*)-flurbiprofen, diclofenac or (*S*)-warfarin – Figure 1) after a primary incubation with various concentrations of tienilic acid, Analog 1, or (±)-suprofen (0.5% total organic v/v) in triplicate in a 96-well plate format at 37°C. Incubations consisted of P450 2C9 Baculosomes® (0.2 μM primary incubation, 0.01 μM secondary incubation) in 100 mM potassium phosphate buffer, pH 7.4, with a NADPH regenerating system (3 mM $MgCl_2$, 1 mM, $NADP^+$, 5 mM isocitric acid, and 1 unit/ml isocitrate dehydrogenase). At selected time intervals (0-5 min for tienilic acid and analog 1, 0-30 min for (±)-suprofen) 15 μl aliquots were taken out of the primary incubation mixture and transferred into 285 μl of a secondary incubation mixture containing (*S*)-flurbiprofen, diclofenac, or (*S*)-warfarin at 25 μM, a concentration >3X the apparent K_m for each reaction, to measure residual activity. The 20-fold dilution and saturating concentration of probe substrate in the secondary incubation served to minimize competitive inhibition and any further inactivation. Secondary incubations (4 min for

diclofenac, 20 min for (*S*)-flurbiprofen and 40 min for (*S*)-warfarin) were quenched with 300 µl of cold acetonitrile containing an internal standard (200 µM tolbutamide). Plates were then centrifuged at 3000 x *g* for 10 min and metabolite formation was analyzed by LC/MS/MS as described below. Observed rates of inactivation (k_{obs}) were calculated for each inactivator concentration by measuring loss in enzyme activity, determined by linear regressions of the ln % remaining activity as a function of primary incubation time using GraphPad PRISM (San Diego, CA), and subsequently re-plotted as k_{obs} as a function of inactivator concentration to estimate inactivation rate constants (K_I and k_{inact}) for each inactivator - substrate combination by nonlinear regression using Sigma Plot 9.0 Systat software (Point Richmond, CA) using either a hyperbolic (Equation 1) or Hill equation (Equation 2):

$$k_{obs} = 0 + \frac{k_{inact} \cdot [I]}{K_I + [I]} \qquad \text{Eq. 1}$$

$$k_{obs} = 0 + \frac{k_{inact} \cdot [I]^n}{K_I + [I]^n} \qquad \text{Eq. 2}$$

where k_{obs} represents the estimated rate constant for inactivation, $k_{obs[I]=0}$ is the apparent inactivation rate measured in the absence of inactivator, k_{inact} is the theoretical maximal rate of inactivation, assessed *in vitro*, K_I is the concentration of inactivator that yields an inactivation rate that equals half k_{inact}, [I] is the concentration of inactivator in the primary incubation (tienilic acid or (±)-suprofen), and n = the Hill coefficient. Comparisons of goodness of the fits were determined by examination of the residuals, R^2 value, residual sum of squares (RSS), and by inspection of Eadie-Hofstee plots.

DMD Fast Forward. Published on October 6, 2008 as DOI: 10.1124/dmd.108.023358
This article has not been copyedited and formatted. The final version may differ from this version.

Spectrophotometric Studies. Spectral binding titration studies were carried out with recombinant P450 2C9 Baculosomes® (0.2 µM) in 100 mM potassium phosphate buffer pH 7.4, containing 20% glycerol, 0.3% CHAPS, 0.1 mM EDTA and 0.2 mM DTT. The mixture was evenly divided into two 10 mm black-walled cuvettes, and experiments were performed at room temperature by titrating in aliquots of tienilic acid (0–70 µM) or (±)-suprofen (0–150 µM) into a sample cuvette, with vehicle controls added to the reference cuvette (DMSO <1% v/v). After each addition, cuvette contents were mixed manually and allowed to sit for 1 minute to allow the samples to reach equilibrium. Difference spectra were recorded between 350 and 500 nm using a Hitachi (Danbury, CT) U3300 dual-beam spectrophotometer with UV Solutions 1.2 software. The spectral binding constants (K_s) were determined by monitoring the type I spectral changes (increase in high spin state at 390 nm with a concomitant decrease in low spin state at 420 nm) in the difference spectra as the enzyme was titrated with each compound. The difference in absorbance between the peak and trough was then plotted vs. the titrant concentration and fit to a hyperbolic curve function (Equation 3) for estimation of the K_s value using GraphPad PRISM (San Diego, CA) software:

$$\Delta A = \frac{B_{max} \cdot [S]}{K_s + [S]} \qquad \text{Eq. 3}$$

Where B_{max} is the maximal binding as measured by the maximal difference in absorbance between 420 and 390 nm, and K_s is the concentration of ligand that yields half B_{max}.

Separation of (±)-Suprofen Enantiomers. Separation of (±)-suprofen enantiomers was performed by preparing a 10 mg/ml stock solution of (±)-suprofen in ethanol, and delivering 1 ml/injection onto a CHIRALPAK AD-H 30 x 250 mm semi-prep column

(Chiral Technologies, West Chester, PA) with 20% ethanol solvent flowing isocratically at 70 ml/min using an SFC (super-critical fluid chromatography) prep system (Berger, multiGram II). Flow was introduced directly into an Agilent UV diode array detector set at 254 nm, followed by an Agilent 1100 single-quadrupole mass spectrometer (Agilent Technologies) fitted with an APCI source operating in positive ion mode (corona voltage 4V, temp 325°). Characterization of (*R*)- and (*S*)-isomers following separation was performed simply by comparison of retention time. The (*R*)-isomer was characterized by a retention time of 4.45 min (agreeing with that of the available (*R*)-suprofen standard), as well as by a protonated ion of $[M+H]^+$ at m/z 261. The (*S*)-isomer of suprofen eluted at 4.94 min, and was also characterized by UV absorption and mass spectrometry as indicated above. Fractions were collected and dried down *in vacuo*, yielding 35 mg (*R*)-suprofen and 33 mg (*S*)-suprofen. Absorption and mass spectral data was processed using ChemStation software (v B.03.01).

Metabolic Stability. The metabolism of tienilic acid, (±)-suprofen, (*R*)-suprofen, and (*S*)-suprofen (1 μM) by recombinant P450 2C9 (0.04 μM) was monitored by substrate depletion in 100 mM potassium phosphate buffer, pH 7.4 at 37°C. Reactions were initiated by the addition of NADPH (2 mM) and at selected time intervals (0-30 min) 50 μl aliquots were taken out of the incubation mixture and quenched by addition to 150 μl of cold acetonitrile containing an internal standard (200 μM tolbutamide). Plates were then centrifuged at 3000 x *g* for 5 min and substrate depletion was analyzed by LC/MS/MS as described below. The *in vitro* half-life ($t_{1/2}$) for each substrate was calculated by linear regression of the natural log (ln) % remaining of substrate as a

function of incubation time using GraphPad Prism (San Diego, CA) software according to equation $t_{1/2}$ = ln 2 / slope.

Partition Ratio. The partition ratio (the molar ratio of inactivator metabolized per P450 2C9 inactivated) was estimated using the titration method, similar to previous work by Lin and Jushchyshyn (Lin et al., 2002; Jushchyshyn et al., 2003). Similar to methods described above, P450 2C9 Baculosomes® (0.2 μM primary incubation) were incubated in the presence of tienilic acid (0-60 μM) or (±)-suprofen (0-160 μM) for 30 min to ensure completion of the inactivation process. Aliquots (15 μl) were then removed from the primary incubation and transferred into 285 μl of the secondary incubation mixture containing 25 μM (*S*)-flurbiprofen to measure residual activity. Metabolite formation was analyzed by LC/MS/MS as described above. The turnover number was estimated by plotting the percent remaining activity as a function of the molar ratio of inactivator to P450 2C9, followed by regression of the linear portions of both the low and the high [inactivator] / [P450 2C9] ratios and extrapolation of their intersection to the *x*-axis. The turnover number includes the one molecule of inactivator (tienilic acid or (±)-suprofen) that inactivates the P450 enzyme and is thus equal to the partition ratio + 1.

LC/MS/MS Analysis. Metabolite formation was analyzed on a PE Sciex API-3000 triple-quadrupole instrument. The mass spectrometer was equipped with an electrospray ionization (ESI) interface connected in line with a Shimadzu LC20AD pump and a Leap Technologies CTC PAL (Carrboro, NC) auto-sampler. Analytes were separated using a Zorbax 3.5 μm Eclipse Plus C_{18} 2.1 x 50 mm column with a gradient elution profile. Mobile phase was flowing at 0.4 ml/min, and the gradient was initiated and held for the first 0.3 min at 95%A: 5%B (A: 0.1% formic acid in H_2O, B: 0.1% formic acid in

acetonitrile), and was then ramped linearly to 5%A: 95%B over the next 2.5 min and held for 1.2 min. The profile was then immediately returned to initial conditions and allowed to re-equilibrate for 2 min. The source temperature was set to 400°C and mass spectral analyses were performed using multiple reaction monitoring (MRM), with transitions for 4`-hydroxyflurbiprofen (*m/z* 259.1→215.2), 4-hydroxydiclofenac (*m/z* 309.9→265.8), 7-hydroxywarfarin (*m/z* 323.1→176.9) and tienilic acid (*m/z* 328.9→270.8) and tolbutamide (*m/z* 269.1→170.0) utilizing a turbo ion spray source in negative ionization mode (-3.5 kV spray voltage). MRM for suprofen (*m/z* 261.06→111.0) was performed utilizing a turbo ion spray source in positive ionization mode (5 kV spray voltage). All data were analyzed using PE Sciex Analyst 1.4.1 software.

DMD Fast Forward. Published on October 6, 2008 as DOI: 10.1124/dmd.108.023358
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Inactivation of P450 2C9. In effort to compare kinetic rates of inactivation with different reporter substrates, both tienilic acid and (±)-suprofen were pre-incubated with P450 2C9 Baculosomes®, and residual enzymatic activity was measured using (*S*)-flurbiprofen, diclofenac, or (*S*)-warfarin as probe substrates. As shown in Table 1, inactivation by tienilic acid and (±)-suprofen was described by comparable k_{inact} and K_I values against P450 2C9-mediated (*S*)-flurbiprofen and diclofenac hydroxylation. In addition, the kinetics of inactivation were described by a sigmoidal kinetic profile (Figure 2 A-D). However, inactivation of (*S*)-warfarin 7-hydroxylation by both tienilic acid and (±)-suprofen was best fit to a standard hyperbolic equation (Figure 2 E and F), which yielded noticeably lower K_I estimates (12.5 and 6.7 μM with tienilic acid and (±)-suprofen, respectively), resulting in moderately higher inactivation efficiencies, in particular for (±)-suprofen (Table 1). Tienilic acid was a markedly better inactivator of P450 2C9 compared to (±)-suprofen, independent of probe substrate, with a 4- to 8-fold higher inactivation efficiency (Table 1). Interestingly, a close structural analog of tienilic acid with the carboxylate functionality replaced by an oxirane ring (Analog 1) did not demonstrate inactivation properties against P450 2C9 (Figure 3).

Spectrophotometric Analysis. The titration of both tienilic acid and (±)-suprofen with P450 2C9 Baculosomes® resulted in a type I difference spectrum, characterized by an absorbance peak ~390 nm, and a trough at ~420 nm (Figure 4 A and B, insets). When fitting data (ΔAbs 420-390 nm vs. tienilic acid or (±)-suprofen) to equation 3, a spectral binding affinity constant (K_s) was estimated to be 2 μM for tienilic acid (Figure 4A) and 21 μM for (±)-suprofen (Figure 4B).

Metabolism by P450 2C9 Baculosomes. When tienilic acid and (±)-suprofen were incubated with P450 2C9 Baculosomes®, there was a substantial difference observed in their metabolic rates, as measured by substrate depletion (Figure 5). The half-life of tienilic acid was about 5 min, while for (±)-suprofen, a 50 min half-life was observed. To examine potential enantiomeric differences between (*R*)- and (*S*)-suprofen metabolism (and potentially, inactivation rates), the enantiomers were separated and their substrate depletion rates compared. There was little evidence for enantio-selective metabolism of suprofen, as the half-life of both enantiomers was comparable (35-38 min), although slightly shorter compared to (±)-suprofen (50 min).

Partition Ratio. To compare the partition ratios (molar ratio of inactivator metabolized per P450 2C9 inactivated) between tienilic acid and (±)-suprofen in the same P450 2C9 enzymatic system, a titration method was used. Tienilic acid (0-60 μM) and (±)-suprofen (0-160 μM) were pre-incubated for 30 min to ensure complete inactivation of (*S*)-flurbiprofen hydroxylation (see Methods). Using the titration methodology demonstrated in Figure 6, the turnover number (the total number of metabolic events that results in inactivation; partition ratio + 1) for tienilic acid inactivation was ~35 (partition ratio of 34), while for inactivation by (±)-suprofen, the turnover number was estimated to be 99 (partition ratio of 98).

Discussion

Tienilic acid and (±)-suprofen are thiophene-containing mechanism-based inactivators of P450 2C9, the former being withdrawn from the market due to hepatotoxicity, proposed to be related to an immune response resulting from covalent modification of the P450 2C9 protein (Lecoeur et al., 1994). The bioactivation mechanism for both tienilic acid and (±)-suprofen involves oxidation of the thiophene ring system (Lopez-Garcia et al., 1994; Koenigs et al., 1999; O'Donnell et al., 2003). Interestingly, while these molecules are structurally related and appear to be bioactivated via similar mechanisms, tienilic acid is noticeably more efficient at inactivating P450 2C9 compared to (±)-suprofen. In separate but related work, the inhibition potency of numerous *competitive* inhibitors of P450 2C9 was shown to be dependent upon the marker substrate used to measure enzymatic activity (Kumar et al., 2006). In addition, Cheng et al. have observed that the mechanism of inhibition of P450 2B4 by the mechanism-based inhibitor 2-ethynylnaphthalene (2EN) was dependent on the probe substrate (Cheng et al., 2007a; Cheng et al., 2007b). Thus, the focus of our research was to use tienilic acid and (±)-suprofen as *in vitro* tools to investigate whether using different probe substrates to measure residual activity *in vitro* would have an impact on mechanism-based inactivation kinetic rates/efficiencies, while also investigating mechanistically why the inactivation kinetics of tienilic acid and (±)-suprofen are so disparate, despite close structural similarities.

To investigate substrate-dependent inactivation kinetics with P450 2C9, three reporter substrates were selected: (*S*)-flurbiprofen, diclofenac, and (*S*)-warfarin. These substrates were strategically selected as representatives of the three sub-groups

designated by a cluster analysis of potency of competitive inhibitors of P450 2C9 (Kumar et al., 2006). While inactivation of both (*S*)-flurbiprofen and diclofenac 4-hydroxylation was best described by a sigmoidal model (Hill Equation), and similar inactivation kinetics, inactivation of (*S*)-warfarin 7-hydroxylation was best described by a classic hyperbolic equation (Table 1), which yielded K_I estimates that were considerably lower than those estimated for inactivation of both (*S*)-flurbiprofen and diclofenac 4-hydroxylation (Table 1). While the lower estimates of K_I when using (*S*)-warfarin as a probe substrate for these inactivation studies are consistent with that reported by Kumar et al., where as much as 20-fold lower inhibition constants (K_I) were observed against (*S*)-warfarin for several competitive inhibitors (Kumar et al., 2006), the magnitude of the observed difference here was not as substantial. The question remains however, whether the observed kinetic differences are significant enough to impact predictions of clinical drug-drug interactions using established methods (Obach et al., 2007). However, this type of analysis is difficult with the selected probe substrates from these studies, as (*S*)-warfarin is the only probe that is primarily cleared by P450 2C9 in humans, with an estimated fraction metabolized through P450 2C9 (f_{m2C9}) of 0.91 (Obach et al., 2007). In addition to P450 2C9-mediated hydroxylation, both (*S*)-flurbiprofen and diclofenac are cleared roughly 50% by direct glucuronidation of the carboxylate functional groups (Kumar et al., 2002; Mano et al., 2007). In the absence of a clear comparison to understand the clinical implications of this data, it may be prudent to consider including (*S*)-warfarin as a reporter substrate *in vitro* when evaluating inactivation of P450 2C9, as this appears to be the most sensitive probe substrate, and is likely to be one of the clinical interactions of most concern due to the known low therapeutic index of this drug.

In addition to the kinetic differences observed for reporter substrates, it was clear that tienilic acid was substantially more effective at inactivating P450 2C9 than (±)-suprofen (Table 1). This was not a surprise, as inactivation kinetics for these compounds have been previously reported, although from separate studies using different enzyme preparations (Lopez-Garcia et al., 1994; O'Donnell et al., 2003; McGinnity et al., 2006). In this direct comparison, we report that the enhanced efficiency of P450 2C9 inactivation by tienilic acid relative to (±)-suprofen is independent of which reporter substrate is used. Considering their structural similarities, these differences are intriguing from a structure-activity relationship (SAR) perspective, and to our knowledge, the underlying causes of these rate differences have yet to be investigated. Firstly, to investigate and compare binding affinity of tienilic acid and (±)-suprofen, difference spectra was collected, which indicated that both inactivators bind in a type I fashion, with characteristic peak and trough absorbances at 390 nm and 420 nm, respectively. This binding is consistent with both tienilic acid and (±)-suprofen being substrates for P450 2C9. However, it is clear upon visual inspection of the spectral data in Figure 4 that (±)-suprofen binds with substantially less affinity, supported by a higher spectral binding affinity constant (K_s) of 21 μM, compared to 2 μM for tienilic acid. It is worth noting that the binding kinetics of tienilic acid and (±)-suprofen were not described by a sigmoidal profile, thus indicating that the sigmoidicity observed for the inactivation of (*S*)-flurbiprofen and diclofenac 4-hydroxylation was likely not due to initial binding properties of tienilic acid and (±)-suprofen alone. Lastly, spectral binding data was consistent with data from incubations in P450 2C9 Baculosomes® showing that tienilic acid is markedly less metabolically stable than (±)-suprofen, with an *in vitro*

DMD Fast Forward. Published on October 6, 2008 as DOI: 10.1124/dmd.108.023358
This article has not been copyedited and formatted. The final version may differ from this version.

disappearance half-life of 5 min, compared to a 50 min half-life for (±)-suprofen (Figure 5). A higher metabolic rate for tienilic acid suggests that it is likely bioactivated more rapidly than is (±)-suprofen, perhaps explaining the disparate inactivation rates displayed by these two inactivators.

Other factors potentially contributing to the inactivation kinetic differences of tienilic acid and (±)-suprofen with P450 2C9 were also investigated. The enantiomers of (±)-suprofen were separated and tested to determine whether there may be enantio-selective metabolism by P450 2C9, which has been observed for other racemic substrates. For example, (*S*)-warfarin is a substrate for P450 2C9 (Rettie et al., 1992), while (*R*)-warfarin is a substrate for P450 1A2 and P450 3A4 (Yamazaki and Shimada, 1997). As shown in Figure 5, there does not appear to be enantio-selective metabolism of (±)-suprofen. While we did not test both suprofen isomers for inactivation of P450 2C9, one can infer based on a similar metabolic half-life (35-38 min) that one isomer likely does not substantially inhibit, or protect the P450 2C9 enzyme from inactivation by the other when the racemic mixture was used in our inactivation studies. The possibility of some minor effects can not be completely discounted, however, since the substrate depletion half-life is slightly longer for (±)-suprofen (50 min). Partition ratios of tienilic acid and (±)-suprofen were also compared using (*S*)-flurbiprofen as the probe substrate. Theoretically, a higher partition ratio (more metabolite formed per enzyme inactivated) would lead to less efficient inactivation. As indicated in Figure 6, the partition ratio of (±)-suprofen is estimated to be ~98, while that for tienilic acid is ~34. It appears then that a higher partition ratio for (±)-suprofen may play a role in less efficient inactivation,

although it seems more likely that slower metabolism, and thus bioactivation, is the primary reason for the decreased inactivation rates relative to tienilic acid.

In an effort to more completely understand the observed differences in inactivation rates with tienilic acid and (±)-suprofen, as well as the probe substrate-dependent kinetic profiles of inactivation, computational studies may be necessary using the crystal structure of P450 2C9 (Wester et al., 2004). Similar approaches would also have to be taken to understand if existing information from the crystal structure of P450 2C9 could explain the fact that both tienilic acid and (±)-suprofen appear to inactivate (*S*)-warfarin 7-hydroxylation with a distinct hyperbolic kinetic profile, yielding up to a 3-fold difference in inactivation efficiency. Interestingly, Melet et al. have shown evidence that the serine residue at position 365 (Ser365) appears to play a role in inactivation of P450 2C9 by tienilic acid, a conclusion based on the observation that replacement of the nucleophilic serine by either alanine (S365A) or glycine (S365G) resulted in attenuation of mechanism-based inactivation of P450 2C9 (Melet et al., 2003). In addition, co-crystallization of (*S*)-warfarin bound to P450 2C9 protein suggests that it lies predominantly in a hydrophobic pocket lined by several residues, including Ser365 (Williams et al., 2003). Although one could argue that this reported binding orientation of (*S*)-warfarin may represent only one potential conformation, especially since this is likely a non-productive binding orientation (i.e. 10 Å from heme), if inactivation by tienilic acid results in covalent modification of Ser365, then perhaps this may offer a reasonable explanation as to the kinetic mechanism for inactivation of (*S*)-warfarin 7-hydroxylation relative to (*S*)-flurbiprofen and diclofenac hydroxylation. Although somewhat speculative at this time, it is possible that covalent modification of P450 2C9

at Ser365 may result in two enzyme pools: adducted P450 2C9 that is inactive against (*S*)-warfarin, but still able to catalyze (*S*)-flurbiprofen and diclofenac metabolism; and un-adducted P450 2C9, which is active against all three substrates used in this study. If this is true, then it may explain the sigmoidal inactivation kinetics of (*S*)-flurbiprofen and diclofenac 4-hydroxylation (i.e. both P450 2C9 enzyme pools active, but distinct kinetically), whereas the inactivation kinetics against (*S*)-warfarin are hyperbolic (i.e. only the un-adducted P450 2C9 enzyme remains active). This is a potentially novel hypothesis as it pertains to inactivation mechanisms for when the apoprotein is covalently modified, and certainly needs to be tested. Wester et al. have also provided evidence that Arg 108 likely plays a role in the high affinity binding of (*S*)-flurbiprofen, and propose that other lipophilic anions such as diclofenac and naproxen likely bind in this mode as well (Wester et al., 2004). The fact that Analog 1 of tienilic acid (minus the carboxylate) did not demonstrate inactivation of P450 2C9 is consistent with these findings, and further suggests that this residue most likely plays a role in the inactivation of tienilic acid and (±)-suprofen. Based on this information, we also hypothesize that the distance between the carboxylate and the bioactivated thiophene, which appears longer for tienilic acid, would allow the thiophene of tienilic acid to position itself closer to the heme prosthetic, relative to the thiophene of (±)-suprofen, may be key in the observed inactivation rate differences. This hypothesis regarding the importance of distance will be the focus of future investigations.

In conclusion, these studies demonstrate that inactivation efficiencies, and to a higher extent, inactivation kinetic profiles, of P450 2C9 by both tienilic acid and (±)-suprofen appear to be substrate-dependent, with (*S*)-warfarin being the most sensitive

reporter substrate. While the differences in inactivation kinetic profile (sigmoidal vs. hyperbolic) are not completely understood, this may have implications for prediction of clinical drug-drug interactions using *in vitro* data, and suggests that (*S*)-warfarin should be considered for use as a reporter substrate when carrying out *in vitro* mechanism-based inactivation studies with P450 2C9.

Acknowledgements

The authors would like to thank Roger Melton for his assistance with development of bioanalytical assays, Sascha Freiwald for bioanalytical assistance, Joe Collins and Robert Hughes for assistance with separation of the enantiomers of (±)-suprofen, and R. Scott Obach for thoughtful comments.

References

Brown HS, Galetin A, Hallifax D and Houston JB (2006) Prediction of in vivo drug-drug interactions from in vitro data : factors affecting prototypic drug-drug interactions involving CYP2C9, CYP2D6 and CYP3A4. *Clin Pharmacokinet* **45:**1035-1050.

Cheng D, Harris D, Reed JR and Backes WL (2007a) Inhibition of CYP2B4 by 2-ethynylnaphthalene: evidence for the co-binding of substrate and inhibitor within the active site. *Arch Biochem Biophys* **468:**174-182.

Cheng D, Reed JR, Harris D and Backes WL (2007b) Inhibition of CYP2B4 by the mechanism-based inhibitor 2-ethynylnaphthalene: inhibitory potential of 2EN is dependent on the size of the substrate. *Arch Biochem Biophys* **462:**28-37.

Giancarlo GM, Venkatakrishnan K, Granda BW, von Moltke LL and Greenblatt DJ (2001) Relative contributions of CYP2C9 and 2C19 to phenytoin 4-hydroxylation in vitro: inhibition by sulfaphenazole, omeprazole, and ticlopidine. *Eur J Clin Pharmacol* **57:**31-36.

Guengerich FP (2002) Update information on human P450s. *Drug Metab Rev* **34:**7-15.

Hutzler JM and Tracy TS (2002) Atypical kinetic profiles in drug metabolism reactions. *Drug Metab Dispos* **30:**355-362.

Hutzler JM, Wienkers LC, Wahlstrom JL, Carlson TJ and Tracy TS (2003) Activation of cytochrome P450 2C9-mediated metabolism: mechanistic evidence in support of kinetic observations. *Arch Biochem Biophys* **410:**16-24.

Jushchyshyn MI, Kent UM and Hollenberg PF (2003) The mechanism-based inactivation of human cytochrome P450 2B6 by phencyclidine. *Drug Metab Dispos* **31:**46-52.

Klose TS, Ibeanu GC, Ghanayem BI, Pedersen LG, Li L, Hall SD and Goldstein JA (1998) Identification of residues 286 and 289 as critical for conferring substrate specificity of human CYP2C9 for diclofenac and ibuprofen. *Arch Biochem Biophys* **357:**240-248.

Koenigs LL, Peter RM, Hunter AP, Haining RL, Rettie AE, Friedberg T, Pritchard MP, Shou M, Rushmore TH and Trager WF (1999) Electrospray ionization mass spectrometric analysis of intact cytochrome P450: identification of tienilic acid adducts to P450 2C9. *Biochemistry* **38:**2312-2319.

Korzekwa KR, Krishnamachary N, Shou M, Ogai A, Parise RA, Rettie AE, Gonzalez FJ and Tracy TS (1998) Evaluation of atypical cytochrome P450 kinetics with two-substrate models: evidence that multiple substrates can simultaneously bind to cytochrome P450 active sites. *Biochemistry* **37:**4137-4147.

Kumar S, Samuel K, Subramanian R, Braun MP, Stearns RA, Chiu SH, Evans DC and Baillie TA (2002) Extrapolation of diclofenac clearance from in vitro microsomal metabolism data: role of acyl glucuronidation and sequential oxidative metabolism of the acyl glucuronide. *J Pharmacol Exp Ther* **303:**969-978.

Kumar V, Wahlstrom JL, Rock DA, Warren CJ, Gorman LA and Tracy TS (2006) CYP2C9 inhibition: impact of probe selection and pharmacogenetics on in vitro inhibition profiles. *Drug Metab Dispos* **34:**1966-1975.

Lecoeur S, Bonierbale E, Challine D, Gautier JC, Valadon P, Dansette PM, Catinot R, Ballet F, Mansuy D and Beaune PH (1994) Specificity of in vitro covalent binding of tienilic acid metabolites to human liver microsomes in relationship to

the type of hepatotoxicity: comparison with two directly hepatotoxic drugs. *Chem Res Toxicol* **7:**434-442.

Lin HL, Kent UM and Hollenberg PF (2002) Mechanism-based inactivation of cytochrome P450 3A4 by 17 alpha-ethynylestradiol: evidence for heme destruction and covalent binding to protein. *J Pharmacol Exp Ther* **301:**160-167.

Lopez-Garcia MP, Dansette PM and Mansuy D (1994) Thiophene derivatives as new mechanism-based inhibitors of cytochromes P-450: inactivation of yeast-expressed human liver cytochrome P-450 2C9 by tienilic acid. *Biochemistry* **33:**166-175.

Lu C, Miwa GT, Prakash SR, Gan LS and Balani SK (2007) A novel model for the prediction of drug-drug interactions in humans based on in vitro cytochrome p450 phenotypic data. *Drug Metab Dispos* **35:**79-85.

Mano Y, Usui T and Kamimura H (2007) Predominant contribution of UDP-glucuronosyltransferase 2B7 in the glucuronidation of racemic flurbiprofen in the human liver. *Drug Metab Dispos* **35:**1182-1187.

McGinnity DF, Berry AJ, Kenny JR, Grime K and Riley RJ (2006) Evaluation of time-dependent cytochrome P450 inhibition using cultured human hepatocytes. *Drug Metab Dispos* **34:**1291-1300.

Melet A, Assrir N, Jean P, Pilar Lopez-Garcia M, Marques-Soares C, Jaouen M, Dansette PM, Sari MA and Mansuy D (2003) Substrate selectivity of human cytochrome P450 2C9: importance of residues 476, 365, and 114 in recognition of diclofenac and sulfaphenazole and in mechanism-based inactivation by tienilic acid. *Arch Biochem Biophys* **409:**80-91.

O'Donnell JP, Dalvie DK, Kalgutkar AS and Obach RS (2003) Mechanism-based inactivation of human recombinant P450 2C9 by the nonsteroidal anti-inflammatory drug suprofen. *Drug Metab Dispos* **31:**1369-1377.

Obach RS, Walsky RL and Venkatakrishnan K (2007) Mechanism-based inactivation of human cytochrome p450 enzymes and the prediction of drug-drug interactions. *Drug Metab Dispos* **35:**246-255.

Obach RS, Walsky RL, Venkatakrishnan K, Gaman EA, Houston JB and Tremaine LM (2006) The utility of in vitro cytochrome P450 inhibition data in the prediction of drug-drug interactions. *J Pharmacol Exp Ther* **316:**336-348.

Paine MF, Hart HL, Ludington SS, Haining RL, Rettie AE and Zeldin DC (2006) The human intestinal cytochrome P450 "pie". *Drug Metab Dispos* **34:**880-886.

Rettie AE, Korzekwa KR, Kunze KL, Lawrence RF, Eddy AC, Aoyama T, Gelboin HV, Gonzalez FJ and Trager WF (1992) Hydroxylation of warfarin by human cDNA-expressed cytochrome P-450: a role for P-4502C9 in the etiology of (S)-warfarin-drug interactions. *Chem Res Toxicol* **5:**54-59.

Riley RJ, Grime K and Weaver R (2007) Time-dependent CYP inhibition. *Expert Opin Drug Metab Toxicol* **3:**51-66.

Stresser DM, Blanchard AP, Turner SD, Erve JC, Dandeneau AA, Miller VP and Crespi CL (2000) Substrate-dependent modulation of CYP3A4 catalytic activity: analysis of 27 test compounds with four fluorometric substrates. *Drug Metab Dispos* **28:**1440-1448.

Tracy TS, Marra C, Wrighton SA, Gonzalez FJ and Korzekwa KR (1996) Studies of flurbiprofen 4'-hydroxylation. Additional evidence suggesting the sole involvement of cytochrome P450 2C9. *Biochem Pharmacol* **52:**1305-1309.

Tracy TS, Marra C, Wrighton SA, Gonzalez FJ and Korzekwa KR (1997) Involvement of multiple cytochrome P450 isoforms in naproxen O-demethylation. *Eur J Clin Pharmacol* **52:**293-298.

Venkatakrishnan K, Obach RS and Rostami-Hodjegan A (2007) Mechanism-based inactivation of human cytochrome P450 enzymes: strategies for diagnosis and drug-drug interaction risk assessment. *Xenobiotica* **37:**1225-1256.

Wang RW, Newton DJ, Liu N, Atkins WM and Lu AY (2000) Human cytochrome P-450 3A4: in vitro drug-drug interaction patterns are substrate-dependent. *Drug Metab Dispos* **28:**360-366.

Wester MR, Yano JK, Schoch GA, Yang C, Griffin KJ, Stout CD and Johnson EF (2004) The structure of human cytochrome P450 2C9 complexed with flurbiprofen at 2.0-A resolution. *J Biol Chem* **279:**35630-35637.

Williams PA, Cosme J, Ward A, Angove HC, Matak Vinkovic D and Jhoti H (2003) Crystal structure of human cytochrome P450 2C9 with bound warfarin. *Nature* **424:**464-468.

Yamazaki H and Shimada T (1997) Human liver cytochrome P450 enzymes involved in the 7-hydroxylation of R- and S-warfarin enantiomers. *Biochem Pharmacol* **54:**1195-1203.

Footnotes

Supported in part by a grant from the National Institutes of Health (GM069753) to T.S.T.

Legends for Figures

Figure 1. Structures of the tested P450 2C9 inactivators tienilic acid and (±)-suprofen, and the P450 2C9 probe substrates (*S*)-flurbiprofen, diclofenac, and (*S*)-warfarin. The chiral carbon in (±)-suprofen is indicated by an asterisk, and the arrows indicate the major site of metabolism for the probe substrates.

Figure 2. Kinetic plots demonstrating observed rates of inactivation (K_{obs}) of P450 2C9 by tienilic acid and (±)-suprofen with diclofenac (A and B), (*S*)-flurbiprofen (C and D), and (*S*)-warfarin (E and F) as probe substrates. Data were fit to either Equation 1 (hyperbolic) or Equation 2 (Hill Equation).

Figure 3. Inactivation of P450 2C9 by tienilic acid and a close structural analog (Analog 1) with the carboxylic acid replaced by an oxirane ring, demonstrating the importance of the carboxylic acid to the inactivation properties of tienilic acid.

Figure 4. Spectral binding titration of tienilic acid (A) and (±)-suprofen (B) with P450 2C9. A type I spectral shift was observed for both inactivators, and a spectral binding affinity constant of 2 μM for tienilic acid and 21 μM for (±)-suprofen was estimated.

Figure 5. Comparison of metabolic rates of tienilic acid (■) and (±)-suprofen (o), as well as the separated enantiomers, (*R*)-suprofen (▲) and (*S*)-suprofen (∇), determined by the substrate depletion method. The calculated *in vitro* half-life of tienilic acid when incubated with P450 2C9 Baculosomes® was 5 min, while that for (±)-suprofen, (*R*)-suprofen and (*S*)-suprofen was 50, 38, and 35 min, respectively.

Figure 6. Titration study showing loss of P450 2C9 enzymatic activity as a function of the ratio of (A) tienilic acid to P450 2C9 ([tienilic acid/P450 2C9]) and (B) (±)-suprofen

to P450 2C9 ([(±)-suprofen /P450 2C9]). A partition ratio of 34 and 98 were estimated for tienilic acid and (±)-suprofen, respectively.

DMD Fast Forward. Published on October 6, 2008 as DOI: 10.1124/dmd.108.023358
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. Kinetic parameters describing the inactivation of P450 2C9 by tienilic acid and (±)-suprofen when different probe substrates are used to measure residual activity. Data were fit to both a hyperbolic equation (Equation 1), and the Hill Equation (Equation 2), as described in the Methods section. Efficiency of inactivation was calculated as the ratio of k_{inact} to K_I.

Inactivator	Substrate	k_{inact} (min^{-1})	K_I (μM)	n	Efficiency (ml/min/μmol)
Tienilic Acid	(*S*)-Flurbiprofen[a]	0.18	19.2	1.6	9.4
	Diclofenac[a]	0.17	20.1	2.1	8.5
	(*S*)-Warfarin[b]	0.13	12.5	-	10.4
(±)-Suprofen	(*S*)-Flurbiprofen[a]	0.03	24.2	1.4	1.2
	Diclofenac[a]	0.03	26.6	1.3	1.1
	(*S*)-Warfarin[b]	0.02	6.7	-	3.0

[a]Data for the inactivation of both (*S*)-flurbiprofen and diclofenac 4-hydroxylation were best fit to Equation 2 (the Hill Equation).

[b]Data for the inactivation of (*S*)-warfarin 7-hydroxylation were best fit to Equation 1.

Figure 1

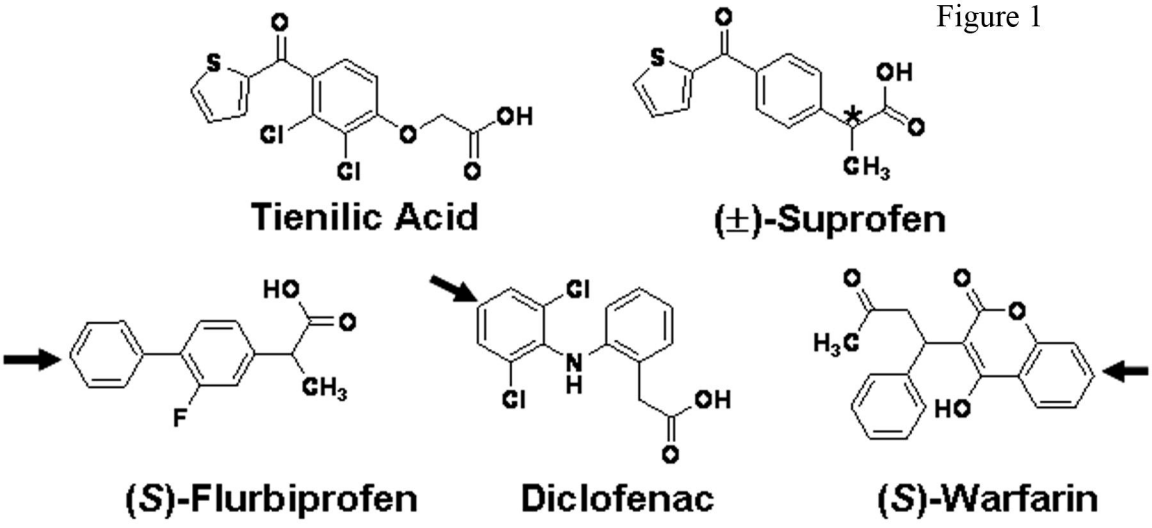

Tienilic Acid
(±)-Suprofen
(S)-Flurbiprofen
Diclofenac
(S)-Warfarin

Figure 2

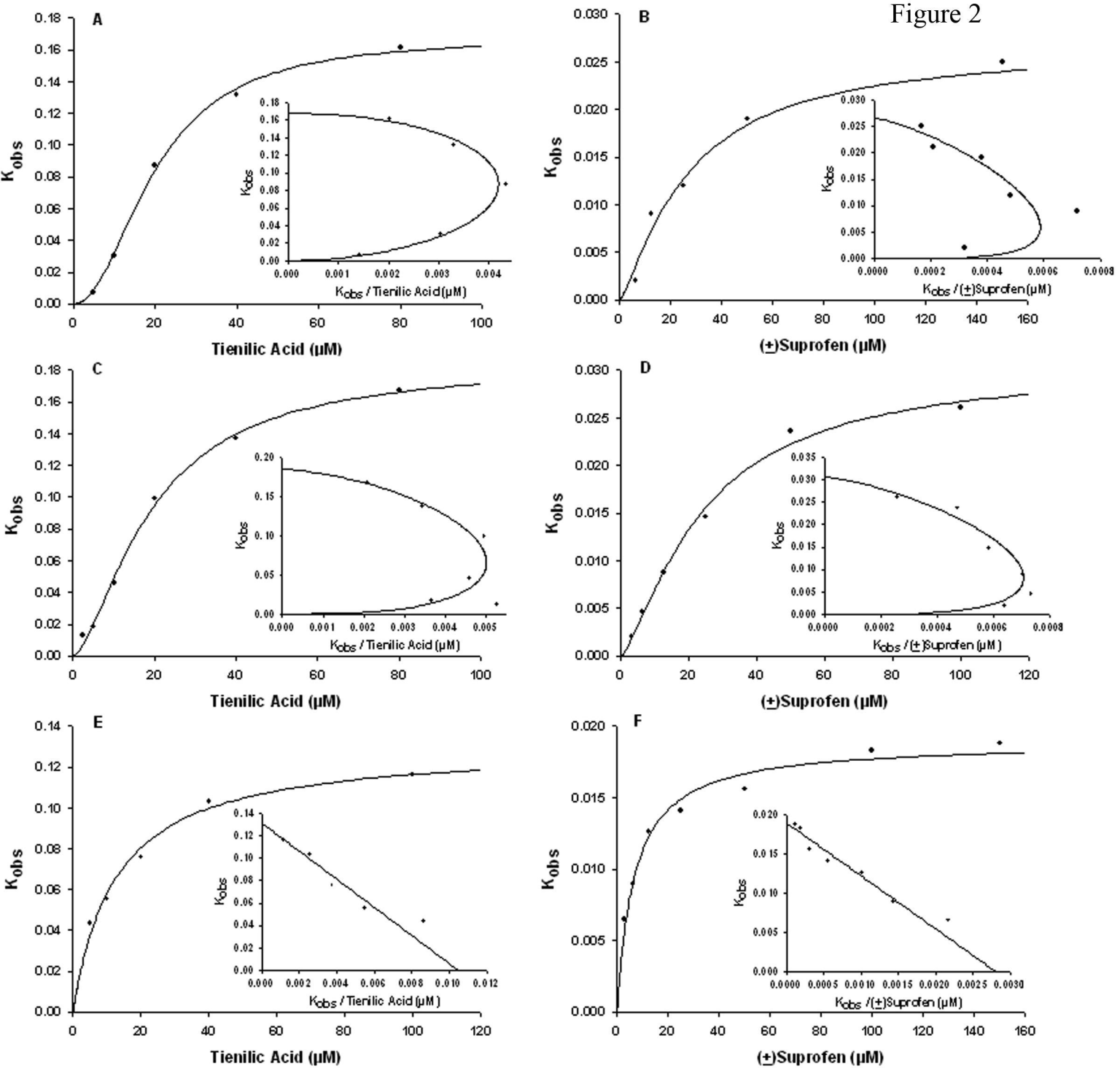

A
Kobs
Tienilic Acid (μM)
Kobs / Tienilic Acid(μM)
B
Kobs
(±)Suprofen (μM)
Kobs /(±)Suprofen(μM)
C
Kobs
Tienilic Acid (μM)
Kobs / Tienilic Acid (μM)
D
Kobs
(±)Suprofen (μM)
Kobs /(±)Suprofen (μM)
E
Kobs
Tienilic Acid (μM)
Kobs / Tienilic Acid (μM)
F
Kobs
(±)Suprofen (μM)
Kobs /(±)Suprofen (μM)

Figure 3

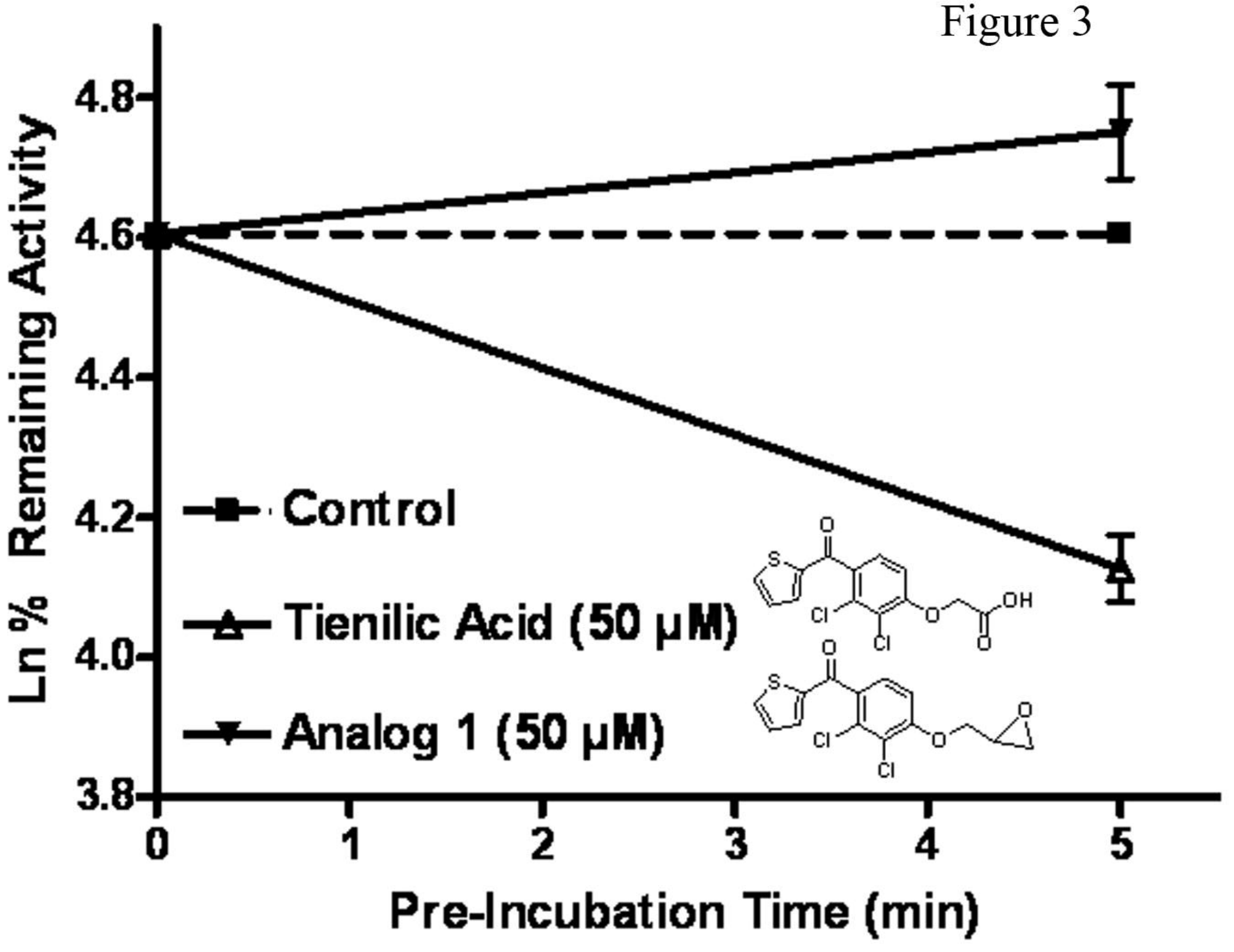

Ln % Remaining Activity
4.8
4.6
4.4
4.2
4.0
3.8
0
1
2
3
4
5
Pre-Incubation Time (min)
Control
Tienilic Acid (50 µM)
Analog 1 (50 µM)
O
S
Cl
Cl
O
OH
O
O
S
Cl
Cl
O
O

Figure 4

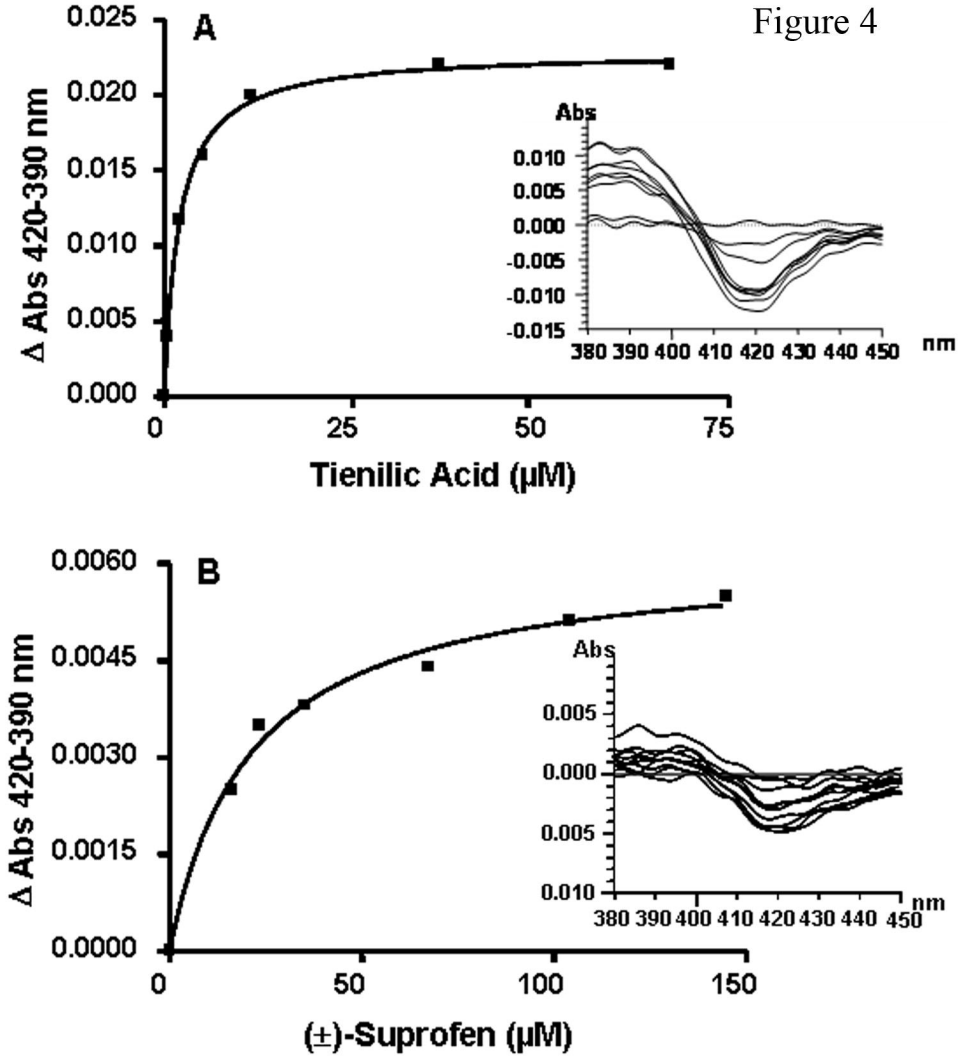

A
Δ Abs 420-390 nm
0.025
0.020
0.015
0.010
0.005
0.000
0
25
50
75
Tienilic Acid (μM)
Abs
0.010
0.005
0.000
-0.005
-0.010
-0.015
380 390 400 410 420 430 440 450
nm
B
Δ Abs 420-390 nm
0.0060
0.0045
0.0030
0.0015
0.0000
0
50
100
150
(±)-Suprofen (μM)
Abs
0.005
0.000
0.005
0.010
380 390 400 410 420 430 440 450
nm

Figure 5

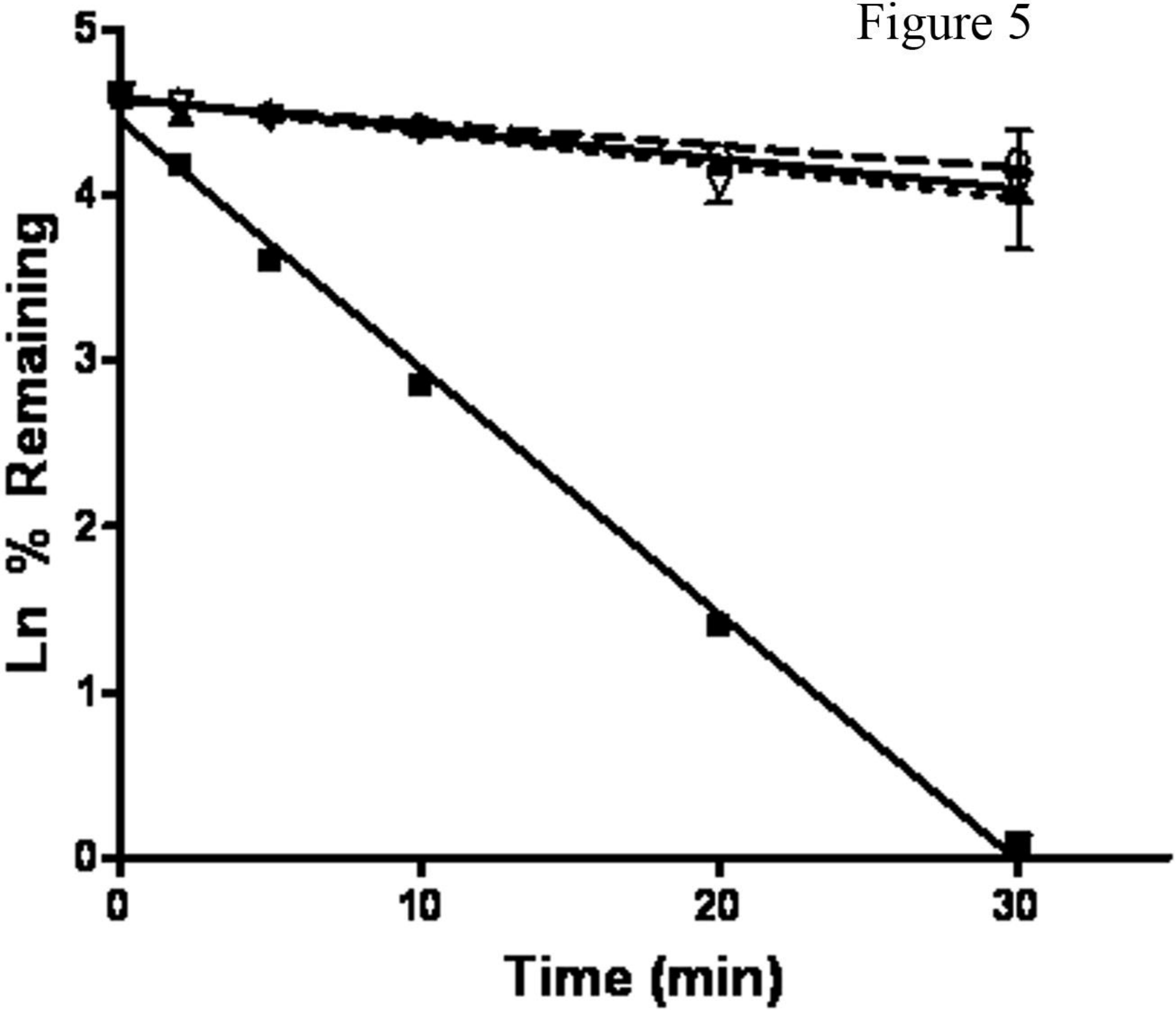
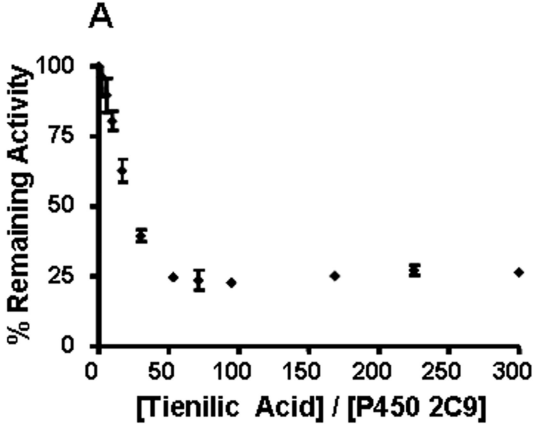
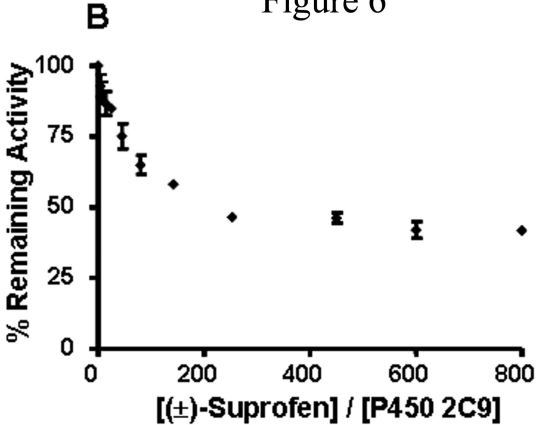


Figure 6


A
% Remaining Activity
100
75
50
25
0
0
50
100
150
200
250
300
[Tienilic Acid] / [P450 2C9]


B
% Remaining Activity
100
75
50
25
0
0
200
400
600
800
[(±)-Suprofen] / [P450 2C9]