PROPOXYPHENE, NORPROPOXYPHENE, AND PROADIFEN (SKF-525A) ARE MECHANISM-BASED INHIBITORS OF CYP3A4, CYP3A5, AND CYP3A IN HUMAN LIVER MICROSOMES

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DEDICATION

Special thanks to my family, and friends (especially MK), and MJK

who helped me during this journey

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ABSTRACT

Anna Ruth Riley

PROPOXYPHENE, NORPROPOXYPHENE, AND PROADIFEN (SKF-525A) ARE MECHANISM-BASED INHIBITORS OF CYP3A4, CYP3A5, AND CYP3A IN HUMAN LIVER MICROSOMES

The purpose of this study is to determine if propoxyphene and norpropoxyphene are mechanism-based (irreversible) inhibitors of CYP3A, and to determine if propoxyphene and norpropoxyphene are reversible inhibitors of CYP3A. Mechanism-based inhibition is a type of irreversible inhibition that results from an inhibitor or its metabolite binding to an enzyme during drug metabolism, which renders the enzyme nonfunctional.

Propoxyphene is an analgesic that is frequently prescribed in the United States and Europe. It is metabolized by CYP3A enzymes, and is an irreversible inhibitor of CYP3A4. The major metabolite of propoxyphene is norpropoxyphene, which has not been extensively studied for enzyme inhibition. Proadifen (SKF-525a) is not a marketed drug, but it is a known CYP inhibitor that is structurally similar to propoxyphene and norpropoxyphene. Propoxyphene, norpropoxyphene, and proadifen were characterized in these studies with CYP3A4(+b5), CYP3A5(+b5) and pooled human liver microsomes. Time-dependent and concentration-dependent loss of activity of CYP3A was measured by formation of testosterone product. Propoxyphene and norpropoxyphene exhibited the greatest inhibition with CYP3A in human liver microsomes, followed by CYP3A4(+b5), and CYP3A5(+b5). Both compounds formed metabolic-inhibitor complexes with

CYP3A4(+b5) and CYP3A5(+b5), but not with human liver microsomes. Proadifen was a more potent inhibitor of CYP3A4(+b5) than of human liver microsomes and CYP3A5(+b5). The K_I values of propoxyphene and CYP3A4(+b5) and human liver microsomes fall within the range of reported therapeutic blood levels of propoxyphene, with reversible inhibition constants (K_i values) above therapeutic blood concentrations for propoxyphene and norpropoxyphene. The K_I values of norpropoxyphene and CYP3A4(+b5) and human liver microsomes are higher than most reported blood levels, except for blood levels after repeated dosing of propoxyphene at high concentrations. The predicted change in the area under the plasma concentration versus time curve of an orally administered CYP3A substrate with propoxyphene (AUC'_{po}/AUC_{po}) was calculated for common CYP3A substrates. The AUC'_{po}/AUC_{po} ratios are four to twenty-five times higher with co-administration of propoxyphene based on *in vitro* kinetic parameters. Propoxyphene and norpropoxyphene may cause adverse events when chronically administered at high doses and/or when co-administered with other CYP3A substrates.

Sherry F. Queener, Ph.D., Chair

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ABBREVIATIONS

ACN	Acetonitrile
AIC	Akaike's Information Criteria
AUC'_{po}/AUC_{po}	Ratio of the area under the concentration versus time curve in the presence of an inhibitor to AUC in the absence of inhibitor, both drugs orally administered
CV	Coefficient of Variation
CYP	Cytochrome P450
CYP3A	Cytochrome P450 3A Family
CYP3A4(+b5)	Cytochrome P450 3A4 with coexpressed cytochrome b5
CYP3A5(+b5)	Cytochrome P450 3A5 with co-expressed cytochrome b5
f_m	Total hepatic elimination of substrate
F_G	Intestinal wall bioavailability of a drug
F'_G	Intestinal wall bioavailability of a drug in the presence of inhibitor
HPLC	High Performance Liquid Chromatography
i.v.	Intravenous administration
I_u	Average steady-state concentration of inhibitor in blood (μM)
K_{deg}	Rate of enzyme degradation (min^{-1})
K_{inact}	Maximal rate of enzyme inactivation in irreversible inhibition experiments (min^{-1})
K_I	Substrate concentration at half K_{inact} (μM)
K_i	Reversible inhibition constant analogous to K_m (μM)
K_m	Substrate concentration at half V_{max} (μM)

$K_{observed}$, K_{obs}	Observed rate of inactivation (min^{-1})
po	Per os, oral administration of a drug (literally “by mouth”)
SBC	Schwartz Bayesian Criterion
SD	Standard deviation of the mean
SE	Standard error of the mean
Uv/vis	Ultraviolet/visible (light)
V_{max}	Maximal velocity of product formation (min^{-1})
v.	Versus
WRSS	Weighted Residual Sum of Squares

CHAPTER 1-INTRODUCTION

Propoxyphene, a commonly used analgesic, may inhibit a major metabolizing enzyme, CYP3A. CYP3A metabolizes an estimated 60% of marketed drugs and endobiotics. CYP3A metabolizes many different types of drugs across multiple chemical classes and therapeutic classifications. Inhibition of CYP3A can reduce and prevent metabolism of CYP3A substrates, including co-administered drugs and chronically administered propoxyphene. Inhibition of CYP3A can lead to higher blood levels of these CYP3A substrates, which may cause an increase in reported side effects or adverse events. Some reported overdoses of propoxyphene may be attributed to mechanism-based inhibition of CYP3A. The purpose of this study was to determine if propoxyphene inhibits CYP3A, and, if so, to characterize the inhibition and determine to what extent the inhibition may affect patients who take the drug

Propoxyphene has been prescribed for over fifty years, but has been associated with numerous overdoses. Propoxyphene, like other opioids, has high abuse potential and is associated with higher rates of self-poisoning than other classes of drugs (Ng and Alvear, 1993). Accidental and suicide deaths have been attributed to the drug and its metabolite, either alone or in combination with other medications, including alcohol, benzodiazepines, acetaminophen, and other painkillers (McBay, 1976). Many cases of accidental overdose have been in combination with alcohol or other pain medication due to additive central nervous system depression (Gram, 1979) or liver toxicity due to acetaminophen overdoses in combined medication (Sheen *et al*, 2002). Propoxyphene has been associated with 2100 reported accidental deaths (38.6% of total propoxyphene deaths) in the US from 1981 to 1999, and 7109 total US deaths from 1999 to 2006. It was

found as one of the top ten drugs identified during autopsies and implicated in 5.6% of drug related deaths from 1981-1999 (DAWN, 2003). Propoxyphene products were withdrawn from the market in the United Kingdom in 2006 due to high numbers of fatalities at approximately 400 deaths per year (Lister, 2005). Norpropoxyphene, the primary metabolite of propoxyphene, has been associated with cardiac deaths in patients, and has a long half-life that allows for accumulation in the body (Inturrisi *et al*, 1982; Holland and Steinberg, 1979). Proadifen is structurally similar to propoxyphene and norpropoxyphene, but is not a marketed drug.

Previous reports of adverse events with propoxyphene administration may be attributed to mechanism-based inhibition of CYP3A. Mechanism-based inhibition occurs when a substrate of an enzyme inhibits it irreversibly during the catalytic cycle, and renders the enzyme permanently inactive. Mechanism-based inhibition can result in a reduction in the total amount of active enzyme. As the active enzyme pool is reduced, fewer substrates may be metabolized until new enzyme is synthesized. As a result, substrates accumulate resulting in elevated blood level concentrations represented by area under the curve, or AUC. AUCs may be above the desired therapeutic window of effectiveness and safety, resulting in toxic concentrations and overdoses.

Purpose of the study:

The focus of this research was to determine if propoxyphene, norporpoxyphene, and proadifen irreversibly inhibit CYP3A4(+b5), CYP3A5(+b5), and CYP3A in human liver microsomes *in vitro*. *In vitro* reversible inhibition of propoxyphene, norpropoxyphene, and proadifen with CYP3A4(+b5), CYP3A5(+b5) and CYP3A in human liver microsomes was also assessed. The second part of this research was to

determine if irreversible inhibition is due to the formation of a metabolic-intermediate complex. Metabolic-intermediate complex formation occurs when an inhibitor irreversibly binds to the CYP enzyme during its catalytic cycle, forming a covalent complex which is visible spectrophotometrically.

The *in vitro* inhibition data were examined comparing the potency of propoxyphene, norpropoxyphene, and proadifen for reversible and irreversible inhibition. The inhibitors were also compared for potency with the different isozymes (CYP3A4 and CYP3A5). The inhibitors were tested for metabolic-intermediate complex formation. Finally, the *in vitro* inhibition data were analyzed in comparison to *in vivo* blood levels of propoxyphene and norpropoxyphene to assess the clinical significance of findings.

Background information on the compounds used in this study:

Propoxyphene is an analgesic that is frequently prescribed in the United States and Europe. It was the seventeenth highest-selling generic drug in the United States in 2006, and ranked thirty-fourth for total retail dollars spent in 2006 at $260 million (Drug Topics, 2007). Marketed under the brand names of Darvon, Distalgesic, Co-proxamol[1], and Darvocet, propoxyphene is administered alone or in combination with non-steroidal anti-inflammatory drugs and/or caffeine to gain synergistic pain relief (Beaver, 1988; PDR, 2000).

Propoxyphene contains two chiral carbon atoms for two pairs of diasteriomers (α-*d,l* and β-*d,l*) (Somogyi *et al*, 2004) (see Table 1, p37). The β-*d,l* racemate is pharmacologically inactive (Nickander *et al*, 1984), but the α-*d* enantiomer (dextropropoxyphene) has analgesic properties (Gruber, 1956) and the α-*l* enantiomer has

[1] Co-proxamol was withdrawn from the market in the United Kingdom in January of 2005 due to drug related suicides and deaths (Lister, 2005).

antitussive properties (Miller *et al*, 1963). The drug product is composed of the α-*d* enantiomer in a hydrochloride or napsylate salt (AHFS, 2007). Dextropropoxyphene relieves pain by targeting opioid receptors in the brain, and is a weak opiate agonist (Miller, 1970). Dextropropoxyphene has one-half to two-thirds the potency of codeine (Gruber, 1977). Its mechanism of action is similar to other narcotic analgesics, such as methadone and codeine, and it shares similar alkylamine chemical structures with these other drugs (Somogyi *et al*, 2004; McMahon, 1961; Feinburg *et al*, 1976) (Tables 1 and 2, p37-38).

Propoxyphene is metabolized by Cytochrome P450 3A (CYP3A) to norpropoxyphene (Somogyi *et al*, 2004). Norpropoxyphene is not a marketed drug, although it has analgesic properties and even greater local anesthetic effects than propoxyphene (Nickander *et al*, 1984). Norpropoxyphene is further metabolized to dinorpropoxyphene (Nash *et al*, 1975). Other minor metabolites of propoxyphene and norpropoxyphene have been identified, but are not commercially available (McMahon *et al*, 1973; Nash *et al*, 1975).

Proadifen has a similar chemical structure and chemical properties to propoxyphene and norpropoxyphene (Table 2, p38). These three compounds are tertiary alkylamines of similar molecular weight. All three compounds are composed of a hydrocarbon chain with an ester, two phenyl substituents, and a di-methylated or di-ethylated nitrogen (Anders and Mannering, 1966; Somogyi *et al*, 2004). Unlike propoxyphene, proadifen was originally developed by Smith Kline and French, but it is not a marketed drug. Proadifen is a potassium channel blocker and a nicotinic acetylcholine receptor blocker (Anders and Mannering, 1966; Buening and Franklin,

1974). It is metabolized to the de-ethylated product SKF 8742-A (Buening *et al*, 1974). Proadifen has been used as a research tool in animals and *in vitro* as a general inhibitor of drug metabolism (Cook *et al*, 1954; Anders and Mannering, 1966; Buening and Franklin, 1974; Jones *et al*, 2007). Proadifen is a known general cytochrome P450 inhibitor (Schenkman *et al*, 1972; Bensoussan *et al*, 1995). Proadifen forms a metabolic-inhibitor complex with CYP3A4 (Jones *et al*, 2007), although its inhibition of CYP3A4 and CYP3A5 has not been extensively characterized.

Background information on enzyme inhibition:

Enzyme inhibition can be categorized as reversible and irreversible[2] (Lin, 1998). In reversible inhibition, the inhibitor and enzyme bind non-covalently. When the enzyme and inhibitor disassociate, the enzyme is still functional. In irreversible inhibition, the enzyme-inhibitor bond is usually covalent, the enzyme has been chemically changed, and the enzyme is no longer functional after binding to the inhibitor (Lin 1998).

An overview of irreversible inhibition is summarized in Schematic I (p44). As shown, the inhibitor can act as a substrate that is metabolized by the enzyme to a product (*i.e.* metabolite), but the enzyme can also be inactivated by the inhibitor (Silverman, 1995). Irreversible inhibition common with Cytochrome P450 enzymes (CYPs) is termed mechanism-based inhibition (MBI), and occurs when a metabolite is reactive and binds to the heme or protein of the CYP that caused its formation (Lin, 1998). The metabolite, or product, binds the CYP enzyme covalently, removing it permanently from the active enzyme pool so that it can no longer metabolize drugs. The enzyme can only be

[2] Some scientists support three types of inhibition including quasi-irreversible inhibition as the third type. Quasi-irreversible inhibition is similar to irreversible inhibition except that the inhibitor can become unbound from the enzyme in an *in vitro* setting chemically (with addition of potassium ferrocyanide) or using radiation (Lin, 1998).

replenished by new synthesis[3]. Mechanism-based inhibitors may form a metabolic-inhibitor complex with CYPs that can be detected experimentally at an absorption spectrum of approximately 450 nm (Murray, 1997)[4]. Metabolic-inhibitor complex formation with the nitrogen atom of an inhibitor and the iron atom of a CYP molecule is proposed in Schematic II (p44). Often alkylamines form a covalent complex with CYPs, particularly with CYP3A (Bensoussan *et al*, 1995). There are several known examples of pharmaceuticals that form metabolic-inhibitor complexes with CYP3A enzymes including some macrolides (oleandomycin, erythromycin) and some protease inhibitors (amprenavir, lopinavir, nelfinavir, ritonavir, and saquinavir) (Polasek and Miners, 2005; Ernest *et al*, 2004). These irreversible inhibitors can reduce the amount of free enzyme available to metabolize other drugs, potentially causing adverse reactions.

During reversible inhibition, the inhibitor binds to the enzyme, the enzyme and inhibitor then disassociate, and the enzyme returns to the active enzyme pool. The inhibitor or metabolite of the inhibitor does not form a permanent complex with the enzyme as occurs in mechanism-based inhibition. As demonstrated by Schematic III (p45), an enzyme (E) can bind with an inhibitor (I) to form the reversible enzyme inhibitor complex (EI), or the enzyme (E) can bind the substrate (S) to form the enzyme-substrate complex (ES) which can yield a product (metabolite), or return to free enzyme (E) and free substrate (S). The K_i is the dissociation constant for reversible inhibition. K_i is a ratio of the amount of free enzyme and inhibitor ([E][I]) to the amount of enzyme and inhibitor complex ([EI]) (Stryer, 1996; Zubay, 1998).

[3] The half life for CYPs in the body is 1-6 days (Dossing, 1983).
[4] Also called an Iron II Metabolite Complex (Naritomi *et al*, 2004).

Background information on enzymes used in this study:

CYPs are heme-based membrane proteins found in bacteria and animals. Eukaryotic CYPs are 480 to 560 amino acids long, and can be found in the endoplasmic reticulum, mitochondria, or cytosol of the cell. Microsomes are self-sealing fragments of endoplasmic reticulum membranes (Stryer, 1996), and the CYPs found in the endoplasmic reticulum of cells are referred to as the microsomal type (Nelson *et al*, 1996). Microsomal CYPs were the focus of this study. CYPs are named for their absorbance peak at 450 nm, (Danielson, 2002). CYP enzymes can be found in the liver (the major site of metabolism) and small intestine, kidney, skin, brain, lungs, gonads, adrenal glands, and other tissues (Goodman and Gillman, 1996). CYPs often metabolize highly lipophilic drugs into more hydrophilic compounds that can then be more readily eliminated from the body in the urine (Danielson, 2002; Goodman and Gillman, 1996).

CYPs require reduced nicotinamide adenine dinucleotide phosphate (NADPH) CYP reductase, NADPH, and molecular oxygen to perform their oxidation/reduction reactions to metabolize drugs. NADPH reductase is a membrane protein located near the CYP that contributes electrons to the oxidation reaction, NADP(H) serves as a cofactor by donating electrons to the reductase, and molecular oxygen can bind the ferric iron of the hemoprotein and eventually combine with the leaving group (*e.g.* N- or O-Dealkylations reactions) or bind to the parent drug (N- or S-oxidations) (Goodman and Gillman, 1996). Although they exist *in vivo*, CYPs and supporting enzymes, and cofactors (CYP reductase, and cytochrome b5, another electron donating group[5]), have

[5] The proposed mechanism of reduced cytochrome b5 in CYP oxidation-reduction reactions is to transfer electrons to P450 after being reduced by NADPH-P450 reductase (Yamazaki *et al*, 1996). It can interact synergistically to boost catalytic efficiency of CYPs (Danielson, 2002).

been recombinantly expressed in bacculovirus cells for *in vitro* testing (Crespi and Penman, 1997).

Thirty different cytochrome P450 enzymes have been identified including twelve families of CYPs in humans (Williams *et al*, 2004). CYPs with greater than 40% amino acid identity belong to the same family (Danielson, 2002). Of all the human CYPs, the CYP3A family is one of the most important for drug metabolism. The CYP3A family in humans metabolizes endobiotics such as testosterone and progesterone (Niwa *et al*, 1998) and approximately sixty percent or more of marketed drugs (Wrighton *et al*, 1990), including propoxyphene (Chow *et al*, 2006).

CYP3A performs N-demethylation and hydroxylation reactions. Propoxyphene and norpropoxyphene are metabolized by CYP3A in the liver to norpropoxyphene and dinorpropoxyphene, respectively (Somogyi *et al*, McMahon *et al*, 1973; Nash *et al*, 1975). Compared to other CYP families, the CYP3A family metabolizes drugs with the largest molecular size (Nagata and Yamazol, 2002). CYP3A is the most abundant CYP in the liver and small intestine (Yamazaki *et al*, 1996); its expression level is thirty to sixty percent of the total CYP content in the human liver (Shimada *et al*, 1994) and comprises sixty to seventy percent of the CYP in the small intestine (Anttila, 1997). The enzymes CYP3A4, CYP3A5, CYP3A7, and CYP3A43 make up the CYP3A family in humans (Wrighton *et al*, 2000).

CYP3A4 and CYP3A5 share an eighty-three percent amino acid sequence identity (Aoyama *et al*, 1989). These two enzymes have similar substrate specificity (Lin *et al*, 2002), with CYP3A5 generally exhibiting a lower metabolic capability than CYP3A4 (Williams *et al*, 2002). Most of the general population expresses CYP3A4

despite the existence of genetic polymorphisms. Keshava *et al*, (2004) found that polymorphisms in CYP3A4 do not contribute to differences in activity. Although Dai *et al*, (2001) found three genotypes associated with differential CYP3A4 expression, *17, *18, and *1B. The CYP3A4*17 genotype corresponds with reduced CYP3A4 expression, and the CYP3A4*18 corresponds with increased CYP3A4 expression as compared with wildtype *1 (Dai *et al*, 2001), (Table 3, p39). Contradictory results have been reported for CYP3A4*1B. CYP3A4*1B expression is associated with increased CYP3A4 activity over wildtype (Kuehl, 2001). CYP3A4*1B is associated with reduced CYP3A4 expression (Wojnoski *et al*, 2002).CYP3A4 is generally considered the most abundant CYP in the liver and the small intestine (Shimada *et al*, 1994). Unlike CYP3A4, CYP3A5 is expressed in the kidney (Eichelman and Burk, 2001), and is the predominant CYP in the lung (Attila, 1997).

CYP3A5 is polymorphic and includes individuals who do not produce functional CYP3A5 enzyme. CYP3A5 is only detectable in twenty to thirty percent of human livers (Eichelbaum and Burk, 2001). Ten to thirty percent of Caucasians, fifty-five to seventy percent of Black Africans and African Americans, and thirty-three percent of the Japanese express CYP3A5 (Kamden *et al*, 2005). Several alleles have been identified for CYP3A5; *1, *2, *3, *5, *6, and *7. The *1 allele is the wildtype allele. The presence of one *1 allele contributes to high expression of CYP3A5. CYP3A5*1 produces ten to thirty times the amount of CYP protein produced from CYP*3/*3 (Kreutz *et al*, 2005). In human liver microsomes, Huang *et al*, (2004) found that individuals carrying the CYP3A5*1 allele, CYP3A5 constituted more than 50% of the total CYP3A expression. Polymorphisms in CYP3A5 can be a causal factor in differential patient responses to

drugs and food products. Chow *et al*, (2006) found that poor CYP3A5 expressers (CYP3A5*3/*3) had higher propoxyphene plasma concentrations and lower clearance rates of propoxyphene than expressers (CYP3A5*1/*3 or CYP3A5*1/*1). CYP3A7 and CYP3A43 are enzymes that have been found in very low levels in some adult livers (Gellner *et. al*., 2001), and CYP3A7 is predominantly a fetal enzyme (Thummel and Wilkinson, 1998). For these reasons, only the CYP3A4 and CYP3A5 enzymes of the CYP3A family are examined along with pooled human liver microsomes.

CHAPTER 2-MATERIALS AND METHODS

An overview of methods used in this study:

Propoxyphene, norpropoxyphene, and proadifen were incubated with recombinant CYP3A4 and recombinant CYP3A5 recombinant or pooled human liver microsomes. Formation of 6β hydroxy testosterone, the major metabolite of testosterone, was quantified with HPLC. Kinetic parameters were estimated from data fit with Windows NonLin non-linear regression data models (version 5.0.1, Pharsight, Mountain View, California). Kinetic parameters calculated from the experimental inhibition data were used to estimate *in vivo* changes in area under the plasma concentration time curve, AUC. Metabolite complexes were detected by ultraviolet/visible spectrophotometry, based upon an absorption maximum of 450 nm.

Chemicals:

Propoxyphene hydrochloride was obtained from the United States Pharmacopeia (Rockville, Maryland). Norpropoxyphene HCl, testosterone, 6β-hydroxytestosterone, desmethyl diazepam, temazepam, ammonium acetate, and NADPH were purchased from Sigma-Aldrich (St. Louis, Missouri). HPLC grade acetonitrile (ACN) and methanol were purchased from J.T. Baker (Phillipsburg, New Jersey), and HPLC grade ethyl acetate was purchased from EMD Chemicals, Inc. (Gibbstown, New Jersey).

Enzymes:

Recombinant CYP3A4(+b5) and recombinant CYP3A5(+b5) were purchased from BD Gentest (Woburn, Massachusetts). Adult human liver microsomes were prepared from human liver tissues (in accordance with protocols approved by the Institutional Review Board of IUPUI). The homogenates of five livers were pooled at 20

mg/ml total protein yielding a CYP concentration of 0.3 nmol/mg protein. (Lowery *et al*, 1951; Gorski *et al*, 1994).

Mechanism-Based (Irreversible) Inhibition Experiments:

Enzyme inhibition was determined by the time-dependent and concentration-dependent loss of 6β-hydroxy-testosterone product formed from testosterone by CYP3A4(+b5), CYP3A5(+b5), or human liver microsomes. The inhibitor was diluted in methanol and subsequently diluted in phosphate buffer (with 5 mM $MgCl_2$, pH 7.4) for a final concentration of < 0.5 % methanol or evaporated to dryness prior to incubation with human liver microsomes (100 µg total protein), CYP3A4(+b5) (20 pmoles), or CYP3A5(+b5) (20 pmoles). The reaction was started with 5 µl of 10 mM NADPH made with phosphate buffer for a final pre-incubation[6] reaction volume of 50 µl at 37 °C in a mixing waterbath. Inhibitor concentrations and inhibitor incubation times were determined from preliminary experiments of single data points. Final pre-incubation experiments were conducted with samples in duplicate.

Immediately following the pre-incubation of inhibitor and enzyme, 950 µL of incubation supplement mixture (containing phosphate buffer as described above, 250 µM[7] testosterone (substrate), and 10 mM NADPH) were added to the tubes in 37 °C water bath for an additional two minute incubation. The reaction was quenched with two mL ice-cold ACN. The internal standard, desmethyl diazepam or temazepam (600 ng), was added and the reaction tubes were mixed on a vortex mixer for 30 seconds and centrifuged at 2500 rpm for 5 minutes. The supernatant was removed from the protein

[6] Pre-incubation refers to the incubation reaction of the inhibitor, enzyme, and NADPH, prior to the addition of substrate.

[7] The concentration of testosterone was well above the V_{max} for 6β hydroxyl testosterone formation with CYP3A4(+b5), CYP3A5(+b5), and HLM.

and added to glass screw-top tubes containing 5 ml of ethyl acetate. The tubes were shaken for 30 minutes and then centrifuged at 2500 rpm for 5 minutes. The organic layer was removed and transferred to 13x100 mm glass culture tubes and evaporated to dryness. That residue was reconstituted with 250 µl mobile phase (40% 30mM Ammonium acetate, pH 6.4: 60% methanol (volume per volume)). Concentrations of 6β-hydroxy testosterone were determined using HPLC with 5 µm C-18(2) Luna Phenomenex (Torrance, California) column with a 1 ml/min flow rate and uv detection at 254 nm.

Reversible Inhibition Experiments:

To test for reversible inhibition, inhibitor and testosterone were diluted in methanol and subsequently diluted in phosphate buffer (with 5 mM $MgCl_2$, pH 7.4), for a final concentration of < 0.5 % methanol, or evaporated to dryness in tubes prior to incubation. Enzyme, substrate, inhibitor and phosphate buffer were combined for a total volume of 900 µL, and 100 µL of 10 mM NADPH solution were added to start the reaction. The tubes were incubated for two minutes in a 37 °C mixing water bath. The reaction was quenched with two mL ice-cold ACN and subsequent extraction and analysis steps were performed (as described above). The concentration of substrate and inhibitor used to calculate K_i values were estimated from preliminary experiments with single samples (see Data Modeling section below). Final experiments were conducted with samples in duplicate. A minimum of four concentrations of testosterone were tested, two concentrations above the K_m and two concentrations below the K_m for each enzyme. Each inhibitor was tested with at least two concentrations above and two concentrations below the estimated K_i value (from preliminary data).

Metabolic-Inhibitor Complex Formation Experiments:

Initial metabolic-inhibitor complex experiments were conducted with a dual beam spectrophotometer (Uvicon 933, Research Instruments). Two 1 mL cuvettes were prepared with 100-200µL enzyme (100-200 pmoles CYP3A4(+b5) or CYP3A5(+b5) and 1 mg of total protein of human liver microsomes), 5 µL of 1 mg/mL[8] inhibitor in methanol (or methanol for reference), and 0.1 M phosphate buffer with 5 mM magnesium chloride each at 37 °C. 100 µL of 10 mM NADPH were added to start the reaction (1 mL total reaction volume). Wavelengths 380-500 nm were scanned at time zero and then at two or five minute intervals, the samples were assessed for an absorbance maximum by subtracting the absorbance at 490 nm from the difference of the absorbance scan at each timepoint and a background absorbance scan. The metabolic-inhibitor complex forms as a characteristic peak at approximately 450 nm that increases with absorbance over time. The method to detect metabolic-inhibitor complex was adapted for a microplate reader (Synergy™ 2, BioTek) for follow-up experiments. In these experiments, 80 µL of enzyme and 260 µL of phosphate buffer (as described above) were combined in a polypropylene tube. Next, 170 µL of enzyme and buffer mixture were aliquoted into each well (experimental and control well). The plate was warmed at 37 °C for two to three minutes, and then 10 µL of inhibitor (in a 1 mg/mL solution of 10% methanol and 90% phosphate buffer) were added to the first well, and 10 µL of 10% methanol 90% phosphate buffer were added to the reference well. The reaction started with the addition of 20 µL 10 mM NADPH (Pershing and Franklin, 1982).

[8] This corresponds to a concentration of approximately 15µM for each.

Data Modeling for Inhibition:

The percentage of enzyme activity remaining after incubation was determined by the amount of 6β-hydroxy testosterone formed relative to the amount formed at 0 time (100%) at each inhibitor concentration. The natural logarithm of percent activity was plotted against pre-incubation time at different inhibitor concentrations. The lines of best fit were determined using Microsoft Excel best fit trendline. The slopes of these lines were used to determine $k_{observed}$ values, or the observed rate of 6β-hydroxy testosterone product decline, at various inhibitor concentrations. Inhibitor pre-incubation time and percent activity (E_t) at various concentrations of inhibitor were modeled with Windows Nonlin Professional to estimate k_{inact}, the rate constant for maximal rate of inactivation, and K_I, the concentration of substrate at half maximal inactivation (Equation 1, p46). From the Windows Nonlin Professional K_I and k_{inact} estimates, $k_{observed}$ was calculated and plotted as a hyperbolic curve (see Equations 2a and 2b). The standard error of the mean (SE), coefficient of variation (CV), Akaike Information Criterion (AIC), and Schwartz Bayesian Criterion (SBC) were used to evaluate each model for best fit. Information on the statistical criteria can be found in the Appendix.

For reversible inhibition, 6β-hydroxy testosterone/min product formation data were modeled with Windows Nonlin Professional. The models estimated V_{max}, K_m, and K_i values. The V_{max} is the maximal velocity of product formation that can be achieved by increasing substrate concentration under the conditions of assay. Preliminary reversible and irreversible inhibition experiments were conducted using singlet data points across multiple concentrations of inhibitor. Experiments were conducted in duplicate and data were modeled in competitive, noncompetitive, and uncompetitive inhibition models (see

Equations 3-5, p46). Inhibition type was determined based on best fit criteria from model (lowest SE[9], CV, AIC, SBC). Final experiments were conducted in duplicate using a minimum of two concentrations below and two concentrations above the estimated K_i value.

Experimental Predictions of *In Vivo* Drug Interactions with Propoxyphene and Norpropoxyphene:

The *in vivo* AUC'_{po}/AUC_{po} of common CYP3A substrates was estimated using Equation 6 (p46) (Wang *et al*, 2005). F_G is the intestinal wall bioavailability (Wang, 2005), and was determined from published data. F'_G is the intestinal wall bioavailability in the presence of the inhibitor. The k_{inact} and K_I values were determined from experiments with human liver microsomes. I_u is the average unbound steady state concentration of each inhibitor. The k_{deg} values, or rate of enzyme degradation, are 0.00128 and 0.00026 min^{-1} based on rat CYP3A and human CYP3A4 in CaCO-2 cells (Correia, 1991; Malhotra *et al*, 2001). Both rates of enzyme degradation were used in the calculation resulting in a range estimates for the AUC'_{po}/AUC_{po}. The equation assumes a maximal inhibition of CYP3A by propoxyphene or norpropoxyphene from the gut wall, and F'_G is equal to 1 (consistent with Wang *et al*, 2005 and Ernest *et al*, 2004). The total CYP3A hepatic elimination of the substrate without inhibitor (f_m) was determined from published *in vivo* data.

[9] See Appendix for more information on statistical criteria used to evaluate inhibition models.

CHAPTER 3-RESULTS

Chromatography Data:

For incubations with CYP3A4(+b5) or CYP3A5(+b5), a mobile phase of 40% 30 mM ammonium acetate (pH 6.3-6.4) was used with desmethyldiazepam or temazepam as internal standards. Non-extracted standards (6β-hydroxy testosterone, internal standard, testosterone), and inhibitors were analyzed by HPLC prior to use in an incubation to verify peak separation and recovery (Figure 1, p47). In some preliminary experiments, metabolite peaks co-eluted with one internal standard, which necessitated the use of the other internal standard for subsequent incubations. The human liver microsomes incubation with testosterone and propoxyphene, norpropoxyphene, or proadifen often resulted in peaks that co-eluted with 6β-hydroxy testosterone and/or both internal standards. The mobile phase was adjusted by increasing the percentage of ammonium acetate and decreasing the percentage of methanol to improve separation, although this increased the run time. Subsequently, the pH of ammonium acetate was adjusted to improve separation and reduce run time. A final mobile phase of 50% ammonium acetate pH 5.6-5.8 and 50% methanol was used for human liver microsomes incubations to improve peak separation (Figure 2, p48).

Irreversible Enzyme Inhibition Data:

Propoxyphene, norpropoxyphene, and proadifen exhibited time and concentration dependent inactivation of CYP3A4(+b5), CYP3A5(+b5), and CYP3A with human liver microsomes (see Table 4, p40). The lowest K_I was achieved by proadifen and CYP3A4(+b5) (0.35 μM). Propoxyphene and human liver microsomes generated the second lowest K_I (0.45 μM). All three compounds were less potent inhibitors of

CYP3A5(+b5) than CYP3A4(+b5) based on K_I values. The K_I values with CYP3A5(+b5) were three to sixty times higher than corresponding values with CYP3A4(+b5) and three to thirty times higher than with human liver microsomes. The K_I values for propoxyphene with human liver microsomes were lower than the K_I values of propoxyphene with CYP3A4(+b5) and CYP3A5(+b5). Norpropoxyphene exhibited inactivation (K_I values) with CYP3A4(+b5) and human liver microsomes at similar concentrations. Proadifen was a more potent inhibitor of CYP3A4(+b5) than human liver microsomes (K_I values were 0.35 and 6.9 μM, respectively).

Proadifen exhibited irreversible inhibition of CYP3A4(+b5), CYP3A(+b5), and CYP3A in human liver microsomes. The K_I values for proadifen and CYP3A5(+b5) were approximately three-fold higher than the K_I values for proadifen and human liver microsomes. The rates of enzyme inactivation are summarized in Table 4, p40. Propoxyphene and norpropoxyphene exhibited the highest rates of inactivation (highest k_{inact}) with CYP3A4(+b5), 0.41 $min^{-1}$and 0.56 min^{-1}, respectively. The highest k_{inact} of all three compounds with CYP3A5(+b5) was achieved by norpropoxyphene (0.21 min^{-1}). The rates of inactivation for proadifen and CYP3A4(+b5) and proadifen and human liver microsomes were similar (0.26 min^{-1} and 0.20 min^{-1}, respectively).

The results of irreversible inhibition experiments are plotted in Figures 3-29 (p49-75). These include graphs of % Activity v. Pre-incubation Time, k_{obs} v. Inhibitor, and % Activity Relative to Control. The percent activity versus pre-incubation time graphs include the averaged data points and lines calculated from model estimates, these are listed in Figures 3-11 (p49-57). Although the pre-incubation times differ, the plots demonstrate that activity decreases as pre-incubation time increases, and activity

decreases with increasing concentrations of inhibitor. The lines in Figures 3-11 were calculated from Equation 2b (p46) with k_{inact} and K_I model estimates. Although not all % activity data points fall on the line, the general trend of the data for each concentration is similar to the predicted line shown in the graph. The slopes of excel best fit lines from Figures 3-11 which represent $k_{observed}$, were plotted versus inhibitor concentration in Figures 12-20 (p58-66). The hyperbolic curve was calculated (Equation 2a, p46) based on k_{inact} and K_I model estimates using percent activity.

Figures 21-29 (p67-75) show percent of control enzyme activity remaining using pre-incubation for control or inhibitor reactions. The concentrations of inhibitor are listed across the x-axis as inhibitor concentration in the total incubation mix (1 mL reaction)[10]. Only norpropoxyphene and CYP3A5(+b5) showed a decrease in % activity relative to control below 75% in these experiments performed without pre-incubation (approximately 70% activity at 4 μM norpropoxyphene, Figure 25, p71).

Metabolic-Inhibitor Complex Formation Data:

Propoxyphene, norpropoxyphene, and proadifen formed metabolic-inhibitor complexes with CYP3A4(+b5) and CYP3A5(+b5) (see Figure 30, p76 for a metabolic-inhibitor complex plot, and Table 4, p40 for metabolic-inhibitor complex formation results for each compound and enzyme). In initial studies with the dual beam spectrophotometer and CYP3A5(+b5) enzyme, only propoxyphene and proadifen formed a metabolic-inhibitor complex with CYP3A5(+b5). Norpropoxyphene had been tested for metabolic-inhibitor complex formation with CYP3A5(+b5) before the K_I was determined by pre-incubation experiments, and the concentration may have been too low to form a

[10]Although this control is not pre-incubated with NADPH prior to the addition of substrate, the total concentration of inhibitor in a 50 μL pre-incubation reaction is listed in parentheses, for ease of comparison to pre-incubated samples.

metabolic-inhibitor complex (norpropoxyphene was tested at 15.3 μM, the K_I was later determined to be 25.2 μM). In follow up experiments with the plate reader, norpropoxyphene was tested at a higher concentration (153 μM) and formed a metabolic-inhibitor complex with CYP3A5(+b5). Propoxyphene and norpropoxyphene did not form a metabolic-inhibitor complex with human liver microsomes, although proadifen did form a metabolic-inhibitor complex with human liver microsomes.

Reversible Enzyme Inhibition Data:

Propoxyphene, norpropoxyphene, and proadifen exhibited reversible inhibition of CYP3A4(+b5), but proadifen was the most potent inhibitor (K_i value was 5 μM) (Table 5, p41). Propoxyphene and norpropoxyphene exhibited reversible inhibition of CYP3A4(+b5) only at higher concentrations (K_i values were 26 μM and 29 μM, respectively). Proadifen was the most potent reversible inhibitor of CYP3A5(+b5) and human liver microsomes, with K_i values of 12 μM and 8 μM, repectively. Norpropoxyphene exhibited reversible inhibition of human liver microsomes (K_i value was 59 μM), but the estimated K_i value of propoxyphene (155 μM) was higher than the highest concentration tested (80μM). The K_i estimates for propoxyphene or norpropoxyphene and CYP3A5(+b5) were also above the highest concentration tested (100 μM). For propoxyphene and CYP3A4(+b5), the competitive model had the lowest SE, CV, AIC, and SBC. For all compounds and enzymes studied, the competitive model yielded the best fit of the data and the lowest overall values for SE, CV, AIC, and SBC.

A Comparison of Irreversible and Reversible Enzyme Inhibition Data:

Propropoxyphene and norpropoxyphene were more potent irreversible inhibitors than reversible inhibitors. The K_i value for propoxyphene and CYP3A4(+b5) is twenty

times higher than the K_I value, and the K_i value for propoxyphene and human liver microsomes is over one hundred and eighty times higher than the K_I value. For propoxyphene and CYP3A5(+b5), the K_i value is more than seven times higher than the K_I value For norpropoxyphene, the K_i value of CYP3A4(+b5) was three times higher than K_I values, and the K_i value for human liver microsomes was seven times higher than the K_I value. Although the K_i value for norpropoxyphene could not be determined, the K_i value is greater than four times the value of K_I value. Unlike propoxyphene and norpropoxyphene, proadifen achieved similar concentrations for K_i value and K_I value with human liver microsomes and CYP3A5(+b5). The K_i value and K_I value for proadifen and human liver microsomes are 8 μM and 7 μM, respectively. The K_i value and K_I value for proadifen and CYP3A5(+b5) are 20 μM and 12 μM, respectively. Irreversible inhibition (K_I value) of CYP3A4(+b5) was ten times greater than reversible inhibition (K_i value) with proadifen, K_I value was 0.4 μM and Ki value was 5 μM (Tables 4 and 5, p40-41). The K_i values of proadifen with CYP3A5(+b5) and human liver microsomes are twelve and eight, respectively, and K_I values are twenty and seven, respectively. The K_i value for proadifen and CYP3A4(+b5) is about fourteen times the K_I value.

CHAPTER 4-DISCUSSION

The results are discussed relative to the distribution of propoxyphene in the body, enzyme selectivity, reversible and irreversible inhibition, *in vivo* propoxyphene and norpropoxyphene concentrations, and drug-drug interactions with other CYP3A substrates. Proadifen results will also be mentioned.

After oral administration, propoxyphene is rapidly distributed to the liver, brain, lungs, and kidneys, and is eliminated as propoxyphene or metabolized product (norpropoxyphene or dinorpropoxyphene) in the urine (Clark, 1986). However, orally-administered propoxyphene must pass through the small intestine and liver before distributing to the rest of the body[11], and is therefore subject to "first-pass" metabolism (Ferrier, 1972). Only eighteen percent of propoxyphene enters the systemic circulation from the oral administration of a 65 mg dose of propoxyphene hydrochloride (Ferrier, 1972). Propoxyphene and norpropoxyphene can be metabolized by CYP3A in the small intestine and liver to dinorpropoxyphene, and/or be irreversibly bound to CYP3A enzymes. CYP3A4 is the most abundant CYP in the liver and small intestine, where CYP3A5 is also found (Shimada *et al*, 1994), although functional protein is only expressed in some individuals (Kamden *et al*, 2005; Huang *et al*, 2004). Propoxyphene or norpropoxyphene that reaches the systemic circulation may be metabolized by CYP3A5 in the kidney (Eichelman and Burk, 2001). Selectivity of CYP3A5 over CYP3A4 would be important to know, as CYP3A4 and CYP3A5 vary in interpersonal expression of functional enzyme and distribution in the body.

[11] The drug is most often taken orally because intravenous and subcutaneous administration result in severe vein and soft tissue damage (Hudson, 1977). Oral administration is also more convenient than *i.v.*

Propoxyphene is a more potent mechanism-based inhibitor of CYP3A4 than of CYP3A5, with K_I's approximately ten times higher for CYP3A4 than CYP3A5 (Table 4, p40). In fact, propoxyphene, norpropoxyphene, and even proadifen were more potent irreversible inhibitors of CYP3A4 than CYP3A5 based on K_I's. CYP3A5 generally exhibits a lower metabolic capability than CYP3A4 (Williams *et al*, 2002), but there are exceptions, such as vincristine and tacrolimus, which are metabolized by CYP3A4 and CYP3A5 with equal efficiency[12] (Dennison *et al*, 2007; Kamden *et al*, 2005). Compounds exhibiting higher catalytic rates of metabolism with CYP3A5 than with CYP3A4 may show increased toxicity in patients who do not express functional CYP3A5. Additionally these compounds may exhibit reduced efficacy due to lower blood levels in patients who express high levels of functional CYP3A5. The clearance rates of tacrolimus and vincristine increased in CYP3A5 high expressers as compared with low expressers (McPhee *et al*, 2002; Dennison *et al*, 2007). Increased adverse events have been associated in cancer patients taking vincristine who have low CYP3A5-mediated metabolism as compared to patients with functional CYP3A5 (Dennison *et al*, 2007).

Patients who express functional CYP3A5 and CYP3A4 may exhibit different drug plasma concentrations and clearance rates than patients who do not express functional CYP3A5, depending on the drug. Individuals expressing functional CYP3A5 (and CYP3A4) possess two enzymes capable of metabolizing one substrate. Chow *et al* (2006) found that poor CYP3A5 expressers (CYP3A5*3/*3) had higher propoxyphene plasma concentrations and lower propoxyphene clearance rates than high expressers

[12] The metabolic capability or efficiency of isozymes can be compared by clearance rates (Williams *et al*, 2002).

(CYP3A5*1/*3 or CYP3A5*1/*1). It is reasonable to conclude that CYP3A5 high expressers (CYP3A5*1/*1) may be less susceptible to propoxyphene-related adverse events, despite propoxyphene's selectivity for CYP3A4 over CYP3A5.

Polymorphisms in CYP3A4 may also affect the clearance and concentration of CYP3A substrates. *In vivo* metabolism of 3A4 substrates may vary up to ten-fold because of differences in CYP3A4 expression (Danielson, 2002). CYP3A4*17, CYP3A4*18, and CYP3A4*1B alleles are associated with differential CYP3A4 expression as compared to wildtype (CYP3A4*1, and propoxyphene and norpropoxyphene concentrations may vary in individuals based on CYP3A4 genotype (Dai *et al*, 2001; Kuehl, 2001). The CYP3A4*17 genotype corresponds with reduced CYP3A4 expression. Accordingly, AUCs for propoxyphene and norpropoxyphene may be higher in individuals that have this genotype, and they may experience an increased incidence of adverse events. Because CYP3A expression and metabolism studies with CYP3A4*1B alleles have produced contradictory results, it is difficult to predict the extent of drug interactions with this genotype. Findings include CYP3A4*1B producing two-fold increased CYP3A activity over wildtype (Kadlubar, 2003), no change in CYP3A4 expression with CYP3A4*1B alleles, and no change in midazolam clearance as compared to wildtype CYP3A4*1 (Rebbeck, 2000). The CYP3A*1B allele is in linkage disequilibrium with the CYP3A5 high expression allele (CYP3A5*1), and high CYP3A5 expression may confound CYP3A drug metabolism results.[13] Propoxyphene and norpropoxyphene blood concentrations may be lower in individuals with the CYP3A4*18 genotype, which corresponds with increased CYP3A4 expression (Dai *et al*, 2001).

[13] The non random association of genes at more than one loci. 80% of Caucasians with CYP3A4*1B allele also possessed one CYP3A5*1A allele (Wojoski *et al*, 2002).

Despite the existence of CYP3A4 and CYP3A5 polymorphisms in the general population, one would expect *in vivo* studies to support the *in vitro* observations that propoxyphene inhibits CYP3A metabolism. The studies by Inturrisi *et al* (1982) support the assertion that propoxyphene (and norpropoxyphene) are possibly mechanism-based inhibitors of CYP3A. Inturrisi *et al* (1982) reported that repeated dosing of propoxyphene resulted in the accumulation of propoxyphene and norpropoxyphene in patients, and blood concentrations of propoxyphene and norpropoxyphene were five to seven times higher than the concentrations achieved after a single dose. They also found that the clearance of propoxyphene and norpropoxyphene decreased with repeated dosing (994 to 508 mL/min and 454 to 210 mL/min, respectively). Inturrisi *et al* (1982) found that the half life of the two compounds increased from 3.3 to 11.8 hours for propoxyphene, and from 6.1 to 39.2 hours for norpropoxyphene with repeated dosing. These characteristics may be attributed to a mechanism-based inhibitor. As CYP3A enzymes are irreversibly inhibited by propoxyphene and norpropoxyphene, the free enzyme pool is depleted, and these compounds may accumulate.

Propoxyphene, norpropoxyphene, and proadifen are mechanism-based inhibitors of CYP3A as measured by inhibition of CYP3A4(+b5), CYP3A5(+b5), and CYP3A in human liver microsomes. Propoxyphene and norpropoxyphene form metabolic-inhibitor complexes with CYP3A4(+b5) and CYP3A5(+b5), but proadifen formed metabolic-inhibitor complexes with the recombinant CYPs and human liver microsomes. Ernest *et al* (2004) observed similar results with protease inhibitors; metabolic-inhibitor complex formation occurred with protease inhibitors and CYP3A4(+b5), but not with human liver microsomes. The pooled liver microsomes express CYP3A4 and CYP3A5 (Wang, 2005),

but the lack of metabolic-inhibitor complex formation could be attributed to the low amount of CYP3A compared to total protein in human liver microsomes. The pooled livers contained approximately three hundred pmoles of CYP (Gorski *et al*, 1994), and approximately thirty pmoles of CYP3A enzyme per mg of total protein (Wang *et al*, 2005). The concentration of CYP3A in human liver microsomes is near the limit of quantitation of twenty-three pmoles (Ernest, 2004). Proadifen is a general CYP inhibitor (Bensoussan *et al*, 1995) and may bind irreversibly to multiple CYPs, therefore it is not surprising that it forms a metabolic-inhibitor complex with human liver microsomes. The binding spectra (450 nm) of other CYPs may resemble the binding spectra of CYP3A. CYP2D6 and paroxetine form a metabolic-inhibitor complex at approximately 450 nm (Bertelsen, 2003). Additionally, proadifen forms a metabolic-intermediate-complex with guinea pig CYP2B6 which may resemble the CYP3A4 spectra (Yamada *et al*, 1992). Therefore it is reasonable that proadifen would form a metabolic-inhibitor complex with human liver microsomes, and propoxyphene and norpropoxyphene did not form a metabolic-inhibitor complex with human liver microsomes.

Propoxyphene and norpropoxyphene are weak reversible inhibitors of CYP3A, with K_I values greater than or equal to 26 μM. Many K_i value estimates were greater than the highest concentrations tested (≥ 80 μM), much higher than drug concentrations in plasma (propoxyphene and CYP3A5(+b5), norpropoxyphene and CYP3A5(+b5), and propoxyphene and human liver microsomes).

Propoxyphene and norpropoxyphene were more potent irreversible inhibitors than reversible inhibitors of CYP3A4(+b5), CYP3A5(+b5), and CYP3A in human liver microsomes. Because irreversible inhibition removes functional enzyme from the enzyme

pool, much of the CYP3A functional protein would theoretically be removed. The K_I values (inhibitor concentration at half k_{inact}) were at least three times lower than the K_i values (concentration of enzyme-inhibitor relative to free enzyme and free inhibitor) for CYP3A4(+b5), CYP3A5(+b5), and human liver microsomes for propoxyphene and norpropoxyphene. The reported therapeutic blood concentrations of propoxyphene are listed in Table 6 (p42) and range from 0.4 to 2.5 μM. The K_I value for propoxyphene (~1 μM) is within range of reported therapeutic blood levels, whereas the K_i value is much higher (~50 μM). Therefore propoxyphene concentrations in the body would not approach the levels needed for reversible inhibition based on *in vitro* data. The toxic blood concentrations of propoxyphene are greater than 1.5 μM, which closely corresponds to the K_I value for CYP3A4 (Merck, 2007). The reported therapeutic concentrations of norpropoxyphene in blood range from 0.9-15 μM (Verbeley and Inturrissi, 1973; Inturrisi *et al*, 1982). These concentrations are within the range of K_I value for norpropoxyphene (~8 μM), whereas the K_i value for norpropoxyphene is approximately 40 μM. Norpropoxyphene blood concentrations would not approach the levels needed for reversible inhibition based on *in vitro* data. *In vivo* inhibition of CYP3A enzymes would probably be due to mechanism-based inhibition and not due to reversible inhibition.

Norpropoxyphene may play a significant role in adverse events attributed to propoxyphene because it has potent anesthetic properties and causes cardiac toxicity and seizures (Nickander *et al*, 1984). Approximately seventy-six percent of propoxyphene deaths are attributed to cardiac toxicity (Whitcomb *et al*, 1989). Norproxyphene causes hypotension, decreased contractability, and interruption of cardiac conduction (Holland

and Steinberg, 1979). Propoxyphene and norpropoxyphene have anti-arrhythmic properties and block sodium channels, but norpropoxyphene is more potent than propoxyphene for cardiac effects (Holland and Steinberg, 1979; Slywka, 1975). Norpropoxyphene also has a longer half life than propoxyphene (30-36 hours versus 6-12 hours), and can accumulate in the body. Norpropoxyphene blood concentrations as high as 15 μM have been found after high oral therapeutic doses of propoxyphene (Inturrisi *et al*, 1982). Toxicity for norpropoxyphene has not been established, but toxicity has been associated with blood concentrations of 0.15 μM propoxyphene (see Table 6, p42).

Inhibition of CYP3A by propoxyphene may cause adverse effects in patients taking high doses of this drug. CYP3A inhibition of propoxyphene may also result in adverse affects in patients concomitantly administered other CYP3A substrates. The use of propoxyphene in elderly patients is limited because of the high number of reported adverse events with propoxyphene in this subpopulation (Beers, 1997), who are often administered multiple drugs over the same time period (polypharmacy) as compared to other subpopulations[14]. Potential drug-drug interactions exist for propoxyphene and other CYP3A substrates. Mechanism-based inhibition of CYP3A by propoxyphene may increase the concentrations of other CYP3A substrates in the body.[15] CYP3A metabolizes up to 60% of marketed drugs including some immunosuppressants, heart medications, and many other drugs (Turgeon *et al*, 1992; Wang *et al*, 2005). Other CYP3A substrates include food products such as caffeine (Tassaneeyakul *et al*, 1993), and grapefruit juice (Bailey *et al*, 1993). Increased plasma concentrations of CYP3A substrates (drugs or

[14] The elderly also often have decreased liver function compared to the general population.
[15] Some drugs are metabolized by multiple CYP isoforms, and co-administration with propoxyphene may not result in higher blood levels. An example is acetaminophen, which is metabolized to N-acetyl-p-benzoquinone imine by CYP2E1, CYP1A2, CYP2A6, CYP2D6, and CYP3A4 (Dong *et al*, 2001).

metabolites) can elicit severe adverse events, such as rhabdomyolysis, with high plasma levels of HMG-CoA reductase inhibitors (Dresser *et al*, 2000) and sedation with benzodiazepines (AHFS, 2007).

It is difficult to determine if inhibition of CYP3A by propoxyphene and norpropoxyphene has contributed to reported adverse events. Accidental and suicide deaths have been attributed to propoxyphene and its metabolite in combination with other medications, including benzodiazepines and other analgesics (McBay, 1976). Several benzodiazepines (alprazolam, diazepam, midazolam, triazolam) and analgesics (cocaine, codeine, fentanyl) are metabolized by CYP3A (Gasche, 2004). Many cases of accidental overdoses with propoxyphene have also occurred in combination with other pain medication (Gram, 1979) such as opioids (Ng and Alvear, 1993), and many opioids are metabolized by CYP3A (Moody, 1996).

Table 7 (p43) lists predicted increases in drug concentrations for some common CYP3A substrates as a result of drug interactions with propoxyphene and norpropoxyphene through inhibition of CYP3A enzymes. The AUC'_{po}/AUC_{po} is a ratio of the area under the plasma concentration versus time curve of an orally administered drug in the presence of inhibitor (AUC'_{po}) to the AUC without inhibitor present (AUC_{po}). The predicted drug interactions (AUC'po/AUCpo's) with propoxyphene and norpropoxyphene were determined for common CYP3A substrates using Equation 6 (p46). The total blood concentrations of propoxyphene and norpropoxyphene (I), were used to calculate the free concentrations (I_u) based on 76.5% plasma protein binding (from an average of 73-80% plasma protein binding based on Giacomini *et al*, 1980). The K_I and k_{inact} kinetic parameters for propoxyphene and norpropoxyphene and human liver microsomes were

incorporated into Equation 6. The estimates assume a maximal inhibition of intestinal wall CYP3A by propoxyphene and norpropoxyphene ($F'_G = 1$), after repeated dosing. The estimates of *in vivo* drug interactions were calculated for low, moderate, and high blood concentrations of propoxyphene and corresponding concentrations of norpropoxyphene, for intravenous midazolam only. Subsequent estimates use only the median concentrations for propoxyphene and norpropoxyphene. The AUC'_{po}/AUC_{po} values were calculated separately for propoxyphene and norpropoxyphene and then added together for net effect (see Table 7, p43), as per Wang *et al*'s studies with verapamil and metabolites (2004).

The predicted AUC'_{po}/AUC_{po} values of orally administered midazolam with propoxyphene are approximately fifteen to twenty-five times the blood concentrations of midazolam administered alone. In general, the lower the intestinal availability of the substrate prior to the addition of inhibitor (F_G) and the greater the fraction metabolized by CYP3A (f_m), the greater the predicted change with propoxyphene co-administration. Sildenafil, triazolam, and R-verapamil have a predicted blood concentrations (AUC) that are at least ten times higher when administered with propoxyphene. Verapamil is a weak CYP3A inducer, and actual blood levels may be slightly lower than predicted (Wang, 2005). All drugs show a predicted increase in AUC by at least four-fold with propoxyphene (and norpropoxyphene).

Although AUC'_{po}/AUC_{po} data were not available for many of the drugs listed in Table 7 (p43), some AUC'_{po}/AUC_{po} values with propoxyphene have been documented. Abernethy *et al* (1985) reported that the AUC'_{po}/AUC_{po} values for alprazolam was 1.6 following three 60 mg propoxyphene doses/day for two days. Using Equation 6, the

predicted AUC'_{po}/AUC_{po} for alprazolam in humans with co-administration of propoxyphene is seven to ten, which is more than three times the reported ratio. The discrepancy between predicted and actual AUC'_{po}/AUC_{po} for alprazolam may be attributed to a lower frequency of propoxyphene administration compared to the dose used for calculations in Table 7 (p43)[16]. Additionally, the discrepancy between the predicted and actual AUC'_{po}/AUC_{po} for alprazolam may be attributed to its low rate of metabolism by the intestine (Obach *et al*, 2006). If the drug is not as affected by propoxyphene-mediated intestinal CYP inhibition, the actual intensity of the drug interaction may be less than predicted by Equation 6 (AUC'_{po}/AUC_{po}).

Equation 6 may be more accurate for midazolam and other CYP3A substrates that are metabolized by both the liver and small intestine (Obach *et al*, 2006). The predicted AUC changes for oral midazolam with propoxyphene co-administration are fifteen to twenty-five times the AUC_{po} of midazolam alone. Also, midazolam is not transported by P-glycoprotein, a transport pump which could reduce the amounts of drug in the intestine. Reducing the amount of drug in the intestine could directly affect the amount of CYP inhibited. (Wang *et al*, 2004). Abernethy *et al* (1985) also reported that AUC changes were not observed for diazepam or lorazepam when co-administered with propoxyphene. These benzodiazepines are not CYP3A substrates as is the case for midazolam, triazolam, and alprazolam.

Propoxyphene has reduced the clearance and increased the half-life of other CYP3A substrates not included in Table 7 (p43) due to lack of sufficient information for

[16] Test subjects taking 65 mg propoxyphene hydrochloride every 6 hours for two and a half-days were administered 1.0 mg of alprazolam once (Abernethy, 1985). Table 6 AUC changes are based on a plasma concentration of 0.6 μM propoxyphene from administration of 65 mg propoxyphene hydrochloride three times a day for four days (Verbeley and Inturrisi, 1973). See Table 5.

calculations with Equation 6[17]. Propoxyphene decreased the total metabolic clearance of antipyrine from 0.53 to 0.63 mL/min, resulting in an increase in the elimination half-life of antipyrine from 12.2 to 15.2 hours (Abernethy, 1982). Abernethy *et al* (1982) found that propoxyphene increased the steady-state plasma levels of doxepin and desmethyldoxepin from 19 to 44 ng/mL and 9 to 20 ng/mL, depressing cognitive function proportionally.

A drug-drug interaction can have serious clinical consequences if the difference between toxic and effective concentrations is small (Lin and Lu, 1998), which is the case for propoxyphene (Inturrisi *et al*, 1982), see Table 6 (p42). Propoxyphene has been associated with many accidental deaths. The high reported rates of accidental overdose may be attributed to the irreversible inhibition of CYP3A enzymes by propoxyphene and norpropoxyphene. The potential drug-drug interactions of propoxyphene through CYP3A inhibition may also have contributed to propoxyphene-related overdoses and adverse events. Inhibition of CYP3A enzymes by propoxyphene and norpropoxyphene may result in higher concentrations of other CYP3A substrates, resulting in adverse events.

Proadifen is not available as a drug but could be used as a positive control for reversible and irreversible inhibition assays with CYP3A. It has been employed in mechanism-based inhibition and metabolic-inhibitor complex formation assays with CYP3A and other CYPs (Yamada *et al*, 1992; Jones *et al*, 2007). Proadifen is more potent than propoxyphene and norpropoxyphene for irreversible inhibition with CYP3A4(+b5). It is also a more potent reversible inhibitor than propoxyphene and norpropoxyphene with CYP3A4(+b5), CYP3A5(+b5), and human liver microsomes. It is

[17] The fraction of the total hepatic elimination due to CYP3A in the absense of inhibitor (f_m) and the intestinal wall bioavailability of the substrate in the absense of inhibitor (F_G) could not be obtained from published literature.

not a controlled substance like propoxyphene (requiring less paperwork and control for laboratory use).

CHAPTER 5-CONCLUSION

Propoxyphene was developed over fifty years ago, before the current knowledge of CYP isozymes, metabolism, and CYP-mediated drug-drug interactions. The results of these studies show that propoxyphene and norpropoxyphene are irreversible inhibitors of CYP3A as measured by *in vitro* experiments with CYP3A4(+b5), CYP3A5(+b5), and human liver microsomes. Propoxyphene and norpropoxyphene exhibit little or no reversible inhibition of CYP3A, and are more potent irreversible inhibitors of CYP3A. Both propoxyphene and norpropoxyphene form metabolic-inhibitor complexes with CYP3A4(+b5) and CYP3A5(+b5). Proadifen, a compound of similar structure to propoxyphene and norpropoxyphene, is a potent irreversible inhibitor of CYP3A4. Proadifen is also an irreversible inhibitor of CYP3A5 and human liver microsomes, and exhibits reversible inhibition with CYP3A4(+b5), CYP3A5(+b5), and human liver microsomes.

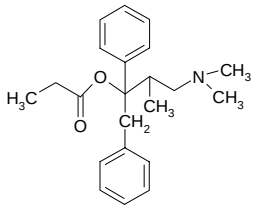
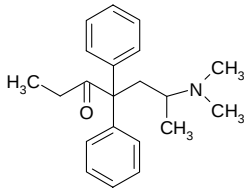
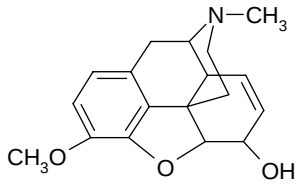
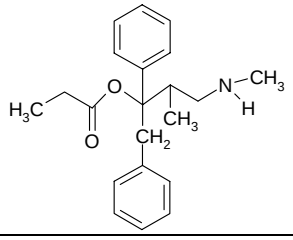
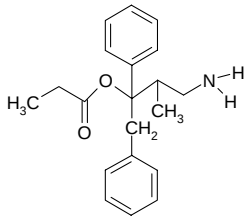
Many reported propoxyphene overdoses may be accidental and attributed to the irreversible inhibition of CYP3A enzymes by propoxyphene, which may result in higher than predicted blood concentrations of the drug or its metabolite, norpropoxyphene. Inhibition of CYP3A enzymes by propoxyphene and norpropoxyphene may result in higher concentrations of other CYP3A substrates, resulting in adverse events.

Future studies may be conducted to provide additional information regarding propoxyphene and norpropoxyphene inhibition of CYP3A. These include testing CYP3A4(+b5) and CYP3A5(+b5) for regeneration of activity following propoxyphene and norpropoxyphene pre-incubation experiments to determine if the irreversible inhibition is completely irreversible. Additionally, propoxyphene and norpropoxyphene

may be examined in combination in irreversible inhibition experiments to assess cumulative, additive, or synergistic effect. A study may also be conducted to monitor concentrations of propoxyphene, norpropoxyphene and dinorpropoxyphene following incubation with CYP3A to assess depletion and product formation. Additionally, propoxyphene and norproxyphene may be tested for induction of CYP3A enzymes *in vitro* using established cell culture models. Although propoxyphene and norpropoxyphene exhibited irreversible inhibition with recombinant CYP3A enzymes, the *in vivo* inhibition may be less than predicted due to CYP3A enzyme induction, which could not be assessed with pre-incubation experiments.

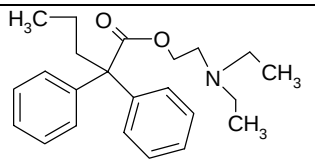
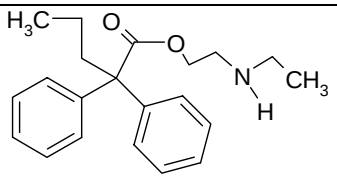
A study may also be conducted to compare theoretical and actual AUC values of patients taking propoxyphene alone and in combination with other CYP3A substrates. As a part of this study, patients may be genotyped to determine if any CYP3A polymorphisms exist. Providing genotyping information would enhance data interpretation, and highlight which sub-populations, if any, are more susceptible to propoxyphene mediated CYP3A inhibition and drug-drug interactions.

Table 1. Chemical Structures of Propoxyphene, Methadone, Codeine, Norpropoxyphene and Dinorpropoxyphene.

Propoxyphene	
Methadone	
Codeine	
Norpropoxyphene	
Dinorpropoxyphene (linear)	

(Feinberg *et al*,1976; McMahon, 1961; Somogyi *et al*, 2004)

Table 2. Chemical Structures of Proadifen (SKF-525a) and SKF-8742

Prodifen (SKF-525a)	
SKF-8742	

(Anders and Mannering, 1966)

Table 3. List of CYP3A4 and CYP3A5 Common Polymorphisms

Enzyme	Expression of Functional Protein in Adult Population	Major Organs	Alleles	Activity Relative to Wild Type
CYP3A4	Majority of population expresses CYP3A4	Liver Small Intestine[f]	CYP3A4*1(A)	Wildtype[f]
			CYP3A4*17	Reduced CYP3A4 expression[a]
			CYP3A4*18	Increased CYP3A4 expression[a]
			CYP3A4*1B	Increased CYP3A4 activity over wildtype[b] Decreased CYP3A4 expression over wildtype[c]
CYP3A5	1-30% of Caucasians[d] 55-75% of Black Africans and African Americans 33% of Japanese[d]	Kidney Lung Liver Colon[f]	CYP3A5*1(A)	Wildtype, Dominant Allele, produced functional protein (*1/*3)[e]
			CYP3A5 *2, *3, *5, *6, *7	Less than 30% of wild type CYP3A5 protein[e]

[a]Dai *et al*, 2001; [b]Kuehl *et al*, 2001; [c]Wojnoski *et al*, 2002; [d]Kamden *et al*, 2005; [e]Kreutz *et al*, 2005; [f]Danielson, 2002

Table 4. Summary of Kinetic Parameters for Enzyme Inactivation (Irreversible Inhibition) with Propoxyphene, Norpropoxyphene, and Proadifen

Tissue	Inhibitor	Kinact (min-1)	KI (μM)	MIC Formation
CYP3A4(+b5)	Propoxyphene	0.41 (+/- 0.03)	1.3 (+/- 0.28)	Yes
	Norpropoxyphene	0.56 (+/- 0.07)	8.8 (+/- 2.1)	Yes
	Proadifen	0.26 (+/- 0.03)	0.35 (+/- 0.16)	Yes
CYP3A5(+b5)	Propoxyphene	0.072 (+/- 0.005)	13 (+/- 3.0)	Yes
	Norpropoxyphene	0.21 (+/-0.03)	25 (+/-8.7)	Yes
	Proadifen	0.11 (+/-0.01)	20 (+/-4.6)	Yes
Human Liver Microsomes	Propoxyphene	0.038 (+/-0.002)	0.45 (+/-0.13)	None detected
	Norpropoxyphene	0.074(+/-0.004)	8.2 (+/-1.4)	None detected
	Proadifen	0.2 (+/-0.04)	6.9 (+/-2.4)	Yes

Table 5. Reversible Inhibition K_i Values

Inhibitor	**Tissue**	**K_i** μM (Standard Error)
Propoxyphene	CYP3A4(+b5)	26 (+/- 3.0)
	CYP3A5(+b5)	>100, estimated 134 (+/-32)
	Human Liver Microsomes	>80, estimated 155 (+/-22)
Norpropoxyphene	CYP3A4(+b5)	29 (+/-5.0)
	CYP3A5(+b5)	>100, estimated 186 (+/-24)
	Human Liver Microsomes	59 (+/-0.049)
Proadifen	CYP3A4(+b5)	5.4 (+/-0.042)
	CYP3A5(+b5)	12 (+/- 1.0)
	Human Liver Microsomes	8.3 (+/- 0.61)

Table 6. Reported Therapeutic (Total) Blood Levels of Propoxyphene and Norpropoxyphene

Dose	**Frequency**	**Duration**	**Propoxyphene** (μg/mL)	**Propoxyphene** (μM)	**Norpropoxyphene** (μg/mL)	**Norpropoxyphene** (μM)
130 mg Propoxyphene hydrochloride [a]	1 time only	once	0.3[a]	0.9	0.3 [a]	0.9
65 mg Propoxyphene hydrochloride [a]	3 doses/day	4 days	0.1-0.2[a]	0.4-0.7	0.6 [a]	1.8
130 mg Propoxyphene hydrochloride [a]	3 doses/day	4 days	0.7-0.9[a]	2-2.5	1.1-1.2 [a]	3.4-3.7
550 mg Propoxyphene hydrochloride [b]	2 dose/Day	12 weeks	0.5[b]	1.5	5 [b]	15
Toxic levels [c]	n/a	n/a	>0.5[c]	>1.5	n/a	n/a
Therapeutic concentration [d]	n/a	n/a	≥ 0.05[d]	≥ 0.15	n/a	n/a

[a]Verbeley and Inturrissi, 1973; [b]Inturrisi *et al*, 1982; [c] Merck Manual, 2007; [d]AHFS, 2007

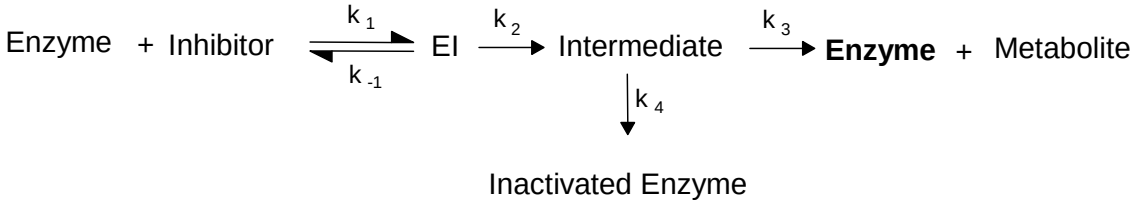
Table 7. Predictions of Propoxyphene and Norproxyphene Interactions with Other CYP3A Substrates (AUC'_{po}/AUC_{po})

Propoxyphene		Norproxyphene		CYP3A Substrates	f_m	F_G	Propoxyphene	Norproxyphene	Propoxyphene + Norproxyphene
I (μM)	I_u (μM)	I (μM)	I_u (μM)				Predicted AUC'_{po}/AUC_{po}	Predicted AUC'_{po}/AUC_{po}	Predicted AUC'_{po}/AUC_{po}
0.6 [a]	0.1	1.8 [a]	0.4	Midazolam-iv [c]	0.9 [c]	1 [d]	5-8	3-6	8-14
2.3 [a]	0.5	3.6 [a]	0.8	Midazolam-iv [c]	0.9 [c]	1 [d]	7-9	4-8	11-17
1.5 [b]	0.4	15 [b]	3.5	Midazolam-iv [c]	0.9 [c]	1 [d]	6-9	6-9	12-18
0.6 [a]	0.1	1.8 [a]	0.4	Midazolam-oral [c]	0.9 [c]	0.4 [c]	8-10	7-15	15-25
0.6 [a]	0.1	1.8 [a]	0.4	Sildenafil [e]	0.8 [f]	0.4 [g]	8-11	6-10	14-21
0.6 [a]	0.1	1.8 [a]	0.4	Alprazolam [o]	0.8 [l]	0.9 [l]	4-5	3-5	7-10
0.6 [a]	0.1	1.8 [a]	0.4	Triazolam [k]	0.8 [l]	0.4 [l]	8-10	6-9	14-19
0.6 [a]	0.1	1.8 [a]	0.4	Trazodone [h]	0.4 [i]	0.8 [j]	2	2	4
0.6 [a]	0.1	1.8 [a]	0.4	R-Verapamil [m]	0.8 [m]	0.5 [n]	5-6	5-9	10-15
0.6 [a]	0.1	1.8 [a]	0.4	S-Verapamil [m]	0.7 [m]	0.5 [n]	7-11	7-11	14-22

[a] Verbeley and Inturrisi, 1973,; [b] AHFS, 2007; [c] Palkama *et al*, 1999; [d] Ernest *et al*, 2004; [e] Muirhead *et al*; [f] Warrington *et al*, 2000; [g] Thummel and Shen, 2001; [h] Greenblatt *et al*, 2003; [i] Jaunch *et al*, 1976; [j] Nilson and Dale, 1992; [k] Greenblatt *et al*, 2000a; [l] Rodrigues *et al*, 2001; [m] Wang *et al*, 2004; [n] Gorski *et al*, 1998; [o] Greenblatt *et al*, 2000b

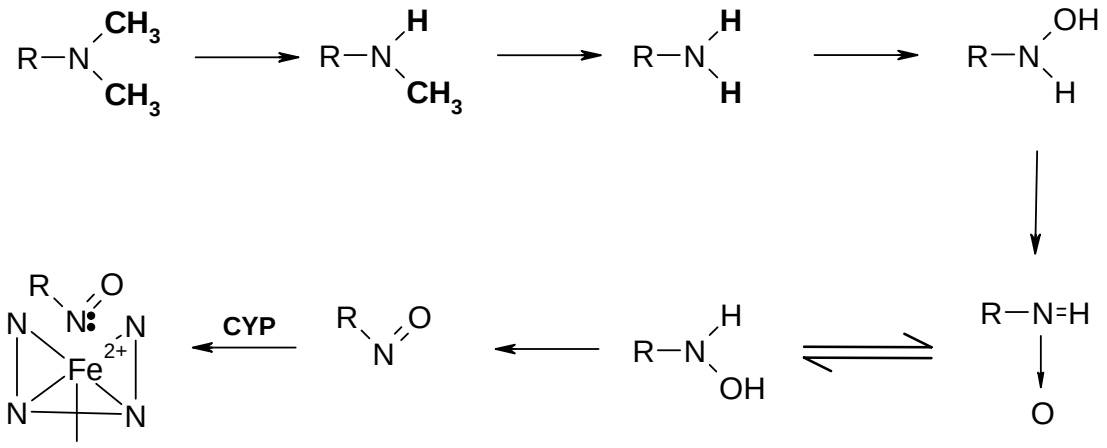
The predicted AUC'_{po}/AUC_{po} values were determined for propoxyphene and norpropoxyphene separately using Equation 6, and then added together. The total blood concentrations of propoxyphene and norpropoxyphene (I), were used to calculate the free concentrations (I_u) based on 76.5% plasma protein binding (Giacomini *et al*, 1978). The fraction of total hepatic elimination of substrate due to CYP3A in the absence of inhibitor is f_m, and was obtained from the scientific literature. F_G is the intestinal wall bioavailability of the substrate in the absence of inhibitor. The endogenous degradation rate of CYP3A (k_{deg}) were 0.00128 and 0.00026 min^{-1} based on rat CYP3A and human CYP3A4 in CaCO-2 cells (Correa, 1991; Malhotra *et al*, 2001).

Schematic I. Irreversible Inhibition



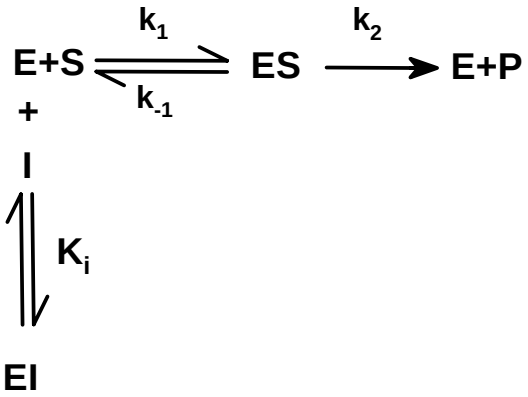
(Silverman, 1995)

Schematic II. Irreversible Inhibition: Formation of Proposed Metabolic-Inhibitor Complex



In this schematic, dialkylamine group of the inhibitor is demethylated twice, oxidized, and finally forms a nitroso group (R-N=O). The two free electrons of the nitrogen in the nitroso group binds to the iron of the heme of the prosthetic group of the CYP.

Schematic III. Reversible Inhibition. Enzyme (E) Binds Substrate (S) or Inhibitor (I).



(Stryer, 1996)

The k_1, k_{-1}, and k_2 rates are listed but are not calculated as part of this thesis.

Equation 1. Irreversible Inhibition Equation for Enzyme Activity (at time (t)), Enzyme Activity at Time (0), and Kobserved (Kobs) at Time (t)

$$E_t = E_0 \times e^{-(Kobs)t}$$

Equation 2a. Irreversible Inhibition Equation for $k_{observed}$ (k_{obs}), k_{inact}, and K_I.

$$k_{obs} = \frac{k_{inact} \times I}{K_I + I}$$

Equation 2b. Irreversible Inhibition Equation for $k_{observed}$, k_{inact}, and K_I and Enzyme Activity

$$E_t = E_0 \times e^{-(\frac{k_{inact} \times I}{K_I + I})t}$$

Equation 3. Competitive Inhibition Equation

$$Y = (V_{max} \text{ x } S)/((K_m \text{ x } (1+I/ K_i)+S)$$

Equation 4. Noncompetitive Inhibition Equation

$$Y = ((V_{max} /(1+I/ K_i))+S)/(K_m +S)$$

Equation 5. Uncompetitive Inhibition Equation

$$Y = (V_{max} \text{ x } S) /(K_m +(S(1+I/ K_i)))$$

Equation 6. Calculating AUC'_{po}/AUC_{po} using kinetic parameters

$$\frac{AUC'_{po}}{AUC_{po}} = \frac{F'_G}{F_G} \text{ x } \frac{1}{\frac{f_m}{1 + \frac{k_{inact} \text{ x } I_u}{k_{deg} \text{ x } (K_I + I_u)}} + (1 - f_m)}$$

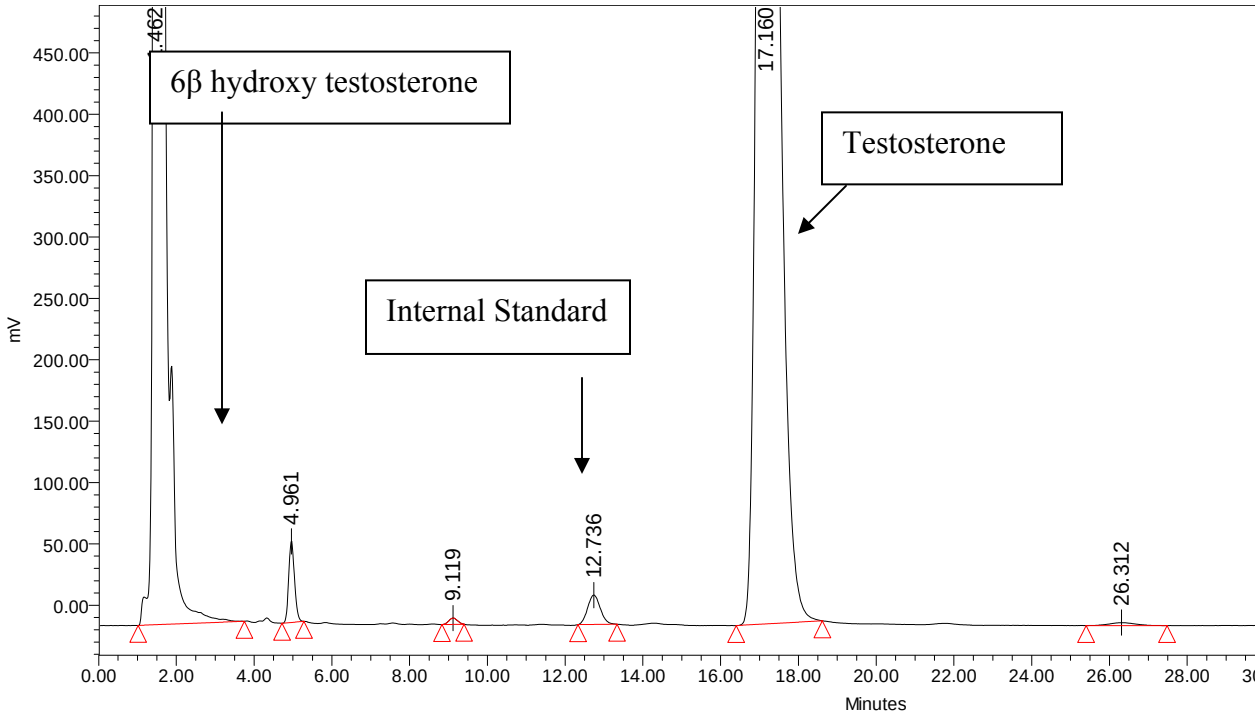


Figure 1. HPLC Chromatogram of Extracted Sample After Incubation with Recombinant CYP. Mobile Phase: 40% 30 mM ammonium acetate pH 6.3-6.4: 60% methanol . HPLC conditions: 5 µm C-18(2) Luna Phenomenex column with a 1 ml/min flow rate and uv detection at 254 nm.

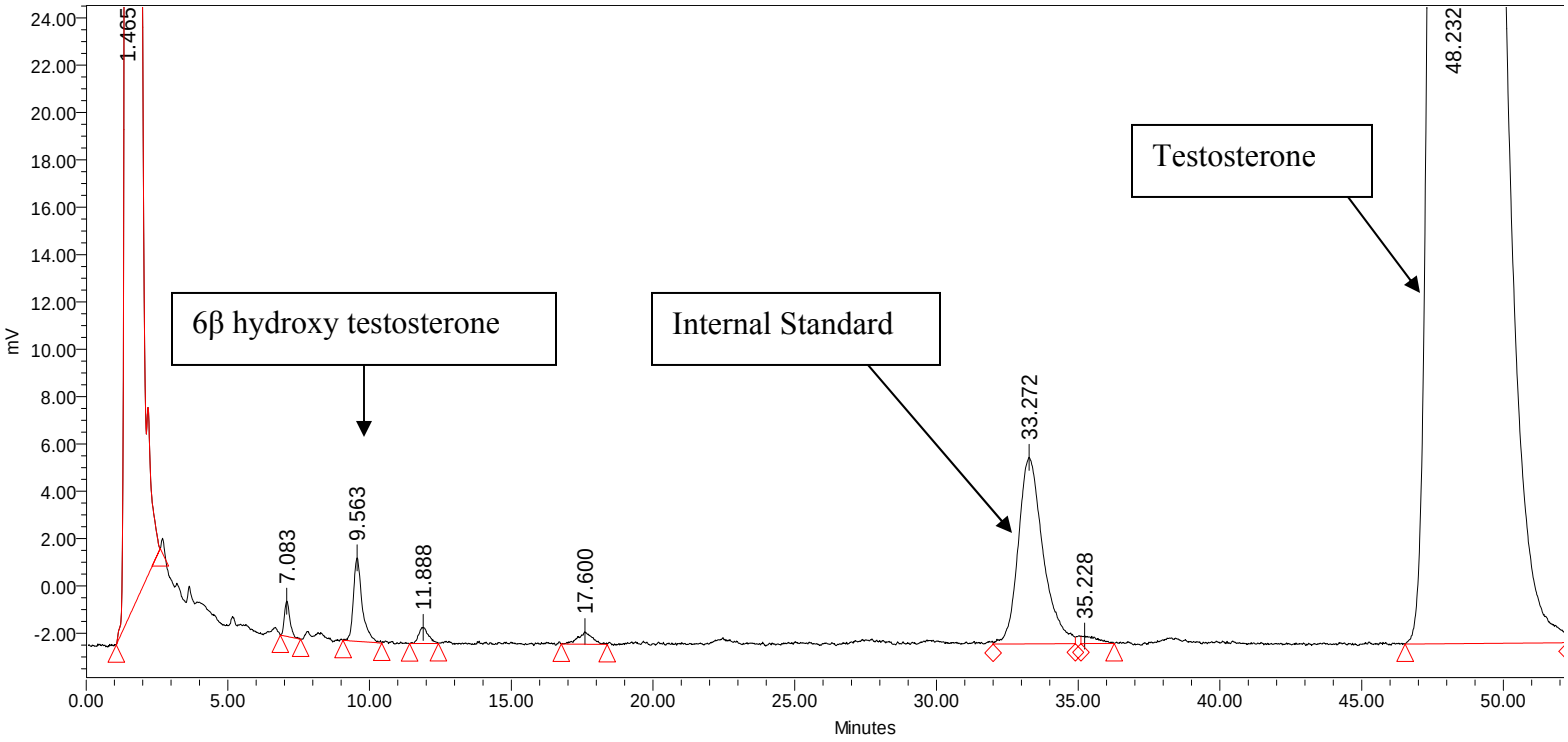


Figure 2. HPLC Chromatogram of Extracted Sample After Incubation with Human Liver Microsomes. Mobile Phase: 40% 30 mM ammonium acetate pH 5.6-5.8: 60% methanol . HPLC conditions: 5 µm C-18(2) Luna Phenomenex column with a 1 ml/min flow rate and uv detection at 254 nm.

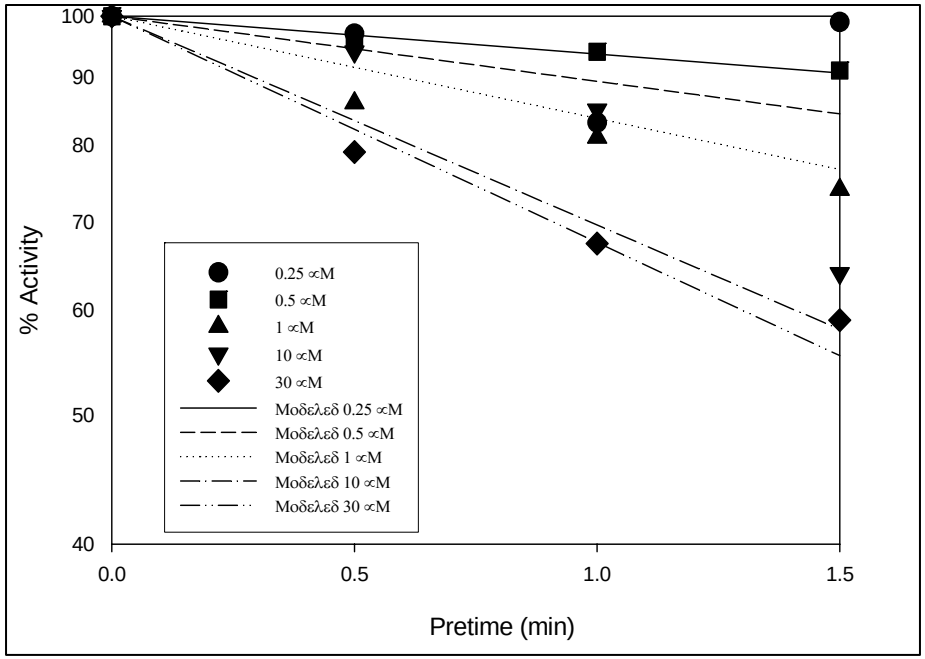


Figure 3. Propoxyphene and CYP3A4(+b5)-Percent Activity v. Pre-incubation Time. Time and concentration dependent inhibition of CYP3A4(+b5) activity by propoxyphene. For each concentration of inhibitor, the remaining testosterone 6β hydroxylase activity is expressed as a percentage relative to control activity. Points are averaged data, and lines are generated from Equation 2b (p46) with k_{inact} and K_I model estimates.

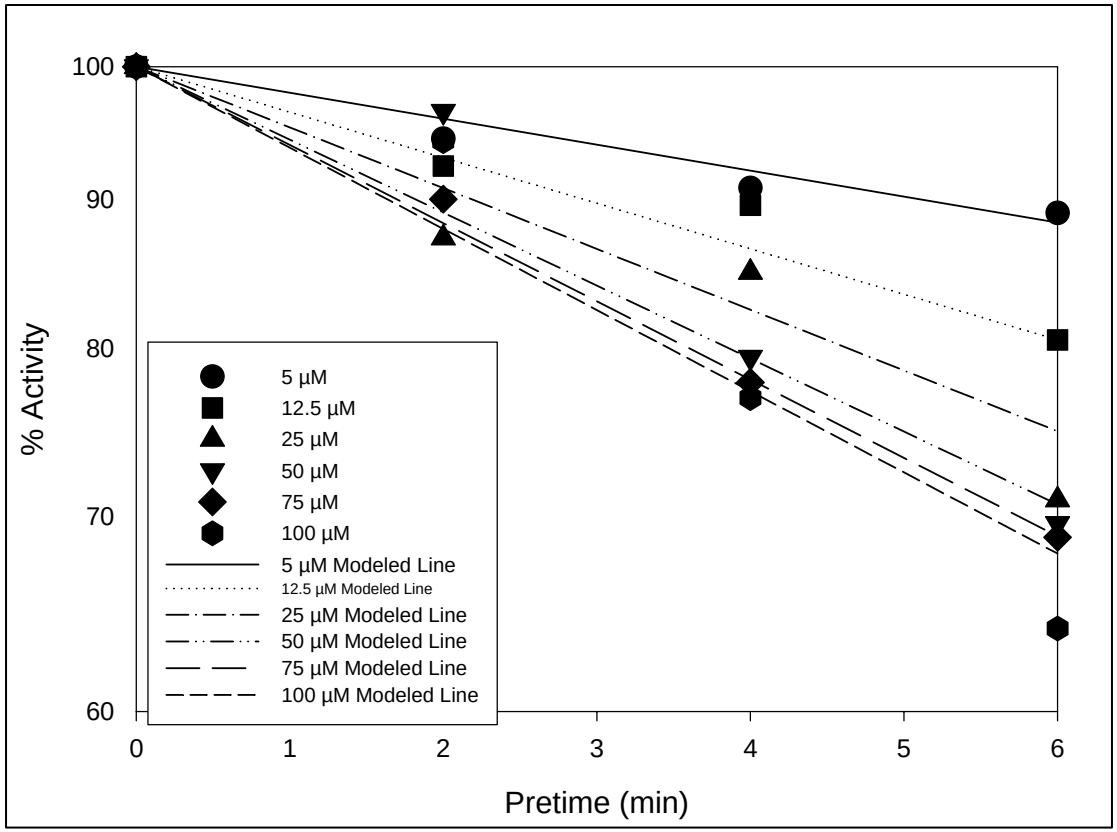


Figure 4. Propoxyphene and CYP3A5(+b5)-Percent Activity v. Pre-incubation Time. Time and concentration dependent inhibition of CYP3A5(+b5) activity by propoxyphene. For each concentration of inhibitor, the remaining testosterone 6β hydroxylase activity is expressed as a percentage relative to control activity. Points are averaged data, and lines are generated from Equation 2b (p46) with k_{inact} and K_I model estimates.

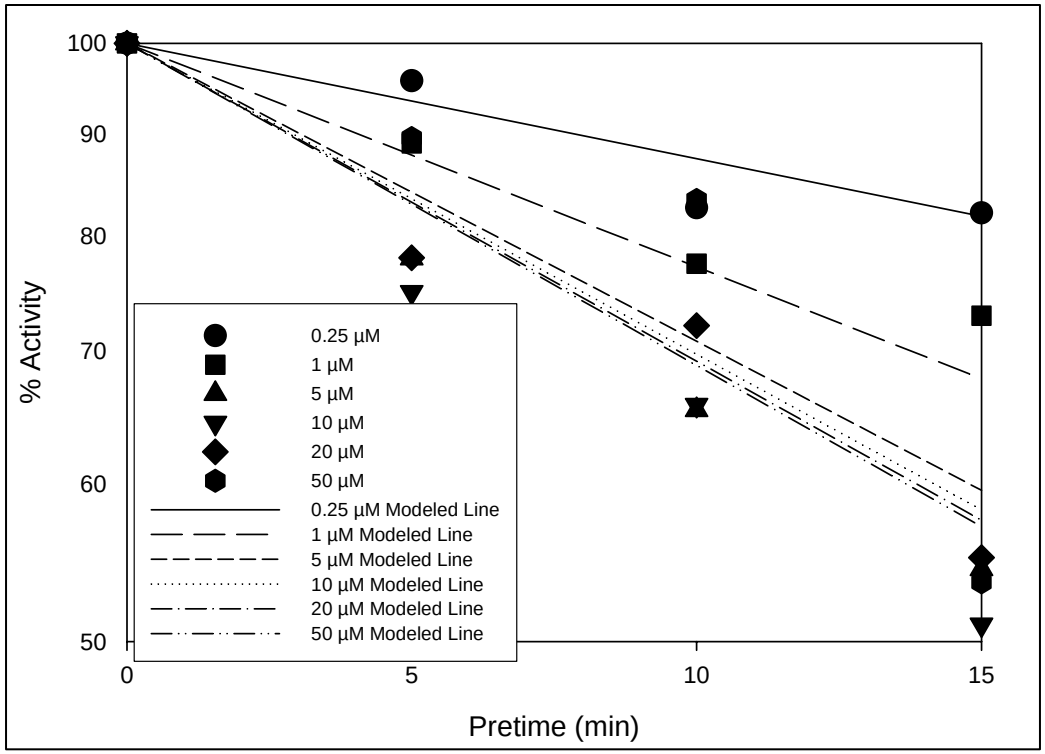


Figure 5. Propoxyphene and Human Liver Microsomes-Percent Activity v. Pre-incubation Time. Time and concentration dependent inhibition of CYP3A activity in human liver microsomes by propoxyphene. For each concentration of inhibitor, the remaining testosterone 6β hydroxylase activity is expressed as a percentage relative to control activity. Points are averaged data, and lines are generated from Equation 2b (p46) with k_{inact} and K_I model estimates.

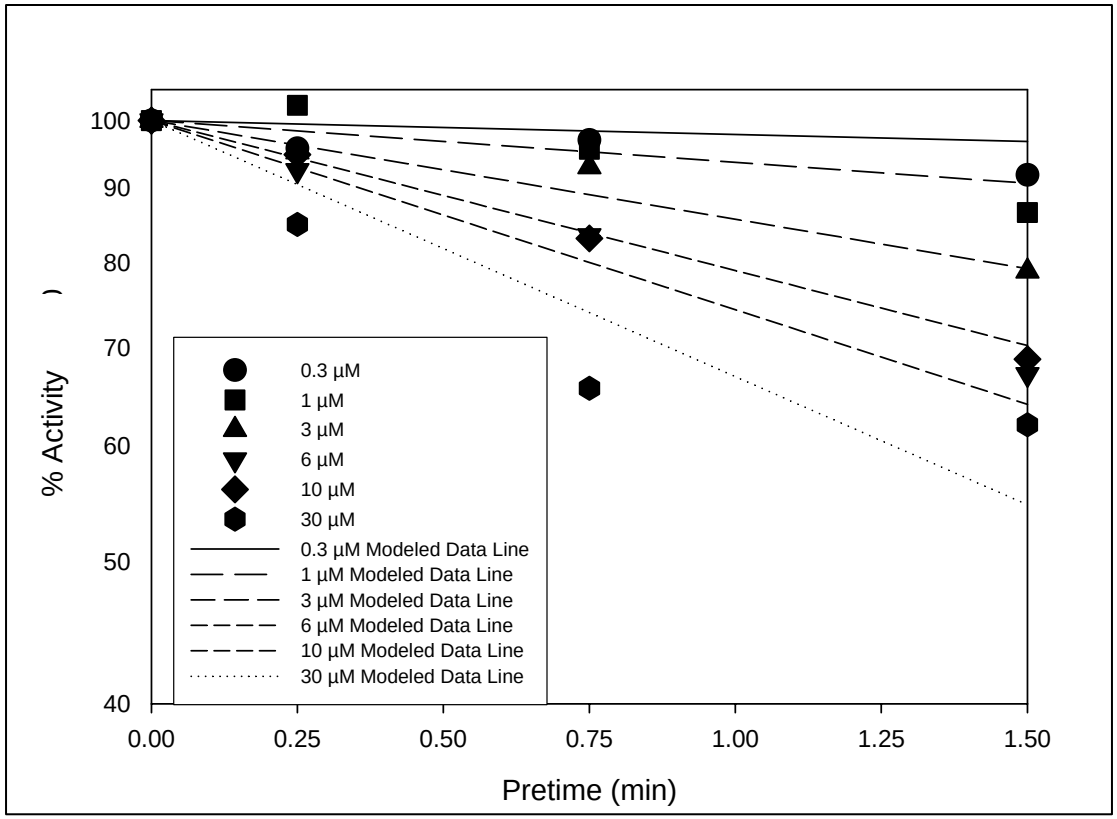


Figure 6. Norpropoxyphene and CYP3A4(+b5)-Percent Activity v. Pre-incubation Time. Time and concentration dependent inhibition of CYP3A4(+b5) activity by propoxyphene. For each concentration of inhibitor, the remaining testosterone 6β hydroxylase activity is expressed as a percentage relative to control activity. Points are averaged data, and lines are generated from Equation 2b (p46) with k_{inact} and K_I model estimates.

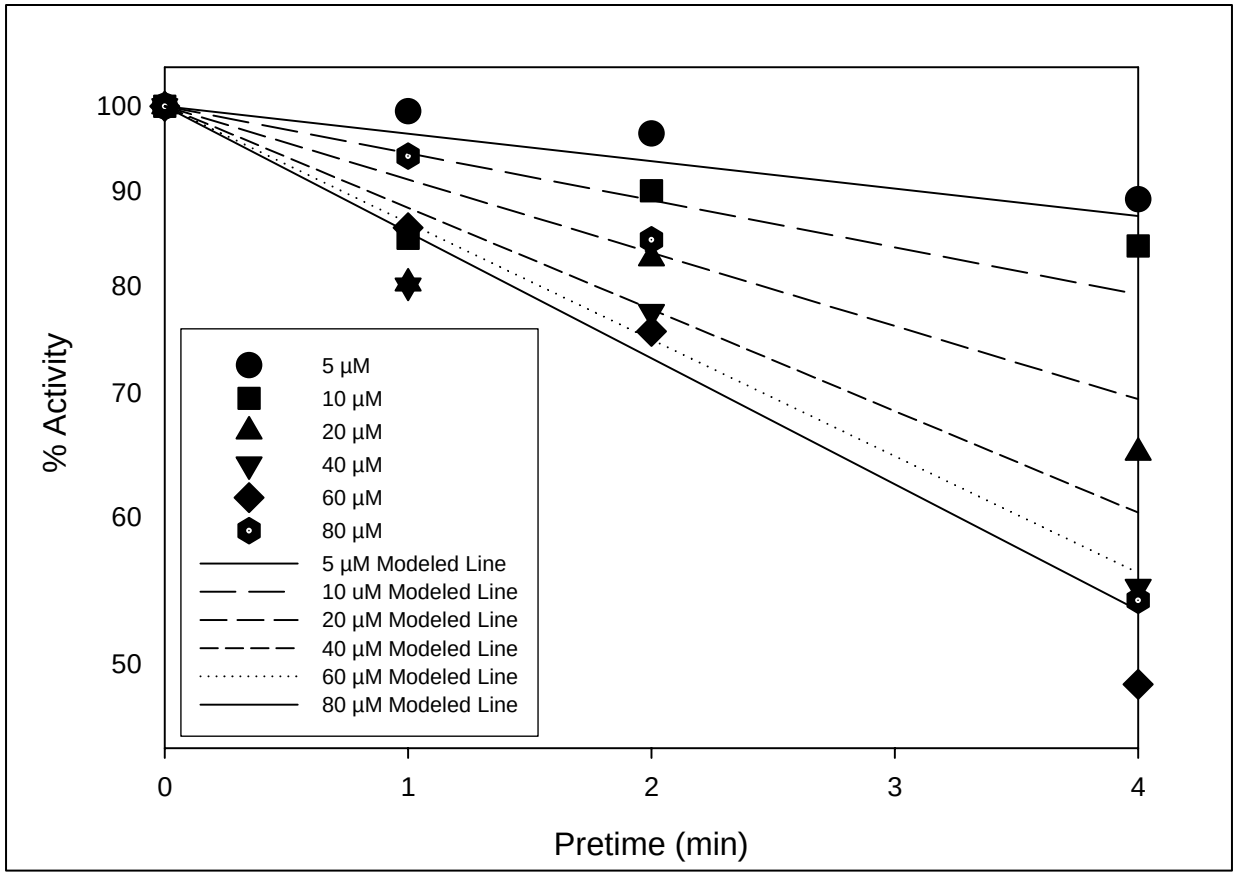


Figure 7. Norpropoxyphene and CYP3A5(+b5)-Percent Activity v. Pre-incubation Time. Time and concentration dependent inhibition of CYP3A5(+b5) activity by propoxyphene. For each concentration of inhibitor, the remaining testosterone 6β hydroxylase activity is expressed as a percentage relative to control activity. Points are averaged data, and lines are generated from Equation 2b (p46) with k_{inact} and K_I model estimates.

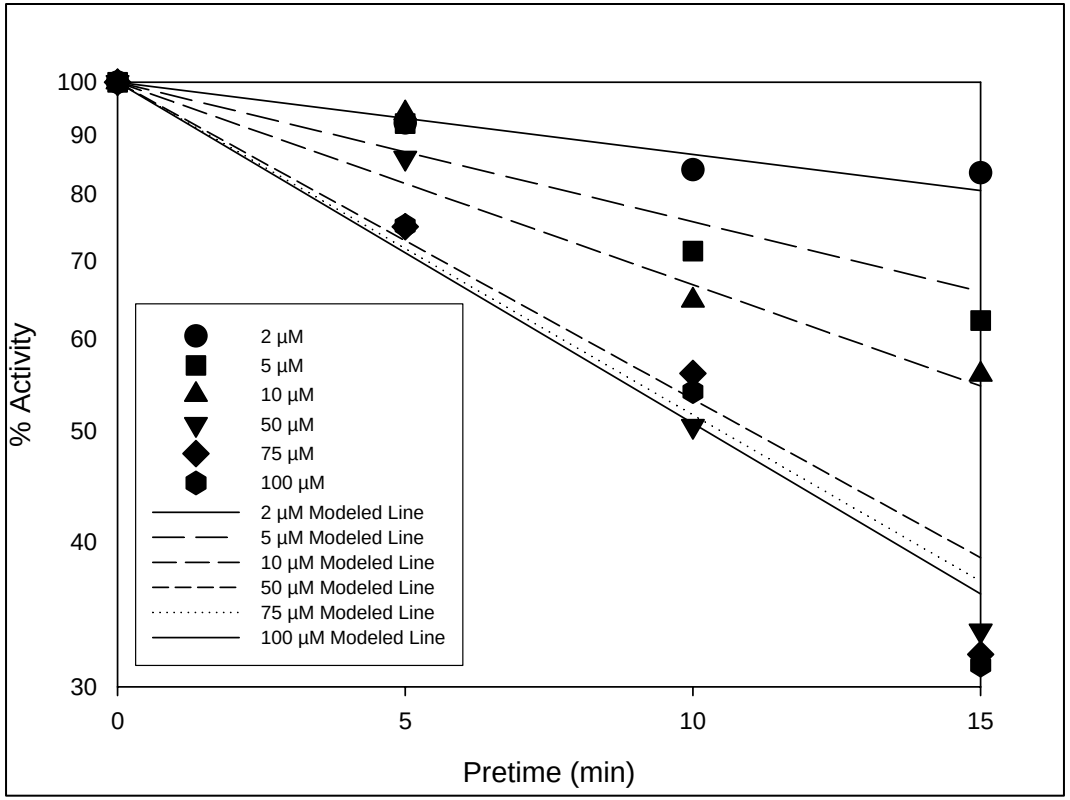


Figure 8. Norpropoxyphene and human liver microsomes-Percent Activity v. Pre-incubation Time. Time and concentration dependent inhibition of CYP3A activity in human liver microsomes by propoxyphene. For each concentration of inhibitor, the remaining testosterone 6β hydroxylase activity is expressed as a percentage relative to control activity. Points are averaged data, and lines are generated from Equation 2b (p46) with k_{inact} and K_I model estimates.

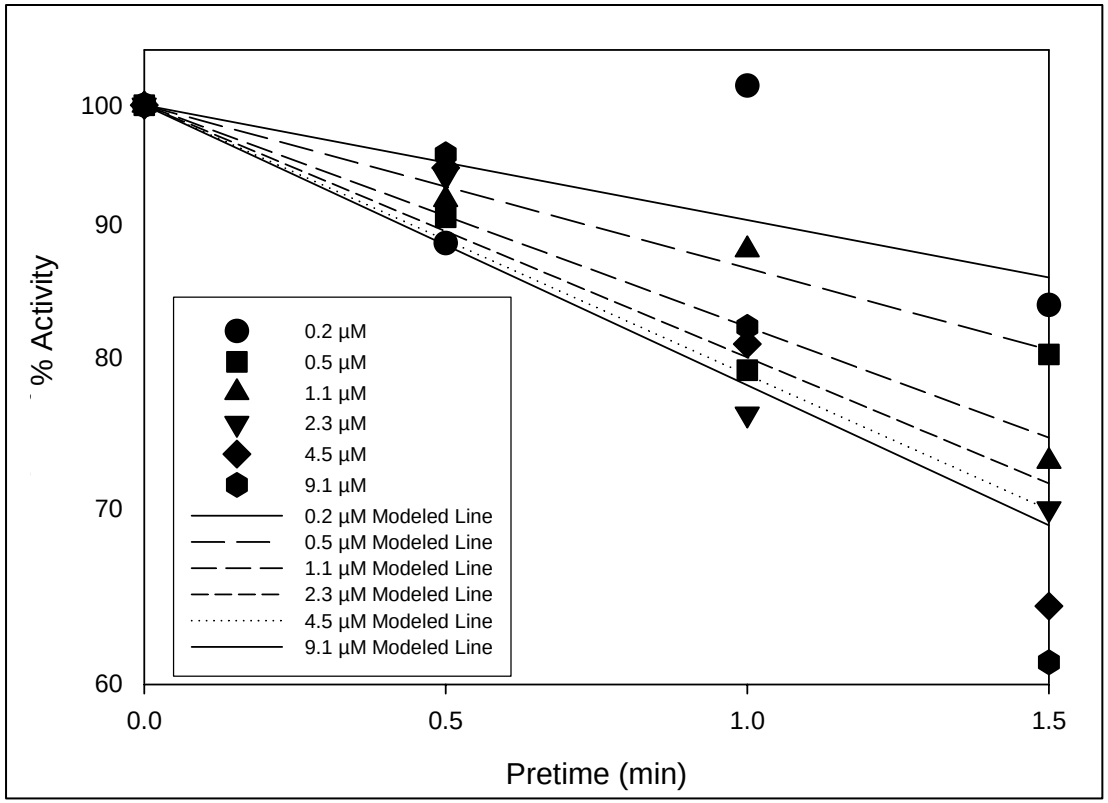


Figure 9. Proadifen and CYP3A4(+b5)-Percent Activity v. Pre-incubation Time. Time and concentration dependent inhibition of CYP3A4(+b5) activity by propoxyphene. For each concentration of inhibitor, the remaining testosterone 6β hydroxylase activity is expressed as a percentage relative to control activity. Points are averaged data, and lines are generated from Equation 2b (p46) with k_{inact} and K_I model estimates.

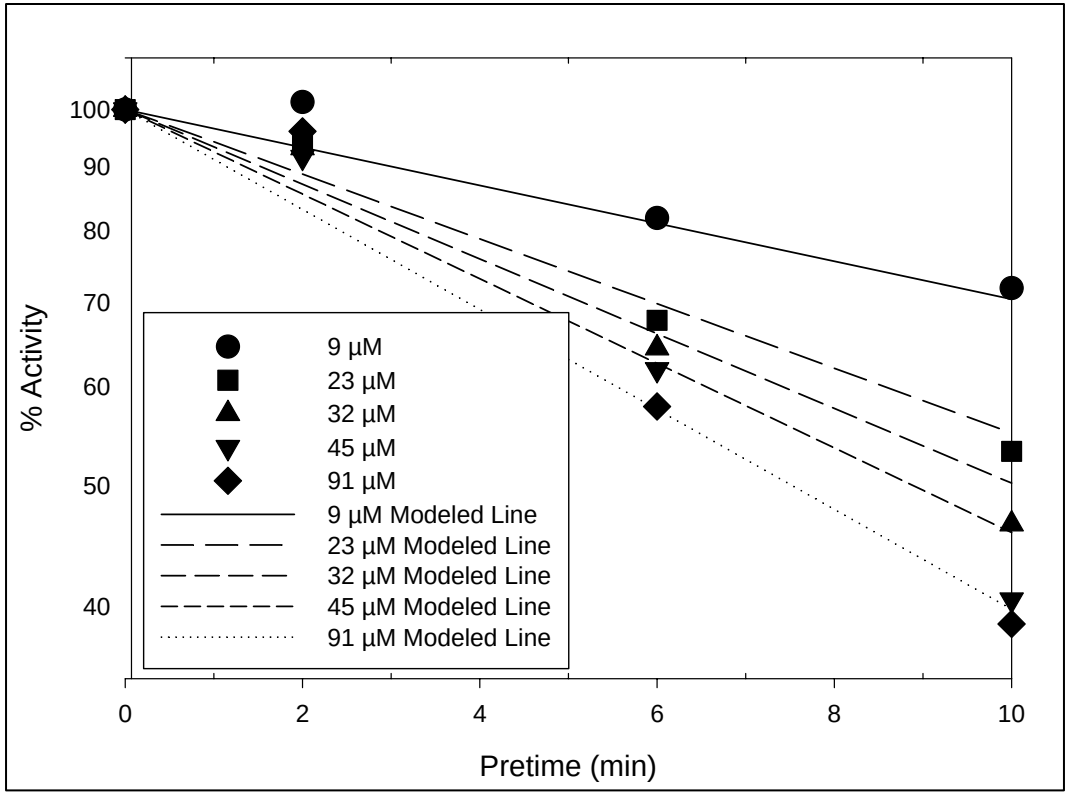


Figure 10. Proadifen and CYP3A5(+b5)-Percent Activity v. Pre-incubation Time. Time and concentration dependent inhibition of CYP3A5(+b5) activity by propoxyphene. For each concentration of inhibitor, the remaining testosterone 6β hydroxylase activity is expressed as a percentage relative to control activity. Points are averaged data, and lines are generated from Equation 2b (p46) with k_{inact} and K_I model estimates.

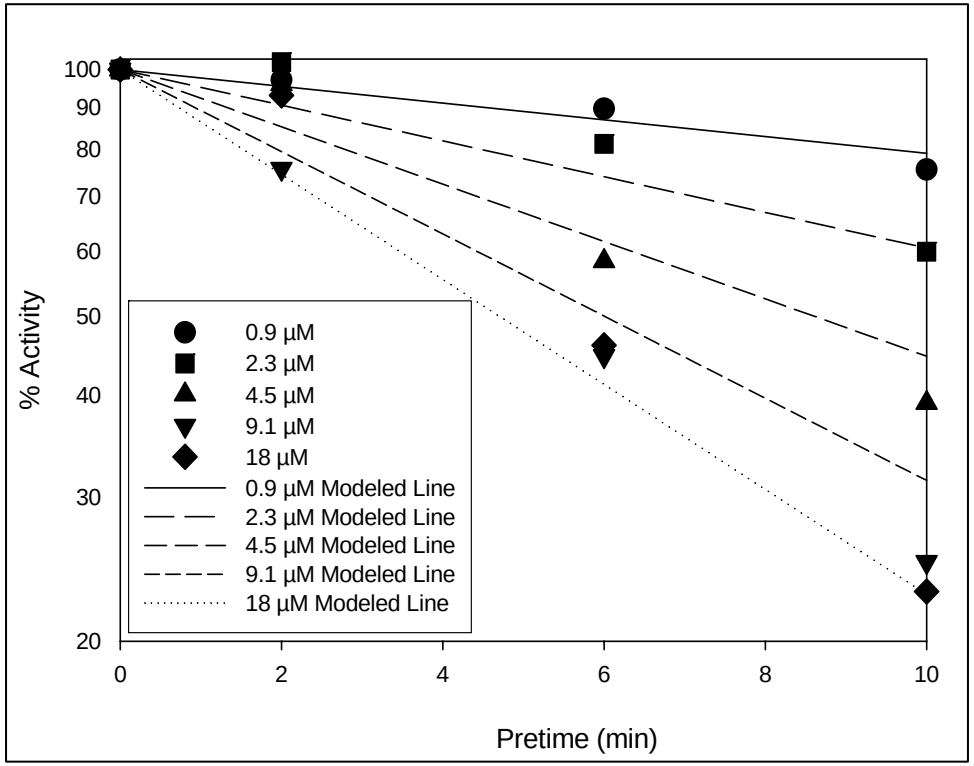


Figure 11. Proadifen and Human Liver Microsomes-Percent Activity v. Pre-incubation Time. Time and concentration dependent inhibition of CYP3A activity in human liver microsomes by propoxyphene. For each concentration of inhibitor, the remaining testosterone 6β hydroxylase activity is expressed as a percentage relative to control activity. Points are averaged data, and lines are generated from Equation 2b (p46) with k_{inact} and K_I model estimates.

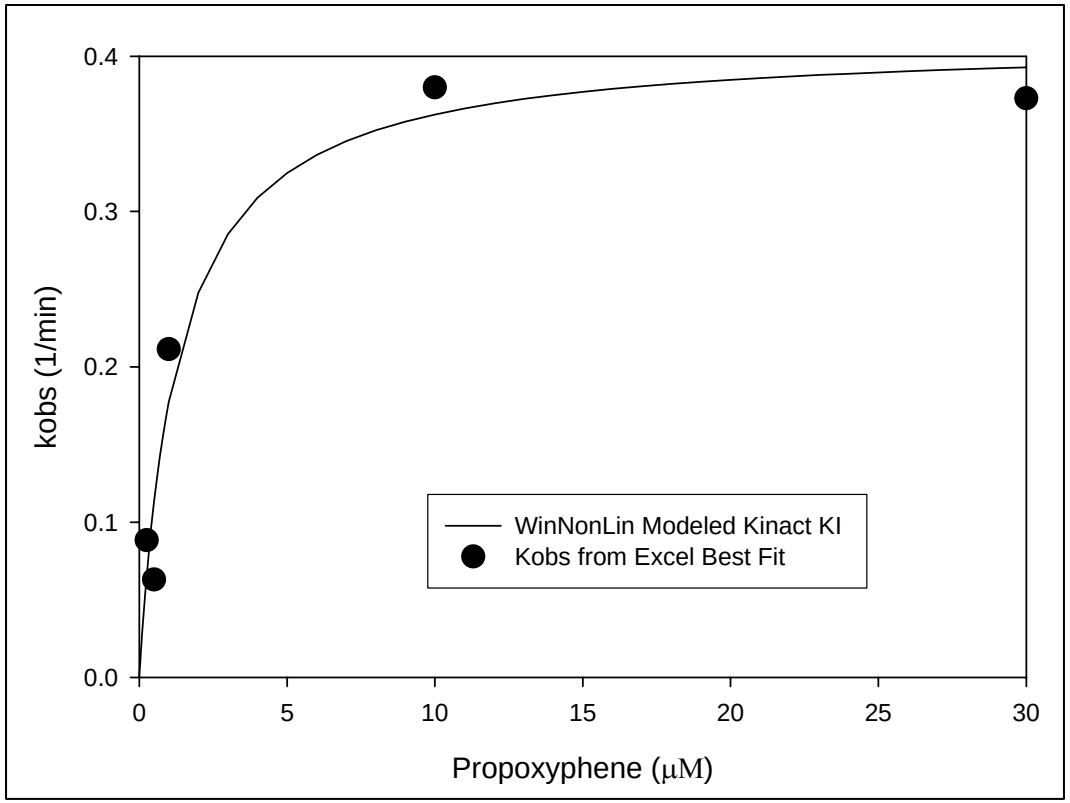


Figure 12. k_{obs} v. Inhibitor Concentration for CYP3A4(+b5) and Propoxyphene. The k_{obs} values were calculated using the slopes of the lines of best fit from percent activity versus pre-incubation time data. The line was calculated using Equation 2a (p46) and the K_I and k_{inact} model estimates.

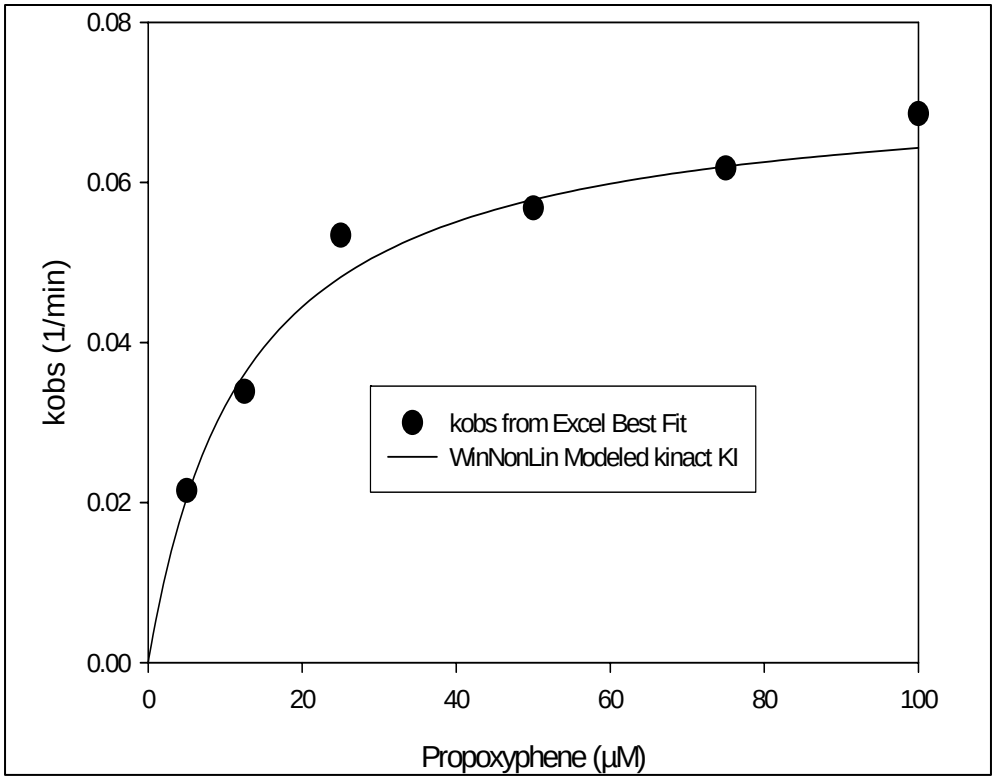


Figure 13. k_{obs} v. Inhibitor Concentration for CYP3A5(+b5) and Propoxyphene. The k_{obs} values were calculated using the slopes of the lines of best fit from percent activity versus pre-incubation time data. The line was calculated using Equation 2a (p46) and the K_I and k_{inact} model estimates.

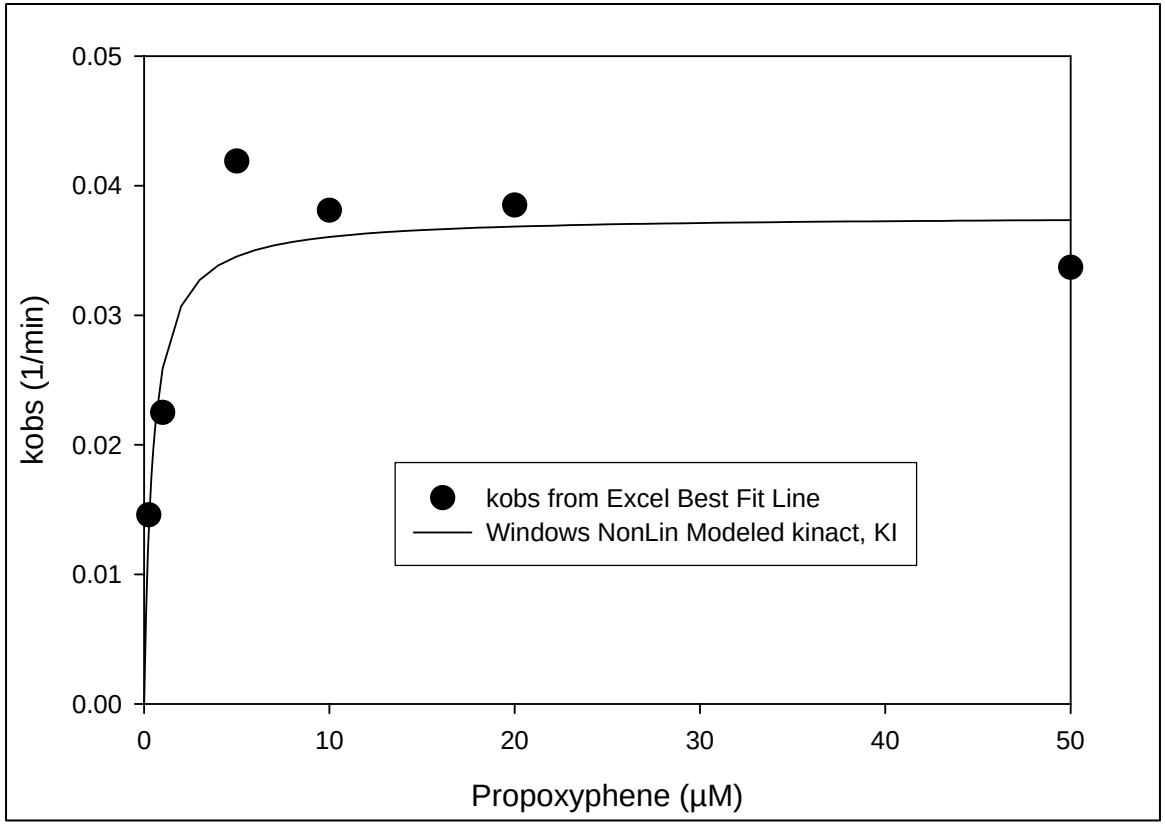


Figure 14. k_{obs} v. Inhibitor Concentration for Human Liver Microsomes and Propoxyphene. The k_{obs} values were calculated using the slopes of the lines of best fit from percent activity versus pre-incubation time data. The line was calculated using Equation 2a (p46) and the K_I and k_{inact} model estimates.

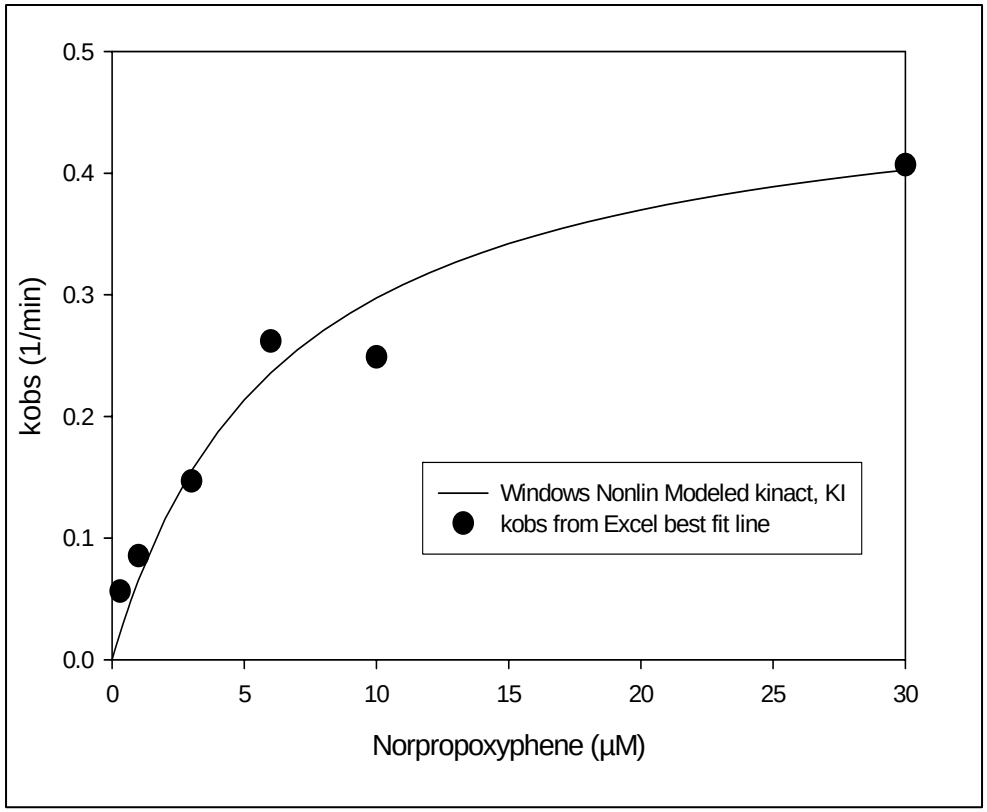


Figure 15. k_{obs} v. Inhibitor Concentration for CYP3A4(+b5) and Norpropoxyphene. The k_{obs} values were calculated using the slopes of the lines of best fit from percent activity versus pre-incubation time data. The line was calculated using Equation 2a (p46) and the K_I and k_{inact} model estimates.

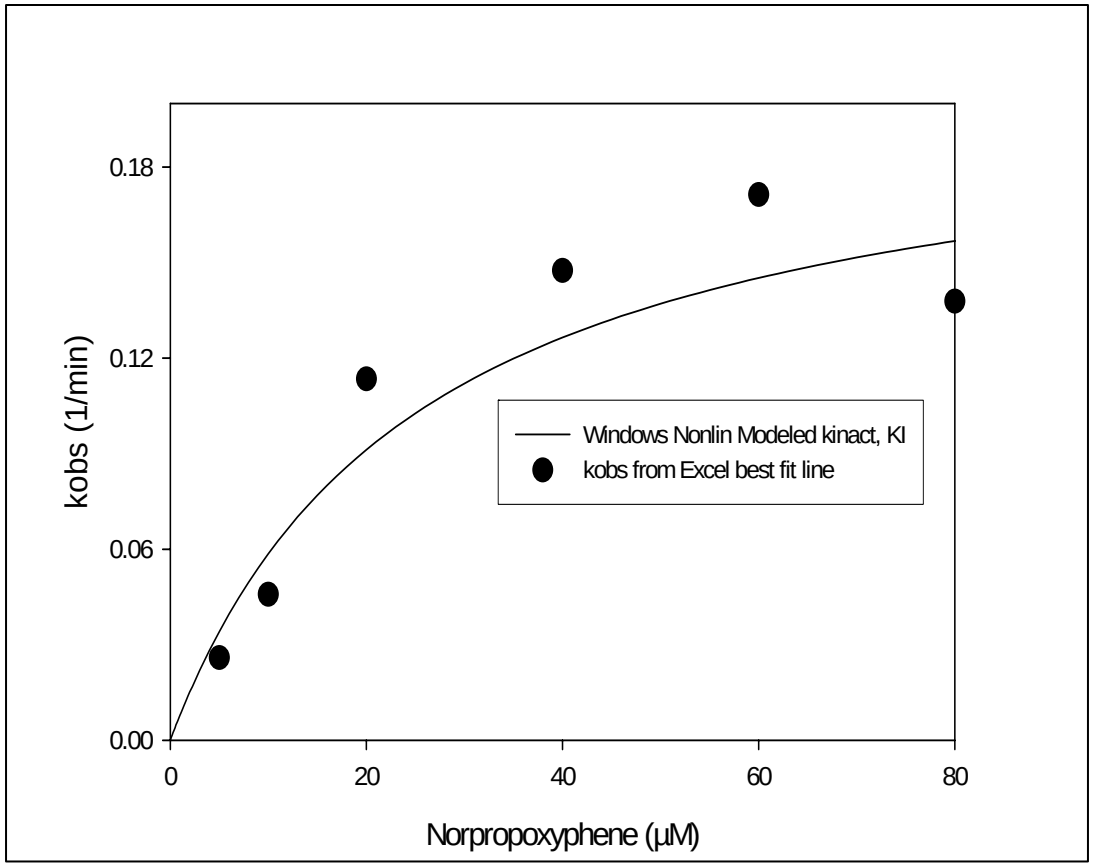


Figure 16. k_{obs} v. Inhibitor Concentration for CYP3A5(+b5) and Norpropoxyphene. The k_{obs} values were calculated using the slopes of the lines of best fit from percent activity versus pre-incubation time data. The line was calculated using Equation 2a (p46) and the K_I and k_{inact} model estimates.

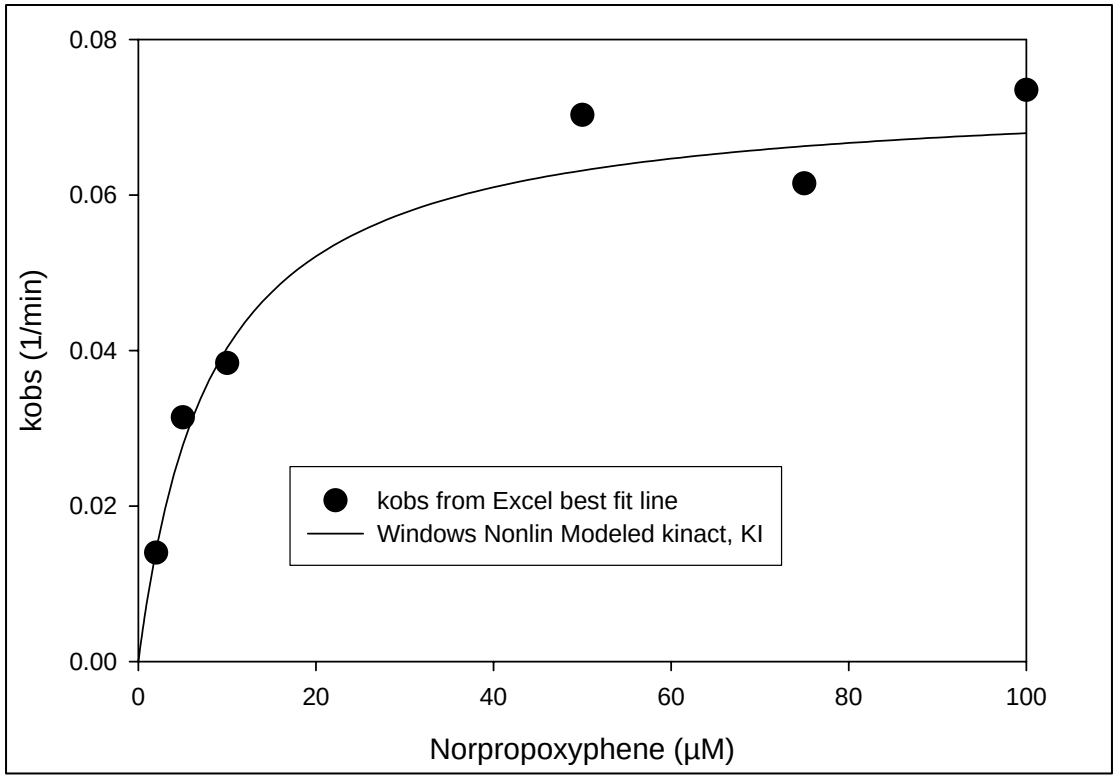


Figure 17. k_{obs} v. Inhibitor Concentration for Human Liver Microsomes and Norpropoxyphene. The k_{obs} values were calculated using the slopes of the lines of best fit from percent activity versus pre-incubation time data. The line was calculated using equation 2a (p46) and the K_I and k_{inact} model estimates.

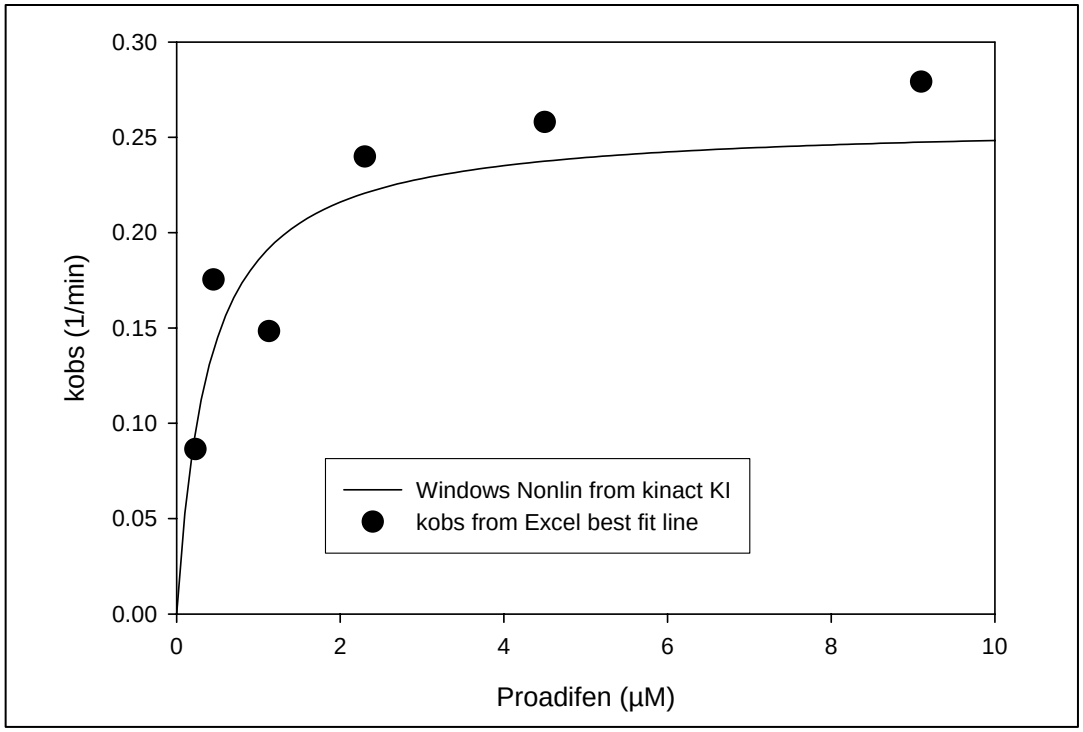


Figure 18. k_{obs} v. Inhibitor Concentration for CYP3A4(+b5) and Proadifen. The k_{obs} values were calculated using the slopes of the lines of best fit from percent activity versus pre-incubation time data. The line was calculated using Equation 2a (p46) and the K_I and k_{inact} model estimates.

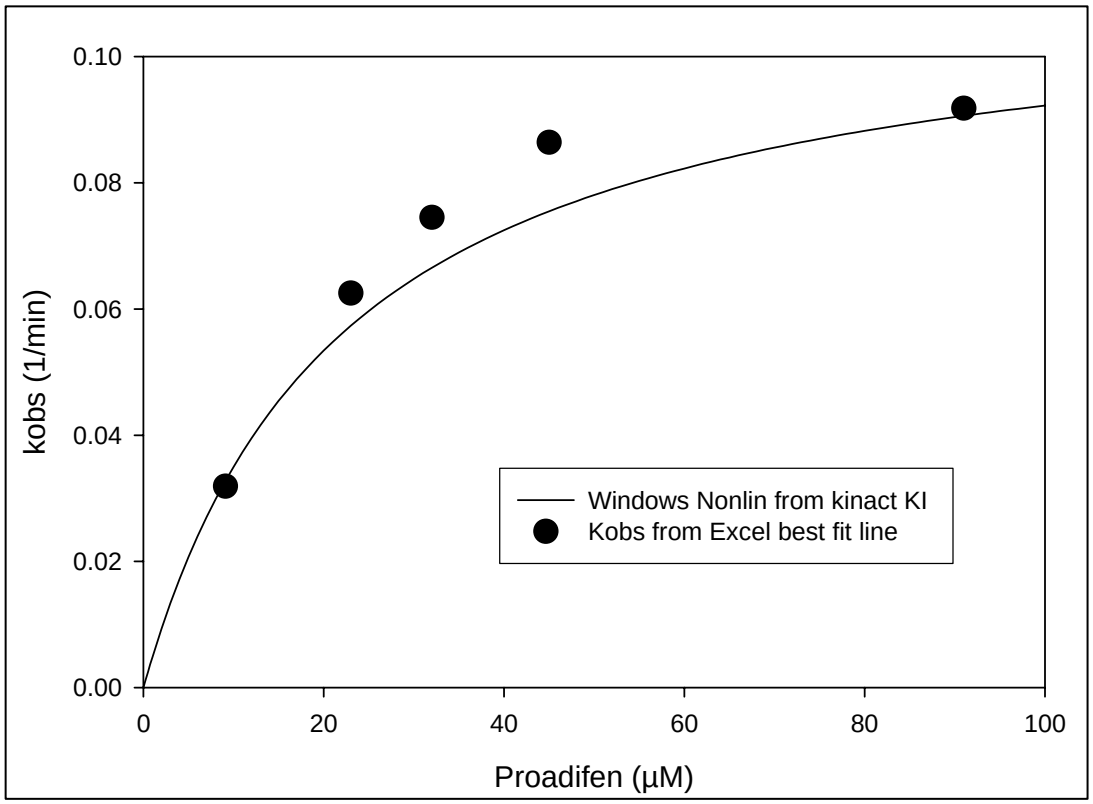


Figure 19. k_{obs} v. Inhibitor Concentration for CYP3A5(+b5) and Proadifen. The k_{obs} values were calculated using the slopes of the lines of best fit from percent activity versus pre-incubation time data. The K_{obs} line was calculated using Equation 2a (p46) and the K_I and k_{inact} model estimates.

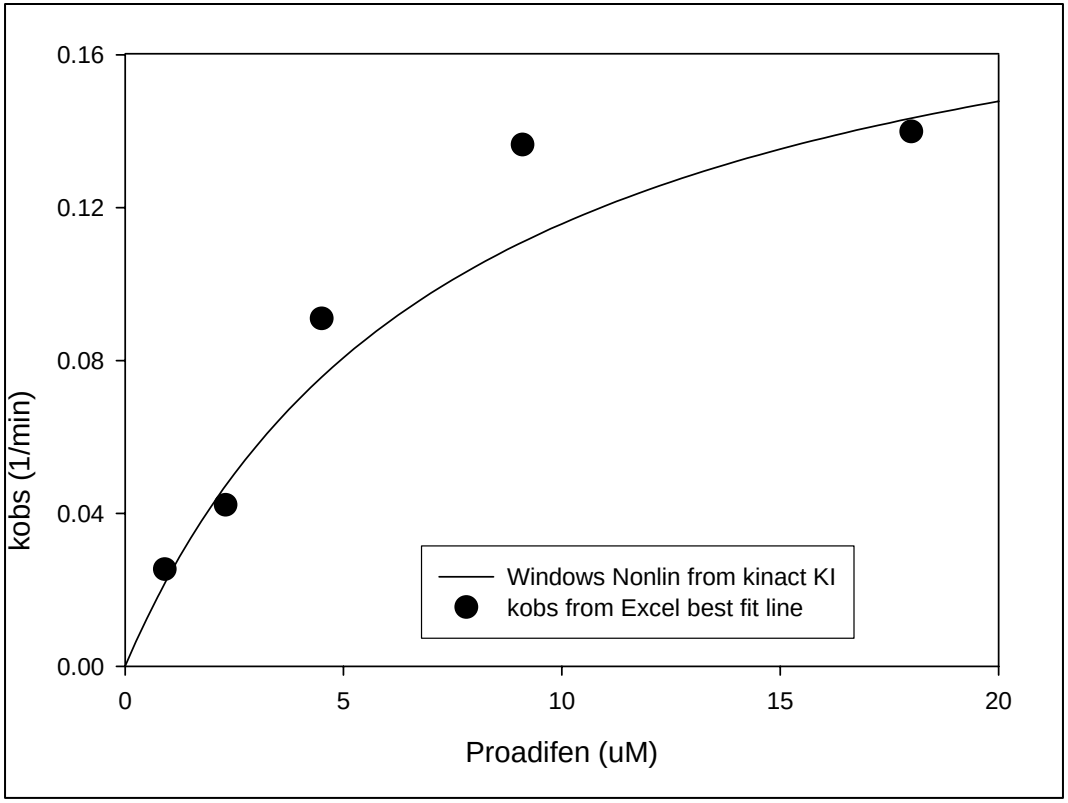


Figure 20. k_{obs} v. Inhibitor Concentration for Human Liver Microsomes and Proadifen. The k_{obs} values were calculated using the slopes of the lines of best fit from percent activity versus pre-incubation time data. The line was calculated using Equation 2a (p46) and the K_I and k_{inact} model estimates.

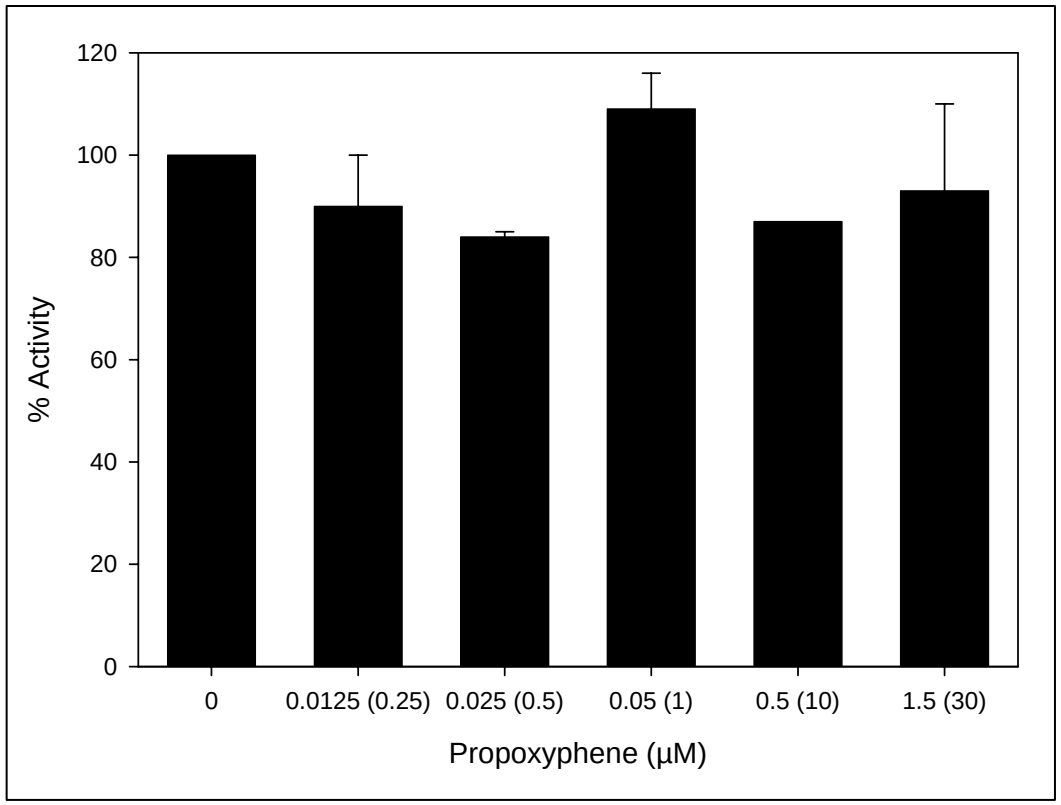


Figure 21. Propoxyphene and CYP3A4(+b5)-Percent Activity Relative to Control. The percentage of initial testosterone 6β hydroxylase activity for each inhibitor concentration without pre-incubation time (time 0) activity was determined relative to the no inhibitor zero pre-incubation time (0,0 control). Concentrations of inhibitor are listed across the x-axis as inhibitor concentration (1 mL reaction) and pre-incubation concentrations are listed in parentheses (50 µL reaction). Error bars are standard deviations of replicates.

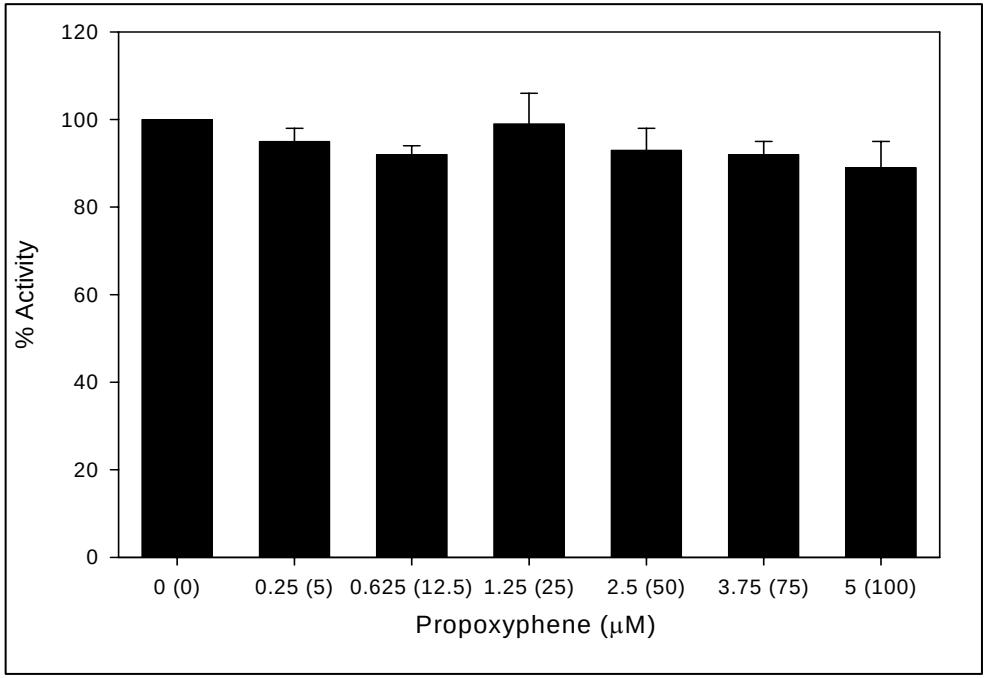


Figure 22. Propoxyphene and CYP3A5(+b5)-Percent Activity Relative to Control. The percentage of initial testosterone 6β hydroxylase activity for each inhibitor concentration without pre-incubation time (time 0) activity was determined relative to the no inhibitor zero pre-incubation time (0,0 control). Concentrations of inhibitor are listed across the x-axis as inhibitor concentration (1 mL reaction) and pre-incubation concentrations are listed in parentheses (50 μL reaction). Error bars are standard deviations of replicates.

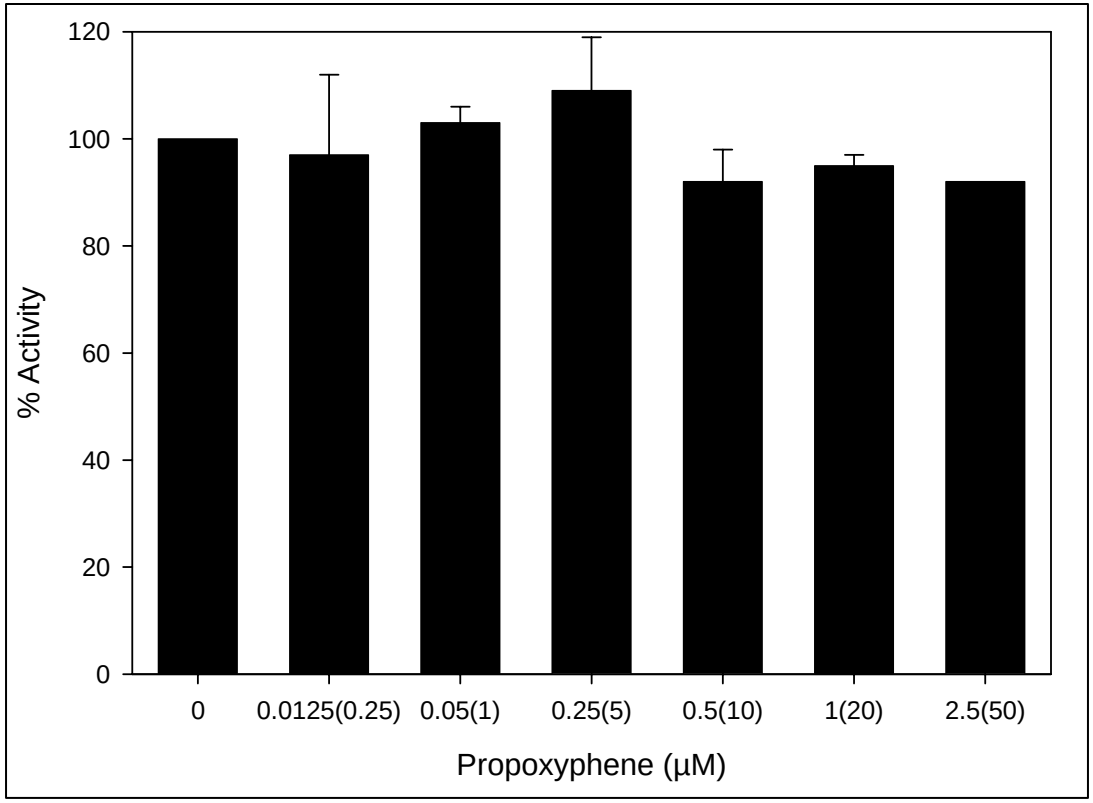


Figure 23. Propoxyphene and Human Liver Microsomes-Percent Activity Relative to Control. The percentage of initial testosterone 6β hydroxylase activity for each inhibitor concentration without pre-incubation time (time 0) activity was determined relative to the no inhibitor zero pre-incubation time (0,0 control). Concentrations of inhibitor are listed across the x-axis as inhibitor concentration (1 mL reaction) and pre-incubation concentrations are listed in parentheses (50 µL reaction). Error bars are standard deviations of replicates.

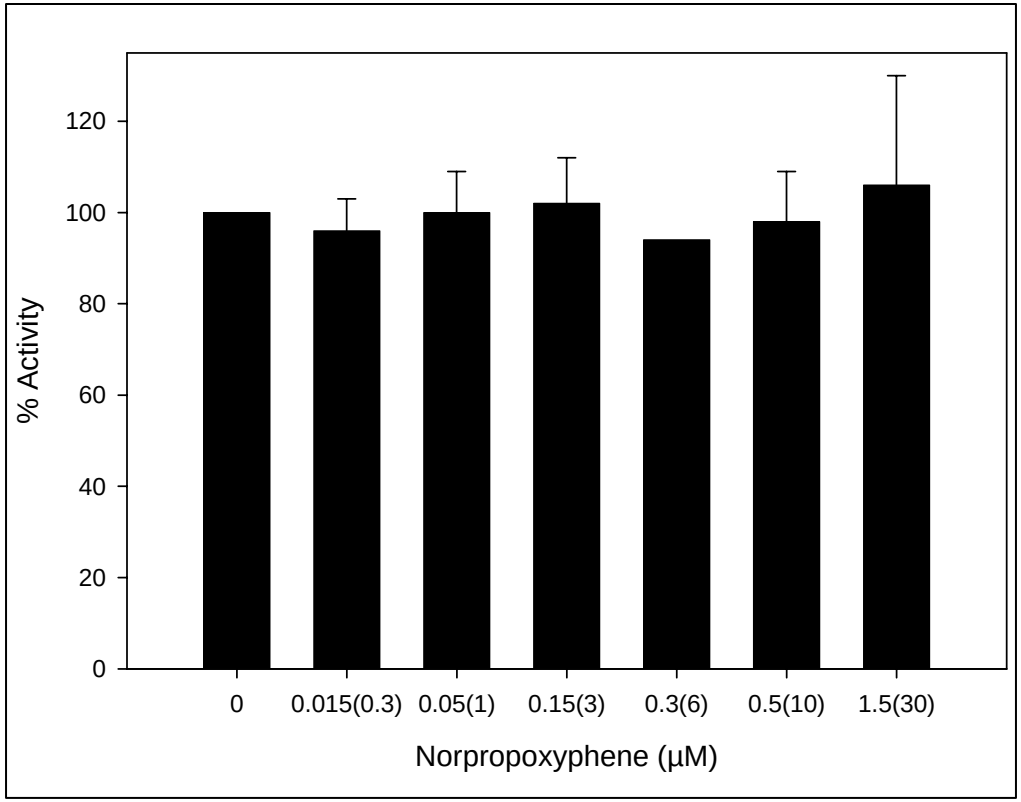


Figure 24. Norpropoxyphene and CYP3A4(+b5)-Percent Activity Relative to Control. The percentage of initial testosterone 6β hydroxylase activity for each inhibitor concentration without pre-incubation time (time 0) activity was determined relative to the no inhibitor zero pre-incubation time (0,0 control). Concentrations of inhibitor are listed across the x-axis as inhibitor concentration (1 mL reaction) and pre-incubation concentrations are listed in parentheses (50 µL reaction). Error bars are standard deviations of replicates.

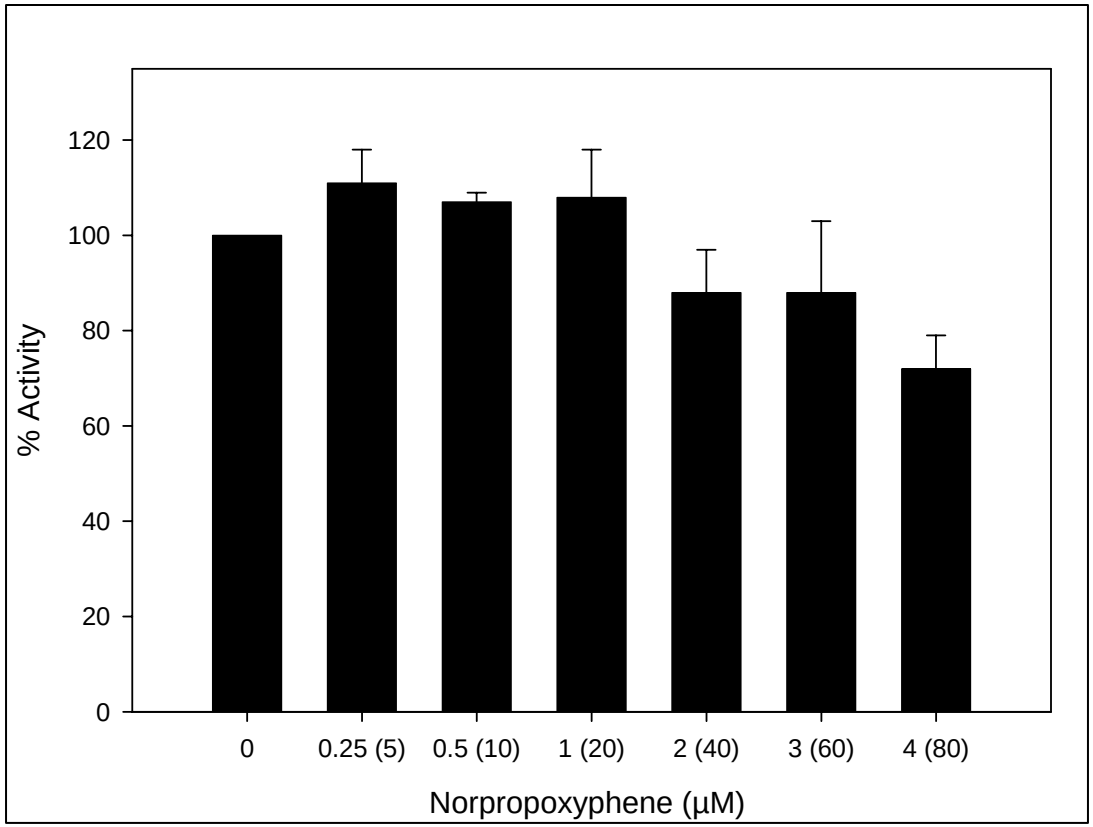


Figure 25. Norpropoxyphene and CYP3A5(+b5)-Percent Activity Relative to Control. The percentage of initial testosterone 6β hydroxylase activity for each inhibitor concentration without pre-incubation time (time 0) activity was determined relative to the no inhibitor zero pre-incubation time (0,0 control). Concentrations of inhibitor are listed across the x-axis as inhibitor concentration (1 mL reaction) and pre-incubation concentrations are listed in parentheses (50 µL reaction). Error bars are standard deviations of replicates.

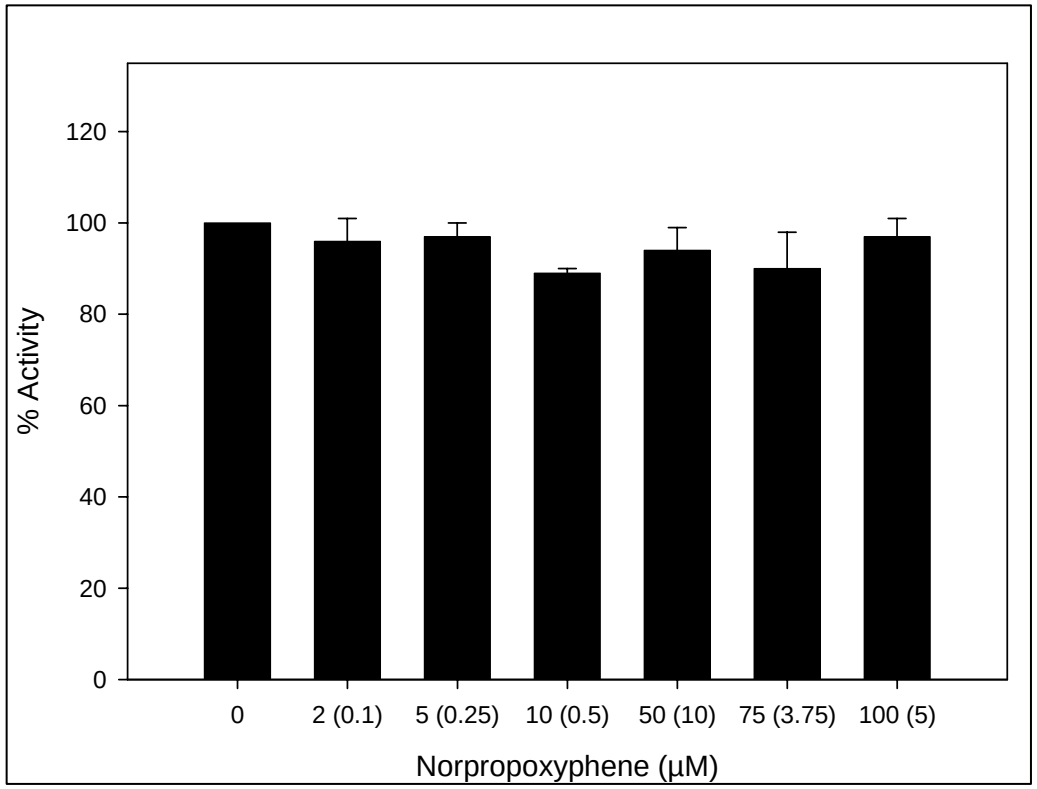


Figure 26. Norpropoxyphene and Human Liver Microsomes-Percent Activity Relative to Control. The percentage of initial testosterone 6β hydroxylase activity for each inhibitor concentration without pre-incubation time (time 0) activity was determined relative to the no inhibitor zero pre-incubation time (0,0 control). Concentrations of inhibitor are listed across the x-axis as inhibitor concentration (1 mL reaction) and pre-incubation concentrations are listed in parentheses (50 µL reaction). Error bars are standard deviations of replicates.

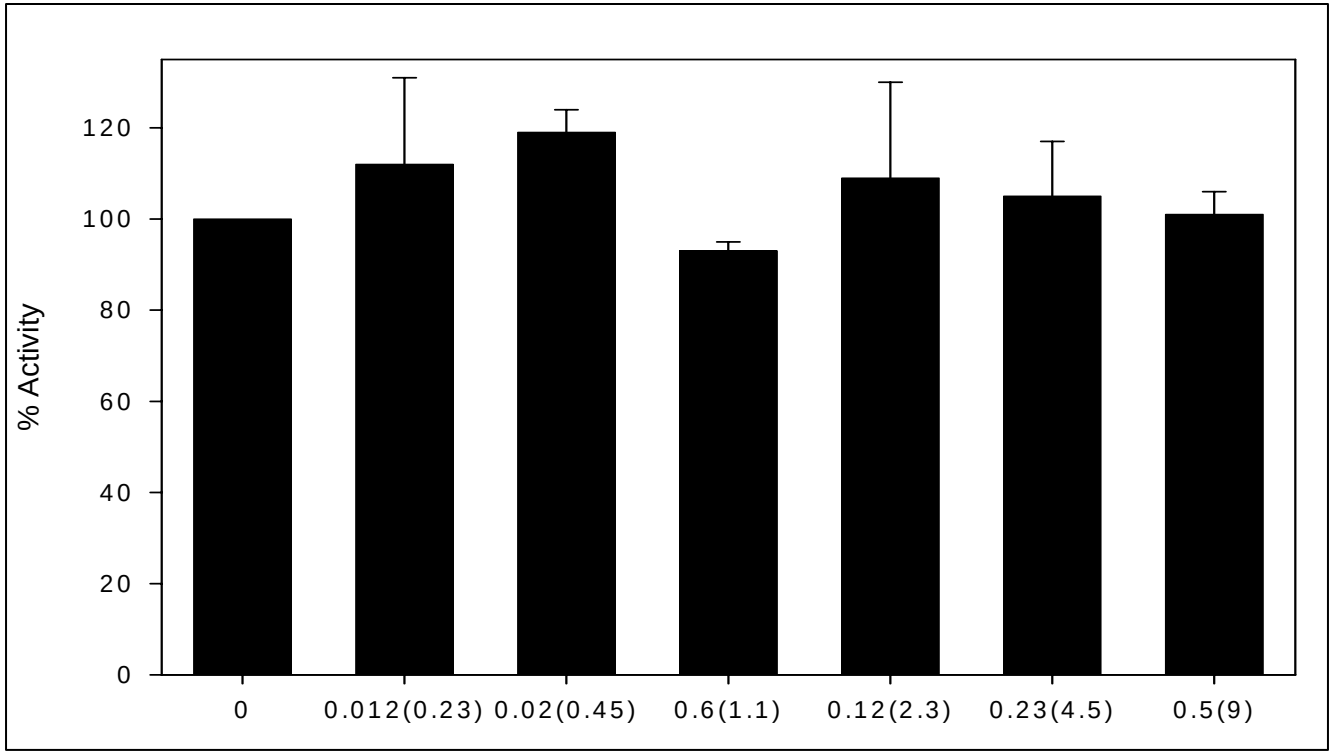


Figure 27. Proadifen and CYP3A4(+b5)-Percent Activity Relative to Control. The percentage of initial testosterone 6β hydroxylase activity for each inhibitor concentration without pre-incubation time (time 0) activity was determined relative to the no inhibitor zero pre-incubation time (0,0 control). Concentrations of inhibitor are listed across the x-axis as inhibitor concentration (1 mL reaction) and pre-incubation concentrations are listed in parentheses (50 µL reaction). Error bars are standard deviations of replicates.

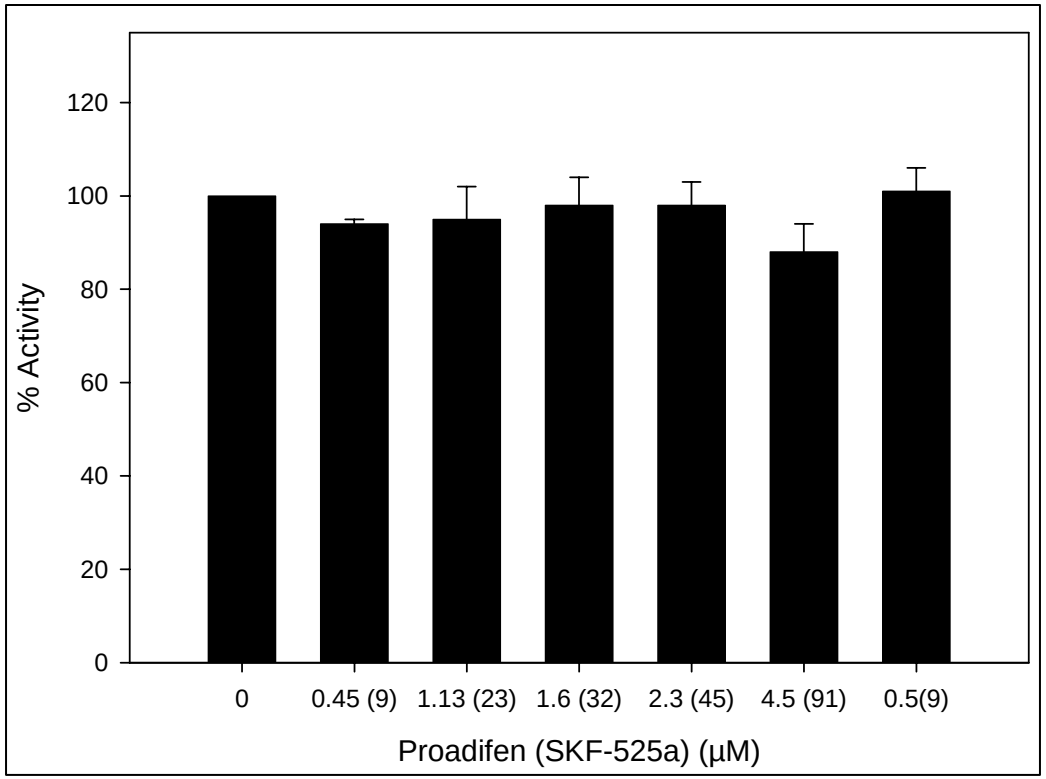


Figure 28. Proadifen and CYP3A5(+b5)-Percent Activity Relative to Control. The percentage of initial testosterone 6β hydroxylase activity for each inhibitor concentration without pre-incubation time (time 0) activity was determined relative to the no inhibitor zero pre-incubation time (0,0 control). Concentrations of inhibitor are listed across the x-axis as inhibitor concentration (1 mL reaction) and pre-incubation concentrations are listed in parentheses (50 µL reaction). Error bars are standard deviations of replicates.

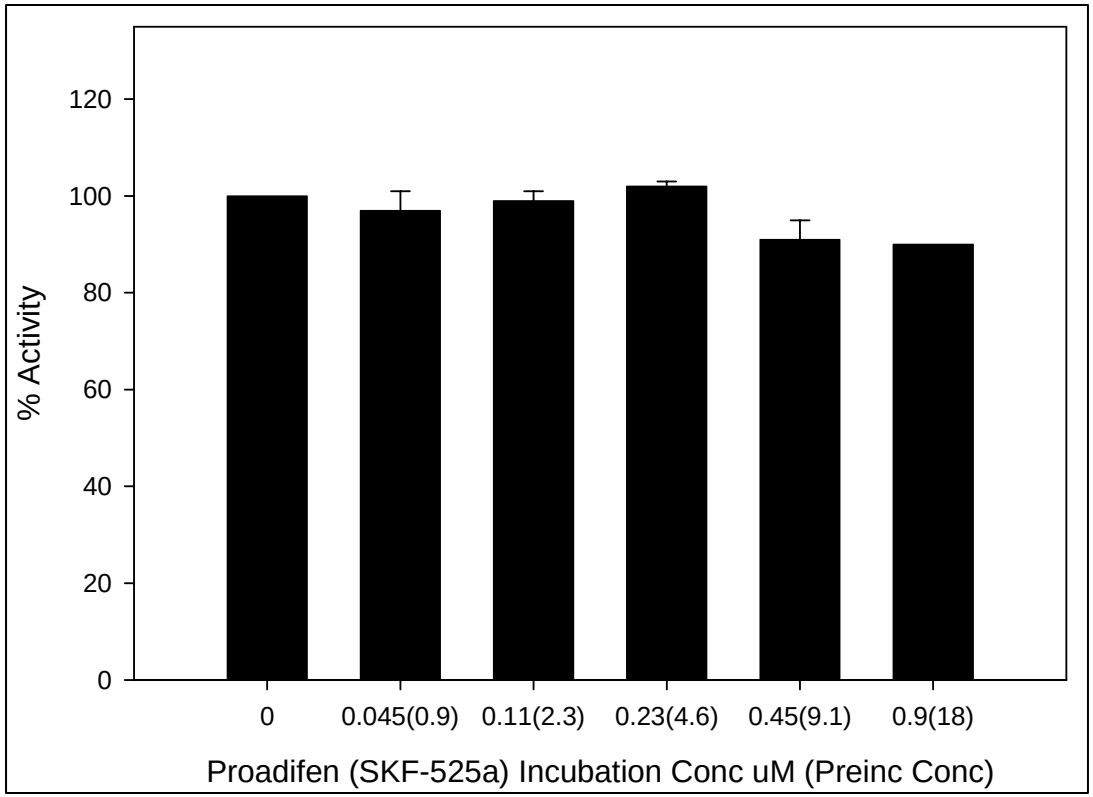


Figure 29. Proadifen and Human Liver Microsomes-Percent Activity Relative to Control. The percentage of initial testosterone 6β hydroxylase activity for each inhibitor concentration without pre-incubation time (time 0) activity was determined relative to the no inhibitor zero pre-incubation time (0,0 control). Concentrations of inhibitor are listed across the x-axis as inhibitor concentration (1 mL reaction) and pre-incubation concentrations are listed in parentheses (50 µL reaction). Error bars are standard deviations of replicates.

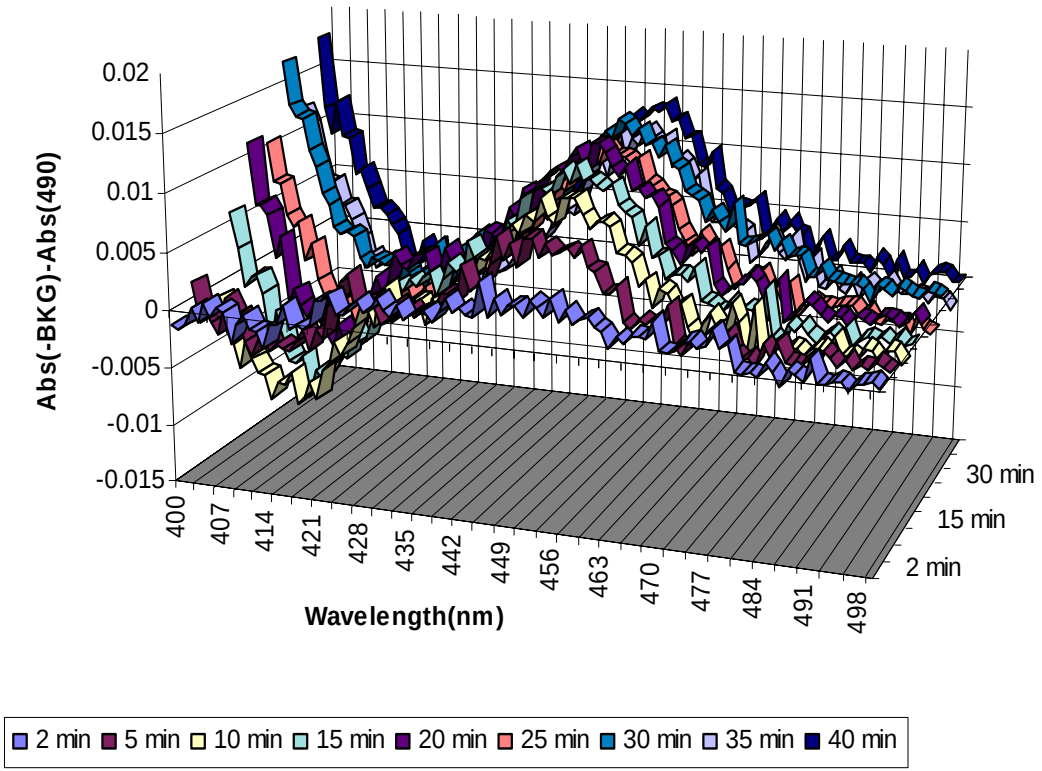


Figure 30. Metabolic-Inhibitor Complex Formation by Propoxyphene with CYP3A4(+b5). Metabolic-inhibitor complex formation was detected by a uv spectrophotometer by calculating the absorbance difference between the experimental cuvette and reference cuvette. Both contained CYP3A4(+b5), phosphate buffer, NADPH, and methanol, but only to a reference cuvette contained propoxyphene. Absorbance was measured scanning wavelengths of 400-500 nm over 30 minutes.

APPENDIX-SUMMARY OF STATISTICAL METHODS

AIC The Akaike Information Criteria determines the goodness of fit for models with different parameters (Akaike, 1987). For comparing multiple models with the same variables (such as competitive, noncompetitive, and uncompetitive modes), the smallest AIC is the best fit.

$$AIC = N_{observations} \times \log (WRSS) + 2 (N_{Parameters})$$

CV The coefficient of variation is calculated by taking the standard deviation divided by the mean and expressed as a percentage. It is a way of comparing the degree of variation of one series to another, even if the means are different from one another.

$$CV = (SD/Mean) \times 100$$

Mean Arithmatic average

SBC The Schwartz Bayesian Criterion measures goodness of fit based on maximum likelihood. For comparing models with similar (or no) weighting, the model with the smallest SBC value is the best fit.

$$SBC = N_{observations} \times \log (WRSS) + Log(N_{observations}) \times N_{Parameters}$$

SD The standard deviation of the mean, it is a measure of dispersion of a data set from its mean. It is calculated as the square root of the sum (Σ) of all differences from the mean ($x_i - \mu$), etc. squared, divided by the number of observations minus one ($n-1$). It is equal to the square root of the variance. (Σ square root $(x_i - \mu))^2 / n-1)$

SE The standard error of the mean, calculated by the dividing the standard deviation by the square root of the sample size (n).

$$SE = SD/(\sqrt{N})$$

WRSS Weighted residual sum of squares, estimates the variance of the residuals. (Residuals are observed minus predicted values, and predicted values come from model fitting). It is the sum of squared deviations from the mean where the means are the predicted parameter values.

(Pharsight Windows Nonlin 5.0.1)

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