# INHIBITION OF THE HUMAN LIVER MICROSOMAL AND HUMAN CYTOCHROME P450 1A2 and 3A4 METABOLISM OF ESTRADIOL BY DEPLOYMENT-RELATED AND OTHER CHEMICALS

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**A list of non-standard abbreviations:**

DEET - *N,N*-diethyl-*m*-toluamide

E2 - estradiol

TST - testosterone

HLM - human liver microsomes

CYP - cytochrome P450

HPLC - high performance liquid chromatography

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## Abstract

Cytochrome P450s (CYPs) are major catalysts in metabolism of xenobiotics and endogenous substrates such as estradiol ($E_2$). It has previously been shown that $E_2$ is predominantly metabolized in humans by CYP1A2 and 3A4 with 2-hydroxyestradiol (2-OHE$_2$) the major metabolite. This study examines effects of deployment-related and other chemicals on $E_2$ metabolism by human liver microsomes (HLM) and individual CYP isoforms. Kinetic studies using HLM, CYP3A4, and CYP1A2 demonstrated similar affinities ($K_m$) for $E_2$ with respect to 2-OHE$_2$ production. $V_{max}$ and CL$_{int}$ values for HLM are 0.32 nmol/min/mg protein and 7.5 μl/min/mg protein, those for CYP3A4 are 6.9 nmol/min/nmole CYP and 291 μl/min/ nmol CYP and those for CYP1A2 are 17.4 nmol/min/nmole CYP and 633 μl/min/ nmol CYP. Phenotyped HLM use demonstrated that individuals with high levels of CYP1A2 and CYP3A4 have the greatest potential to metabolize $E_2$. Preincubation of HLM with a variety of chemicals, including those used in military deployments, resulted in varying levels of inhibition of $E_2$ metabolism. The greatest inhibition was observed with organophosphorus compounds, including chlorpyrifos and fonofos, with up to 80% inhibition for 2-OHE$_2$ production. Carbaryl, a carbamate pesticide, and naphthalene, a jet fuel component, inhibited ca. 40% of $E_2$ metabolism. Preincubation of CYP1A2 with chlorpyrifos, fonofos, carbaryl, or naphthalene resulted in 96, 59, 84, and 87% inhibition of $E_2$ metabolism, respectively. Preincubation of CYP3A4 with chlorpyrifos, fonofos, deltamethrin or permethrin resulted in 94, 87, 58 and 37% inhibition of $E_2$ metabolism. Chlorpyrifos inhibition of $E_2$ metabolism is shown to be irreversible.

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## Introduction

The cytochrome P450 (CYP) monooxygenase system is comprised of a superfamily of heme-containing enzymes, expressed in many mammalian tissues with the highest levels found in liver, and capable of catalyzing the metabolism of a wide range of both endogenous and exogenous substrates (Nelson et al., 1996). Human CYP3A4 and CYP1A2 are two of the most important and abundant drug-metabolizing CYP isoforms in human liver microsomes. On average, CYP3A4 and CYP1A2 account for approximately 40% and 13% of the total CYP in human liver microsomes, respectively (Shimada et al, 1994; Lehman et al., 1998). Human CYP3A4 and CYP1A2 not only metabolize xenobiotics but are also responsible for the metabolism of endogenous compounds, such as steroid hormones. Both human CYP1A2 and CYP3A4 are involved in the metabolism of estradiol ($E_2$) and estrone ($E_1$) (Lee et al., 2001, 2003), whereas CYP3A4 plays a predominant role in the metabolism of testosterone (TST), androstenedione (AD), and progesterone (Waxman et al., 1988; Usmani et al., 2003).

In the human female, $E_2$ is the most potent primary circulating estrogen of a group of endogenous estrogen steroids which includes $E_1$ and estriol ($E_3$). During female development, $E_2$ stimulates the growth of female sex organs, regulates and sustains female sexual development and reproductive function, promotes hypertrophy in female breast and male muscle during puberty, initiates the synthesis of specific proteins, and controls fat deposition and distribution in subcutaneous tissues, thereby determining the characteristic female figure. Like TST in males, estrogens are the primary cause of the growth spurt, maturation of long bones and development of secondary sexual characteristics in females. In the adult, $E_2$ regulates events during the menstrual cycle (growth of endometrial lining), is important during pregnancy and lactation and

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contributes to the maintenance of sexual drive and female personality (Wilson et al., 1998; and

Constanti et al., 1998). Maintaining hormonal balance relies upon a number of variables

including the rate of hormone synthesis, interactions among hormones, and rates of secretion,

transport, and metabolism by phase I and phase II enzymes. CYPs (phase I) are a major element

in the maintenance of a proper steroid hormone levels in mammalian systems and are the sole

subject of this investigation. Exposure to foreign compounds may exert changes in endocrine

function both directly (hormone agonists or antagonists) or indirectly (altering circulating levels

of hormones by influencing rates of hormone synthesis or metabolism) that can severely affect

steroid hormone action (Wilson and LeBlanc, 1998). It follows that perturbation of the CYP

system by xenobiotics may in turn affect the subsequent metabolism and disposition of $E_2$.

Perturbations in $E_2$ metabolism may affect levels of circulating $E_2$ with possible reproductive and

other consequences, including further modulation of the expression of some CYP proteins.

The uses of deployment-related chemicals are essential for the health and well-being of

deployed forces during both peace keeping and wartime missions. Human hazards associated

with chemicals used to protect personnel during peace and wartime are poorly understood since

the definition of cause and effect relationships depends on knowledge of the mechanisms of toxic

action and interaction in humans. Following the Gulf War some veterans reported illnesses

which may have been the result of chemical exposures. Some studies of these veterans have

concluded that significant correlations between perceived illnesses and chemical use exist (Haley

and Kurt, 1997). The reported chemical exposures included the insect repellent *N,N*-diethyl-*m*-

toluamide (DEET), insecticides such as permethrin and chlorpyrifos used to protect against

insect borne diseases and the neuroprotective agent pyridostigmine bromide used to protect

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against possible nerve gas attack. It has been reported that chlorpyrifos and DEET are metabolized by human CYPs (Tang et al., 2001; Usmani et al., 2002) and that interactions of deployment-related chemicals can inhibit or induce the CYPs involved in their metabolism (Usmani et al., 2002). In a recent study examining the effects of various deployment-related chemicals on TST metabolism, Usmani et al. (2003) reported that organophosphorus pesticides are potent non-competitive and irreversible inhibitors of TST metabolism due to CYP3A4 inhibition. Other studies have reported that interaction of Gulf War related chemicals could produce greater than additive toxicity in rats and mice (McCain et al., 1997; Chaney et al., 1997), increased neurotoxicity in hens associated with increased inhibition of brain acetylcholinesterase and Neurotoxicity Target Esterase (Abou-Donia et al., 1996a,b) and neurobehavioral deficits associated with significant inhibition of brainstem acetylcholinesterase activities in rats (Abou-Donia et al., 2001). Possible detrimental interactions as a result of altered estrogen metabolism have not yet been examined relative to these chemicals nor have studies been carried out to examine the induction or inhibition by these or related compounds of human CYP-mediated metabolism of estrogens, such as $E_2$.

The objective of present study was to study the inhibition or activation by various deployment-related chemicals of the metabolism of $E_2$ by HLM, CYP1A2, and CYP3A4.

**Materials and Methods**

**Chemicals.** Chlorpyrifos, fonofos, phorate, DEET, fipronil, imidacloprid, deltamethrin, permethrin, carbofuran, carbaryl, and naphthalene were purchased from ChemService (West Chester, PA). Pyridostigmine bromide was purchased from Roche (Indianapolis, IN). Testosterone (TST), 17β-estradiol ($E_2$), 2-hydroxyestradiol (2-$OHE_2$), and 6β-hydroxytestosterone were purchased from Steraloids (Newport, RI). HPLC grade water, methanol, acetonitrile, and tetrahydrofuran were purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals, if not specified, were purchased from Sigma (St. Louis, MO). The structures of all chemicals tested as inhibitors are shown in Fig 1.

**Human liver microsomes and human CYP isoforms.** Pooled human liver microsomes (HLM) (pooled from 21 donors), single-donor HLM, and the human CYP isoforms, 1A2 and 3A4, expressed in baculovirus infected insect cells (Sf9) (BTI-TN-5B1-4) were purchased from BD Biosciences (Woburn, MA).

**Assay conditions.** All assays were conducted in triplicate and all the reactions were pre-warmed for 5 min at 37°C. All the reactions contained NADPH-regenerating system (0.25 mM NADP, 2.5 mM glucose 6-phosphate, and 2 U/ml glucose-6-phosphate dehydrogenase) and 100 mM potassium phosphate buffer (pH 7.4) containing 5 mM ascorbic acid and 3.3 mM $MgCl_2$. Final concentrations of HLM were 1 mg/ml while final concentrations of CYP3A4 and CYP1A2 were 50 pmol/ml. The final reaction volume was 250 μl and all reactions were terminated after 20 min by the addition 150 μl of methanol and vortexing. After 5 min centrifugation at 21,000 g in a microcentrifuge, the supernatants were analyzed for $E_2$ metabolite concentrations by HPLC. The protein concentrations and incubation times used in the assays were found to be in the linear

range in preliminary experiments.  No metabolites were detected when incubations were carried out in the absence of an NADPH-generating system.

*In vitro* **$E_2$ metabolism.**  Enzyme kinetic assays using HLM or the recombinant isoforms CYP1A2 or 3A4 were performed by incubation of serial concentrations of $E_2$ (final concentrations 3.125-200 µM) with an NADPH-generating system in potassium phosphate buffer and incubated at $37^{\circ}C$ for 5 min.  The reactions were initiated by the addition of ice cold HLM (final protein concentration 1 mg/ml), CYP1A2 or 3A4. No measurable lag phase was observed.

To determine the metabolic activities of individual HLM, incubations of $E_2$ (final concentration 50 µM) were conducted for 20 min at $37^{\circ}C$ using 1 mg protein/ml.  To differentiate between the relative concentrations of CYP1A2 and CYP3A4 to the metabolism of $E_2$ in individual HLM, a specific CYP3A4 inhibitor, ketoconazole (final concentration 2 µM), was added to the reaction mixture simultaneously with 50 µM $E_2$.

**Preliminary inhibition studies.**  The effects of test chemicals (chlorpyrifos, fonofos, phorate, DEET, fipronil, imidacloprid, deltamethrin, permethrin, carbofuran, carbaryl, naphthalene, and pyridostigmine bromide) on $E_2$ metabolism were examined using HLM, CYP1A2, or 3A4 after preincubation with the test compounds in otherwise complete mixtures lacking only the substrate, $E_2$.  The HLM, CYP3A4, or CYP1A2 were incubated with individual test compounds (final concentration 50 µM) for 5 min at $37^{\circ}C$ before adding $E_2$ (final concentration 50 µM).  All inhibitors, except pyridostigmine bromide, were added in 2.5 µl of acetonitrile with an equal volume of acetonitrile being added to the control incubations. Pyridostigmine bromide was added in 2.5 µl of water. Because of the likelihood that

phosphorothioates are metabolized to irreversible CYP inhibitors, preincubation is tested as a variable with these chemicals. Preliminary experiments indicated little or no difference between pre- and co-incubation for other potential inhibitors. Only those chemicals which were capable of greater than 50% inhibition of $E_2$ metabolism at concentration of 50 µM were studied further.

**Inhibition kinetics of deployment-related chemicals on $E_2$ metabolism by CYP3A4 and CYP1A2.** The chemicals for which $IC_{50}$ values were below 25 µM as determined by preliminary incubation studies were analyzed further for inhibitory potential. Range finding assays were conducted for chlorpyrifos, fonofos, phorate, deltamethrin, and permethrin (0.39–200 µM) by incubating with CYP3A4 for 5 min at $37^{o}C$ before adding $E_2$ (final concentration 50 µM). The kinetic study of $E_2$ using varying $E_2$ concentrations (6.25 – 100 µM) was performed using CYP3A4, which was preincubated with chlorpyrifos or fonofos (0.78, 1.56 or 3.125 µM) for 5 mins at $37^{o}C$.

For CYP1A2, range finding assays were conducted for chlorpyrifos and fonofos as described above. Concentrations of chlorpyrifos (0.5 and 2.0 µM) and fonofos (12.5 and 50.0 µM) were preincubated with CYP1A2 for 5 min at $37^{o}C$ before adding $E_2$ (final concentration 3.125-100 µM) in order to measure their inhibition of CYP1A2 metabolism of $E_2$. In all inhibition studies involving preincubation with the inhibitor, the NADPH generating system was present during the preincubation.

*Pre-incubation time and concentration-dependent inactivation by chlorpyrifos of CYP3A4 metabolism of estradiol.* These studies were conducted using the same conditions as described in the assay conditions (50 µM $E_2$; 20 min. incubation at $37^{o}C$) but varying concentrations ( 0, 1, 2, 5, 10 and 50 µM of CPS) and pre-incubation times (0, 1, 2, 3, 4 and 5 min.). The generation of 2-

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OHE$_2$ (the major metabolite of E$_2$) was compared in this assay, conducted in duplicate.

Inactivation parameters (k$_{inact}$ and K$_I$) were estimated as described in previous studies

(Silverman, 1995, Heydari et al, 2004). The rate constant for initial inactivation (k$_{obs}$) at each

concentration of inhibitor (CPS) was calculated from the slopes in the regression plots (-k$_{obs}$)

(natural logarithm of remaining activity as % of control activity versus pre-incubation time). The

inactivation values (k$_{inact}$ and K$_I$) were calculated as described in Heydari et al. (2004); k$_{obs}$ =

k$_{inact}$ x [$I$] / (K$_I$ + [$I$]) where [$I$] is the inhibitor concentration, k$_{inact}$ is the maximum rate constant

for inactivation and K$_I$ is the inhibitor concentration that produces half maximal rate of

inactivation.

*Inhibition kinetics of carbaryl and its metabolites on E$_2$ metabolism by CYP1A2*. Before the $K_i$

(inhibition constant) determination was done, range finding assays were conducted for the

inhibition by carbaryl and its metabolites of the metabolism of E$_2$ by CYP1A2.  Varying

concentrations (1.56–50 µM) of carbaryl, 4-hydroxycarbaryl, 5-hydroxycarbaryl, and carbaryl

methylol were pre- or co-incubated with CYP1A2.  Preincubation experiments with inhibitors

were conducted similarly to those described above.  For coincubation experiments, carbaryl or its

metabolites were incubated with E$_2$ (final concentration 50 µM).  The reactions were initiated by

the addition of ice-cold CYP1A2.  For Michaelis-Menten plots, carbaryl (6.25 and 12.5 µM), 4-

hydroxycarbaryl (6.25 and 12.5 µM), and carbaryl methylol (12.5 and 25 µM) were coincubated

with E$_2$ (final concentration 3.125-100 µM).  The reactions were initiated by the addition of ice-

cold CYP1A2.

*Inhibition kinetics of naphthalene and its metabolites on E$_2$ metabolism by CYP1A2.*

Before the $K_i$ determination was done, range finding assays were conducted for the inhibition by

naphthalene and its metabolites of the metabolism of $E_2$ by CYP1A2.  Varying concentrations of

naphthalene (3.125 – 25 µM), 1-naphthol (0.78 - 25 µM), 2-naphthol (25 µM), *trans*-1,2-

dihydro-1,2-naphthalenediol (25 µM), 1,2-napthoquinone (25 µM), 1,4-naphthoquinone (25

µM), and 1,4-dihydroxynapthlene (25 µM) were pre- or co-incubated with CYP1A2.  The pre-

and co-incubation experiments with naphthalene and its metabolites were conducted similarly as

described above.  For Michaelis-Menten plots, naphthalene (1.56, 3.125, and 6.25 µM) was

preincubated with CYP1A2 for 5 min at $37^{\circ}$C before adding $E_2$ (final concentration 3.125-100

µM).

For Michaelis-Menten plots, 1-naphthol (1.56 and 3.125 µM) was coincubated with $E_2$

(final concentration 3.125-100 µM).  The reactions were initiated by the addition of ice-cold

CYP1A2.

To demonstrate whether 1-naphthol inhibition is reversible or irreversible, incubations

with and without 1-naphthol (3.125 µM) were conducted with $E_2$ (final concentration 50 µM).

The reactions were initiated by the addition of varying concentrations of ice-cold CYP1A2

(3.125-12.5 pmol).

**Analysis of 2-OHE$_2$ and 6β-hydroxytestosterone by HPLC.**  Analysis of the $E_2$

metabolite 2-OHE$_2$ was performed with an HPLC system coupled with in-line UV detection as

described previously (Suchar et al., 1995).  The HPLC system consisted of a Waters 2690

separation module and a Waters UV photodiode array detector (model 2996).  All system

components were controlled through the Waters powerline firmware.  Data was collected via a

Waters system controller and analyzed using Waters Empower software.  The solvent system for

separation of $E_2$ and 2-OHE$_2$ consisted of acetonitrile (solvent A), 0.1% acetic acid in water

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(solvent B), and 0.1% acetic acid in methanol (solvent C).  The solvent gradient (solvent

A/solvent B/solvent C) used for eluting $E_2$ and 2-OHE$_2$ was as follows: 8 min of isocratic at

16:68:16, 7 min of a concave gradient (curve number 9) to 18:64:18, 13 min of a concave

gradient (curve number 8) to 20:59:21, 10 min of a convex gradient (curve number 2) to

22:57:21, 13 min of a concave gradient (curve number 8) to 58:21:21, followed by a 0.1 min step

to 92:5:3 and a 3.9 min isocratic period at 92:5:3. The gradient was returned to the initial

condition (16:68:16) for 2 min and held for 3 min before analyzing the next sample.  The flow

rate was 1.2 ml/min.  $E_2$ and 2-OHE$_2$ were separated by an Ultracarb 5 ODS column (150 x 4.6

mm, Phenomenex, Rancho Palos Verdes, CA) at 30$^o$C and detected at 280 nm.  The limits of

detection for 2-OHE$_2$ was approximately 0.04 μM.  Concentrations of 2-OHE$_2$ were obtained

from the chromatographic peak area from a standard curve (0.15 μM – 20.0 μM).

TST and its metabolite, 6β-hydroxytestosterone, were separated using the method

described by Usmani et al. (2003).  $E_2$ and its metabolites eluted after 6β-hydroxytestosterone

and TST.

**Data Analysis and statistics.**  The apparent $K_m$ and $V_{max}$ parameters were calculated

using the SigmaPlot Enzyme Kinetics Module for Windows, version 1.1 (SPSS Inc., Chicago,

IL).  The $K_i$ values were estimated by nonlinear regression analysis by fitting different models of

enzyme inhibition to the kinetic data using SigmaPlot Enzyme Kinetics Module for Windows,

version 1.1 (SPSS Inc., Chicago, IL).  The mode of inhibition was established by comparing the

statistical results including the $r^2$ information criterion values of different inhibition models and

by selecting the one with the best fit.  Furthermore, the mode of inhibition was established by

comparing the 95% confidence intervals that did not overlap for $K_m$ and $V_{max}$ values.  Significant

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differences between data sets were determined by student t-test method using SigmaPlot for

Windows, version 8.0 (SPSS Inc., Chicago, IL).

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## Results

**Enzyme kinetics.** HLM, CYP3A4, and CYP1A2 all displayed similar $K_m$ values for metabolism of $E_2$ to 2-OHE$_2$ (Table 1). $V_{max}$ and CL$_{int}$ values for HLM were 0.32 nmol/min/mg protein and 7.5 μl/min/ mg protein, those for CYP3A4 were 6.9 nmol/nmole CYP and 291 μl/min/ nmol CYP and those for CYP1A2 were 17.4 nmol/min/nmole CYP and 633 μl/min/ nmol CYP.

**$E_2$ metabolism in single-donor HLM.** Incubations of $E_2$ with individual donor HLM (6 male and 11 female) demonstrated as much as 36-fold variability in metabolism among individuals (Fig. 2). Phenotype data based on metabolic activities of these individuals was used to derive correlations between CYP isoform content and $E_2$ metabolic activity. CYP3A4 was the isoform with the best correlation ($r^2 = 0.87$). All other correlations between $E_2$ hydroxylation activity and specific CYP isoforms were less than 0.40. To determine the CYP1A2 contribution to $E_2$ metabolism among individual HLM, ketoconazole (2μM), a specific CYP3A4 inhibitor, was used to inhibit CYP3A4 activity. Preliminary observations with ketoconazole indicated that 2 μM inhibited less than 5% of CYP1A2 activity (data not shown). In the presence of ketoconazole, variation among individuals was reduced to 12-fold and the correlation of CYP1A2 with $E_2$ metabolism increased to 0.81 (Fig. 2).

**Inhibition of $E_2$ metabolism.** The effects of various deployment-related and other chemicals on $E_2$ (50 μM) metabolism were investigated by preincubating 50 μM concentrations of each chemical with pooled HLM (Fig. 3). The organophosphorus compounds, chlorpyrifos fonofos, and phorate all significantly inhibited $E_2$ metabolism to 2-OHE$_2$, although inhibition by chlorpyrifos and fonofos was profound (ca. 80%) compared to phorate (ca. 16.5%).

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Preincubation of pooled HLM with carbaryl, a carbamate, and naphthalene, a jet fuel component, also resulted in significant inhibition (ca. 40%) of $E_2$ metabolism to 2-OHE$_2$. Fipronil, a phenyl pyrazole insecticide also significantly inhibited $E_2$ metabolism, though to a lesser degree (ca. 18%).

To further explore the inhibition of $E_2$ metabolism observed in HLM, assays were also conducted using CYP3A4 and CYP1A2. Preincubation of CYP3A4 with a variety of chemicals resulted in varying levels of activation and inhibition of $E_2$ metabolism (Fig. 4). $E_2$ metabolism was significantly inhibited by the organophosphorus compounds, chlorpyrifos and fonofos, with up to ca. 90% inhibition of $E_2$ metabolism to 2-OHE$_2$. Preincubation of CYP3A4 with pyrethroids, deltamethrin and permethrin resulted in ca. 58 and 37% inhibition of $E_2$ metabolism to 2-OHE$_2$, respectively. In contrast, preincubation of CYP3A4 with DEET, an insect repellent, and carbofuran, a carbamate, resulted in the production of small, but not significantly greater, levels of 2-OHE$_2$.

Similarly, preincubation of CYP1A2 resulted in varying levels of activation and inhibition of $E_2$ metabolism (Fig. 5). Preincubation of chlorpyrifos and fonofos resulted in ca. 96% and 59% inhibition of $E_2$ metabolism, respectively. In contrast with CYP3A4, preincubation of CYP1A2 with carbaryl and naphthalene resulted in significant (ca. 85%) inhibition of $E_2$ metabolism. Some chemicals, such as fipronil and deltamethrin appeared to produce small, but not significantly higher, levels of 2-OHE$_2$.

**Mechanism of inhibition.** To investigate the type of inhibition of CYP3A4 by chlorpyrifos and fonofos in the production of 2-OHE$_2$, a major $E_2$ metabolite, different concentrations of chlorpyrifos and fonofos were preincubated for 5 min with CYP3A4 before

adding varying concentrations of $E_2$. Michaelis-Menten plots showed that the $V_{max}$ values were significantly reduced without affecting $K_m$ values, indicative of a non-competitive inhibition of $E_2$ metabolism by chlorpyrifos and fonofos (Fig. 6).

In a similar manner, inhibition of CYP1A2 metabolism of $E_2$ by chlorpyrifos and fonofos involved a 5 minute preincubation prior to the addition of varying concentrations of $E_2$. As shown for CYP3A4 (Fig. 5), Michaelis-Menton plots for CYP1A2 also indicated non-competitive inhibition (Fig. 7).

The production of 2-OHE$_2$ by CYP3A4 was significantly inhibited by CPS in a time and concentration-dependent manner (Fig. 8A). Inactivation parameters ($k_{inact}$ and $K_I$) determined for CYP3A4 metabolism of estradiol by CPS were 0.3 min$^{-1}$ and 4.9 $\mu$M, respectively (Fig. 8B). Even at a pre-incubation of 0', the metabolic activity of CYP3A4 for estradiol was, apparently, significantly decreased by CPS, in a dose-dependent manner, indicating a very rapid initial rate of inhibition..

For IC$_{50}$ values, varying concentrations of carbaryl and its metabolites were pre- and co-incubated with CYP1A2. Under preincubation conditions but not with coincubation, carbaryl, 4-hydroxycarbaryl, and carbaryl methylol caused small increases in the production of 2-OHE$_2$ at the low concentrations (1.56, 3.125, and 6.25 $\mu$M) whereas at high concentrations (12.5 - 50 $\mu$M) inhibition of $E_2$ metabolism was observed (data not shown). Under preincubation conditions but not with coincubation, 5-hydroxycarbaryl caused small increases in the production of 2-OHE$_2$ at all concentrations (1.56 - 50 $\mu$M) (data not shown). Under coincubation conditions, carbaryl, 4-hydroxycarbaryl, 5-hydroxycarbaryl, and carbaryl methylol all caused inhibition of $E_2$ metabolism at all concentrations tested (1.56 - 50 $\mu$M) (data not shown). To determine the $K_i$

value and investigate the type of inhibition of CYP1A2 metabolism of $E_2$ by carbaryl and its

metabolites, different concentrations of carbaryl, 4-hydroxylcarbaryl, and carbaryl methylol were

coincubated with CYP1A2 and varying concentrations of $E_2$.  Michaelis-Menten plots showed

that the $V_{max}$ values were significantly reduced without affecting $K_m$ values, indicative of a non-

competitive inhibition of $E_2$ metabolism by carbaryl and its metabolites (Fig. 9).  The inhibition

constants ($K_i$) indicated that carbaryl is the most potent inhibitor of $E_2$ metabolism with a $K_i$

value of 7.7 µM, 4-hydroxycarbaryl is the second best inhibitor with a $K_i$ value of 10.2 µM,

while carbaryl methylol is a weak inhibitor with a $K_i$ value of 29.0 µM.

In the determination of $IC_{50}$ values, varying concentrations of naphthalene and its

metabolites were pre- and co-incubated with CYP1A2.  Preincubation of naphthalene increased

the inhibition of $E_2$ metabolism by nearly 2-fold in comparison with the coincubation of

naphthalene with $E_2$, indicating that a reactive metabolite is involved (data not shown).  No

differences were observed in the $IC_{50}$ values when 1-naphthol was pre- or co-incubated with $E_2$

(data not shown).  2-Naphthol, 1,2-napthoquinone, 1,4-naphthoquinone, and 1,4-

dihydronaphthalene (25 µM) inhibited  $E_2$ (50 µM) metabolism ca. 85 – 100%, whereas trans-

1,2-dihydro-1,2-naphthalenediol had no inhibitory effect on $E_2$ metabolism (data not shown).  To

determine the $K_i$ value and to investigate the type of inhibition of CYP1A2 by naphthalene on $E_2$

metabolism, naphthalene was preincubated for 5 min with CYP1A2 before adding varying

concentrations of $E_2$.  The Michaelis-Menten plot showed that the $V_{max}$ values were significantly

reduced without affecting $K_m$ values, indicative of a non-competitive inhibition of $E_2$ metabolism

by naphthalene (Fig. 10).  To investigate the type of inhibition of CYP1A2 by 1-naphthol on $E_2$

metabolism, 1-naphthol was coincubated with CYP1A2 and varying concentrations of $E_2$.  1-

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Naphthol, a predominant naphthalene metabolite, was shown to be a noncompetitive inhibitor of

$E_2$ metabolism (Fig. 11).  Further investigation of noncompetitive reversible or nonreversible

inhibition data revealed that the inhibition of 2-OHE$_2$ by 1-naphthol is nonreversible (data not

shown).

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### Discussion

CYP-dependent hydroxylation is a major pathway of oxidative metabolism of $E_2$ in

mammalian liver. The studies carried out using human CYP isoforms provided further insight

into the range of $E_2$ hydroxylation reactions that can be catalyzed by human CYP enzymes.

Human CYP1A2 and CYP3A4 are the major isoforms responsible for $E_2$ metabolism and 2-

OHE$_2$ is the major $E_2$ metabolite, as previously demonstrated (Yamazaki et al., 1998; Lee et al.,

2001, 2003). The phenotyped HLM data and the mean metabolic intrinsic clearance rates, as

estimated by $V_{max}/K_m$, corroborates earlier findings (Yamazaki et al., 1998; Lee et al., 2001,

2003) that both CYP3A4 and CYP1A2 play major roles in the metabolism of $E_2$ in human liver

and the content of these two CYPs in HLM determine which isoform is more important.

However, CYP3A4 may play a more important role than CYP1A2 because on average CYP3A4

accounts for approximately 40% of the total CYP in HLM. Our kinetic data presented in this

study for CYP1A2 and CYP3A4 are in general agreement with the previous reports showing that

CYP1A2 is more active than CYP3A4 in $E_2$ metabolism (Yamazaki et al., 1998; Badawi et al.,

2001).

CYP3A4 is one of the most important and abundant CYP isoforms in human liver and

has broad substrate specificity. CYP3A4 not only metabolizes xenobiotics but is also

responsible for the metabolism of endogenous compounds such as steroid hormones, including

TST and $E_2$ (Usmani et al., 2003; Lee et al., 2003). Because TST and $E_2$ are predominantly

metabolized by CYP3A4, both can potentially compete for the same catalytic site of CYP3A4.

The usual interaction between two different substrates for the same enzyme is competitive

inhibition. But because the $K_m$ values for TST and $E_2$ are quite different ($K_m = 108$ μM and 24

μM for TST and $E_2$, respectively), interactions between these substrates may be complicated due

to the allosteric characteristics of CYP3A4 (Shimada and Guengerich 1989; Lee et al., 1995;

Usmani et al., 2003; Williams et al., 2004). The recent demonstration of a relatively large

substrate binding catalytic site by crystal structure elucidation is consistent with the capacity of

CYP3A4 to accommodate large molecules and possibly more than one substrate (Yano et al.,

2004).

Endogenous steroids, such as $E_2$, always exist in vivo and considerable amounts of these

steroids are metabolized by the CYPs expressed in the human liver, where foreign compounds

are mainly metabolized. Since TST and $E_2$ both have important, and different effects in vivo, the

inhibition of the metabolism of both of these steroid hormones must be considered in detail. If

xenobiotics substantially alter enzymes such as CYP3A4 and CYP1A2, they may affect the rate

of $E_2$ metabolism, ultimately disrupting $E_2$ homeostasis. Preincubation of pooled HLM,

CYP3A4, and CYP1A2 with organophosphorus compounds, such as chlorpyrifos and fonofos,

resulted in the extensive inhibition of the production of $2\text{-OHE}_2$, a major $E_2$ metabolite.

Chlorpyrifos and fonofos inhibited $2\text{-OHE}_2$ formation noncompetitively and are among the most

potent inhibitors of the CYP-dependent oxidation of $E_2$ yet described. Organophosphorus

pesticides, such as chlorpyrifos and fonofos are activated by a CYP-catalyzed desulfuration

reaction (Fukuto, 1990). The sulfur atom released from these pesticides in this reaction is highly

reactive and is believed to bind immediately to the heme iron of P450 and inhibit its activity

(Norman et al, 1974, Halpert et al., 1980, Neal, 1980, Neal and Halpert, 1982. Butler and

Murray, 1997). Our previous study demonstrated that organophosphorus pesticides are potent

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non-competitive and irreversible inhibitors of TST metabolism by HLM and by CYP3A4

(Usmani et al., 2003).

Enzyme activity is often modulated by inhibition or induction, either condition modifying

the extent to which xenobiotics or endogenous substrates are metabolized (Guengerich, 1997;

Szklarz and Halpert, 1998). Inhibition may, in some interactions, be more serious than enzyme

induction since inhibition occurs more rapidly than induction (Guengerich, 1997). Preincubation

of CYP3A4 with chlorpyrifos and fonofos resulted in almost complete inhibition of $E_2$

metabolism to 2-OHE$_2$. Preincubation of CYP1A2 with chlorpyrifos resulted in almost complete

inhibition of 2-OHE$_2$ formation, whereas fonofos was not as potent an inhibitor. The kinetics of

inactivation of $E_2$ metabolism by CPS confirms that CPS is a mechanism based inactivator of

CYP3A4 in these studies. Pre-incubation of CPS in the presence of an NADPH-generating

system increased the inhibitory effect in a time and concentration-dependent manner. The

significant reduction of 3A4 activity toward $E_2$ metabolism by CPS at time 0' suggests that the

binding affinity of the inhibitor (CPS) is higher than that of the substrate ($E_2$) with the CYP3A4

isoform and that the initial rate of inhibition is rapid. The relatively low $K_I$ value (high inhibitory

potency) of CPS toward CYP3A4 metabolism of $E_2$ appears to confirm the strong binding

affinity of CPS with this isoform. The possibility exists, therefore, that inhibition of CYP3A4

and CYP1A2 by these chemicals could lead to higher levels of $E_2$ and alter hormonal properties.

The *in vivo* importance of these observations will require further studies.

Carbaryl is a widely used anticholinesterase carbamate insecticide. In an *in vitro* study,

5-hydroxycarbaryl, 4-hydroxycarbaryl, and carbaryl methylol were identified as the major

metabolites of carbaryl produced by HLM (Tang et al., 2002). CYP isoform data indicated that

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CYP1A1, 1A2, 2B6, 2C19, and 3A4 are the most active isoforms in human metabolism of

carbaryl (Tang et al., 2002). In our preliminary inhibition studies we found that preincubation of

pooled HLM and CYP1A2 with carbaryl significantly inhibited $E_2$ metabolism. Because

carbaryl is metabolized by CYP1A2 while also demonstrating significant inhibition of $E_2$

metabolism we expected competitive interactions between $E_2$ and carbaryl or its metabolites.

However, carbaryl and its metabolites inhibited formation of 2-OHE$_2$ noncompetitively in our

coincubation studies. The $K_i$ values indicated that carbaryl and 4-hydroxycarbaryl were much

better inhibitors of 2-OHE$_2$ than carbaryl methylol. While there is no obvious explanation of the

slight activation of $E_2$ at low concentrations of carbaryl, 4-hydroxycarbaryl and carbaryl

methylol, it may be noted that this is a common phenomenon in the oxidation of TST by human

liver microsomes (Usmani et al., 2003).

The polycyclic aromatic hydrocarbon naphthalene is an industrial chemical, an

environmental pollutant, and a component of jet fuel and has considerable toxicological

importance because of widespread human exposure and its potential to form toxic and

carcinogenic metabolites in humans (Wilson et al., 1996). We recently demonstrated that

CYP1A2 is the most efficient of the 15 different isoforms tested for their ability to metabolize

naphthalene to its two major metabolites in HLM, 1-naphthol and 1,2-dihydro-1,2-

naphthalenediol (Cho et al., 2006). In this study, preincubations of naphthalene with pooled

HLM and CYP1A2 significantly inhibited $E_2$ metabolism. Because naphthalene is metabolized

by CYP1A2 and also significantly inhibited $E_2$ metabolism we expected a competitive

interaction between $E_2$ and naphthalene or its metabolite. However, this study demonstrated

noncompetitive inhibition of $E_2$ metabolism. Coincubation of 1-naphthol, a well known

cytotoxic metabolite of naphthalene, resulted in noncompetitive and irreversible inhibition of $E_2$ metabolism.

In conclusion, the hydroxylation of $E_2$ by CYP3A4 and CYP1A2 isoforms indicates important functions for these enzymes other than detoxification of xenobiotics. The deployment-related and other chemicals used in this study, including pesticides, caused a marked modification of CYP mediated $E_2$ metabolism *in vitro*. Organophosphorus pesticides were very potent inhibitors of the production of 2-OHE$_2$ and inhibited $E_2$ metabolism noncompetitively. Carbaryl and some of its metabolites inhibited $E_2$ metabolism non-competitively. Naphthalene inhibited $E_2$ metabolism noncompetitively, an effect probably due to 1-naphthol, a naphthalene metabolite. 1-Naphthol inhibits $E_2$ metabolism noncompetitively and irreversibly. It should be noted that in vivo toxicokinetic data are not available for these chemicals in humans and thus the effects noted indicate only the potential for in vivo effects.

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Part of the studies were presented at the 7th International ISSX meeting in Vancouver, Canada,

2004 and part were presented at the 44th Annual meeting of SOT in New Orleans, 2005.

**Numbered footnote:**

[1]Deceased, Dr Rose died in a tragic accident on May 23rd, 2006.

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**Legends for Figures**

Figure 1. Structures of all chemicals tested as inhibitors of estradiol metabolism.

Figure 2. Metabolism of $E_2$ to 2-OHE$_2$ by single-donor HLM in the presence and absence of

ketoconazole. Solid bars = no ketoconazole and open bars = 2 μM ketoconazole.

Figure 3. Effects of deployment-related and other chemicals on $E_2$ metabolism by pooled HLM.

* Statistically significant different when compared with control ($P < 0.01$).

Figure 4. Effects of deployment-related and other chemicals on $E_2$ metabolism by CYP3A4.

* Statistically significant different when compared with control ($P < 0.01$).

♦ Statistically significant different when compared with control ($P < 0.05$).

Figure 5. Effects of deployment-related and other chemicals on $E_2$ metabolism by CYP1A2.

* Statistically significant different when compared with control ($P < 0.01$).

Figure 6. Michaelis-Menten plots for the inhibition of CYP3A4 mediated $E_2$ hydroxylation by

chlorpyrifos (A) and fonofos (B). Kinetic constants for the uninhibited (no inhibitor

present) enzyme are shown in Table 1.

Figure 7. Michaelis-Menten plots for the inhibition of CYP1A2 mediated $E_2$ hydroxylation by

chlorpyrifos (A) and fonofos (B). Kinetic constants for the uninhibited (no inhibitor

present) enzyme are shown in Table 1.

Figure 8. Time and concentration-dependent inactivation of CYP3A4 metabolism of $E_2$ by CPS.

A. Correlation between preincubation time with various concentrations of CPS and

CYP3A4 activity. B. Plot of inactivation rate constant as a function of CPS concentration

to determine $k_{inact}$ and $K_I$ values.

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Figure 9.  Michaelis-Menten plots for the inhibition of CYP1A2 mediated $E_2$ hydroxylation by

carbaryl (A), 4-hydroxycarbaryl (B), and carbaryl methylol (C). Kinetic constants for the

uninhibited (no inhibitor present) enzyme are shown in Table 1.

Figure 10.  Michaelis-Menten plot for the inhibition of CYP1A2 mediated $E_2$ hydroxylation by

naphthalene. Kinetic constants for the uninhibited (no inhibitor present) enzyme are

shown in Table 1.

Figure 11.  Michaelis-Menten plot for the inhibition of CYP1A2 mediated $E_2$ hydroxylation by

1-naphthol.

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Table 1.  Kinetic parameters for 2-hydroxylation of $E_2$ by pooled HLM, CYP3A4, and CYP1A2

| | $K_m$ | $V_{max}$ | $V_{max}/K_m$ |
|---|---|---|---|
| | μM | nmol/min/mg protein | μl/min/mg protein |
| HLM | $42.8 \pm 9.40$ | $0.32 \pm 0.02$ | 7.5 |
| | | nmol/min/nmol CYP | μl/min/nmol CYP |
| CYP3A4 | $23.7 \pm 3.00$ | $6.9 \pm 0.35$ | 291 |
| CYP1A2 | $27.5 \pm 2.16$ | $17.4 \pm 0.54$ | 633 |

Values are expressed as mean $\pm$ S.E.M. ($n = 3$).

Fig. 1
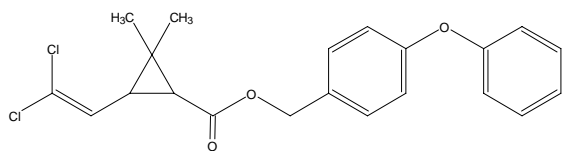


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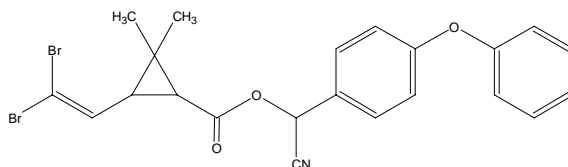
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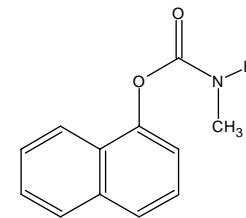
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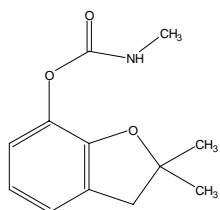
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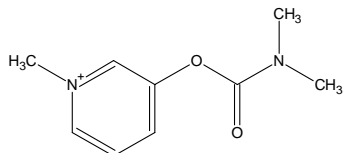
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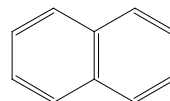
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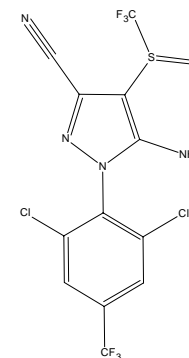
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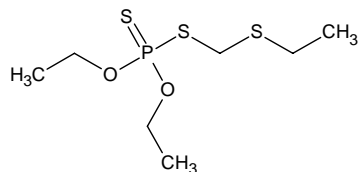
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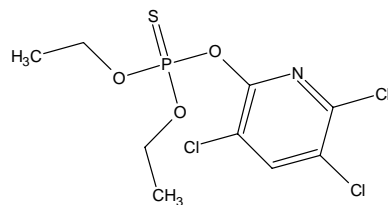
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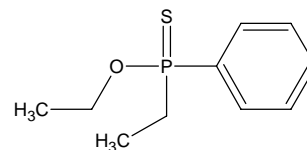
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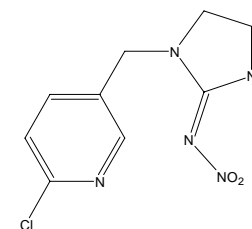
Imidacloprid

**Figure 2.**

**Figure 3.**
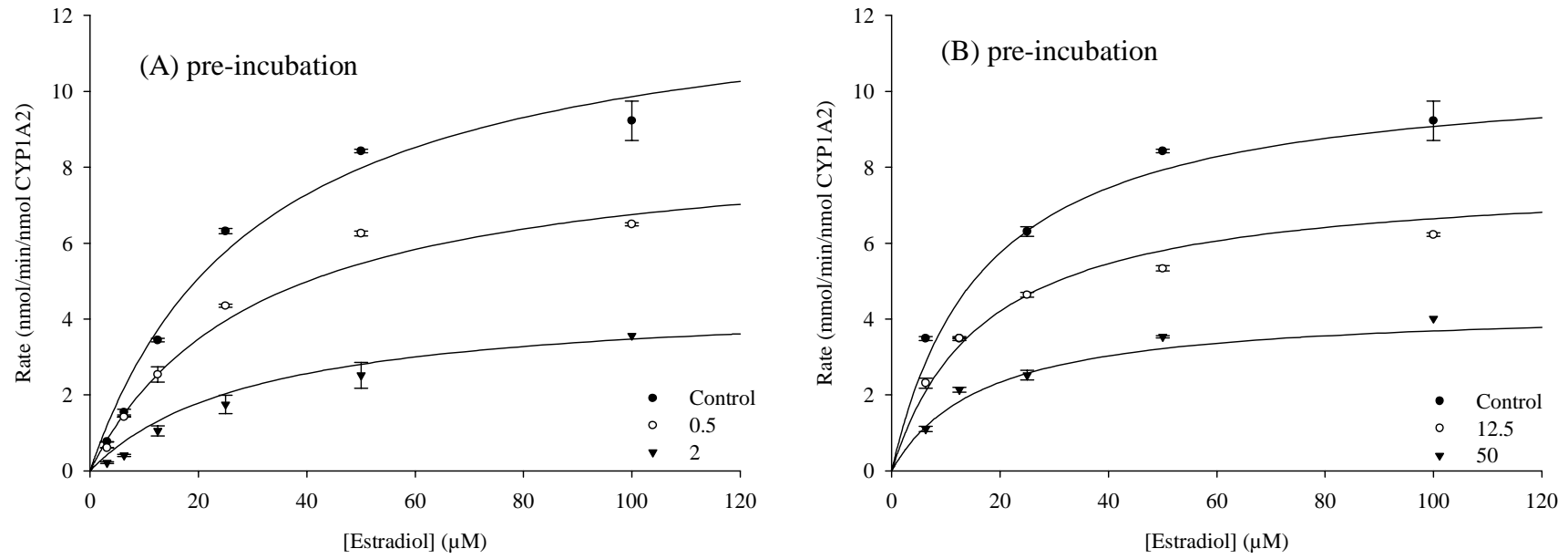


2-OHE$_2$
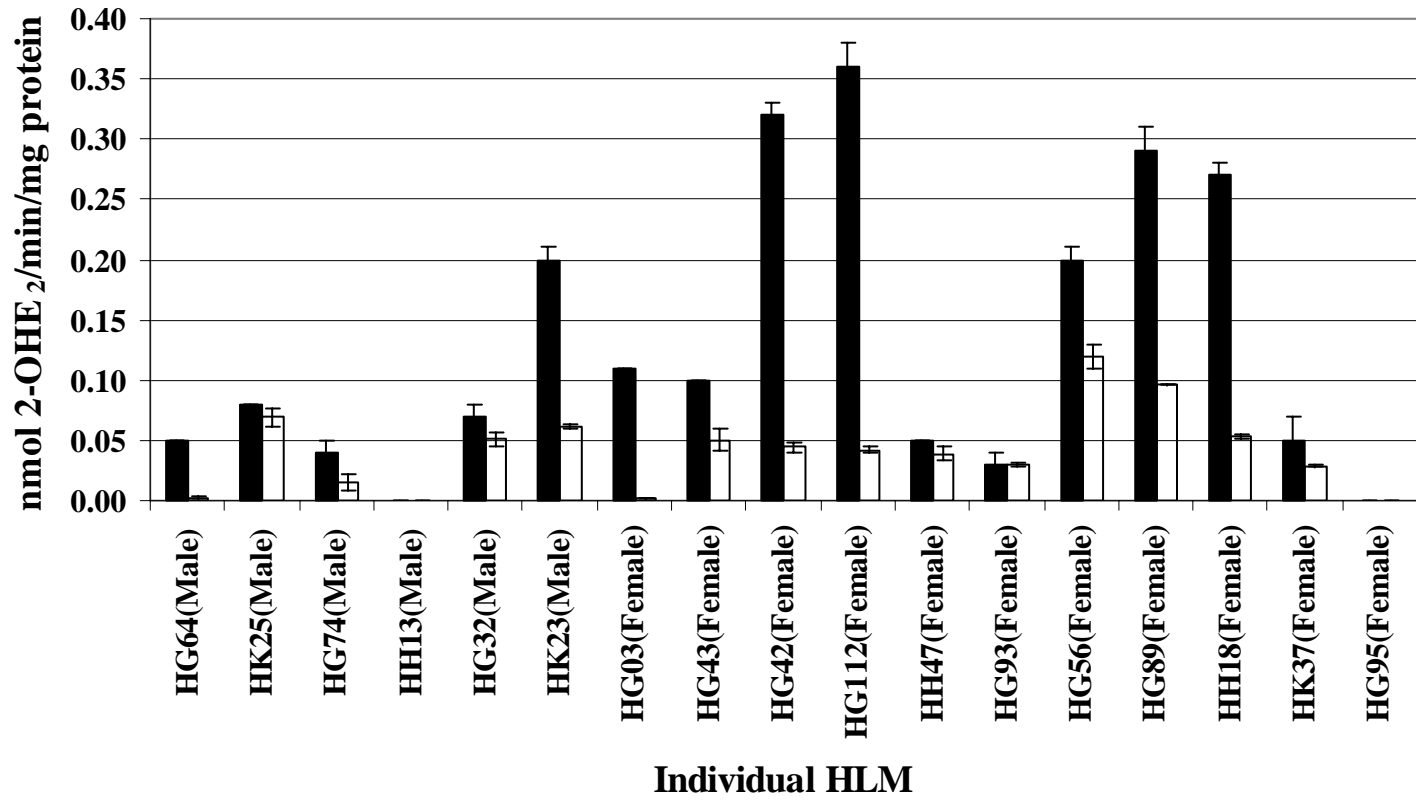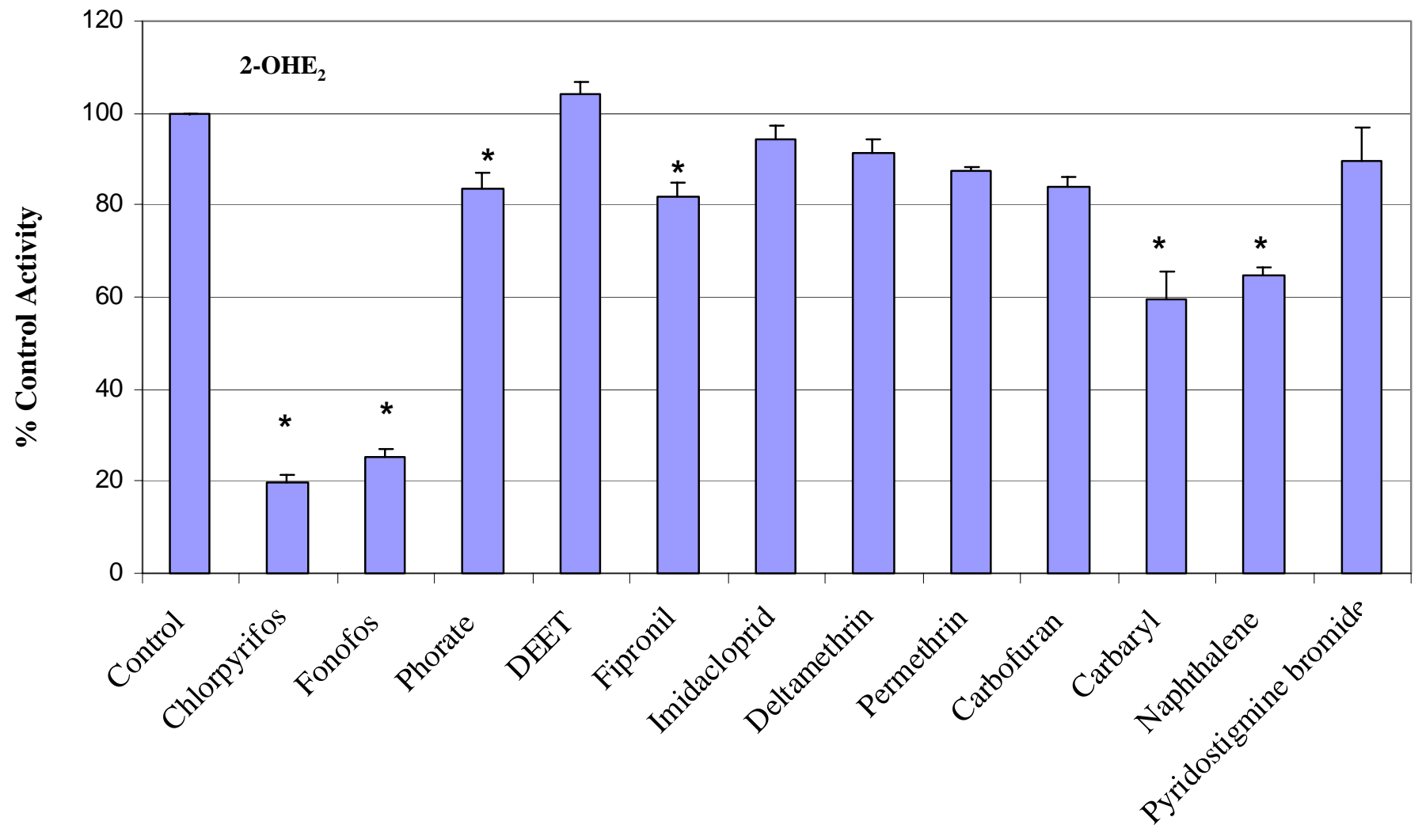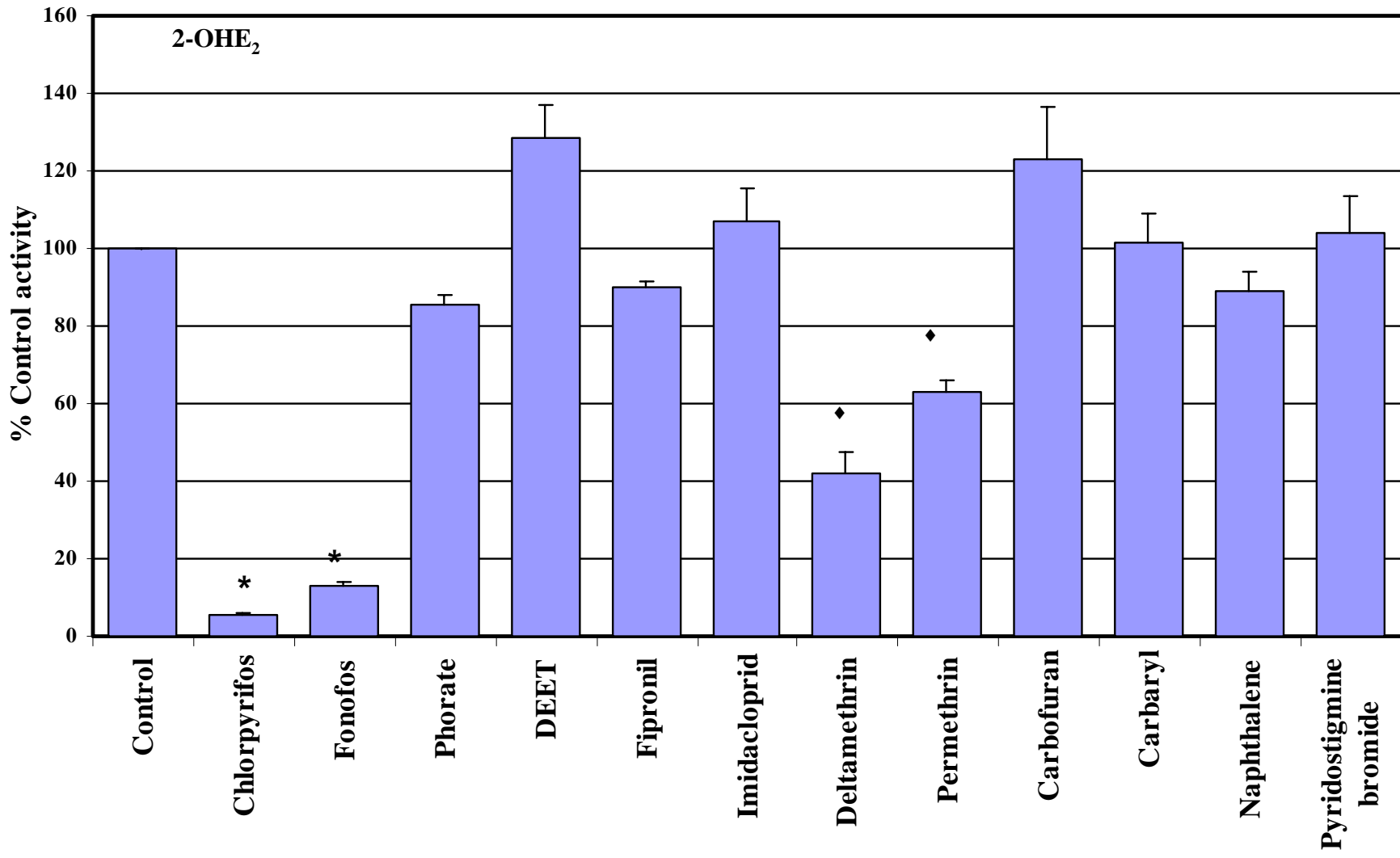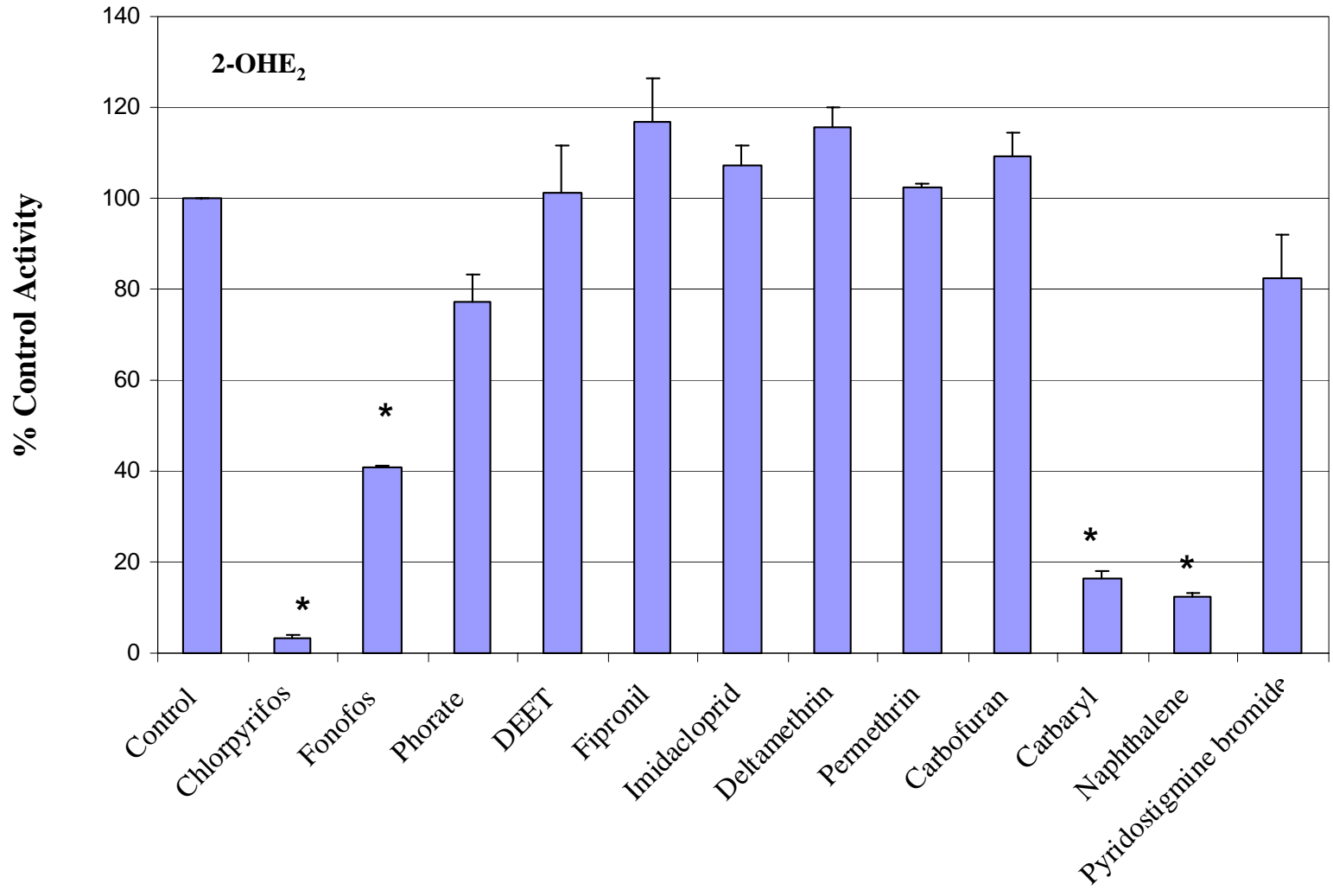
**Figure 4.**

**Figure 5.**
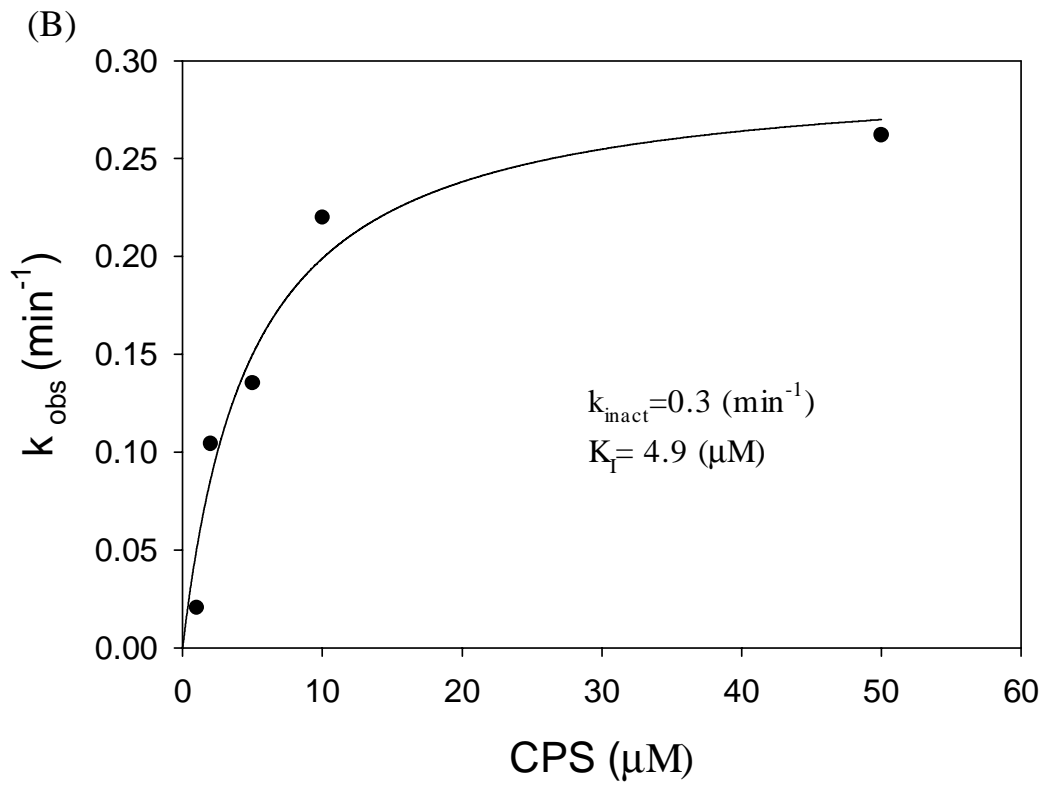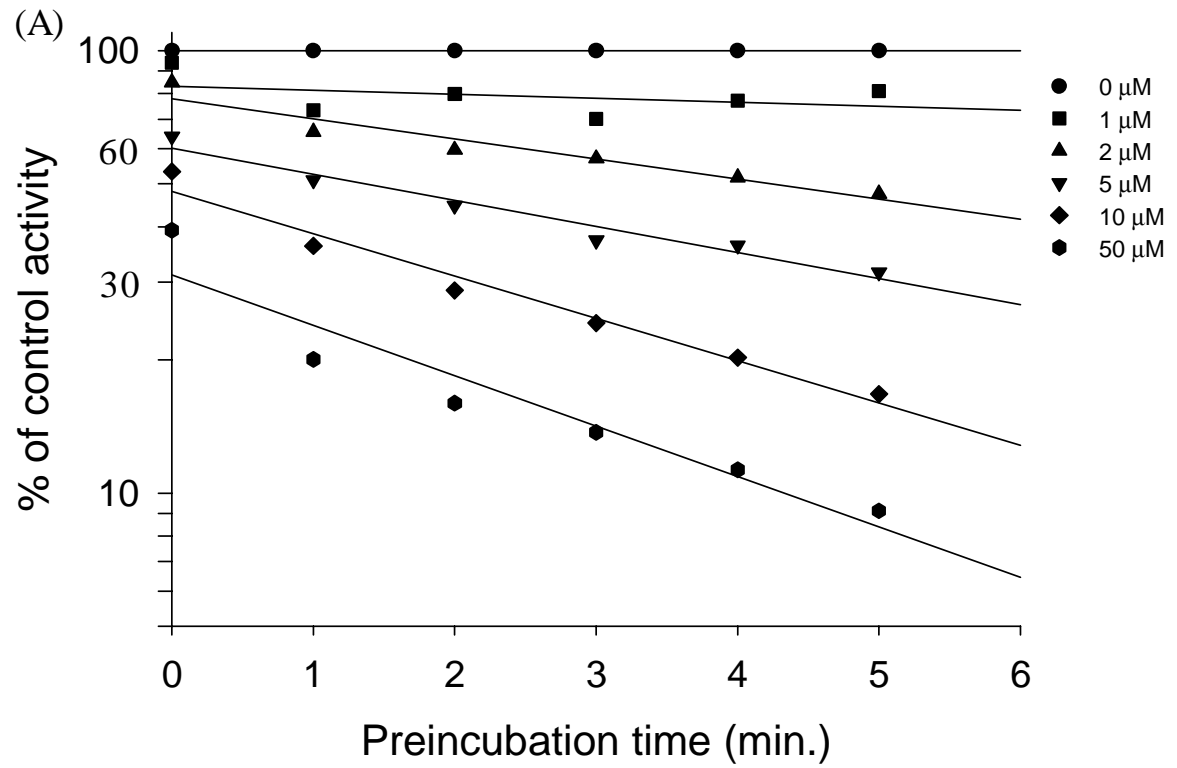
Figure 6.

Figure 7.

Figure 8.

(A)

(B)



$k_{inact}$=0.3 (min$^{-1}$)

$K_I$= 4.9 (µM)

Figure 9.

Fig. 10.

Figure 11.



co-incubation

Rate (nmol/min/nmol CYP1A2)

[Estradiol] (μM)

• Control
○ 1.56
▼ 3.125