INHIBITION OF *TRANS*-PERMETHRIN HYDROLYSIS IN HUMAN LIVER FRACTIONS BY CHLOROPYRIFOS OXON AND CARBARYL

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SUMMARY

Permethrin, a pyrethroid insecticide, is one of several deployment-related chemicals that have been suggested as causative agents for Gulf War related illnesses. Hydrolysis of *trans*-permethrin (tPMT) is a major route of detoxication and a potential locus for interactions with chemicals with similar use patterns. This study examined the potential inhibitory effects of chlorpyrifos, carbaryl, pyridostigmine bromide and the insect repellent *N,N*-diethyl-*m*-toluamide (DEET) on tPMT hydrolysis in human liver fractions. Although chlorpyrifos was not inhibitory, its toxic metabolite, chlorpyrifos oxon, strongly and irreversibly inhibited tPMT hydrolysis at low concentrations (cytosolic and microsomal K_i values of 3 and 16 nM, respectively). Carbaryl, a known anticholinesterase agent, showed non-competitive inhibition kinetics, with K_i values two orders of magnitude higher than those for chlorpyrifos oxon. Although DEET was much less effective than either chlorpyrifos oxon or carbaryl, equimolar concentrations inhibited up to 45% of tPMT hydrolysis. Pyridostigmine bromide showed no inhibitory effects. This study suggests that interaction potential between organophosphorus and pyrethroid insecticides should be

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considered in safety assessments when both insecticides are deployed simultaneously.

KEY WORDS

permethrin, chlorpyrifos oxon, *N,N*-diethyl-*m*-toluamide (DEET), pyridostigmine bromide, inhibition, ester hydrolysis, pesticide metabolism

INTRODUCTION

Adverse effects caused by drug interactions are elusive to define and in many cases cannot be predicted by routine animal based toxicology tests. Human metabolic pathways and metabolites have not been defined for many pesticides and insect repellents, making predictions of metabolic interactions in humans difficult. Our recent studies have begun to identify human metabolic pathways and the enzymes involved for several pesticides including chlorpyrifos /1/, carbaryl /2/, permethrin /3/, fipronil /4/, a variety of sulfur-containing pesticides /5/, and *N,N*-diethyl-*m*-toluamide (DEET) /6/.

Permethrin [3-phenoxy-benzyl (±) *cis/trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylate] is a photo-stable synthetic pyrethroid insecticide which exerts its neurotoxic effects by interacting with the sodium channel of neuronal cells of the nervous system /7/. During the Gulf War, troops were exposed to permethrin through permethrin impregnated uniforms, which were issued for their repellent and insecticidal action /8/. Studies examining potential causes of Gulf War related illnesses have suggested that interactions among deployment-related chemicals, including permethrin, chlorpyrifos, pyridostigmine bromide and DEET, are possible causes of these illnesses /9-11/. In recent studies of the human metabolism of DEET /6/ and carbaryl /2/ it was found that there are potential chemical interactions between these and other pesticides as a result of their ability to induce or inhibit xenobiotic metabolizing enzymes involved in their metabolism.

To date, most studies of the metabolism of permethrin have been carried out using rodents /12,13/. These studies show that the major initial permethrin detoxification step is the hydrolysis of the ester

linkage in the parent compound by esterases, producing phenoxybenzyl alcohol (PBOH) and 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid. PBOH is further transformed to phenoxybenzaldehyde and subsequently to phenoxybenzoic acid (PBCOOH) with concomitant hydroxylations and conjugations /12/. More recently, human *in vitro* studies of permethrin metabolism have demonstrated that the metabolic pathway from PBOH involves sequential metabolism by alcohol and aldehyde dehydrogenases to form PBCOOH /3/.

Chlorpyrifos [O,O-diethyl-O-(3,5,6-trichloro-2-pyridinyl)-phosphorothioate] is a widely used organophosphorus insecticide which is activated through a cytochrome P450 (CYP)-catalyzed desulfuration reaction to chlorpyrifos-oxon (CPO), a potent anticholinesterase agent /14/. The fact that permethrin and chlorpyrifos may often be used in temporal and physical proximity with one another combined with the knowledge that an active metabolite of chlorpyrifos is known to be an effective esterase inhibitor suggested the importance of determining whether there are metabolic interactions between chlorpyrifos and permethrin in humans. In the present study, we examined the potential of several deployment-related chemicals, including chlorpyrifos, CPO, DEET and pyridostigmine bromide, to interact with permethrin metabolizing enzymes in human liver fractions. Carbaryl, a carbamate insecticide with known esterase inhibitory properties, was also included in the study.

MATERIALS AND METHODS

Chemicals and human liver fractions

Trans-permethrin (98.1% purity) and phenoxybenzaldehyde (98% purity) were purchased from Sigma (St. Louis, MO). PBOH (98% purity), PBCOOH (99% purity), carbaryl (99%), chlorpyrifos (99.2%), CPO (98%), DEET (98%) and pyridostigmine bromide were purchased from ChemService (West Chester, PA). HPLC grade acetonitrile and water were purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals, if not specified, were purchased from Sigma (St. Louis, MO). Pooled human liver microsomes and cytosol were purchased from BD Biosciences Company (Woburn, MA). Micro-

somal and cytosolic fractions were derived from the same batch of 13 pooled human liver samples.

Assay of *trans*-permethrin metabolism in the presence of chlorpyrifos, CPO, DEET or carbaryl

Incubations of *trans*-permethrin with human liver microsomal or cytosolic fractions were conducted as previously described /3/ with minor modifications. Reactions were conducted in 0.1 M Tris buffer (pH 7.4) containing either microsomal (60 μg protein) or cytosolic (200 μg protein) fractions in the presence of 5 mM $MgCl_2$ and 3 mM EDTA in total volumes of 250 μl. Assays were initiated in a 37°C water bath by the addition of tPMT (200 μM final concentration) to an incubation tube. Following 20 min incubation, reactions were stopped by the addition of 250 μl of cold acetonitrile followed by 5 min centrifugation at 20,000 *g*. Supernatants were decanted and stored at 4°C until HPLC analysis was carried out.

To study inhibition, varying concentrations of substrate were pre-incubated with the appropriate microsomal or cytosolic fraction for 5 minutes at room temperature prior to the addition of tPMT. Concentrations of CPO varied from 2.5-120 nM, while concentrations of all other substrates (chlorpyrifos, carbaryl, DEET and pyridostigmine bromide) ranged from 2.5-120 μM. Assays conducted with chlorpyrifos in the microsomal fraction also included the use of an NADPH regenerating system as previously described /3/.

Kinetic assays were performed by incubation of a series of concentrations of permethrin (2.5-120 μM) with cytosolic and microsomal fractions for 20 minutes as described above. Based on preliminary estimates of IC_{50} values obtained for CPO and carbaryl, 20 and 80 nM concentrations of CPO were used for the cytosolic and microsomal fractions, respectively, while 10 μM was used with both fractions for carbaryl.

Analysis of metabolites by HPLC

Analyses of tPMT and two primary metabolites (PBOH and PBCOOH) were conducted using the HPLC method previously described /3/. Briefly, analytes were separated on a Phenomenex Luna column (Torrence, CA) and detected at 270 nm on a Shimadzu SPD-10AV-VP UV-VIS detector. The gradient elution previously described

fully resolved permethrin (10.2 min) and its two metabolites, PBOH (3.7 min) and PBCOOH (4.1 min). Concentrations were calculated using peak areas and a standard curve for each compound.

Enzyme kinetics and statistical analysis

V_{max} and apparent K_m values were determined by a non-linear regression method using GraphPad software (GraphPad Software, Inc., San Diego, CA) and K_i values were determined using a formula based on effects on V_{max} and K_m for a particular inhibition type /15/. Significant statistical differences for V_{max} and K_m were apparent when 95% confidence intervals did not overlap. The type of inhibition was determined based on statistically significant differences in V_{max} or K_m values after inhibitor treatments. In the graphical representation of inhibition kinetics, most values are expressed as the mean ± SEM (n = 3).

RESULTS

In range finding assays, tPMT hydrolysis in human liver fractions was significantly inhibited by CPO, carbaryl and DEET, but not by pyridostigmine bromide (data not shown). For both CPO and carbaryl, concentrations as low as 10 μM produced significant inhibition, while inhibition by DEET was approximately 45% at 200 μM. IC_{50} values calculated from inhibition curves following CPO inhibition of tPMT hydrolysis were 35 and 60 nM for human liver cytosol and microsomal fractions, respectively (data not shown). In contrast, the IC_{50} values for carbaryl were two orders of magnitude less potent than those for CPO (3.5 and 3.75 μM for cytosol and microsomes, respectively). In the case of CPO, complete inhibition of tPMT hydrolysis occurred at concentrations two-fold greater than the IC_{50} values (data not shown). In contrast, substantially greater amounts of carbaryl (*ca.* 40-fold) were required to obtain complete inhibition of tPMT hydrolysis in either microsomes (Fig. 1A) or cytosol (Fig. 1B).

A 5 min pre-incubation of the cytosolic fraction with 20 nM CPO significantly reduced both the V_{max} and the K_m values from their respective control values (from 0.51 to 0.24 nmole/mg/min for V_{max} and from 5.54 to 2.42 for K_m) (Table 1). In a similar manner, pre-incubation of 80 nM CPO with the microsomal fraction also resulted

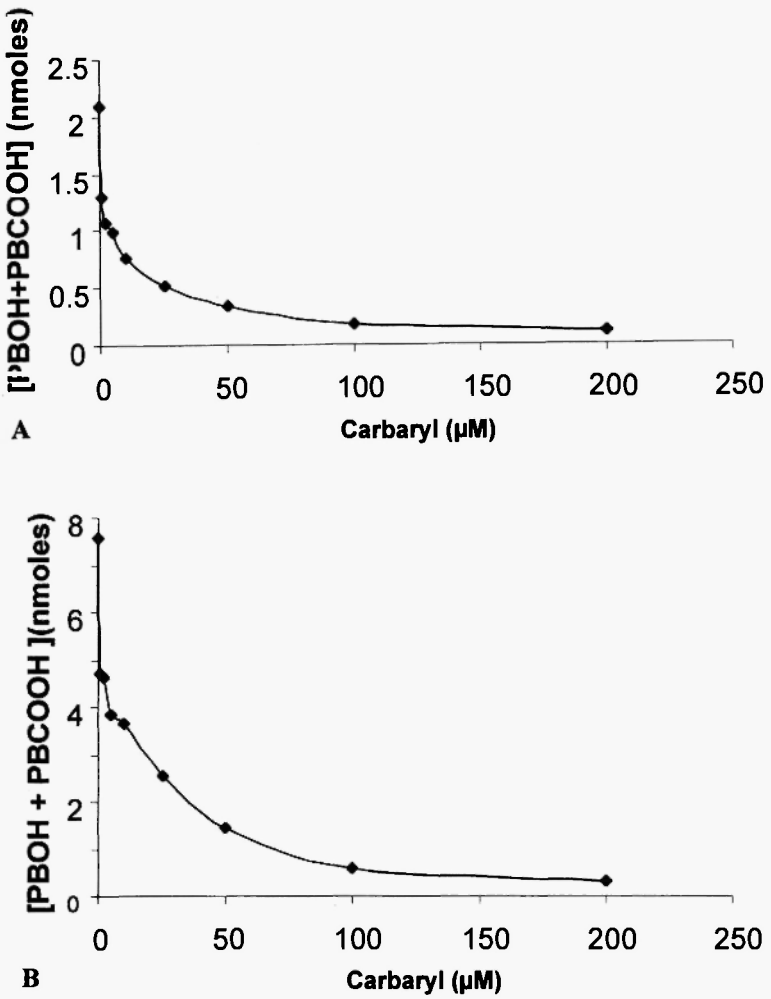

Fig. 1: Carbaryl inhibition of tPMT hydrolysis in human liver microsomes (**A**) and cytosol (**B**).

in significant decreases in both the V_{max} and K_m values (from 4.21 to 0.73 nmole/mg/min for V_{max} and from 11.57 to 4.94 for K_m) (Table 1 and Fig. 2A).

In contrast to inhibition by CPO, similar studies involving pre-incubations of 10 μM concentrations of carbaryl in both cytosolic and microsomal human liver fractions resulted in significant decreases in

TABLE 1

Inhibition of *trans*-permethrin hydrolysis in human liver fractions by chlorpyrofos oxon (CPO)

	V_{max} (nmole/mg/min)	95% CI V_{max}	K_m (μM)	95% CI (μM)	K_i (nM)	95% CI (nM)
Cytosol	0.51	0.49-0.52	5.54	4.72-6.37	NA	NA
Cytosol w/CPO (20 nM)	0.24	0.23-0.25	2.42	1.81-3.02	2.97	1.07-4.87
Microsomes	4.21	3.79-4.63	11.57	7.11-16.02	NA	NA
Microsomes w/CPO (80 nM)	0.73	0.48-0.97	4.94	3.04-12.92	16.13	4.0-28.27

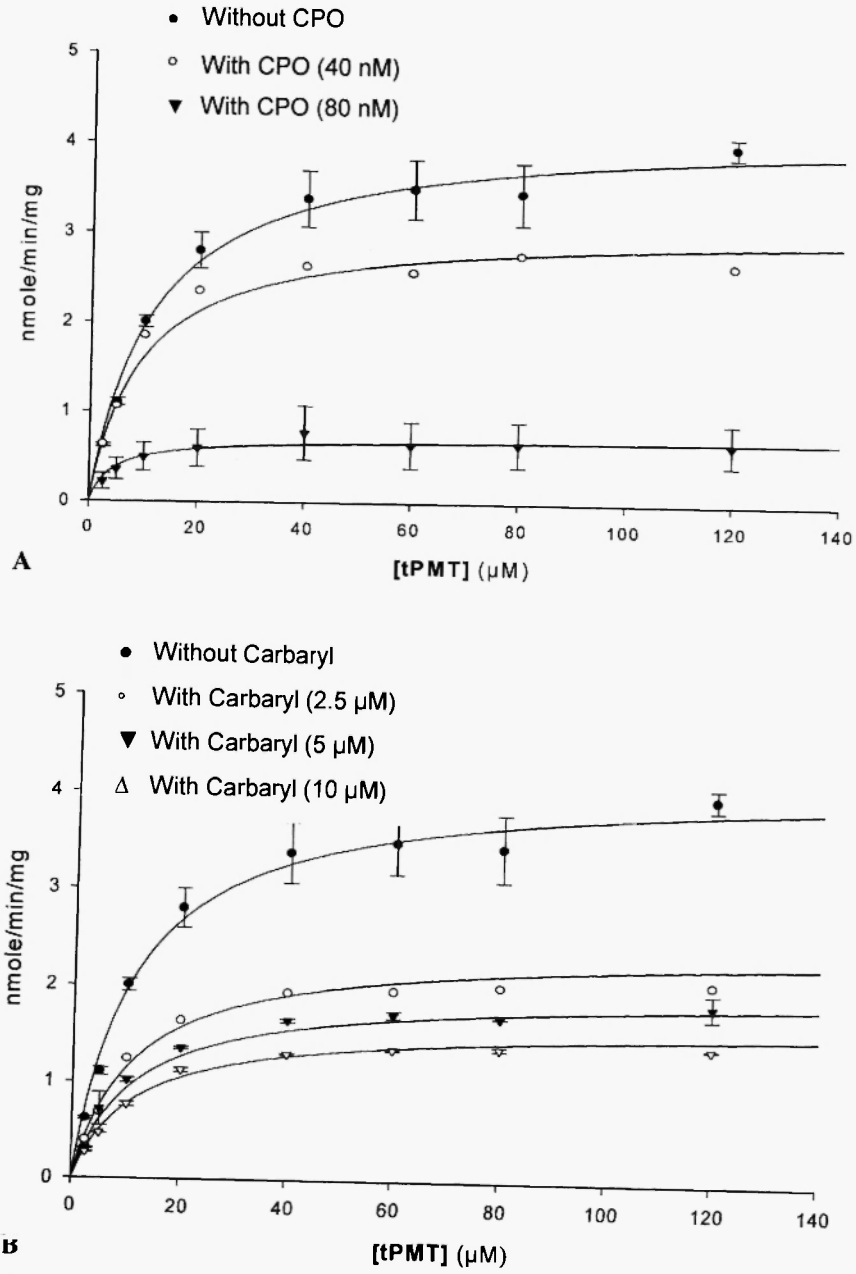

Fig. 2: Inhibition of tPMT hydrolysis by chlorpyrifos oxon (40 and 80 nM) (**A**) or by carbaryl (2.5, 5, and 10 nM) (**B**). Values accompanied by error bars represent the mean ± SEM (n = 3).

V_{max} values while exerting relatively minor changes in K_m values (Table 2 and Fig. 2B).

The inhibition constants (K_i), indicators of inhibitory affinity to the target enzyme, were calculated from V_{max} and K_m values for CPO and carbaryl (Tables 1 and 2, respectively). For CPO, K_i values were 3 and 16 nM for the cytosolic and microsomal fractions, respectively (Table 1). For carbaryl, the K_i values were 0.5 and 5.7 μM in the cytosolic and microsomal fractions, respectively (Table 2). The values for CPO were approximately one hundred times lower than those for carbaryl, indicating the higher inhibitory potential for CPO.

In contrast to effects observed with CPO, incubation of chlorpyrifos with human liver microsomes and cytosol did not inhibit tPMT hydrolysis. However, inclusion of an NADPH regenerating system with human liver microsomes which were incubated with chlorpyrifos did lead to inhibitory effects (data not shown).

The irreversibility of CPO inhibition of tPMT hydrolysis is illustrated in Figure 3A in which no hydrolysis products were produced from tPMT by up to 30 μg of microsomal protein in the presence of 80 nM CPO. In contrast, incubations of 10 μM carbaryl with decreasing concentrations of microsomal protein failed to completely inhibit tPMT hydrolysis, indicating that carbaryl acts as a reversible non-competitive inhibitor (Fig. 3B).

DISCUSSION

Since *trans*-permethrin is much more actively metabolized in human liver fractions than *cis*-permethrin /3/, this study focused on the effect of esterase inhibitors on tPMT hydrolysis. Our previous study demonstrated that the initial detoxification step for tPMT in human liver fractions, namely hydrolysis of the parent compound, is not mediated by CYPs /3/. Based on the nature of the chemical reaction and earlier animal studies it was assumed that the hydrolysis process was mediated by esterases.

Esterases are commonly categorized based on their sensitivity to inhibition by organophosphorus compounds /16/. A-esterases hydrolyze organophosphate compounds; B-esterases are inhibited by them; and C-esterases neither hydrolyze nor are they inhibited by these compounds. Chlorpyrifos, an organophosphorus insecticide, inhibits many esterases after CYP-mediated metabolic activation to CPO.

TABLE 2

Inhibition of *trans*-permethrin hydrolysis in human liver fractions by carbaryl

	V_{max} (nmole/mg/min)	95% CI V_{max}	K_m (μM)	95% CI (μM)	K_i (nM)	95% CI (nM)
Cytosol	0.51	0.49-0.52	5.54	4.72-6.37	NA	NA
Cytosol w/carbaryl (10 μM)	0.17	0.17-0.18	5.91	5.2-6.63	0.51	0.34-0.69
Microsomes	4.21	3.79-4.63	11.57	7.11-16.02	NA	NA
Microsomes w/carbaryl (10 μM)	1.57	1.52-1.63	9.79	8.31-11.27	5.68	5.20-6.16

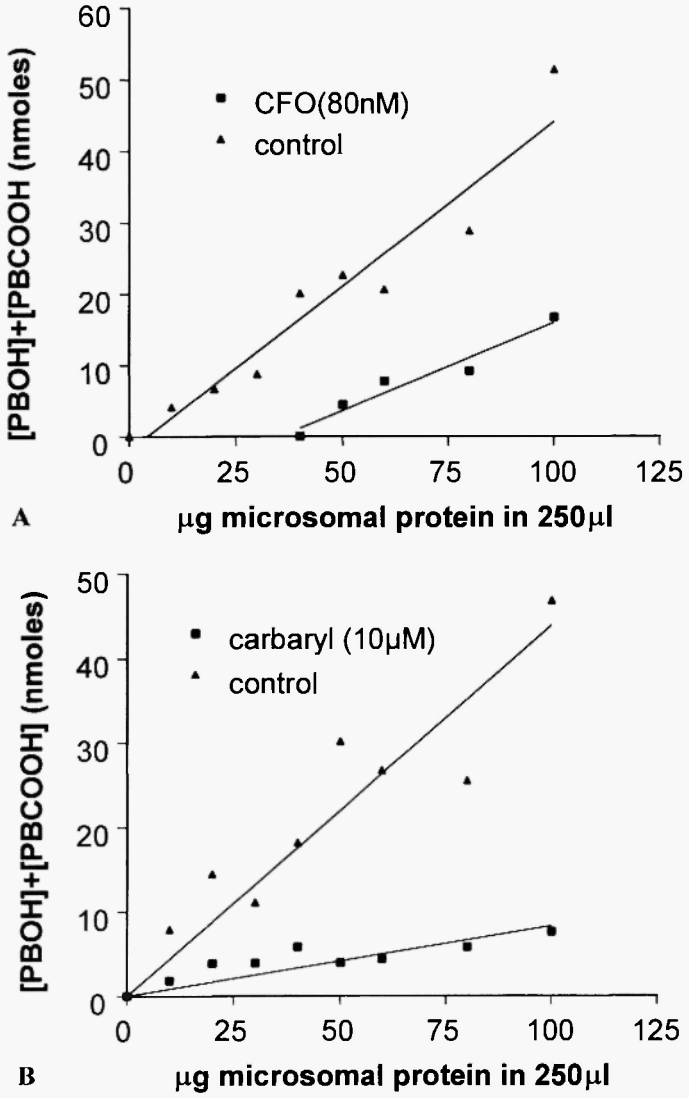

Fig. 3: Non-competitive vs irreversible inhibition by chlorpyrifos oxon (**A**) or by carbaryl (**B**).

CPO completely inhibited tPMT hydrolysis with very low K_i values in both human liver microsomal and cytosolic fractions, indicating involvement of B-esterases in tPMT hydrolysis. In comparison with CPO, the parent compound, chlorpyrifos, and the other major

chlorpyrifos metabolite (3,5,6-trichloro-2-pyridinol) showed minimal levels of inhibition in either fraction. The observation that pre-incubation of chlorpyrifos with an NADPH regenerating system in microsomes substantially increased tPMT inhibition confirmed that CPO is the chemical species responsible for the inhibition of tPMT hydrolysis. These findings are in agreement with previous studies which demonstrate that metabolic bioactivation of organophosphates can increase their anticholinesterase potency by as much as three orders of magnitude /17/.

The proposed mechanism for the inhibition of acetylcholinesterase by CPO is *trans*-esterification, resulting in the formation of a strong covalent bond between the oxon and a serine residue in the esterase active site. For physiological substrates, such as acetylcholine, a transient bond which is readily cleaved by deacylation is formed in place of the covalent bond /17/. The observed inhibition kinetics with respect to chlorpyrifos oxon inhibiton of permethrin hydrolysis (reduced V_{max} and K_m) and the irreversible nature of inhibition strongly imply that the inhibition of human liver esterases hydrolyzing tPMT is mediated by the same mechanism.

The discovery that CPO was inhibitory to permethrin hydrolysis led to the inclusion of carbaryl in the study. Carbaryl shows a different pattern of inhibition from CPO, namely, typical non-competitive inhibition. This result is in accord with the fact that carbamate compounds are reversible and less persistent inhibitors compared to organophosphorus compounds /14/. This also explains why the K_i values for carbaryl are two orders of magnitude less potent than those for CPO.

Another important observation is that, in contrast to CPO, carbaryl does not completely inhibit tPMT hydrolysis even at high concentrations. Incomplete inhibition at high concentrations of carbaryl suggests that there are multiple hydrolytic enzymes involved in tPMT hydrolysis in human liver fractions, with at least two species (or groups) of B-esterases involved, one with higher sensitivity to carbaryl inhibition and the other with lower or no sensitivity to carbaryl, but with both being sensitive to inhibition by CPO.

It is of some interest that high concentrations of DEET were capable of significant inhibition of tPMT hydrolysis. There is no evidence that DEET is metabolized by esterases; however, DEET is metabolized by several cytochrome P450 isoforms that have been

identified in humans /6/. Interestingly, preincubation of human CYP2B6 with chlorpyrifos, but not chlorpyrifos oxon, completely inhibited DEET metabolism to its primary metabolite /6/. Pyridostigmine bromide, another deployment-related chemical tested, did not inhibit tPMT hydrolysis, regardless of the presence or absence of an NADPH regenerating system.

In conclusion, we report that hydrolysis of tPMT in human liver fractions is strongly inhibited by CPO and to a lesser extent by carbaryl. The differential inhibition patterns of CPO and carbaryl indicates that multiple B-esterases are probably involved in the hydrolysis of tPMT in human liver fractions. This study suggests that the potential for metabolic interactions between pyrethroids and organophosphorus chemicals should be considered when safety is assessed in situations in which humans are likely to be exposed to members of both chemical classes.

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