METABOLISM OF CHLORPYRIFOS BY HUMAN CYTOCHROME P450 ISOFORMS AND HUMAN, MOUSE, AND RAT LIVER MICROSONES

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ABSTRACT:

One of the factors determining the toxicity of chlorpyrifos (CPS), an organophosphorus (OP) insecticide, is its biotransformation. CPS can be activated by cytochrome P450 (CYP) through a desulfuration reaction to form chlorpyrifos-oxon (CPO), a potent anticholinesterase. CPS can also be detoxified by CYP through a dearylation reaction. Using pooled human liver microsomes (HLM), a $K_{\text{m app}}$ of 30.2 μM and $V_{\text{max app}}$ of 0.4 nmol/min/mg of protein was obtained for desulfuration, and a $K_{\text{m app}}$ of 14.2 μM and a $V_{\text{max app}}$ of 0.7 nmol/min/mg of protein was obtained for dearylation. These activities are lower than those obtained from rat liver microsomes. Gender differences in humans were also observed with female HLM possessing greater activity than male HLM. Use of human CYP isoforms expressed in human lymphoblastoma cells demonstrated that CYP1A2, 2B6, 2C9*1, 2C19, and 3A4 are involved in CPS metabolism. CYP2B6 has the highest desulfuration activity, whereas dearylation activity is highest for 2C19. CYP3A4 has high activity for both dearylation and desulfuration. The use of pheno-typed individual HLM demonstrated that predictions of metabolic activation and/or detoxication could be made based on relative amounts of CYP2B6, 2C19, and 3A4 in the microsomes. Thus, individuals with high CYP2C19 but low 3A4 and 2B6 are more active in dearylation than in desulfuration. Similarly, individuals possessing high levels of CYP2B6 and 3A4 have the greatest potential to form the activation product. These differences between individuals suggest that differential sensitivities to CPS may exist in the human population.

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Chlorpyrifos [O,O-diethyl-O-(3,5,6-trichloro-2-pyridinyl)-phosphorothioate] (CPS) is a widely used organophosphorus (OP) insecticide. It has numerous agricultural applications and, until recently, has been used for termite control in foundations and for the control of nuisance insects and disease vectors in homes and during military deployments. The extensive use of CPS inevitably results in human exposure and has the potential to cause toxic effects.

The in vivo toxicity of CPS is a result of its bioactivation by cytochrome P450 (CYP)-mediated monooxygenases to a more potent cholinesterase inhibitor, chlorpyrifos-oxon (CPO). This oxidation reaction, which proceeds through a possible phosphooxythiiran intermediate, can result in either a desulfuration reaction that generates the oxon or a dearylation reaction that degrades the parent compound (Chambers, 1992) (Fig. 1).

Studies of parathion, a related organophosphate, have shown that parathion oxidation is catalyzed by human CYP1A2, 2B6, and 3A4 and that its oxidation is highly correlated to CYP3A4 activity in human liver microsomes (HLM) (Butler and Murray, 1997; Mutch et al., 1998).

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Abbreviations used are: CPS, chlorpyrifos; CPO, chlorpyrifos-oxon; TCP, 3,5,6-trichloro-2-pyridinol; OP, organophosphorus; HLM, human liver microsomes; RLM, rat liver microsomes; MLM, mouse liver microsomes; CYP, cytochrome P450; HPLC, high-performance liquid chromatography.

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FIG. 1. Cytochrome P450-dependent metabolism of phosphorothioates illustrated with chlorpyrifos.
Dearylation and desulfuration activities toward chlorpyrifos in HLM, RLM, or MLM

Activities are expressed as mean ± S.E.M. (n = 3 determinations), means in RLM (pooled from three male rats) and MLM (pooled from three male mice) significantly different than HLM (pooled from 10 donors) are indicated by *p < 0.05 or **p < 0.01.

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<th>Desulfuration</th>
<th>Dearylation</th>
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<td></td>
<td>μM</td>
<td>Vmax/app</td>
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<tr>
<td>HLM</td>
<td>30.2 ± 1.7</td>
<td>0.4 ± 0.1</td>
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<tr>
<td>RLM</td>
<td>6.1 ± 1.1**</td>
<td>1.0 ± 0.2*</td>
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<td>MLM</td>
<td>24.4 ± 2.4</td>
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Other studies using specific chemical inhibitors for human CYP isoforms demonstrated that both CYP2D6 and CYP3A4 were active in the desulfuration of parathion, CPS, and diazinon (Sams et al., 2000).

The present study was designed to 1) determine oxidation activities toward CPS in human, mouse, and rat liver microsomes in the same assay system, 2) identify the human CYP isoforms and CYP polymorphic forms responsible for CPS oxidation, and 3) examine the differences in CPS oxidation activities among liver microsomes from selected individual humans.

Materials and Methods

Chemicals. CPS, CPO, and 3,5,6-trichloro-2-pyridinol (TCP) were purchased from ChemService (West Chester, PA). HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals, if not specified, were purchased from Sigma (St. Louis, MO).

Rodent Liver Microsome Preparation. Rat liver microsomes (RLM) and mouse liver microsomes (MLM) were prepared from adult male Long-Evans rats and adult male CD-1 mice (Charles River Laboratories, Raleigh, NC), respectively, according to the method of Cook and Hodgson (1983). Briefly, immediately after sacrificing the animals, the fresh livers were removed, weighed, minced, and then homogenized with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) in 50 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and 1.15% potassium chloride. The homogenate was centrifuged at 10,000g for 15 min. The supernatant was filtered through glass wool and centrifuged at 100,000g for 1 h. The pellet was resuspended in 50 mM potassium phosphate buffer (pH 7.5) containing 0.1 mM EDTA and 0.25 M sucrose. All processes were performed at 0 to 4°C. The micromosomal preparation was aliquoted and stored at −80°C until use. Protein concentration was determined using a BCA kit (Pierce, Rockford, IL).

Human Liver Microsomes and Human Cytochrome P450 Isoforms.

Pooled HLM (pooled from 10 donors), individual HLM, and human lymphoblast-expressed CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9*1 (Arg144→Ile150), 2C9*2 (Cys99→Ser), 2C19, 2D6*1 (2D6-Val), 2E1, 3A4, and 4A11 were purchased from XenoTech, LLC (Kansas City, KS). Different mutant alleles of human CYP2C19 were expressed in Escherichia coli, according to the method of Luo et al. (1998). NADPH-CYP reductase was obtained from Oxford Biomedical Sciences (Oxford, MI).

In Vitro Chlorpyrifos Metabolism. Enzyme kinetic assays for microsomes were performed by incubation of serial concentrations of CPS (final concentration range, 2–100 μM) with microsomes in 100 mM Tris-HCl buffer (pH 7.4 at 37°C) containing 5 mM MgCl2 and 3 mM EDTA for 5 min. The micromosomal protein concentrations used in assays were 1.5 mg/ml for HLM, 0.5 mg/ml for RLM, and 1 mg/ml for MLM. After preincubation at 37°C for 3 min, reactions were started by the addition of an NADPH-generating system (0.25 mM NADP, 2.5 mM glucose-6-phosphate, and 2 U/ml glucose-6-phosphate dehydrogenase). The controls were identical except for the absence of an NADPH-generating system. Reactions were terminated by adding an equal volume of ice-cold methanol and vortexing. After 5 min of centrifugation at 15,000 rpm in a microcentrifuge, the supernatants were analyzed for CPO and TCP concentrations by HPLC.

Metabolic activity assays for human lymphoblast-expressed CYP isoforms were performed by incubation of CPS (final concentration, 100 μM) with CYP isoforms (final protein concentration, 0.9 mg/ml; final P450 contents, 23.4–180 pmol/ml) for 20 min in CYP-specific buffers recommended by the supplier (GENTEST). For CYP1A1, 1A2, 2D6, and 3A4, 100 mM potassium phosphate buffer with 3.3 mM MgCl2 (pH 7.4) was used. For CYP2B6, 2C8, 2C19, and 2E1, 50 mM potassium phosphate buffer with 3.3 mM MgCl2 (pH 7.4) was used. For CYP2C9*1, 2C9*2, and 4A11, the buffer was 100 mM Tris-HCl buffer with 3.3 mM MgCl2 (pH 7.5), whereas for CYP2A6, 50 mM Tris-HCl buffer with 3.3 mM MgCl2 (pH 7.4) was used.

The metabolic activity assays for E. coli-expressed human CYP2C19s were performed according to the method of Klose et al. (1998). Briefly, l-α-phosphatidylcholine (0.3 μg/pmol of P450), CYP reductase (4 pmol/pmol of P450), and P4C2C19 (24 pmol) were combined and preincubated at 37°C for 5 min. This mixture then was incubated with 100 μM CPS in 50 mM potassium phosphate buffer with 3.3 mM MgCl2 (pH 7.4) for 10 min. The reaction was initiated with NADPH-generating system as described previously.

Assays of individual HLM (final protein concentration, 1.5 mg/ml) with CPS (final concentration, 100 μM) were described previously.

Analysis of Metabolites by HPLC. The HPLC system used in this study consisted of two Shimadzu (Kyoto, Japan) pumps (LC-10AT), a Shimadzu auto injector (SIL-10AD VP), and a Waters 486 tunable absorbance detector (Milford, MA). The mobile phase for pump A was 10% acetonitrile, 89% water, and 1% phosphoric acid, whereas for pump B was 99% acetonitrile and 1% phosphoric acid. A gradient system was initiated at 20% pump B and increased to 100% pump B in 20 min. The flow rate was 1 ml/min. Metabolites were separated by a C18 column (Luna 5 μ, 150 × 3 mm; Phenomenex, Rancho Palos Verdes, CA) and detected at 230 nm. Using this system, the retention times obtained for TCP, CPO, and CPS were 8.5, 12, and 17 min, respectively. The limits of detection for TCP and CPO were 0.03 and 0.04 μM, respectively, at an injection volume of 15 μl. Concentrations of metabolites were obtained by extrapolation of peak height from a standard curve. Kinetic parameters were obtained using a Hanes-Woolf plot (Segel, 1975).

Results

The protein concentrations and incubation times used in the assays were within linear ranges determined in preliminary experiments. No metabolites were detected when incubations were carried out in the absence of an NADPH-generating system.

HLM displayed lower affinity (i.e., higher Km) and lower reaction velocity toward CPS for both desulfuration and dearylation than RLM (Table 1). Compared with HLM, MLM exhibited similar affinities but a higher reaction velocity toward CPS (Table 1). Both RLM and MLM have higher values of clearance terms (Vmax/Km) than HLM. Pooled female HLM showed significantly higher metabolic activity toward CPS than pooled male HLM (Table 2).

A screen of several human CYP isoforms demonstrated that CYP1A2, 2B6, 2C9*1, 2C19, and 3A4 were involved in CPS metabolism (Table 3), whereas no oxidation activity toward CPS was detected using CYP1A1, 2A6, 2C8, 2C9*2, 2D6, 2E1, and 4A11. Desulfuration and dearylation activities were greatest for CYP2B6.
and CYP2C19, respectively. Marked decreases in metabolic activity toward CPS were observed with different polymorphic alleles of CYP2C19 (Table 4).

To determine the variation range of CPS metabolism between individuals, we examined CPS metabolism from five individuals representing contrasting activities of some important CYP isoforms (Table 5). Individuals with high levels of CYP2B6 and 3A4 (HG042 and 112) had high-desulfuration activity; individuals with low levels of CYP2B6 and 3A4 (HG006, 2043, and 434) had low-desulfuration activity. The dearylation pathway was more predominant in the individual (HG043) with high-CYP2C19 but low-3A4 levels. No particular change in either metabolite was observed in the individual (HG023) with high levels of CYP2D6.

**Discussion**

The metabolic intrinsic clearance rates ($V_{max}/K_m$) indicate that liver microsomes from all three species more readily produce a detoxication product (i.e., TCP) than an activation product (i.e., CPO). These observations are similar to previous reports on rodents (Sultatos and Murphy, 1983; Ma and Chambers, 1995). The clearance rates also demonstrate that pooled HLM are less active than RLM and MLM in both desulfuration and dearylation, suggesting that less TCP and CPO are generated in the human liver than in the rodent liver immediately after exposure to CPS.

Consistent with data on other substrates provided by the supplier (XenoTech, LLC) regarding gender differences in CYP activity, pooled female HLM showed higher activities in both desulfuration and dearylation of CPS than pooled male HLM. Note that this gender difference was demonstrated using only one pool of 10 males and 10 females, respectively. It is not known whether this difference would also be true in a larger population. These data contrast with CYP activities in rats because males are more active in CPS desulfuration than females (Chambers and Chambers, 1989; Sultatos, 1991).

Our results show that human lymphoblast-expressed CYP1A2, 2B6, 2C9*1, 2C19, and 3A4 are responsible for both dearylation and desulfuration of CPS, whereas CYP1A1, 2A6, 2C8, 2C9*2, 2D6*1, 2E1, and 4A11 did not display detectable activities toward CPS oxidation. These results are similar to those of a parahion metabolism study (Butler and Murray, 1997), where CYP1A2, 2B6, and 3A4 were shown to have high-desulfuration activities toward parathion. CYP2B6 more readily generates the oxon, similar to phenobarbital-induced CYP2B isoforms in rodents (Fabrizi et al., 1999; Levi et al., 1988). CYP2C19 exhibits the greatest dearylation activity and relatively low-desulfuration activity. Genetic polymorphisms have been identified in CYP2C19 (Demoraiz et al., 1994), and differential metabolic activities toward CPS by different variants of CYP2C19 were observed in this study. Dearylation by the polymorphic CYP2C19 alleles was significantly less than that of the wild-type forms, which could influence the in vivo toxicity of CPS in individuals possessing these alleles.

CYP3A4 is also a highly active, although not the most active, isoform in CPS metabolism. The fact that CYP3A4 is the most abundant CYP isoform in human liver (Shimada et al., 1994) suggests that this isoform plays a significant role in vivo metabolism of the CYP2C9*1 (Arg144) showed some activities in both desulfuration and dearylation of CPS, whereas CYP2C9*2 (Cys144), a single amino acid difference, showed no detectable activity toward CPS. CYP2C9*1 and 1A2, although not the most active isoforms for either desulfuration or dearylation, may play a role in vivo. A previous study using specific chemical inhibitors and human lymphoblastoid cell-expressed CYP2D6 suggested that CYP2D6 plays a significant role in desulfuration of parathion, CPS, and diazinon (Sams et al., 2000). Our work with CPS did not reveal any metabolites using human lymphoblast-expressed CYP2D6, nor did HLM with high-CYP2D6 activity (HG023) produce a significant amount of the oxon metabolite.

Potential differences in the human population with respect to CPS metabolism were further examined using different individual HLM.
Individuals with varying levels of CYP2B6, 2C19, 2D6, and 3A4 were selected to represent contrasting levels of predicted metabolic activity. Thus, individuals (HG042 and HG112) possessing high levels of CYP2B6 and CYP3A4 would be expected to possess greater ability to form the desulfuration product than those (HG006, HG023, and HG043) with lower levels of these isoforms, as observed (Table 5). Similarly, individuals with greater levels of CYP2C19 or CYP3A4, such as HG042, HG043, and HG112, would also be expected to produce more of the dearylation product than those with significantly lower levels of these isoforms (HG006 and HG023). Because individuals with contrasting levels of CYP2B6 and 3A4 were not available, we were unable to differentiate the extent of their contribution to desulfuration separately. However, based on its content in human liver, CYP3A4 should contribute significantly to both dearylation and desulfuration, as has been previously observed (Butler and Murray, 1997; Mutch et al., 1999; Sams et al., 2000).

Using our selection of five individuals, the variations between individual HLM in desulfuration of CPS were around 8-fold and that for dearylation was 3-fold and would presumably be greater if more samples were examined. For parathion, the difference between individual HLM in dearylation and desulfuration is as great as 10- and 16-fold, respectively (Butler and Murray, 1997; Mutch et al., 1999). Considerations of metabolic differences between individuals should also consider the contributions of esterases, which are also major factors determining the in vivo toxicities of OP compounds (Maxwell et al., 1987; Chambers et al., 1990; Costa et al., 1990).

In conclusion, HLM use the same pathways as RLM and MLM to metabolize CPS, although activities were generally lower in humans than in rodents. From pools of 10 individuals, female HLM displayed a higher activity in CPS metabolism than male HLM. Human lymophoblast-expressed CYP1A2, 2B6, 2C9*1, 2C19, and 3A4 showed oxidation activities toward CPS, whereas no activities were detected for CYP1A1, 2A6, 2C8, 2C9*2, 2D6, 2E1, and 4A11. Although CYP2C19 and 2B6 displayed the greatest dearylation and desulfuration activities, respectively, 3A4 was highly active in both reactions. Activities of CYP2B6, 2C19, and 3A4 greatly affect CPS metabolism in HLM.

References


Diego, CA.


