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Enzymes and Inhibitors in Neonicotinoid Insecticide Metabolism

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Neonicotinoid insecticide metabolism involves considerable substrate specificity and regioselectivity of the relevant CYP450, aldehyde oxidase, and phase II enzymes. Human CYP450 recombinant enzymes carry out the following conversions: CYP3A4, 2C19, and 2B6 for thiamethoxam (TMX) to clothianidin (CLO); 3A4, 2C19, and 2A6 for CLO to desmethyl-CLO; 2C19 for TMX to desmethyl-TMX. Human liver aldehyde oxidase reduces the nitro substituent of CLO to nitroso much more rapidly than it does that of TMX. Imidacloprid (IMI), CLO, and several of their metabolites do not give detectable *N*-glucuronides but 5-hydroxy-IMI, 4,5-diol-IMI, and 4-hydroxythiacloprid are converted to *O*-glucuronides in vitro with mouse liver microsomes and UDP-glucuronic acid or in vivo in mice. Mouse liver cytosol with *S*-adenosylmethionine converts desmethyl-CLO to CLO but not desmethyl-TMX to TMX. Two organophosphorus CYP450 inhibitors partially block IMI, thiacloprid, and CLO metabolism in vivo in mice, elevating brain and liver levels of the parent compounds while reducing amounts of the hydroxylated metabolites.

KEYWORDS: Aldehyde oxidase; clothianidin; CYP450; glucuronide; imidacloprid; methylation; neonicotinoid insecticide; thiacloprid; thiamethoxam

INTRODUCTION

The safe and effective use of pesticides requires an understanding of the products and mechanisms of their metabolism. This study considers thiamethoxam (TMX), clothianidin (CLO), imidacloprid (IMI), and thiacloprid (THI) (**Figure 1**), which are four of the seven commercial neonicotinoids, the newest major class of insecticides (*1*−*3*). Many of their in vivo metabolites are identified in mice, rats, goats, and hens (see refs *4* and *5* and references cited therein) as models or predictors for humans. The extrapolation of these findings to people is improved by understanding the fate of the compounds in human enzyme systems and the molecular mechanisms involved (*6*).

Two of the neonicotinoids, TMX and CLO, have a special precursor−product relationship (**Figure 2**) relevant to their mechanisms of insecticidal and carcinogenic activity. Some TMX is converted to CLO in insects and plants, leading to the suggestion that TMX is a proinsecticide (*7*, *8*). TMX is classified as a likely human carcinogen on the basis of the increased incidence of hepatocellular adenomas and carcinomas with male and female mice (but not rats) in chronic feeding studies (*9*). In contrast, there is no evidence of carcinogenicity for CLO (*10*). The hepatocarcinogenicity of TMX in mice is attributed to the metabolites desmethyl-TMX (TMX-dm) and desmethyl-CLO (CLO-dm) by a mechanism that is arguably not as relevant in rats or humans (*11*, *12*). CYP450 enzymes play a major role in pesticide metabolism as seen in studies in vivo and with specific recombinant enzymes (*13*). CYP3A4 is the most abundant P450 in humans with a dominant role in xenobiotic metabolism (*14*, *15*). It is therefore important to define differences in TMX and CLO metabolism in human liver with recombinant CYP450 enzymes. In addition, CLO (but not TMX) is also metabolized by aldehyde oxidase (AOX) as established with rabbit liver cytosol (*16*). The present study uses human recombinant CYP450 enzymes to study TMX conversion to CLO and TMX-dm and CLO metabolism to CLO-dm. It further examines human liver AOX to consider possible nitro reduction reactions of TMX and CLO.

Phase II metabolism involves glucuronidation and methylation among other reactions. No glucuronidation has been reported for the parent compounds or metabolites active as nicotinic agonists. N-Methylation of CLO-dm but not TMX-dm has been observed in mice (*5*). Accordingly, the substrate specificity of glucuronidation and methylation reactions of neonicotinoid metabolites were examined in vitro using human liver microsomes (HLM) or mouse liver microsomes (MLM) and cytosol and in vivo in mice.

The effectiveness of most organic insecticides is increased or synergized by CYP450 inhibitors (*17*). Two organophosphorus (OP) compounds are of particular interest, one for potency and the other for simple structure. *O*-(*n*-Propyl) *O*-(2-propynyl) phenylphosphate (PPP) is a very potent neonicotinoid synergist in houseflies (*18*) and [for the *O*-(2-methylpropyl) analogue] CYP450 inhibitor in mice (*19*). The other OP synergist *O*,*O*-diethyl *O*-phenyl phosphorothionate (also known as SV_1 or dietholate) (*20*, *21*) possibly forms a hydrodisulfide at the CYP450 catalytic site on substrate desulfuration (*22*). The effects

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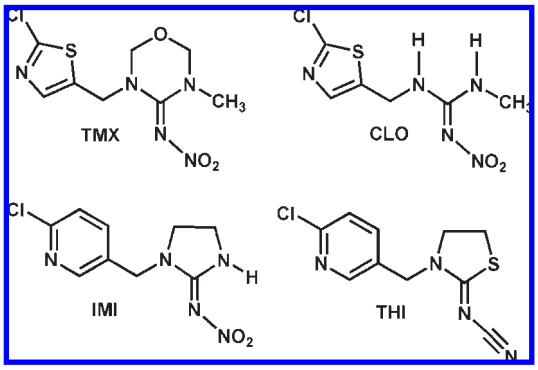

Figure 1. Structures of thiamethoxam (TMX), clothianidin (CLO), imidacloprid (IMI), and thiacloprid (THI).

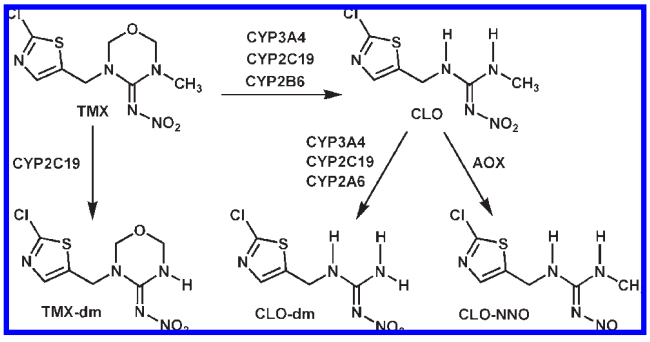

Figure 2. Precursor−product relationships and CYP450 regioselectivity in metabolism of TMX and CLO.

of PPP and SV_1 were therefore examined on the tissue levels and metabolism of IMI, THI, and CLO in mice to elucidate the role of CYP450 synergists in governing persistence and toxicity.

On a general basis, this study considers the enzymes and inhibitors in neonicotinoid insecticide metabolism. More specifically, it defines the regioselectivity of human recombinant CYP450 enzyme oxidations of TMX and CLO, examines the involvement of phase II glucuronidation and methylation in neonicotinoid metabolism, and establishes the OP-synergist sensitive in vivo reactions in mice.

MATERIALS AND METHODS

Chemicals. The neonicotinoids and metabolites were available from our earlier studies (*4*, *5*, *16*, *23*, *24*). PPP synthesized as described (*25*) was purified by column chromatography [silica gel, methylene chloride/ethyl acetate (2:1)]. SV_1 prepared by coupling *O*,*O*-diethyl phosphorochloridothioate with phenol (*26*) was purified on a silica gel column [hexane/ethyl acetate (4:1)]. All other chemicals were obtained from Sigma-Aldrich (Milwaukee, WI).

Metabolic Phenotyping Using Recombinant CYP450 Enzymes. Human recombinant CYP450 Supersomes (1A2, 2A6, 2B6, 2C9, 2C19, 2D6*1, 2E1, and 3A4) coexpressed with P450 reductase and (except for CYP1A2 and CYP2D6*1) cytochrome b_5 were obtained from BD Gentest (Woburn, MA). Supersomes expressing only P450 reductase and cytochrome b_5 were used as a negative control. TMX or CLO (132 μM) was incubated with each Supersome (15 pmol) and NADPH (1 mM) in 250 μL of Tris-HCl buffer (50 mM, pH 7.4 for CYPs 2A6 and 2C9) or potassium phosphate buffer (100 mM, pH 7.4, for the other enzymes) for 1 h at 25 °C. Samples were then extracted with an equal volume of ethyl acetate as previously described (*24*), evaporated to dryness, and resuspended in acetonitrile/water (50:50). LC-MS/MS was then used to measure the formation of CLO and TMX-dm from TMX and that of CLO-dm from CLO. A 100 mm × 2 mm i.d., 3 μm, Luna C8(2) column was used for both compounds with 0.1% formic acid in water (mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 0.2 mL/min. TMX-dm and CLO were resolved using a 17% B isocratic method, whereas CLO-dm was analyzed at 25% B, in each case with IMI as the internal standard. Samples were analyzed using selective ion monitoring on a Finnigan TSQ 700 electrospray mass spectrometer (Thermo Finnigan, San Jose, CA) operated in the positive mode with the following settings: collision gas, 2.3 torr; voltage, 1700 V; collision energies, 25, 30, and 20 eV for TMX-dm, CLO, and CLO-dm, respectively. TMX-dm was detected using the m/z 319 → 132 transition, whereas 291 → 169 and 277 → 155 transitions were used for CLO and CLO-dm, respectively.

Measurement of Enzyme Kinetics. Human recombinant CYP450 Supersomes were used to measure the kinetic parameters for conversion of TMX to TMX-dm and CLO and that of CLO to CLO-dm. Only the enzymes identified in the phenotyping study were tested, and except for varying substrate concentrations, incubation conditions were identical. Metabolites were quantified as previously described (*16*).

Metabolism by Human Liver CYP450 Enzymes and AOX. Reaction mixtures of pooled HLM (BD Gentest) (1 mg of protein/mL), neonicotinoid (200 μM), and NADPH (1 mM) in phosphate buffer (100 mM, pH 7.4, 200 μL final volume) were incubated for 1 h at 37 °C prior to extraction and analysis as described (*16*). AOX activity was assayed as before (*16*, *24*) using 5 mg of protein/mL human liver cytosol instead of rabbit liver cytosol.

Glucuronide Metabolites. In vitro investigations used described methods for assay of UDP-glucuronosyltransferase activity (*27*, *28*) adapted for the present conditions. The reaction mixture consisting of MLM (2.5 mg of protein) or pooled HLM (1 mg of protein), substrate (2 mM), UDP-glucuronic acid (UDPGlcUA) (5 mM), $MgCl_2$ (10 mM), alamethicin (20 μg), and 1,4-saccharolactone (8.5 mM) in Tris buffer (50 mM, pH 8.9, 100 μL) was incubated for 120 min at 37 °C in a shaking water bath. As controls, samples were assayed in the absence of UDPGlcUA. *p*-Nitrophenol glucuronidation was used as a standard. The reaction was stopped by cooling on ice and adding ice-chilled acetonitrile (100 μL). The resulting mixture was centrifuged at 10000*g* for 15 min. The supernatant was passed through a 0.45 μm nylon membrane in an Acrodisk syringe filter (13 mm) before analysis by LC-MS. The Luna C18 column was used with a precolumn filter, an injection volume of 30 μL, and a flow rate of 0.8 mL/min. The mobile phase gradient between acetonitrile containing 0.1% formic acid and water began with 5% acetonitrile for 2 min and then steadily increased to 80% acetonitrile over a period of 30 min, finishing with an additional 10 min of 80% acetonitrile.

For in vivo studies, mice were treated intraperitoneally (ip) with the test compound at 10 mg/kg, and after 2 h the liver, kidney, and brain were used for analysis as above. Alternatively, urine was collected (*4*, *5*) for 6 h after injection of IMI or 6-chloropyridinyl-3-carboxylic acid (CPCOOH) at 10 mg/kg. A mixture of 100 μL of urine and 100 μL of ice-chilled acetonitrile was vortexed and centrifuged at 10000*g* for 15 min. Filtration of the supernatant through a 0.45 μm nylon membrane was followed by LC-MS analysis as before (*4*, *5*, *8*). The tentative assignment of glucuronide metabolites was based on three criteria: mass of the parent plus 176 with a ^{35}Cl:^{37}Cl isotope ratio of 3:1; detection in enzyme incubations in the presence but not in the absence of added UDPGlcUA; t_R value the same for in vitro and in vivo studies. Attempted synthesis of the *N*-glucuronides of IMI and IMI-NH yielded products with LC-MS peaks of appropriate mass.

Methylated Metabolites. A system based on those of Seifert et al. (*29*) and Maurer et al. (*30*) was used to examine possible methylation of CLO-dm and TMX-dm by freshly prepared mouse liver cytosol. The final incubation mixture in 300 μL of 50 mM Tris buffer (pH 7.4) consisted of liver cytosol (1 mg of protein) as the enzyme source, CLO-dm or TMX-dm (0.5 mM) as the substrate, *S*-adenosylmethionine (AdoMet) *p*-toluenesulfonate salt (4 mM) as the methyl donor, and $MgCl_2$ (1 mM) as the activator. The mixture was incubated for 120 min at 37 °C and then filtered for LC-MS analysis as above using synthetic standards for comparison (*4*, *5*, *8*).

Effects of OP Synergists on Neonicotinoid Metabolism in Mice. Male albino Swiss−Webster mice (25−30 g) (*4*, *5*) were pretreated with PPP (150 mg/kg) or SV_1 (300 mg/kg) by ip administration with Me_2SO as the carrier vehicle (1 μL/g of mouse weight). After 1 h, IMI, THI, or CLO in Me_2SO was administrated ip at either 10 mg/kg (IMI and THI) or 20 mg/kg (CLO). Using isoflurane for anesthesia, whole fresh brain or liver was sampled 60 min after treatment and placed in 5 mL of acetonitrile

Table 1. Regioselectivity of Human Recombinant CYP450 Enzymes in Metabolism of Thiamethoxam to Clothianidin and Desmethylthiamethoxam and of Clothianidin to Desmethylclothianidin

	pmol of metabolite/pmol of P450/h[b] (mean ± SD, n = 3)		
	TMX metabolism		CLO metabolism
CYP450[a]	CLO	TMX-dm	CLO-dm
1A2	ND[c]	ND[c]	ND[c]
2A6	ND	ND	3.4 ± 0.2
2B6	0.66 ± 0.15	ND	ND
2C9	ND	ND	ND
2C19	14 ± 1	3.3 ± 0.7	4.1 ± 0.2
2D6*1	ND	ND	ND
2E1	ND	ND	ND
3A4	89 ± 26	ND	6.9 ± 0.7
reductase	ND	ND	ND

[a] Coexpressed with CYP450 reductase and (except for CYP1A2 and CYP2D6*1) with cytochrome b_5. [b] Incubation with 15 pmol of P450 for 1 h. [c] Not detected at a discriminating level of 8 pmol for CLO, 2 pmol for TMX-dm, and 23 pmol for CLO-dm.

Table 2. Kinetics of Thiamethoxam and Clothianidin Metabolism by Human Recombinant CYP450 Enzymes

CYP450	substrate	product	V_{max}(pmol/min/pmol of P450)	K_m (μM)	Cl_{int}(μL/min/pmol of P450)
2A6	CLO	CLO-dm	1.0	4200	2.5×10^{-4}
2C19	TMX	CLO[a]	0.73	270	27×10^{-4}
	TMX	TMX-dm	0.17	210	8.0×10^{-4}
	CLO	CLO-dm	0.71	3400	2.1×10^{-4}
3A4	TMX	CLO[a]	13	4500	29×10^{-4}
	CLO	CLO-dm	5.1	2700	19×10^{-4}

[a] A portion of the CLO formed is further metabolized to CLO-dm.

containing 250 mg of sodium chloride. A homogenate was prepared with a sonic dismembrator (Fisher Scientific, Pittsburgh, PA) followed by mixing with a vortex. The acetonitrile extract, recovered after centrifugation at 2000*g* for 15 min, was reduced to dryness using a Savant SVC 200H centrifugal evaporator (Farmingdale, NY). Acetonitrile/water/trifluoroacetic acid (75:25:0.1) (300 μL) was added followed by sonication and filtration for LC-MS analysis (*4*, *5*). Tissue levels of metabolites are reported as equivalents based on the standard curves of the parent compounds.

RESULTS AND DISCUSSION

Human CYP450 Regioselectivity. Considerable regioselectivity is observed in human recombinant CYP450 oxidation of TMX and CLO. Eight human recombinant CYP450 enzymes were used to determine the identities of the isoforms responsible for conversion of TMX to TMX-dm and CLO and that of CLO to CLO-dm (**Table 1**; **Figure 2**). Only three of these enzymes were active with TMX, each forming CLO in an activity order of 3A4 > 2C19 > 2B6. 2C19 also converted TMX to TMX-dm. Demethylation of CLO was catalyzed by, in order of specific activity, 3A4 > 2C19 > 2A6. In terms of Cl_{int} (V_{max}/K_m), conversions of TMX to CLO by 3A4 and 2C19 were the major reactions with nearly equivalent magnitudes (**Table 2**). Interestingly, however, CYP3A4 demonstrated a very high V_{max} and seems to be the predominant enzyme with respect to CLO demethylation. Conversion of TMX to CLO by CYP2C19 appears to be favored over TMX demethylation by an almost 4-fold ratio. Given the importance of CYP3A4 and CYP2C19 in liver and intestine, they are probably the principal CYP450 enzymes in TMX and CLO metabolism.

An experiment with selective inhibitors supports the observation above with recombinant CYP450s that the principal human liver microsomal enzyme for TMX and CLO metabolism is CYP3A4. Thus, HLM were incubated with 200 μM TMX or CLO and 1.5 mM NADPH to determine the effect of inhibitors (*14*, *15*) on conversion to CLO or CLO-dm, respectively, the major reactions involved under these assay conditions. The diagnostic CYP3A4 and CYP2A6 inhibitors ketoconazole and tranylcypromine gave 31−50% inhibition at 10 μM with both substrates, whereas the less selective coumarin and orphenadrine gave little or no inhibition at 1000−3000 μM.

Table 3. Specific Activities of Thiamethoxam and Clothianidin Metabolism by Human Liver Microsomes and Cytosol

substrate	product	subcellular fraction[a]	specific activity (pmol/min/mg of protein) (mean ± SD, n = 3)
TMX	TMX-dm	microsomes	ND[b]
TMX	CLO[c]	microsomes	35 ± 2
TMX	TMX-NNO	cytosol	ND[d]
CLO	CLO-dm	microsomes	20 ± 0.4
CLO	CLO-NNO	cytosol	69 ± 7

[a] Microsomes (1.5 mg of protein/mL) with 1.5 mM NADPH and cytosol (5 mg of protein/mL) with 10 mM *N*-methylnicotinamide. [b] Not detected at a discriminating level of 0.1 pmol/min/mg of protein. [c] A portion of the CLO formed is further metabolized to CLO-dm. [d] No metabolism detected by LC-MS (*16*).

Earlier studies showed that mouse, rat, and human microsomes differ in their rates of TMX and CLO metabolism, with mouse microsomes much more active than rat or human microsomes in converting TMX to TMX-dm and CLO-dm (*12*), the two metabolites important in TMX-related mouse liver tumors (*12*, *31*). With IMI, CYP3A4 is selective for imidazolidine oxidation, whereas CYP1A2, CYP2B6, CYP2D6, and CYP2E1 are selective for nitro reduction (*32*). The relative importance of CYP450 and AOX in IMI reduction in vivo remains to be determined.

Metabolism of TMX and CLO by Human Liver Enzymes. Microsomes and cytosol with NADPH and *N*-methylnicotinamide, respectively, were examined for activity in terms of the demethylation and nitro reduction of TMX and CLO (**Table 3**). Specific activities under the assay conditions were lower with microsomes for TMX to CLO and CLO to CLO-dm than with cytosol for CLO to CLO-NNO. The rates of nitro reduction were compared for CLO and TMX with human AOX. Human liver cytosol converted CLO to CLO-NNO but not TMX to TMX-NNO (**Table 3**).

Possible Glucuronidation of Neonicotinoids and Metabolites. Several of the neonicotinoids and their metabolites have −NH or −OH functionalities (**Figures 3**−**5**) that might undergo glucuronidation in mice. Glucuronidation of any neonicotinoid or nicotinic agonist metabolite thereof would presumably be a detoxification process based on all available structure−activity information and binding site models. The LC-MS results failed to show *N*-glucuronide formation from IMI, CLO, CLO-dm, and $CPCH_2NH_2$ with MLM or HLM or in vivo in mice. However, *O*-glucuronides were formed by both MLM and HLM from IMI-5-OH, IMI-4,5-diol, THI-4-OH, $CPCH_2OH$, and CPCOOH and with some of the compounds in vivo in mice on the basis of urine analysis (**Table 4**). Glucuronides were not observed with MLM and UDPGlcUA for IMI, IMI-$NCONH_2$, IMI-ole, IMI-NNH_2, CLO, CLO-dm, $CPCH_2NH_2$, CTCOOH, or nicotine (see **Figure 5** for some of the structures). Furthermore, no *N*-glucuronide was observed with the HLM−UDPGlcUA system for IMI, IMI-NH, CLO-dm, and $CPCH_2NH_2$. In addition, *N*-glucuronides were not observed with mouse in vivo for IMI, CLO, CLO-dm, and TMX-dm. Nicotine and cotinine undergo N-glucuronidation with HLM but not MLM, pointing out the importance of interspecies hepatic variation (*27*, *28*).

Enzymatic Methylation of CLO-dm. Mouse liver cytosol (but not microsomes) with AdoMet and Mg^{2+} (but not without Mg^{2+}) converted CLO-dm (t_R 15.96 min) to about 5% CLO (t_R 17.06 min) with the appropriate increase in mass of 14 from m/z 235 to 249. CLO-dm also undergoes methylation to CLO in mice (*5*), thereby providing a potential cyclic situation in which CLO undergoes both CYP450 demethylation and enzyme-dependent remethylation, perhaps helping thereby to prolong the persistence. Surprisingly, TMX-dm was not converted to TMX under the same conditions. In a preliminary study, mouse brain and kidney cytosol could not be substituted for liver cytosol for conversion of CLO-dm to CLO.

In Vivo Effects of Two OP CYP450 Inhibitors on Neonicotinoid and Metabolite Levels in Mice. A mouse model was used to consider the effect of CYP450 inhibitors on in vivo neonicotinoid metabolism. Neonicotinoids are quickly metabolized to a variety of products on ip administration to mice (*4*, *5*), allowing the simultaneous evaluation of the inhibition of multiple metabolic pathways. The metabolites in brain and liver were analyzed at 60 min after IMI, THI, and CLO administration when the plasma levels of parent compounds were 33−66% of those at 15 min (*4*, *5*) to examine synergist-induced changes while metabolism was still proceeding rapidly. If CYP450 enzymes are major contributors to neonicotinoid metabolism in vivo, then CYP450 inhibitors should alter the levels of neonicotinoids and their metabolites in tissues. The synergists and their metabolites did not interfere in any way with analysis of the neonicotinoids or metabolites, such as by overlapping LC-MS peaks. The neonicotinoid levels were elevated by the OP CYP450 inhibitors to a greater extent in brain than in liver and with PPP versus SV_1 (**Table 5**). PPP practically doubled the level of IMI in brain without significantly changing the IMI level in liver. THI and CLO levels in brain were increased by almost the same factor as the IMI level by the synergists. The liver is probably the principal site of CYP450 metabolism and inhibition, resulting in elevated neonicotinoid levels entering the brain, where the metabolism is slower.

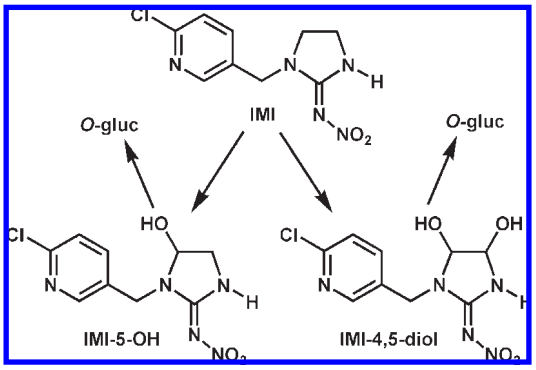

Figure 3. Hydroxylation and glucuronidation of the imidazolidine moiety of IMI in vitro and in vivo.

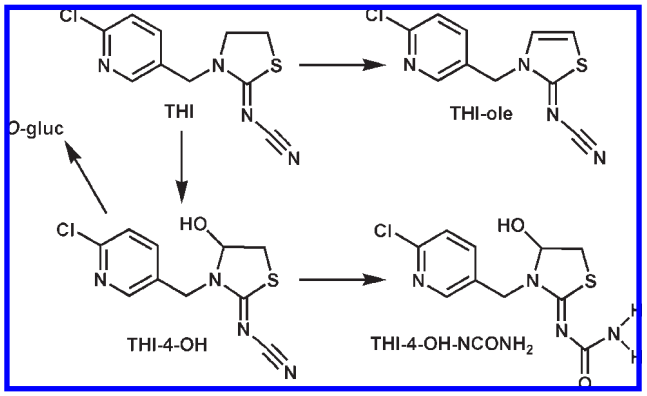

Figure 4. Hydroxylation, hydration, glucuronidation, and desaturation of THI in vitro and in vivo.

The livers of mice treated with IMI, THI, and CLO contain metabolites formed by imidazolidine hydroxylation, thiazolidine hydroxylation and desaturation, and N- demethylation (**Figures 2−4**). The participation of CYP450 in these processes was evaluated by determining the in vivo effects of PPP and SV_1 (**Table 6**). N-Demethylation of CLO in brain and liver was strongly inhibited by both synergists. Imidazolidine and thiazolidine hydroxylations were generally inhibited by SV_1 somewhat and by PPP to a much greater extent. The olefin and urea ($CONH_2$) derivatives were also lowered by both synergists.

Importance of Understanding Neonicotinoid Metabolism. Neonicotinoid metabolism is a major contributor in insects to the development of resistant strains (*33*, *34*) and the action of synergists (*18*), in mammals to certain adverse effects under

Table 4. Glucuronides of Some Neonicotinoid Metabolites Formed in Vivo in Mice or in Vitro in Mouse and Human Liver Microsome−UDPGlcUA Systems

	LC-MS t_R (min)		glucuronide formation	
compound administered or candidate substrate	parent	gluc[a]	mouse in vivo	MLM− and HLM− UDPGlcUA
IMI-5-OH	15.85	17.61	+[b]	+
IMI-4,5-diol	15.06	19.31 (18.51, 20.01)[c]	+[b]	+
IMI-NH	3.25	4.65[d]	−	−
THI-4-OH	18.82	20.06, 21.43	NT[e]	+
$CPCH_2OH$	13.95	15.77	NT[e]	+
CPCOOH	19.41	18.27	+	+
4-nitrophenol[f]	20.41	14.02	NT[e]	+

[a] *O*-Glucuronide (mass of parent compound + 176) of appropriate $^{35}Cl/^{37}Cl$ isotope. [b] Also observed with IMI administration. [c] Proposed to be individual isomers. [d] t_R from chemical synthesis (see Supporting Information) but not observed as a metabolite in vitro or in vivo. [e] Not tested. [f] Positive control or standard.

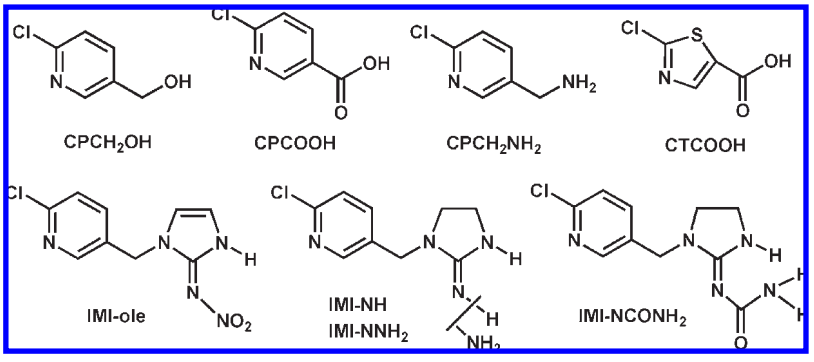

Figure 5. Glucuronidation observed for $CPCH_2OH$ and CPCOOH but not the other candidate metabolites in vitro or in vivo.

Table 5. In Vivo Effects of Two Organophosphorus CYP450 Inhibitors in Elevating IMI, THI, and CLO Levels in Brain and Liver of Mice 60 min after Intraperitoneal Administration

	neonicotinoid level		
tissue and synergist pretreatment[a]	IMI	THI	CLO
	ppm equiv (mean ± SD, n = 3)		
brain			
control	2.9 ± 0.4	2.8 ± 0.4	4.5 ± 0.7
PPP	5.4 ± 0.4***	6.1 ± 0.9***	8.8 ± 1.6**
SV_1	3.8 ± 0.7	4.0 ± 0.8	6.0 ± 1.6
liver			
control	13 ± 1	7.8 ± 1.1	15 ± 2
PPP	15 ± 1	13 ± 1**	20 ± 1**
SV_1	13 ± 1	9.2 ± 31	19 ± 3
	fold increase		
brain			
PPP	1.9	2.2	2.0
SV_1	1.3	1.4	1.3
liver			
PPP	1.2	1.7	1.3
SV_1	1.0	1.2	1.3

[a] PPP at 150 and SV_1 at 300 mg/kg 60 min before IMI, THI, or CLO at 10, 10, or 20 mg/kg, respectively. **, $P < 0.01$; ***, $P < 0.001$.

Table 6. In Vivo Effects of Two Organophosphorus CYP450 Inhibitors on Levels of IMI, THI, and CLO Metabolites in Brain and Liver of Mice 60 min after Intraperitoneal Administration[a]

	metabolite level		
tissue and neonicotinoid metabolite	control	PPP	SV_1
	ppm equiv (mean ± SD, n = 3)		
brain			
CLO-dm	0.44 ± 0.13	<0.01***	0.08 ± 0.02***
liver			
IMI-5-OH	0.74 ± 0.12	0.22 ± 0.02**	0.67 ± 0.28
THI-4-OH	0.24 ± 0.06	0.08 ± 0.03*	0.31 ± 0.12
THI-4-OH-$CONH_2$	0.06 ± 0.01	<0.01*	0.02 ± 0.03
THI-ole	0.05 ± 0.01	0.02 ± 0.01	0.03 ± 0.02
CLO-dm	4.1 ± 1.2	0.18 ± 0.07***	0.57 ± 0.01***
	relative to control		
brain			
CLO-dm	1.0	<0.02	0.18
liver			
IMI-5-OH	1.0	0.30	0.91
THI-4-OH	1.0	0.33	1.3
THI-4-OH-$CONH_2$	1.0	<0.17	0.33
THI-ole	1.0	0.40	0.60
CLO-dm	1.0	0.04	0.14

[a] See **Table 5** for conditions and **Figures 2**−**4** for structures. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

laboratory conditions (*2*, *11*, *12*), and in plants to neonicotinoid residue dissipation (*8*). A wide diversity of metabolic reactions takes place with each neonicotinoid, and although the products are known, the specific enzymes involved are often not defined. The phase I enzymes considered earlier are CYP450 and AOX, but no studies have been made until now on the phase II enzymes with neonicotinoid substrates. The present study considers the human CYP450 regioselective oxidations, the glucuronidation and methylation aspects of phase II metabolism, and some inhibitors of neonicotinoid metabolism. A glutathione *S*-transferase is presumably also involved because the chloro substituent of an IMI metabolite is ultimately cleaved for a portion of the dose in mammals yielding *S*-substituted-cysteinyl and *S*-methyl derivatives (*4*). The relationship of TMX metabolism to its carcinogenicity in mice but not rats may be related to the balance of activating and detoxifying enzymes (*11*, *12*, *31*). Knowledge of the enzymes and inhibitors in neonicotinoid metabolism will help ensure that these insecticides are used safely and effectively.

ABBREVIATIONS USED

AdoMet, *S*-adenosylmethionine; AOX, aldehyde oxidase; CLO, clothianidin; CLO-dm, desmethylclothianidin; dm, desmethyl; HLM, human liver microsomes; IMI, imidacloprid; MLM, mouse liver microsomes; OP, organophosphate; ppm equiv, parts per million equivalents based on the absorbance at 254 nm and recovery values of the parent compounds; PPP, *O*-(*n*-propyl) *O*-(2-propynyl) phenylphosphate; SV_1, *O*,*O*-diethyl *O*-phenyl phosphorothionate; THI, thiacloprid; TMX, thiamethoxam; TMX-dm, desmethylthiamethoxam; UDPGlcUA, UDP-glucuronic acid.

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Supporting Information Available: Descriptions of determining tissue levels of neonicotinoids and metabolites, synthesis of *N*-glucuronides from IMI and IMI-NH, effect of inhibitors on metabolic demethylation of TMX and CLO, and enzymatic methylation of CLO-dm. This material is available free of charge via the Internet at http://pubs.acs.org.

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