Published in final edited form as:

J Agric Food Chem. 2009 June 10; 57(11): 4861–4866. doi:10.1021/jf900250f.

Enzymes and Inhibitors in Neonicotinoid Insecticide Metabolism

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Abstract

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 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-hydroxy-thiacloprid 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 the 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 (4,5 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 based on 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

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hepatocarcinogenicity of TMX in mice is attributed to the metabolites desmethyl-TMX (TMX-dm) and desmethyl-CLO (CLO-dm) by a mechanism which is arguably not as relevant in rats or humans (11,12). CYP450 enzymes play a major role in pesticide metabolism based on 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) analog] CYP450 inhibitor in mice (19). The other OP synergist *O,O*-diethyl *O*-phenyl phosphorothionate (also known as SV₁ or dietholate) (20,21) possibly forms a hydrodisulfide at the CYP450 catalytic site on substrate desulfuration (22). The effects of PPP and SV₁ 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)]. SV₁ 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 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 formation of CLO and TMX-dm from TMX and 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, while 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 319 → 132 *m/z* transition while 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 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 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 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 protein) or pooled HLM (1 mg protein), substrate (2 mM), UDP-glucuronic acid (UDPGlcUA) (5 mM), MgCl₂ (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 10,000 g for 15 min. The supernatant was passed through a 0.45 µm nylon membrane in an Acrodisc syringe filter (13 mm) before analyzing 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, 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 i.p. 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 urine and 100 µL ice-chilled acetonitrile was vortexed and centrifuged at 10,000g 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 ³⁵Cl:³⁷Cl isotope ratio of 3:1; detected 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 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 μ L 50 mM Tris buffer (pH 7.4) consisted of liver cytosol (1 mg 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 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 i.p. administration with Me_2SO as the carrier vehicle (1 μ L/g mouse weight). After 1 h, IMI, THI or CLO in Me_2SO was administered i.p. 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 acetonitrile containing 250 mg sodium chloride. An homogenate was prepared with a sonic dismembrator (Fisher Scientific, Pittsburgh, PA) followed by mixing with a vortex. The acetonitrile extract, recovered after centrifugation at 2,000g 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 μ L) 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 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), conversion 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 predominate 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 μ M 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 tranilcypromine gave 31 to 50% inhibition at 10 μ M with both substrates while the less selective coumarin and orphenadrine gave little or no inhibition at 1000 to 3000 μ M.

Earlier studies showed that mouse, rat and human microsomes differ in their rates of TMX and CLO metabolism with mouse much more active than rat or human 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 while 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) which 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 CPCH₂NH₂ 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, CPCH₂OH and CPCOOH and with some of the compounds *in vivo* in mice based on urine analysis (Table 4). Glucuronides were not observed with MLM and UDPGlcUA for IMI, IMI-NCONH₂, IMI-ole, IMI-NNH₂, CLO, CLO-dm, CPCH₂NH₂, CTCOOH or nicotine (see Figure 5 for some of the structures). Further, no *N*-glucuronide was observed with the HLM-UDPGlcUA system for IMI, IMI-NH, CLO-dm and CPCH₂NH₂. 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 Mg²⁺ (but not without Mg²⁺) 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 i.p. 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 liver and with PPP versus SV₁ (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.

The liver of mice treated with IMI, THI and CLO contains metabolites formed by imidazolidine hydroxylation, thiazolidine hydroxylation and desaturation, and *N*-demethylation (**Figures 2, 3 and 4**). The participation of CYP450 in these processes was evaluated by determining the *in vivo* effects of PPP and SV₁ (Table 6). *N*-Demethylation of CLO in brain and liver was strongly inhibited by both synergists. Imidazolidine and thiazolidine hydroxylations were generally inhibited by SV₁ somewhat and PPP to a much greater extent. The olefin and urea (CONH₂) 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 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 since 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 insure that these insecticides are used safely and effectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

We thank our Berkeley laboratory colleagues Motohiro Tomizawa, Tami Clark, and Alex Gulevich for advice and assistance. We acknowledge Rita Nichiporuk of the Mass Spectrometry Facility, Department of Chemistry at Berkeley, for valuable suggestions.

This manuscript was supported by the Major State Basic Research Development Program of China (973, 2006CB102003), the National Natural Science Foundation of China (NSFC30771426), the Program for China New Century Excellent Talents in University (NCET-06-0113), and the China Scholarship Council (CSC). This study was also supported by a University of California Toxic Substances Research and Teaching Fellowship to K.A.F., and Grant R01 ES08424 from the National Institute of Environmental Health Sciences, National Institutes of Health, and the University of California, Berkeley William Muriece Hoskins Chair in Chemical and Molecular Entomology to J.E.C.

ABBREVIATIONS USED

AdoMet, *S*-adenosylmethionine
AOX, aldehyde oxidase

CLO, clothianidin
 CLO-dm, desmethyl clothianidin
 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, SV₁, *O,O*-diethyl *O*-phenyl phosphorothionate
 THI, thiacloprid
 TMX, thiamethoxam
 TMX-dm, desmethyl thiamethoxam
 UDPGlcUA, UDP-glucuronic acid

LITERATURE CITED

- (1). Tomizawa M, Casida JE. Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. *Annu. Rev. Entomol* 2003;48:339–364. [PubMed: 12208819]
- (2). Tomizawa M, Casida JE. Neonicotinoid insecticide toxicology: mechanisms of selective action. *Annu. Rev. Pharmacol. Toxicol* 2005;45:247–268. [PubMed: 15822177]
- (3). Tomlin, CDS., editor. *The Pesticide Manual*. Vol. 14th ed.. British Crop Protection Council, Alton; Hampshire, U.K.: 2006. p. 1350
- (4). Ford KA, Casida JE. Chloropyridinyl neonicotinoid insecticides: diverse molecular substituents contribute to facile metabolism in mice. *Chem. Res. Toxicol* 2006;19:944–951. [PubMed: 16841963]
- (5). Ford KA, Casida JE. Unique and common metabolites of thiamethoxam, clothianidin, and dinotefuran in mice. *Chem. Res. Toxicol* 2006;19:1549–1556. [PubMed: 17112244]
- (6). Hodgson, E.; Levi, PE. Metabolism of pesticides. In: Krieger, RI., editor. *Handbook of Pesticide Toxicology*. Vol. 2nd Ed.. Vol. 1. Academic Press; San Diego: 2001. p. 531-562.
- (7). Nauen R, Ebbinghaus-Kintscher U, Salgado VL, Kaussman M. Thiamethoxam is a neonicotinoid precursor converted to clothianidin in insects and plants. *Pestic. Biochem. Physiol* 2003;76:55–69.
- (8). Ford KA, Casida JE. Comparative metabolism and pharmacokinetics of seven neonicotinoid insecticides in spinach. *J. Agric. Food Chem* 2008;56:10168–10175. [PubMed: 18922014]
- (9). Environmental Protection Agency. Thiamethoxam; pesticide tolerance. *Fed. Regist* 2002;68:66561–66571.
- (10). Environmental Protection Agency. Clothianidin; pesticide tolerance. *Fed. Regist* 2003;68:32390–32400.
- (11). Green T, Toghil A, Lee R, Waechter F, Weber E, Noakes J. Thiamethoxam induced mouse liver tumors and their relevance to humans. Part 1: Mode of action studies in the mouse. *Toxicol. Sci* 2005;86:36–47. [PubMed: 15716477]
- (12). Green T, Toghil A, Lee R, Waechter F, Weber E, Pepper R, Noakes J, Robinson M. Thiamethoxam induced mouse liver tumors and their relevance to humans. Part 2: Species differences in response. *Toxicol. Sci* 2005;86:48–55. [PubMed: 15716476]
- (13). Hodgson E. In vitro human phase I metabolism of xenobiotics I: Pesticides and related compounds used in agriculture and public health, May 2003. *J. Biochem. Mol. Toxicol* 2003;17:201–206. [PubMed: 12898643]
- (14). Guengerich, FP. Human cytochrome P450 enzymes. In: Ortiz de Montellano, PR., editor. *Cytochrome P450: Structure, Mechanism, and Biochemistry*. Kluwer Academic/Plenum Publ.; New York: 2005. p. 377-530.

- (15). Correia, MA.; Ortiz de Montellano, PR. Inhibition of cytochrome P450 enzymes. In: Ortiz de Montellano, PR., editor. Cytochrome P450: Structure, Mechanism, and Biochemistry. Kluwer Academic/Plenum Publ; New York: 2005. p. 247-322.
- (16). Dick RA, Kaane DB, Casida JE. Substrate specificity of rabbit aldehyde oxidase for nitroguanidine and nitromethylene neonicotinoid insecticides. *Chem. Res. Toxicol* 2006;19:38–43. [PubMed: 16411654]
- (17). Casida JE. Mixed-function oxidase involvement in the biochemistry of insecticide synergists. *J. Agric. Food Chem* 1970;18:753–772. [PubMed: 4919838]
- (18). Liu M-Y, Lanford J, Casida JE. Relevance of [³H]imidacloprid binding site in house fly head acetylcholine receptor to insecticidal activity of 2-nitromethylene- and 2-nitroimino-imidazolidines. *Pestic. Biochem. Physiol* 1993;46:200–206.
- (19). Škrinjarčić-Špoljar M, Matthews HB, Engel JL, Casida JE. Response of hepatic microsomal mixed-function oxidases to various types of insecticide chemical synergists administered to mice. *Biochem. Pharmacol* 1971;20:1607–1618.
- (20). Oppenoorth FJ, Voerman S, Welling W, Houx NWH, Wouters van den Oudenweyer J. Synergism of insecticidal action by inhibition of microsomal oxidation with phosphorothionates. *Nature New Biology* 1971;233:187–188.
- (21). Weed Science Society of America. *Herbicide Handbook*. Vol. 6th Ed.. Champaign, IL: 1989. Dietholate; p. 102-103.
- (22). Neal RA, Rosen RD, Magee PS, Casida JE. Chemical mechanisms of the cytochrome P-450 monooxygenase - catalyzed metabolism of phosphorothionate triesters. *Amer. Chem. Soc. Symp. Ser* 1981;158:19–34. Sulfur in Pesticide Action and Metabolism
- (23). Kanne DB, Dick RA, Tomizawa M, Casida JE. Neonicotinoid nitroguanidine insecticide metabolites: synthesis and nicotinic receptor potency of guanidines, aminoguanidines, and their derivatives. *Chem. Res. Toxicol* 2005;18:1479–1484. [PubMed: 16167841]
- (24). Dick RA, Kanne DB, Casida JE. Identification of aldehyde oxidase as the neonicotinoid nitroreductase. *Chem. Res. Toxicol* 2005;18:317–323. [PubMed: 15720138]
- (25). Yamamoto I, Tomizawa M, Saito T, Miyamoto T, Walcott EC, Sumikawa K. Structural factors contributing to insecticidal and selective actions of neonicotinoids. *Arch. Insect Biochem. Physiol* 1998;37:24–32. [PubMed: 9397513]
- (26). Fletcher JH, Hamilton JC, Hechenbleikner I, Hoegberg EI, Sertl BJ, Cassaday JT. Preparation of O, O-diethyl O-*p*-nitrophenyl thiophosphate (parathion). *J. Am. Chem. Soc* 1948;70:3943–3944.
- (27). Kuehl GE, Murphy SE. *N*-Glucuronidation of nicotine and cotinine by human liver microsomes and heterologously expressed UDP-glucuronosyltransferases. *Drug Metab. Disposition* 2003;31:1361–1368.
- (28). Ghosheh I, Hawes EM. *N*-Glucuronidation of nicotine and cotinine in human: formation of cotinine glucuronide in liver microsomes and lack of catalysis by 10 examined UDP-glucuronosyltransferases. *Drug Metab. Disposition* 2002;30:991–996.
- (29). Seifert R, Hoshino J, Kröger H. Nicotinamide methylation: tissue distribution, developmental and neoplastic changes. *Biochim. Biophys. Acta* 1984;801:259–264. [PubMed: 6236853]
- (30). Maurer HH, Bickeboeller-Friedrich J, Kraemer T. Gas chromatographic-mass spectrometric procedures for determination of the catechol-*O*-methyltransferase (COMT) activity and for detection of unstable catecholic metabolites in human and rat liver preparations after COMT catalyzed *in statu nascendi* derivatization using *S*-adenosylmethionine. *J. Chromatogr. B* 2008;739:325–335.
- (31). Pastoor T, Rose P, Lloyd S, Peffer R, Green T. Case study: weight of evidence evaluation of the human health relevance of thiamethoxam-related mouse liver tumors. *Toxicol. Sci* 2005;86:56–60. [PubMed: 15716475]
- (32). Schulz-Jander DA, Casida JE. Imidacloprid insecticide metabolism: human cytochrome P450 isozymes differ in selectivity for imidazolidine oxidation versus nitroimine reduction. *Toxicol. Lett* 2002;132:65–70. [PubMed: 12084621]
- (33). Daborn P, Boundy S, Yen J, Pittendrigh B, ffrench-Constant R. DDT resistance in *Drosophila* correlates with *Cyp6g1* over-expression and confers cross-resistance to the neonicotinoid imidacloprid. *Mol. Genet. Genomics* 2001;266:556–563. [PubMed: 11810226]

- (34). Rauch N, Nauen R. Identification of biochemical markers linked to neonicotinoid cross resistance in *Bemisia tabaci* (Hemiptera: Aleyrodidae). Arch. Insect Biochem. Physiol 2003;54:165–176. [PubMed: 14635178]

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.

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*.

Figure 5.
Glucuronidation observed for CPCH₂OH and CPCOOH but not the other candidate metabolites *in vitro* or *in vivo*.

Table 1

Regioselectivity of Human Recombinant CYP450 Enzymes in Metabolism of Thiamethoxam to Clothianidin and Desmethyl-thiamethoxam and of Clothianidin to Desmethyl-clothianidin

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

^a Coexpressed with CYP450 reductase and (except for CYP1A2 and CYP2D6*1) also with cytochrome *b*₅.

^b Incubation with 15 pmol 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 P450	K_m μ M	Cl_{int} μ L/min/pmol P450
2A6	CLO	CLO-dm	1.0	4200	2.5×10^{-4}
	TMX	CLO ^a	0.73	270	27×10^{-4}
2C19	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.

Table 3

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

substrate	product	subcellular fraction ^a	specific activity <i>pmol/min/mg protein</i> (mean $\pm$ SD, n = 3)
TMX	TMX-dm	microsomes	ND ^b
TMX	CLO ^c	microsomes	35 $\pm$ 2
TMX	TMX-NNO	cytosol	ND ^d
CLO	CLO-dm	microsomes	20 $\pm$ 0.4
CLO	CLO-NNO	cytosol	69 $\pm$ 7

^aMicrosomes (1.5 mg protein/mL) with 1.5 mM NADPH and cytosol (5 mg protein/mL) with 10 mM *N*-methylnicotinamide.

^bNot detected at a discriminating level of 0.1 pmol/min/mg protein.

^cA portion of the CLO formed is further metabolized to CLO-dm

^dNo metabolism detected by LC/MS (16)

Table 4

Glucuronides of Some Neonicotinoid Metabolites Formed *In Vivo* in Mice or *In Vitro* in Mouse and Human Liver Microsome-UDPGlcUA Systems

compound administered or candidate substrate	LC/MS t_R (min)		glucuronide formation	
	parent	Gluc ^a	mouse <i>in vivo</i>	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 ₂ OH	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 ³⁵Cl/³⁷Cl isotope ratio.

^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

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 I.P. Administration

tissue and synergist pretreatment ^a	neonicotinoid level		
	IMI	THI	CLO
ppm equiv (mean ± SD, n = 3)			
<u>brain</u>			
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
<u>liver</u>			
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			
<u>brain</u>			
PPP	1.9	2.2	2.0
SV ₁	1.3	1.4	1.3
<u>liver</u>			
PPP	1.2	1.7	1.3
SV ₁	1.0	1.2	1.3

^aPPP at 150 and SV₁ 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 I.P. Administration^a

tissue and neonicotinoid metabolite	metabolite level		
	control	PPP	SV ₁
	ppm equiv (mean ± SD, n = 3)		
<u>brain</u>			
CLO-dm	0.44 ± 0.13	<0.01 ^{***}	0.08 ± 0.02 ^{***}
<u>liver</u>			
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	
<u>brain</u>			
CLO-dm	1.0	<0.02	0.18
<u>liver</u>			
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

^aSee Table 5 for conditions and **Figures 2-4** for structures.

* P < 0.05

** P < 0.01

*** P < 0.001