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Neonicotinoid Insecticide Metabolism and Mechanisms of Toxicity in Mammals

by Tami Lynn Swenson

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Molecular Toxicology in the Graduate Division of the University of California, Berkeley

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Abstract

Neonicotinoid Insecticide Metabolism and Mechanisms of Toxicity in Mammals

by

Tami Lynn Swenson

Doctor of Philosophy in Molecular Toxicology

University of California, Berkeley

Professor John E. Casida, Chair

Neonicotinoids are the most important class of insecticides. Seven commercial neonicotinoids currently account for approximately 25% of the total insecticide market and are increasing in use as they replace other major classes such as organophosphates and methylcarbamates. Neonicotinoids are extensively metabolized in plants and mammals to produce over 100 metabolites. The overall goal of this study was to better understand three aspects of neonicotinoid metabolism in mice: the importance of aldehyde oxidase (AOX) *in vivo*, the fate of a new neonicotinoid, cycloxaprid (CYC), and the production of formaldehyde-generating intermediates from the hepatotoxicant, thiamethoxam (TMX).

Neonicotinoids are metabolized in vitro by cytochrome P450s (CYPs) via oxidation reactions and by AOX on reduction of the nitroimino group. AOX metabolizes many xenobiotics in vitro but its importance in vivo is unknown relative to CYPs and other detoxification systems. Here we establish the relative importance of AOX and CYPs in vivo in neonicotinoid metabolism using the mouse model. AOX activity was reduced in mice by 45% with tungsten, 61% with hydralazine and 81% in AOX-deficient mice relative to controls and CYP activity was not affected. When mice were treated intraperitoneally with the major neonicotinoid imidacloprid (IMI), metabolism by CYPoxidation reactions was not appreciably affected whereas the AOX-generated nitrosoguanidine metabolite was decreased by 30% with tungsten, 56% with hydralazine and 86% in the AOX-deficient mice. Another IMI nitroreduction metabolite, desnitro-IMI, was decreased by 55, 65, and 81% with tungsten, hydralazine and in the AOX-deficient mice, respectively. Thus, decreasing liver AOX activity by three quite different procedures gave a corresponding decrease for in vivo reductive metabolites in the liver of IMI-treated mice. Possible AOX involvement in IMI metabolism in insects was evaluated using AOX-expressing and AOX-deficient Drosophila, but no differences were found in IMI nitroreduction or sensitivity between the two strains. This is the first study to establish the in vivo relevance of AOX in neonicotinoid metabolism in mammals and one of the first for xenobiotics in general.

The candidate novel insecticide, CYC, has a unique heterocyclic ring system and *cis*nitro substituent. Although it is not yet registered for use, it is proposed to control IMIresistant pests by binding to a different site on the nicotinic acetylcholine receptor. CYC is potentially a proinsecticide, metabolized to the active nitromethylene-imidazole (NMI) analog of IMI. The metabolic pathways of CYC and NMI are unknown. Metabolites in the brain, liver and plasma of CYC- or NMI-treated mice were analyzed by liquid chromatography/ mass spectrometry at 15 and 120 min post-treatment. The major metabolites of CYC were mono- and dihydroxylation products (CYC m/z +16 and +32) and NMI. All metabolites dissipated by 24 h. NMI was metabolized only to a small extent to one hydroxylation and one nitroso product. Although CYC may be a proinsecticide, the major metabolic pathways in mice do not involve high or persistent levels of NMI as an intermediate.

Not all neonicotinoid metabolites are detoxification products. TMX, one of the most commonly-used neonicotinoids, is hepatotoxic and hepatocarcinogenic in mice but not rats. Earlier studies established that TMX is a much better substrate for mouse liver microsomal CYPs than the corresponding rat or human enzymes in forming desmethyl-TMX (dm-TMX), which is also hepatotoxic, and clothianidin (CLO), which is not hepatotoxic or hepatocarcinogenic. It was proposed that TMX hepatotoxicity/ hepatocarcinogencity is due to dm-TMX and a further metabolite, desmethyl-CLO (dm-CLO) (structurally analogous to a standard inducible nitric oxide synthase inhibitor), acting synergistically. Here we considered formation of formaldehyde (HCHO) and Nmethylol intermediates as an alternative mechanism of TMX hepatotoxicity/ hepatocarcinogenicity. Comparison of neonicotinoid metabolism by mouse, rat and human microsomes with NADPH showed two important points. First, TMX and dm-TMX yield more HCHO than any other commercial neonicotinoid. Second, mouse microsomes give much higher conversion than rat or human microsomes. These observations provide an alternative hypothesis of HCHO and N-methylol intermediates from CYP-mediated oxidative oxadiazinane ring cleavage as the bioactivated hepatotoxicants. However, the proposed mono-N-methylol CYP metabolites are not observed, possibly further reacting in situ. Thoroughly characterizing the metabolism and mechanisms of toxicity of neonicotinoids is important for future pesticide design especially as the demand for and use of these compounds continues to increase.

Dedication

I dedicate this dissertation to my family without whom I would not have made it this far. They have provided me endless support and guidance throughout my education. To my parents, Tim and Cheryl Clark who have always encouraged me to work to my fullest potential and never give up: from my scary first days of pre-school all the way through my PhD education (they continue to provide motivation across thousands of miles). To my niece, Kendall for reminding to stay bright, smile and be carefree and to my sister, Holly Ertmer for being a positive role model and someone to always look up to. Finally, to my husband, Joel Swenson for his endless and tireless support, providing intriguing scientific discussions (and challenges) and for always being someone to emulate with his positive attitude and outlook on life. He has kept me sane and shaped me into the scientist and person I have always wanted to be.

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Abbreviations

ACE	acetamiprid
ACh	acetylcholine
AChBP	acetylcholine binding protein
AChE	acetylcholinesterase
ANOVA	analysis of variance
ACN	acetonitrile
AOX	aldehvde oxidase
AOX-/-	AOX-deficient Drosophila
AOX ^{+/+}	AOX-expressing Drosophila
CLO	clothianidin
CNA	6-chloronicotinic acid
CNS	central nervous system
CYC	cycloxaprid
CYP	cytochrome P450
	dichlorodinhenvltrichloroethane
	dinotefuran
dm_CLO	desmethyl_clothianidin
dm-TMX	desmethyl-thiamethoxam
	n_dimethylaminocinnamaldehyde
	dimethylsulfovide
	2.4 dipitrophonylbydrazino
	2,4-unincophenyinyurazine
	formaldabuda dabudraganaaa
GADA	gamma-ammobulync aciu
	nours
	Iormaldenyde
	tormic acid
HPLC	nign performance liquid chromatography
IMI-4-OH	4-nydroxy-imidacloprid
IMI-5-OH	5-hydroxy-imidacloprid
IMI-de	desethano-imidacloprid
IMI-diol	4,5-dihydroxy-imidacloprid
IMI-NH	desnitro-imidacloprid
IMI-NNH ₂	aminoguanidine-imidacloprid
IMI-NNO	nitrosoguanidine-imidacloprid
IMI-ole	imidacloprid olefin
IMI-tri	imidacloprid methyltriazinone
IMI-urea	imidacloprid urea
iNOS	inducible nitric oxide synthase
ip	intraperitoneal
L-NAME	N ^G -L-nitroarginine
LC/MS	liquid chromatography/ mass spectrometry

LD ₅₀	median lethal dose
mAChR	muscarinic acetylcholine receptor
min	minutes
nAChR	nicotinic acetylcholine receptor
NH ₂ -CYC	amino-cycloxaprid
NIT	nitenpyram
NMI	nitromethylene-imidazole
NMN	N-methylnicotinamide
nNOS	neuronal nitric oxide synthase
NO-CYC	nitroso-cycloxaprid
NO-NMI	nitroso-nitromethylene-imidazole
NOAEL	no-observed adverse effect level
NOS	nitric oxide synthase
(OH) ₂ -CYC	dihydroxy-cycloxaprid
OH-CYC	hydroxy-cycloxaprid
OH-NMI	hydroxy-nitromethylene-imidazole
PBS	phosphate buffered saline
PNS	peripheral nervous system
r	Pearson correlation coefficient
rCYP2C19	recombinant CYP 2C19
rCYP3A4	recombinant CYP 3A4
SE	standard error
sec	seconds
THI	thiacloprid
THI-4-OH	4-hydroxy-thiacloprid
THI-ole	thiacloprid olefin
TMX	thiamethoxam
t _R	retention time
UDPGA	uridine 5'-diphosphoglucuronic acid

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Chapter 1

Introduction: Neonicotinoid Insecticides

1.1 Insect pests and insecticides

Pesticides are natural or synthetic chemicals used to control pests. In order to support an expanding population there is a continuous need for pesticides (such as insecticides, herbicides, fungicides and acaricides). Worldwide, there are thousands of pests including insects, weeds, fungi, bacteria, viruses, mycoplasma and nematodes that destroy crops, transmit diseases and compete for resources. One of the first written records of pesticide use was from around 1000 B.C. when Homer described the use of sulfur to control pests by farmers. Many natural pesticides and botanicals were used since that initial discovery: arsenic, mercury, lead, nicotine, pyrethrum and rotenone. However, insect resistance and safety issues for these inorganics and botanicals led to synthetic the production and use of the first organic insecticide. dichlorodiphenyltrichloroethane (DDT) discovered in 1939 by Paul Müller (first synthesized in 1873). Currently, there are over 40,000 different pesticide products for retail sales with different formulations (e.g. sprays, dusts or granulars) and control mechanisms (e.g. neuroactive agents, defoliants, dessicants, growth regulators, attractants or repellents).

Since the major discovery of DDT, advances have continued with the synthesis and commercialization of hundreds of pesticides including five major neuroactive insecticide classes all with unique toxicity profiles (in both insects and mammals) and target sites: chlorinated hydrocarbons (organochlorines), pyrethroids, carbamates, organophosphates and neonicotinoids (Table 1.1). Chlorinated hydrocarbons (DDT) and pyrethroids are insecticidal through their ability to destabilize voltage-gated sodium ion channels (or as antagonists of the gamma-aminobutyric acid (GABA) receptor for some chlorinated hydrocarbons). DDT has low acute toxicity to mammals, but is persistent in the environment which ultimately led to it being banned in the US in 1972. Other problems from DDT include its potential carcinogenicity, thinning of bird eggshells and fish death. Pyrethroids, modeled from natural pyrethrin compounds from the Chrysanthemum flower, are relatively non-toxic and are less stable in the environment than DDT. Carbamates and organophosphates both inhibit acetylcholinesterase (AChE) leading to accumulation of acetylcholine and overstimulation of the nervous system. Carbaryl was at one time the most commonly used carbamate with low mammalian toxicity and broad-spectrum use and selectivity. Organophosphates are related to potent nerve agents (e.g. sarin). Often highly toxic to mammals, they are metabolized and detoxified readily. Neonicotinoids, the most important class of insecticides, have favorable mammalian and environmental toxicology and now account for approximately 25 percent of the worldwide insecticide market value.



Table 1.1 Comparison of five major neuroactive insecticide classes with examples and their biological targets.

1.2 Nicotine, the cholinergic system and the nicotinic receptor

1.2.1 Nicotine

Nicotine is an alkaloid found in the leaves of the genus *Nicotiana* (of the *Solanaceae* family) and has long been recognized for its insecticidal and pharmacological properties. It can be extracted from the plant with petroleum ether, ether, trichloroethylene or benzene and used as a contact poison, fumigant or ingestion (when in the form of salts) insecticide. Due to its non-systemic, volatile and highly toxic properties (to insects and mammals), nicotine use as an insecticide has been phased out and replaced by neonicotinoids (Negherbon, 1959; Tomlin, 2003).

1.2.2 The cholinergic system and the nicotinic receptor

Neonicotinoids target the cholinergic system within the central nervous system (CNS) of insects. However, in mammals the cholinergic system exists in both the peripheral nervous system (PNS) and the CNS (Yamamoto and Casida, 1999). Within the cholinergic system there are two types of acetylcholine receptors: nicotinic acetylcholine receptors (nAChR) (ionotropic and nicotine-responsive) and muscarinic acetylcholine receptors (mAChR) (metabotropic and muscarine-responsive) (**Fig. 1.1**).

Acetylcholine (ACh) is the important excitatory neurotransmitter of the cholinergic system. When ACh is released from presynaptic neurons it binds to acetylcholine receptors (AChRs) on the presynaptic or postsynaptic neuron within the CNS or at the neuromuscular junction within the PNS. In the case of the nAChR, ACh binding causes the ligand-gated ion-channel to open, allowing Na⁺ (and sometimes Ca²⁺) influx and K⁺ efflux. Activation of the mAChR results in a G-protein-complex secondary messenger cascade in the postsynaptic neuron. ACh is then degraded by AChE in the synaptic cleft.



Figure 1.1 The cholinergic system consists of nAChRs (ligand-gated ion channels; depicted here as $\alpha 4\beta 2$ and $\alpha 7$) and mAChRs (G-protein coupled receptors; depicted here as M1-M5) (Figure from Jones *et al.*, 2012).

The nAChR is part of the superfamily of neurotransmitter-gated ion channels along with GABA, glycine and 5-HT₃ serotonin receptors. The structure of the nAChR is better understood in mammals compared to insects. The mammalian nAChR consists of five subunits with combinations from ten α , four β , and one each of δ , γ and ϵ subunits (Tomizawa and Casida, 2003; Yamamoto and Casida, 1999). Different subunit combinations result in varying degrees of sensitivity to ACh or other agonists such as abungarotoxin. The most common subtypes in the vertebrate brain are the $\alpha 4\beta 2$ (two $\alpha 4$ and three β 2 subunits) and the α 7. Ligands bind within the nAChR to a conserved core of aromatic amino acids at the interface region between subunits (Dougherty, 2008; Tomizawa and Casida, 2003, 2005). In insects, the subunit combinations and related pharmacology of the nAChR subtypes have not been completely resolved. However, in general, the insect nAChR is distributed in the neuropil regions of the CNS and consists of a combination of α and β subunits to form a five-subunit transmembrane complex (Tomizawa and Casida 2003, 2005). Currently, the best functional model of the insect nAChR consists of expressed *Drosophila* α subunits with vertebrate β subunits. Subunit variations and alternative splicing lead to a variety of nAChRs with different affinities to ACh and neonicotinoids. The mammalian nAChR is also a target for therapeutic agents for a variety of neurological conditions including analgesia, schizophrenia, depression and anxiety (Tomizawa and Casida, 2005).

1.3 Neonicotinoid discovery

1.3.1 Chronology of nicotinoids and neonicotinoids

In an attempt to understand the mechanism of action of nicotine, Izuru Yamamoto discovered that insecticidal activity depends on ionization or basicity of the nitrogen of nicotine and all nicotine-related compounds (termed 'nicotinoids') (Yamamoto *et al.*, 1962; Yamamoto and Casida, 1999). Yamamoto and colleagues realized that although ionization prevents penetration of the CNS of insects which decreases insecticidal activity, these insecticides needed to be ionized to interact with the nAChR.

The search began for synthetic insecticides with high insecticidal activity, low mammalian toxicity and the ability to penetrate the insect CNS (not ionized) yet basic enough to interact with the nAChR. Nithiazine, a nitromethylene heterocycle, was the first neonicotinoid prototype developed by Shell Development Company in 1978 (Yamamoto and Casida, 1999). It had excellent insecticidal activity, good systemic action in plants and low mammalian toxicity. However, nithiazine was highly photolabile (rapidly broke down in sunlight). Shinzo Kagabu and colleagues modified the structure of nithiazine and synthesized a series of compounds with varying ring structures and substituents and screened them for insecticidal activity against the major rice pest, the green rice leafhopper. This led to the discovery of the first highly active neonicotinoid, imidacloprid (IMI), in 1985 (Kagabu, 2011). IMI has 12 times higher insecticidal activity than nicotine, is more systemic and photostable and therefore was commercialized by Bayer in 1991. Other first-generation chloropyridinyl-containing neonicotinoids include nitenpyram (NIT), acetamiprid (ACE) and thiacloprid (THI) commercialized in 1995, 1996 and 2000, respectively (Yamamoto and Casida, 1999). Further derivatization and optimization lead to the discovery of the two second-generation neonicotinoids, thiamethoxam (TMX) and clothianidin (CLO) by Novartis and Takeda, respectively (Yamamoto and Casida, 1999). Finally, the only tetrahydrofuranyl-containing neonicotinoid, dinotefuran (DIN), was commercialized by Mitsui Chemical Company in 2002. The term "neonicotinoid" was proposed in 1993 to cover all of these compounds (Yamamoto and Casida, 1999) and now this category contains seven commerciallyused insecticides (Fig. 1.2) with more compounds in the development and early registration stages (Tomizawa and Yamamoto, 1992).



Figure 1.2 Nicotine, the neonicotinoid prototype (nithiazine) and the seven commercial neonicotinoids with structures and abbreviations.

1.3.2 Uses

Neonicotinoids have become the most important class of insecticides since synthetic pyrethroids. They have been increasing in use due to resistance and toxicity of other major pesticide classes including the organophosphates and methylcarbamates (Jeschke *et al.*, 2011; Tomizawa and Casida, 2003). Neonicotinoids are registered for use in over 120 countries for control of sucking and chewing insect pests and animal health. In 2010, IMI was among the five most-used insecticides by acres treated in California. Their statewide use continues to increase to control pests that have become resistant to other pesticides including chlorpyrifos (California Department of Pesticide Regulation, 2010).

Neonicotinoids are highly systemic and can be applied as a wettable powder to soil or directly to the crop or seed to be taken up through the plant to treat sucking and chewing pests such as aphids, Colorado potato beetles, rice hoppers, thrips and whiteflies (Meister, 2005). Neonicotinoids are used on a variety of crops including cereal, cotton, fruit, maize, potatoes, rice, sugar beets, turf and vegetables (Meister, 2005). The most common crops in California to which neonicotinoids are applied include grapes, lettuce and cotton (California Department of Pesticide Regulation, 2010). Due to the systemic activity of neonicotinoids, crops have the potential to maintain high levels of the parent compound and its potentially toxic metabolites. An additional common use for neonicotinoids, particularly CLO, NIT and IMI, is in pet collars such as Advantage to control fleas on dogs and cats.

1.4 Neonicotinoid enzymatic metabolism

1.4.1 Cytochrome P450s and phase II reactions



Figure 1.3 Sites of metabolite attack on IMI and 6-chloronicotinic acid (CNA) for phase I and phase II reactions (Figure from Casida, 2011).

Neonicotinoids undergo phase I and phase II biotransformation in insects. mammals and plants (Fig. 1.3). In vitro neonicotinoid metabolism studies have indicated the importance of cytochrome P450s (CYPs) in neonicotinoid oxidation and reduction. IMI is oxidized to the 5-hydroxy (IMI-5-OH) and olefin (IMI-ole) metabolites and reduced to the nitrosoguanidine (IMI-NNO), aminoguanidine (IMI-NNH₂), desnitro (IMI-NH) and urea (IMI-urea) metabolites by a variety of human CYP isozymes (Fig. 1.4) (Schulz-Jander and Casida, 2002; Schulz-Jander et al., 2002). The most active CYP isozyme for oxidation of the imidazolidine moiety of IMI is CYP3A4 (the most abundant CYP in humans) followed by CYP2C19, 2A6 and 2C9 and for nitroreduction, CYP1A2, 2B6, 2D6 and 2E1. Flavin monooxygenases with NADPH are not likely involved in neonicotinoid metabolism (Schulz-Jander and Casida, 2002). Interestingly, IMI is not only metabolized by human or rabbit liver microsomes in the presence of NADPH, but it is also reduced by rabbit liver cytosol without added cofactor. These and other observations lead to the discovery that the cytosolic enzyme, aldehyde oxidase (AOX) is involved in neonicotinoid nitroreduction (Fig. 1.4) (Dick et al., 2005; Schulz-Jander et al., 2002).



Figure 1.4 Partial *in vitro* metabolic pathways for IMI to its reduction and oxidation metabolites. ^a AOX produces IMI-NNO and IMI-NNH₂ *in vitro* (Figure from Swenson and Casida, 2013a).

TMX is metabolized to CLO mostly by CYP3A4 and to a smaller extent by CYP2C19 and 2B6 and is demethylated by 2C19. CLO is demethylated by CYP3A4, 2C19 and 2A6 (Shi *et al.*, 2009). *In vivo*, selective organophosphorus CYP inhibitors demonstrated that neonicotinoids are metabolized by CYPs to their hydroxylated metabolites including IMI-5-OH from IMI, 4-hydroxy-THI (THI-4-OH), the amide of THI-4-OH, the olefin (THI-ole) from THI and demethylated-CLO (dm-CLO) from CLO (Shi *et al.*, 2009). There are very few reports concerning phase II metabolism of neonicotinoids which have many potential reactive -NH or -OH functionalities. However, *O*-glucuronides have been detected from IMI-5-OH and 4,5-dihydroxy-IMI (IMI-diol) in IMI-treated mice and from THI-4-OH, CNA and chloropyridinylalcohol *in vitro* with mouse liver microsomes and uridine 5'-diphosphoglucuronic acid (UDPGA) (Shi *et al.*, 2009).

1.4.2 Aldehyde oxidase

AOX is a cytosolic molybdo-flavoenzyme important in xenobiotic metabolism. Many studies have implicated its significance in *in vitro* metabolism of pharmaceuticals containing aldehyde, nitro or *N*-heterocyclic moieties (Kitamura *et al.*, 2006; Pryde *et al.*, 2010). This enzyme is expressed mainly in liver but is also present in many other tissues with variations in activity depending on species, gender, age, drug usage and disease states (AI-Salmy, 2002; Beedham, 1987; Garattini *et al.*, 2008; Pryde *et al.*, 2010).

The nitroreduction of neonicotinoids can occur by CYP-mediated NADPHdependent reactions and with rabbit liver cytosol independent of NADPH (which is sensitive to AOX-specific inhibitors such as menadione and is decreased in an aerobic atmosphere) (Dick *et al.*, 2005; Schulz-Jander and Casida, 2002). Nitroguanidinecontaining neonicotinoids (IMI, CLO and DIN) are reduced by partially-purified AOX (from rabbit liver cytosol) to their nitrosoguanidine and aminoguanidine metabolites (*e.g.* IMI-NNO and IMI-NNH₂) in the presence of electron donor substrates such as *N*methylnicotinamide (NMN) (Dick *et al.*, 2005). This nitroreduction by AOX occurs by a two-electron reaction to form nitrosoguanidine metabolites and a six-electron reaction to form aminoguanidines (Dick *et al.*, 2006).

The nitroguanidine neonicotinoids, with the exception of TMX, are better substrates for AOX compared to the nitromethylene, NIT, which is reduced by AOX only to its nitroso metabolite. Of the four nitroguanidines, CLO is the most rapidly reduced by AOX and TMX is a poor substrate possibly due to the presence of a unique tertiary nitrogen (Dick *et al.*, 2006). Other AOX substrate preferences include acyclic neonicotinoids over cyclics (NIT versus nitromethylene-IMI), chlorothiazolyls over tetrahydrofuryls (CLO versus DIN) and secondary nitrogens over tertiary nitrogens (dm-TMX versus TMX). Finally, IMI-NNO is metabolized by AOX to a form that can covalently bind proteins and irreversibly inactivate AOX in a time- and NMN-dependent manner (Dick *et al.*, 2007).

1.5 *In vivo* metabolic pathways

1.5.1 Insects

CYPs are involved in neonicotinoid metabolism in many insect species. IMIresistant insects (*Drosophila*) have increased expression of *CYP6G1*, indicating this CYP isozyme is involved in detoxifying IMI and potentially other neonicotinoids (Daborn *et al.*, 2001). AOX is unlikely to play a major role in neonicotinoid metabolism in insects (Swenson and Casida, 2013a). TMX is 10,000-fold less potent than other neonicotinoids at the nAChR, but acts as a proinsectide by being metabolized rapidly to CLO in *Spodoptera frugiperda* larvae (Nauen *et al.*, 2003).

1.5.2 Mammals

There are limited early studies examining the *in vivo* metabolic pathways of neonicotinoids with the exception of IMI. After oral exposure of radiolabeled IMI in rats, two major metabolic pathways were identified. The first route, accounting for 30% of the administered radiolabel, is oxidative cleavage to form CNA then conjugation with glycine and dechlorination of the chloropyridinyl ring producing 6-hydroxynicotinic acid and its methylmercapturic acid derivative (likely via a glutathione conjugate). The second pathway is hydroxylation of the imidazolidine ring at the 4 or 5 position to yield IMI-4-OH and IMI-5-OH (16% of the radiolabel) and then loss of water to produce IMI-ole. All of these metabolites were detected in both urine and feces of treated rats. IMI-NH is a

minor metabolite and was only identified in feces. About 15% of the radiolabel was in the form of the parent compound. (Klein, 1987a; Tomlin, 2003)

Early studies also examined metabolite formation in major organs including kidney and liver of orally-dosed rats. The same metabolites were identified in the kidney as urine and feces. However, in the liver the glycine conjugate of CNA, IMI-ole and IMI-5-OH were not observed. Unique liver metabolites included IMI-NH, IMI-urea and the methyltriazinone of IMI (IMI-tri). CNA was detected in both organs (Advisory Committee on Pesticides, 1993).

The most recent studies thoroughly examined metabolite production and persistence of all seven neonicotinoids in liver, brain, urine and feces of intraperitoneally (ip)-treated mice (Ford and Casida, 2006a,b). These studies identified a diverse set of neonicotinoid metabolites. Multiple common cleavage products of the chloropyridinyl moiety of IMI, ACE, NIT and THI appeared in urine: CNA, its methylthio- and *N*-acetylcysteinyl derivatives and glycine, *O*-glucuronide and sulfate conjugates (**Fig. 1.5**). Of those cleavage products, CNA and its glycine conjugate were the most prominent metabolites in urine. Other IMI metabolites include IMI-NH (brain, liver), IMI-NNO (brain, liver, plasma), IMI-ole (liver, plasma) and IMI-5-OH (liver, plasma). Metabolites uniquely detected in the liver include IMI-NNH₂, IMI-tri, and IMI-diol (**Fig. 1.6**). Within 24 h post-treatment, 22% of unmetabolized IMI was excreted in urine along with IMI-5-OH, IMI-NH and the chloropyridinyl cleavage products.



Figure 1.5 Common chloropyridinyl cleavage metabolites from IMI, NIT, THI and ACE identified in ip-treated mice. Metabolites specifically mentioned in the text include c= CNA, d= O-glucuronide, e= *N*-acetylcysteinyl acid derivative, f= methylthio acid derivative, g= 6-hydroxynicotinic acid, h and i= glycine conjugates, j= sulfate conjugate (Figure from Ford and Casida, 2006a).



Figure 1.6 Partial *in vivo* metabolic pathway of IMI in ip-treated mice (b=brain, l=liver, p=plasma, u=urine) (Figure from Ford and Casida, 2006a).

NIT *in vivo* metabolism involves *N*-demethylation (brain, liver, plasma, urine), followed by addition of a carboxylic acid (brain, liver), formation of a cyano metabolite (liver) and cleavage of the nitromethylene portion of the molecule (urine). Of the administered compound, 46% was excreted in urine. THI metabolites include descyano-THI (brain, liver), THI-ole (brain, liver), THI-4-OH (brain, liver, urine), a sulfate methyl derivative (brain, liver, urine), an amide after cyano cleavage (urine) and other metabolites from cyano modification were detected in liver. ACE metabolism involves *N*-demethylation (brain, liver, plasma, urine), cyano hydrolysis to its demethylated amide product (urine) and formation of an acetamide and/or its demethylated products (liver, urine, brain). Only 1.6% of unmetabolized ACE was identified in urine 24 h after treatment. All four chloropyridinyl neonicotinoids reached peak tissue and plasma levels at approximately 15 min post-treatment followed by a steady decline. The exception is ACE which was persistent in tissues for up to 240 min following ip treatment.

The second study by Ford and Casida (2006b) examined the metabolism of the two chlorothiazolyl neonicotinoids, TMX and CLO, and the tetrahydrofuranyl, DIN after ip treatment in mice. TMX is considered a proinsecticide since it undergoes *O*-methylene hydroxylation followed by oxadiazinane cleavage to yield CLO. *N*-Demethylation of either TMX or CLO produces desmethyl-TMX (dm-TMX) or desmethyl-

CLO (dm-CLO). The nitro substituent on both neonicotinoids is reduced to their corresponding nitrosoguanidine, aminoguanidine, desnitro and urea metabolites (all found in the liver and some in brain, plasma and urine). The aminoguanidine metabolites can also conjugate with pyruvate to give a methyltriazinone metabolite. There are three cleavage products in common with TMX, CLO and DIN: nitroguanidine, desnitroguanidine and desmethylnitroguanidine. However, the presence of the oxadiazinane ring on TMX allows for the production of unique oxadiazinane-containing nitroguanidine products. Chlorothiazolymethyl moiety cleavage products (including the carboxaldehyde and carboxylic acid), produced during oxidation of the methylene bridge of TMX or CLO, were found in the liver and some in the brain, plasma and urine. Parent compound levels, TMX and CLO, peaked by 60 min in tissues followed by peak metabolite levels by 120 min after ip treatment in mice. Interestingly, dm-CLO is persistent in tissues. Twenty-four h following treatment, 19-27% of TMX or CLO were excreted in urine.

DIN undergoes *N*-demethylation, nitroreduction and hydroxylation on the tetrahydrofuranyl ring, *N*-methylene hydroxylation (to yield the carboxaldehyde and carboxylic acid) and amine cleavage to yield a complex metabolic pathway. DIN is rapidly metabolized and levels decrease quickly in the brain, liver and plasma. However, desmethyl-DIN exceeded parent levels in the brain by 60 min. Within 24 h following treatment, 55% of DIN was excreted in urine and tissue persistence was among the lowest of all neonicotinoids.

1.5.3 Plants

The metabolism, persistence and uptake of all seven neonicotinoids in spinach seedlings vary indicating the diverse chemical properties of neonicotinoids. When spinach seedlings were hydroponically treated with each of the seven neonicotinoids. nitroreduction. metabolites identified in leaves indicated cvano hvdrolvsis. demethylation, sulfoxidation, imidazolidine and thiazolidine hydroxylation followed by olefin formation, oxadiazinane hydroxylation and ring opening and chloropyridinyl dechlorination (Ford and Casida, 2008). Phase II metabolites include many O- and Nglucosides and gentiobiosides and amino acid conjugates (Ford and Casida, 2008). NIT is the least persistent neonicotinoid likely due to its photoinstability and THI is among the most persistent.

1.6 Neonicotinoids and the nicotinic receptor

1.6.1 Binding site interactions

Neonicotinoid insecticidal activity is due to their action as insect nAChR agonists (Tomizawa and Yamamoto, 1992). In order for nicotine and neonicotinoids to elicit their effects in insects, they must cross the CNS ion barrier. At physiological pH, neonicotinoids are not protonated and can easily penetrate the insect CNS (**Fig. 1.7**).



Figure 1.7 IMI readily crosses the insect ion barrier and binds the nAChR. Nicotine only crosses the ion barrier when not ionized (Figure from Tomizawa and Casida, 2003).

After entering the CNS, neonicotinoids bind the nAChR resulting in ion-channel opening, continual neural transmission and overstimulation of the cholinergic system (**Fig. 1.8**). The neonicotinoid binding site on the insect nAChR is the same as nicotine and ACh, is conserved between many insect species and is potentially localized at the interface between two subunits. Understanding the molecular binding interactions between neonicotinoids and the nAChR has been facilitated by chemical and structural analyses of ACh binding proteins (AChBP) from the saltwater mollusk *Aplysia californica* and the freshwater snail *Lymnaea stagnalis* (Tomizawa and Casida, 2009). The *Aplysia* AChBP serves as a structural surrogate for the insect nAChR since it is sensitive to neonicotinoids whereas the *Lymnaea* AChBP is less sensitive to

neonicotinoids and therefore is used as a model for the vertebrate nAChR. Ligandreceptor interactions were elucidated by photoaffinity labeling and X-ray crystallography with the AChBPs. Neonicotinoids are coplanar between the substituted guanidine or amidine and the nitro or cyano moiety. This spatial orientation provides electronic conjugation and a partial negative charge on the nitro or cyano tip allowing neonicotinoids to interact with cationic residues (lysine, arginine or histidine) within the insect nAChR (Kagabu, 1997; Matsuda *et al.*, 2005; Tomizawa and Casida, 2005). Other important contacts within the nAChR binding site include the chloropyridinyl nitrogen of neonicotinoids as an H-bond acceptor and the chlorine as a hydrophobic contact (Kagabu, 2011).



Figure 1.8 Interaction of IMI at the nAChR (Figure from Tomizawa and Casida, 2003).

1.6.2 Structure-activity relationships

Neonicotinoids are more selective for the insect nAChR than mammalian due to critical pharmacophore differences between species. The mammalian nAChR has a conserved core of electron-rich aromatic amino acid residues within the extracellular loops (A-C from α subunits) preventing the electronegative tip of neonicotinoids from interacting with the receptor. However, nicotinoids, such as nicotine, are cationic and are consequently selective for the mammalian nAChR (Tomizawa and Casida, 2005). Neonicotinoid metabolites lacking the electronegative tip can become selective for the mammalian nAChR, particularly the $\alpha4\beta2$ subtype at the neuromuscular junction (**Fig. 1.9**). For example, conversion of the nitroguanidine substituent (of IMI, TMX, CLO and DIN) to the guanidine (e.g. IMI-NH) or aminoguanidine (e.g. IMI-NNH₂) is detoxifying in *Drosophila*, but is bioactivating in mammals by increasing the affinity for the vertebrate

 α 4 β 2 AChR (Kanne *et al.*, 2005). Removal of the cyano moiety of THI and possibly ACE results in the same increased interaction with the vertebrate nAChR causing neurotoxic agonist effects (Kanne *et al.*, 2005; Tomizawa and Casida, 2003). Insect versus mammalian nAChR-neonicotinoid interactions verify the importance of the electron-withdrawing nitro and cyano groups in producing a partial-positive charge on the imidazolidine neonicotinoid allowing selective interaction with the insect nAChR.



Figure 1.9 The electron-rich nitro or cyano moiety of neonicotinoids interacts with cationic amino acids of the insect nAChR. Metabolites lacking these moieties (IMI-NH) can interact with electron-rich amino acids of the mammalian nAChR (Figure from Tomizawa and Casida, 2005).

1.7 Neonicotinoid toxicity

1.7.1 Absorption, distribution and excretion of neonicotinoids

Neonicotinoids are rapidly absorbed in the intestine after oral exposure. For example, in orally-treated rats (20 mg/kg of ¹⁴C-IMI), IMI is quickly absorbed in the intestinal lumen followed by distribution from the plasma into the body (Klein, 1987a; Tomlin, 2003). The specific transporters involved in intestinal absorption of neonicotinoids are unknown. *In vitro* studies using human intestinal Caco-2 cells demonstrated that IMI and ACE are both absorbed in a concentration-dependent manner and cellular transport is not saturable (up to 200 μ M) (Brunet *et al.*, 2004, 2008). Since depleting sodium from the cell media did not affect IMI uptake, but the addition of sodium azide or trypsin did, it was concluded that IMI is transported by an ATP-dependent (and sodium-independent) protein (Brunet *et al.*, 2004). However, ACE is likely absorbed via a sodium-dependent transporter (Brunet *et al.*, 2008).

In general, neonicotinoids are rapidly distributed and excreted after oral exposure. For IMI, after oral exposure in rats (20 mg/kg), the distribution was followed by autoradiography on X-ray film from one to 48 h (Klein, 1987b). Once the radiolabel was absorbed, it was quickly distributed to tissues and organs (within one h for oral treatment and five min for intravenous injection), but levels dissipated within 24 h of treatment. This study revealed that IMI readily permeates most tissues except for fatty tissues, the CNS and the mineral part of bone. Highest concentrations of IMI were found in the kidney (indicating renal excretion as the major route), the thyroid gland and adrenals.

Various lines of evidence indicate rapid excretion of IMI. After rats were exposed intravenously to one mg/kg of ¹⁴C-IMI, 92% was excreted, primarily in the form of urine, within 48 h (Klein, 1987a). As further verification, when rats were treated orally with IMI, 96% was excreted in urine and feces within 48 h with more than 90% of the urinary excretion occurring within 24 h (Klein, 1987a). Approximately 15% of IMI is eliminated in the form of the parent compound (Tomlin, 2003). Metabolites in urine are discussed in section 1.5.2. TMX is also quickly absorbed, distributed and eliminated (mostly in urine). After exposure in rats, highest tissue concentrations were in skeletal muscle (10-15% of administered dose) and 84-95% was excreted in urine within 24 h (Environmental Protection Agency, 2007a).

1.7.2 Acute and chronic toxicity

There is a wide range of LD_{50} values of IMI depending on the route of exposure and the species as seen in **Table 1.2**. Toxic symptoms in rats and mice are primarily due to the agonistic effect on the nAChR resulting in unsteady or uncoordinated gait, reduced locomotion, trembling and spasms (Sheets, 1994). The no-observed adverse effect level (NOAEL) for these acute neurotoxic effects is estimated to be 42 mg/kg of IMI. For chronic toxicity (in rats), the NOAEL for IMI is 5.7-9.8 mg/kg/day (Tomizawa and Casida, 2005). **Table 1.2** Toxicological profiles in mammals, birds and fish of the neonicotinoids compared to nicotine (Table from Tomizawa and Casida, 2005).

		Mammal ^b		Bird ^f	Fish ^g
Compound	Acute oral ^c LD ₅₀ (mg/kg)	NOAEL ^d (mg/kg/day)	Carcinogen ^e	Acute oral LD ₅₀ (mg/kg)	LC ₅₀ (ppm)
Neonicotinoids					
Acetamiprid	182	7.1	No	180	>100
Clothianidin	>5000	9.8	No	>2000	>100
(±)-Dinotefuran	2400	127	No	>2000	>40
Imidacloprid	450	5.7	No	31	211
Nitenpyram	1628	_	_	>2250	>1000
Nithiazine	300	_	_		150
Thiacloprid	640	1.2	Yes	49	31
Thiamethoxam	1563	0.6	Yes	1552	>100
Nicotinoid					
(-)-Nicotine	50–60	_	_	Toxic	4

^a References are given by Tomizawa and Casida, 2005.

^b Dermal LD₅₀ values of neonicotinoids are >2000 to >5000 mg/kg (rat) except for (-)-nicotine 50 mg/kg (rabbit).

^c Average data for male and female rats with sex differences less than twofold.

^d NOAEL for chronic toxicity studies in rats. This value also applies to all adverse effects in chronic toxicity studies with mice and dogs.

^e Thiacloprid gives thyroid and uterine tumors in rats and ovary tumors in mice. Thiamethoxam gives heptocellular adenomas and carcinomas in male and female mice. They are considered to be likely human carcinogens.

¹ Japanese or bobwhite quail. ⁹ Rainbow trout or carp.

Neonicotinoids do not cause reproductive or developmental toxicity at low doses (Becker *et al.*, 1988). However, the reproductive NOAEL (based on decreased Wistar/ Han rat pup weight gain) of IMI in diet is 100 ppm (8 mg/kg/day) and the LOAEL is 250 ppm (19 mg/kg/day) (Suter *et al.*, 1990). IMI has been shown to have adverse effects such as decreased neurobehavioral performance on the offspring of ip-treated (337 mg/kg) Sprague-Dawley rats (Abou-Donia *et al.*, 2008). These offspring also develop altered binding patterns of brain proteins and increased AChE activity in the midbrain, cortex and brainstem. The effects seen in the offspring are mediated by a complex array of multiple pathways due to dysfunction in the CNS by IMI. The exact mechanism is thought to be due to an influx of Ca²⁺ ions upon activation of the nAChR followed by induction of neuronal apoptosis.

1.7.3 Genotoxicity and carcinogenicity

To assess genotoxicity of neonicotinoids, cytogenetic and chromosomal damage assays such as the reverse mutation, comet, micronucleus and sister chromatid exchange tests have been done with neonicotinoids prior to registration. Through these assays, neonicotinoids (with and without bioactivation by addition of the hepatic S9 fraction) do not appear to be genotoxic or clastogenic. However, IMI causes chromosomal damage *in vitro* in human peripheral blood lymphocytes at 0.05 mg/L and higher concentrations in a dose-dependent manner (Costa *et al.*, 2009; Feng *et al.*, 2005). Metabolic activation after addition of S9 to IMI slightly increases DNA damage (Costa *et al.*, 2009).

TMX and THI are the only two neonicotinoids shown to have carcinogenic effects. TMX is a hepatotoxicant and hepatocarcinogen in mice but not rats or dogs (Green *et al.*, 2005a,b; Tomizawa and Casida, 2005). In a study conducted by Syngenta Central Toxicology Laboratories, when mice were fed TMX (500-2500 ppm) daily for 18 months, an increased incidence of liver tumors was observed. The mode of action was determined to initially involve a reduction in plasma cholesterol (seen within one week), then single cell necrosis and increased apoptosis (by 10 weeks) followed by an increase in hepatic cell replication rates (by week 20) (**Fig. 1.10**). These adverse effects occurred in a dose-dependent manner above 500 ppm. The metabolite responsible for the carcinogenic effects of TMX was proposed to be dm-TMX since a similar chronic feeding experiment with this metabolite produced the same liver pathology (Green *et al.*, 2005a).



Figure 1.10 Sequence of hepatic physiological events in mice fed 2500 ppm TMX. % change refers to: cholesterol= % decrease compared to control; apoptosis and necrosis= % increase in the number of animals showing these effects. LI indicates reparative cell division (Figure from Green *et al.*, 2005a).
Liver microsomal metabolism is greater for mice than rats or humans in the production of TMX metabolites, dm-TMX, CLO and dm-CLO (**Fig. 1.11**) (Green *et al.*, 2005b). These early studies proposed that the mouse-specific adverse effects of TMX are due to dm-TMX production exacerbated by dm-CLO mimicking the structure of N^G-L-nitroarginine (L-NAME) (**Fig. 1.12**), a standard inhibitor of inducible nitric oxide synthase (iNOS) (Green *et al.*, 2005a). There are two other forms of NOS in mammals, neuronal (nNOS) and endothelial NOS all of which catalyze the formation of nitric oxide from L-arginine. Although nitric oxide has many biological functions, it has been shown to play a regulatory role in the development of hepatotoxicity (Kuo and Slivka, 1994). The degree of inhibition of iNOS and nNOS by dm-CLO and alternative hypotheses regarding TMX hepatotoxicity/ hepatocarcinogenicity are discussed in Chapter 4.



Figure 1.11 Previous proposed metabolism and mechanism of hepatotoxicity and hepatocarcinogenicity of TMX (formation of dm-TMX and inhibition of iNOS by dm-CLO) (Green *et al.*, 2005a).



Figure 1.12 Structural comparison between dm-CLO and L-NAME, a standard NOS inhibitor (Figure from Swenson and Casida, 2013b).

Although THI lacks the oxadiazinane substituent, it is also a carcinogen but the lesions are of a different type than those from TMX (Tomizawa and Casida, 2005). THI is currently classified as "likely to be carcinogenic to humans" by the Environmental Protection Agency based on increased uterine tumors in rats, thyroid follicular adenomas in rats and ovarian tumors in mice (Environmental Protection Agency, 2013).

1.8 Statement of the problem

Neonicotinoids currently account for approximately 25% of the total insecticide market share. They are the most important class of insecticides introduced to the market since synthetic pyrethroids and are now extensively used for crop protection, consumer/ professional products and animal health. As of 2009, IMI was the largest selling insecticide in the world (at U.S. \$1091 million) (Jeschke *et al.*, 2011). Worldwide use of neonicotinoids continues to expand as pest populations develop resistance to the once-widely-used pesticide classes including the organophosphates and methylcarbamates. The most commonly used neonicotinoids are IMI and TMX, the primary focus of these studies.

The overall goal is to further understand the metabolism of neonicotinoids relative to: *in vivo* importance of AOX, metabolic pathways of the novel neonicotinoid, cycloxaprid (CYC) and mechanisms of TMX hepatotoxicity and hepatocarcinogenicity. CYPs have been shown to be involved in *in vitro* and *in vivo* neonicotinoid metabolism, but the relative *in vivo* importance of AOX is unknown. AOX is implicated to play a role in the nitroreduction of *N*-nitroguanidine neonicotinoids, the most prominent subclass. There is considerable variability in the activity of AOX between species and individuals which may be reflected in differences in neonicotinoid metabolism and detoxification. Secondly, CYC is a new neonicotinoid that is under development to control IMI-resistant pests. However, its metabolic pathway has yet to be determined, particularly in reverting to its potent nAChR agonist precursor, nitromethylene-imidazole (NMI).

Finally, TMX is the only neonicotinoid to produce liver toxicity and tumors in chronically-treated mice, but not rats. Earlier studies concluded that formation of dm-TMX and iNOS inhibition by dm-CLO is likely the mechanism of TMX toxicity. Furthermore, differences in metabolic rates between species may explain the mouse-specific toxicity. However, the molecular mechanism of TMX or dm-TMX hepatotoxicity/ hepatocarcinogenicity remains unclear. It is critical to fully understand the metabolic/ enzymatic pathways of neonicotinoids and mechanisms of toxicity as their use continues to increase and for future pesticide design.

Chapter 2

Aldehyde Oxidase Importance in Imidacloprid Nitroreduction in Mice

* The work in this chapter has been previously published in Swenson, T.L., and Casida, J.E. (2013a). Aldehyde oxidase importance *in vivo* in xenobiotic metabolism: imidacloprid nitroreduction in mice. *Toxicol. Sci.* **133**, 22-28. Content is reproduced here by permission from Oxford University Press.

2.1 Introduction

The nitro substituent on neonicotinoids is important relative to their potency and selectivity for the insect nAChR. From the seven commercial neonicotinoids, approximately 100 metabolites have been identified in plants and mammals, some of which are bioactivated and can interact with the mammalian nAChR (*e.g.* IMI-NH) (Ford and Casida, 2006a, b, 2008). A number of studies have demonstrated the importance of CYPs in neonicotinoid metabolism *in vitro* and *in vivo* (Schulz-Jander and Casida, 2002; Shi *et al.*, 2009). However, the role of AOX in neonicotinoid metabolism has yet to be established *in vivo*, especially in the oxidative- and CYP-rich environment of the liver.

AOX is important in xenobiotic metabolism. This enzyme is expressed mainly in liver but is also present in many other tissues with variations in activity depending on species, gender, age, drug usage and disease states (Al-Salmy, 2002; Beedham, 1987; Garattini et al., 2008; Pryde et al., 2010). Tungsten (Rivera et al., 2005) or hydralazine (Critchley et al., 1994; Johnson et al., 1985) in the diet or drinking water results in reduced AOX activity in guinea pigs, rabbits and mice. There are even notable differences in AOX activity between strains of mice (Al-Salmy, 2002), e.g. compared to CD-1 mice, the DBA/2 strain is deficient in the expression of AOX homologue 1 (AOH1) and homologue 2 and has reduced expression of AOX1 (Vila et al., 2004). Since AOH1 and AOX1 are the primary AOX genes expressed in mouse liver (Garattini et al., 2008), DBA/2 mice are an appropriate AOX-deficient model for studies on in vivo mammalian xenobiotic metabolism. The wide range of inter- and intra-species AOX activity may result in different rates of neonicotinoid metabolism and detoxification in mammals and insects. Despite the increasing significance of AOX, there have been very few studies examining the in vivo contribution of this enzyme to xenobiotic metabolism. Mice can serve as a surrogate for humans since AOX activity in IMI nitroreduction in vitro is comparable between these two species (Dick et al., 2005). This study uses chemical inhibitors and genetic deficiency for mice and Drosophila melanogaster to evaluate the relevance of AOX in neonicotinoid metabolism in vivo.

2.2 Materials and methods

2.2.1 Chemicals

IMI, THI, NMN, sodium tungstate dihydrate, hydralazine hydrochloride, *p*-dimethylaminocinnamaldehyde (DMAC) and 7-ethoxycoumarin were from Sigma-Aldrich (St. Louis, MO). Phosphate-buffered saline, pH 7.4 (PBS) was from Invitrogen (Grand Island, NY).

2.2.2 Organisms

AOX-expressing systems were compared to AOX-inhibited or -deficient systems (mice and Drosophila) in IMI metabolism. Male Swiss Webster (25-35 g), DBA/2 (20-21 g) and CD-1 (30-38 g) mice were obtained from Charles River Laboratories (Wilmington, MA). In all of the following studies, mice were housed and maintained according to the National Research Council Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) and procedures were performed under an Institutional Animal Care and Use Committee-approved protocol. Animals were housed in a temperature-controlled room (18-26°C) under a 12-h light-dark cycle. Food and water were provided ad libitum. Experiments involved three mouse treatment sets: 1) Swiss Webster (control) versus tungsten-treated Swiss Webster, 2) Swiss Webster (control) versus hydralazine-treated Swiss Webster and 3) CD-1 (control) versus DBA/2 (AOX-deficient). All mice within each treatment set were the same age and involved the same number of mice per treatment (n= 5 or 13). Swiss Webster mice were employed in sets one and two since this strain was used in earlier in vivo IMI metabolism studies (Ford and Casida, 2006a). CD-1 mice were employed as controls for comparison with DBA/2 mice in set three since these two strains were used in earlier studies in comparing AOX expression (Vila et al., 2004). AOX-expressing (AOX^{+/+}) (wild-type Oregon R strain) and AOX-deficient (AOX^{-/-}) (ry² Po^{lpo} Aldox-1ⁿ¹ Sb^{sbd-2}) Drosophila were obtained from Carolina Biological Supply Company (Burlington, NC).

2.2.3 Mouse studies: treatments

Swiss Webster mice were used for studies involving tungsten or hydralazine treatment compared with controls. For the tungsten study, control mice were given regular drinking water and the treatment group was given drinking water supplemented with tungsten (0.7 mg/mL) for 14 days. For the hydralazine study, control mice were given drinking water containing 5 mM potassium phosphate, pH 6 and the treatment group was given a solution of hydralazine hydrochloride (0.1 mg/mL) in 5 mM potassium phosphate, pH 6 for 7 days. DBA/2 and CD-1 mice received regular drinking water. Following these treatment schedules, the mice were administered either IMI (ip, 10 mg/kg) in dimethylsulfoxide (DMSO) (1 μ L/g body weight) or carrier solvent alone. Livers were removed one h after IMI or DMSO treatments and analyzed for AOX activity, CYP activity and IMI metabolite levels as described below.

2.2.4 Liver enzyme assays

Mouse liver cytosol and microsomes were prepared by homogenizing liver (250 mg) in ice-cold PBS (1.7 mL) using a Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) followed by centrifugation of the homogenate at 1,000g for 10 min and then the supernatant at 10,000g for 30 min. An aliquot of the 10,000g supernatant was recovered for AOX activity analysis and the remainder was centrifuged at 100,000g for 1 h to collect the CYP-containing microsomal pellet fraction which was resuspended in PBS for protein measurement (Bradford, 1976) and the CYP activity assay.

The oxidative activity of AOX was assayed spectrophotometrically using DMAC as the substrate (Maia and Mira, 2002). Mouse liver cytosol (15 μ L, 14-20 mg/mL protein) was added to 50 μ M DMAC solution (200 μ L in PBS) and the reaction monitored by an absorbance decrease using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA) at 398 nm for 5 min with an average control value of -18.4 mOD/min.

7-Ethoxycoumarin is a broad-specificity substrate used to measure the activity of many CYP enzymes by monitoring the oxidation to 7-hydroxycoumarin (Waxman and Chang, 2007). Microsomes (20 µL, 8 mg/mL protein in PBS) were mixed with 50 mM 7ethoxycoumarin (4 µL in methanol) in assay buffer (156 µL, 100 mM potassium phosphate, pH 7.4 containing 20% (v/v) glycerol and 0.1 mM EDTA) and prewarmed at 37°C for 5 min. After addition of 10 mM NADPH (20 µL in assay buffer), reactions were incubated at 37°C for 30 min in a shaking water bath. Ice-cold 2 M HCI (25 µL) was added to stop reactions and the mixture was vortexed and placed on ice. Samples were extracted with chloroform (450 µL), briefly vortexed, then centrifuged at 3,000g for 5 min. The organic phase (bottom layer) was removed (300 µL) and added to 30 mM sodium borate (1 mL, pH 9.2) and vortexed. Following centrifugation at 3,000g for 5 min, the upper layer was recovered and plated (200 µL) on a Costar 96-well black plate and fluorescence read at an excitation wavelength of 370 nm and an emission wavelength of 460 nm using a SpectraMax M2 Microplate Reader (Molecular Devices, Sunnyvale, CA) with an average control value of 11.2 nmol 7-hydroxycoumarin/mg protein.

2.2.5 Liver IMI metabolite analysis

Metabolites were recovered for analysis by homogenizing liver (500 mg) in icecold acetonitrile (ACN) (2 mL containing 10 nmol THI as an internal standard) and centrifuging at 900*g* for 15 min. The supernatant was evaporated to dryness under nitrogen at 25°C, resuspended in 10:90:0.1 ACN/water/formic acid (HCO₂H) (300 μ L) and filtered through 0.2 μ m nylon for LC/MS analysis.

For metabolite analyses in all experimental groups (except tungsten and its control set), an Agilent 1100 series LC was used with a Luna C-18 column (250 x 2.0 mm, 5 μ m) and a Waters LCT Premier XE mass spectrometer. Electrospray ionization was in the positive mode with source parameters as follows: capillary voltage 1300 V; sampling cone voltage 54 V; source temperature 90°C; dessolvation temperature

200°C. The mobile phase consisted of ACN/water containing 0.1% HCO_2H beginning with 5% ACN for 3 min and increasing to 100% by 25 min at a flow rate of 0.2 mL/min. A final 10 min wash with 5% ACN eluted interfering materials.

For the tungsten set, metabolites were quantified by selected reaction monitoring using an Agilent G6410B QQQ instrument with a Gemini reverse-phase C-18 column (50 mm x 4.6 mm, 5 µm). For LC separation, mobile phase A consisted of 95:5 water:methanol and mobile phase B consisted of 60:35:5 isopropanol:methanol:water, both containing 0.1% HCO₂H. Samples (10 µL) were injected into the LC starting with a flow rate of 0.1 mL/min at 0% B for 5 min and increasing to 100% B (at 0.4 mL/min) by 20 min, held for 8 min (at 0.5 mL/min) followed by 0% B from 28 to 35 min. t_R values for all analytes were verified with analytical standards. IMI nitroreduction and oxidation metabolite levels were quantified by comparing peak areas with the THI internal standard.

2.2.6 Drosophila studies: in vitro metabolism and analysis

Drosophila were frozen at -80°C and homogenized using a mortar and pestle (120 mg/ 1 mL ice-cold PBS). The AOX-containing supernatant was collected after centrifugation at 16,000*g* for 30 sec. To verify $AOX^{-/-}$ Drosophila had negligible AOX activity, cytosol was assayed with DMAC. In order to obtain detectable levels of IMI reductive metabolites, saturating conditions of IMI and the cofactor, NMN, were used (Dick *et al.*, 2006). An aliquot of the cytosolic supernatant (1 mg protein) was incubated with IMI (1 mM) and NMN (10 mM) in PBS (200 µL total volume) for 20 min at 37°C in a shaking water bath. Ice-cold ACN (400 µL containing 10 nmol THI as an internal standard) was added to terminate reactions and incubated on ice for 10 min. Following centrifugation at 16,000*g* for 5 min, the supernatant was evaporated to dryness under nitrogen and analyzed by LC/MS.

2.2.7 *Drosophila* sensitivity

Drosophila adults (15-20) were placed in glass test tubes (16 x 100 mm) containing filter paper strips (10 x 80 mm) and covered with parafilm. Solutions of IMI (5 μ g in 50 μ L water) were injected through the parafilm onto the filter paper of each vial and *Drosophila* were monitored for adverse effects (twitching, immobilization or death) from 15 to 165 min. The percentage of adversely affected *Drosophila* was used to determine sensitivity to IMI.

2.2.8 Statistical analysis

Within each mouse treatment set, experiments were performed in at least triplicate (including controls) and reported as percent of control (mean) \pm standard error (SE). Significant differences between AOX-expressing and AOX-inhibited or -deficient groups were analyzed by Student's *t*-test using Microsoft Excel. A *p* value < 0.05 was considered statistically significant. For the correlation analyses, the Pearson correlation coefficient (r) and r² were calculated using R software (version 2.15.2).

2.3 Results

2.3.1 AOX activity in tungsten- or hydralazine-treated mice and the DBA/2 strain

The first goal was to generate or obtain mice with reduced cytosolic AOX activity but normal microsomal CYP activity. Two diagnostic inhibitors, tungsten and hydralazine, were evaluated in 7- or 14-day treatments for *in vivo* AOX inhibition by measuring the oxidation of DMAC by liver cytosol. Tungsten or hydralazine treatment did not result in any signs of apparent toxicity or changes in body weight or water consumption. Cytosolic AOX activity in tungsten (14-day drinking water) and hydralazine (7-day drinking water) treated mice was significantly reduced: 45±4% and 61±3% less than control mice, respectively (**Fig. 2.1**). DBA/2 mice, a strain known to be deficient in liver AOX activity, had significantly lower (81±2% less) liver cytosolic AOX activity compared to CD-1 mice (**Fig. 2.1**). In further studies, two specific and potent *in vitro* AOX inhibitors, raloxifene and menadione (Obach *et al.*, 2004), did not reduce AOX activity in Swiss Webster mice as analyzed 15-90 min after ip treatment (25-40 mg/kg raloxifene; 25 mg/kg menadione). Additionally, IMI treatment (ip, 10 mg/kg for 1 h) of Swiss Webster, CD-1 or DBA/2 mice did not affect AOX activity compared to mice treated with carrier solvent alone.



Figure 2.1 Effect of tungsten or hydralazine treatment or AOX-deficiency (DBA/2 mice) compared to control mice (Swiss Webster given regular drinking water or CD-1 mice) on liver cytosolic AOX activity using DMAC as the substrate. Values are presented as mean \pm SE as percent of the control. n=5 (tungsten or AOX-deficient), n=13 (hydralazine). **p < 0.01 or ***p < 0.001 compared to control.

2.3.2 CYP activity in tungsten- or hydralazine-treated mice and the DBA/2 strain

To evaluate if the candidate AOX inhibitor treatments or mouse strain differences affected CYP activity, the conversion of 7-ethoxycoumarin to 7-hydroxycoumarin was monitored in liver microsomal fractions. Tungsten or hydralazine treatment had little or no significant effect on liver microsomal CYP activity compared to control mice (**Fig. 2.2**). CYP activity was also not significantly different between DBA/2 and CD-1 mice (**Fig. 2.2**) or from IMI treatment as above.



Figure 2.2 Effect of tungsten or hydralazine treatment or AOX-deficiency (DBA/2 mice) compared to control mice (Swiss Webster given regular drinking water or CD-1 mice) on liver microsomal CYP activity using 7-ethoxycoumarin as the substrate. Values presented as mean \pm SE as percent of the control. n=5 (tungsten or AOX-deficient), n=13 (hydralazine). Differences are not significant.

2.3.3 IMI-NNO and IMI-NH as nitroreduction metabolites

To test if the mice with reduced AOX activity also had decreased metabolism of the IMI nitroguanidine substituent, both reduction and oxidation metabolites in liver were analyzed by LC/MS (**Figs. 2.3** and **2.4**). For this study, IMI nitroreduction metabolites included IMI-NNO and IMI-NH. Although studies by Dick *et al.* (2005) reported *in vitro* AOX-catalyzed IMI nitroreduction to IMI-NNO and IMI-NNH₂, the latter metabolite was not detected here by LC/MS analysis likely due to its high reactivity with aldehyde-containing solvent impurities or liver components (Dick *et al.*, 2005, 2006). Oxidation metabolites of IMI included IMI-5-OH and IMI-ole (**Fig. 1.4**). A further nitroreduction metabolite, IMI-urea was consistently detected in *Drosophila in vitro* reactions.



Figure 2.3 AOX-produced IMI nitroreduction metabolites include IMI-NNO, IMI-NNH₂, IMI-NH and IMI-urea. IMI-NNO, IMI-NH and IMI-urea (*Drosophila* only) were analyzed in this study.



Figure 2.4 Representative LC/MS chromatograms of liver metabolites from AOX (CD-1) mice versus AOX-deficient (DBA/2) mice showing (**A** and **B**) differences in AOXdependent reductive metabolites (IMI-NH *m/z* 211, t_R 8.0 min; IMI-NNO *m/z* 240, t_R 20.9 min) and (**C** and **D**) similarities in AOX-independent oxidative metabolites (IMI-ole *m/z* 254, t_R 21.3 min; IMI-5-OH *m/z* 272, t_R 21.9 min).

2.3.4 Nitroreduction and oxidation IMI metabolites in tungsten- or hydralazine-treated mice and the DBA/2 strain

Tungsten and hydralazine treatments not only resulted in significantly less AOX activity (**Fig. 2.1**) but also decreased IMI nitroreduction. Tungsten treatment resulted in 30±15% less IMI-NNO and 55±6% less IMI-NH production and hydralazine treatment resulted in 56±5% less IMI-NNO and 65±5% less IMI-NH relative to controls (**Fig. 2.5**). Compared to CD-1 mice, DBA/2 mice formed 86±1% less IMI-NNO and 81±3% less IMI-NH (**Fig. 2.5**). All differences were significant relative to controls except the IMI-NNO levels after tungsten treatment. Levels of IMI and IMI oxidation metabolites, IMI-5-OH and IMI-ole, were not significantly affected by either hydralazine or tungsten treatment or in DBA/2 mice (**Fig. 2.6**). This strain difference in reduction versus oxidation is readily apparent on comparing the LC/MS chromatograms in **Fig. 2.4**.



Figure 2.5 Effect of tungsten or hydralazine treatment or AOX-deficiency (DBA/2 mice) compared to control mice (Swiss Webster given regular drinking water or CD-1 mice) on IMI nitroreduction to IMI-NNO and IMI-NH. Values are presented as mean \pm SE as percent of the control. n=4 (tungsten or AOX-deficient), n=11 (hydralazine). *p < 0.05 or **p < 0.01 compared to control.



Figure 2.6 Effect of tungsten or hydralazine treatment or AOX-deficiency (DBA/2 mice) compared to control mice (Swiss Webster given regular drinking water or CD-1 mice) on IMI levels and IMI oxidation to IMI-5-OH and IMI-ole. Values are presented as mean \pm SE as percent of the control. n=4 (tungsten or AOX-deficient), n=11 (hydralazine). Differences are not significant.

2.3.5 IMI metabolism and sensitivity in Drosophila

Other studies considered if insect AOX is important in IMI nitroreduction and detoxification. *Drosophila* were used as a model organism to define IMI metabolism and sensitivity. When tested for AOX activity using the DMAC assay, AOX^{-/-} *Drosophila* had less than one percent of the activity of AOX^{+/+} insects. Incubations of IMI and NMN with homogenate cytosol from AOX^{+/+} or AOX^{-/-} *Drosophila* produced comparable levels of IMI nitroreduction metabolites (IMI-NNO, IMI-NH and IMI-urea) (**Fig. 2.7**). IMI metabolite levels were independent of NMN further verifying that their formation was not via AOX. For sensitivity assays, 5 µg IMI was chosen as a discriminating dose resulting in intermediate toxicity (symptoms) that could be easily monitored over time. Although there was considerable variability in response, there was no significant difference between AOX^{+/+} and AOX^{-/-} *Drosophila* in the sensitivity to IMI (**Fig. 2.8**).



Figure 2.7 Comparison of AOX^{+/+} and AOX^{-/-} *Drosophila* in *in vitro* metabolism of IMI to nitroreduction metabolites IMI-NNO, IMI-NH, IMI-urea. Differences are not significant.



Figure 2.8 Comparison of AOX^{+/+} and AOX^{-/-} *Drosophila* in sensitivity on exposure to 5 μ g IMI from 15 to 165 min. Differences are not significant.

2.4 Discussion

AOX is a potentially important factor in drug metabolism with many studies examining its *in vitro* inhibition and the proposed effects on xenobiotic action (Garattini *et al.*, 2008; Obach, 2004; Pryde *et al.*, 2010). There is a wide range of AOX activity between species with rabbits, monkeys and humans the highest, mice intermediate and rats and dogs having the lowest activity (Pryde *et al.*, 2010). This same species-dependent relationship is also observed for *in vitro* IMI nitroreduction by liver cytosol (Dick *et al.*, 2005).

Tungsten and hydralazine treatments provide a way to reduce AOX activity *in vivo* in mammals to evaluate its relevance in xenobiotic metabolism (Critchley *et al.*, 1994; Johnson *et al.*, 1985; Rivera *et al.*, 2005). Tungsten replaces molybdenum at the active center of AOX, rendering it inactive (Rivera *et al.*, 2005), but the mechanism of AOX inactivation by hydralazine is unknown (Johnson *et al.*, 1985). The goal of this study was to reduce AOX activity without affecting CYP activity *in vivo*. The level of AOX inhibition by tungsten treatment in mice (45%) was less than that by hydralazine (61%), a difference reflected in their effect on IMI metabolism. Hydralazine treatment resulted in significantly reduced IMI metabolism to IMI-NNO and IMI-NH, but tungsten treatment only significantly reduced IMI metabolism to IMI-NH.

There are four AOX genes in mice with two of the variants being expressed in the liver, *AOH1* and *AOX1*. DBA/2 mice are completely deficient in the expression of *AOH1* and have low expression of *AOX1* compared to CD-1 mice (Vila *et al.*, 2004). Our data also establish that DBA/2 mice have significantly lower AOX activity in the liver and further show that the reduced AOX activity decreased IMI metabolism to IMI-NNO and IMI-NH, but not to IMI-5-OH or IMI-ole.

The AOX-generated IMI metabolites are not all detoxification products. IMI-NH is a likely contributor to the nicotinic effects of IMI. It is over 300 times more potent than IMI at the mammalian nAChR (vertebrate $\alpha4\beta2$ IC₅₀= 8.2 nM for IMI-NH; 2600 nM for IMI) and the mouse ip toxicity is also increased several fold (Chao and Casida, 1997; Tomizawa and Casida, 2003, 2005). IMI-NNO retains insecticidal activity (Nauen *et al.*, 1998) and as an *N*-nitroso compound it was subjected to extensive toxicological tests and cleared of potential problems (Advisory Committee on Pesticides, 1993). Our study concludes that reduced AOX activity is tightly correlated with reduced IMI metabolism to IMI-NNO and IMI-NH (**Fig. 2.9**) indicating that these products are mostly from AOX, not CYPs. Based on the metabolic sequence and relevant correlations, IMI-NH is mostly formed via IMI-NNO rather than another pathway.



Figure 2.9 Relationship between reduced AOX activity and **(A)** reduced IMI-NNO and **(B)** reduced IMI-NH formation in mice treated ip with IMI. Each data point represents one mouse. n= 18. r = Pearson's correlation coefficient. Three data points for IMI-NNO and one for IMI-NH indicated by open symbols are most divergent from the plotted correlation lines. When these data points are removed, the correlation coefficients given in the figures are improved to r = -0.94, r² = 0.88 for IMI-NNO and r = -0.76, r² = 0.58 for IMI-NH.

Insect AOX is implicated in insecticide detoxification and resistance (Hemingway *et al.*, 2000). However, AOX^{+/+} and AOX^{-/-} *Drosophila* did not display any differences in IMI nitroreduction or sensitivity. These limited data suggest a greater importance of AOX for the nitroreduction of IMI in mice rather than *Drosophila*. This species specificity is not surprising since the proposed *Drosophila* orthologs to mouse *AOX1* and *AOH1* (Garattini *et al.*, 2008) have 30% or less sequence identity (online DRSC Integrative Ortholog Prediction Tool) (Hu *et al.*, 2011) which may contribute to differences in substrate selectivity and activity.

AOX is the most important mouse IMI hepatic nitroreductase *in vivo*. Mouse AOX activity is similar to that of humans in metabolizing IMI and some other substrates and therefore is a preferred model for human AOX. Although these studies used ip treatment, IMI-NH and IMI-NNO are produced *in vivo* when rats or mice are exposed to IMI orally (Advisory Committee on Pesticides, 1993) and may be relevant to people with dietary exposure. Differences in AOX expression and nitroreduction may result in species differences in toxicity and residue dissipation. This is the first conclusive evidence of AOX, not just CYP, involvement in neonicotinoid metabolism *in vivo* and more generally is also one of the first studies showing the toxicological importance of AOX in *in vivo* xenobiotic metabolism.

Chapter 3

Cycloxaprid and Nitromethylene-Imidazole Metabolism in Mice

3.1 Introduction

China is a critical pesticide producer and user and plays an especially important role in the research and development of neonicotinoids (Shao *et al.*, 2013). A recent discovery, CYC, is among a set of novel neonicotinoids under development for practical use (Qian *et al.*, 2010, Shao *et al.*, 2010, 2011a,b). It is proposed to protect crops against IMI-resistant pests including the brown planthopper by binding to and activating a different site on the nAChR (Cui *et al.*, 2012; Shao *et al.*, 2010, 2011a). The unique interaction of CYC with the nAChR is due to the nitro substituent being in the *cis* configuration whereas in all other commercial neonicotinoids the nitro is in the *trans* configuration (Cui *et al.*, 2012; Shao *et al.*, 2011a,b). However, CYC is hydrolyzed to its precursor, NMI (Xusheng Shao, personal communication). NMI and similar nitromethylene neonicotinoid analogs are potent nAChR agonists in insects with lower potency in mammals (Tomizawa *et al.*, 2001). The metabolic pathways of CYC in mammals, particularly relative to NMI, are unknown. Here we compare the *in vivo* metabolites of CYC and NMI in mice at 15 and 120 min after ip treatment to identify the metabolic routes and the extent of CYC metabolism to NMI.

3.2 Methods

3.2.1 Mice treatment

Male Swiss Webster mice (25-35 g) were administered either CYC (ip, 20 mg/kg) or NMI (ip, 3 mg/kg) in DMSO (1 μ L/g body weight) and tissues were collected by the procedures of Ford and Casida (2006a,b). The lower dose of NMI was used to reduce adverse nicotinic symptoms. Blood was collected by cardiac puncture 15 or 120 min after treatment then mice were euthanized by cervical dislocation. Livers and brains were harvested, flash frozen in liquid nitrogen and stored in -80°C until metabolite analysis. Plasma was isolated from blood samples by centrifuging at 5000*g* for 10 min at 4°C and stored in -80°C until analysis. Some CYC- or NMI-treated mice were kept in glass metabolism cages to collect excreta (urine and feces) for up to 24 h.

3.2.2 Sample preparation

Metabolites were recovered for analysis by homogenizing liver (500 mg), whole brain (350-450 mg), plasma (100 μ L), urine (200 μ L) or feces (100 mg) in ice-cold ACN (750 μ L containing 10 nmol THI as an internal standard) using a Sonic Dismembrator. THI was not added to urine or feces. Samples were centrifuged at 900*g* for 15 min and the supernatant evaporated to dryness under nitrogen at 25°C. Residues were resuspended in 5:95 ACN/ 5 mM NH₄OH, pH 9 buffer (250 μ L) and filtered through 0.2 μ m nylon for LC/MS analysis.

3.2.3 Metabolite analysis

CYC and NMI metabolites were analyzed on an Agilent 1100 series LC with a Phenomenex Kinetex XB-C18 column (100 x 2.10 mm, 2.6 μ m) and a Waters LCT Premier XE mass spectrometer. Electrospray parameters were the same as indicated in section 2.2.5. The LC/MS mobile phase (pH 9) was selected to minimize aqueous breakdown of CYC to NMI. The mobile phase consisted of ACN/ 5 mM NH₄OH, pH 9 beginning with 5% ACN for 3 min and increasing to 100% by 25 min at a flow rate of 0.18 mL/min. A final 10 min wash with 5% ACN eluted interfering materials. Levels were quantified by comparing peak areas with the internal standard, THI.

3.2.4 Metabolite structure assignments and quantitation

Metabolite structures were assigned based on calculated *m/z* values, characteristic chlorine isotope patterns and absence of the peak(s) in DMSO-treated control mice. The structures are based on analogy with established neonicotinoid metabolic pathways (Ford and Casida, 2006a,b). Metabolite levels were quantified based on standard curves obtained from CYC or NMI standards versus THI and calculated using GraphPad Prism (version 6.0). CYC levels much higher than any of the metabolites were not quantitated.

3.3 Results

3.3.1 CYC metabolism

CYC metabolites detected in liver were primarily oxidation products assigned as isomers of hydroxyl addition in the 6-, 7-, 10- or 11- position (**Fig. 3.1**). The next most abundant product was NMI followed by small amounts of the diol ((OH)₂-CYC), nitroso-CYC (NO-CYC) and amino-CYC (NH₂-CYC) (**Figs. 3.2** and **3.3**, **Table 3.1**). CYC and all metabolite levels decreased from 15 to 120 min and no metabolites were detected by 24 h with the exception of NMI (less than 2% of the level observed at 15 min).



Figure 3.1 Metabolites of (R/S)-CYC and NMI in mice. Numbers in the (R/S)-CYC structure refer to ring positions. Mono- and dihydroxylation sites are arbitrarily shown as carbons 10 or 11 or both although carbons 6 and 7 are also possible. Sites of metabolite detection: I= liver, b=brain, p=plasma.

¹ The number of possible isomers are four for OH-NMI and eight for OH-(R/S)-CYC and (OH)₂-(R/S)-CYC. ² OH-NMI and NO-NMI were detected in NMI- but not CYC-treated mice.



Figure 3.2 Representative LC/MS chromatogram of CYC metabolites in mouse liver 15 min after CYC ip administration.



Figure 3.3 CYC and CYC metabolite levels in mouse liver, brain and plasma 15 and 120 min after CYC ip administration at 20 mg/kg. The CYC level was higher than any metabolite and was not quantitated at 15 or 120 min for liver and at 15 min for brain and plasma. OH-CYC (b) was not observed in brain.

Table 3.1	Metabolites of CYC and NMI in mice	

	LC/MS		detection sites ¹	
Compound	m/z	t _R (min)	15 min	120 min
CYC treatment				
CYC	322.6	13.5	l, b, p	l, b, p
NMI	254.7	8.5	l, b, p	l, b, p
OH-CYC ²				
а	338.6	7.0	I	
b	338.6	10.7	l, p	I
С	338.6	12.0	l, b, p	l, b, p
d	338.6	19.6	l, b, p	l, b, p
е	338.6	20.0	I	I
(OH) ₂ -CYC	354.5	6.3	I	I
NO-CYC	306.6	7.1	I	
NH ₂ -CYC	292.6	8.3	I	I
NMI treatment				
NMI	254.7	8.5	l, b, p	l, b, p
OH-NMI	270.6	5.4	l	-
NO-NMI	238.7	3.3	I	I

¹ Sites of metabolite detection: I= liver, b=brain, p=plasma

² Multiple peaks (a-e) of m/z 338.6 refer to different OH-CYC isomers

Metabolites detected in the brain of CYC-treated mice included NMI and two hydroxyl (CYC m/z +16) products with retention times corresponding to OH-CYC (c) and OH-CYC (d) found in liver (**Fig. 3.3**, **Table 3.1**). NMI levels remained after 120 min, but hydroxyl product levels decreased. No metabolites were detected at 24 h.

Major plasma metabolites included NMI and three hydroxyl products with retention times corresponding to OH-CYC (b), OH-CYC (c) and OH-CYC (d) found in the liver (**Fig. 3.3, Table 3.1**). NMI levels remained at 120 min, but hydroxylation product levels decreased. Finally, no metabolites were detected in plasma at 24 h. Urine contained CYC, NMI, 4 hydroxylation products, (OH)₂-CYC, NO-CYC, NH₂-CYC, one NMI hydroxylation product (OH-NMI) and one peak of a mass corresponding to loss of an oxygen. Feces contained all the products identified in urine except only two hydroxylation products were found.

3.3.2 NMI metabolism

When mice were administered NMI, only the parent compound, OH-NMI and one nitroso product (NO-NMI) were found in liver (**Fig. 3.1**, **Table 3.1**). Only the parent compound (NMI) was identified in brain and plasma. All compound levels were greatly decreased by 120 min. Urine contained both NMI and OH-NMI, while feces only had NMI.

3.4 Discussion

This is the first report on the metabolism of CYC with an emphasis on in vivo metabolites in mice at 15 and 120 min post-treatment. Metabolites are not conclusively identified since synthetic standards were not available. However, based on calculated m/z values and characteristic chlorine isotope patterns, it is concluded that CYC is converted in part to NMI, but is mostly oxidized to multiple mono- and dihydroxylation products within 15 min and dissipate by 120 min in liver. There are many possible isomeric CYC oxidation products from hydroxylation on the 6-, 7-, 10- or 11- position in each case with two possible stereoisomers of which five are detected as distinct peaks by LC/MS. Minor products include (OH)₂-CYC and nitroreduction on the nitro group to NO-CYC and NH₂-CYC. CYC is readily hydrolyzed to NMI. Therefore, extraction and analytical conditions were carefully chosen to limit degradation. However, minor amounts of NMI detected may be due to degradation of CYC rather than its in vivo metabolism (e.g. 24 h liver samples). NMI per se was not extensively metabolized. Only minor amounts of one hydroxylation and one nitroso product were evident. Detection methods may limit observing the extent of metabolism since only 3 mg/kg was administered to mice (compared to 20 mg/kg for CYC).

The findings reported here lay the background for future studies on characterization of metabolites and *in vitro* species comparisons. *In vitro* conditions will allow detection of more metabolites (by using saturating conditions) and the enzymes responsible for their formation can be determined (by comparing reactions with and without added cofactor).

Chapter 4

Formaldehyde Generation as a Possible Mechanism of Mouse-Specific Hepatotoxicity/ Hepatocarcinogenicity of Thiamethoxam

* A portion of this work presented in this chapter has been previously published in Swenson, T.L., and Casida, J.E. (2013b). Neonicotinoid formaldehyde generators: possible mechanism of mouse-specific hepatotoxicity/ hepatocarcinogenicity of thiamethoxam. *Toxicol. Lett.* **216**, 139-145. Content is reproduced here with permission from Elsevier Ltd.

4.1 Introduction

TMX undergoes metabolic activation to CLO in insects, plants and mice (Casida, 2010; Ford and Casida, 2006, 2008; Honda *et al.*, 2006; Nauen *et. al.*, 2003). The neonicotinoids generally have favorable mammalian toxicology with the exception of TMX which is a hepatotoxicant and hepatocarcinogen in mice but not rats or dogs (Green *et al.*, 2005a,b; Tomizawa and Casida, 2005). The mechanism of this TMX- and mouse-specific hepatotoxicity/ hepatocarcinogenicity is of considerable interest relative to neonicotinoid risk assessment. Green *et al.* (2005b) observed that liver microsomal metabolic rates are greater for mouse than rat or human in the production of TMX metabolites, dm-TMX, CLO and dm-CLO. They also found that mouse-specific adverse effects of TMX are due to dm-TMX exacerbated by dm-CLO which mimics the structure of L-NAME, a standard inhibitor of iNOS, an enzyme with a regulatory role in the development of hepatotoxicity (**Fig. 1.12**)(Green *et al.*, 2005a).

The structure-activity relationships of neonicotinoids as hepatotoxicants or hepatocarcinogens help focus mechanistic hypotheses on specific molecular substituents (**Fig. 4.1** and **Table 4.1**). TMX and dm-TMX are hepatotoxicants/ hepatocarcinogens and contain the oxadiazinane substituent uniquely among the neonicotinoids so this moiety is of particular interest. *N*-Methyl substituents on five of the neonicotinoids are not the hepatotoxic moiety because dm-TMX lacks this group. The Green *et al.* (2005a) hypothesis is that dm-TMX is the hepatotoxicant exacerbated by dm-CLO as an iNOS inhibitor. The present study examines an alternative hypothesis that the unique aspect of the oxadiazinane moiety is its metabolic conversion to HCHO and *N*-methylol intermediates (**Fig. 4.2**), the ultimate hepatotoxicants and hepatocarcinogens which may be synergized by dm-CLO as a NOS inhibitor.



Figure 4.1 Neonicotinoids examined with arrows to designate sites of CYP oxidation (Casida, 2011) leading to *N*-methylol or HCHO liberation from oxadiazinane and/or *N*-methyl substituents.

Table 4.1 Structural features of neonicotinoids as hepatotoxicants/ hepatocarcinogens

 in mice and HCHO generators with mouse liver microsomal CYPs.

Neonicotinoid	Moiety Oxadiazinane	/ N-Methyl	Hepatotoxicant/ hepatocarcinogen	HCHO generator [⊳]
ТМХ	+	+	++	+++
dm-TMX	+	-	++	++
CLO	-	+	-	+
DIN	-	+	-	+/-
ACE	-	+	-	+
NIT	-	+	-	+/-
IMI	-	-	-	+/-
THI	-	-	+ ^a	+/-

^a Lesions induced by THI are of a distinctly different type and localization (Tomizawa and Casida, 2005).

^b Based on this study (Section 4.3.2)



Figure 4.2 Potential *N*-methylol and other HCHO generating intermediates in the CYP conversion of TMX to dm-TMX, CLO and dm-CLO. Proposed *N*-methylol intermediates at the right in the bracketed region have m/z values of 280 (R= CH₃) and 266 (R= H) for *N*-methylols and 308 (R= CH₃) and 294 (R= H) for *N*-methylol formamides.

4.2 Materials and methods

4.2.1 Chemicals

Sources for the neonicotinoids were as follows: TMX, ACE, and NIT from Sigma-Aldrich (St. Louis, MO); CLO and DIN from Valent (Walnut Creek, CA); dm-TMX from Novartis (Basel, Switzerland); dm-CLO was synthesized in the ECTL (Berkeley, CA).

4.2.2 In vitro NOS inhibition

iNOS inhibition by dm-CLO (0-1000 μ M) and L-NAME (0-1000 μ M) was analyzed by a high-throughput oxymyoglobin assay by following the procedure of Dawson and Knowles (1999). To determine nNOS inhibition, HEK293 cells overexpressing rat nNOS (Fang and Silverman, 2009) were cultured in Dulbecco's modified Eagle's medium containing 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.4 mg/mL geneticin (Invitrogen, Grand Island, NY). For enzyme activity assays, cells were cultured in Costar 96-well black plates (1x10⁵ cells/well) and treated with 5 μ M A23187 ionophore (Sigma-Aldrich, St. Louis, MO) (5 μ L in DMSO) to activate nNOS then with dm-CLO or L-NAME (0-1000 μ M in PBS) for 8 h. To measure nitric oxide production, cell media was removed and replaced with reaction buffer containing 1 mM L-arginine, 10 μ M 4,5-diaminofluorescein diacetate (Cayman Chemical, Ann Arbor, MI), 1 mM NADPH and dm-CLO or L-NAME (0-1000 μ M) (in 1.2 mM MgSO₄, 129 mM NaCl, 5 mM KCl, 2.8 mM CaCl₂, 10 mM glucose in 10 mM sodium phosphate buffer, pH 7.4). After a 2-h incubation in the dark, fluorescence was read at an excitation wavelength of 490 nm and an emission wavelength of 520 nm using a SpectraMax M2 Microplate Reader.

4.2.3 Liver microsomal and recombinant CYP metabolism

Mouse liver microsomes were prepared by homogenizing livers from male albino Swiss Webster mice in PBS (2 mL/g liver) followed by differential centrifugation of the supernatant (1,000g for 10 min, 10,000g for 30 min and finally 100,000g for 1 h). The microsomal 100,000g pellet was resuspended in PBS and protein concentration measured (Bradford, 1976). Liver microsomes for species comparison studies (mouse, rat, human) were from BD Biosciences (San Jose, CA). Recombinant CYP3A4 (rCYP3A4) was compared to recombinant CYP2C19 (rCYP2C19), the two isoforms previously shown to be responsible for TMX metabolism (Shi *et al.*, 2009). Each neonicotinoid (300 μ M final concentration) was incubated with microsomes from mouse, rat or human (1 mg protein) or rCYP isoform (25 pmol) and 0 or 1 mM NADPH in PBS (600 μ L total volume) for 1 h at 37°C. Alachlor and hexamethylphosphoramide (HMPA), compounds known to produce HCHO via *N*-methylol intermediates on activation by CYPs (Jacobsen *et al.*, 1991; Terry and Bořkovec, 1970), were also incubated under the same conditions with mouse liver microsomes alone or with NADPH.

4.2.4 In vivo TMX metabolism

Male albino Swiss Webster mice (25-35 g) were individually treated with either TMX (ip, 15-25 mg/kg, 1-3 doses 45 min apart) in DMSO (1 μ L/g body weight) or carrier solvent alone. Livers were extracted 30 min after the last dose and homogenized in PBS (4 mL/g liver) and 600 μ L analyzed for HCHO levels as with the enzymatic reactions below.

4.2.5 HCHO analysis

Enzymatic reactions were terminated by addition of 25% ZnSO₄ aqueous solution (50 µL, containing 25 nmol benzaldehyde as an internal standard for HPLC analysis) and saturated Ba(OH)₂ aqueous solution (50 µL). Samples were briefly vortexed and placed on ice for 5 min then centrifuged at 18,000g for 5 min. HCHO levels were analyzed after conversion to the 2,4-dinitrophenylhydrazine (DNPH) derivative (Jacobsen et. al., 1991). An aliquot of the supernatant (500 µL) was mixed with 7.2 mM DNPH (350 µL in 2M HCI) and incubated at room temperature for 30 min followed by addition of carbon tetrachloride (350 µL), vortexing for 30 seconds and a final incubation at room temperature for 30 min. The lower organic layer was evaporated to dryness under N₂ at 25°C and resuspended in 80:20:0.1 ACN/water/HCO₂H (500 µL) and filtered through 0.2 µm nylon for HPLC analysis. Samples were analyzed on a Waters Alliance 2695 HPLC equipped with an Agilent Zorbax SB-C18 column (100 x 4.6 mm, 3.5 µm) and Waters Alliance 2487 dual UV absorbance detector. The mobile phase consisted of ACN and water containing 0.1% trifluoroacetic acid beginning with 65% and increasing to 100% over 11 min at 1 mL/min. A final wash at 65% ACN for 6 min eluted interfering materials. The t_R values for HCHO-DNPH and benzaldehyde-DNPH were verified with analytical standards.

Formaldehyde dehydrogenase (FDH) was used to determine if the HCHO produced was bound or free (which can be oxidized by FDH to formate). Microsomal incubations (containing 1 mM TMX or alachlor) after 1 h were treated with 0.025 units FDH (Sigma-Aldrich), 40 mM NAD+ (15 μ L) and 80 mM reduced glutathione (15 μ L) followed by a 20-min incubation at 37°C then analysis for HCHO as above.

4.2.6 Neonicotinoid metabolite analysis

Neonicotinoid metabolites were analyzed by LC/MS. Microsomal incubations were stopped by addition of ice-cold ACN (1200 μ L containing 10 nmol of DIN as an internal standard) and placed on ice for 10 min. After centrifuging at 1,000*g* for 5 min, the supernatant was evaporated to dryness under N₂ at 25°C and resuspended in 10:90:0.1 ACN/water/HCO₂H (300 μ L) and filtered through 0.2 μ m nylon. Agilent 1100 series LC and Waters LCT Premier XE mass spectrometer conditions were used as in section 2.2.5. t_R values for analytes were verified with analytical standards. Metabolite levels were quantified by comparing peak areas with the internal standard.

4.2.7 Direct analysis of *N*-methylol and *N*-formamide intermediates

Some experiments were specifically designed to detect *N*-methylol and *N*formamide intermediates if present. TMX or dm-TMX was incubated with mouse, rat or human liver microsomes or rCYP isoforms (3A4 or 2C19) (or alachlor or HMPA with mouse liver microsomes) with or without NADPH and analyzed by LC/MS as in 2.2.5 searching for m/z values corresponding to proposed *N*-methylol and *N*-formamide intermediates.

4.2.8 Methylation of *N*-methylol intermediates

Some *N*-methylol metabolites such as diuron or monuron *N*-methylol can be detected after methylation to form a *N*-methoxymethyl derivative (Suzuki and Casida, 1981). Following the Suzuki procedure, TMX, dm-TMX or diuron-microsomal-NADPH incubations were extracted four times with ethyl ether (600 μ L) which was then evaporated under N₂ at 25°C to 1 mL and 100 μ L of methanol and 20 μ L of concentrated sulfuric acid were added. After shaking for 1 min at room temperature, reactions were extracted with ice-cold water (600 μ L) and the ether layer recovered. The aqueous fraction was further extracted with ethyl ether (two times with 600 μ L). The ether fractions were combined, evaporated to dryness and analyzed as in section 2.2.5 for methylated *N*-methylol intermediates, but none were identified.

4.2.9 Glucuronidation of *N*-methylol intermediates

An attempt was made to enzymatically trap *N*-methylol intermediates as glucuronides by the procedure of Shi *et al.* (2009) with modifications. Mouse liver microsomes (2 mg), MgCl₂ (5 mM) and alamethicin (20 μ g/mL) were incubated on ice for 15 min then TMX or *p*-nitrophenol (300 μ M or 1 mM), NADPH (1 mM) and UDPGA (5 or 8 mM) were added and incubated for another 30-120 min at 37°C. All solutions were in 100 mM Tris-HCl, pH 7.5. Reactions were stopped as in section 4.2.6 and analyzed by LC/MS with peak comparison between samples containing UDPGA and those without. Glucuronides of TMX metabolites were not found, but the glucuronide of *p*-nitrophenol was observed.

4.2.10 Preparation and metabolism of proposed *N*-methylols

CLO or dm-CLO (2 µmol) was treated with an excess of HCHO (450 µmol; 40 µL of a 37% solution) in HCO₂H (40 µL) for 40 min at 80°C, evaporated to dryness under N₂ at 25°C then resuspended in 10:90:0.1 ACN/water/ HCO₂H (500 µL) and analyzed by LC/MS monitoring for mono- and di-*N*-methylols at *m*/z 280 and 310 from CLO and 266 and 296 from dm-CLO. The stability of the aforementioned products was determined in enzyme incubation conditions. The synthetic reaction mixtures were evaporated to dryness under N₂ at 25°C then resuspended in 100 µL PBS. An aliquot (30 µL) was added to mouse liver microsomes (1 mg protein) and 0 or 1 mM NADPH in PBS (100 µL total volume) and incubated for 1 h at 37°C. Reactions were stopped by

addition of ice-cold ACN (100 μ L) and analyzed by LC/MS as in section 2.2.5 comparing the original synthetic reaction mixture with the enzyme incubations in the presence and absence of NADPH.

4.2.11 Statistical analysis

Data are presented as mean \pm SE and analyzed using one-way ANOVA followed by Tukey's post hoc test for comparison of groups using GraphPad Prism. LC/MS metabolite data were considered matched sets based on the day of analysis to account for interday instrument or enzyme variability. A value of p < 0.05 was considered statistically significant.

4.3 Results

4.3.1 Dm-CLO and iNOS or nNOS activity

Preliminary data indicate that compared to L-NAME, dm-CLO did not potently inhibit iNOS *in vitro* (**Fig. 4.3**). The approximate IC₅₀ for dm-CLO was 400 μ M compared to less than 10 μ M for L-NAME. Upon analysis of nNOS, dm-CLO had no effect on enzymatic activity. However, L-NAME inhibited nNOS with an approximate IC₅₀ of 20 μ M.



Figure 4.3 Inhibition of iNOS by dm-CLO and L-NAME. Approximate IC_{50} values for dm-CLO is 400 μ M and L-NAME (depicted as "nitro-Arg") is <10 μ M.
4.3.2 Structural features of neonicotinoids as HCHO generators

Inhibition of iNOS by dm-CLO does not appear to play a major role in TMX hepatotoxicity. Therefore, the formation of other reactive metabolites upon microsomal metabolism was analyzed. Mouse liver microsomes were used with the seven commercial neonicotinoids and dm-TMX to determine structural features conferring high HCHO yields (**Fig. 4.4**). The two oxadiazinanes TMX and dm-TMX yielded more HCHO than the four other *N*-methyl neonicotinoids pointing to the oxadiazinane moiety as a major contributor. More HCHO was observed with CLO than the other *N*-methyl compounds whereas IMI and THI, lacking a *N*-methyl group, gave only background levels (average 0.3 nmol). HCHO liberation from the oxadiazinane and *N*-methyl compounds was always NADPH-dependent. Alachlor with NADPH produced 17.7 \pm 1.7 nmol HCHO.



Figure 4.4 Comparison of neonicotinoids as HCHO generators on mouse liver microsomal CYP metabolism. Values are presented as mean \pm SE. n=3. **p < 0.01 for TMX compared to all other compounds.

4.3.3 Species and isozyme differences in TMX and dm-TMX metabolism

Mouse, rat and human liver microsomes were compared for differences in the metabolism of TMX or dm-TMX to HCHO (**Fig. 4.5A**). Mouse yielded significantly more HCHO from TMX and dm-TMX compared to rat or human. Analyses of other metabolites confirmed Green *et al.* (2005b) in showing the same species-dependent relationship in TMX metabolism to dm-TMX, CLO and dm-CLO (**Fig. 4.5B**), and in the conversion of dm-TMX to dm-CLO (**Fig. 4.5C**). The relative amount of HCHO liberated is closely correlated with the relative CLO yield (correlation coefficient, r^2 =0.97) (**Fig. 4.6**) as expected since the TMX to CLO conversion is the primary pathway of HCHO formation.





Figure 4.5 Species differences in liver microsomal CYP metabolism of (A) TMX and dm-TMX to HCHO, (B) TMX to dm-TMX, CLO and dm-CLO and (C) dm-TMX to dm-CLO. Values are presented as mean \pm SE. n= 3-6. *p < 0.05 or ***p < 0.0001 for mouse compared to rat and #p < 0.05, ##p < 0.01 or ###p < 0.0001 for mouse or rat compared to human.



Figure 4.6 HCHO liberation from TMX correlates with CLO formation from mouse, rat and human liver microsomal CYPs. Relative amounts: CLO 100% = 4.3 nmol and HCHO 100% = 14.6 nmol for female mouse microsomes.

To determine which human CYP isoform contributes to TMX or dm-TMX metabolism, reactions of rCYP3A4 or rCYP2C19 with NADPH were compared for metabolite and HCHO formation (**Fig. 4.7**). rCYP3A4 metabolized TMX more efficiently than rCYP2C19 (with CLO being the major product in each case). There was no difference between isoforms for the dm-TMX to dm-CLO conversion and both isoforms produced similar amounts of HCHO from TMX and dm-TMX.



4.3.4 Nature and reactions of the liberated HCHO

The HCHO observed from TMX metabolism could be free, protein-bound or liberated from an *N*-methylol during analysis. Most of the detected HCHO with TMX, mouse liver microsomes and NADPH was free since addition of FDH and the cofactors (NAD+ and reduced glutathione) to the incubation mixture to destroy all the free HCHO reduced the total HCHO yield by $58 \pm 4\%$ (**Fig. 4.8**). Addition of FDH to microsomal-NADPH incubations containing alachlor (1 mM) reduced HCHO levels by 21% compared to 51% in an earlier report (Jacobsen *et al.*, 1991) attributed to different experimental conditions.



Figure 4.8 HCHO levels after FDH addition to microsomal reactions containing 1 mM TMX. FDH reduced HCHO formation by $58 \pm 4\%$ compared to control. Values are presented as mean \pm SE. n=6. **p<0.01 for FDH-containing samples compared to control.

4.3.5 HCHO as a TMX metabolite in vivo

Attempts to detect elevated HCHO levels in the liver of TMX-treated mice were inconclusive due to variable background readings and interference from other liver components with the DNPH method.

4.3.6 Attempts to observe *N*-methylol and *N*-formamide metabolites

N-Methylol and *N*-formamide metabolites should be recognizable by LC/MS as *m*/z 280 and 308 from TMX and as *m*/z 266 and 294 from dm-TMX (**Fig. 4.2**). LC/MS products corresponding to these masses (with appropriate chlorine isotope patterns) or modified (methylated or glucuronidated) *N*-methylol intermediates were not observed in TMX or dm-TMX liver microsomal or rCYP/ NADPH incubation systems with various extraction methods (including methylation and glucuronidation). Systems containing alachlor or HMPA also did not produce detectable *N*-methylol metabolites.

4.3.7 Synthesis of *N*-methylol intermediates

dm-TMX can be prepared by reaction of dm-CLO with HCHO (Maienfisch *et al.*, 2001) presumably via *N*-methylol intermediates (**Fig. 4.9**). When an excess of HCHO was reacted with dm-CLO, in addition to dm-TMX, products of *m*/*z* of 266 and 296 were recognized and with CLO, in addition to TMX, products of *m*/*z* of 280 and 310 were observed (**Fig. 4.10** and **Table 4.2**). The 310 and 296 products are proposed di-*N*-methylol intermediates (3a and 3b in **Fig. 4.9**) formed prior to cyclization to the oxadiazinane ring. When the mixture of reaction products was further incubated with mouse liver microsomes with and without NADPH in PBS for an additional h, the proposed mono- and di-*N*-methylol products (266 and 296 from dm-CLO and 280 and 310 from CLO) were still observed, *i.e.* were not degraded by mouse microsomal enzymes.



Figure 4.9 Proposed pathways for conversion of CLO or dm-CLO to TMX or dm-TMX on reaction with HCHO and HCO₂H showing mono- and di-*N*-methylol intermediates (for analogous reactions involving HCHO cyclization of symmetrically substituted ureas see Petersen, 1973).



Figure 4.10 Partial LC/MS chromatograms of the reaction products of (**A**) CLO and (**B**) dm-CLO with HCHO and HCO₂H showing proposed mono-*N*-methylol intermediates (m/z 280 and 266) and end products (TMX and dm-TMX).

Reaction and compounds	m/z	t _R (min)
<u>CLO → TMX</u> CLO TMX 1a, 1b	250 292 280	22.4 21.3 16.2, 18.8ª
3a	310	18.2, 19.6, 20.4 ^{a,b}
dm-CLO → dm-TMX dm-CLO dm-TMX 2a, 2b 3b	236 278 266 296	21.6 23.2 21.7, 22.1 ^a 22.1, 22.4, 22.6 ^{a,b}

Table 4.2 *N*-Methylol intermediates observed in the conversion of CLO or dm-CLO to TMX or dm-TMX on reaction with HCHO and HCO_2H .

^a Proposed structures for the mono-*N*-methylols (1a, 1b, 2a, 2b) and di-*N*-methylols (3a, 3b) are shown in Fig. 4.9.

^b Structures for two additional dimethylols are not assigned but are likely formed by HCHO addition at alternate sites, *e.g.* NCH₂OCH₂OH.

4.4 Discussion

TMX is a hepatotoxicant and hepatocarcinogen in mice and its metabolite, dm-TMX, is also hepatotoxic in mice with its toxicity exacerbated by dm-CLO as an iNOS inhibitor (Green *et al.*, 2005a). Importantly, these unfavorable toxicological features are not evident in rats, raising the question of whether mice or rats are the better model for humans (Pastoor *et al.*, 2005). Comparative metabolism may be a factor since mouse liver microsomes form dm-TMX and dm-CLO much more efficiently than rat or human liver microsomes. TMX was initially categorized as a "likely human carcinogen" based on the mouse model (Environmental Protection Agency, 2002; Tomizawa and Casida, 2005) but this was modified to "not likely to be carcinogenic to humans" based on species differences in metabolism (Environmental Protection Agency, 2007b; Pastoor *et al.*, 2005).

Initial preliminary studies focused on the *in vitro* inhibition of two isoforms of NOS (inducible and neuronal) by dm-CLO. Reducing the formation of nitric oxide via NOS inhibition is known to enhance hepatotoxicity of other toxicants (Kuo and Slivka, 1994; Liu and Waalkes, 2005) and may explain the hepatotoxicity observed in TMX-treated mice. However, the tentative conclusion is that dm-CLO does not potently inhibit either iNOS or nNOS *in vitro*. Therefore the focus of further studies turned to analyzing the formation of other reactive metabolites (HCHO and *N*-methylols) from TMX and dm-TMX.

TMX is the only one of the seven commercial neonicotinoids to induce hepatotoxicity or hepatocarcinogenicity in mice or rats. The unique structural feature of TMX and its hepatotoxic metabolite dm-TMX is the oxadiazinane moiety, which is a potential source of HCHO and *N*-methylol metabolites. In this first study of HCHO as a neonicotinoid CYP metabolite we find that of all commercial neonicotinoids TMX and dm-TMX are the most efficient HCHO generators and much more so with mouse than rat or human CYPs. Our results on species differences in HCHO liberation fully agree with the findings of Green *et al.* (2005b) on dm-TMX, CLO and dm-CLO formation from the rest of the molecule. The observed species differences in metabolism of TMX or dm-TMX are likely due to substrate specificity and expression differences of various CYP enzymes, with mice having the highest number of *CYP* genes compared to rats and humans (Nelson, 2009). The present study therefore confirms the preference for the rat over the mouse model in TMX human risk assessment.

The hepatotoxicant/ hepatocarcinogen candidates from TMX and dm-TMX metabolism are HCHO and *N*-methylol intermediates. HCHO is a known human carcinogen (International Agency for Research on Cancer, 2012; National Toxicology Program, 2011). To test if free HCHO was formed, FDH was added to TMX- mouse microsomal incubation reactions. Based on FDH-induced HCHO loss, most of the HCHO formed by CYPs from TMX was free, but the remaining HCHO could be protein-bound or released from *N*-methylol intermediates during analysis. A similar result was obtained for HCHO liberated from the NCH₂OCH₃ moiety of alachlor under comparable conditions (Jacobsen *et al.*, 1991).

Attempts to detect *N*-methylol metabolites in the liver of TMX-treated mice were unsuccessful *in vivo* (Ford and Casida, 2006) as well as *in vitro* (this study) possibly due to instability on formation and analysis. The same applies to *in vivo* detection of HCHO which is very bioreactive (International Agency for Research on Cancer, 2012). Although not detailed here, white blood cells from the same groups of TMX-treated and control mice were analyzed for DNA-protein crosslinks induced by HCHO production from TMX using the method of Zhitkovich and Costa (1992). These attempts were unsuccessful with the assay method having low sensitivity (requiring more than 100 μ M HCHO for *in vitro* detection of DNA-protein crosslinks).

Many compounds including pesticides proposed or established to be carcinogens yield *N*-methylol metabolites (Fig. 4.11) some of which may contribute to their toxicity. The inability to detect N-methylol metabolites from alachlor or HMPA in our studies agree with previous literature indicating the difficulty of their analysis due to high reactivity and short half-lives (Jacobsen et al., 1991; Terry and Bořkovec, 1970). The candidate N-methylol intermediates from TMX and dm-TMX were therefore synthesized by reaction of CLO and dm-CLO with HCHO and HCO₂H. Although individual intermediates were not isolated and characterized, the CLO and dm-CLO reactions with HCHO in HCO₂H appear to give two mono-*N*-methylols and three further addition compounds with m/z equivalent to the addition of two methylols per molecule as the reaction proceeds ultimately ending up as TMX and dm-TMX. The proposed mono-Nmethylols from synthesis were found to be stable not only to extraction and LC/MS but also to an additional incubation with mouse liver microsomes. This creates an apparent contradiction wherein the synthetic *N*-methylols are adequately stable for study but the proposed CYP-formed *N*-methylols are not observed. This anomaly is rationalized by the different mechanisms and environment of N-methylol formation in the chemical synthesis and enzymatic CYP systems. Perhaps the N-methylols as they are enzymatically formed further react in the CYP active site containing ferrous iron, cysteine thiol and imidazole N-H.



References to N-methylol metabolites:

- 1. This study. Arbitrary selection of one from several possible *N*-methylols (see Fig. 4.2)
- 2. Dorough and Casida, 1964
- 3. Ashby and Lefevre, 1982; Holden et al., 1982; Terry and Bořkovec, 1970; Tsuyuki et al., 1955
- 4. Jacobsen *et al.*, 1991
- 5. Ross et al., 1981

References to proposed or established carcinogens:

- 6. Environmental Protection Agency, 2002, 2007; Tomizawa and Casida, 2005
- 7. Environmental Protection Agency, 2008
- 8. Lee and Trochimowicz, 1982
- 9. Environmental Protection Agency, 1987
- 10. National Toxicology Program, 1988

Figure 4.11 *N*-Methylol metabolites of pesticides proposed or established to be carcinogens in rodents.

In summary, the oxadiazinane moiety of TMX provides a slow-release reservoir for the production of HCHO and potentially *N*-methylols as candidate hepatotoxicants and hepatocarcinogens with higher yields in mice than rats or humans. Final products of TMX-oxadiazinane moiety metabolism are CLO and dm-TMX acting as nAChR agonists and insecticides (Nauen *et al.*, 2003).

Conclusions

The overall aim of this study was to further characterize the metabolism and mechanisms of toxicity of neonicotinoid insecticides with the following major findings: 1) AOX is important in the *in vivo* nitroreduction of IMI and potentially other neonicotinoids in mice, 2) CYC is metabolized mostly to hydroxylation products and NMI *in vivo* while NMI only produces minor metabolites and 3) TMX is specifically hepatotoxic/ hepatocarcinogenic to mice possibly due to its metabolism to HCHO and *N*-methylols.

CYPs were thought, until now, to be the primary phase I enzymes responsible for neonicotinoid metabolism. AOX, a cytosolic liver enzyme with considerable species variation in activity, is shown here to play a major role in neonicotinoid metabolism in mice, but not *Drosophila*. While there are multiple isoforms of CYPs that can metabolize neonicotinoids, there are significantly fewer isoforms of AOX in mice and Drosophila. Consequently, genetic differences between species resulting in variations in AOX substrate selectivity and activity can lead to differences in neonicotinoid detoxification. AOX in vivo importance was only determined for IMI, but may convert other nitroguanidine neonicotinoids such as CYC to its NO-CYC and NH₂-CYC products. The in vivo enzymatic pathways of CYC and NMI were analyzed in mice. In addition to nitroreduction products, CYC was primarily oxidized to a series of mono- and dihydroxylation products and the oxabridge was cleaved to form NMI. The rapid metabolism and distribution (to brain and plasma) shown by these initial studies indicate that the pharmacokinetics of CYC may be similar to other neonicotinoids. However, further studies need to be conducted to determine the relative contribution of CYPs and AOX to CYC and NMI metabolism.

Rodents are generally good models for the analysis of *in vivo* metabolism and toxicity of xenobiotics. For example, the reported metabolic pathways of neonicotinoids are similar for mice, rats, dogs and hens (Ford and Casida, 2006a,b). However, we have shown here that in some cases mice are not a good model for determining human health relevance of TMX exposure resulting in hepatotoxicity/ hepatocarcinogenicity. Mouse liver microsomes are more efficient than rat or human at metabolizing TMX to its major products, including HCHO. These differences may mostly be attributed to variable CYP expression and glutathione levels between species.

The use of neonicotinoids for pest control in agriculture and animal health is expected to increase as other major neuroactive insecticide classes are phased out due to resistant pest populations and toxicological problems. During pesticide registration efforts, focus is primarily on examining the toxicity of parent compounds. A thorough analysis of the metabolic pathways (in mammals and plants) is typically a secondary consideration. However, farmers and the general public are often exposed to both the parent chemicals and their metabolic products. The understanding of which enzymes are involved in *in vivo* metabolism, the metabolic pathways and which metabolites contribute to adverse effects is critical for the future design and use of pesticides.

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