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NEONICOTINOID INSECTICIDE TOXICOLOGY: Mechanisms of Selective Action

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■ **Abstract** The neonicotinoids, the newest major class of insecticides, have outstanding potency and systemic action for crop protection against piercing-sucking pests, and they are highly effective for flea control on cats and dogs. Their common names are acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid, and thiamethoxam. They generally have low toxicity to mammals (acute and chronic), birds, and fish. Biotransformations involve some activation reactions but largely detoxification mechanisms. In contrast to nicotine, epibatidine, and other ammonium or iminium nicotinoids, which are mostly protonated at physiological pH, the neonicotinoids are not protonated and have an electronegative nitro or cyano pharmacophore. Agonist recognition by the nicotinic receptor involves cation-π interaction for nicotinoids in mammals and possibly a cationic subsite for interaction with the nitro or cyano substituent of neonicotinoids in insects. The low affinity of neonicotinoids for vertebrate relative to insect nicotinic receptors is a major factor in their favorable toxicological profile.

INTRODUCTION

Pest insect control, an essential component of crop protection and public health, has evolved over a recorded history of three millennia (1, 2). Sulfur was first referred to by Homer in 1000 BC as a fumigant for pest control, and, in California, it is still used in larger amounts than any other pesticide. Nicotine in the form of tobacco extracts was reported in 1690 as the first plant-derived insecticide, followed by the pyrethrins from pyrethrum flowers and rotenone from derris roots in the early 1800s. Synthetic organics in the 1940s to the 1970s largely replaced inorganics and botanicals with the introduction of organophosphates, methylcarbamates, organochlorines, and pyrethroids. With each new chemical class, resistant strains were soon selected to limit their effectiveness. Genetically modified crops expressing *Bacillus thuringiensis* (*Bt*) δ-endotoxin were introduced for pest insect

control in 1995. Many of the remaining gaps in pest control capabilities were filled recently by the neonicotinoids (Figure 1), which combine outstanding effectiveness with relatively low toxicity to vertebrates (3–7).

NEONICOTINOIDS

The current synthetic organic insecticides were discovered by modifying natural products, e.g., using the pyrethrins as a prototype for synthetic pyrethroids, or by screening hundreds of thousands of structurally diverse compounds for novel leads. Nicotine is still used as a minor insecticide, particularly in China, but attempts to improve its insecticidal activity, e.g., 3′,4′-dehydronicotine and 3-(alkylaminomethyl)-pyridines (8), were not successful. The lead for the neonicotinoids, 2-(dibromonitromethyl)-3-methylpyridine, was discovered in 1970 by Shell Development Company in California to have modest activity against house flies and pea aphids (9–11). Molecular modifications to achieve optimal potency on corn earworm larvae (a major lepidopterous pest) culminated with nithiazine, but unfortunately it could not be commercialized for crop protection due to photoinstability (10, 12). This shortcoming relegated nithiazine to a niche market for fly abatement in poultry and animal husbandry (11). A major improvement of its structure was made by Nihon Tokushu Noyaku Seizo in Japan (presently Bayer Crop Science Japan) by introducing a chloropyridinylmethyl group, leading to a nitromethylene prototype of outstanding potency on green rice leafhopper (a major pest of rice and vegetables). However, photoinstability again prevented its use for crop protection. Further structure-activity studies established that good activity was retained on replacement of the imidazolidine by thiazolidine or oxadiazinane or acylic counterpart, and the chloropyridinylmethyl by chlorothiazolylmethyl or tetrahydrofuranmethyl. Changing the nitromethylene to nitroguanidine or cyanoamidine afforded photostability and produced highly effective compounds in field conditions (3, 13, 14). The current neonicotinoids and their year of patent are the heterocyclics nithiazine (1977), imidacloprid (IMI) (1985), thiacloprid (1985), and thiamethoxam (1992); and the acyclics nitenpyram (1988), acetamiprid (1989), clothianidin (1989), and dinotefuran (1994). The physical properties of the neonicotinoids and nicotine are compared in Table 1. Molecular weights range from 160 to 292 and log P values from −0.66 to 1.26. The compounds vary in water solubility from 0.185–0.61 g/l for clothianidin, IMI, and thiacloprid, to infinite for nicotine.

The neonicotinoids are the only major new class of insecticides developed in the past three decades. Worldwide annual sales of neonicotinoids are approximately one billion dollars, accounting for 11%–15% of the total insecticide market. They are readily absorbed by plants and act quickly, at low doses, on piercing-sucking insect pests (aphids, leafhoppers, and whiteflies) of major crops. The neonicotinoids are poorly effective as contact insecticides and for control of lepidopterous larvae. They are used primarily as plant systemics; when applied to seeds, soil, or foliage they move to the growing tip and afford long-term protection from piercing-sucking

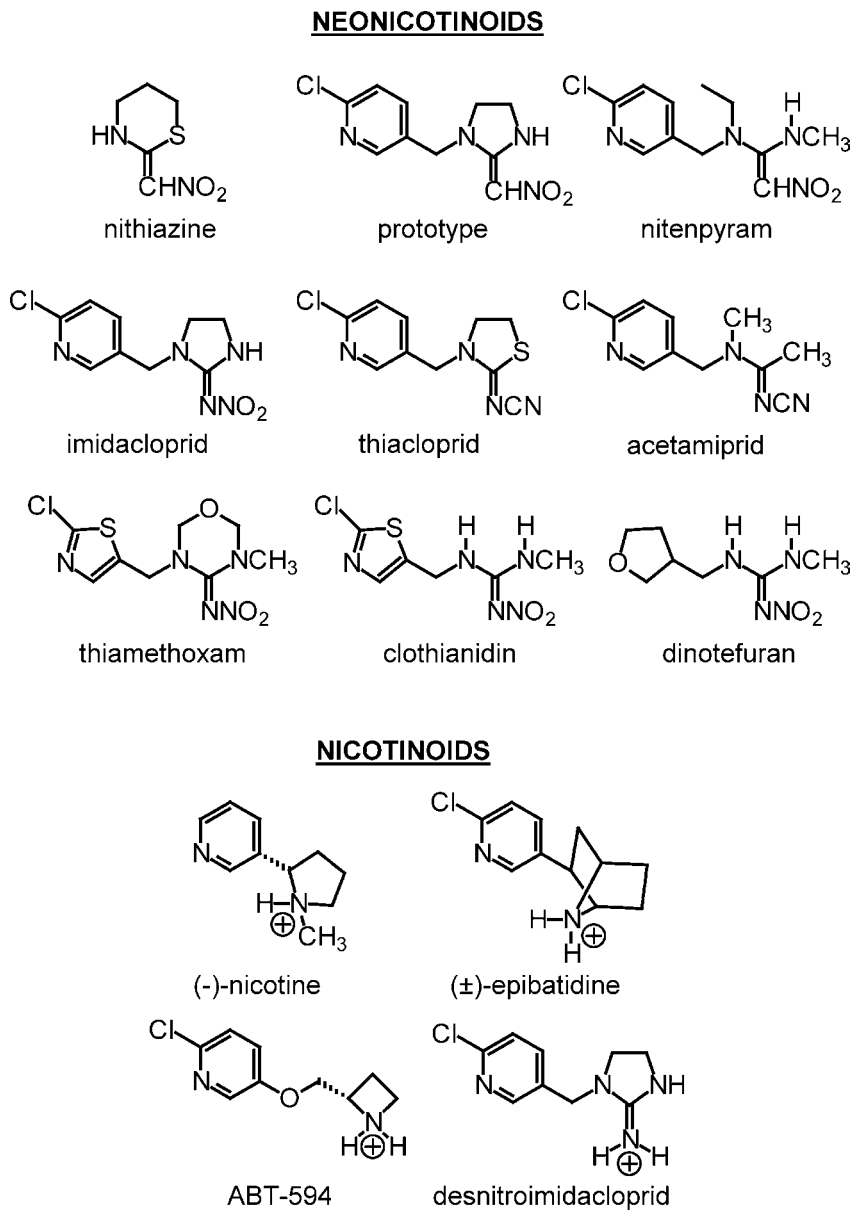

Figure 1 Nine neonicotinoid insecticides and four nicotinoids. The neonicotinoids are nitromethylenes (C=$CHNO_2$), nitroguanidines (C=NNO_2), and cyanoamidines (C=NCN). Compounds with 6-chloro-3-pyridinylmethyl, 2-chloro-5-thiazolylmethyl, and 3-tetrahydrofuranmethyl moieties are referred to as chloropyridinyls (or chloronicotinyls), chlorothiazolyls (or thianicotinyls), and tefuryl, respectively. The nicotinoids are naturally occurring [(−)-nicotine and (−)-epibatidine] and synthetics (ABT-594 and desnitroimidacloprid).

TABLE 1 Physical properties of the neonicotinoids and nicotine

Compound	Molecular weight	Water solubility (g/l)	Log P[a]
Neonicotinoids			
Acetamiprid	222.7	4.25	0.80
Clothianidin	249.7	0.30–0.34	0.7
(±)-Dinotefuran	202.2	54.3	−0.64
Imidacloprid	255.7	0.61	0.57
Nitenpyram	270.7	>590	−0.66
Nithiazine	160.1	200	−0.60
Thiacloprid	252.7	0.185	1.26
Thiamethoxam	291.7	4.1	−0.13
Nicotinoid			
(−)-Nicotine	162.2	∞	0.93 (free base)

Data from References 15–17.

[a]P = 1-octanol/water partition.

insects, e.g., for 40 days in rice. Some organophosphates and methylcarbamates also have good systemic activity but their use is declining due to selection of resistant insect strains and increasing restrictions based on human safety considerations. The expanding importance of crops expressing *Bt* δ-endotoxin encourages neonicotinoid use because the types of pests not controlled by the endotoxin are often those highly sensitive to neonicotinoids. Although crop protection is the major use for neonicotinoids, pest insect control on pets or companion animals is also a significant market. IMI and nitenpyram are highly effective flea control agents on cats and dogs, and are administered as oral tablets or topical spot treatments (see, for example, Reference 18).

While the nicotinoids are structurally similar to the neonicotinoids, they primarily differ by containing an ionizable basic amine or imine substituent (Figure 1). Notable nicotinoids other than nicotine are two very potent candidate analgesic agents, i.e., epibatidine, which was isolated from the skin of an Ecuadoran frog (19, 20), and ABT-594 (21, 22). Interestingly, the same chloropyridinyl substituent appears in both these nicotinoids and the optimized neonicotinoid insecticides. Desnitro-IMI, an iminium metabolite of IMI, fits the nicotinoid category (23).

TOXICOLOGY

The neonicotinoids have unique physical and toxicological properties as compared with earlier classes of organic insecticides (Table 2). They generally have the lowest log P values, which is consistent with their outstanding plant systemic activity shared by some organophosphates and methylcarbamates but not by the more lipophilic organochlorines and pyrethroids. The neonicotinoids and pyrethroids

TABLE 2 Comparison of neonicotinoids with other classes of insecticides[a]

Class	Log P	Systemic action	Nerve target[b]	Potency (LD_{50}, mg/kg)[c]		Selectivity factor
				Insects	Rats	
Neonicotinoids	−0.7 to 1.3	+	nAChR	2.0	912	456
Organophosphates	1 to 5.5	±	AChE	2.0	67	33
Methylcarbamates	−1 to 3	±	AChE	2.8	45	16
Organochlorines	5.5 to 7.5	−	Na^+ or Cl^- channels	2.6	230	91
Pyrethroids	4 to 9	−	Na^+ channel	0.45	2000	4500

[a]Data from Reference 24 except for neonicotinoids.

[b]Insecticide examples are the organophosphates parathion and malathion (as their oxon metabolites) and methylcarbamates carbaryl and aldicarb inhibiting AChE, the organochlorine DDT and the pyrethroid deltamethrin acting on the voltage-sensitive sodium channel, and the organochlorines endosulfan and lindane blocking the γ-aminobutyric acid (GABA)-gated chloride channel.

[c]Geometric means of large data sets (11 to 83 items each) for rat acute oral and insect topical (principally four species) LD_{50} values for all classes of compounds except neonicotinoids (24). Values for the neonicotinoids are geometric means for rat oral LD_{50} data in Table 3 and arbitrary for insects to reflect similar potency of neonicotinoids and organophosphates on the same target insects.

have higher selectivity factors for insects versus mammals than the organophosphates, methylcarbamates, and organochlorines. This is attributable to both target site specificity and detoxification, which are considered later. The neonicotinoids act as agonists at the nicotinic acetylcholine receptors (nAChRs) of insects and mammals (particularly the $\alpha4\beta2$ subtype) (7).

The toxicological profiles of the individual neonicotinoids and nicotine are compared in Table 3. The acute oral LD_{50} values (mg/kg) for rats range from 50–60 for nicotine to >5000 for clothianidin. When ranked on the basis of chronic toxicity to rats, reported as no-observed-adverse-effect-level (NOAEL; the principal toxicological parameter used in risk assessment), thiacloprid and thiamethoxam have the lowest values (0.6–1.2 mg/kg/day) and are rated as likely human carcinogens. Intermediate values (5.7–9.8 mg/kg/day) are observed for acetamiprid, clothianidin, and IMI, whereas dinotefuran has the highest value. The EPA has not followed a cumulative risk approach in determining pesticide tolerances for neonicotinoids and has not assumed that each neonicotinoid has a common mechanism of toxicity with other substances (25–30). Of the commercial neonicotinoids, acetamiprid, IMI, and thiacloprid are the most toxic to birds, and thiacloprid to fish. Several neonicotinoids are harmful to honeybees, either by direct contact or ingestion, but potential problems can be minimized or avoided by treating seeds and not spraying flowering crops (15).

The mammalian toxicity of neonicotinoids is considered to be centrally mediated because the symptoms of poisoning are similar to those of nicotine. Toxicity correlates with agonist action and binding affinity at the vertebrate $\alpha4\beta2$ nAChR,

TABLE 3 Toxicological profiles of the neonicotinoids and nicotine[a]

Compound	Mammal[b]			Bird[f]	Fish[g]
	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]Data from References 9, 15, 25–30.

[b]Dermal LD_{50} 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 difference less than twofold.

[d]No-observed-adverse-effect-level (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 hepatocellular adenomas and carcinomas in male and female mice. They are considered to be likely human carcinogens.

[f]Japanese or bobwhite quail.

[g]Rainbow trout or carp.

the primary target in brain (31). Chronic exposure to neonicotinoid insecticides, and certain metabolites as well as nicotine, upregulates $\alpha4\beta2$ nAChR levels without altering the sensitivity of the binding site. This upregulation in M10 cells is initiated by receptor-insecticide interaction (32). Neonicotinoids and metabolites also elicit acute intracellular responses, particularly in relation to signal integration pathways in mammalian cells. In mouse neuroblastoma cells, low levels of these compounds activate the extracellular-regulated (also called mitogen-activated) protein kinase cascade via the nAChR and intracellular calcium mobilization, leading to possible attenuation of neuronal functions (33). Nicotine and other nicotinoids are candidate therapeutic agents as analgesics and for treatment of neurodegenerative diseases (21, 22, 34). The potential activity of neonicotinoids is therefore of interest. A nitromethylene neonicotinoid, with modest agonist action on the $\alpha4\beta2$ nAChR, is as potent as nicotine in inducing antinociceptive activity in preclinical pain models in mice. This effect persists longer than that of nicotine or epibatidine and appears to involve a different mechanism of action (31). However, other neonicotinoid insecticides and metabolites (even with high agonist potency at the $\alpha4\beta2$ nAChR) fail to induce analgesia, perhaps owing to adverse nociceptive and toxic effects (21, 35, 36, 37) or insufficient subtype selectivity (23, 38).

There are no specific antidotes for neonicotinoid poisoning in mammals (39). Treatment with an acetylcholinesterase (AChE)-reactivating oxime (e.g., pralidoxime important in organophosphate poisoning) or a nicotinic antagonist might be either ineffective or contraindicated. Symptomatic treatment is recommended for any possible acute poisoning case.

BIOTRANSFORMATIONS

Metabolism of the commercial neonicotinoids has been extensively studied in crops, rats, lactating goats, and laying hens (15, 40). These studies are part of the EPA registration requirements for approved uses. Extensive data has been published for IMI (28, 41), thiacloprid (29, 42), clothianidin (26, 43, 44), and to a lesser extent, nithiazine (45), nitenpyram (40), acetamiprid (40), thiamethoxam (30), and dinotefuran (27). Owing to their relatively high water solubility and slow metabolism in mammals, some (IMI and thiacloprid) to almost all (clothianidin, dinotefuran, and nitenpyram) of an oral neonicotinoid dose is excreted unchanged in urine. The chemical fate of neonicotinoids in and on crops is governed both by metabolic and photochemical reactions. These processes may produce identical or different products depending on the mechanisms involved.

Analysis of neonicotinoid residues to enforce crop tolerances and registered uses involves the parent compound and toxic metabolites. IMI residues are determined as the parent compound plus metabolites with the chloropyridinyl moiety. Thiacloprid is combined with an amide and a hydroxy derivative in evaluating residues. Clothianidin and acetamiprid residues are regulated as the parent compounds. Thiamethoxam residues are considered along with those of its principal metabolite clothianidin. Dinotefuran residues are combined with those of its guanidine and urea metabolites. The analyses are achieved by various combinations of high-performance liquid chromatography or gas chromatography with UV, electron capture, or mass spectrometry for detection and characterization.

Most neonicotinoids undergo metabolic alterations at multiple sites (Figure 2). For convenience, different parts of the molecules are considered separately, indicating the known or presumed effects of the reactions on bioactivity. In Figure 2*A*, oxidation of the nitromethylene carbon of nithiazine is likely a detoxification mechanism (45). IMI is hydroxylated in the imidazolidine moiety at either one of the two methylene substituents, which is followed by conjugation or dehydration to form the olefin, apparently with little or no ring opening; these unconjugated metabolites retain insecticidal activity or insect nAChR potency (46–48). Some *N*-demethylation is observed in each case with compound-dependent effects on product potency. It greatly increases insecticidal and/or receptor activity for *N*-methyl-IMI (a model compound) (16), nitenpyram (49), and thiamethoxam (50, 51). Thiamethoxam is readily converted to clothianidin by ring methylene hydroxylation in insects and plants (52), whereas clothianidin undergoes *N*-demethylation (43, 44). The thiazolidine ring of thiacloprid is opened and the sulfur oxidized

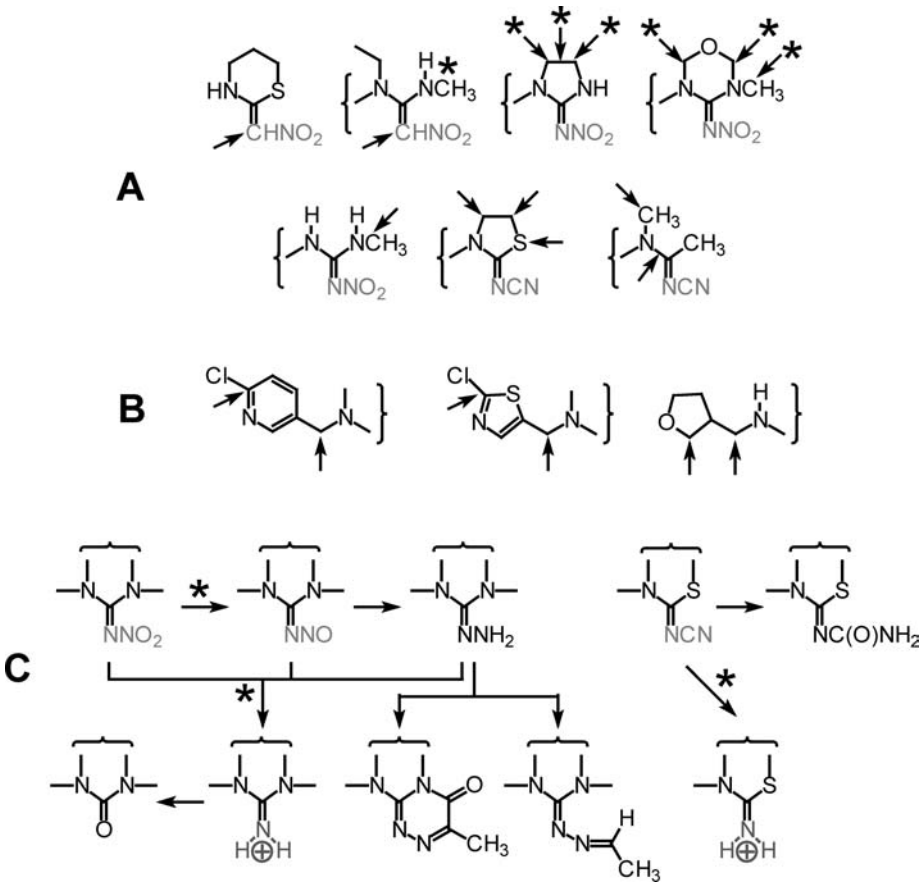

Figure 2 Neonicotinoid biotransformations shown by arrows as sites of metabolic attack (*A* and *B*) and substituent modifications (*C*) on three moieties (see Figure 1 for full structures and text for references). Asterisks designate sites of change leading to active metabolites based on nAChR potency or toxicity, whereas all other sites yield or are presumed to give low activity or inactive metabolites. The biotransformation reactions shown are in mammalian systems unless indicated otherwise in the text.

and methylated (42). Acetamiprid undergoes *N*-demethylation and cleavage of the *N*-cyanoacetamidine linkage in plants (40). The chloropyridinylmethyl, chlorothiazolylmethyl, and tetrahydrofuranmethyl substituents (Figure 2*B*) undergo *N*-methylene hydroxylation and cleavage, followed by aldehyde oxidation to the corresponding carboxylic acids, which are commonly excreted as glycine derivatives following conjugation. The chloro substituent is displaced presumably by glutathione and ultimately leads (via cysteine and -SH derivatives) to the methylsulfide. With dinotefuran, hydroxylation of the tetrahydrofuran moiety leads to

ring opening and liberation of an aldehyde that forms cyclic derivatives (27). The C═N─NO_2 (nitroguanidine) moiety (Figure 2*C*) of IMI is reduced to C═N─NO (nitrosoguanidine) and C═N─NH_2 (aminoguanidine), and cleaved to the C═NH (guanidine) and C═O (urea) derivatives. The aminoguanidine of clothianidin is also conjugated with pyruvic or acetic acid, followed by cyclization (26). The nitrosoguanidine metabolite of IMI has moderate to high insecticidal activity and insect nAChR potency (53), whereas the guanidine metabolite is highly activated against mammalian but deactivated against insect nAChRs (23, 38, 54).

Cytochrome P450 (CYP450) isozymes are involved in oxidative IMI metabolism. Based on studies with individual recombinant enzymes, human CYP3A4 is the predominant IMI *N*-methylene hydroxylase (55). The insecticidal activity of many neonicotinoids is strongly synergized by CYP450 inhibitors, such as piperonyl butoxide and *O*-propyl *O*-propynyl phenylphosphonate, suggesting that these enzymes limit their efficacy (16, 56). Fruit flies (*Drosophila melanogaster*) overexpressing CYP6G1 are resistant to IMI, probably owing to enhanced detoxification (57, 58). Several human P450s also reduce IMI to the nitroso derivative in an oxygen-sensitive manner (55). A relatively oxygen-insensitive "neonicotinoid nitro reductase" of rabbit liver cytosol (59) was recently identified as aldehyde oxidase and readily converts IMI to nitrosoguanidine and aminoguanidine (60). The aminoguanidine of IMI (a hydrazone) is further derivatized to an acetaldehyde imine upon incubation with mammalian liver cytosol (60) and to a triazinone and other conjugates in vitro and in vivo (59, 60a). The C═N─CN moiety of thiacloprid is hydrolyzed to the amide [C═NC(O)NH_2] and also undergoes N─CN cleavage. Descyanothiacloprid (a plant metabolite) (42) is a particularly potent mammalian nAChR agonist (23).

Toxicokinetic studies in mammals, so important in pharmaceutical research, are often given lower priority in pesticide investigations. As an example, it is not clear if the toxicity of IMI in mammals is due to the parent compound or the desnitro metabolite (which enters the brain following direct intraperitoneal administration in mice) (54). IMI is highly absorbed in a human intestinal cell model, suggesting potential effects in mammals following ingestion (61).

NICOTINIC RECEPTORS

The vertebrate nAChR is an agonist-gated ion channel responsible for rapid excitatory neurotransmission. It is a pentameric transmembrane complex in the superfamily of neurotransmitter-gated ion channels, including γ-aminobutyric acid ($GABA_A$ and $GABA_C$), glycine, and 5-HT_3 serotonin receptors. The nAChR consists of diverse subtypes assembled in combinations from ten α, four β, γ, δ, and ε subunits. The skeletal muscle or electric ray (*Torpedo*) subtype is made up of two $\alpha 1$ subunits and one each of $\beta 1$, γ, and δ (or ε in adult muscle) subunits. Neuronal nAChR subtypes expressed in vertebrate brain and ganglia are assembled in combinations of $\alpha 2$–10 and $\beta 2$–4, and are pharmacologically classified into two groups based on sensitivity to α-bungarotoxin (α-BGT). The $\alpha 2$–6 and

β2–4 subunits are involved in assembling the α-BGT-insensitive subtypes, whereas α7–10 subunits are responsible for α-BGT-sensitive receptors. Of these, the most abundant subtypes in vertebrate brain are $\alpha4\beta2$ and $\alpha7$ (α-BGT-insensitive and -sensitive, respectively). The $\alpha4\beta2$ subtype consists of two $\alpha4$ and three $\beta2$ subunits (heteropentameric) and the $\alpha7$ subtype is considered to be a homopentameric structure (34, 62, 63). The agonist or drug-binding site is localized at the interface region between subunits. Specific subunit combinations confer differences in sensitivity to acetylcholine (ACh) and/or pharmacological profiles among the nAChR subtypes. The ligand-binding site in all subtypes consists of a conserved core of aromatic amino acid residues (64–67). Neighboring variable residues are considered to confer individual pharmacological properties to each subtype (62). The mammalian nAChR is a potential target for therapeutic agents for analgesia, neurodegenerative diseases, cognitive dysfunction, schizophrenia, depression, and anxiety (22). The most potent nicotinic agonist is epibatidine (20). An important aspect of nicotinic drug development is the discovery of highly subtype-selective agents (e.g., ABT-594; Figure 1) (21, 22, 68–70).

Neonicotinoids have little or no effect on the vertebrate peripheral nAChR $\alpha1\gamma\alpha1\delta\beta1$ subtype (23, 71–73) or some neuronal subtypes [$\alpha3\beta2$ (and/or $\beta4$)$\alpha5$, $\alpha4\beta2$, and $\alpha7$] (16, 23, 54, 72, 74–77). Minor structural modifications of neonicotinoids confer differential subtype selectivity in vertebrate nAChRs. Nitromethylene analogs with high insecticidal activity display comparable or higher affinity than that of nicotine to the $\alpha3\beta2\beta4\alpha5$ or $\alpha7$ subtype (Table 4). Toxicological evaluations of insecticide safety should consider both the nAChRs as a whole and as major subtypes (23, 38).

The insecticidal activity of the neonicotinoids is due to their action as insect nAChR agonists. This was first demonstrated by electrophysiological and [^{125}I]α-BGT binding studies with nithiazine and the cockroach nerve cord (78, 79). It was verified with IMI using binding studies with insect brain membranes and [^{3}H]- or [^{125}I]α-BGT (80–83). More definitive confirmations were obtained with [^{3}H]IMI (84) by structure-activity correlations for displacement of binding potency with knockdown activity (56, 85) and electrophysiological responses (86).

Insect nAChRs are less well understood than their vertebrate counterparts as to functional architecture and diversity (87), as illustrated here with *Drosophila*. They are widely distributed in the synaptic neuropil regions of the insect central nervous system. In *Drosophila*, genes for four α (Dα1–4) and three β (Dβ1–3) subunits have been identified, and several additional candidate genes for nAChR subunits are predicted from genome data (7, 87–89). On expression in *Xenopus* oocytes, human embryonic kidney 293 cells, or *Drosophila* S2 cells, the four α subunit genes (Dα1–4) and three β subunit genes (Dβ1–3), alone or in various combinations, never produce an electrophysiological response or [^{3}H]epibatidine binding. However, functional ion channel property or [^{3}H]epibatidine binding is clearly observed when any of the four α subunits is coexpressed with chick or rat $\beta2$ or with rat $\beta4$ subunit (90–93). Also, Dα1/Dα2/chick $\beta2$ ternary receptor can be coassembled within a single receptor complex, although functional channel property is unclear

TABLE 4 Specificity of neonicotinoids and nicotinoids for insect and vertebrate $\alpha4\beta2$ nicotinic receptors

	IC_{50}, nM		
Compound[a]	**Insect**[b]	**Vertebrate $\alpha4\beta2$**[b,c]	**Selectivity ratio**
Neonicotinoids			
Acetamiprid	8.3	700	84
Clothianidin	2.2	3500	1591
(±)-Dinotefuran	900	>100,000	>111
Imidacloprid	4.6	2600	565
Nitenpyram	14	49,000	3500
Nithiazine	4800	26,000	5.4
Prototype	0.24	210	875
Thiacloprid	2.7	860	319
Thiamethoxam	5000	>100,000	>20
Nicotinoids			
Desnitroimidacloprid	1530	8.2	0.005
Descyanothiacloprid	200	4.4	0.022
(−)-Nicotine	4000	7.0	0.002
(±)-Epibatidine	430	0.04	0.0001

[a]For structures see Figure 1.

[b]IC_{50} values for displacing [^{3}H]imidacloprid binding to the house fly (*Musca domestica*) (acetamiprid), aphid (*Myzus persicae*) (thiamethoxam), and fruit fly (the other neonicotinoids) receptor, and [^{3}H]nicotine binding to the vertebrate $\alpha4\beta2$ nAChR.

[c]IC_{50} values (μM) for the vertebrate $\alpha7$ nAChR subtype (assayed by [^{125}I]α-BGT binding) are acetamiprid, 290; clothianidin, 190; (±)-dinotefuran, >1000; imidacloprid, 270; nitenpyram, >300; nithiazine, >300; prototype neonicotinoid, 6.1; thiacloprid, 100; thiamethoxam, >300; desnitroimidacloprid, 9.9; descyanothiacloprid, 6.0; (−)-nicotine, 21; and (±)-epibatidine, 0.031.

(94). Three *Drosophila* β subunits (Dβ1–3), each coassembled within Dα3/rat $\beta2$ or rat $\alpha4\beta2$ receptor hybrid complexes, modulate [^{3}H]epibatidine binding activity (95). Thus, coexpression of any *Drosophila* α subunit with a vertebrate β subunit constitutes the best available model at present. However, these hybrid receptors do not faithfully reflect native insect nAChRs. [^{3}H]Epibatidine is generally not useful as a radioligand for native insect receptors (5), except for that of the American cockroach (96). As expected, epibatidine is a weak displacer of [^{3}H]IMI binding to the *Drosophila* receptor and shows very low toxicity to insects (23). Immunological approaches suggest that two *Drosophila* subtypes exist consisting of Dα1/Dα2/Dβ2 and Dα3/Dβ1 (97, 98). Protein biochemical approaches to native *Drosophila* nAChR subunits, involving neonicotinoid affinity chromatography and photoaffinity labeling, reveal the existence of several subunits, including Dα2 as the main neonicotinoid-binding component (7, 49, 98–100).

Distinctive pharmacological profiles are observed for hybrid nAChRs consisting of various combinations of *Drosophila* α and vertebrate $\beta2$ subunits (7, 87, 89). For example, ACh-evoked current is blocked by α-BGT in hybrid nAChRs consisting

of *Drosophila* Dα1 or Dα3 and chick β2 subunits expressed in *Xenopus* oocytes; however, the electrophysiological response is not blocked by α-BGT in a hybrid receptor of Dα2 and chick β2 subunits (90, 92). Similarly, [^{125}I]α-BGT recognizes either Dα1/rat β2 or Dα3/rat β2, but not Dα2/rat β2 hybrid receptor expressed in a *Drosophila* S2 clonal cell line, yet [^{3}H]IMI and [^{3}H]epibatidine bind to all three of these hybrid receptors with high affinities (101). In contrast, IMI is totally ineffective in generating an electrophysiological response with the Dα1/chick β2 hybrid receptor expressed in *Xenopus* oocyte (77). A Dα4/rat β2 hybrid receptor demonstrates [^{3}H]epibatidine but not [^{125}I]α-BGT binding activity (93). Native *Drosophila* nAChRs contain distinct binding sites for IMI and α-BGT, but it is not clear if they are on the same or different receptors (N. Zhang, M. Tomizawa, J.E. Casida, unpublished observations).

MOLECULAR FEATURES OF NICOTINIC AGONISTS

Neonicotinoid insecticides display excellent selectivity profiles that are largely attributable to specificity for insect versus mammalian nAChRs (7) (Table 4). Neonicotinoids and nicotinoids have common structural features (Figure 1) but different protonation states at physiological pH. The neonicotinoids (e.g., IMI) are not protonated and selective for the insect nAChR, whereas the nicotinoids (e.g., nicotine) are cationic in nature and consequently selective for the mammalian nAChR. Therefore, neonicotinoids and their analogs are excellent probes to help define the mechanisms of selectivity and ultimately the topological divergence between insect and vertebrate binding sites.

The nicotinic pharmacophore model for mammals is derived from nicotinoid structure-activity relationships as compared with ACh, the endogenous agonist. The pK_a of nicotine (pyrrolidinyl nitrogen) is 7.90; therefore, at physiological pH, 89% will be protonated (8). (−)-Nicotine and ACh share three structural elements: a quaternary (sp^3) nitrogen atom, a hydrogen bond acceptor (the pyridine nitrogen of nicotine and the carboxyl oxygen of ACh), and a dummy point (a receptor-related feature that imposes directionality to the pyridine nitrogen of nicotine or the corresponding oxygen of ACh). The center of the sp^3 nitrogen atom is situated approximately 5.9 Å from the van der Waals surface of the hydrogen-bond acceptor (102, 103). The internitrogen (N-N) distance of (−)-epibatidine (5.5 Å) does not coincide with that of (−)-nicotine (4.8 Å) (104, 105). This is rationalized by placing an additional directional requirement on the ammonium nitrogen atom, which, in modeling studies, confers reasonable overlap of (−)-nicotine and (−)-epibatidine by orienting the pyridine nitrogen atom proximal to the sp^3 nitrogen atom (N─N distance of 4.79 Å) (68, 106). The iminium ($^{+}C{-}NH_2 \leftrightarrow C{=}{}^{+}NH_2$) metabolites of IMI and thiacloprid (desnitro and descyano, respectively) are mostly protonated at physiological pH (23).

Neonicotinoids have, instead of an easily protonated nitrogen, the nitro or cyano or equivalent electronegative pharmacophore, and they demonstrate a coplanarity

between this tip and the substituted guanidine/amidine moiety (Figure 3) (23, 53, 107, 108). The guanidine moiety of IMI has pK_a values of 1.56 and 11.12 for protonation and deprotonation, respectively (109), indicating less than 0.0002% protonation at physiological pH. The coplanarity allows a conjugated electronic system that facilitates negative charge flow toward the electronegative tip to consolidate the binding. Interestingly, high affinity and insect receptor selectivity are retained in nitroso (C═N─NO) analogs of neonicotinoids, suggesting that only one (O1) of the electronegative oxygen atoms is essential (53). The role of N1 (Figure 3) in neonicotinoid action is of particular interest. The distance between the van der Waals surfaces of the two nitrogen atoms in nicotine (102) is the same as that between the pyridinyl and N1 nitrogen atoms in the neonicotinoids (5.45–6.06 Å). Additionally, a partial positive charge (δ^+) for the N1 nitrogen atom is conferred by the electron-withdrawing nitro or cyano substituent (74, 83, 107) as supported by comparative molecular field analysis (110) and semiempirical molecular orbital theory (PM3) calculation (111). The same relationship in the above distance is also observed between N1 and O1 of the neonicotinoids (107). However, the N1 atom does not have a significant positive charge in PM3 and high-level ab initio calculations (17, 53, 112), and it can be replaced by a carbon atom with retention of significant binding activity to the *Drosophila* receptor and toxicity to insects (108, 113). The pyridin-3-ylmethyl substituent or equivalent moiety greatly enhances the binding affinity (82, 108). Thus, in terms of binding, there is a crucial role for the nitro or cyano group, an important contribution from the pyridine nitrogen, and a complementary role for N1 of the neonicotinoids (23, 53). Other molecular features also affect the selectivity. The *N*-methyl group of thiamethoxam favors a specific receptor interaction particularly at low temperature with *Myzus* compared with *Drosophila* or other insects (114, 114a); the preference of insect nAChRs for chloropyridinyl versus chlorothiazolyl moieties depends on the rest of the molecule (Figure 1) (51); introducing azido or amino at the 5-position of the 6-chloropyridin-3-yl moiety of neonicotinoids and epibatidine reduces the potency for *Drosophila* but not for $\alpha4\beta2$ and $\alpha7$ nAChRs (115, 116).

BINDING SITE AND SUBSITE SPECIFICITY

Agonist ligands acting at vertebrate neurotransmitter-gated ion channels are characteristically cationic in nature. The iminium cation of the *N*-unsubstituted imine analogs of neonicotinoids (e.g., desnitro-IMI, Figures 3 and 4), or ammonium nitrogen of nicotine, epibatidine, or ACh, binds to a π-electron-rich subsite composed of aromatic residues, including the critical tryptophan in loop B of the α subunit. The cation makes van der Waals contact with the π-electrons (δ^-) of the aromatic residues (62, 64–66, 117–119). A supplementary role is proposed for aspartate 152 and/or 200 as an anionic residue from loop B and/or loop C of the $\alpha1$ subunit (120, 121). These structural features are also conserved in a snail ACh-binding protein (122, 123). The crystal structure of the snail ACh-binding

protein as a complex with nicotine reveals the following molecular features: the carbonyl oxygen of a tryptophan in loop B contacts through a hydrogen bond with the ammonium nitrogen of nicotine; the carbonyl oxygen of a leucine and amide nitrogen of a methionine (both from complementary loop E) make hydrogen bonds with the pyridine nitrogen of nicotine through a bridging water molecule (123a).

The neonicotinoids are an anomaly for the nicotinoid cation-π interaction model (53). The electronegative pharmacophore, crucial for optimum potency of the neonicotinoids, is proposed to associate with a cationic subsite (possibly lysine, arginine, or histidine) in the insect nAChR (Figure 4) (23, 53, 108). Lysine and arginine are prominent (and histidine minor) in the extracellular domain of Dα2, the main neonicotinoid-binding subunit (94, 98–100). Although no direct information is available on the actual location of the relevant residue(s), photoaffinity labeling with a suitable neonicotinoid ligand (115) coupled with computer-assisted docking simulation (118) may help define the orientation of the neonicotinoid electronegative tip in the binding domain. A point mutation (glutamine to arginine or lysine) on the avian α7 subunit confers enhanced electrophysiological response for IMI at 3 mM compared to that in the wild type (although the affinity of IMI on this

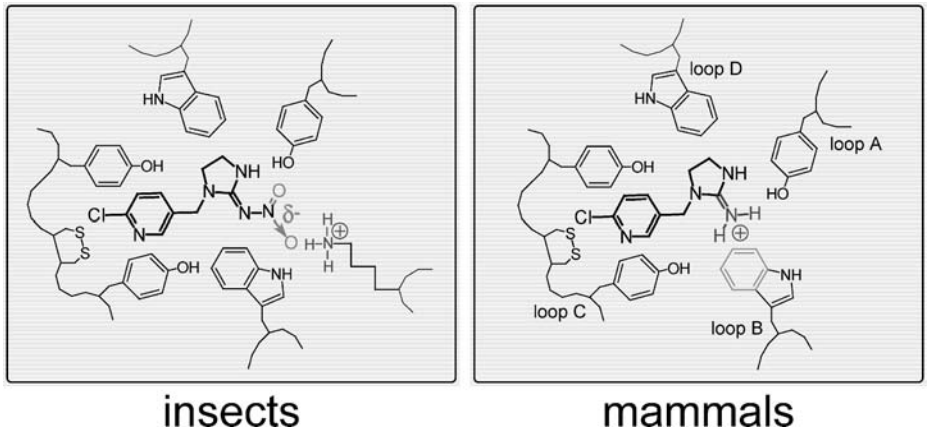

Figure 4 Binding subsite specificity shown as hypothetical schematic models for neonicotinoid imidacloprid binding in the insect nAChR and nicotinoid desnitroimidacloprid binding in the mammalian nAChR, each at the ACh agonist site. The positioning of desnitroimidacloprid and the interacting amino acids in the mammalian site is based on earlier modeling with ACh and nicotinoids (23, 53, 62, 65, 117, 118). In the mammalian binding site, loops A-C are from an α subunit and loop D from a complementary subunit. The insect (*Drosophila*) nAChR subunits conserve the aromatic and vicinal cysteine residues suitably positioned from homology modeling. Imidacloprid is arbitrarily placed in the same way as desnitroimidacloprid and a lysine (or alternatively arginine or histidine) cationic residue is introduced to interact with the negatively charged (δ^-) tip important in selectivity for insect versus mammalian nicotinic receptors.

mutated receptor remains unchanged) (124), providing possible support for a binding model featuring the role of the neonicotinoid electronegative pharmacophore (23, 53). These relationships provide a testable model for the hypothesis that specific subsite differences between insect and vertebrate receptors confer neonicotinoid selectivity.

MECHANISMS OF SELECTIVE ACTION

The neonicotinoids are the newest major class of insecticides. They are structurally distinct from all other synthetic and botanical pesticides and exhibit favorable selectivity (7, 39, 125). As plant systemics they are increasingly replacing organophosphates and methylcarbamates to control piercing-sucking insect pests, and are also highly effective flea control agents for cats and dogs. They generally have low acute toxicity to mammals, birds, and fish, but display some chronic toxicities in mammals. Biotransformations involve initial oxidation or reduction as both activation and detoxification mechanisms. The neonicotinoids are nicotinic agonists that interact with the nAChR in a very different way than nicotine, which confers selectivity to insects versus mammals. The neonicotinoids are not protonated but instead have an electronegative tip consisting of a nitro or cyano pharmacophore that imparts potency and selectivity, presumably by binding to a unique cationic subsite of the insect receptor. This is in marked contrast to the action of protonated nicotinoids, which require a cation-π interaction for binding to the vertebrate receptor. These differences provide the neonicotinoids with favorable toxicological profiles.

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LITERATURE CITED

1. Ware GW. 2000. *The Pesticide Book.* Fresno, CA: Thomson Publ. 418 pp.
2. Casida JE, Quistad GB. 1998. Golden age of insecticide research: past, present, or future? *Annu. Rev. Entomol.* 43:1–16
3. Kagabu S. 1997. Chloronicotinyl insecticides—discovery, application and

future perspective. *Rev. Toxicol.* 1:75–129

4. Yamamoto I, Casida JE, eds. 1999. *Nicotinoid Insecticides and the Nicotinic Acetylcholine Receptor.* Tokyo: Springer-Verlag. 300 pp.
5. Nauen R, Ebbinghaus-Kintscher U, Elbert A, Jeschke P, Tietjen K. 2001. Acetylcholine receptors as sites for developing neonicotinoid insecticides. In *Biochemical Sites of Insecticide Action and Resistance*, ed. I Ishaaya, pp. 77–105. Berlin, Heidelberg: Springer
6. Kagabu S. 2003. Molecular design of neonicotinoids: past, present and future. In *Chemistry of Crop Protection, Progress and Prospects in Science and Regulation*, ed. G Voss, G Ramos, pp. 193–212. Weinheim, Ger.: Wiley-VCH
7. Tomizawa M, Casida JE. 2003. Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. *Annu. Rev. Entomol.* 48:339–64
8. Yamamoto I. 1965. Nicotinoids as insecticides. In *Advances in Pest Control Research*, ed. RL Metcalf, vol. 6, pp. 231–60. New York: Wiley
9. Soloway SB, Henry AC, Kollmeyer WD, Padgett WM, Powell JE, et al. 1978. Nitromethylene heterocycles as insecticides. In *Pesticide and Venom Neurotoxicity*, ed. DL Shankland, RM Hollingworth, T Smyth Jr, pp. 153–58. New York: Plenum
10. Soloway SB, Henry AC, Kollmeyer WD, Padgett WM, Powell JE, et al. 1979. Nitromethylene insecticides. In *Advances in Pesticide Science*, ed. H Geissbüehler, vol. 2, pp. 206–17. Oxford: Pergamon
11. Kollmeyer WD, Flattum RF, Foster JP, Powell JE, Schroeder ME, Soloway SB. 1999. Discovery of the nitromethylene heterocycle insecticides. In *Nicotinoid Insecticides and the Nicotinic Acetylcholine Receptor*, ed. I Yamamoto, JE Casida, pp. 71–89. Tokyo: Springer-Verlag.
12. Kleier D, Holden I, Casida JE, Ruzo LO. 1985. Novel photoreactions of an insecticidal nitromethylene heterocycle. *J. Agric. Food Chem.* 33:998–1000
13. Kagabu S, Medej S. 1995. Stability comparison of imidacloprid and related compounds under simulated sunlight, hydrolysis conditions, and to oxygen. *Biosci. Biotechnol. Biochem.* 59:980–85
14. Kagabu S, Akagi T. 1997. Quantum chemical consideration of photostability of imidacloprid and related compounds. *J. Pestic. Sci.* 22:84–89
15. Tomlin CDS, ed. 2003. *The Pesticide Manual.* Alton, Hampshire, UK: Br. Crop Protection Council. 1344 pp. 13th ed.
16. Yamamoto I, Tomizawa M, Saito T, Miyamoto T, Walcott EC, Sumikawa K. 1998. Structural factors contributing to insecticidal and selective actions of neonicotinoids. *Arch. Insect Biochem. Physiol.* 37:24–32
17. Jeschke P, Moriya K, Lantzsch R, Seifert H, Lindner W, et al. 2001. Thiacloprid (Bay YRC 2894)—a new member of the chloronicotinyl insecticide (CNI) family. *Pflanzenschutz-Nachr. Bayer* 54:147–60
18. Schenker R, Tinembart O, Humbert-Droz E, Cavaliero T, Yerly B. 2003. Comparative speed of kill between nitenpyram, fipronil, imidacloprid, selamectin and cythioate against adult *Ctenocephalides felis* (Bouché) on cats and dogs. *Vet. Parasitol.* 112:249–54
19. Spande TF, Garraffo HM, Edwards MW, Yeh HJC, Pannell L, Daly JW. 1992. Epibatidine: a novel (chloropyridyl)azabicycloheptane with potent analgesic activity from an Ecuadoran poison frog. *J. Am. Chem. Soc.* 114: 3475–78
20. Badio B, Daly JW. 1994. Epibatidine, a potent analgesic and nicotinic agonist. *Mol. Pharmacol.* 45:563–69
21. Decker MW, Meyer MD. 1999. Therapeutic potential of neuronal nicotinic acetylcholine receptor agonists as

novel analgesics. *Biochem. Pharmacol.* 58:917–23
22. Lloyd GK, Williams M. 2000. Neuronal nicotinic acetylcholine receptors as novel drug targets. *J. Pharmacol. Exp. Ther.* 292:461–67
23. Tomizawa M, Lee DL, Casida JE. 2000. Neonicotinoid insecticides: molecular features conferring selectivity for insect versus mammalian nicotinic receptors. *J. Agric. Food Chem.* 48:6016–24
24. Elliott M. 1977. Synthetic pyrethroids. In *Synthetic Pyrethroids*. ACS Symposium Series 42, ed. M Elliott, pp. 1–28. Washington, DC: Am. Chem. Soc.
25. Environmental Protection Agency. 2003. Acetamiprid; pesticide tolerance. *Fed. Regist.* 68:52343–53
26. Environmental Protection Agency. 2003. Clothianidin; pesticide tolerance. *Fed. Regist.* 68:32390–400
27. Environmental Protection Agency. 2003. Dinotefuran; notice of filing a pesticide petition to establish a tolerance for a certain pesticide chemical in or on food. *Fed. Reg.* 68:39547–54
28. Environmental Protection Agency. 2003. Imidacloprid; pesticide tolerances. *Fed. Reg.* 68:35303–15
29. Environmental Protection Agency. 2003. Thiacloprid; pesticide tolerances. *Fed. Reg.* 68:55503–13
30. Environmental Protection Agency. 2002. Thiamethoxam; pesticide tolerance. *Fed. Reg.* 67:66561–71
31. Tomizawa M, Cowan A, Casida JE. 2001. Analgesic and toxic effects of neonicotinoid insecticides in mice. *Toxicol. Appl. Pharmacol.* 177:77–83
32. Tomizawa M, Casida JE. 2000. Imidacloprid, thiacloprid, and their imine derivatives up-regulate the $\alpha 4\beta 2$ nicotinic acetylcholine receptor in M10 cells. *Toxicol. Appl. Pharmacol.* 169:114–20
33. Tomizawa M, Casida JE. 2002. Desnitroimidacloprid activates the extracellular signal-regulated kinase cascade via the nicotinic receptor and intracellular calcium mobilization in N1E-115 cells. *Toxicol. Appl. Pharmacol.* 184:180–86
34. Cordero-Erausquin M, Marubio LM, Klink R, Changeux J-P. 2000. Nicotinic receptor function: new perspectives from knockout mice. *Trends Pharmacol. Sci.* 21:211–17
35. Hamann SR, Martin WR. 1992. Opioid and nicotinic analgesic and hyperalgesic loci in the rat brain stem. *J. Pharmacol. Exp. Ther.* 261:707–15
36. Khan IM, Yaksh TL, Taylor P. 1997. Epibatidine binding sites and activity in the spinal cord. *Brain Res.* 753:269–82
37. Khan IM, Stanislaus S, Zhang L, Taylor P, Yaksh TL. 2001. A-85380 and epibatidine each interact with disparate spinal nicotinic receptor subtypes to activate analgesia and nociception. *J. Pharmacol. Exp. Ther.* 297:230–39
38. Tomizawa M, Casida JE. 1999. Minor structural changes in nicotinoid insecticides confer differential subtype selectivity for mammalian nicotinic acetylcholine receptors. *Br. J. Pharmacol.* 127:115–22
39. Sheets LP. 2002. The neonicotinoid insecticides. In *Handbook of Neurotoxicology*, ed. EJ Massaro, vol. 1, pp. 79–87. Totowa, NJ: Humana
40. Roberts TR, Hutson DH, eds. 1999. *Metabolic Pathways of Agrochemicals. Part. 2: Insecticides and Fungicides*. Cambridge, UK: R. Soc. Chem. 1475 pp.
41. Thyssen J, Machemer L. 1999. Imidacloprid: toxicology and metabolism. In *Nicotinoid Insecticides and the Nicotinic Acetylcholine Receptor*, ed. I Yamamoto, JE Casida, pp. 213–22. Tokyo: Springer-Verlag
42. Klein O. 2001. Behaviour of thiacloprid (YRC 2894) in plants and animals. *Pflanzenschutz-Nachr. Bayer* 54:209–40
43. Klein O. 2003. Behaviour of clothianidin (TI-435) in plants and animals. *Pflanzenschutz-Nachr. Bayer* 56:75–101
44. Yokota T, Mikata K, Nagasaki H, Ohta K. 2003. Absorption, tissue distribution,

excretion, and metabolism of clothianidin in rats. *J. Agric. Food Chem.* 51: 7066–72

45. Reed WT, Erlam GJ. 1978. The house fly metabolism of nitromethylene insecticides. In *Pesticide and Venom Neurotoxicity*, ed. DL Shankland, RM Hollingworth, T Smyth Jr, pp. 159–69. New York: Plenum
46. Nauen R, Tietjen K, Wagner K, Elbert A. 1998. Efficacy of plant metabolites of imidacloprid against *Myzus persicae* and *Aphis gossypii* (Homoptera: Aphididae). *Pestic. Sci.* 52:53–57
47. Nauen R, Reckmann U, Armborst S, Stupp H-P, Elbert A. 1999. Whitefly-active metabolites of imidacloprid: biological efficacy and translocation in cotton plant. *Pestic. Sci.* 55:265–71
48. Nauen R, Ebbinghaus-Kintscher U, Schmuck R. 2001. Toxicity and nicotinic acetylcholine receptor interaction of imidacloprid and its metabolites in *Apis mellifera* (Hymenoptera: Apidae). *Pest Manag. Sci.* 57:557–86
49. Tomizawa M, Latli B, Casida JE. 1996. Novel neonicotinoid-agarose affinity column for *Drosophila* and *Musca* nicotinic acetylcholine receptors. *J. Neurochem.* 67:1669–76
50. Wiesner P, Kayser H. 2000. Characterization of nicotinic acetylcholine receptors from the insects *Aphis craccivora, Myzus persicae*, and *Locusta migratoria* by radioligand binding assays: relation to thiamethoxam action. *J. Biochem. Mol. Toxicol.* 14:221–30
51. Zhang A, Kayser H, Maienfisch P, Casida JE. 2000. Insect nicotinic acetylcholine receptor: conserved neonicotinoid specificity of [^{3}H]imidacloprid binding site. *J. Neurochem.* 75:1294–303
52. Nauen R, Ebbinghaus-Kintscher U, Salgado VL, Kaussmann M. 2003. Thiamethoxam is a neonicotinoid precursor converted to clothianidin in insects and plants. *Pestic. Biochem. Physiol.* 76:55–69
53. Tomizawa M, Zhang N, Durkin KA, Olmstead MM, Casida JE. 2003. The neonicotinoid electronegative pharmacophore plays the crucial role in the high affinity and selectivity for the *Drosophila* nicotinic receptor: an anomaly for the nicotinoid cation-π interaction model. *Biochemistry* 42:7819–27
54. Chao SL, Casida JE. 1997. Interaction of imidacloprid metabolites and analogs with the nicotinic acetylcholine receptor of mouse brain in relation to toxicity. *Pestic. Biochem. Physiol.* 58:77–88
55. Schulz-Jander DA, Casida JE. 2002. Imidacloprid insecticide metabolism: human cytochrome P450 isozymes differ in selectivity for imidazolidine oxidation versus nitroimine reduction. *Toxicol. Lett.* 132:65–70
56. Liu M-Y, Lanford J, Casida JE. 1993. Relevance of [^{3}H]imidacloprid binding site in house fly head acetylcholine receptor to insecticidal activity of 2-nitromethylene- and 2-nitroimino-imidazolidines. *Pestic. Biochem. Physiol.* 46:200–6
57. Daborn P, Boundy S, Yen J, Pittendrigh B, ffrench-Constant R. 2001. DDT resistance in *Drosophila* correlates with *Cyp6g1* over-expression and confers cross-resistance to the neonicotinoid imidacloprid. *Mol. Genet. Genomics* 266:556–63
58. Le Goff G, Boundy S, Daborn PJ, Yen JL, Sofer L, et al. 2003. Microarray analysis of cytochrome P450 mediated insecticide resistance in *Drosophila*. *Insect Biochem. Mol. Biol.* 33:701–8
59. Schulz-Jander DA, Leimkuehler WM, Casida JE. 2002. Neonicotinoid insecticides: reduction and cleavage of imidacloprid nitroimine substituent by liver microsomal and cytosolic enzymes. *Chem. Res. Toxicol.* 15:1158–65
60. Dick RA, Kanne DB, Casida JE. 2004. Aldehyde oxidase catalyzes the reduction of the nitroimine moiety of the

neonicotinoid insecticide imidacloprid. *Drug Metab. Rev.* 36 (Suppl. 1):270

60a. Klein O. 1994. The metabolism of imidacloprid in animals. *IUPAC Int. Congr. Pestic. Chem., 8th, Washington, DC* Abstr. 367

61. Brunet J-L, Maresca M, Fantini J, Belzunces LP. 2004. Human intestinal absorption of imidacloprid with Caco-2 cells as enterocyte model. *Toxicol. Appl. Pharmacol.* 194:1–9

62. Corringer J-P, Le Novère N, Changeux J-P. 2000. Nicotinic receptors at the amino acid level. *Annu. Rev. Pharmacol. Toxicol.* 40:431–58

63. Elgoyhen AB, Vetter DE, Katz E, Rothlin CV, Heinemann SF, Boulter J. 2001. $\alpha 10$: a determinant of nicotinic cholinergic receptor function in mammalian vestibular and cochlear mechanosensory hair cells. *Proc. Natl. Acad. Sci. USA* 98:3501–6

64. Dougherty DA. 1996. Cation-π interactions in chemistry and biology: a new view of benzene, Phe, Tyr, and Trp. *Science* 271:163–68

65. Zhong W, Gallivan JP, Zhang Y, Li L, Lester HA, Dougherty DA. 1998. From *ab initio* quantum mechanics to molecular neurobiology: a cation-π binding site in the nicotinic receptor. *Proc. Natl. Acad. Sci. USA* 95:12088–93

66. Dougherty DA, Lester HA. 2001. Snails, synapses and smokers. *Nature* 411:252–55

67. Zacharias N, Dougherty DA. 2002. Cation-π interactions in ligand recognition and catalysis. *Trends Pharmacol. Sci.* 23:281–87

68. Holladay MW, Dart MJ, Lynch JK. 1997. Neuronal nicotinic acetylcholine receptors as targets for drug discovery. *J. Med. Chem.* 40:4170–94

69. Romanelli MN, Gualtieri F. 2003. Cholinergic nicotinic receptors: competitive ligands, allosteric modulators, and their potential applications. *Med. Res. Rev.* 23:393–426

70. Bunnelle WH, Dart MJ, Schrimpf MR. 2004. Design of ligands for the nicotinic acetylcholine receptors: the quest for selectivity. *Curr. Top. Med. Chem.* 4:299–334

71. Methfessel C. 1992. Effect of imidacloprid on the acetylcholine receptor of rat muscle. *Pflanzenschutz-Nachr. Bayer* 45:369–80

72. Zwart R, Oortgiesen M, Vijverberg HPM. 1994. Nitromethylene heterocycles: selective agonists of nicotinic receptors in locust neurons compared to mouse N1E-115 and BC3H1 cells. *Pestic. Biochem. Physiol.* 48:202–13

73. Tomizawa M, Otsuka H, Miyamoto T, Yamamoto I. 1995. Pharmacological effects of imidacloprid and its related compounds on the nicotinic acetylcholine receptor with its ion channel from the *Torpedo* electric organ. *J. Pestic. Sci.* 20: 49–56

74. Yamamoto I, Yabuta G, Tomizawa M, Saito T, Miyamoto T, Kagabu S. 1995. Molecular mechanism for selective toxicity of nicotinoids and neonicotinoids. *J. Pestic. Sci.* 20:33–40

75. Nagata K, Aistrup GL, Song JH, Narahashi T. 1996. Subconductance-state currents generated by imidacloprid at the nicotinic acetylcholine receptor of PC 12 cells. *NeuroReport* 7:1025–28

76. D'Amour KA, Casida JE. 1999. Desnitroimidacloprid and nicotine binding site in rat recombinant $\alpha 4\beta 2$ neuronal nicotinic acetylcholine receptor. *Pestic. Biochem. Physiol.* 64:55–61

77. Ihara M, Matsuda K, Otake M, Kuwamura M, Shimomura M, et al. 2003. Diverse actions of neonicotinoids on chicken $\alpha 7$, $\alpha 4\beta 2$ and *Drosophila*-chicken SAD$\beta 2$ and ALS$\beta 2$ hybrid nicotinic acetylcholine receptors expressed in *Xenopus laevis* oocytes. *Neuropharmacology* 45:133–44

78. Schroeder ME, Flattum RF. 1984. The mode of action and neurotoxic properties of the nitromethylene heterocyclic

insecticides. *Pestic. Biochem. Physiol.* 22:148–60

79. Sattelle DB, Buckingham SD, Wafford KA, Sherby SM, Bakry NM, et al. 1989. Actions of the insecticide 2(nitromethylene)tetrahydro-1,3-thiazine on insect and vertebrate nicotinic acetylcholine receptors. *Proc. R. Soc. London Ser. B* 273:501–14
80. Abbink K. 1991. The biochemistry of imidacloprid. *Pflanzenschutz-Nachr. Bayer* 44:183–195
81. Bai D, Lummis SCR, Leicht W, Breer H, Sattelle DB. 1991. Actions of imidacloprid and a related nitromethylene on cholinergic receptors of an identified insect motor neurone. *Pestic. Sci.* 33:197–204
82. Tomizawa M, Yamamoto I. 1992. Binding of nicotinoids and the related compounds to the insect nicotinic acetylcholine receptor. *J. Pestic. Sci.* 17:231–36
83. Tomizawa M, Yamamoto I. 1993. Structure-activity relationships of nicotinoids and imidacloprid analogs. *J. Pestic. Sci.* 18:91–98
84. Liu M-Y, Casida JE. 1993. High affinity binding of [^{3}H]imidacloprid in the insect acetylcholine receptor. *Pestic. Biochem. Physiol.* 46:40–46
85. Tomizawa M, Latli B, Casida JE. 1999. Structure and function of insect nicotinic acetylcholine receptors studied with nicotinoid insecticide affinity probes. In *Nicotinoid Insecticides and the Nicotinic Acetylcholine Receptor*, ed. I Yamamoto, JE Casida, pp. 271–92. Tokyo: Springer-Verlag
86. Nishimura K, Kanda Y, Okazawa A, Ueno T. 1994. Relationship between insecticidal and neurophysiological activities of imidacloprid and related compounds. *Pestic. Biochem. Physiol.* 50: 51–59
87. Gundelfinger ED, Schulz R. 2000. Insect nicotinic acetylcholine receptors: genes, structure, physiological and pharmacological properties. In *Handbook of Experimental Pharmacology. Vol. 144: Neuronal Nicotinic Receptors*, ed. F Clementi, D Fornasari, C Gotti, pp. 497–521. Berlin: Springer
88. Littleton JT, Ganetzky B. 2000. Ion channels and synaptic organization: analysis of the *Drosophila* genome. *Neuron* 26:35–43
89. Tomizawa M, Casida JE. 2001. Structure and diversity of insect nicotinic acetylcholine receptors. *Pest Manag. Sci.* 57:914–22
90. Bertrand D, Ballivet M, Gomez M, Bertrand S, Phannavong B, Gundelfinger ED. 1994. Physiological properties of neuronal nicotinic receptors reconstituted from the vertebrate $\beta 2$ subunit and *Drosophila* α subunits. *Eur. J. Neurosci.* 6:869–75
91. Lansdell SJ, Schmitt B, Betz H, Sattelle DB, Millar NS. 1997. Temperature-sensitive expression of *Drosophila* neuronal nicotinic acetylcholine receptors. *J. Neurochem.* 68:1812–19
92. Schulz R, Sawruk E, Mülhardt C, Bertrand S, Baumann A, et al. 1998. Dα3, a new functional α subunit of nicotinic acetylcholine receptors from *Drosophila*. *J. Neurochem.* 71:853–62
93. Lansdell SJ, Millar NS. 2000. Cloning and heterologous expression of Dα4, a *Drosophila* neuronal nicotinic acetylcholine receptor subunit: identification of an alternative exon influencing the efficiency of subunit assembly. *Neuropharmacology* 39:2604–14
94. Schulz R, Bertrand S, Chamaon K, Smalla K-H, Gundelfinger ED, Bertrand D. 2000. Neuronal nicotinic acetylcholine receptors from *Drosophila*: two different types of α subunits coassemble within the same receptor complex. *J. Neurochem.* 74:2537–46
95. Lansdell SJ, Millar NS. 2002. Dβ3, an atypical nicotinic acetylcholine receptor subunit from *Drosophila*: molecular cloning, heterologous expression and

coassembly. *J. Neurochem.* 80:1009–18

96. Orr N, Shaffner AJ, Watson GB. 1997. Pharmacological characterization of an epibatidine binding site in the nerve cord of *Periplaneta americana*. *Pestic. Biochem. Physiol.* 58:183–92
97. Chamaon K, Schulz R, Smalla K-H, Seidel B, Gundelfinger ED. 2000. Neuronal nicotinic acetylcholine receptors of *Drosophila melanogaster*: the α-subunit Dα3 and the β-type subunit ARD coassemble within the same receptor complex. *FEBS Lett.* 482:189–92
98. Chamaon K, Smalla K-H, Thomas U, Gundelfinger ED. 2002. Nicotinic acetylcholine receptors of *Drosophila*: three subunits encoded by genomically linked genes can co-assemble into the same receptor complex. *J. Neurochem.* 80:149–57
99. Tomizawa M, Casida JE. 1997. [^{125}I]Azidonicotinoid photoaffinity labeling of insecticide-binding subunit of *Drosophila* nicotinic acetylcholine receptor. *Neurosci. Lett.* 237:61–64
100. Tomizawa M, Wen Z, Chin H-L, Morimoto H, Kayser H, Casida JE. 2001. Photoaffinity labeling of insect nicotinic acetylcholine receptors with a novel [^{3}H]azidoneonicotinoid. *J. Neurochem.* 78:1359–66
101. Lansdell SJ, Millar NS. 2000. The influence of nicotinic receptor subunit composition upon agonist, α-bungarotoxin and insecticide (imidacloprid) binding affinity. *Neuropharmacology* 39:671–79
102. Beers WH, Reich E. 1970. Structure and activity of acetylcholine. *Nature* 228: 917–22
103. Sheridan RP, Nilakantan R, Dixon JS, Venkataraghavan R. 1986. The ensemble approach to distance geometry: application to the nicotinic pharmacophore. *J. Med. Chem.* 29:899–906
104. Glennon RA, Herndon JL, Dukat M. 1994. Epibatidine-aided studies toward definition of a nicotinic receptor pharmacophore. *Med. Chem. Res.* 4:461–73
105. Abreo MA, Lin N-H, Garvey DS, Gunn DE, Hettinger A-M, et al. 1996. Novel 3-pyridyl ethers with subnanomolar affinity for central neuronal nicotinic acetylcholine receptors. *J. Med. Chem.* 39: 817–25
106. Meyer MD, Decker MW, Rueter LE, Anderson DJ, Dart MJ, et al. 2000. The identification of novel structural compound classes exhibiting high affinity for neuronal nicotinic acetylcholine receptors and analgesic efficacy in preclinical models of pain. *Eur. J. Pharmacol.* 393:171–77
107. Kagabu S, Matsuno H. 1997. Chloronicotinyl insecticides. 8. Crystal and molecular structures of imidacloprid and analogous compounds. *J. Agric. Food Chem.* 45:276–81
108. Zhang N, Tomizawa M, Casida JE. 2004. α-Nitro ketone as an electrophile and nucleophile: synthesis of 3-substituted 2-nitromethylenetetrahydrothiophene and -tetrahydrofuran as *Drosophila* nicotinic receptor probes. *J. Org. Chem.* 69:876–81
109. Chamberlain K, Evans AA, Bromilow RH. 1996. 1-Octanol/water partition coefficient (K_{ow}) and pK_a for ionizable pesticides measured by a pH-metric method. *Pestic. Sci.* 47:265–71
110. Okazawa A, Akamatsu M, Ohoka A, Nishiwaki H, Cho W-J, et al. 1998. Prediction of the binding mode of imidacloprid and related compounds to house-fly head acetylcholine receptors using three-dimensional QSAR analysis. *Pestic. Sci.* 54:134–44
111. Matsuda K, Buckingham SD, Kleier D, Rauh JJ, Grauso M, Sattelle DB. 2001. Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. *Trends Pharmacol. Sci.* 22:573–80
112. Nakayama A, Sukekawa M. 1998. Quantitative correlation between molecular similarity and receptor-binding activity

of neonicotinoid insecticides. *Pestic. Sci.* 52:104–10

113. Boëlle J, Schneider R, Gérardin P, Loubinoux B, Maienfisch P, Rindlisbacher A. 1998. Synthesis and insecticidal evaluation of imidacloprid analogs. *Pestic. Sci.* 54:304–7
114. Kayser H, Lee C, Decock A, Baur M, Haettenschwiler J, Maienfisch P. 2004. Comparative analysis of neonicotinoid binding to insect membranes: I. A structure-activity study of the mode of [^{3}H]imidacloprid displacement in *Myzus persicae* and *Aphis craccivora*. *Pest Manag. Sci.* 60:945–58

114a. Wellmann H, Gomes M, Lee C, Kayser H. 2004. Comparative analysis of neonicotinoid binding to insect membranes: II. An unusual high affinity site for [^{3}H]thiamethoxam in *Myzus persicae* and *Aphis craccivora*. *Pest Manag. Sci.* 60:959–70

115. Zhang N, Tomizawa M, Casida JE. 2002. Structural features of azidopyridinyl neonicotinoid probes conferring high affinity and selectivity for mammalian $\alpha4\beta2$ and *Drosophila* nicotinic receptors. *J. Med. Chem.* 45:2832–40
116. Zhang N, Tomizawa M, Casida JE. 2003. 5-Azidoepibatidine: an exceptionally potent photoaffinity ligand for neuronal $\alpha4\beta2$ and $\alpha7$ nicotinic acetylcholine receptors. *Bioorg. Med. Chem. Lett.* 13:525–27
117. Foucaud B, Perret P, Grutter T, Goeldner M. 2001. Cysteine mutants as chemical sensors for ligand-receptor interactions. *Trends Pharmacol. Sci.* 22:170–73
118. Le Novère N, Grutter T, Changeux J-P. 2002. Models of the extracellular domain of the nicotinic receptors and of agonist- and Ca^{2+}-binding sites. *Proc. Natl. Acad. Sci. USA* 99:3210–15
119. Schmitt JD, Sharples CGV, Caldwell WS. 1999. Molecular recognition in nicotinic acetylcholine receptors: the importance of π-cation interactions. *J. Med. Chem.* 42:3066–74
120. O'Leary ME, White MM. 1992. Mutational analysis of ligand-induced activation of the *Torpedo* acetylcholine receptor. *J. Biol. Chem.* 267:8360–65
121. Sugiyama N, Boyd AE, Taylor P. 1996. Anionic residue in the α-subunit of the nicotinic acetylcholine receptor contributing to subunit assembly and ligand binding. *J. Biol. Chem.* 271:26575–81
122. Brejc K, van Dijk WJ, Klaassen RV, Schuurmans M, van der Oost J, et al. 2001. Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature* 411:269–76
123. Sixma TK, Smit AB. 2003. Acetylcholine binding protein (AChBP): a secreted glial protein that provides a high-resolution model for extracellular domain of pentameric ligand-gated ion channels. *Annu. Rev. Biophys. Biomol. Struct.* 32:311–34

123a. Celie PHN, van Rossum-Fikkert SE, van Dijk WJ, Brejc K, Smit AB, Sixma TK. 2004. Nicotine and carbamylcholine binding to nicotinic acetylcholine receptors as studied in AChBP crystal structures. *Neuron* 41:907–14

124. Shimomura M, Okuda H, Matsuda K, Komai K, Akamatsu M, Sattelle DB. 2002. Effects of mutations of a glutamine residue in loop D of the $\alpha7$ nicotinic acetylcholine receptor on agonist profiles for neonicotinoid insecticides and related ligands. *Br. J. Pharmacol.* 137:162–69
125. Sheets LP. 2001. Imidacloprid: a neonicotinoid insecticide. In *Handbook of Pesticide Toxicology*, ed. R Krieger, Vol. 2, pp. 1123–30. San Diego: Academic

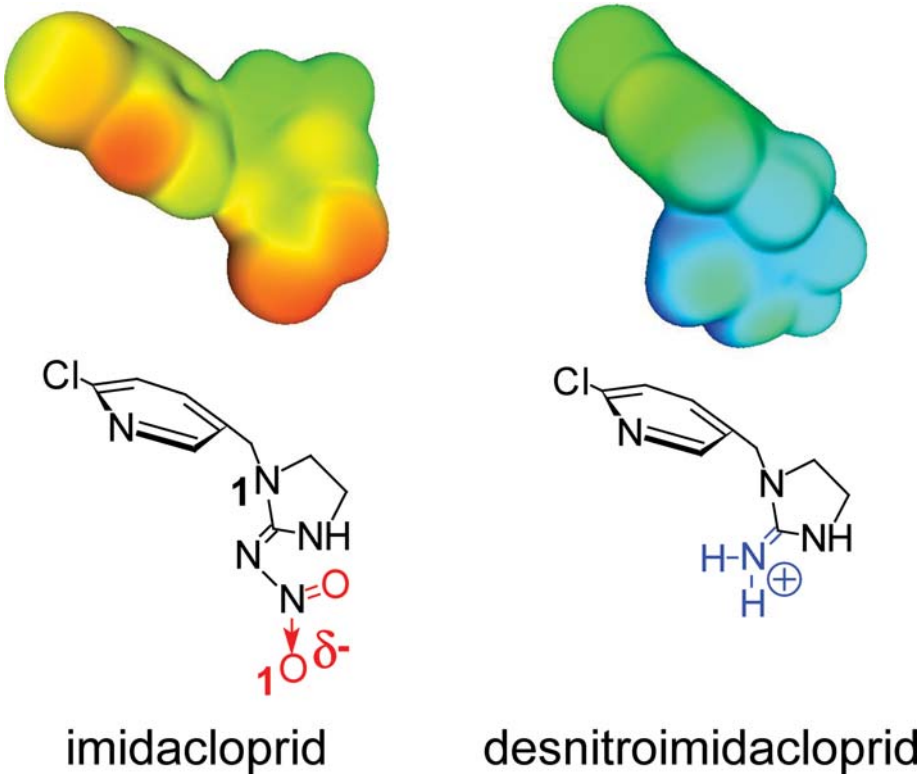

Figure 3 Molecular features of nicotinic agonists shown as electrostatic potential (ESP) mapping on the molecular surfaces of insect-selective imidacloprid and mammalian-selective desnitroimidacloprid (protonated at physiological pH) obtained in the gas phase by high-level *ab initio* calculation (53). ESP surfaces are shown as red for negative graded through orange, yellow, and green to blue for positive with an overall energy range of –60 to +160 kcal/mol. The strong electronegative tip illustrated for the nitro moiety of imidacloprid is also evident for the cyano substituent of thiacloprid (17).

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