Neonicotinoid Insecticides: Molecular Features Conferring Selectivity for Insect versus Mammalian Nicotinic Receptors

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The favorable selective toxicity of neonicotinoid insecticides (represented here by imidacloprid, thiacloprid, and a nitromethylene analogue) for insects versus mammals is not shared by three of their *N*-unsubstituted imine derivatives or by nicotine or epibatidine. The same selectivity pattern is evident at the receptor level, i.e., the insect nicotinic acetylcholine receptor (nAChR) versus mammalian nAChR subtypes (α1, α3, α4, and α7) assayed independently. The insect-selective compounds are not protonated with a nitroimine, cyanoimine, or nitromethylene group and the mammalian-selective compounds are ionized at physiological pH. We propose that the negatively charged tip of the nitro or cyano group (not a partial positive charge at imidazolidine N-1 as suggested earlier) interacts with a putative cationic subsite of the insect nAChR. This contrasts with the mammalian nAChRs where the iminium cation ($^{+}C{-}NH_2 \leftrightarrow C{=}^{+}NH_2$) of the neonicotinoid imine derivatives or ammonium nitrogen of nicotine or epibatidine interacts with the anionic subsite.

Keywords: *Desnitroimidacloprid; epibatidine; imidacloprid; neonicotinoid insecticides; nicotine; nicotinic acetylcholine receptors; thiacloprid*

INTRODUCTION

The major insecticides are nerve poisons acting on the voltage-dependent sodium channel, the γ-aminobutyric acid receptor, and the cholinergic system as inhibitors of acetylcholinesterase or agonists at the nicotinic acetylcholine receptor (nAChR) (Casida and Quistad, 1998). Selective toxicity involving low hazard for mammals and high potency to pests is an essential requirement for safe and effective pesticides. Nicotine (the classical nAChR agonist) was used for centuries to control sucking insects despite relatively low efficacy and high toxicity to mammals. The neonicotinoids, which are also nAChR agonists, are more toxic to insects and less toxic to mammals, providing an excellent example of selective toxicity (Kagabu, 1997; Yamamoto and Casida, 1999).

The nAChR is an agonist-regulated ion channel complex that is responsible for rapid neurotransmission. The mammalian nAChR consists of diverse subtypes assembled as five subunits in combinations from α1−9, β1−4, δ, γ, and ε. Differences in nAChR subtypes play an important role in neonicotinoid toxicology in mammals (Tomizawa and Casida, 1999). The skeletal muscle (or *Torpedo*) subtype is made up of two α1 subunits and one each of β1, δ, γ (or ε) subunits and is best understood relative to the ligand-binding site environment (Arias, 1997). Neuronal nAChR subtypes expressed in mammalian brain and ganglia are assembled in combinations of α2−9 and β2−4 and are pharmacologically classified into two branches based on sensitivity to α-bungarotoxin (α-BGT). The α-BGT-insensitive branch is made up of subtypes with combinations of α2−6 and β2−4 subunits. Of these, the α3β2β4α5 subtype is mainly found in ganglia and the α4β2 is the most abundant subtype in brain. The α7−9 subunits are involved in the α-BGT sensitive subtypes in brain and ganglia, and the amount of α7-containing receptor is comparable to that of the α4β2 subtype in brain (Holladay et al., 1997; Lindstrom, 1997; Pereira et al., 1998). In comparison, insect nAChRs are poorly understood relative to diversity and functional architecture (Tomizawa et al., 1999; Lansdell and Millar, 2000; Schulz et al., 2000; Tomizawa, 2000).

The goal of this study is to define the structural basis for the selective toxicity of neonicotinoid insecticides to insects and some of their derivatives to mammals. Eight compounds are considered (Figure 1). Three are potent neonicotinoid insecticides, i.e., the nitroimine imidacloprid (IMI) and the cyanoimine thiacloprid (THIA) (both commercial compounds) and a nitromethylene analogue (THPCHI) (Kagabu, 1997; Shiokawa et al., 1987). Another three are *N*-unsubstituted imines, i.e., the desnitro metabolite (Thyssen and Machemer, 1999) of IMI (DNIMI), the descyano derivative of THIA (DCTHIA), and a potent olefin analogue (DCTHIA-O) (Latli et al., 1999; Tomizawa and Casida, 2000). Two standards are included: (±)-epibatidine (from frog skin) (Badio and Daly, 1994) and (−)-nicotine. This investigation examines four hypotheses: selectivity at the organismal level is conferred by the receptor itself; protonation is an important determinant; selectivity is dependent on charge distribution of the neonicotinoid or analogue; variations between insects and mammals in neonicotinoid nAChR affinity are due to fundamental structural differences in their binding sites.

MATERIALS AND METHODS

Chemicals. The neonicotinoids and analogues studied are given in Figure 1. They were available from previous studies in this laboratory (Latli et al., 1999; Tomizawa and Casida, 1999, 2000). Other chemicals were obtained from various sources: (3-[^{125}I]*iodotyrosyl*)α-BGT ([^{125}I]α-BGT, >215 Ci/

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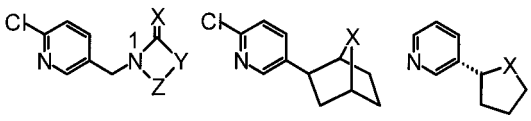


neonicotinoids (±)-epibatidine (-)-nicotine

compound	X	Y	Z
selective for insects			
IMI	NNO_2	NH	$(CH_2)_2$
THIA	NCN	S	$(CH_2)_2$
THPCHI	$CHNO_2$	NH	$(CH_2)_3$
selective for mammals			
DNIMI	NH	NH	$(CH_2)_2$
DCTHIA	NH	S	$(CH_2)_2$
DCTHIA-O	NH	S	CH=CH
(±)-epibatidine	NH	—	—
(-)-nicotine	NCH_3	—	—

Figure 1. Neonicotinoid insecticides selective for insects and analogues selective for mammals. Imidacloprid (IMI) and thiacloprid (THIA) are commercial insecticides and the nitromethylene analogue THPCHI also has high insecticidal activity. The *N*-unsubstituted imines are an IMI metabolite (DNIMI) and the descyano derivatives of THIA and an olefin analogue (DCTHIA and DCTHIA-O). (±)-Epibatidine and (−)-nicotine are used here as standards.

mmol) was obtained from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ); (−)-nicotine hydrogen tartrate and (±)-epibatidine dihydrochloride were purchased from Sigma (St. Louis, MO); dimethyl-d_6 sulfoxide (DMSO-d_6) and deuterium oxide (D_2O) were from Cambridge Isotope Laboratories, Inc. (Andover, MA); and Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), calf serum (CS), L-glutamine, and penicillin-streptomycin were obtained from Gibco Life Technologies (Grand Island, NY). Sigma was the source of monoclonal antibodies (mAbs) for the following nAChR subunits: mAb 210 for α1 (Tzartos et al., 1987), mAb 35 for α3 (Conroy et al., 1992), mAb 299 for α4 (Whiting and Lindstrom, 1988), and mAb 306 for α7 (Schoepfer et al., 1990).

HPLC Analysis and NMR Spectroscopy. The HPLC analysis method was essentially the same as that described by Kagabu and Medej (1995). Separation was carried out on an Ultrasphere ODS reversed-phase C_{18} column (4.6 × 250 mm) from Beckman (Fullerton, CA). Mobile phases were H_2O/MeOH (1:1) for neutral; H_2O/MeOH containing 0.1% trifluoroacetic acid (TFA) (1:1) for acidic; and 20 mM Na_2HPO_4 (pH 9.9)/MeOH (1:1) for basic conditions. Peak elution was monitored at 254 nm. ^{13}C NMR spectra were recorded at 75 MHz sequentially for samples (100 mg) in DMSO-d_6 (1.0 mL), then following addition of D_2O (50 μL), and finally after addition of TFA (20 μL). ^{15}N NMR spectra were recorded at 500 MHz for samples (300 mg) in DMSO-d_6 (1.0 mL) with about 3300 scans, using nitromethane as the external standard. NMR spectra for compounds in DMSO-d_6 were assigned for ^{13}C according to Kagabu et al. (1998) and for ^{15}N based on Philipsborn and Müller (1986) and Yamamoto et al. (1995).

Cell Culture. Cultures of human medulloblastoma TE671 cells expressing the α1γα1δβ1 nAChR subtype (Luther et al., 1989) (from the American Type Culture Collection, Rockville, MD) and human neuroblastoma SH−SY5Y cells expressing the α7-containing nAChR subtype (Peng et al., 1994) (from the Department of Molecular and Cell Biology, University of California at Berkeley) were maintained in DMEM supplemented with 2 mM L-glutamine, 100 units/mL penicillin, 0.1 mg/mL streptomycin and 10% FBS (for SH−SY5Y cells) or 10% CS (for TE671 cells) at 37 °C in 5% CO_2/95% air atmosphere with a medium change every 2−3 days. Some of the data cited were obtained with SH−SY5Y cells expressing the α3β2β4α5 nAChR subtype(s) and mouse fibroblast M10 cells transfected with the α4β2 subtype cultured as described by Tomizawa and Casida (1999, 2000).

Table 1. Toxicity of Neonicotinoids and Analogues to Housefly and Mouse

compound	housefly LD_{50} (μg/g)[a]	mouse LD_{50} (mg/kg)[b]
IMI	0.02−0.07[c,d]	40−50
THIA	0.03	25−30
THPCHI	0.08[c]	10−15
DNIMI	>5	6−9
DCTHIA	>5	0.7−1.5
DCTHIA-O	>5	0.7−1.5
(±)-epibatidine	>25[e]	0.06−0.09
(−)-nicotine	>50[d]	6−8

[a] Pretreated with 100 μg/g *O*-propyl *O*-(2-propynyl) phenylphosphonate as a metabolic detoxification inhibitor. [b] Estimated LD_{50} range from Tomizawa and Casida (unpublished data). [c] Data from Liu et al. (1993). [d] Data from Yamamoto et al. (1998). [e] Data from Liu et al. (1995).

Receptor Preparation. Receptor preparation and radioligand binding experiments were performed as described previously (Tomizawa and Casida, 1999, 2000). Cells in phosphate-buffered saline (PBS) (100 mM sodium chloride, 10 mM sodium phosphate, pH 7.5) were harvested with a cell lifter. The harvested cells were disrupted by brief vortexing in four volumes of lysis buffer (2% w/v Triton X-100, 50 mM sodium chloride, 5 mM EDTA, 5 mM EGTA, 2 mM phenylmethanesulfonyl fluoride, 5 mM benzamidine, and 5 mM iodoacetamide in 50 mM sodium phosphate, pH 7.5). After 20 min of gentle rotation on a rocking platform at 4 °C, the sample was centrifuged for 20 min at 4 °C in an Eppendorf microcentrifuge, and the supernatant was recovered for assay.

Radioligand Binding. MAb 35, 210, 299, or 306 (2−3 μg per well) in 0.1 mL of 10 mM sodium bicarbonate buffer (pH 8.8) was coupled to Immulon 4HBX Removawells (Dynex Technologies, Chantilly, VA) overnight at 4 °C. After three washes with 0.2 mL of the bicarbonate buffer, wells were quenched with 0.3 mL of 3% bovine serum albumin in PBS-Tween 20 buffer (0.05% Tween 20 in PBS) for 4 h at 4 °C. The wells were then washed three times with 0.3 mL of PBS-Tween 20. Supernatant from each cell preparation (0.1 mL) was added to the mAb-coated well and incubated overnight at 4 °C. The wells were washed three times with 0.2 mL of PBS-Tween 20, and then treated with the test compound in PBS-Tween 20 and after 20 min with [^{125}I]α-BGT (0.2 nM for α1 or 2 nM for α7 subtype) in 0.1 mL final volume with incubation overnight at 4 °C. The wells were then washed three times with 0.2 mL of PBS-Tween 20 and radioactivity remaining was subjected to liquid scintillation counting. Background binding was determined using wells lacking mAb. Data for α3β2β4α5 and α4β2 subtypes expressed in SH−SY5Y and M10 cells, respectively, are taken from our earlier studies (Tomizawa and Casida, 1999, 2000). Insect receptor preparation from fruit fly (*Drosophila melanogaster*) heads and [^{3}H]IMI binding were performed according to our previous procedure (Tomizawa et al., 1996). IC_{50} values (molar concentrations of test compounds for 50% inhibition of specific radioligand binding) were determined by iterative nonlinear least-squares regression using the Sigmaplot program (Jandel Scientific Software, San Rafael, CA).

Toxicity. Toxicity to mice was determined by the intraperitoneal route. Insecticidal activity assays with adult houseflies (*Musca domestica*) were performed by intrathoracic injection as before (Liu et al., 1993; Yamamoto et al., 1998).

RESULTS

Selective Toxicity (Figure 1, Table 1). As appropriate for selective insecticides, the nitroimine (IMI), cyanoimine (THIA), and nitromethylene (THPCHI) were over a hundred times more toxic to houseflies than mice under the test conditions. The corresponding *N*-unsubstituted imines were moderately toxic (DNIMI) to highly toxic (DCTHIA and DCTHIA-O) to mice, with little or no toxicity to houseflies. The secondary amine (±)-

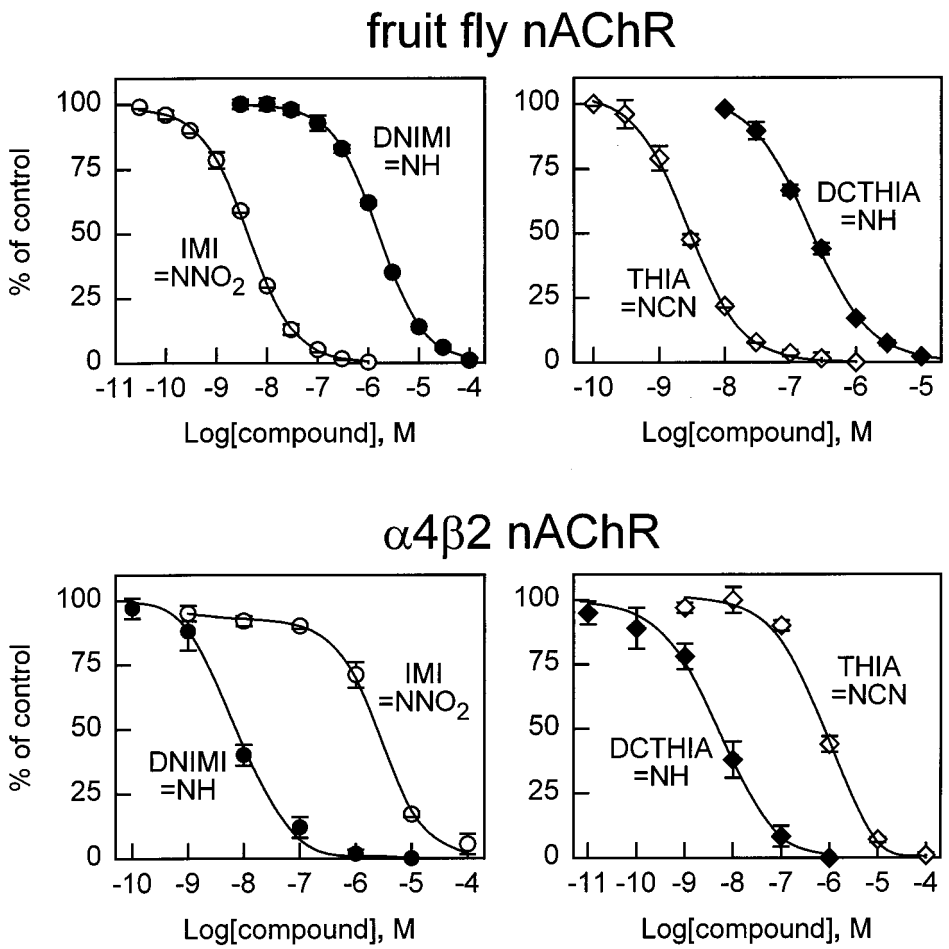


Figure 2. Displacement by neonicotinoids (IMI and THIA) and their imine analogues (DNIMI and DCTHIA) of 3 nM [^{3}H]IMI binding from fruit fly head nAChR and of 10 nM [^{3}H]nicotine binding from α4β2 nAChR (immuno-isolated by mAb 299 from mouse fibroblast M10 cells). Data for α4β2 nAChR are replotted from Tomizawa and Casida (2000). ═NH refers to *N*-unsubstituted imine, and ═NNO_2 or ═NCN refers to nitroimine or cyanoimine, respectively.

Table 2. Potency of Neonicotinoids and Analogues on Insect and Mammalian Nicotinic Acetylcholine Receptors

	insect	mammal[b]			
	[^{3}H]IMI binding IC_{50}±SD (nM, *n*=3)	[^{125}I]α-BGT binding IC_{50}±SD (μM, *n*=3)		[^{3}H]nicotine binding IC_{50}±SD (nM, *n*=3)	
compound[a]	fruit fly	α1	α7	α3[c]	α4[d]
IMI	4.6 ± 0.5	>300 (39%)	270 ± 80	14000 ± 4000	2600 ± 85
THIA	2.7 ± 0.4	120 ± 12	100 ± 2	NT	860 ± 31
THPCHI	0.37 ± 0.03	81 ± 9	1.0 ± 0.4	180 ± 50	80 ± 8
DNIMI	1530 ± 66	11 ± 0.5	9.9 ± 0.2	14 ± 5	8.2 ± 1.5
DCTHIA	200 ± 27	6.0 ± 0.5	6.0 ± 0.9	NT	4.4 ± 1.8
DCTHIA-O	185 ± 20	8.0 ± 0.8	4.0 ± 0.9	NT	1.4 ± 0.5
(±)-epibatidine	430 ± 20	0.16 ± 0.05	0.031 ± 0.004	NT[e]	0.037 ± 0.005
(−)-nicotine	4000 ± 165	21 ± 3	21 ± 1	45 ± 10	7.0 ± 2.1

[a] Chemical structures are given in Figure 1. [b] Mammalian nAChR subtypes expressed in cell lines were immuno-isolated: α1 (α1γα1$\delta\beta$1) in TE671 by mAb 210; α3 (α3β2β4α5) in SH−SY5Y by mAb 35; α4 (α4β2) in M10 by mAb 299; α7 (α7 homomer or heteromer with unknown subunit) in SH−SY5Y by mAb 306. [c] Data from Tomizawa and Casida (1999). [d] Data from Tomizawa and Casida (2000). [e] Picomolar affinity for both (+)- and (−)-epibatidine (Gerzanich et al. 1995). NT, not tested. Value in parentheses is percent inhibition at indicated concentration.

epibatidine and tertiary amine (−)-nicotine were respectively highly and moderately toxic to mice with much lower toxicity to houseflies. On this basis the neonicotinoids and analogues are classified as selective to insects for the first three compounds and to mammals for the remaining derivatives.

Selective Action on Insect versus Mammalian nAChR (Figure 2, Table 2). The studies next tested the hypothesis that the selective toxicity of the two sets of compounds might also be evident as selectivity in their action at insect and mammalian nAChRs. The insect nAChR was very sensitive to IMI, THIA, and THPCHI with IC_{50}s of 0.37−4.6 nM in marked contrast to their *N*-unsubstituted imine analogues (DNIMI, DCTHIA, and DCTHIA-O), (±)-epibatidine, and (−)-nicotine with IC_{50}s of 185−4000 nM.

The mammalian nAChRs, examined as four subtypes (individually containing α1, α3, α4, and α7 subunits), had the opposite selectivity with higher sensitivity to the *N*-unsubstituted imines, (±)-epibatidine, and (−)-nicotine compared with that to the *N*-substituted imines IMI and THIA. Interestingly, THPCHI with the nitro-

Table 3. Comparison of HPLC Retention Times for Neonicotinoids and Analogues under Neutral, Acidic, and Basic Conditions

	retention time (min)[a]		
compound	neutral	acidic	basic
IMI	4.84	4.87	4.82
THIA	6.06	6.07	6.05
DNIMI	2.90	2.80	4.24
DCTHIA	3.00	2.84	4.23
DCTHIA-O	3.02	2.98	4.21
(−)-nicotine	3.18	3.16	11.23

[a] Mobile phases: neutral, H_2O/MeOH (1:1); acidic, H_2O/MeOH containing 0.1% TFA (1:1); basic, 20 mM Na_2HPO_4 (pH 9.9)/MeOH (1:1). Flow rate 0.7 mL/min. Void volume 2.46 min. Retention times are the mean of three runs under each condition.

methylene moiety showed 21-fold higher affinity compared with (−)-nicotine to the α7 nAChR, although THPCHI had low affinity to the other three nAChR subtypes. (±)-Epibatidine was confirmed here as the best ligand among the tested compounds for all of the mammalian receptor subtypes.

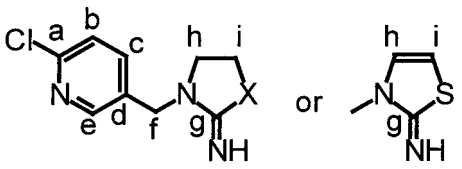
Basicity Determined by HPLC (Table 3). The biological observations above divide the compounds into two sets which may differ in basicity. Accordingly, the neonicotinoids and analogues were examined by reversed-phase HPLC under neutral and acidic conditions in which the ionized compounds would not be retained and under basic conditions in which the free base would be retained for a longer period. IMI and THIA gave almost the same retention times under neutral, acidic, and basic conditions. The chromatographic peaks for DNIMI, DCTHIA, DCTHIA-O, and (−)-nicotine appeared immediately after the void volume under neutral and acidic conditions as protonated ionic molecules. However, the retention times were significantly delayed (about 1.2−1.3 min for the *N*-unsubstituted imine analogues and 8.1 min for nicotine) as nonprotonated free bases under basic conditions with ionization suppressed.

Molecular Features Based on ^{13}C and ^{15}N NMR Spectroscopy. NMR spectral analyses were carried out to determine differences in molecular features for the two sets of compounds. ^{13}C NMR signals for the imino carbons of DNIMI, DCTHIA, and DCTHIA-O in DMSO-d_6 were shifted 1.6−1.8 ppm downfield when D_2O was added without significant changes for the signals of the other carbons (Table 4). This deshielding was therefore due to full or partial ionization of the imino moiety by protonation. The downfield shift was even more pronounced (4−8 ppm) on the addition of TFA to DCTHIA and DCTHIA-O (Table 4, footnote). No data were obtained for DNIMI with TFA as decomposition was observed with this imine in the DMSO/D_2O/TFA NMR solvent system.

Comparison of the ^{13}C NMR chemical shift of the imino or equivalent carbon atom (column **g** in Table 5) revealed little or no difference in the presence and the absence of an electron-withdrawing nitro or cyano substituent (i.e., IMI, CNIMI, and the nitromethylene analogue versus DNIMI) (Table 5). The ^{13}C NMR signal of the imino carbon (column **g**) in the thiazolidine THIA was shifted 12.8 ppm downfield compared with that in DCTHIA, but this relationship was not observed in the imidazolidine series (IMI, CNIMI, and DNIMI) (Table 5). In IMI and analogues, the nitro- or cyanoimino or nitromethylene group is coplanar with the imidazolidine ring plane (Kagabu and Matsuno, 1997). By analogy, in THIA, the steric hindrance between the cyano substituent and the large sulfur atom may distort the coplanar relationship between the cyanoimino group and the thiazolidine ring plane as a possible explanation for the unique downfield shift with THIA. The electron-donating effect of the sulfur atom in THIA, DCTHIA, and DCTHIA-O was clearly evident by comparing adjacent carbon **i** with the next carbon **h** involving an upfield shift of 18.8 to 26.6 ppm whereas in the imidazolidine series carbon **i** was only 2.9−3.8 ppm upfield of **h** in IMI, DNIMI, and CNIMI (Tables 4 and 5).

Only three of the expected four ^{15}N NMR signals were observed for DNIMI (Table 6), probably because of the equivalency of two of the nitrogen atoms of the guanidine moiety as reported earlier for arginine (Kanamori et al., 1978). The imine nitrogens of DNIMI (−302.1 ppm) and DCTHIA (−183.1 ppm) were shifted upfield in comparison to that of IMI (−144.7 ppm) as a result of the loss of the strongly electron-withdrawing nitro and cyano groups, respectively. Chemical shifts for the other nitrogen atoms in the imidazolidine or thiazolidine ring of DNIMI or DCTHIA were similar to those of IMI

Table 4. ^{13}C NMR Chemical Shifts of Neonicotinoid Analogues

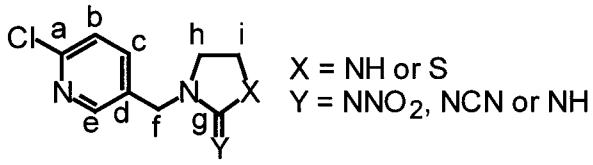


X = NH or S

	δ (ppm)								
compound	**a**	**b**	**c**	**d**	**e**	**f**	**g**	**h**	**i**
DNIMI									
DMSO-d_6	149.5	124.2	139.6	133.7	149.1	47.8	161.2	45.9	42.6
+D_2O	150.1	125.1	140.6	133.9	149.9	48.3	163.0[a]	46.2	42.6
DCTHIA									
DMSO-d_6	149.5	124.1	139.6	133.1	149.2	50.9	162.0	45.4	26.6
+D_2O	150.1	124.8	140.2	133.5	149.9	51.7	163.6[a]	46.0	27.2
DCTHIA-O									
DMSO-d_6	149.2	127.5	139.1	132.8	149.1	44.7	161.3	124.0	97.4
+D_2O	150.0	128.3	139.9	133.5	149.9	45.5	163.0[a]	124.8	98.5

[a] With TFA, DNIMI unstable, DCTHIA 166.2 ppm, and DCTHIA-O 169.2 ppm.

Table 5. ^{13}C NMR Chemical Shifts of Neonicotinoids and Analogues



	δ (ppm)[a]								
compound	**a**	**b**	**c**	**d**	**e**	**f**	**g**[b]	**h**	**i**
IMI	149.5	124.3	139.4	131.5	149.3	46.0	160.3	44.5	41.6
DNIMI	149.5	124.2	139.6	133.7	149.1	47.8	161.2	45.9	42.6
CNIMI[c,d]	149.4	124.2	139.3	131.7	149.2	46.2	163.4	44.2	40.4
THIA[d]	149.8	124.4	139.6	131.0	149.5	52.8	174.8	46.2	27.4
DCTHIA	149.5	124.1	139.6	133.1	149.2	50.9	162.0	45.4	26.6

[a] All samples were determined in DMSO-d_6. [b] δ for carbon **g** of nitromethylene analogue of IMI (X = NH, Y = $CHNO_2$) is 158.5 ppm (Kagabu et al., 1998). [c] Data for the cyano analogue of IMI (X = NH, Y = NCN) from Kagabu et al. (1998). [d] δ for cyano carbons of CNIMI and THIA are 111.7 and 117.2 ppm, respectively.

Table 6. ^{15}N NMR Chemical Shifts of Neonicotinoids and Analogues

	δ (ppm)[a]				
				imidazolidine[b] or thiazolidine	
compound	nitro N	pyridine N	imine N	1N	3N
IMI[c]	−12.0	−72.9	−144.7	−291.2	−288.0
DNIMI		−74.1	−302.1	−278.6	−302.1
DCTHIA		−74.1	−183.1	−286.4	

[a] Nitromethane as external standard (0 ppm). [b] δ (ppm) for the imidazolidine ring nitrogens of various neonicotinoid analogues having a nitroimino, cyanoimine, nitromethylene, or cyanomethylene group are −288.7 to −293.2 for the 1-position and −284.7 to −290.6 for the 3-position (Yamamoto et al., 1995). [c] Data from Yamamoto et al. (1995).

and also to its analogues with an electron-withdrawing substituent (Yamamoto et al., 1995).

Mulliken Atomic Charges of Neonicotinoids (Table 7). The Mulliken atomic charges of the neonicotinoid molecules were calculated as an index of charge distribution. The oxygen atoms (**g** and **h** in Table 7) of the nitro group and the imino nitrogen (**e**) of IMI or methylene carbon atom (**i**) of THPCHI had strong negative charges, whereas the nitrogen atom (**f**) of the nitro group had a strong positive charge. Interestingly, all of the atoms in the cyanoimino moiety of CNIMI and THIA (**d**, **e**, **j**, and **k**) were negatively charged to a moderate degree. The nitrogen atom (**a**) of the imidazolidine or thiazolidine ring of DNIMI or DCTHIA was slightly negative, whereas it was slightly positive in IMI, CNIMI, THIA, or THPCHI. The imino nitrogen atoms (**e**) of DNIMI and DCTHIA were negative.

DISCUSSION

Selectivity for Insects versus Mammals. Selective toxicity was considered for houseflies versus mice treated by injection and selective action at the nAChR by comparing fruit fly nAChR with various mammalian expressed nAChR subtypes. On this basis, the three neonicotinoids selectively toxic to insects are also selective for the insect nAChR whereas the *N*-unsubstituted imine and amine compounds examined are selective for mammalian toxicity and mammalian nAChRs, i.e., the receptor data in this context are predictive of selective toxicity.

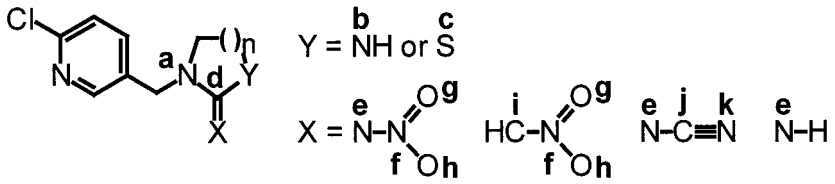
The neonicotinoids and analogues studied fall into two types with respect to basicity and action on insects versus mammals. IMI, THIA, and THPCHI (which are not protonated) are selectively toxic to insects and more potent at the insect receptor. The proton-accepting compounds [DNIMI, DCTHIA, DCTHIA-O, (−)-nicotine, and (±)-epibatidine] are selectively toxic to mammals and more potent at the mammalian nAChR subtypes (α1, α3, α4, and α7) than at the insect nAChR. It is therefore of interest to consider in more detail other molecular aspects relative to selective action.

Molecular Features of Nitro- and Cyanoimines and Nitromethylene. The insecticidal neonicotinoids have several features in common on comparing compounds with imidazolidine and isosteric moieties (thiazolidine, oxazolidine, tetrahydropyrimidine, or pyrrolidine) and nitroimino, cyanoimino, or nitromethylene substituents. When each molecule is first superimposed onto the most active compound so as to maximize the molecular shape similarity, the electrostatic similarity correlates well with the binding affinity (Nakayama and Sukekawa, 1998) and a similar correlation is obtained by comparative molecular field analysis (CoMFA) (Okazawa et al., 1998). Particularly high potency for the nitroimines, cyanoimines, and nitromethylenes with a negative electrostatic potential implies a positive electrostatic potential for the corresponding insect nAChR recognition site (Nakayama and Sukekawa, 1998).

Considerable attention has been given to the possible involvement of imidazolidine N-1 (Figure 1) or equivalent position of the thiazolidine ring in neonicotinoid action. X-ray crystal structure analysis of IMI and related compounds indicates that the precise distances between the van der Waals surface of the nitrogen of the 3-pyridinylmethyl moiety and the atomic center of N-1 of the imidazolidine are 5.45−6.06 Å. This value coincides with the distance between the ammonium nitrogen and carbonyl oxygen of acetylcholine and between the nitrogen atoms of (−)-nicotine (Kagabu and Matsuno, 1997), although the binding affinity of (−)-nicotine to insect nAChR is much lower than that of neonicotinoid insecticides.

^{15}N NMR chemical shifts are determined in part by the electronic status of the nitrogen atoms important in ligand−receptor interactions (Philipsborn and Müller, 1986). An earlier study, comparing neonicotinoids and analogues with (−)-nicotine and other 3-pyridinylmethylamines, led to the proposed importance of a partial positive charge at N-1 of the imidazolidine or equivalent moiety (intext figure **A**, below) due to the strong electron-withdrawing effect of the neighboring

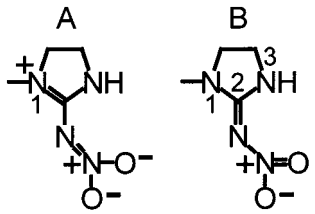
Table 7. Mulliken Atomic Charges of Neonicotinoids



compound	Mulliken atomic charge[a]						
	a	**b** or **c**	**d**	**e** or **i**	**f** or **j**	**g**	**h** or **k**
IMI	+0.0315	+0.0235	+0.113	−0.570	+1.306	−0.671	−0.567
CNIMI[b]	+0.0129	+0.0142	−0.0085	−0.0775	−0.0998		−0.110
THIA	+0.0118	+0.1210	−0.1086	−0.0056	−0.1088		−0.0747
THPCHI	+0.0355	+0.0266	+0.1035	−0.749	+1.358	−0.612	−0.682
DNIMI	−0.0063	−0.0205	−0.0606	−0.1892			
DCTHIA	−0.0076	+0.0493	−0.1402	−0.1203			

[a] Calculated by MNDO-PM3 with SPARTAN program. [b] Cyanoimino analogue of IMI. IMI (X = NNO_2, Y = NH, n = 1); CNIMI (X = NCN, Y = NH, n = 1); THIA (X = NCN, Y = S, n = 1); THPCHI (X = $CHNO_2$, Y = NH, n = 2); DNIMI (X = NH, Y = NH, n = 1); DCTHIA (X = NH, Y = S, n = 1). Atomic charges for pyridine nitrogens of the above six compounds were −0.0578 to −0.0688.

nitro or cyano group. On this basis it was suggested that the partial positively charged N-1 nitrogen and pyridine nitrogen interact with the anionic subsite and hydrogen-bonding site, respectively, in the insect receptor (Tomizawa and Yamamoto, 1993; Yamamoto et al., 1995). This binding model is supported by CoMFA (Okazawa et al., 1998). However, in the present investigation comparing ^{15}N NMR signals of the imidazolidine N-1 position of IMI and many analogues (Yamamoto et al., 1995) versus DNIMI and DCTHIA, little or no effect is noted on introducing the electron-withdrawing group. ^{13}C NMR studies support this observation on finding little or no difference in the chemical shift of the imino carbon atom in the presence (IMI and CNIMI) and the absence (DNIMI and DCTHIA) of an electron-withdrawing nitro or cyano substituent.



The Mulliken atomic charges of IMI, CNIMI, THIA, and THPCHI imply that the charge status of the heterocyclic nitrogen atom (N-1 or **a** in Table 7) in the above molecules is marginally positive in comparison with those of DNIMI and DCTHIA. Further, the Mulliken atomic charge or electrostatic potential-derived charge at the N-1 position does not correlate with the binding affinity (Nakayama and Sukekawa, 1998). Thus, it appears that the effect of the electron-withdrawing group is not sufficient to confer a strong positive charge at N-1 (intext figure **A**), and the N-1 nitrogen atom may not greatly contribute to insect receptor binding. The Mulliken atomic charge distribution of IMI and THPCHI reveals that the nitrogen atom of the nitro group is strongly positive but the imino nitrogen, methylene carbon and oxygen atoms are strongly negative. Interestingly, all of the atoms in the cyanoimino moiety of CNIMI and THIA are negatively charged to a moderate degree. On the other hand, the urea analogue of IMI (=O instead of =NNO_2) shows no biological activity, despite providing a localized negative charge (data not shown). This can be attributed to the difference in distance to a negative locus between the urea analogue and IMI. In addition, the distance from the imino nitrogen (or methylene carbon) atom to the oxygen atoms of the =NNO_2 (or =$CHNO_2$) moiety coincides with that from the imino nitrogen atom to the cyano nitrogen atom of =NCN (Kagabu and Matsuno, 1997). It is therefore plausible that the negatively charged tip of the nitro group or negatively charged region of the cyano group (but not the negatively charged imino nitrogen or methylene carbon) is more important in providing affinity for the insect nAChR than the positively charged nitrogen atom of the nitro group (intext figure **B**).

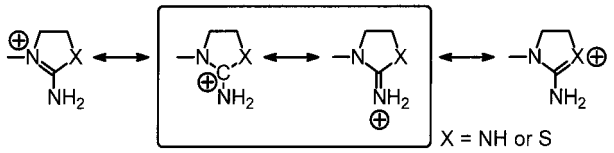


Figure 3. Resonance structures of protonated neonicotinoid imine analogues (X = NH for DNIMI and X = S for DCTHIA) with proposed form under physiological conditions in box.

Molecular Features of Ionized *N*-Unsubstituted Imines. The differences in potency and selectivity for the nitro- and cyanoimines versus the *N*-unsubstituted imines prompted a comparison of the molecular features for the two sets of compounds. The basicity of the whole molecule is evident in the base-induced shift in HPLC retention time, relative to that in neutral or acidic condition, for the neonicotinoid imine analogues (DNIMI, DCTHIA, and DCTHIA-O) and for the tertiary amine (−)-nicotine but not for the nitroimine (IMI) and cyanoimine (THIA). The neonicotinoid imine analogues, as (−)-nicotine itself, are therefore probably mostly ionized at physiological pH.

The site of protonation in the *N*-unsubstituted imines is evident from their ^{15}N and ^{13}C NMR spectral features. ^{15}N NMR shows that the electron density of the imino nitrogen is higher for DNIMI and DCTHIA than it is for IMI, i.e., the *N*-unsubstituted imino nitrogen is a potential proton acceptor. This is also confirmed by the calculated Mulliken atomic charges. In ^{13}C NMR experiments, the addition of D_2O or TFA results in a change only in the imino carbon which is deshielded, establishing the imino moiety as the site of protonation. Figure 3 shows possible resonance structures conferring a positive charge on the imino nitrogen and/or carbon atom under physiological conditions.

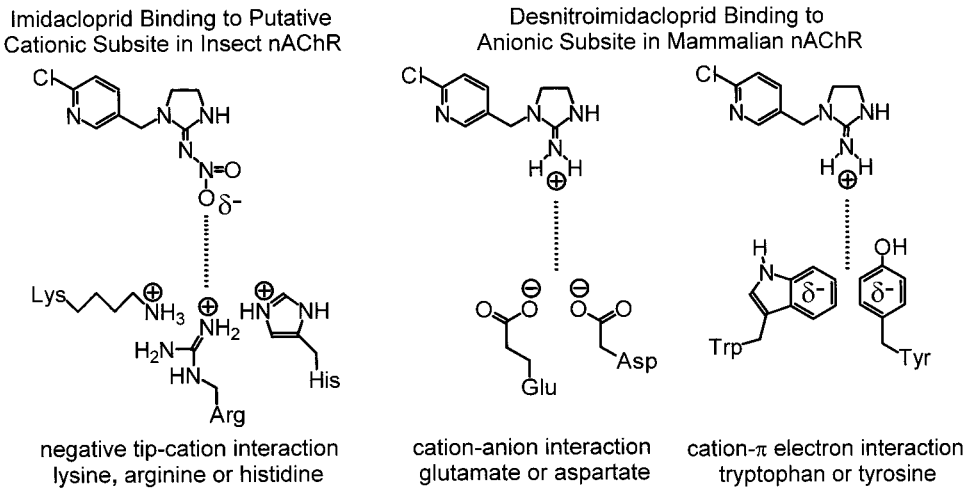


Figure 4. Schematic representation of the interaction of an imidacloprid (IMI) nitro-oxygen atom with the putative cationic subsite of insect nAChR and of the desnitroimidacloprid (DNIMI) iminium cation with the anionic subsite of mammalian nAChR. Although a nitro group is shown, the negatively charged tip or region can also be a cyano group. The nitrogen atom of the 3-pyridinyl moiety is also important for interaction with a hydrogen-bonding subsite of the receptor. Two hypotheses are shown for the anionic subsite based on acetylcholine involving cation−anion interaction (Karlin and Akabas, 1995) and cation−π electron interaction (Zhong et al., 1998). The site of ionization in DNIMI is considered to be either the imino nitrogen or carbon (see Figure 3).

Insect versus Mammalian nAChRs. There are distinct selectivity differences in the action of neonicotinoid insecticides and their *N*-unsubstituted imino derivatives at the insect versus mammalian nAChRs. Neonicotinoids with nitroimino and cyanoimino substituents are selective for insect receptors, and their *N*-unsubstituted imino derivatives (and also nicotine and epibatidine) with iminium or ammonium moieties are selective for mammalian nAChRs. In both cases the pyridinyl nitrogen also makes an important contribution, e.g. comparing insect nAChR affinity of tetrahydro-2-(nitromethylene)-2*H*-1,3-thiazine and 2-nitromethyleneimidazolidine with IMI and many analogues containing the 3-pyridinylmethyl moiety (Tomizawa and Yamamoto, 1992; Liu and Casida, 1993; Yamamoto and Casida, 1999).

We suggest that the critical feature for insect nAChR binding is the negatively charged tip or region of the nitro or cyano group (not a partial positive charge at imidazolidine N-1 as suggested earlier); this hypothesis is based on directly comparing the effect of electron-withdrawing moieties on selectivity for insect versus mammalian nAChRs. It is also proposed that this negatively charged tip interacts with cationic amino acid residue(s) such as lysine, arginine, or histidine in the neonicotinoid binding region of the insect nAChR, making it a putative cationic subsite (Figure 4). Lysine and arginine residues are prominent in the relevant 149−220 (extracellular) region from the *N*-termini of fruit fly α and β subunits ALS, Dα2, ARD, and SBD, and one or two histidine residues are also found (Gundelfinger and Hess, 1992). This contrasts with the mammalian nAChRs where the neonicotinoid iminium cation ($^{+}C-NH_2 \leftrightarrow C=^{+}NH_2$) or ammonium nitrogen of nicotine or epibatidine interacts with the anionic subsite (Figure 4). There are two hypotheses relative to amino acid residue(s) in the anionic subsite. The first suggests cation interaction with the carboxyl anion from glutamate and aspartate residues on the γ or δ subunit (the agonist-binding site is localized at the interface between α1-γ or α1-δ subunits) (Karlin and Akabas, 1995). The second hypothesis proposes cation-π electron interaction with the aromatic amino acid residues of tryptophan and tyrosine on the α1 subunit as the possible anionic subsite [the quaternary ammonium head makes van der Waals contact with the π-electron (δ^-) of the aromatic ring] (Zhong et al., 1998). Photoaffinity labeling by nicotinic ligands of several aromatic amino acid residues may support this hypothesis (Arias, 1997). These differences between insects and mammals in neonicotinoid binding provide an important safety feature for this major class of insecticides.

ABBREVIATIONS USED

α-BGT, α-bungarotoxin; CNIMI, cyano analogue of IMI; DCTHIA and DCTHIA-O, descyanothiacloprid and corresponding olefin analogue; DNIMI, desnitroimidacloprid; D_2O, deuterium oxide; DMSO-d_6, dimethyl-d_6 sulfoxide; IMI, imidacoprid; mAb, monoclonal antibody; nAChR, nicotinic acetylcholine receptor; TFA, trifluoroacetic acid; THIA, thiacloprid; THPCHI, tetrahydropyrimidine analogue with nitromethylene group.

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