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# Insect Nicotinic Acetylcholine Receptors: Neonicotinoid Binding Site Specificity Is Usually but Not Always Conserved with Varied Substituents and Species

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The diversity of neonicotinoid insecticides acting as insect nicotinic acetylcholine (ACh) receptor (nAChR) agonists is illustrated by imidacloprid (IMI) with chloropyridinylmethyl (CPM) and *N*-nitroimine substituents, dinotefuran (DIN) with tetrahydrofurylmethyl (TFM) and *N*-nitroimine moieties, and acetamiprid (ACE) with CPM and *N*-cyanoimine groups. These three neonicotinoids are used here as radioligands to test the hypothesis that they all bind to the same site in the same way in both fruit flies (*Drosophila melanogaster*) and a leafhopper pest (*Homalodisca coagulata*): that is, neonicotinoid binding site specificity is conserved in the insect nAChRs. Multiple approaches show that [$^3$H]IMI and [$^3$H]ACE interact with an identical site in both species. However, although [$^3$H]DIN binds with high affinity in both insects, its pharmacological profile in *Homalodisca* is surprisingly unique, with high sensitivity to some TFM-containing compounds and ACh. The TFM moiety of DIN may bind in a different orientation compared to the CPM group of IMI and ACE.

**KEYWORDS: Acetamiprid; dinotefuran; *Drosophila*; *Homalodisca*; imidacloprid; neonicotinoids; nicotinic acetylcholine receptors**

## INTRODUCTION

The nicotinic acetylcholine (ACh) receptor (nAChR) is an agonist-regulated ion channel in the insect central nervous system. It is responsible for rapid excitatory neurotransmission and constitutes a major target for insecticide action (*1*). The first botanical insecticide, nicotine, acts on the nAChR as an agonist but with limited insecticidal efficacy and spectrum and rather high risk to people. Nicotine and nicotinoids have a predominantly protonated nitrogen atom at physiological pH, resulting in poor to moderate affinity for the insect receptor and low insecticidal activity but conferring high potency for vertebrate nAChRs and high mammalian toxicity (*1*−*3*). In marked contrast, neonicotinoids, also acting as nicotinic agonists, have greatly improved effectiveness for pest management and favorable toxicological features (*1*−*5*). The distinctive structural aspect of neonicotinoids is a *N*-nitroimine, *N*-cyanoimine, or 2-nitromethylene moiety. This nonprotonatable and electronegative pharmacophore plays a crucial role in the high affinity and selectivity for the insect nAChR (*6*, *7*).

The neonicotinoids created a renaissance in the investigation of insect nAChRs. Knowledge from structure−activity relationships and species specificity had potential immediate applications in improving the effectiveness and safety of pest control. There are three important heterocyclic methyl substituents, 6-chloropyridin-3-ylmethyl (CPM), 2-chlorothiazol-5-ylmethyl (CTM), and tetrahydro-3-furylmethyl (TFM), coupled with six cyclic or acyclic *N*-nitroimine (**1**, **2**, and **6**), *N*-cyanoimine (**3** and **4**), or 2-nitromethylene (**5**) moieties (**Figure 1**). Seminal advances were made by direct neonicotinoid radioligand binding studies for various insect species primarily with [$^3$H]imidacloprid (IMI, CPM-1) (*1*, *8*, *9*) and secondarily for house fly (*Musca domestica*) with a [$^3$H]nitromethyleneimidazolidine (CTM-5) (*10*) and peach−potato and cowpea aphids (*Myzus persicae* and *Aphis craccivora*, respectively) with [$^3$H]thiamethoxam (TMX, CTM-6) (*11*). Other unique moieties are represented in two important neonicotinoids prepared as candidate radioligands, that is, [$^3$H]acetamiprid (ACE, CPM-4) (*12*) and [$^3$H]dinotefuran (DIN, TFM-2) (*13*) (**Figure 2**). The high insecticidal activities of DIN and its TFM analogues (*14*) are somewhat unexpected from earlier biochemical evaluations based on [$^3$H]IMI, [$^3$H]-epibatidine (EPI), or [$^3$H]α-bungarotoxin (α-BGT) binding to *Drosophila*, *Myzus*, *Musca*, and American cockroach (*Periplaneta americana*) nAChRs (*15*−*17*) and from electrophysiological response in an insect/vertebrate hybrid receptor (*18*). The results of these studies suggest a different binding mode for TFM compounds, a proposal supported by the unique pharmacological profile of the [$^3$H]DIN binding site in *Periplaneta* nerve cord nAChR with significantly lower sensitivity to IMI than to DIN itself (*13*). Although the *N*-cyanoimine neonicotinoids including ACE and thiacloprid (CPM-3) generally behave similarly to the *N*-nitroimine compounds, this has not been tested directly with an *N*-cyanoimine radioligand. The goal of this investigation is

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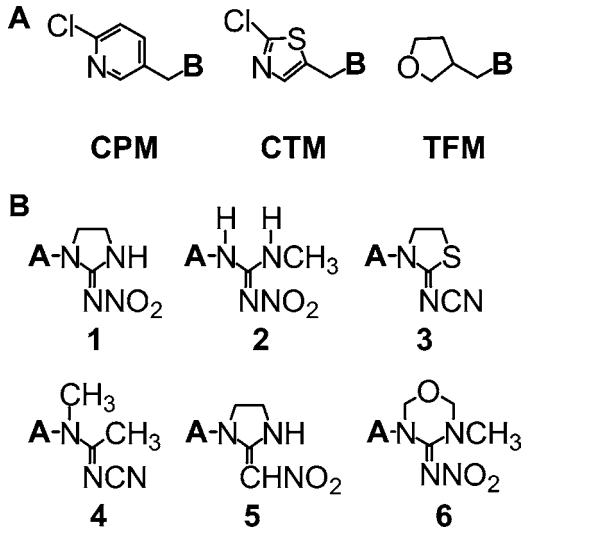


**Figure 1.** Three heterocyclic methyl substituents (**A**) and six cyclic or acyclic *N*-nitroimine, *N*-cyanoimine, or 2-nitromethylene moieties (**B**) important in commercial insecticides or radioligands.

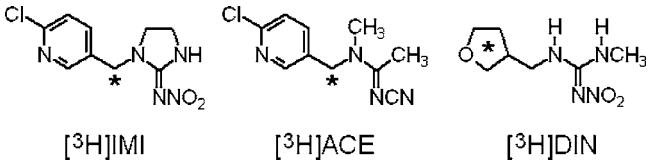


**Figure 2.** Three neonicotinoid radioligands for insect nAChRs. Asterisks indicate positions of tritium.

to determine if IMI, DIN, ACE, and other neonicotinoids with varied substituents all interact at the same site in the same way, a possibility tested directly here by [$^3$H]IMI, [$^3$H]DIN, and [$^3$H]-ACE binding in the fruit fly (*Drosophila melanogaster*) as the representative insect and the glassy-winged sharpshooter (*Homalodisca coagulata*) as a target pest for neonicotinoids.

## MATERIALS AND METHODS

**Chemicals.** Sources were as follows: [$^3$H]IMI (32 Ci/mmol) (*8*) and [$^3$H]ACE (23 Ci/mmol) (*12*) were from the Berkeley laboratory; [2,3,4,5-$^3H_4$-tetrahydrofuran]-(±)-DIN ([$^3$H]DIN, 63 Ci/mmol) was from Mitsui Chemicals, Inc. (Tokyo, Japan); α-BGT was from Sigma (St. Louis, MO); and (±)-EPI was from TOCRIS (St. Louis, MO). The neonicotinoids and desnitro-IMI were from previous studies in this laboratory (*1*, *2*, *15* and papers cited therein) except for two DIN analogues (TFM-1 and -3) synthesized according to the method of Wakita et al. (*14*).

**Receptors.** The insects used were laboratory-cultured *Drosophila* adults, field-collected (Bakersfield, CA) *Homalodisca* adults (the leafhopper vector of the bacterium *Xylella fastidiosa* that causes Pierce's disease of grape), and greenhouse-collected *Bemisia tabaci* adults (whitefly pests of cotton, B-biotype). Other nAChRs used were *Myzus* α2 and rat β2 hybrid receptor (Mpα2/Rβ2) expressed in *Drosophila* S2 cells (*19*, *20*); chick α4β2 and human α7 receptors expressed in mouse fibroblast M10 and human neuroblastoma SH-SY5Y cells, respectively; and α1 (α1γα1δβ1) subtype from *Torpedo californica* electric organ (*21*, *22*).

**Radioligand Binding.** Procedures for receptor preparation and radioligand binding were according to published methodologies: *Drosophila* (*23*); *Homalodisca* (*24*); *Bemisia* (*25*); Mpα2/Rβ2 (*20*); α4β2 (*21*); α7 and α1 (*22*). As a general protocol, the receptor preparation (100−300 μg of protein) was incubated for 60 min at 25 °C with one or two radioligands alone or plus unlabeled displacer. The binding reaction was terminated by rapid filtration on a GF/B filter presoaked in 0.1% polyethylenimine, three rinses with ice-cold saline, and scintillation counting. Specific binding of the [$^3$H]neonicotinoids was determined with 10 μM IMI. Values for $IC_{50}$, molar concentration for 50% displacement of specific radioligand binding, were determined by iterative nonlinear least-squares regression using the Sigmaplot program (SPSS Inc., Chicago, IL). All experiments were repeated three or more times to give the mean and standard deviation (SD) values reported.

**Table 1.** [$^3$H]Neonicotinoid Binding Profiles of Native and Recombinant Insect and Vertebrate nAChRs

| receptor and radioligand (nM) | specific binding fmol/mg of protein (mean ± SD, $n = 4$) | % |
|---|---|---|
| native insect | | |
| *Drosophila* | | |
| [$^3$H]IMI (50) | 1400 ± 20 | 96 |
| [$^3$H]ACE (50) | 1700 ± 70 | 94 |
| [$^3$H]DIN (200) | 1200 ± 50 | 91 |
| *Homalodisca* | | |
| [$^3$H]IMI (50) | 270 ± 8 | 88 |
| [$^3$H]ACE (50) | 390 ± 15 | 89 |
| [$^3$H]DIN (100) | 100 ± 14 | 70 |
| *Bemisia* | | |
| [$^3$H]IMI (50) | 240 ± 25 | 76 |
| [$^3$H]ACE (50) | 440 ± 30 | 70 |
| [$^3$H]DIN (100) | 170 ± 36 | 74 |
| recombinant insect | | |
| *Myzus* α2/rat β2 hybrid | | |
| [$^3$H]IMI (50) | 1500 ± 23 | 93 |
| [$^3$H]ACE (50) | 300 ± 75 | 75 |
| [$^3$H]DIN (50) | not detected | 0 |
| vertebrate | | |
| recombinant chick α4β2[a] | | |
| [$^3$H]IMI (50) | 86 ± 11 | 52 |
| [$^3$H]ACE or [$^3$H]DIN (50) | not detected | 0 |
| native human α7[a] | | |
| [$^3$H]IMI, [$^3$H]ACE, or [$^3$H]DIN (50) | not detected | 0 |
| native *Torpedo* α1[a] | | |
| [$^3$H]IMI (50) | 31 ± 12 | 20 |
| [$^3$H]ACE or [$^3$H]DIN (50) | not detected | 0 |

[a] Binding activities (fmol/mg of protein and % specific binding) of the three vertebrate receptor preparations were confirmed by [$^3$H]EPI (5 nM) for α4β2 (600 and 99%), [$^{125}$I]-α-BGT (1 nM) for α7 (22 and 98%), and [$^3$H]-α-BGT (1 nM) for α1 (>1000 and 92%) (unpublished data).

## RESULTS

**[$^3$H]Neonicotinoid Binding Profiles of Native and Recombinant Insect and Vertebrate nAChRs (Table 1).** Binding profiles of the three [$^3$H]neonicotinoid radioligands were compared with those of four native and recombinant insect receptors and three vertebrate nAChR subtypes. With *Drosophila*, the three radioligands showed high and similar levels of specific binding activities and percentages. With *Homalodisca* and *Bemisia*, they also gave definite and similar specific binding parameters. Interestingly with *Myzus* examined as recombinant *Myzus* α2 and rat β2 hybrid receptor, the binding of [$^3$H]ACE was 5 times less than that of [$^3$H]IMI, and [$^3$H]DIN failed to give specific binding activity. With the three vertebrate nAChR subtypes, [$^3$H]IMI gave a little activity for the α4β2 and α1 receptors and no binding to the α7 nAChR, and [$^3$H]ACE, and [$^3$H]DIN gave no detectable binding.

**Pharmacological Profiles of [$^3$H]IMI, [$^3$H]ACE, and [$^3$H]-DIN Binding Sites in *Drosophila* nAChR (Table 2).** Potencies as $IC_{50}$ values of neonicotinoids, nicotinoids, and other nicotinic agents were compared for the three radioligands. The first remarkable feature is that practically identical $IC_{50}$ values are obtained with [$^3$H]IMI at 3 nM and with [$^3$H]ACE at 10 nM, not only for the nine neonicotinoids but also for the three nicotinoids and three other nicotinic agents. Although not

**Table 2.** Pharmacological Profiles of [$^3$H]IMI, [$^3$H]ACE, and [$^3$H]DIN Binding Sites in *Drosophila* nAChR

| | $IC_{50}$ (nM) ± SD ($n = 3$) | | |
|---|---|---|---|
| compound | 3 nM [$^3$H]IMI | 10 nM [$^3$H]ACE | 5 nM [$^3$H]DIN |
| neonicotinoids | | | |
| thiacloprid | 2.7 ± 0.4[a] | 2.9 ± 0.8 | 0.8 ± 0.2 |
| IMI | 4.6 ± 0.5[a] | 6.9 ± 2.7 | 1.8 ± 1.2[b] |
| CLO | 6.1 ± 0.7 | 7.8 ± 1.8 | 1.6 ± 0.5 |
| ACE | 11 ± 1 | 11 ± 5 | 3.5 ± 0.4[b] |
| nitenpyram | 14 ± 1[a] | 18 ± 4 | 2.9 ± 0.9 |
| DIN | 130 ±6 | 140 ± 32 | 22 ± 7[b] |
| nithiazine | 1100 ± 260 | 1000 ± 180 | 150 ± 60 |
| *N*-methyl-IMI | 5900 ± 540 | 8600 ± 600 | 1800 ± 100 |
| TMX | 6200 ± 840 | 6500 ± 1500 | 1700 ± 840 |
| nicotinoids | | | |
| EPI | 430 ± 20[a] | 500 ± 80 | 80 ± 20 |
| desnitro-IMI | 1500 ± 70[a] | 2200 ± 180 | 450 ± 200 |
| nicotine | 4000 ± 170[a] | 3900 ± 170 | 650 ± 190 |
| other nicotinic agents | | | |
| α-BGT | 710 ± 60[a] | 980 ± 40 | 500 ± 100 |
| carbamoylcholine | 9600 ± 600 | 11600 ± 1300 | 1800 ± 500 |
| ACh[c] | 660 ± 30 | 940 ± 100 | 140 ± 50 |

[a] Data from refs *2* and *20*. [b] $IC_{50}$ values of IMI, ACE, and DIN in assays with 20 nM [$^3$H]DIN were 1.7 ± 0.4, 4.7 ± 1, and 36 ± 10 nM, respectively, showing no significant potency difference conferred by the 4-fold increase in radioligand concentration. [c] Co-incubated with 100 μM paraoxon to inhibit ACh esterase.

**Table 3.** Neonicotinoid Substituent Effects on Potency for Displacing [$^3$H]IMI, [$^3$H]ACE, and [$^3$H]DIN Binding in *Drosophila* nAChR

| | $IC_{50}$ (nM) ± SD ($n = 3$) | | |
|---|---|---|---|
| compound[a] | 3 nM [$^3$H]IMI | 10 nM [$^3$H]ACE | 5 nM [$^3$H]DIN |
| chloropyridinylmethyl | | | |
| CPM-1 (IMI) | 4.6 ± 0.5 | 6.9 ± 2.7 | 1.8 ± 1.2 |
| CPM-2 | 18 ± 3 | 25 ± 1 | 5.5 ± 2.9 |
| CPM-3 (thiacloprid) | 2.7 ± 0.4 | 2.9 ± 0.8 | 0.8 ± 0.2 |
| CPM-4 (ACE) | 11 ± 1 | 11 ± 5 | 3.5 ± 0.4 |
| chlorothiazolylmethyl | | | |
| CTM-1 | 8.2 ± 0.5 | 12 ± 0.8 | 1.9 ± 0.2 |
| CTM-2 (CLO) | 6.1 ± 0.7 | 7.8 ± 1.8 | 1.6 ± 0.5 |
| CTM-3 | 15 ± 2 | 17 ± 2 | 2.7 ± 0.8 |
| CTM-4 | 57 ± 8 | 60 ± 3 | 8.5 ± 0.6 |
| tetrahydrofurylmethyl | | | |
| TFM-1 | 190 ± 13 | 220 ± 15 | 34 ± 6 |
| TFM-2 (DIN) | 130 ± 6 | 140 ± 32 | 22 ± 7 |
| TFM-3 | 2400 ± 280 | 2250 ± 450 | 480 ± 65 |

[a] Substituents are shown in **Figure 1**.

specifically shown, the $IC_{50}$ ratios of the [$^3$H]ACE and [$^3$H]-IMI assays are essentially the same values (0.91−1.5) with all compounds. The second striking observation is that all of the test compounds showed lower $IC_{50}$ values (higher potencies) with [$^3$H]DIN (at 5 nM) than with [$^3$H]IMI and [$^3$H]ACE. This phenomenon was also evident even with a 4 times higher concentration of [$^3$H]DIN (20 nM), which gave $IC_{50}$ values for IMI, ACE, and DIN almost the same as those with the 5 nM [$^3$H]DIN assay (**Table 2**, footnote). Thus, the $IC_{50}$ ratios in both [$^3$H]IMI/[$^3$H]DIN and [$^3$H]ACE/[$^3$H]DIN comparisons were found to be 2.6−7.3 (except for the antagonist α-BGT).

**Neonicotinoid Substituent Effects on Potency for Displacing [$^3$H]IMI, [$^3$H]ACE, and [$^3$H]DIN Binding in *Drosophila* nAChR (Table 3).** Major neonicotinoids are assembled from various combinations of heterocyclic moieties such as CPM, CTM, or TFM with *N*-nitroimine or *N*-cyanoimine coupled to imidazolidine, thiazolidine, or an acyclic group. The potency order for displacing the specific binding of three [$^3$H]neonicotinoids was generally CPM ≥ CTM > TFM, but for the rest of the molecules depended on the heterocyclic or acyclic counterparts. $IC_{50}$ ratios between two CPM radioligands ([$^3$H]ACE/[$^3$H]IMI) were 1.0−1.5, whereas those between TFM ([$^3$H]DIN) and the two CPM radioligands were distinctly higher (2.6−7.0).

**Table 4.** Pharmacological Profiles of [$^3$H]IMI, [$^3$H]ACE, and [$^3$H]DIN Binding Sites in *Homalodisca* nAChR

| | $IC_{50}$ (nM) ± SD ($n = 3$) | | |
|---|---|---|---|
| compound[a] | 10 nM [$^3$H]IMI | 10 nM [$^3$H]ACE | 20 nM [$^3$H]DIN |
| neonicotinoids | | | |
| IMI | 10 ± 1[a] | 4.6 ± 0.5 | 4.1 ± 1.9 |
| ACE | 11 ± 1 | 8.1 ± 1.3 | 1.6 ± 0.9 |
| *N*-methyl-IMI | 530 ± 90 | 380 ± 70 | 1000 ± 630 |
| CLO | 19 ± 1 | 16 ± 2 | 1.5 ± 0.1 |
| TMX | 350 ± 50 | 170 ± 9 | 800 ± 400 |
| DIN | 45000 ± 14000[a] | 14000 ± 3000 | 23 ± 4 |
| TFM-1 | 2700 ± 500 | 2300 ± 140 | 69 ± 9 |
| nithiazine | 25000 ± 8600 | 19000 ± 4700 | 100 ± 49 |
| others | | | |
| EPI | 97 ± 16 | 77 ± 3 | 100 ± 30 |
| ACh[b] | 16000 ± 1200 | 10500 ± 1000 | 38 ± 2 |

[a] *Bemisia* receptor gave $IC_{50}$ values for IMI and DIN against 10 nM [$^3$H]IMI binding of 7.6 ± 0.1 and 430 ± 10 nM, respectively. [b] Co-incubated with 100 μM paraoxon to inhibit ACh esterase.

**Pharmacological Profiles of [$^3$H]IMI, [$^3$H]ACE, and [$^3$H]-DIN Binding Sites in *Homalodisca* nAChR (Table 4).** With *Homalodisca*, as with *Drosophila*, IMI ($IC_{50}$ = 4.1−10 nM) and ACE ($IC_{50}$ = 1.6−11 nM) had high affinities using the three neonicotinoid radioligands and DIN, and its analogue TFM-1 showed high potency ($IC_{50}$ = 23−69 nM) for the [$^3$H]-DIN site. However, unlike in *Drosophila*, DIN and TFM-1 had greatly diminished potency for competing with [$^3$H]IMI and [$^3$H]-ACE in *Homalodisca* ($IC_{50}$ = 2300−45000 nM). ACh showed markedly lower potencies ($IC_{50}$ = 10500−16000 nM) at the [$^3$H]IMI and [$^3$H]ACE sites in *Homalodisca* than those in *Drosophila* ($IC_{50}$ = 660−940 nM). In sharp contrast, ACh in *Homalodisca* had 276−421-fold higher potency ($IC_{50}$ = 38 nM) for the [$^3$H]DIN site than for the [$^3$H]IMI and [$^3$H]ACE sites, a relationship very similar to those observed for DIN and TFM-1. This is of particular interest because the acetyl group of ACh served as a lead moiety for the tetrahydrofuryl part of DIN (*14*). The nicotinoid EPI with a chloropyridinyl moiety exhibited similar levels of potency for binding sites of the three [$^3$H]-neonicotinoids ($IC_{50}$ = 77−100 nM). As with *Drosophila*, clothianidin (CLO, CTM-2) showed high potencies ($IC_{50}$ = 1.5−19 nM) in the three radioligand assays in *Homalodisca*. *N*-Methyl-IMI (with a methyl substituent on the 3-position of the imidazolidine ring) and TMX (CTM-6) (with a methyl group on the 3-position of the oxadiazinane ring) were moderately potent ($IC_{50}$ = 170−530 nM) at the [$^3$H]IMI and [$^3$H]ACE sites but had a slightly diminished effectiveness ($IC_{50}$ = 800−1000 nM) at the [$^3$H]DIN site. These relationships are different from those in *Drosophila* ($IC_{50}$ = 1700−8600 nM) (**Table 2**). The potency of nithiazine (lacking a CPM, CTM, or TFM group) in *Homalodisca* showed a pattern similar to that in *Drosophila*, although the $IC_{50}$ values for [$^3$H]IMI and [$^3$H]ACE assays were ∼20-fold higher than those in *Drosophila*. In *Bemisia* receptor, IMI and DIN showed levels of potency for the [$^3$H]IMI site similar to those in *Drosophila* (**Table 4**, footnote).

**Simultaneous Dual Radioligand Binding in *Drosophila* and *Homalodisca* nAChRs (Table 5).** Dual probe binding experiments were conducted in which the simultaneous binding or direct competition of two radioligands was examined in the same receptor preparation. This method can provide direct evidence that two radioligands bind either to distinct sites or to the same

**Table 5.** Simultaneous Dual Radioligand Binding in *Drosophila* and *Homalodisca* nAChRs

| radioligand | assay level[a] (nM) | specific binding (dpm/mg of protein) | dual binding (% of expected)[b] |
|---|---|---|---|
| *Drosophila* | | | |
| [$^3$H]IMI | 50 | 73800 ± 2200 | 41.0 |
| [$^3$H]ACE | 100 | 106400 ± 5800 | 59.0 |
| [$^3$H]IMI + [$^3$H]ACE | 50 + 100 | 102100 ± 4600 | 56.7 |
| [$^3$H]IMI | 50 | 73900 ± 890 | 45.7 |
| [$^3$H]DIN | 200 | 87900 ± 5200 | 54.3 |
| [$^3$H]IMI + [$^3$H]DIN | 50 + 200 | 83300 ± 7900 | 51.6 |
| [$^3$H]IMI[c] | 20 | 60700 ± 970 | 43.7 |
| [$^3$H]EPI[c] | 100 | 78000 ± 2800 | 56.3 |
| [$^3$H]IMI + [$^3$H]EPI[c] | 20 + 100 | 133900 ± 6600 | 96.5 |
| *Homalodisca* | | | |
| [$^3$H]IMI | 50 | 12200 ± 1050 | 46.3 |
| [$^3$H]DIN | 100 | 14100 ± 3000 | 53.7 |
| [$^3$H]IMI + [$^3$H]DIN | 50 + 100 | 12600 ± 4600 | 47.8 |

[a] Concentrations are near or at saturation levels. [b] Expected is the theoretical total of 100% defined as the sum of the dpm/mg of protein for each individual radioligand. [c] Data from ref *20*.

domain (or closely coupled sites) (*20*, *26*). In *Drosophila*, [$^3$H]-IMI and [$^3$H]ACE bindings were found to be 41 and 59%, respectively, of that for the theoretical total of 100%. The two radioligands together conferred only 57% of the expected value. Similarly, simultaneous use of [$^3$H]IMI and [$^3$H]DIN reached only 52% of the theoretical total of 100%. Therefore, there is clear interference in the simultaneous binding of [$^3$H]IMI and [$^3$H]ACE and/or [$^3$H]DIN. In contrast, the dual binding experiment with neonicotinoid [$^3$H]IMI and nicotinoid [$^3$H]EPI recovered 97% of the expected total of 100% (*20*); that is, there is no interference by one radioligand in the binding of the other one. In *Homalodisca*, [$^3$H]IMI and [$^3$H]DIN bindings were 46 and 54%, respectively, of the theoretical total of 100%. The two radioligands together displayed 48% of the expected value; that is, on this basis the binding site for [$^3$H]IMI overlaps with that for [$^3$H]DIN, and they compete with each other for the same domain.

## DISCUSSION

**Neonicotinoid Binding Sites in Insect nAChRs.** The nAChR is a pentameric transmembrane complex consisting of diverse subtypes assembled in combinations of various subunits. Specific subunit combinations confer differences in sensitivity to ACh and/or pharmacological profiles among the vertebrate nAChR subtypes (*27*). In insects, genes are identified encoding multiple nAChR subunits, suggesting the existence of diverse insect receptor subtypes. However, despite the importance in understanding insecticide action, the structure and diversity of insect nAChRs are still poorly defined (*1*, *2*, *28*, *29*). As with vertebrate receptors, the binding domain for nicotinic agonists/antagonists in insect nAChRs is located at the subunit interface and both α and non-α subunits influence the pharmacological properties (*19*, *20*, *30*). In *Drosophila*, the [$^3$H]IMI binding site is distinct from that for either [$^3$H]EPI or [$^3$H]-α-BGT, and the latter two radioligands are suggested to interact with the same domain or closely coupled sites (*20*, *26*).

A high-affinity [$^3$H]IMI binding site of conserved neonicotinoid sensitivity and specificity is found across a broad range of insects including *Drosophila*, *Musca*, *Myzus*, *Aphis*, *Bemisia*, green rice leafhopper (*Nephotettix cincticeps*), brown planthopper (*Nilaparvata lugens*), *Homalodisca*, *Periplaneta*, migratory

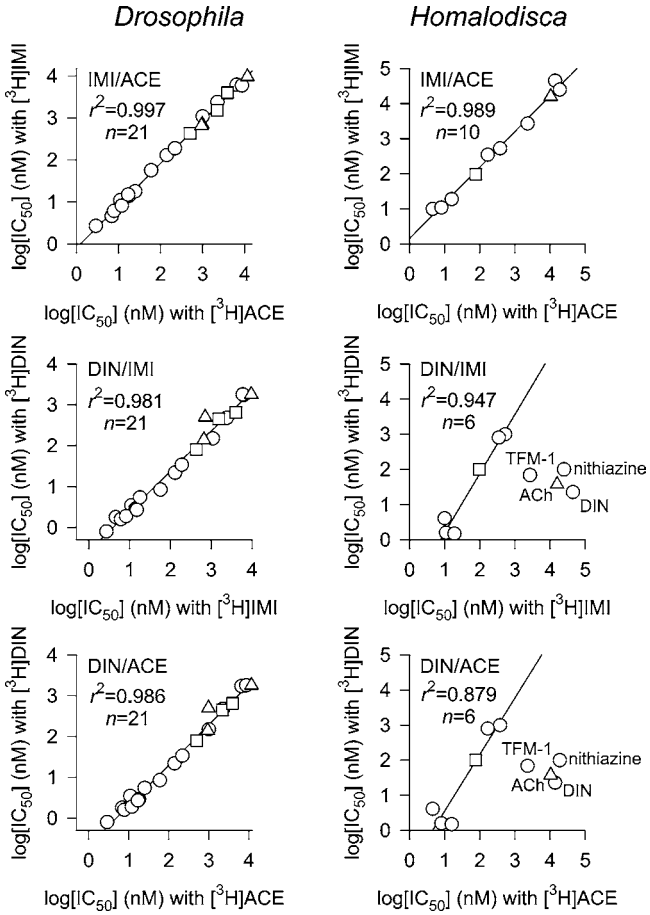


**Figure 3.** Correlation plots of potencies of neonicotinoids (○), nicotinoids (□), and other nicotinic agents (△) for [$^3$H]IMI, [$^3$H]ACE, and [$^3$H]DIN binding sites in *Drosophila* and *Homalodisca* nAChRs. Data are from **Tables 2**–**4**.

locust (*Locusta migratoria*), tobacco hornworm (*Manduca sexta*), and honeybee (*Apis mellifera*) (*1* and papers cited therein, *25*, *31*). As with [$^3$H]IMI, this study shows distinct binding sites for [$^3$H]ACE and [$^3$H]DIN are also present in *Drosophila*, *Homalodisca*, and *Bemisia*, whereas poor or no detectable binding activity was found in the three vertebrate receptor subtypes. These biochemical properties support the observed potent insecticidal activities and selective toxicities of these neonicotinoids.

**Neonicotinoid Binding Site Specificity Is Usually Conserved.** Binding sites for the two CPM neonicotinoids ([$^3$H]-IMI and [$^3$H]ACE with heterocyclic *N*-nitroimine and acyclic *N*-cyanoimine moieties, respectively) were compared in their pharmacology and structure−activity relationships and in direct competition experiments. Potencies of 15 neonicotinoids, 3 nicotinoids, and 3 other nicotinic ligands for the [$^3$H]ACE binding site are completely correlated with those for the [$^3$H]-IMI site in *Drosophila* ($r^2 = 0.997$, $n = 21$) (**Figure 3**). Simultaneous dual binding experiments with [$^3$H]IMI and [$^3$H]-ACE also clearly establish that these radioligands share the same binding domain on the *Drosophila* nAChR. This relationship also extends to *Homalodisca* ($r^2 = 0.989$, $n = 10$) (**Figure 3**). The binding properties of a [$^3$H]nitromethyleneimidazolidine (CTM-5) are consistent with those of [$^3$H]IMI in *Musca* (*10*). In six *N*-cyanoimine neonicotinoid analogues, potencies as displacers of [$^3$H]IMI binding are clearly correlated with those as knockdown agents for synergist-pretreated *Musca* (*32*). In *Myzus*, an azido radioligand with CPM and acyclic *N*-nitroimine moieties (5-azido-CPM-2) is identical to [$^3$H]IMI in binding

parameters and pharmacological profiles (*24*). Therefore, these relationships suggest that *N*-cyanoimine, in the same way as *N*-nitroimine or nitromethylene (*6*, *7*), serves as an electronegative pharmacophore contributing to the high affinity and selectivity of insect nAChRs.

**Neonicotinoid Binding Site Specificity Is Not Always Conserved.** *Similarities and Differences in DIN Binding.* DIN is unique in having a TFM moiety, which sets it apart from other aromatic CPM and CTM neonicotinoids. The TFM conformation for DIN overlays well with those for the CPM of IMI and the CTM of CLO, and the tetrahydrofuryl oxygen may function as the hydrogen acceptor, similar to the nitrogen of the pyridine or thiazole (*33*). Although not specifically considered here, the (*S*)-(+)- and (*R*)-(−)-enantiomers of DIN have different potencies, but (*S*)-(+)- and (*RS*)-(±)-enantiomers show almost equal effectiveness in binding and toxicity evaluations (*16*, *17*).

In *Drosophila*, the potency order of all of the test compounds in the [$^3$H]DIN assays directly correlates with that in either [$^3$H]-IMI or [$^3$H]ACE binding ($r^2 = 0.981-0.986$, $n = 21$) (**Figure 3**) and [$^3$H]DIN is a competitor of [$^3$H]IMI at the same binding region. These observations suggest that the [$^3$H]DIN site is fundamentally the same as the [$^3$H]IMI or [$^3$H]ACE site in *Drosophila*. It is noteworthy that all of the test compounds show higher potencies in the [$^3$H]DIN binding assay than in the [$^3$H]-IMI or [$^3$H]ACE system, indicating at first glance that [$^3$H]DIN binds less effectively than [$^3$H]IMI or [$^3$H]ACE. However, high specific [$^3$H]DIN binding is clearly evident even at low concentrations of the radioligand (e.g., 86−93% specific binding at 0.5−1.0 nM [$^3$H]DIN), implying an analogous but yet somewhat different association or dissociation process for [$^3$H]-DIN compared with [$^3$H]IMI and [$^3$H]ACE in *Drosophila*.

In *Homalodisca*, the potency order of the 10 compounds competing for the [$^3$H]DIN site does not follow that for either the [$^3$H]IMI or [$^3$H]ACE assay, although good correlations are evident when results for 6 of the 10 compounds with the chloropyridine or chlorothiazole ring (IMI, ACE, *N*-methyl-IMI, CLO, TMX, and EPI) are plotted ($r^2 = 0.879-0.947$, $n = 6$) (**Figure 3**). The four remaining compounds (DIN, TFM-1, nithiazine, and ACh) constitute a set distinct from the well-correlated group of six. DIN and TFM-1 are much less potent inhibitors for the [$^3$H]IMI and [$^3$H]ACE binding sites in *Homalodisca* than in *Drosophila*. However, these two TFM compounds are very active at the [$^3$H]DIN binding site. Although the direct competition study indicates that the overall binding domain for [$^3$H]DIN coincides with that for [$^3$H]IMI and [$^3$H]DIN binding is readily displaced by CPM and CTM neonicotinoids, the marked contrast in DIN and TFM-1 sensitivities suggests that in *Homalodisca* the binding orientation or recognition site for the tetrahydrofuryl moiety may not be identical to that for the pyridinyl or thiazolyl group. Very interestingly, in *Homalodisca* the endogenous agonist ACh is a poor displacer at the [$^3$H]IMI and/or [$^3$H]ACE site, whereas, as with DIN and TFM-1, ACh is highly active at the [$^3$H]DIN site. These observations indicate that the binding direction or subsite for the acetyl moiety of ACh may overlap with that for the tetrahydrofuryl moiety of DIN but not that for their CPM and CTM counterparts. These relationships may be species dependent because in *Periplaneta* ACh shows much lower potency as an inhibitor of [$^3$H]DIN binding (*13*) than in *Homalodisca*. Furthermore, nithiazine, which has no CPM, CTM, or TFM moiety, also has an apparent anomalous binding behavior, which presumably resembles those of the two TFM compounds and ACh in *Homalodisca*.

*Diversity in Mode of Neonicotinoid Binding Depending on Insect Species.* In *Myzus* and *Aphis* receptors, an apparent anomalous neonicotinoid target site behavior has been proposed for the binding mechanism of [$^3$H]TMX (or *N*-methyl-IMI), which is noncompetitive with [$^3$H]IMI and vice versa (*11*, *34*). In *Homalodisca* TMX and *N*-methyl-IMI have much higher potencies for the [$^3$H]IMI and/or [$^3$H]ACE site than for those in *Drosophila* (present paper) and aphids (*34*), suggesting that these compounds might act at least in part directly on the *Homalodisca* receptor. In *Periplaneta*, both TMX and CLO are highly potent for [$^3$H]EPI binding (*16*). In addition, the apparent [$^3$H]DIN binding site in *Periplaneta* nerve cord has a diminished IMI sensitivity, and the pharmacological and kinetic profiles are different from those of the [$^3$H]EPI site, which has high sensitivities for CPMs IMI, and ACE (*13*, *16*), although the neural effect of DIN in *Periplaneta* is comparable to those of IMI and CLO (*35*). Another aspect of these relationships involves potential bioactivation. Thus, TMX and *N*-methyl-IMI are metabolically activated (undergoing *N*-desmethylation and/or conversion from TMX to CLO) by some lepidopteran insects and rat and human CYP450s, allowing potent interaction with insect receptors (*3*, *36*, *37*), but this bioactivation may not be necessary in the case of *Periplaneta* (*38*).

**Concluding Remarks.** CPM *N*-nitroimine [$^3$H]IMI and CPM *N*-cyanoimine [$^3$H]ACE and the available corresponding unlabeled CTM analogues bind to the identical site in the same way in *Drosophila* and *Homalodisca*. However, TFM *N*-nitroimine [$^3$H]DIN appears to have an anomalous and unique mode of interaction, particularly in *Homalodisca*. Therefore, varied and minor structural differences in neonicotinoid molecules may confer diversity in their binding modes depending upon insect species. Although the insect nAChR is generally conserved in high neonicotinoid sensitivity and specificity, the exceptions are of particular interest for the most effective use of neonicotinoids in pest management.

## ABBREVIATIONS USED

ACE and [$^3$H]ACE, acetamiprid and its tritiated form; ACh, acetylcholine; α-BGT and [$^3$H]-α-BGT, α-bungarotoxin and its tritiated form; CLO, clothianidin; CPM, 6-chloropyridin-3-ylmethyl; CTM, 2-chlorothiazol-5-ylmethyl; DIN and [$^3$H]DIN, dinotefuran and its tritiated form; EPI and [$^3$H]EPI, epibatidine and its tritiated form; $IC_{50}$, molar concentration for 50% displacement of specific radioligand binding; IMI and [$^3$H]IMI, imidacloprid and its tritiated form; nAChR, nicotinic ACh receptor; SD, standard deviation; TFM, tetrahydro-3-furylmethyl; TMX and [$^3$H]TMX, thiamethoxam and its tritiated form.

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