JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

# Nereistoxin and Cartap Neurotoxicity Attributable to Direct Block of the Insect Nicotinic Receptor/Channel

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Nereistoxin (NTX) (4-dimethylamino-1,2-dithiolane) is the naturally occurring prototype for cartap [the bis(thiocarbamate) derivative of the NTX dithiol], which is generally regarded as a proinsecticide reverting to NTX. The aim of this study is to define the target site(s) for dithiolanes and dithiol esters. The affinity of [$^3$H]NTX was not suitable for binding assays with honeybee (*Apis mellifera*) head membranes. However, NTX and cartap are equally potent, direct-acting, and competitive displacers of [$^3$H]thienylcyclohexylpiperidine binding at the noncompetitive blocker (NCB) site of the *Apis* nicotinic acetylcholine receptor (nAChR)/channel. NTX also binds at the *Apis* [$^3$H]imidacloprid agonist site, but cartap does not. As candidate metabolic pathways, sequential *N*-desmethylation and *S*-oxidation of NTX progressively reduce its potency at the NCB site and toxicity to houseflies. A P450 inhibitor reduces the toxicity of NTX and enhances it with cartap. Surprisingly, cartap is not just a pro-NTX but instead directly induces inhibitory neurotoxicity by blocking the nAChR/channel, whereas NTX may have dual NCB and agonist targets.

**KEYWORDS: Cartap; inhibitory neurotoxicity; nereistoxin; nicotinic acetylcholine receptor and ion channel; noncompetitive or ion channel blocker**

## INTRODUCTION

The nicotinic acetylcholine receptor (nAChR) is a neurotransmitter-gated ion channel. It is assembled from five homologous subunits and penetrates the synaptic membrane. The primary structures of the nAChR subunits predict hydrophilic extracellular domains encompassing a binding site for cholinergic agonists or competitive blockers and four transmembrane hydrophobic segments. The second transmembrane domain of the five subunits forms the lumen of the ion channel pore and involves a binding site for noncompetitive blockers (NCBs, ion channel blockers) (*1*). The nAChR is an important target for insecticide action, although the functional architecture and diversity of the insect receptor are poorly understood compared to those of mammalian receptors (*2*−*4*). The neonicotinoids act as agonists at the insect nAChR (*4*, *5*). The NCBs may act at an alternative insecticide target affecting the insect nAChR/channel function, but there is limited knowledge on structure−activity relationships of NCBs and their toxicological relevance.

Nereistoxin (NTX), a naturally occurring insecticide in the marine worm *Lumbriconereis heteropoda* (*6*, *7*), was the lead compound optimized for the synthetic insecticides cartap (**Figure 1**) and bensultap (*8*−*10*). Cartap and its dithiol ester analogues may be proinsecticides which are converted metabolically in the insect body to NTX (*10*, *11*), which then competes with acetylcholine (ACh) to block the nAChR-mediated signal (*12*, *13*). In contrast to nAChR agonists causing excitatory effects, NTX induces inhibitory neurotoxicity (*10*). Multiple actions of NTX are reported with relatively high concentration in vertebrate nAChRs: i.e., (1) a blocker of frog and rat muscle endplates and chick retina (*14*−*16*); (2) a partial agonist (*15*, *17*, *18*); (3) an NCB for the open status of the nAChR/channel from the *Torpedo* electric organ (*15*, *17*, *18*). Dual actions are also proposed at the honeybee (*Apis mellifera*) nAChR because NTX binds to both the NCB and ACh sites with high and low affinities, respectively (*19*). However, these studies did not consider the relevance of target site potency to insecticidal action of NTX and its analogues.

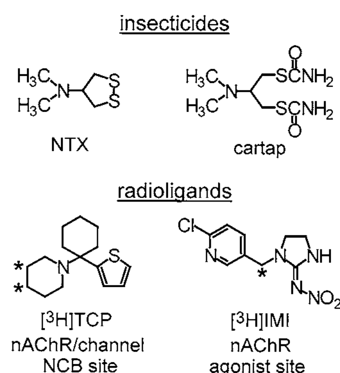


**Figure 1.** Structures of insecticides NTX and cartap and radioligands [$^3$H]TCP and [$^3$H]IMI for NCB and agonist sites of nAChR/channel. Asterisks indicate positions of $^3$H labeling.

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10.1021/jf021149s CCC: $25.00 © 2003 American Chemical Society
Published on Web 03/27/2003

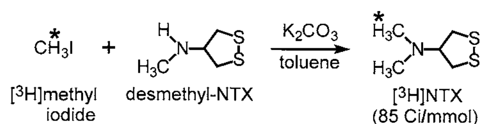


**Figure 2.** Radiosynthesis of high-specific-activity [$^3$H]NTX. Asterisks indicate positions of $^3$H labeling.

The goal of the present study is to define the target site interactions of NTX and its candidate metabolites and analogues and of cartap relative to their neurotoxic effects. Three radioligands are used, i.e., first [$^3$H]NTX (**Figure 2**) and then [$^3$H]thienylcyclohexylpiperidine (TCP) and [$^3$H]imidacloprid (IMI), as probes for the NCB and agonist sites, respectively (**Figure 1**). The experiments primarily consider the nAChR of *Apis* and toxicity to houseflies (*Musca domestica*) as models using established systems (*19*). More specifically, NCB and agonist site potencies and toxicities are determined for NTX and its candidate metabolites and analogues and for cartap and bensultap. We find that cartap binds directly and selectively to the NCB site of the *Apis* nAChR/channel, whereas NTX shows dual actions.

## MATERIALS AND METHODS

**Radioligands, Pharmacological Probes, and Metabolic Inhibitors.** [$^3$H]*N*-[1-(2-Thienyl)cyclohexyl]piperidine ([$^3$H]TCP; 42 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA). [$^3$H]IMI (32 Ci/mmol) and IMI were available from previous studies in this laboratory (*20*). Pharmacological probes were obtained as follows: carbachol chloride, chlorpromazine hydrochloride, ethidium bromide, mecamylamine hydrochloride, and physostigmine hemisulfate from Sigma (St. Louis, MO); chlorisondamine diiodide from TOCRIS (Ellisville, MO). The candidate metabolic inhibitors were available from earlier investigations cited later.

**Dithiolanes and Dithiol Esters.** The dithiolanes used and references for their synthesis are as follows: NTX (**3**) and its didesmethyl (**1**) and desmethyl (**2**) analogues (*21*); NTX sulfoxide (NTX-SO, **4**) (*22*); NTX sulfone (NTX-$SO_2$, **5**) (*23*) (a modified synthesis procedure is given below); the morpholino analogue of NTX (**7**) (*24*). NTX oxalate as a standard was from Wako Pure Chemical (Kyoto, Japan). The commercial dithiol ester insecticides cartap hydrochloride and bensultap were from Takeda Chemical Industries (Tsukuba, Japan).

**[$^3$H]Nereistoxin.** [$^3$H]NTX was prepared as a candidate radioligand at high specific activity (Figure 2). [$^3$H]Methyl iodide (0.12 $\mu$mol, 10 mCi, 85 Ci/mmol, from Amersham Pharmacia Biotech, Piscataway, NJ) in toluene (1 mL) was added to **2** (0.6 mg, 4 $\mu$mol) and $K_2CO_3$ (2 mg) in toluene (100 $\mu$L) under stirring and a $N_2$ atmosphere in a liquid $N_2$ bath. After being stirred for 48 h at room temperature, the reaction mixture was chromatographed on a silica gel column (30 × 1 cm) with methylene chloride/methanol (25/1) to obtain labeled [$^3$H]NTX in the 16−20 mL eluent fraction to which trifluoroacetic acid (TFA) (10 $\mu$L) was added prior to evaporation to form the salt and thereby prevent volatility loss. TLC cochromatography with standard unlabeled compound [silica gel, methylene chloride/methanol (25/1), $R_f$ = 0.25] validated the identity of [$^3$H]NTX.

**4-Dimethylamino-1,2-dithiolane 1,1-Dioxide (NTX-$SO_2$, 5).** To a solution of **4** (0.12 g, 0.72 mmol) in acetone/water (1/1; 14 mL) at −5 °C were added $MgSO_4$ (0.24 g, 2 mmol) and $KMnO_4$ (0.090 g, 0.57 mmol). The mixture, after being stirred for 24 h at room temperature, was extracted with ethyl acetate and the organosoluble fraction purified on a silica gel column with methylene chloride/methanol/ether (40/1/1) to give **5** (20 mg, 15% yield): $^1$H NMR ($CDCl_3$) $\delta$ 3.76−3.68 (1H, m), 3.64−3.52 (3H, m), 3.40 (1H, dd, $J$ = 9.2, 12.3 Hz), 2.36 (6H, s); $^{13}$C NMR ($CDCl_3$) $\delta$ 63.5, 56.8, 41.9, 37.6; EI-MS $m/z$ calcd for $C_5H_{11}NO_2S_2$ ($M^+$) 181, found 181.

**4-*n*-Butylamino-1,2-dithiolane (6).** 2-(Benzylthio)-1-(benzylthiomethyl)-ethyl-*n*-butylamine (0.17 g, 0.96 mmol) was prepared by reaction of 1,3-bis(benzylthio)-2-propanone (0.59 g, 1.95 mmol) (*25*) with *n*-butylamine (0.71 g, 9.75 mmol) and $NaBH_3CN$ (0.12 g, 1.95 mmol) in methanol (10 mL) containing TFA (1 mL) for 24 h at 25 °C with product isolation by silica gel chromatography using methylene chloride/methanol (35/1). It was then reacted with diphenyl sulfoxide (0.47 g, 2.36 mmol) and methyltrichlorosilane (1.4 g, 9.4 mmol) in TFA (30 mL) for 2 h at 5 °C. TFA was removed under reduced pressure, and saturated aqueous $NaHCO_3$ solution (100 mL) was added followed by extraction with ethyl acetate. Purification on a silica gel column with methylene chloride/methanol/ether (30/1/1) yielded **6** (20 mg, 11% yield): $^1$H NMR ($CDCl_3$) $\delta$ 3.90−3.88 (1H, m), 3.18−3.08 (4H, m), 2.61 (2H, t, $J$ = 7.2 Hz), 1.51−1.41 (2H, m), 1.39−1.26 (2H, m), 0.92 (3H, t, $J$ = 7.2 Hz); $^{13}$C NMR ($CDCl_3$) $\delta$ 64.5, 47.2, 43.6, 32.3, 20.4, 13.9; FAB-MS $m/z$ calcd for $C_7H_{16}NS_2$ ($MH^+$) 178, found 178.

**Preparation of *Apis* and *Musca* Head Membranes.** Heads from frozen bees maintained at −80 °C were cut off with a small scissors, and those from flies were obtained by a frozen-rupture and sieving procedure (*26*). Membranes were prepared at 4 °C. The heads for [$^3$H]NTX and [$^3$H]TCP binding assays were homogenized using a Polytron in nine volumes of 20 mM Tris−HCl buffer (pH 7.4) containing 0.02% sodium azide and 0.1 mM diisopropyl fluorophosphate (DFP) (to inhibit acetylcholinesterase) for 30 s, and following a 60 s pause, the homogenization and pause cycle was repeated two times. The homogenate was filtered through four layers of cheesecloth. The filtrate was centrifuged at 1000*g* for 10 min and the supernatant at 40000*g* for 30 min. The pellet was suspended in the above buffer at 2 mg of protein (*27*)/mL. The same procedure was used for membrane preparations for [$^3$H]NTX and [$^3$H]IMI binding except with 10 mM sodium phosphate buffer (pH 7.5) containing 100 mM NaCl without further additives.

**Radioligand Bindings.** Three radioligands were used, [$^3$H]NTX, [$^3$H]TCP, and [$^3$H]IMI, with emphasis on [$^3$H]TCP (**Figures 1** and **2**). [$^3$H]NTX (2 nM) was assayed under the conditions indicated below with both *Apis* and *Musca* head membranes (0.1−1.0 mg of protein/assay) using 1 mM unlabeled NTX or mecamylamine to determine nonspecific binding. [$^3$H]TCP (*28*) was used in place of [$^3$H]phencyclidine ([$^3$H]PCP) (*18*, *19*) as a reporter molecule for the NCB site in the *Apis* nAChR/channel. [$^3$H]PCP binding in *Apis* head membranes is conveniently assayed as a single high-affinity site by using a 60 s incubation time (*19*, *29*). The *Apis* head membranes (0.3 mg of protein/assay) were incubated for 60 s at 25 °C with [$^3$H]TCP at 5 nM (for potency evaluation of toxicants) or 0.1−100 nM (for saturation isotherm experiments) with or without a test chemical in 0.25 mL of 20 mM Tris−HCl buffer (pH 7.4) containing 0.02% sodium azide and 0.1 mM DFP. The binding reaction was terminated by rapid filtration on a GF/B filter that had been presoaked in 0.1% polyethylenimine. The GF/B filter was rinsed three times with 2.5 mL of ice-cold saline and transferred into a counting vial. The radioactivity remaining on the filter was counted after overnight incubation at room temperature in 3 mL of scintillation cocktail (OptiPhase Hi Safe 2; Wallac Oy, Turku, Finland). Specific binding was considered to be the difference in bound radioactivity in the absence and presence of the NCB mecamylamine at 1 mM. Nonspecific binding was usually less than 10% of the total binding. [$^3$H]IMI (3 nM, 60 min of incubation) was assayed according to Tomizawa et al. (*26*) with >90% specific binding defined by 10 $\mu$M unlabeled IMI. The $IC_{50}$ value for a test chemical, the molar concentration necessary for 50% displacement of specific radioligand binding, was determined by iterative nonlinear least-squares regression using the Sigmaplot program (Jandel Scientific Software, San Rafael, CA). The binding parameters [dissociation constant ($K_D$), maximal binding capacity ($B_{max}$), and Hill coefficient ($n_H$)] based on saturation isotherm experiments were calculated by the EBDA program (Biosoft, Cambridge, U.K.). Significant differences between the control and treated groups were analyzed by an unpaired Student's *t* test.

**Toxicity to Houseflies (*M. domestica*).** Adult female houseflies (20−25 mg each) of a standard susceptible laboratory colony were individually treated with metabolic inhibitors (*30*) including 2 $\mu$g/fly of *O*-propyl *O*-(2-propynyl) phenylphosphonate (PPP) (to inhibit P450), 5 $\mu$g/fly of piperonyl butoxide (PB) (to inhibit P450), 2 $\mu$g/fly of *S*,*S*,*S*-tributyl phosphorotrithioate (DEF) (to inhibit P450 and esterases), or 2 $\mu$g/fly of 2-phenyl-4*H*-1,3,2-benzodioxaphosphorin 2-oxide (PBDPO) (to inhibit esterases, amidases, and glutathione-*S*-transferase) (*31*, *32*) in 0.5 $\mu$L of acetone applied topically to the ventrum of the abdomen. After 2 h the test chemical in 0.22 $\mu$L of water or 25% dimethyl

**Table 1.** Pharmacological Characterization of High-Affinity [$^3$H]TCP Binding in *Apis* nAChR/Channel

| ligand | $IC_{50}$ ($\mu$M) ± SD ($n = 3$) |
|---|---|
| Channel Blocker | |
| mecamylamine | 1.2 ± 0.3 |
| chlorpromazine | 2.5 ± 0.8 |
| ethidium | 8.6 ± 0.7 |
| chlorisondamine | 80 ± 10 |
| Agonist | |
| IMI[a] | 110 ± 15 |
| carbachol | >1000 |
| Anticholinesterase | |
| physostigmine[b] | 10 ± 1 |

[a] $IC_{50} = 3.4 \pm 0.2$ nM ($n = 3$) for binding of [$^3$H]IMI at 3 nM. [b] $IC_{50} = 2.0 \pm 0.8$ $\mu$M ($n = 3$) for binding of [$^3$H]IMI at 3 nM.

sulfoxide or ethanol/water solution was administrated by intrathoracic injection. Due to solubility limitations, bensultap (3 $\mu$g in 0.5 $\mu$L of acetone/fly) was topically applied on the notum. Knockdown or mortality was observed 1, 3, 5, 10, and 24 h after toxicant treatment.

## RESULTS

**[$^3$H]NTX as a Candidate Radioligand.** The attempted use of [$^3$H]NTX for direct assay of its binding site was not successful. This included comparison of *Apis* and *Musca*, Tris−HCl and phosphate buffers, the presence and absence of DFP [or phenylmethanesulfonyl fluoride (100 $\mu$M)] and/or carbachol (100 $\mu$M), and incubation times of 1−60 min. No specific binding was observed in any case with combinations of these variations.

**Pharmacological Characterization of the [$^3$H]TCP Binding Site (Table 1)**.Three channel blockers (i.e., mecamylamine, chlorpromazine, and ethidium) acting at the nAChR/channel were potent inhibitors ($IC_{50} = 1-9$ $\mu$M) of [$^3$H]TCP binding to *Apis* head membranes, while chlorisondamine showed moderate potency ($IC_{50} = 80$ $\mu$M). The nicotinic agonists IMI and carbachol were less active and inactive, respectively, while physostigmine was a potent inhibitor ($IC_{50} = 10$ $\mu$M).

**Dithiolanes and Dithiol Esters as Inhibitors of [$^3$H]TCP and [$^3$H]IMI Binding Sites (Table 2, Figure 3)**. Seven dithiolanes and two dithiol esters were compared for potencies at the [$^3$H]TCP and [$^3$H]IMI binding sites. NTX is progressively less potent upon *N*-desmethylation in the order NTX (**3**), desmethyl-NTX (**2**), and didesmethyl-NTX (**1**) at both the NCB and agonist sites. It is also less potent on *S*-oxidation in the series NTX (**3**), NTX-SO (**4**), and NTX-$SO_2$ (**5**) at the NCB and agonist sites with the single exception of the unexpected potency of NTX-$SO_2$ at the agonist site ($IC_{50} = 37$ $\mu$M). On this basis, NTX-SO and NTX-$SO_2$ have opposite selectivity properties, NTX-SO being more effective at the NCB site (49 $\mu$M) than the agonist site (630 $\mu$M) and NTX-$SO_2$ the reverse with $IC_{50} = 240$ $\mu$M at the NCB site and 37 $\mu$M at the agonist site. The *N*-butyl analogue (**6**) displayed the highest affinity to the NCB site (1.8 $\mu$M) and the least at the agonist site; i.e., it was most selective among all of the test compounds. The morpholino analogue (**7**) was much less active than NTX.

On considering the dithiol esters, the most interesting finding is that cartap showed a target site selectivity different from that of the corresponding dithiolane NTX: i.e., cartap preferred the NCB site (4.3 $\mu$M) to the agonist site (>1000 $\mu$M), while NTX had dual actions at NCB and agonist sites with $IC_{50}$ values of 4.5 and 48 $\mu$M, respectively (**Figure 3**, **Table 2**). Another dithiol ester insecticide, bensultap, was moderately effective on the NCB site (83 $\mu$M) but ineffective on the agonist site.

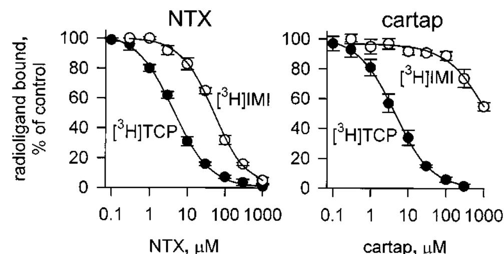


**Figure 3.** Displacement curves for NTX and cartap of high-affinity [$^3$H]-TCP and [$^3$H]IMI sites in *Apis* nAChR/channel. Data points are with the SD of three independent experiments.

**Table 2.** Dithiolanes and Dithiol Esters as Inhibitors of [$^3$H]TCP Binding at 5 nM and [$^3$H]IMI Binding at 3 nM to the NCB and Agonist Sites, Respectively, in *Apis* nAChR/Channel

| toxicant | | $IC_{50}$ ($\mu$M) ± SD ($n = 3$) | |
|---|---|---|---|
| R or R′ | n | NCB site | agonist site |
| Dithiolanes $RCHCH_2SSO_nCH_2$ | | | |
| NTX and Candidate Metabolites | | | |
| (**1**) $H_2N$– | 0 | 640 ± 140 | >1000 ($I = 43\%$)[a] |
| (**2**) $H_3CHN$– | 0 | 53 ± 4.3 | >1000 ($I = 17\%$)[a] |
| (**3**) $(H_3C)_2N$– (NTX) | 0 | 4.5 ± 0.5 (5.1 ± 1.3)[b] | 48 ± 5.7 |
| (**4**) $(H_3C)_2N$– (NTX-SO) | 1 | 49 ± 14 (35 ± 6.5)[b] | 630 ± 27 |
| (**5**) $(H_3C)_2N$– (NTX-$SO_2$) | 2 | 240 ± 15 | 37 ± 3.0 (37 ± 2.3)[b] |
| NTX Analogues | | | |
| (**6**) *n*-BuHN– | 0 | 1.8 ± 0.3 | >1000 ($I = 0\%$)[a] |
| (**7**) $O(CH_2CH_2)_2N$– | 0 | 52 ± 3.9 | >1000 ($I = 12\%$)[a] |
| Dithiol Esters $(CH_3)_2NCH(CH_2SR')_2$ | | | |
| (**8**) –C(O)$NH_2$ (cartap) | | 4.3 ± 0.2 (5.1 ± 0.5)[b] | >1000 ($I = 47\%$)[a] |
| (**9**) –$SO_2C_6H_5$ (bensultap) | | 83 ± 4.2 | >1000 ($I = 16\%$)[a] |

[a] Percent inhibition at indicated concentration. [b] Pretreatment for 30 min before the binding reaction was started by addition of [$^3$H]TCP or [$^3$H]IMI. The binding reaction was then terminated after 60 s or 60 min of incubation, respectively.

The standard assay of 60 s with [$^3$H]TCP and inhibitor before filtration gives a very short time for equilibrium and potential site derivatization. Comparison was therefore made between the standard 60 s assay ([$^3$H]TCP binding) and preincubation of the membranes for 30 min with the toxicant before the binding reaction was started by addition of [$^3$H]TCP or [$^3$H]IMI. This preincubation experiment did not change the potency of NTX, NTX-SO, or cartap at the NCB site or NTX-$SO_2$ at the agonist site.

**Competitive Interaction of Cartap with the [$^3$H]TCP Binding Site (Figure 4)**. A Scatchard plot for specific [$^3$H]-TCP binding revealed a single high-affinity site with $K_D = 31 \pm 2$ nM, $B_{max} = 588 \pm 13$ fmol/mg of protein, and $n_H = 1.001 \pm 0.003$. The $K_D$ value in the presence of 3.5 $\mu$M cartap (66 ± 6 nM) was significantly different from that in the absence of inhibitor ($P = 0.0054$), whereas both $B_{max}$ and $n_H$ (573 ± 69 fmol/mg of protein and 0.999 ± 0.0024, respectively) were almost identical to those of the control ($P = 0.789$ and 0.494, respectively).

**Effect of Metabolic Inhibitors on the Toxicity of NTX, Cartap, and Mecamylamine to *Musca* (Figure 5).** NTX and cartap injected at 0.5 $\mu$g/fly gave high knockdown, but varied effects were found for candidate metabolic inhibitors on knockdown or mortality. The response to NTX was not affected by PB, but recovery was facilitated by DEF or PPP; i.e., they reduced the ultimate toxicity. In contrast, flies knocked down by cartap mostly recover in 10−24 h, but apparently more slowly with a metabolic inhibitor, which in this manner appeared to increase its toxicity. Mecamylamine gave little or no effect

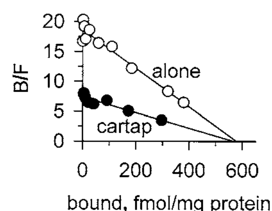


**Figure 4.** Scatchard plots indicate competitive interaction of cartap with the [$^3$H]TCP binding site in *Apis* nAChR/channel. Binding reactions with various concentrations (0.1–100 nM) of [$^3$H]TCP were initiated by addition of *Apis* head membranes and incubation for 60 s. The plots represent typical data from three independent determinations. In the absence of inhibitor (○), $K_D = 31 \pm 2$ nM, $B_{max} = 588 \pm 13$ fmol/mg of protein, and $n_H = 1.001 \pm 0.003$. In the presence of 3.5 $\mu$M cartap (●), $K_D = 66 \pm 6$ nM [significant difference between the control and treatment ($P < 0.01$)], $B_{max} = 573 \pm 69$ fmol/mg of protein, and $n_H = 0.999 \pm 0.0024$.

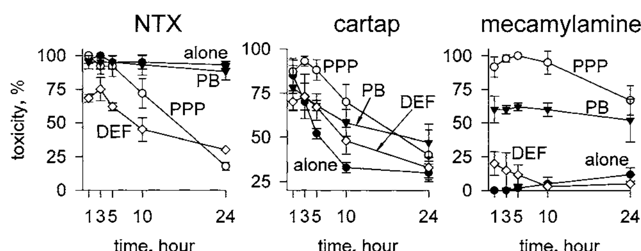


**Figure 5.** Effect of metabolic inhibitors on the toxicity (knockdown or mortality) of NTX, cartap, and mecamylamine to *Musca*. The metabolic inhibitor was applied topically 2 h before intrathoracic injection of the toxicant at 0.5 $\mu$g/fly. Although not primarily a P450 oxidase inhibitor, the results for cartap with PBDPO were superimposable on those with PB and are therefore not shown. Data points are with the SD of three independent experiments.

**Table 3.** Toxicity of Dithiolanes and Dithiol Esters to *Musca* Alone and with PPP

| toxicant: R or R′ | n | knockdown (%) ± SD ($n = 3$)[a]: alone | knockdown: PPP | mortality (%) ± SD ($n = 3$)[a]: alone | mortality: PPP |
|---|---|---|---|---|---|
| Dithiolanes $RCHCH_2SSO_nCH_2$ | | | | | |
| NTX and Candidate Metabolites | | | | | |
| (**1**) $H_2N$– | 0 | 6 ± 2 | 27 ± 7 | 0 ± 0 | 5 ± 2 |
| (**2**) $H_3CHN$– | 0 | 7 ± 3 | 67 ± 6 | 2 ± 3 | 25 ± 5 |
| (**3**) $(H_3C)_2N$– (NTX) | 0 | 95 ± 5 | 92 ± 8 | 93 ± 3 | 18 ± 3 |
| (**4**) $(H_3C)_2N$– (NTX-SO) | 1 | 88 ± 8 | 80 ± 9 | 73 ± 7 | 35 ± 5 |
| (**5**) $(H_3C)_2N$– (NTX-$SO_2$) | 2 | 0 ± 0 | 80 ± 5 | 0 ± 0 | 8 ± 2 |
| NTX Analogues | | | | | |
| (**6**) *n*-BuHN– | 0 | 52 ± 9 | 100 ± 0 | 2 ± 2 | 63 ± 9 |
| (**7**) $O(CH_2CH_2)_2N$– | 0 | 100 ± 0 | 100 ± 0 | 95 ± 5 | 100 ± 0 |
| Dithiol Esters $(CH_3)_2NCH(CH_2SR')_2$ | | | | | |
| (**8**) –C(O)$NH_2$ (cartap) | | 52 ± 3 | 88 ± 6 | 30 ± 5 | 40 ± 15 |
| (**9**) –$SO_2C_6H_5$ (bensultap)[b] | | 3 ± 5 | 5 ± 4 | 0 ± 0 | 0 ± 0 |

[a] Toxicant administered at 0.5 $\mu$g/fly by intrathoracic injection with observations for knockdown at 5 h and mortality 24 h later. [b] Topically applied (3 $\mu$g/fly). For comparison, the $LD_{50}$ value of topically applied NTX is 0.49 ± 0.07 $\mu$g/fly (without PPP pretreatment) (*19*).

alone or with DEF but increased knockdown and mortality with PB and particularly PPP. On the basis of these observations, PPP was compared with no metabolic inhibitor for all other toxicity evaluations of NTX and analogues.

**Toxicity of Dithiolanes and Dithiol Esters (Table 3).** Toxicity was evaluated by knockdown and mortality 5 and 24 h after dithiolane or dithiol ester injection alone or following PPP pretreatment. The toxicity of NTX decreased with *N*-desmethylation and *S*-oxidation in the order NTX > desmethyl-NTX > didesmethyl-NTX and NTX > NTX-SO > NTX-$SO_2$, respectively. The three most potent dithiolanes (**3**, **4**, and **7**) induced knockdown, which was unaffected by PPP, although the oxidase inhibitor reduced mortality for **3** and **4**. Dithiolanes **1**, **2**, **5**, and **6** were synergized in knockdown or mortality by PPP. Morpholino compound **7** was usually the most active in all assays. Cartap was generally less effective than NTX except for knockdown with PPP. Bensultap applied topically was ineffective at 3 $\mu$g/fly, which would be a nearly complete lethal dose for NTX in this assay.

**Signs of Intoxication in *Musca*.** All of the dithiolanes, cartap, and mecamylamine induced anesthetic-like effects or complete suppression of the movement of flies similar to those induced by ether or carbon dioxide. These intoxication signs were clearly different from the excitation induced by nicotinic agonists or anticholinesterases and are considered to result from inhibitory neurotoxicity.

## DISCUSSION

**nAChR/Channel as a Target for Insecticide Action.** The major insecticides by market value are neurotoxicants acting at the voltage-dependent sodium channel (pyrethroids, 20%), the $\gamma$-aminobutyric acid receptor (polychlorocycloalkanes and fipronil, 7%), and the cholinergic nervous system as acetylcholinesterase inhibitors (organophosphorus compounds and methylcarbamates, 53%) and nAChR agonists (neonicotinoids, 9%) (*4*, *33*). Selection for resistance continues to erode the effectiveness of insecticides working at each of these sites. The NCB site in the insect nAChR/channel is a potential underutilized target for the action of new insecticides, thereby circumventing current target site cross-resistance. Accordingly, we focus here on the mechanism of the neurotoxic action of NTX and cartap and their analogues as the present and first generation of NCB insecticides.

**[$^3$H]NTX Binding.** [$^3$H]NTX was prepared at 85 Ci/mmol for possible direct assay of the binding site rather than indirect measurement with an alternative radioligand, e.g., [$^3$H]TCP or [$^3$H]IMI. Unfortunately, the affinity of [$^3$H]NTX was not sufficient for filtration binding assay with *Apis* or *Musca* head membranes. Accordingly, the studies proceeded with the alternative and established radioligands.

**Pharmacological Characterization of the [$^3$H]TCP Binding Site in *Apis*.** TCP and PCP act at the NCB site of the vertebrate nAChR/channel (*28*, *34*) and at the analogous target in the insect nAChR/channel on the basis of high-affinity [$^3$H]TCP and [$^3$H]-PCP binding in *Apis* (*19*, this study) and electrophysiological experiments with the American cockroach (*Periplaneta americana*) (*35*). In the present investigation high-affinity [$^3$H]TCP binding in *Apis* is displaceable by various NCBs known to act at the vertebrate nAChR/channel (mecamylamine, chlorpromazine, ethidium, and chlorisondamine) (*34*) at lower concentrations than by the agonists IMI and carbachol. As expected, these profiles for high-affinity [$^3$H]TCP binding are fundamentally the same as those with [$^3$H]PCP, which measures the NCB site in the *Apis* nAChR/channel (*19*).

TCP and PCP binding in the insect nAChR involves the high-affinity NCB site and the low-affinity agonist site. For differentiation with [$^3$H]PCP and *Apis*, short-term incubation (60 s or less) reveals only the high-affinity NCB site as a single binding component, whereas long-term incubation (more than 30 min) also gives the low-affinity agonist site (*19, 29*). A single high-affinity site is also observed in the present [$^3$H]TCP study

with 60 s incubation, and the $K_D$ and $B_{max}$ values (31 nM and 590 fmol/mg of protein, respectively) are comparable to those reported for [$^3$H]PCP binding to the *Apis* nAChR/channel (20 nM and 760 fmol/mg of protein) (*29*). These observations establish that the binding site for [$^3$H]TCP is the same as that for [$^3$H]PCP, i.e., the NCB site in the channel pore of *Apis* nAChR.

Physostigmine is known to be an open-channel blocker (*36*) and a noncompetitive agonist (the binding site is different from ACh) in vertebrate nAChRs (*37*−*39*). This anticholinesterase has dual actions at the *Apis* nAChR/channel with $IC_{50}$ values of 10 and 2 $\mu$M to the NCB and agonist sites, respectively.

**Target Sites for Dithiolanes and Dithiol Esters.** Dithiolanes **1**−**4**, **6**, and **7** bind preferentially to the NCB site relative to the agonist site as noted in this study and for **3** by Tomizawa et al. (*19*). In contrast, **5** is selective for the agonist site. *N*-Substituents of dithiolanes greatly affect their relative potency at these sites, with **6** showing the highest potency to the NCB site and least to the agonist site in the *Apis* nAChR/channel. This increased potency of *N*-butyl versus *N*-methyl at the NCB site with a decrease at the agonist site is similar to that observed for acyclic nicotinoids (*29*) and neonicotinoids (*40*) for the two target sites. **7** (with a moderate level of affinity to the NCB site and a good selectivity profile) is highly toxic to *Musca* in both the absence and the presence of PPP. Compound **7** is also 2.3-fold more potent than NTX against the Adzuki bean weevil (*Callosobruchus chinensis*), but 5-fold less potent for the rice stem borer (*Chilo suppressalis*) (*8*).

Cartap has the same potency as NTX and bensultap about 19-fold lower potency at the *Apis* NCB site. The two dithiol esters are almost inactive at the agonist site. NTX competitively interacts with the NCB site in the *Apis* nAChR/channel (*19*), and with equal potency cartap also acts in the same manner (this investigation). In rat PC12 cells, coapplication of ACh with cartap shortens the open time of the ion channel (*41*).

There is a controversy on whether NTX and cartap act directly or only after ultimate reduction to NTX dithiol. Thus, it is proposed that competitive blocking by NTX involves reduction and cross-linking of the NTX-derived thiol with a receptor thiol (*11*, *42*, *43*), in a manner similar to that of the reducing reagent dithiothreitol, although this may only be relevant at high micromolar concentrations (*16*, *44*). The observations here that (1) cartap acts competitively and with the same low micromolar potency as NTX at the NCB site and (2) cartap does not form NTX under the assay conditions (data not shown) do not support the cartap → NTX → dithiol → receptor derivatization hypothesis but instead favor the direct action of NTX and cartap at low micromolar levels at the NCB site.

**Inhibitory Neurotoxicity.** The dithiolanes and cartap induce reversible and anesthetic-like intoxication signs similar to those for the NCBs mecamylamine (present study), PCP, TCP, and the *N*,*N*-dibutyl analogue of an acyclic nicotinoid (*19*, *40*). NTX at higher concentration causes excitatory signs such as leg tremor, suggesting partial agonistic action (*15*, *17*−*19*). We find that NTX also binds to the agonist site. Thus, the toxicity signs produced by these compounds are consistent with their proposed primary NCB and secondary agonist target sites.

**Potencies of Candidate Metabolites.** NTX metabolism is likely to involve *S*-oxidation (*42*, *45*) or *N*-desmethylation or both (Figure 6). NTX-SO is considered to be a possible metabolite of NTX (*42*). Each reaction sequence [S → SO → $SO_2$ and $N(CH_3)_2$ → $NHCH_3$ → $NH_2$] progressively decreases the potency at the NCB and agonist sites and the toxicity (with the single exception of NTX-$SO_2$ at the agonist site). These

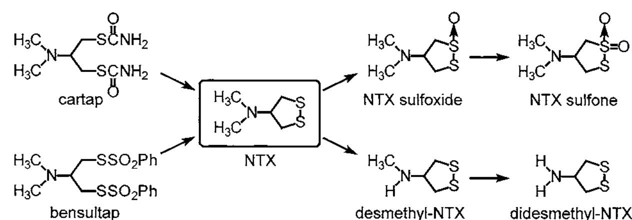


**Figure 6.** NTX and potential precursors and metabolites. *S*-Oxidation and *N*-desmethylation are generally detoxification reactions for NTX.

SARs for toxicity (except for NTX-$SO_2$, which was not studied) are also observed with lepidopterous larvae (*42*). The P450 oxidase inhibitor PPP decreases the mortality of *Musca* from NTX and NTX-SO but increases it from four other dithiolanes, indicating that these sets of compounds undergo metabolic activation and detoxification, respectively. More generally, metabolic oxidation of the dithiolanes may result in either activation or detoxification with toxicity from not only the parent compound and possible metabolites but also dual targets of primarily the NCB site and secondarily the agonist site.

Cartap is usually less potent than NTX to *Musca*, and the toxicity may be increased somewhat but not dramatically by the oxidase inhibitors. In three insect species [$^{35}$S]cartap is converted in part to [$^{35}$S]NTX on the basis of chromatographic analysis (*11*). The primary effect of PPP on cartap toxicity is probably due to increasing the persistence of the parent compound. In conclusion, cartap, without the requirement of metabolic activation, acts directly and selectively at the NCB site of the insect nAChR/channel, producing inhibitory neurotoxicity.

**Summary.** Cartap and bensultap are used to control chewing and sucking insects, particularly Lepidoptera and Coleoptera, which discontinue feeding and die of starvation (*46*). NTX was discovered because of its toxicity to *Musca* (*6*). *Apis* and *Musca* are studied here as experimental models and may be less sensitive than some of the pests under normal use conditions (*46*). However, the mechanisms observed in this investigation for dithiolanes and dithiol esters are probably applicable to pest species. The assays and relationships described here may be relevant in the discovery and development of a new generation of NCB insecticides.

## ABBREVIATIONS USED

ACh, acetylcholine; DEF, *S*,*S*,*S*-tributyl phosphorotrithioate; DFP, diisopropyl fluorophosphate; IMI or [$^3$H]IMI, imidacloprid or its tritiated form; nAChR, nicotinic ACh receptor; NCB, noncompetitive blocker; NTX or [$^3$H]NTX, nereistoxin or its tritiated form; NTX-SO, sulfoxide analogue of NTX; NTX-$SO_2$, sulfone analogue of NTX; PBDPO, 2-phenyl-4*H*-1,3,2-benzodioxaphosphorin 2-oxide; PCP or [$^3$H]PCP, phencyclidine or its tritiated form; TCP or [$^3$H]TCP, thiophene analogue of PCP or its tritiated form.

## ACKNOWLEDGMENT

We thank Takeda Chemical Industries for the donation of cartap and bensultap. We received valuable advice and assistance from our laboratory colleagues Nanjing Zhang and Gary Quistad.

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**Received for review November 26, 2002. Revised manuscript received February 3, 2003. Accepted February 3, 2003. This work was supported in part by Grant R01 ES08424 from the National Institute of Environmental Health Science (NIEHS), NIH, and its contents are solely the responsibility of the authors and do not necessarily represent the official views of NIEHS, NIH.**

JF021149S