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Minor structural changes in nicotinoid insecticides confer differential subtype selectivity for mammalian nicotinic acetylcholine receptors

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1 The major nitroimine insecticide imidacloprid (IMI) and the nicotinic analgesics epibatidine and ABT-594 contain the 6-chloro-3-pyridinyl moiety important for high activity and/or selectivity. ABT-594 has considerable nicotinic acetylcholine receptor (AChR) subtype specificity which might carry over to the chloropyridinyl insecticides. This study considers nine IMI analogues for selectivity in binding to immuno-isolated α_1, α_3 and α_7 containing nicotinic AChRs and to purported $\alpha_4\beta_2$ nicotinic AChRs.

2 α_1- and α_3-Containing nicotinic AChRs (both immuno-isolated by mAb 35, from *Torpedo* and human neuroblastoma SH-SY5Y cells, respectively) are between two and four times more sensitive to DN-IMI than to (−)-nicotine.

3 With immuno-isolated α_3 nicotinic AChRs, the tetrahydropyrimidine analogues of IMI with imine or nitromethylene substituents are 3–4 fold less active than (−)-nicotine. The structure-activity profile with α_3 nicotinic AChRs from binding assays is faithfully reproduced in agonist potency as induction of ^{86}rubidium ion efflux in intact cells.

4 α_7-Containing nicotinic AChRs of SH-SY5Y cells (immuno-isolated by mAb 306) and rat brain membranes show maximum sensitivity to the tetrahydropyrimidine analogue of IMI with the nitromethylene substituent.

5 The purported $\alpha_4\beta_2$ nicotinic AChRs [mouse (Chao & Casida, 1997) and rat brain] are similar in sensitivity to DN-IMI, the tetrahydropyrimidine nitromethylene and nicotine.

6 The commercial insecticides (IMI, acetamiprid and nitenpyram) have low to moderate potency at the α_3 and purported $\alpha_4\beta_2$ nicotinic AChRs and are essentially inactive at α_1 and α_7 nicotinic AChRs.

7 In conclusion, the toxicity of the analogues and metabolites of nicotinoid insecticides in mammals may involve action at multiple receptor subtypes with selectivity conferred by minor structural changes.

Keywords: Chloropyridinyl nicotinic ligands; human neuroblastoma SH-SY5Y cells; imidacloprid; nicotinic AChR subtypes; nicotinoid insecticides; $^{86}Rb^+$ efflux

Abbreviations: AAP, acetamiprid; AChR, acetylcholine receptor; α-BGT or [^{125}I]α-BGT, α-bungarotoxin or its 125-iodine labelled ligand; CH-IMI, nitromethylene analogue of IMI; Cl-TMNI, chlorothiazolyl analogue of CH-IMI; DMEM, Dulbecco's modified Eagle's medium; DN-IMI, desnitro metabolite of IMI; DN-THP, tetrahydropyrimidine analogue of DN-IMI; EC_{50}, molar concentration of test compound to induce 50% specific $^{86}Rb^+$ efflux; FBS, foetal bovine serum; IC_{50}, molar concentration of test compound for 50% inhibition of specific radioligand binding; IMI, imidacloprid; mAb, monoclonal antibody; NTP, nitenpyram; PBS, phosphate-buffered saline; PMSF, phenylmethanesulphonyl fluoride; $^{86}Rb^+$, ^{86}rubidium ion; SCH-IMI, thiazolidine analogue of CH-IMI; THPCH-IMI, tetrahydropyrimidine analogue of CH-IMI

Introduction

Nicotinic acetylcholine receptors (AChRs) consist of diverse subtypes formed from five homologous subunits in combinations from nine α, four β, γ, δ and ε subunits. Advances in knowledge of nicotinic AChR structure and function provide a means to establish the specific receptor subtypes conferring selectivity for nicotinic drugs. The 6-chloro-3-pyridinyl moiety is present in some of the most potent and/or selective nicotinic agonists as to subtype specificity, e.g. the analgesics epibatidine and ABT-594 (Badio & Daly, 1994; Holladay *et al*., 1997; Bannon *et al*., 1998), and it is also important in a new class of synthetic nicotinoid insecticides (Shiokawa *et al*., 1995) (Figure 1). Imidacloprid (IMI) is the best known example of these highly effective new insecticides and others are acetamiprid (AAP) and nitenpyram (NTP) (Figure 2). These nicotinoid insecticides and their analogues might also be selective in their action on nicotinic AChR subtypes.

α_1-Containing nicotinic AChRs expressed in skeletal muscle and *Torpedo* electric organ are $\alpha_1\gamma$(or ε in adult)$\alpha_1\delta\beta_1$ heteromers; they are the best understood nicotinic AChRs as to the ligand binding site environment (Karlin & Akabas, 1995; Arias, 1997). Neuronal nicotinic AChR subtypes in brain and ganglia are assembled in combinations of α_{2-9} and β_{2-4} subunits and are pharmacologically classified into two main groups based on sensitivity to α-bungarotoxin (α-BGT) (Sargent, 1993; Lindstrom, 1997). The α-BGT-insensitive subtypes are formed from combinations of α_2, α_3, α_4 and α_6 with β_2 or β_4 subunits (sometimes with α_5 or β_3). The most prominent subtype of this group is $\alpha_4\beta_2$ which represents >90% of high affinity tritiated agonist binding sites in brain (Whiting & Lindstrom, 1986; Flores *et al*., 1992). α_3-Containing nicotinic AChRs ($\alpha_3\beta_4\alpha_5$ and $\alpha_3\beta_2\alpha_5$) are expressed in

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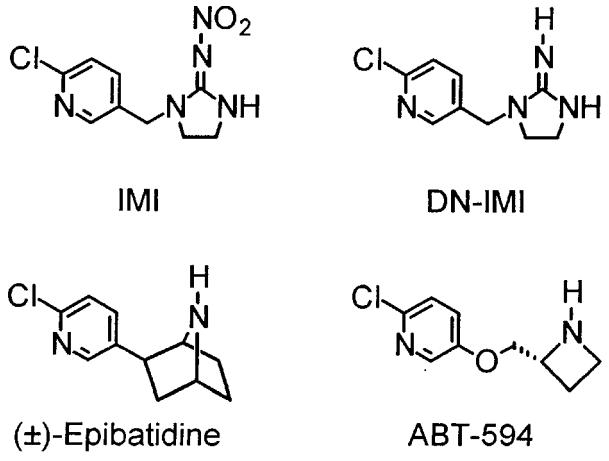

Figure 1 Structural similarity between 6-chloro-3-pyridinyl-containing nicotinic ligands. Imidacloprid (IMI) is an insecticide and desnitro-imidacloprid (DN-IMI) is one of its metabolites. Epibatidine (from the skin of an Ecuadorian frog) and ABT-594 (from structure-activity optimization studies) are analgesics of outstanding potency and/or nicotinic AChR subtype specificity.

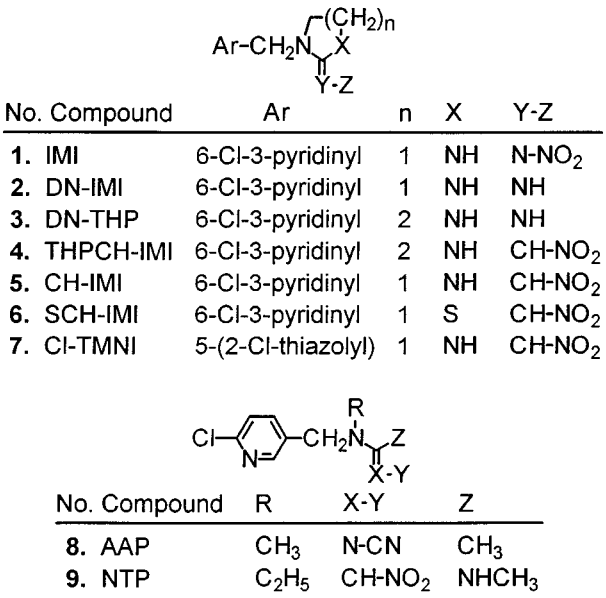

Figure 2 Nicotinoid insecticides (1, 8 and 9) and a metabolite (2) and analogues (3–7). Eight of the compounds contain the 6-chloro-3-pyridinyl substituent with a methylene bridge to an imidazolidine (IMI, DN-IMI, CH-IMI and Cl-TMNI), tetrahydropyrimidine (DN-THP and THPCH-IMI), thiazolidine (SCH-IMI) or acyclic replacement for the heterocyclic ring (AAP and NTP). They include nitroimines (N-NO_2), imines (NH), nitromethylenes (CH-NO_2) and a cyanoimine (N-CN). A chlorothiazole moiety replaces the chloropyridine in Cl-TMNI. All of these chemicals, except DN-IMI and DN-THP, are highly potent insecticides.

peripheral ganglia and limited regions of the brain (Conroy & Berg, 1995; Lindstrom, 1997). α-BGT-sensitive neuronal nicotinic AChR subtypes have α_7, α_8 and α_9 subunits (Lindstrom, 1997). The abundance of α_7-containing nicotinic AChRs in brain is comparable to that of the $\alpha_4\beta_2$ subtype (Clarke *et al*., 1985; Whiting & Lindstrom, 1988; Lindstrom, 1997). The α_7 nicotinic AChRs are also coexpressed with multiple α_3-containing receptors ($\alpha_3\beta_4\alpha_5$ and $\alpha_3\beta_2\alpha_5$) in ganglia as well as in human neuroblastoma cells such as SH-SY5Y (Lukas *et al*., 1993; Peng *et al*., 1994; Lindstrom, 1997). The α_8 subunit is found only in chickens and the α_9 in limited regions of the rat nervous system (Schoepfer *et al*., 1990; Keyser *et al*., 1993; Elgoyhen *et al*., 1994). The native α_7-containing nicotinic AChRs are considered to be assembled either as a homomer (Couturier *et al*., 1990; Chen & Patrick, 1997; Lindstrom, 1997) or as heteromers with unknown subunit(s) (Whiting & Lindstrom, 1987; Gotti *et al*., 1991; Anand *et al*., 1993b).

The structure and function of insect nicotinic AChRs have been investigated with biochemical, molecular biological and immunohistochemical approaches but are poorly understood relative to those of animals. Although several candidate genes encoding the α and non-α subunits are identified from fruit flies (*Drosophila melanogaster*) and migratory locusts (*Locusta migratoria*), their functional coexpression has not been successful in any combination, implying the involvement of unidentified subunit(s) in assembling the native insect receptors (Gundelfinger & Hess, 1992; Tomizawa *et al*., 1996; 1999; Tomizawa & Casida, 1997; Hermsen *et al*., 1998). Interestingly, functional ion channel properties are clearly observed when either of two *Drosophila* α type subunits is coexpressed with chick β_2 subunit and the two reconstituted *Drosophila* α/chick β receptors display different sensitivities to α-BGT (Bertrand *et al*., 1994). It is proposed for the cockroach (*Periplaneta americana*) that the α-BGT-sensitive and -insensitive nicotinic AChRs are expressed in the dorsal unpaired median neurons and that both subtypes are affected by IMI, based on electrophysiology studies (Lapied *et al*., 1990; Buckingham *et al*., 1997).

IMI and its desnitro metabolite (DN-IMI) (Figure 1) differ greatly in binding site specificity: IMI is highly potent at insect but not mammalian nicotinic AChRs (Liu & Casida, 1993; Zwart *et al*., 1994; Yamamoto *et al*., 1998) whereas DN-IMI is much more active in mammals than insects (Liu *et al*., 1993; Chao & Casida, 1997; Nauen *et al*., 1998). On this basis, minor structural variations in nicotinoid insecticides may also alter the subtype specificity for mammalian nicotinic AChRs. The objective of this study is to determine the contribution of α_1-, α_3-, α_7- and $\alpha_4\beta_2$-containing nicotinic AChRs to the specificity of nicotinoid insecticide action.

Methods

Chemicals

Structures and abbreviations for the nicotinoids studied are given in Figure 2. They were available from our previous studies (Liu *et al*., 1995; Tomizawa *et al*., 1996; Chao & Casida, 1997) except for DN-THP which was synthesized by a procedure analogous to that used for DN-IMI (Latli *et al*., 1996). The purity of these compounds was >95% and they were stored in amber bottles (under nitrogen atmosphere if needed) at room temperature, and the test sample was freshly prepared for each experiment.

Sources for other chemicals were as follows: (3-[^{125}I]*iodotyrosyl)*α-BGT ([^{125}I]α-BGT, >277 Ci mmol^{-1}) and ^{86}rubidium chloride (1.7 mCi mg^{-1} Rb, $^{86}Rb^+$) from Amersham Life Science (Arlington Heights, IL, U.S.A.); L-[*N*-methyl-^{3}H]-nicotine ([^{3}H]-nicotine, 81.5 Ci mmol^{-1}) from NEN Life Science Products (Boston, MA, U.S.A.); α-BGT, (−)-nicotine hydrogen tartrate and poly-L-lysine hydrobromide from Sigma (St. Louis, MO, U.S.A.); Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS) and penicillin-streptomycin from Gibco Life Technologies (Grand Island, NY, U.S.A.). The nicotinic AChR monoclonal antibodies (mAb) used were mAb 35 against α_1, α_3 and α_5 subunits (Conroy *et al*., 1992) and

mAb 306 against the α_7 subunit (Schoepfer *et al*., 1990) from Research Biochemicals International (Natick, MA, U.S.A.).

Torpedo *receptor preparation*

Torpedo electric organ (Biofish Associates, Georgetown, MA, U.S.A.) was homogenized in four volumes of 50 mM sodium phosphate buffer (pH 7.5) containing (in mM): NaCl 1000, EDTA 5, EGTA 5, phenylmethanesulphonyl fluoride (PMSF) 2, benzamidine 5 and iodoacetamide 5 at 4°C using a Polytron for three 30 s periods with 60 s intervals in between. The homogenate was filtered through four layers of cheesecloth and the filtrate was centrifuged at $40{,}000 \times g$ for 30 min at 4°C. The pellet was suspended in lysis buffer (in mM): sodium phosphate (pH 7.5) containing 2% Triton X-100 50, NaCl 50, EDTA 5, EGTA 5, PMSF 2, benzamidine 5 and iodoacetamide 5 (same volume as the homogenate) and the suspension was solubilized by rotation on a rocking platform for 60 min at 4°C. Insoluble material was removed by centrifugation at $40{,}000 \times g$ for 30 min at 4°C.

Human neuroblastoma cell receptor preparation

Cultures of SH-SY5Y cells (Department of Molecular and Cell Biology, University of California, Berkeley) were maintained in DMEM supplemented with 10% FBS, 50 u ml^{-1} penicillin and 50 $\mu g\ ml^{-1}$ streptomycin at 37°C in 5% CO_2/95% air atmosphere with a medium change every 2–3 days. The cells were harvested with a cell lifter in phosphate-buffered saline (PBS, NaCl 100 mM, sodium phosphate buffer 10 mM, pH 7.5). The harvested cells were disrupted by brief vortexing in five volumes of lysis buffer as above. After 20 min 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.

Radioligand binding

Supernatants from *Torpedo* electric organ or SH-SY5Y cell preparations were used for immuno-isolation of receptor subtypes (Anand *et al*., 1993a; Peng *et al*., 1997) then radioligand binding assay. mAb 35 or 306 (immunoglobulin at 5 mg ml^{-1}) was coupled to Immulon 4HBX Removawells (Dynex Technologies, Chantilly, VA, U.S.A.) by incubating 4–5 μg mAb (per well) in 0.1 ml of 10 mM sodium bicarbonate buffer (pH 8.8) overnight at 4°C. After three washes with 0.2 ml of the bicarbonate buffer, the wells were quenched with 0.2 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 0.2 ml of the PBS-Tween 20. The receptor preparation (0.1 ml) was added to each mAb-precoated well and incubated overnight at 4°C. The wells were washed three times with 0.2 ml of the PBS-Tween 20 buffer and then treated with various concentrations of test compound for 20 min. Radioligand binding assay was initiated by addition to this medium of [^{3}H]-nicotine (20 nM for α_3 nicotinic AChRs) or [^{125}I]-α-BGT (0.2 nM for α_1 nicotinic AChRs or 2 nM for α_7 nicotinic AChRs) and incubation in 0.1 ml final volume for 60 min (for [^{3}H]-nicotine binding) or overnight (for [^{125}I]-α-BGT binding) at 25 or 4°C, respectively. The wells were then rinsed three times with 0.2 ml PBS-Tween 20 buffer and the radioactivity remaining was subjected to liquid scintillation counting. Every experiment included (−)-nicotine as a standard at 50 nM (for α_3 nicotinic AChRs with [^{3}H]-nicotine) or 10 μM (for α_7 nicotinic AChRs with [^{125}I]-α-BGT). Background binding was determined using wells lacking mAb. For comparison, the binding affinity for (−)-nicotine in [^{3}H]-nicotine binding to immuno-isolated α_3 receptors from SH-SY5Y cells is 0.02 μM (Peng *et al*., 1997), and for α-BGT and (−)-nicotine in [^{125}I]-α-BGT binding to immuno-isolated α_7 receptors from the same cells are 0.00106 and 2.6 μM, respectively (Peng *et al*., 1994).

Membranes from male rat whole brain were prepared and assayed for 2 nM [^{125}I]-α-BGT binding by the method of Marks *et al*. (1986) and for 5 nM [^{3}H]-nicotine binding as described by Yamamoto *et al*. (1995). Data for [^{3}H]-nicotine binding to mouse brain membranes are from Chao & Casida (1997).

$^{86}Rb^+$ *efflux assay*

Agonist-induced cation flux in SH-SY5Y cells has been attributed to α_3-containing nicotinic AChRs; α-BGT-sensitive

Table 1 Structure-activity relationships of nicotinoid insecticide action on muscle and neuronal nicotinic AChR subtypes

		Muscle	*Neuronal*					
		Torpedo *electric organ*	*Human neuroblastoma cell (SH-SY5Y)*			*Rodent brain membranes*		
		α_1[a]	α_3[a]		α_7[b]	*Rat putative* α_7	*Mouse (or rat) putative* $\alpha_4\beta_2$	
		[^{125}I]-α-BGT binding	*[^{3}H]-Nicotine binding*	*$^{86}Rb^+$ efflux*[c]	*[^{125}I]-α-BGT binding*	*[^{125}I]-α-BGT binding*	*[^{3}H]-Nicotine binding*[d]	*Toxicity mice rating*[d,e]
No.	*Compound*	IC_{50} μM	IC_{50} μM	EC_{50} μM	IC_{50} μM	IC_{50} μM	IC_{50} μM	
1.	IMI	>300 (29%)[f]	14±4	320±85	210±75	42±6	0.81	+
2.	DN-IMI	13±3	0.014±0.006	2.4±0.6	12±4	2.6±0.4	0.015	+ + +
3.	DN-THP	>1000 (41%)[f]	0.14±0.05	12±8	33±14	NT[g]	0.21[b]	−[i]
4.	THPCH-IMI	120±32	0.18±0.05	13±5	1.2±0.3	0.31±0.04	0.012	+ + + +
5.	CH-IMI	680[j]	2.3±0.6	140±16	6.1±1.2	0.63±0.13	0.033	+ + + +
6.	SCH-IMI	NT[g]	38±10	>300 (38%)[k]	11±1	5.4±1.3	0.093	+ +
7.	Cl-TMNI	NT[g]	35±5	320±100	18±6	4.1±0.5	0.25	+ + + +
8.	AAP	>300 (13%)[f,j]	20±2	350±130	290±48	19±4	0.68[h]	+ +[i]
9.	NTP	>300 (2%)[f,j]	48±20	>300 (23%)[f,k]	>300 (22%)[f]	130±60	49[h]	−[i]
10.	(−)-Nicotine	25±1	0.045±0.010	10±3	25±3	1.9±0.5	0.009	+ + + +
11.	α-BGT	NT[g]	>1.0 (5%)[f]	NT[g]	0.001±0.0002	0.004±0.001	NT[g]	NG[g]

Chemical structures and abbreviations are given in Figure 2. (−)-Nicotine and α-BGT are included as standards. IC_{50} and EC_{50} values are mean±s.d. based on three experiments. [a]α_1- or α_3-Containing nicotinic AChRs immuno-isolated by mAb 35. [b]α_7-Containing micotinic AChRs immuno-isolated by mAb 306. [c]Assayed with intact cell. [d]Data from Chao & Casida (1997). [e]LD_{50} (i.p., mg kg^{-1}) ranges: −, ⩾50; +, 35–49; + +, 25–34; + + +, 16–24; + + + +, 7–15. [f]Per cent inhibition at indicated concentration of test compound. [g]Not tested. [h]Data from the present determination with rat brain membranes. [i]Data from the present study. [j]Data from [^{3}H]-α-BGT binding to *Torpedo* membranes (Tomizawa *et al*., 1995). [k]Specific $^{86}Rb^+$ efflux (relative to 0.1 mM nicotine) at 0.3 mM of test compound.

receptors do not contribute detectably to the ion flux measured in this assay (Lukas *et al.*, 1993). SH-SY5Y cells were therefore used to assay α_3-containing receptor function by the procedure of Lukas (1989) with minor modification. The cells were seeded in 24-well (18.5 mm diameter) culture plates at a density of 10^6 cells well^{-1} following the coating of each well by treatment with poly-L-lysine (30 μg ml^{-1}) then aspiration off. At confluence, the cells attached to the culture plates were loaded with 0.2 μCi of $^{86}Rb^+$ in DMEM supplemented with 10% FBS, 50 u ml^{-1} penicillin and 50 μg ml^{-1} streptomycin (0.5 ml) and incubated overnight at 37°C and 5% CO_2/95% air atmosphere. The medium containing $^{86}Rb^+$ was removed by aspiration, the cells were rinsed twice with 0.5 ml of fresh medium and then exposed to 0.25 ml of medium with or without a test compound for 5 min. (−)-Nicotine (0.1 mM) was tested as a standard in each experiment. After exposure to the test compound, the assay medium was immediately transferred into a vial for Cerenkov counting. For validation in a preliminary experiment, we confirmed that 0.1 mM *d*-tubocurarine gave 97–100% blockage of $^{86}Rb^+$ efflux induced by 0.1 mM (−)-nicotine as reported by Lukas *et al.* (1993).

Data calculation

IC_{50} (molar concentration of test compound for 50% inhibition of specific radioligand binding) and EC_{50} [molar concentration of test compound to induce 50% specific $^{86}Rb^+$ efflux relative to 0.1 mM (−)-nicotine] values were determined by iterative nonlinear least-squares regression using the SigmaPlot program (Jandel Scientific Software, San Rafael, CA, U.S.A.).

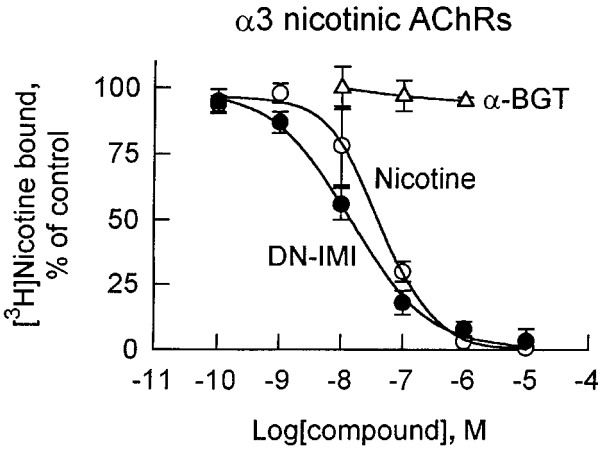

Figure 3 Displacement by DN-IMI and (−)-nicotine of [^{3}H]-nicotine binding to α-BGT-insensitive α3 nicotinic AChRs immunoisolated from human neuroblastoma SH-SY5Y cells. The extracted cell membranes with lysis buffer were reacted with mAb 35-precoated wells overnight at 4°C, and then the immunoprecipitated α_3 nicotinic AChRs were incubated for 60 min at 25°C with 20 nM of [^{3}H]-nicotine in competition with a test compound. Data points represent means of three experiments with s.d.

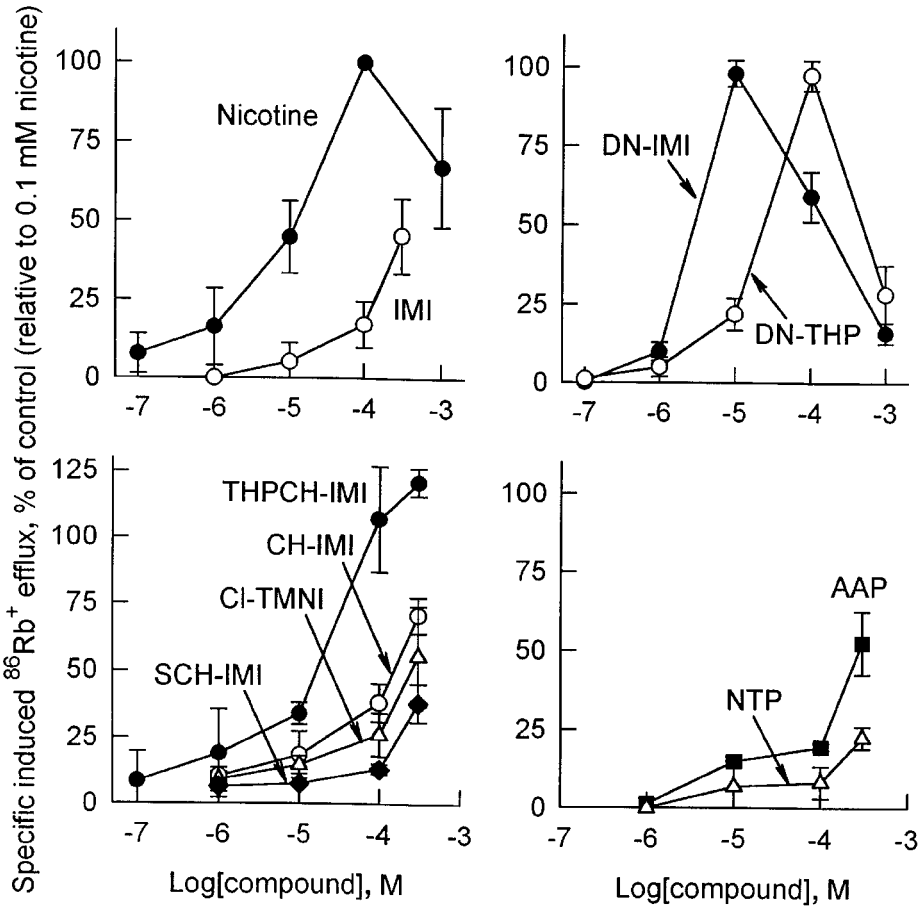

Figure 4 Induction by nicotinoids of specific $^{86}Rb^+$ efflux from intact human neuroblastoma SH-SY5Y cells. Data for agonist potency are given on a percentage basis relative to 0.1 mM (−)-nicotine as 100% in the same experiment. The intact SH-SY5Y cells preloaded with 0.2 μCi of $^{86}Rb^+$ were exposed to medium with or without a test agonist for 5 min. The value for the (−)-nicotine standard ranged from 6000–7000 c.p.m. versus a background without agonist of 1000–1200 c.p.m. Data points represent means of three experiments with s.d.

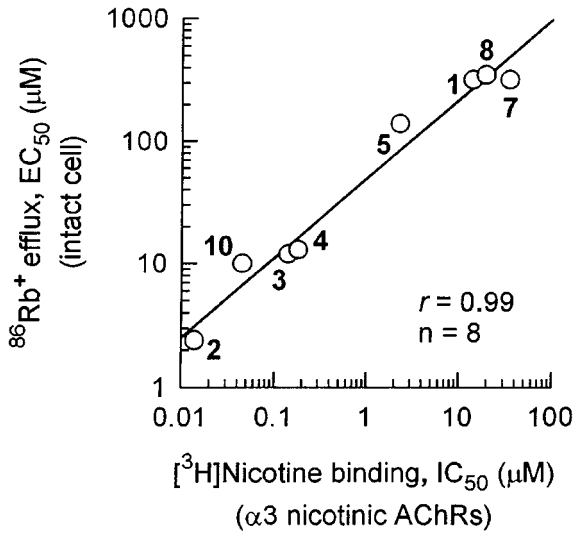

Figure 5 Correlation for nicotinoids of inhibitory potency for [^{3}H]-nicotine binding to immuno-isolated α3 nicotinic AChRs and of agonist potency to induce specific $^{86}Rb^+$ efflux in cultured human neuroblastoma SH-SY5Y cells. Numbers on graph refer to compounds in Figure 2 and Table 1.

Toxicity

Male albino Swiss-Webster mice (20–25 g) were treated i.p. with the test compounds dissolved in water or dimethyl sulphoxide with mortality observations at 24 h as described by Chao & Casida (1997). Toxicity data are from Chao & Casida (1997) for seven compounds and the present determination for three compounds. These studies were carried out in accordance with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health.

Results

Interaction with α_1 nicotinic AChRs

The *Torpedo* electric organ was used as the source of α_1 nicotinic AChRs with immuno-isolation by mAb 35 and binding assay with [^{125}I]-α-BGT. DN-IMI is 2 fold more potent than nicotine with IC_{50} values of 13 and 25 μM, respectively (Table 1). THPCH-IMI has low activity (IC_{50} 120 μM) and all the other nicotinoids are essentially inactive (Table 1).

Interaction with α_3 nicotinic AChRs

Human neuroblastoma SH-SY5Y cells express multiple $\alpha_3\beta_2\beta_4\alpha_5$ nicotinic AChRs; the α_3 receptors were immuno-isolated with mAb 35 for [^{3}H]-nicotine binding. DN-IMI is the most potent compound (IC_{50} 0.014 μM) and nicotine is 3 fold less active (Figure 3, Table 1). The potency order for the other nicotinoids is DN-THP and THPCH-IMI (IC_{50} 0.14–0.18 μM) > CH-IMI (IC_{50} 2.3 μM) > IMI, AAP, Cl-TMNI, SCH-IMI and NTP (IC_{50} 14–48 μM) (Table 1).

Induced $^{86}Rb^+$ efflux with intact SH-SY5Y cells provides another means to evaluate nicotinoid agonist effect attributable to α_3-containing receptor function. DN-IMI with an EC_{50} of 2.4 μM is 4–5 fold more potent than nicotine, DN-THP and THPCH-IMI (EC_{50} 10–13 μM), and DN-IMI and DN-THP display steep efflux induction curves (Table 1, Figure 4).

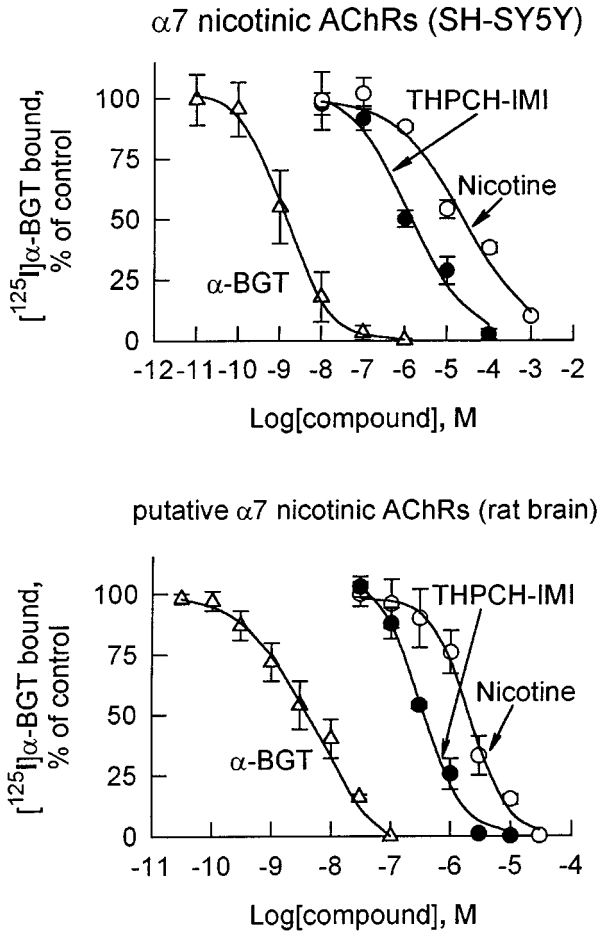

Figure 6 Displacement by THPCH-IMI, (−)-nicotine and α-BGT of [^{125}I]-α-BGT binding to α_7 nicotinic AChRs immuno-isolated from human neuroblastoma SH-SY5Y cells (top) and to putative α_7 nicotinic AChRs from rat whole brain membranes (bottom). The extracted cell membranes with lysis buffer were reacted with mAb 306-precoated wells overnight at 4°C, and then the immuno-precipitated α_7 nicotinic AChRs were incubated overnight at 4°C with 2 nM of [^{125}I]-α-BGT in competition with a test compound. Rat whole brain membranes (200 μg protein) were incubated with 2 nM of [^{125}I]-α-BGT for 4 h at 37°C in the absence and the presence of the test compound. Data points represent means of three experiments with s.d.

THPCH-IMI induces higher $^{86}Rb^+$ efflux than that induced by 0.1 mM (−)-nicotine. The remaining chemicals do not induce an efflux response that reaches the maximum of the nicotine standard.

The inhibitory potency of eight nicotinoids with immuno-isolated α_3 nicotine AChRs of SH-SY5Y cells is highly correlated ($r = 0.99$) with that for agonist-induced $^{86}Rb^+$ efflux from intact cells (Figure 5).

Interaction with α_7 nicotinic AChRs

[^{125}I]-α-BGT binding was determined to mAb 306 immuno-isolated α_7 nicotinic AChRs of SH-SY5Y cells and in putative α_7 nicotinic AChRs of rat brain membranes. The α_7 nicotinic AChRs of the human neuroblastoma cells are most sensitive to THPCH-IMI (Figure 6) and then CH-IMI (IC_{50}s 1.2 and 6.1 μM, respectively), least sensitive to IMI, AAP and NTP (IC_{50}s 210–>300 μM) and with intermediate sensitivity to nicotine and the other five nicotinoids (IC_{50}s 11–33 μM) (Table 1). The same structure-activity relationships are obtained with the several-fold more sensitive rat brain putative

α_7 nicotinic AChR (Figure 6 and Table 1) as clearly apparent by a correlation plot for inhibition of the SH-SY5Y and rat brain receptors (Figure 7).

Interaction with putative $\alpha_4\beta_2$ nicotinic AChRs

[^{3}H]-Nicotine binding in mouse or rat brain membranes was used to determine putative $\alpha_4\beta_2$ nicotinic AChRs. Data for mouse [^{3}H]-nicotine binding were taken from our previous report (Chao & Casida, 1997). There are four potent inhibitors in this assay (IC_{50} 0.009–0.033 μM for nicotine, THPCH-IMI, DN-IMI and CH-IMI) with moderate activity for SCH-IMI, DN-THP and Cl-TMNI (IC_{50} 0.093–0.25 μM), lower activity for IMI and AAP and the lowest activity for NTP.

Toxicity

The i.p. toxicity rating in mice of two chloropyridinyl compounds (CH-IMI and THPCH-IMI) and one chlorothiazolyl compound (Cl-TMNI) is similar to that of nicotine while DN-IMI is a little less toxic than nicotine and the others are much less active (Table 1). The poisoning signs at an LD_{50} dose included tremors and seizures and appeared to be consistent with action on nicotinic AChRs.

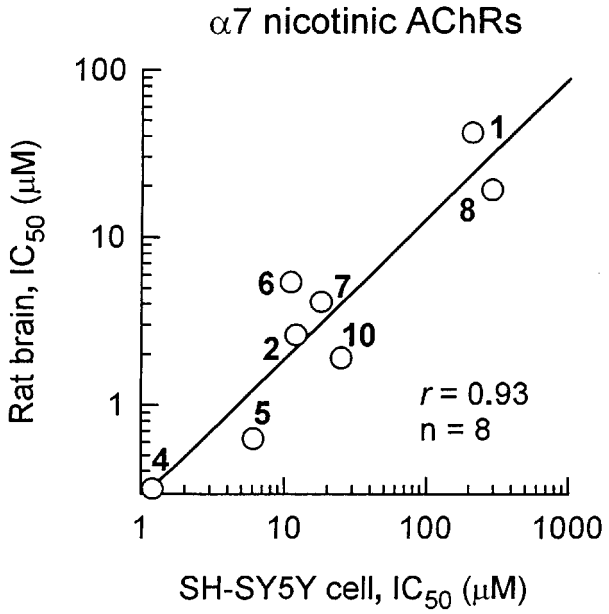

Figure 7 Correlation for nicotinoids of inhibitory potency for [^{125}I]-α-BGT binding to immuno-isolated α_7 nicotinic AChRs from human neuroblastoma SH-SY5Y cells and to putative α_7 nicotinic AChRs from rat brain membranes. Numbers on graph refer to compounds in Figure 2 and Table 1.

Discussion

The chloropyridinyl nicotinoid insecticide IMI has little or no activity in vertebrate systems based on six observations: (1) the failure to recognize [^{3}H]-IMI specific binding site(s) in brain from several mammalian and avian species and the electric eel (Liu & Casida, 1993); (2) low potency as an inhibitor of [^{3}H]-α-BGT binding and low agonistic effect in muscle-type nicotinic AChR from *Torpedo* electric organ (not only for IMI but also for AAP and NTP) (Tomizawa *et al.*, 1995); (3) little activity as an inhibitor of [^{3}H]-nicotine binding to rat and mouse brain membranes (Yamamoto *et al.*, 1995; Chao & Casida, 1997); (4) very weak agonistic action in mouse N1E-115 neuroblastoma and BC3H1 muscle cells (IMI and an analogue) (Zwart *et al.*, 1992; 1994); (5) low activity in ion channel activation compared to acetylcholine with rat $\alpha_4\beta_2$ and α_7 subtypes expressed in *Xenopus* oocytes (Yamamoto *et al.*, 1998); (6) weak or partial agonistic nature with recombinant chick $\alpha_4\beta_2$ receptor (Matsuda *et al.*, 1998). The present study extends

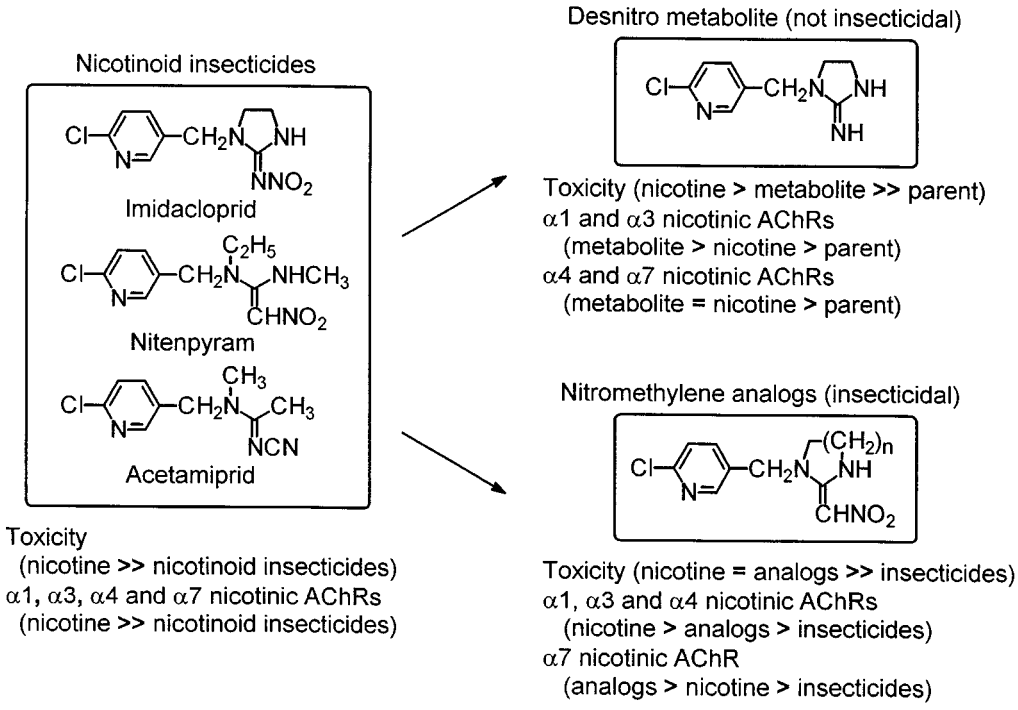

Figure 8 Toxicity of nicotinoid insecticides and selected analogues in mammals involves differential action at multiple receptor subtypes conferred by minor structural changes. The potency rankings in parentheses are generalizations.

these relationships for IMI, AAP and NTP to include very low apparent affinity to α_1, α_3 and α_7 nicotinic AChR subtypes. However, these conclusions are based only on the parent insecticide which might undergo metabolic activation such as the case of IMI to DN-IMI (Klein, 1994; Chao & Casida, 1997). The imine metabolite DN-IMI is much more effective than the parent IMI, not only for putative $\alpha_4\beta_2$ receptors (Chao & Casida, 1997) but also for α_1, α_3 and α_7 nicotinic AChRs (this study).

The chloropyridinyl group is an important structural feature for several nicotinic agonists conferring outstanding potency but little selectivity with epibatidine (Holladay *et al*., 1997) and remarkable $\alpha_4\beta_2$ nicotinic AChR specificity with ABT-594 (Bannon *et al*., 1998). The binding affinities of analogues without the chlorine atom in the insect receptor are several-fold less than those with the chlorine atom (Liu *et al*., 1993; Tomizawa & Yamamoto, 1993). The chlorothiazolyl replacement for the chloropyridinyl moiety (Cl-TMNI versus CH-IMI) greatly reduces potency in the mammalian receptor assays but not the toxicity to mammals or activity at the insect nicotinic AChR (Liu *et al*., 1993; Chao & Casida, 1997).

Differential nicotinic AChR subtype selectivity is conferred by minor structural changes in the chloropyridinyl nicotinoid insecticides (Figure 8). The desnitro analogues favour the α_1, α_3 and putative $\alpha_4\beta_2$ receptor subtypes and the nitromethylene analogues the α_7 nicotinic AChRs. THPCH-IMI and CH-IMI are much more potent than nicotine on α_7 nicotinic AChRs while SCH-IMI and Cl-TMNI are less active than the first two compounds. All four of these nitromethylenes are much less active than nicotine on α_1 and α_3 nicotinic AChRs. Interestingly, the two desnitro analogues DN-IMI and DN-THP prefer the α_3 over the α_7 nicotinic AChRs. The change from a five-membered imidazolidine to a six-membered tetrahydropyrimidine ring greatly reduces the potency of the imines (DN-IMI versus DN-THP) but increases the activity of the nitromethylenes (CH-IMI versus THPCH-IMI) in all nicotinic AChR subtypes.

The mammalian toxicity of the nicotinoid insecticides and analogues studied on an overall basis is most closely related to their potency at α_7 nicotinic AChRs with decreasing relationships sequentially at the $\alpha_4\beta_2$, α_3 and α_1 nicotinic AChRs. More specifically, the nitromethylenes are more potent in the α_7-containing receptors while DN-IMI is particularly potent at α_1, α_3 and putative $\alpha_4\beta_2$ receptors (Figure 8). Thus, the toxicity of the nicotinoid insecticides in mammals may involve action at multiple receptor subtypes with selectivity conferred by minor structural changes.

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