SESSION 3C

NEONICOTINOID INSECTICIDES – CURRENT STATUS AND FUTURE PROSPECTS

Chairman & Session Organiser: Dr R Nauen
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Papers: 3C-1 to 3C-4

Cyanotropanes: novel chemistry interacting at the insect nicotinic acetylcholine receptor

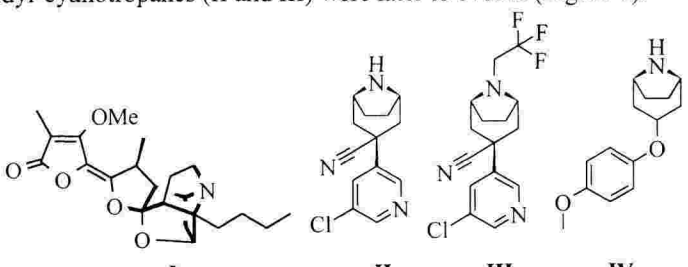
R J Lind, D T Greenhow, J Blythe, J Goodchild, E Hirst, S J Dunbar and F G P Earley
Syngenta, Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY

ABSTRACT

Cyanotropane chemistry was inspired by the natural product stemofoline that shares its mode of action as an agonist at insect nicotinic acetylcholine receptors (nAChR). Metabolic studies in larval *Heliothis virescens* strongly suggests a bioactivation of cyanotropanes. Further evidence for a proinsecticide mechanism and nAChR mode of action is found collectively in radioligand binding and electrophysiological experiments. The cyanotropane pharmacology, determined in peach potato aphid (*Myzus persicae*) membranes, resembles that of the snake toxin α-bungarotoxin, but is distinct from imidacloprid. Taken together, these results demonstrate a nicotinic mechanism of action for cyanotropanes and strongly suggest a propesticidal action.

INTRODUCTION

The insect nicotinic acetylcholine receptor (nAChR) has become a key molecular target in the last decade with the discovery of the fourth largest commercialised class of insecticides, the neonicotinoids (Matsuda *et al.*, 2001). Their success results from rapid and high biological activity, systemic properties and a novel mode of action among commercial synthetic insecticides as agonists of the nAChR. The genomic and molecular examination of this target site has revealed considerable heterogeneity in the subunit composition of this pentameric ligand gated ion channel (Tomizawa and Casida, 2001). The discovery of new nicotinic ligands progresses our understanding of insect nAChR pharmacology which is important for the future discovery and use of neonicotinoids or other chemistries acting on nAChR's.
Cyanotropane chemistry originated from synthesis around the natural product stemofoline (I) (Figure 1) isolated from the leaves and stem of the vine *Stemona japonica* (Ujváry, 1999). Stemofoline's mode of action was quickly determined as a potent agonist at insect nAChRs using biochemical and electrophysiological approaches. Stemofoline itself required a further boost in biological potency and thus screening began for substructures of the natural product that retained high potency against insect *in vitro* nAChR screens and which correlated with *in vivo* screen activity against insects. This led to the discovery of the tropane ethers (IV) from which the pyridyl-cyanotropanes (II and III) were later to evolve (Figure 1).



I II III IV

Figure 1. Structure of stemofoline (I), in which the tropane substructure contained in (II and III) and ether (IV) is shaded.

The cyanotropanes share the same types of neuronal receptors as sites of actions with the neonicotinoids, exemplified by imidacloprid (IMI) and thiamethoxam (Maienfisch, 2001a, 2001b). We report here on the cyanotropanes which have distinct chemistry, nAChR pharmacology and propesticidal properties demarking this series from classical generations of neonicotinoids.

MATERIALS AND METHODS

Chemicals

Cyanotropanes II, [^{3}H]-II (40Ci/mmol), III and IMI were supplied by Jealott's Hill International Research Centre (UK). All other reagents were purchased from Amersham International, Sigma-Aldrich (Poole, UK) or Tocris Cookson (Bristol, UK).

Radioligand binding experiments

Classical radioligand binding methods to determine ligand affinity, binding site populations, displacement and rate kinetics were employed as described by *Lind* et al, 1998. Membranes of the peach potato aphid *Myzus persicae* and the blowfly *Lucilia sericata* were used in this study. Briefly, binding assays utilised 100µg insect membranes in a final volume of 200µl, reactions terminated using rapid filtration and radioactivity quantified by a beta plate counter.

Saturation binding was determined at concentrations of [^{3}H]-II ranging between 0.01 and 30nM on membranes of *M. persicae* and *L. sericata.* An incubation period of 3 hours was employed to allow equilibrium. Non-specific binding was below 10% at concentrations <5nM. Ligands employed for competition binding studies were added to *M. persicae* membranes 30 min prior to the addition of radioligand, namely either radio-labelled II, α-Bungarotoxin (α-BgTx), methyllycaconitine (MLA), epibatidine (EPI) or IMI.

A concentration of 1nM [^{3}H]-II was used to investigate association and dissociation rate kinetics in *M. persicae* membranes at 4°C. Association rates were determined by incubating for time periods from 0.5 to 180 min. The experiment was terminated by filtration. To investigate the kinetics of dissociation, membranes were allowed to associate with [^{3}H]-II for 3 hours before isotopic dissociation was initiated by the addition of 1µM II, MLA, IMI or EPI to investigate allosteric interactions. Residual bound radioligand was determined between 0.5 and 60 min, in the presence and absence of 1µM II for non-specific and total binding respectively.

Data analysis was performed using non-linear regression analysis with Microsoft Excel's solver macro program (Lind *et al.,* 1998) and unless otherwise stated, experiments were performed in triplicate. Non-specific binding was determined using a final concentration of 1µM of the unlabelled ligand corresponding to the labelled version.

Electrophysiology

Central neuronal effects of the cyanotropanes on adult cockroaches were determined by extracellular recording of multi-synaptic EPSP activity using a suction electrode positioned between the 5th and 6th abdominal ganglia of cercal preparations. Such preparations have been widely described previously (Miller 1979). CNS activity determinations in *Heliothis virescens*

were carried out using extracellular recording one or two nodes posterior to the metathoracic ganglion in whole isolated nerve-cord preparations of third or fourth instar larvae. In desheathed preparations, the perineurium was carefully torn using fine watchmakers forceps or sharpened glass capillaries. Compounds were administered by incorporation in the bathing medium and unstimulated spike frequencies before and during exposure were counted using a Digitimer D-130 Spike Processor.

Depolarizing effects of the compounds were determined by intracellular recording from unidentified somata in dissociated neuronal preparations of brain and thoracic ganglia from *Periplaneta americana* and *Schistocerca gregaria,* prepared using methods described previously (Pinnock and Sattelle 1987). Compounds were dissolved in bathing medium at 10μM and applied by pressure ejection from a patch pipette positioned 50-100μm from the soma.

Pharmacokinetics

One microlitre of a solution of technical material of either III or II (dissolved in DMSO at a concentration of 1000ppm) was injected into the haemoceol of 5th instar *H. virescens*. At time periods of 0, 2, 6 hrs & 20 hours treated caterpillars (4 replicates per time point) were homogenised in 1 ml of acetonitrile. Samples were centrifuged at 6000 rpm for 5 minutes and the resulting supernatant decanted directly into HPLC vials prior to LCMS analysis to quantify concentrations of compounds III and II.

RESULTS

Radioligand binding experiments

Saturation binding of [^{3}H]-II is consistent with a single binding component with an affinity (K_d) of 0.48nM and a maximal binding capacity (B_{max}) of 322 fmol/mg and an associated Hill value of 0.94 in membranes of *M. persicae* (Figure 2). This was very similar to the K_d determined in *L. sericata* membranes of 0.9nM.

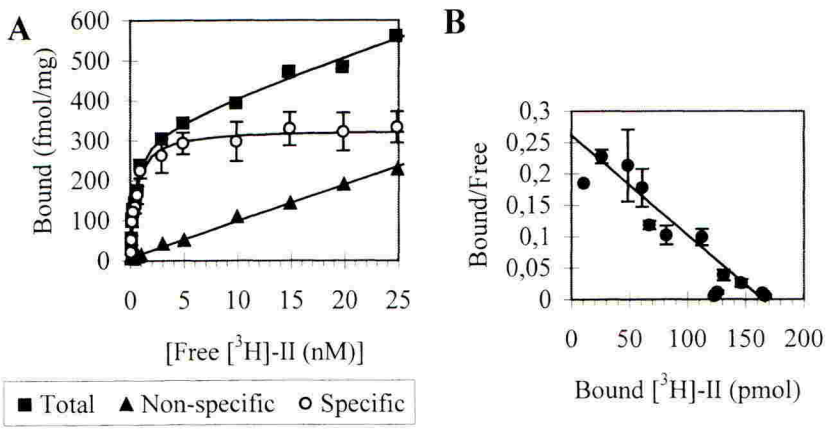


Figure 2. Saturation (A) and Scatchard (B) plot of [^{3}H]-II binding to *M. persicae* membranes. Data shown gives the mean±SEM.

The pharmacological profile (Figure 3 and Table 2) demonstrates a nicotinic character, and is most similar to that determined previously for α-BgTx binding in *M. persicae* (Lind *et al.*, 1999a). ACh and (-)-nicotine were only poor displacers of [^{3}H]-II with IC_{50} values respectively more than 4 and 2 orders of magnitude higher than that for II itself. α-BgTx, imidacloprid and epibatidine were found to be more effective displacers of [^{3}H]-II, with IC_{50} values of approximately 16, 19 and 27-fold greater than for II. MLA was slightly more potent than II itself which is probably related to its kinetic properties as a ligand (Lind *et al.*, 2001). II, MLA, epibatidine and (-)-nicotine all gave Hill values of approximately 1, whereas imidacloprid, α-BgTx and ACh all exhibited Hill values markedly less than 1. Displacement of [^{3}H]-α-BgTx by II and III gave IC_{50} values of 1.5 and 58900nM respectively indicating over a 4-fold order of magnitude difference in activity.

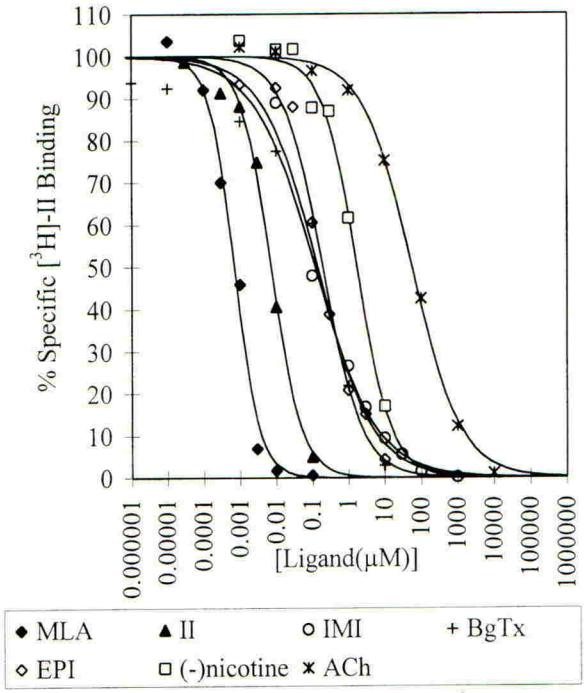


Figure 3. Displacement curves for nicotinic ligands against [^{3}H]-II binding in *M. persicae* membranes.

Kinetic analysis to record the 'on' and 'off' rates produced biphasic responses giving an estimated K_d for the fast components of 0.13nM which is in good agreement with that derived from equilibrium binding experiments. The dissociation kinetics can be used to study the allosteric interactions between ligands (Figure 4). The dissociation of [^{3}H]-II by addition of II or MLA is similar while data using IMI or EPI demonstrates an allosteric interaction interfering with the dissociation of [^{3}H]-II. Interactions with α-BgTx are hampered by this ligand's slow kinetic rates so its precise interactions can not be determined.

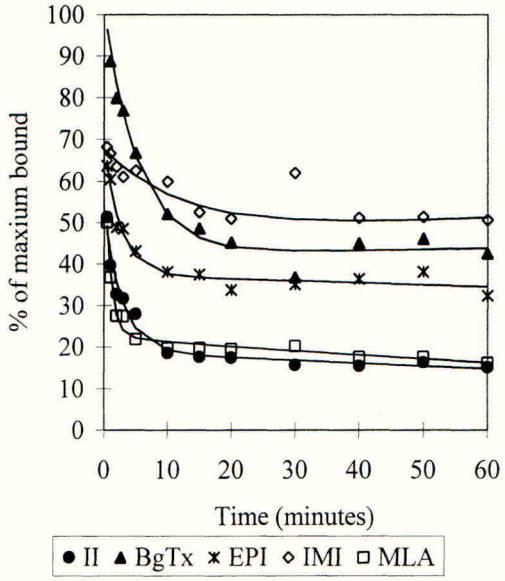


Figure 4. Isotopic dissociation curves of [^{3}H]-II binding to *M. persicae* membranes demonstrating allosteric interactions with other nicotinic ligands.

Electrophysiology

Both I and II elicit increases in spontaneous spike frequency in desheathed 6th abdominal ganglion preparations of cockroach characteristic of nicotinic agonists with a potency roughly equal to that of IMI (breakpoints 0.2 to 1μM). Likewise, they were equipotent in their ability to cause excitation in desheathed CNS of *H. virescens*, although on sheathed preparations, I was an order of magnitude more active than II, suggesting that the perineurium presents a greater barrier to the penetration of the secondary amine. Application of these compounds to naked neurones of cockroach or locust revealed that both I and II strongly elicited depolarizations caused by cation-carried inward currents whereas III was only very weakly active.

Pharmacokinetics

Analysis of insects treated with compounds II and III showed half lives of approximately 2.5 and 1 hours respectively (Figure 5a). In the insects treated with compound III significant levels of compound II were observed to accumulate as a primary metabolite (Figure 5b).

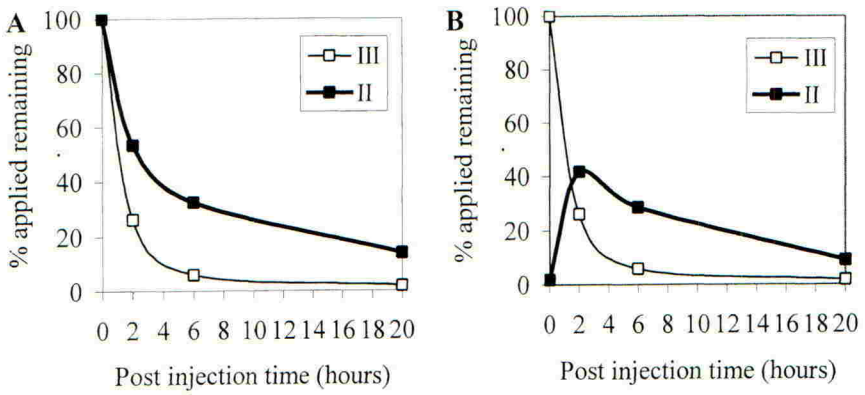


Figure 5. **A**. Relative stability of III and II in 5th instar *H. virescens*. **B**. Conversion of III to II in 5th instar *H. virescens*.

DISCUSSION

The cyanotropanes represent novel insecticidal compounds which interact with the insect nAChR but which are chemically distinct from the neonicotinoids. Evidence from metabolism studies in *H. virescens* indicates that compound III is readily degraded in a propesticidal manner to compound II. Further evidence for a propesticide action is given by biochemical evidence that the *in vivo* active parent (III) is virtually inactive at the insect nAChR while the more stable metabolite (II) is highly active *in vitro*. Furthermore, electrophysiological evidence on the insect CNS is consistent with agonist actions on insect nAChR demonstrating again that II is more active than III.

Radioligand binding experiments utilising [^{3}H]-II have characterised a single binding site among the population of insect nAChRs which has a pharmacology similar to that of the snake toxin α-BgTx (Table 2). This study complements earlier work (Table 1) to investigate the heterogeneity in native populations of nAChR binding sites in *M. persicae* and allows some tentative conclusions to be drawn.

Table 1. Comparison of saturable binding of [^{3}H]-II, [^{3}H]-MLA, [^{125}I]-α-BgTx, [^{3}H]-IMI and [^{3}H]-EPI in *M. persicae* membranes providing K_d and B_{max} values. [1]Data from Lind *et al.* (2001) [2]Data from Lind *et al.* (1999a) [3]Data from Lind *et al.* (1998) [4]Data from Lind *et al.* (1999b)

	High affinity		Low affinity		
Ligand	K_d (nM)	B_{max} (fmol/mg)	K_d (nM)	B_{max} (fmol/mg)	Summed B_{max} values
[^{3}H]-II	0.48	322			322
[^{3}H]-MLA[1]	0.95	1290			1290
[^{125}I]-α-BgTx[2]	1.18	167	33.7	640	807
[^{3}H]-IMI[3]	0.14	284	12.6	883	1167
[^{3}H]-EPI[4]	0.89	344	18.4	904	1248

MLA, IMI, EPI and α-BgTx appear to interact with a similar number of binding sites, if both high and low affinity binding components are taken into consideration. [^{3}H]-II is distinct from the other ligands and appears to label a single high affinity binding site. The density of this site (B_{max}) is very similar to that of the high affinity sites of EPI, IMI and α-BgTx. However, no low affinity sites were observable with the methodology used. However this doesn't mean that the missing sites do not exist, merely that they were undetectable in this study possibly because their affinity is very low. [^{3}H]-MLA is able to interact with similar affinity with all of the sites labelled by the other radioligands and provides a useful tool to characterise total numbers of binding sites (Lind *et al.*, 2001).

The pharmacology of the [^{3}H]-II binding site can be compared with earlier work on *M. persicae* membranes to begin to elucidate a model of binding sites (Table 2).

Table 2. Pharmacological comparisons of cross pairing of ligands in *M. persicae* membranes giving K_i values in nM. [1]Data from Lind *et al.* (2001) [2]Data from Lind *et al.* (1999a) [3]Data from Lind *et al.* (1998) [4]Data from Lind *et al.* (1999b)

	Labelled Ligand					
Unlabelled Ligand	II	[2]α-BgTx	[3]IMI	[4]EPI	[1]MLA (High)	[1]MLA (low)
II	2.4	1.5	40.5	10.6	20.2	-
α-BgTx	39.2	0.7	13.7	4.1	10.0	1,238
IMI	45.7	703	0.2	0.3	0.3	419
EPI	63.6	26.1	4.8	1.2	3.2	704
MLA	0.2	1.6	3.3	1.4	0.6	-
(-)nicotine	568	670	141	53.9	607	11,351
ACh	18,454	2,000	522	287	131,910	1057,426

The pharmacological data suggest that binding sites for II and α-BgTx are very similar, being sensitive to displacement by each other and by MLA whilst being resistant to displacement by IMI and EPI. IMI and EPI have a distinct pharmacological profile. MLA is particularly interesting in its position in this model in that it is equipotent at all binding sites, which is consistent with the saturation data presented in table 1. Moreover, the [^{3}H]-II dissociation data are consistent with the displacement study model such that II and MLA share a common site, whereas IMI and EPI interact allosterically with the II binding site in a cooperative manner indicating they are on the same nAChR but spatially distinct.

In summary, the cyanotropanes act as agonists of insect nAChR. The high *in vivo* activity of III correlates strongly with a propesticidal model that it is converted to II with evidence stemming from metabolic, radioligand binding and electrophysiological experiments. [^{3}H]-II demonstrates specific binding to an apparently single binding component present in *M. persicae* and *L. sericata*. Furthermore, [^{3}H]-II behaves as a specific ligand labelling a sub population of binding sites in *M. persicae*. These are postulated to share a high affinity binding site with α-BgTx based on similarities in pharmacology but which are distinct from that of the high affinity IMI and EPI binding sites. The heterogeneity observed in *M. persicae* is mirrored in its genomic diversity of subunit genes for nAChR (Tomizawa and Casida, 2001). This makes [^{3}H]-II a specific biochemical tool for defining a sub-population of nAChR in insects, and for investigating the binding behaviour of ligands for the future discovery of new neonicotinoids.

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