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

Neonicotinoid insecticides - retrospective consideration and prospects

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ABSTRACT

Neonicotinoids (chloronicotinyls) are increasing worldwide as a novel class of insecticides. As agonists they act selectively on nicotinic acetylcholine receptors (nAChRs) of insects and are part of a single mode of action (MOA) group as defined by the Insecticide Resistance Action Committee (IRAC) for pest management purposes. Retrospective considerations regarding bioisosteric segments of the commercialized neonicotinoids gave insight into general structural requirements for all the different ring systems and non-cyclic structures. However, for the well known practical application methods of most neonicotinoids, such as soil- and seed treatment as well as foliar application, the uptake and translocation in plants is in many cases important for their excellent insecticidal activity. Therefore, not only individual molecular features of neonicotinoids but also their physicochemical properties such as logP-value can have a remarkable influence on the distribution of these a.i. in plants. Prospects of neonicotinoid chemistry will now be focused on the differentiation of new compounds through access to new insecticidal market segments, on the use of target assays to detect novel chemistry with different physicochemical properties and on the structure elucidation of the insect nAChR as a target useable for molecular modelling studies.

INTRODUCTION

Up to now, the insecticide world market (> 7 bn $ at the beginning of the 1990s) is dominated by acetylcholinesterase (AChE) inhibitors. AChE is one of the most essential enzymes in the central nervous system (CNS) of insects, responsible for the degradation of the predominant neurotransmitter acetylcholine (ACh). However, the market share of these AChE inhibitors, i. e. organophosphates and carbamates, decreased from 71% in 1987 to some 47% in 1999 (Table 1). Together the AChE inhibitors and those insecticides that act on the voltage-gated sodium channel, in particular the pyrethroids, account for more than 64% of the world market, i.e. these two modes of action account for two-thirds of the market.
One of the insecticide molecular target sites of growing importance is the nicotinic acetylcholine receptor (nAChR). In spite of the use of the alkaloid nicotine as natural insecticide (aqueous tobacco extracts) for a long time, the nAChR has been an underexploited biochemical target for modern insecticides, with an estimated total insecticide world market share < 2% up to 1991 (Nauen *et al.*, 2001). Because of its high mammalian toxicity and relatively low level of insecticidal activity no major insecticidal class could be established through taking nicotine as a lead structure. On the other hand, registered compounds active on the nAChR include the very small group of so-called nereistoxin analogues such as Takeda's cartap (1964) and bensultap (1968), and Sandoz' thiocyclam (1977). These products are in fact pro-insecticides which are converted metabolically into the natural product nereistoxin.

Table 1. Mode of action of the top 100 selling insecticides/acaricides and their world marked share[a]

Mode of action	1987 (%)	1999 (%)	Change (%)
Acetylcholinesterase	71.2	47.4	-23.8
Voltage-gated sodium channel	16.5	16.4	-0.1
Nicotinic acetylcholine receptor	**1.5**	**10.6**	**+9.1**
GABA-gated chloride channel	5.0	7.7	+2.7
Chitin biosynthesis	2.1	2.8	+0.7

[a] Excluding fumigants, endotoxins and those with unknown mode of action.

Neonicotinoid history

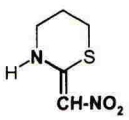
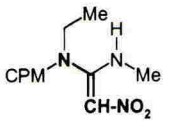
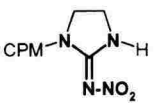
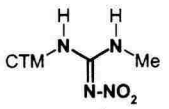
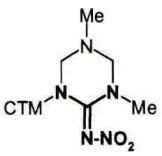
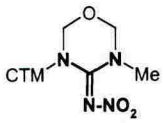
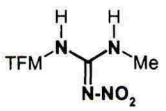
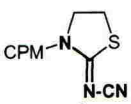
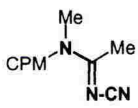
In the early 1970s, Shell invented a new class of nitromethylene heterocyclic compounds acting on the nAChR. The most active compound of that class was the six-membered tetrahydro-1,3-thiazine, nithiazine (SKI-71) - the 1st generation of the so-called neonicotinoid insecticides (Table 2, the term of neonicotinoids was proposed by Tomizawa & Yamamoto, 1993). Because of the photolabile 2-nitromethylene group (electronic absorption: λ_{max} = 348 nm), nithiazine was never commercialized for broad agricultural use (Soloway *et al.*, 1979; Kollmeyer *et al.*, 1999). In the early 1980s synthesis work was initiated at Nihon Bayer on the basis of this first neonicotinoid lead structure. By introduction of a nitrogen-containing hetarylmethyl group as a N-substituent of the 2-nitromethylene-imidazolidine 5-ring system as described for the derivative NTN32692, the insecticidal activity could be remakably enhanced (Kagabu, 1997). After preparation of about 2000 compounds, imidacloprid, a so-called chloronicotinyl or 2nd generation neonicotinoid, was selected for commercial use based on its photostability and residual activity in greenhouse and field trials, its extremely high intrinsic insecticidal potency, broad insecticidal spectrum, excellent systemic properties, plant compatibility and favorable safety profile, especially low mammalian toxicity. The differences in photostability between the 2-nitromethylene group in compound NTN32692 (λ_{max} = 323 nm) and the 2-nitroimino group in imidacloprid (λ_{max} = 269 nm) was examined by quantum chemical methods and *ab initio* calculations (Kagabu, 1997). This breakthrough to a novel systemic insecticide was achieved by coupling a special heterocyclic group, the 6-chloro-pyrid-3-yl-methyl (CPM) residue, to the 2-(N-nitroimino)-imidazolidine building block. Imidacloprid was introduced to the market in 1991 as the first 2nd generation neonicotinoid, and has since become the largest selling insecticide worldwide in the last decade for crop protection (trade names: Admire®, Confidor®, Gaucho®, Provado®) and animal health applications (Advantage®), to control ectoparasitic insects (Mencke & Jeschke, 2002).

STRUCTURAL DIVERSITY OF 2nd GENERATION NEONICOTINOIDS

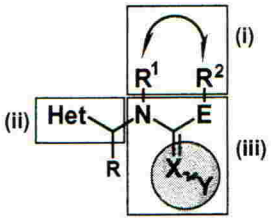
In connection with these excellent results, an extensive research program led also to the discovery and development of thiacloprid, the second member of the chloronicotinyl insecticide group (Jeschke *et al.*, 2001). Attracted by Bayer's success with imidacloprid, several different companies such as Takeda, Nippon Soda, Mitsui Toatsu (now Mitsui Chemicals, Inc.)

and Ciba Geigy (now Syngenta) developed their own neonicotinoid insecticides. The activities within these research-based companies were facilitated by the fact that the neonicotinoid chemistry showed a relatively broad practical variability (Wollweber & Tietjen 1999). Therefore, it is not surprising that two of the subsequently commercialized 2nd generation neonicotinoids, nitenpyram (Takeda) and acetamiprid (Nippon Soda) also have the characteristic CPM-moiety. In other structural types of neonicotinoids like thiamethoxam (Syngenta), the not commercialized compound from Agro Kanesho, and clothianidin (Takeda/Bayer), the CPM-moiety is replaced by the 2-chloro-1,3-thiazol-5-yl (CTM) group. In (±)-dinotefuran, a novel neonicotinoid of the nitroguanidine type discovered by Mitsui Chemicals, Inc., the CPM residue is replaced by a *(R,S)*-tetrahydrofur-3-yl (TFM) moiety. In general, all these commercialized or developed 2nd generation neonicotinoids differ regarding their structural segments (Table 2 & Figure 1).

Table 2. Chemical structures of the 1st generation neonicotinoid nithiazine and 2nd generation neonicotinoids displaying the different types of pharmacophores

Pharmacophores [-N-C(E)=X-Y]	Structure type: Ring system (R^1-R^2, R^1-Z-R^2)	Structure type: Non-cyclic structure (R^1, R^2)
Nitroenamines (E = S, N) (Nitromethylenes) [-N-C(E)=CH-NO_2]	 Nithiazine	 Nitenpyram (1986)[a]
Nitroguanidines (E = N) [-N-C(N)=N-NO_2]	 Imidacloprid (1985)[a]	 Clothianidin[b] (1989)[a]
	 Agro Kanesho	 Thiamethoxam[b] (1992)[a]
		 (±)-Dinotefuran[b] (1993)[a]
Cyanoamidines (E = S, Me) [-N-C(E)=N-CN]	 Thiacloprid[b] (1986)[a]	 Acetamiprid (1988)[a]

[a] Year of the first patent application covering the insecticide.
[b] ISO draft proposal.



(i) Ring systems (R^1-R^2, R^1-Z-R^2; Z = O, NMe) or non-cyclic structures (R^1, R^2),

(ii) Heterocyclic N-substituents [HetCHR ; R = H > alkyl] (*e.g.* CPM, CTM, TFM) and

(iii) Different types of pharmacophores [-N-C(E)=X-Y] (*e.g.* nitroenamines [-N-C(E)=CH-NO_2] with E = S, N; nitroguanidines [-N-C(N)=N-NO_2] and cyanoamidines [-N-C(E)=N-CN] with E = S, Me)

Figure 1. Structural segments for 2nd generation neonicotinoids

The influence of these different structural features on the insecticidal activity of neonicotinoids has already been described (Nauen *et al.*, 2001).

Correlation between electrophysiology and radioligand binding studies

All 2nd generation neonicotinoids act as agonists on insect nAChR and they are part of a single mode of action (MOA) group as defined by Insecticide Resistance Action Committee (IRAC) for resistance management purposes. However, the binding potency and agonistic efficacy of these compounds were quite different, as shown in tobacco budworm (*Heliothis virescens)* preparations (Table 3).

Table 3. Comparison between electrophysiological and [^{3}H]imidacloprid displacement potencies for different neonicotinoids on insect nAChRs (Nauen *et al.*, 2001)

Neonicotinoid	(n)	EC_{50} (µM)	Relative efficacy (1 mM ACh = 1)	[^{3}H]imidacloprid (pI_{50})
Imidacloprid	4	0.31 ± 0.15	0.14 ± 0.02	9.3
Clothianidin	3	0.33 ± 0.03	0.99 ± 0.08	9.2
Acetamiprid	3	1.07 ± 0.37	0.56 ± 0.05	8.7
Nitenpyram	3	1.66 ± 0.38	0.98 ± 0.07	8.6
Nithiazine	4	9.60 ± 3.20	0.79 ± 0.06	6.8

For example, imidacloprid and clothianidin were the most potent compounds in this preparation with an EC_{50} of 0.3 µM. The electrophysiological data (EC_{50} and relative [agonist] efficacy) were obtained on neuron cell bodies isolated from the CNS of *H. virescens.*

Syntheses of the heterocyclic substituents, ring systems *vs* non-cyclic structures

The key intermediates for the above mentioned CPM- or CTM-substituted 2nd generation neonicotinoids are 6-chloro-3-chloromethyl-pyridine (CCMP) and 2-chloro-5-chloromethyl-1,3-thiazol (CCMT), respectively. Up to now numerous syntheses have been studied to develop practical and economic processes for both intermediates based on different commercially available starting materials. Therefore a number of patent applications (25 and 19) and publications (10 and 2) have been described in literature for the preparation of CCMP and CCMT respectively. On the other hand, different synthetic methods for the preparation of the nico-

tinyl insecticides are also known so far, e. g. by ring closure reaction between N-nitroguanidine and N-(6-chloropyrid-3-yl-methyl)-ethylenediamine (imidacloprid) or by N-alkylation reaction of ring systems such as 2-(N-cyanoimino)-1,3-thiazolidine, 2-(N-nitroimino)-1,3-imidazolidine with CCMP (imidacloprid, thiacloprid) or 4-(N-nitroimino)-3-methyl-1,3,5-triazine and -1,3,5-oxadiazine with CCMT (Agro Kanesho, thiamethoxam). The preparation of non-cyclic systems is possible, e.g. by ring cleavage reaction of appropriate N-substituted 4-(N-nitroimino)-3-alkyl-1,3,5-triazines (clothianidine, (±)-dinotefuran) or by nucleophilic substitution reaction of commercially available building blocks like methyl (or ethyl) N-cyanoacetimidate or N-methyl-1-(methylthio)-2-nitro-1-ethenamine with CPM-amine or its derivatives (acetamiprid, nitenpyram) (Wollweber & Tietjen, 1999, Nauen *et al.*, 2001).

BIOISOSTERIC SEGMENTS OF 2nd GENERATION NEONICOTINOIDS

Retrospective considerations regarding bioisosteric segments of the developed and commercialized 2nd generation neonicotinoids gave insight into general structural requirements (segments i-iii, Figure 1) for all the different ring systems and non-cyclic structures. Several common molecular features when comparing compounds with imidazolidine and the isosteric alternatives like thiazolidine, perhydro-1,3,5-oxadiazine or hexahydro-1,3,5-triazine and N-nitroimino, N-cyanoimino or nitromethylene substituents of these insecticides have already been described (Tomizawa *et al.*, 2000). After superposition of the most active derivatives, first statements regarding the molecular shape similarity of the 2nd generation neonicotinoids were possible. It was found that electrostatic similarity of the most active compounds correlates well with the binding affinity (Nakayama & Sukekawa, 1998), and a similar correlation was obtained by comparative molecular field analysis (CoMFA) (Okazawa *et al.*, 1998).

(i) Ring systems *vs* non-cyclic structures: In comparison to the corresponding ring systems the non-cyclic structures exhibit similar broad insecticidal activity by forming a so-called quasi cyclic conformation when binding to the insect nAChR. Based on the CoMFA results, a binding model for imidacloprid was described. This model clarified that the nitrogen of the CPM moiety interacts with a hydrogen-donating site of nAChR, and the nitrogen atom at the 1-position of the imidazolidine ring interacts with the negatively charged domain (Okazawa *et al.*, 1998). Furthermore the binding activity of non-cyclic structures (e.g. acetamiprid, nitenpyram and related compounds) to the nAChR of houseflies was measured and the results were analyzed using CoMFA. Superposition of stable conformations of nitenpyram, acetamiprid and imidacloprid shows that the preferred regions for negative electrostatic potentials near the oxygen atoms of the nitro group as well as the sterically forbidden regions beyond the imidazolidine 3-nitrogen atom of imidacloprid are important for binding (Okazawa *et al.*, 2000). The area around the 6-chloro atom of the CPM moiety is described as a sterically permissible region. Apparently the steric interactions were more important for non-cyclic neonicotinoids than for the cyclic derivative, imidacloprid. Finally, it was also demonstrated that the non-cyclic structures bind to the nAChR recognition site in a manner similar to ring structures like imidacloprid, and that the electrostatic properties of the non-cyclic amino- and cyclic imidazolidine structures are affecting their binding activity.

(ii) Isosteric alternatives to the heterocyclic N-substituents: The nitrogen-containing hetarylmethyl group as N-substituent (CPM, CTM) has a remarkably strong influence on the insecticidal activity. X-Ray crystal structure analysis of imidacloprid and related neonicotinoids indicated that the distances between the van der Waals surface of the nitrogen of the CPM

moiety and the atomic center of the pharmacophoric nitrogen are 5.45-6.06 Å (Tomizawa *et al.*, 2000). This range coincides with the distance between the ammonium nitrogen and carbonyl oxygen of acetylcholine and between the nitrogen atoms of nicotine (Kagabu, 1997). On the other hand, the CPM and CTM moieties were assumed to be able to participate in hydrogen bonding like the pyridine ring of nicotine, and that this is important for the insecticidal activity. Surprisingly, replacing both CPM and CTM by an oxygen-containing 5-membered heterocycle resulted in a novel N-substituent TFM and finally led to the development of the insecticide (±)-dinotefuran. In an attempt to understand this, the hydrogen bonding regions of CPM, CTM, and TFM were projected onto their respective Connolly surfaces. It was confirmed by measurements that the nitrogen-containing hetarylmethyl groups are stronger hydrogen bond acceptors whereas the TFM group, especially the better fitting *(S)*-isomer, is a markedly weaker acceptor.

(iii) Bioisosteric pharmacophores - thiacloprid as an example: The particularly high potency of the neonicotinoids bearing N-nitroimino, N-cyanoimino, or nitromethylene moieties, which have a negative electrostatic potential, implies a positive electrostatic potential for the corresponding insect nAChR recognition site (Nakayama & Sukekawa, 1998). Therefore, considerable attention has been given to the possible involvement of the pharmacophoric nitrogen in neonicotinoid action. As described in an alternative binding model for imidacloprid, the interatomic distance of 5.8 Å between the oxygen of the nitro group (at the van der Waals surface) and the nitrogen in 1-position was also noted as adequate (Kagabu, 1997). That means that the oxygen of the nitro group and the cyano nitrogen are well suited as acceptors for hydrogen bonding with the nAChR in place of ring nitrogen atom in CPM and CTM or the ring oxygen in TFM. Thus the π-conjugated system composed of a N-nitroimino or N-cyanoimino group and the conjugated nitrogen in 1-position are considered the essential moieties for the binding of neonicotinoids to the putative cationic subsite in insect nAChR (Figure 2a, X = N). Recently, the geometry of thiacloprid has been determined by two-dimensional nmr spectroscopy (i. e. HMQC, HMBC) and X-ray analysis (Figure 2b).

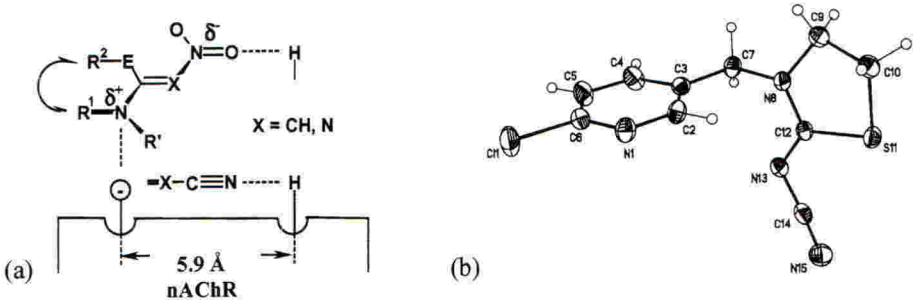


Figure 2. (a) Binding to putative cationic subsite in insect nAChR (Kagabu, 1997) and the (b) Ortep plot of thiacloprid (monoclinic, space group $P2_1/c$) (Jeschke *et al.*, 2001)

Starting from forcefield methods (MMFF94), the final minimum energies, geometries and properties were obtained at DFT (BP functional, TZVP basis, COSMO-RS for solvent effects) level of theory (Jeschke *et al.*, 2001). It was found that in the gas phase the Z-configuration of thiacloprid is about 4 kcal mol^{-1} lower in energy than the E-isomer. The preference for the Z-configuration results mainly from steric factors; the nitrile moiety has virtually no close contacts to any other atom (sulfur to nitrile-carbon distance 3.1 Å). The molecular dipole moment (derived from the electric field dependence of the DFT wave function)

is calculated to be quite high. Both electrostatics and the spatial relation of the pharmacophoric feature [-N-C(E)=X-Y] are very much in line with the pharmacophore model of 2nd generation neonicotinoids like imidacloprid (Jeschke *et al.*, 2001). Besides its influence on biological activity, the pharmacophoric group is not only responsible for the photolytic stability but also for some specific properties such as degradation in soil, metabolism in plants and lack of toxicity to different animals and beneficals (Nauen *et al.*, 2001).

WATER SOLUBILITY AND SYSTEMICITY OF NEONICOTINOIDS

For the well known practical application methods of 2nd generation neonicotinoids, such as soil- and seed treatment as well as foliar application, the uptake and translocation in plants is crucial for their insecticidal activity. Thereby not only the above mentioned bioisosteric segments of neonicotinoids but also the whole molecule shape (Figure 1) including the resultant water solubility has to be considered. In the case of soil- and seed treatment the systemic imidacloprid (Confidor®, Gaucho®) has excellent root uptake, xylem translocation and plant compatibility properties. The optimum exploitation of these properties can be achieved by application methods that work via the root system like drench, drip irrigation, in-furrow and float applications. Therefore it is important for effective pest control by these methods that the roots can take up a sufficient quantity of a.i.. Furthermore, imidacloprid offers good residual efficacy as a foliar spray, too. When sprayed on leaves, part of the a.i. is taken up translaminarily by the leaf from the upper to the lower surface and acropetally towards the tip of the leaf. In contrast to the extremely high water solubility of the neonicotinoids nitenpyram (840 g/l at 20 °C) and nithiazine (200 g/i at 23 °C), acetamiprid (4.2 g/l at 25 °C), imidacloprid (0.61 g/l at 20 °C) and clothianidin (0.327 g/l at 20 °C) have a more balanced physicochemical profile. The latter a.i.s are evenly distributed within the whole leaf lamina while the former neonicotinoids tend to accumulate more rapidly at the leaf margins in an acropetal direction.

PROSPECTS OF NEONICOTINOID CHEMISTRY

The discovery of the 2nd generation neonicotinoids as a new class of ligands of the nAChR can be considered a milestone in insecticide research and permits an understanding of the functional properties of insect nAChRs. Prospects of neonicotinoid chemistry will be focused on:

- the differentiation of new compounds, giving access to new insecticidal market segments (e.g. biting insects such as noctuid larvae),
- the use of target assays to detect novel chemistry with different physicochemical (and structural) properties and
- the structure elucidation of the insect nAChR as target with adequate resolution (e.g. exploitable for rational design).

Up to now the the most current information regarding nAChRs originated from research with vertebrate receptors. From the comparison of the known amino acid sequences and properties it may be assumed that insect receptors have a similar construction to vertebrate receptors. However the real folding pattern of the protein is unknown so far. Recently the pentameric crystal structure of a molluscan acetylcholine-binding protein (AChBP) was described, a structural and functional homologue of the amino-terminal ligand-binding domain of a

nAChR subunit (Brejc *et al.*, 2001). With this structure the first detailed three-dimensional information about the folding and arrangement of the acetylcholine binding site was obtained. Today, the neonicotinoids are the fastest growing group of insecticides (estimated marked share in 2005: c.15 %), with widespread use in most countries in many agronomic cropping systems. The relatively low risk and target-specificity of the products combined with their suitability for a range of application methods will maintain them as important insecticides also in integrated pest management (IPM) strategies.

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Cyanotropanes: novel chemistry interacting at the insect nicotinic acetylcholine receptor

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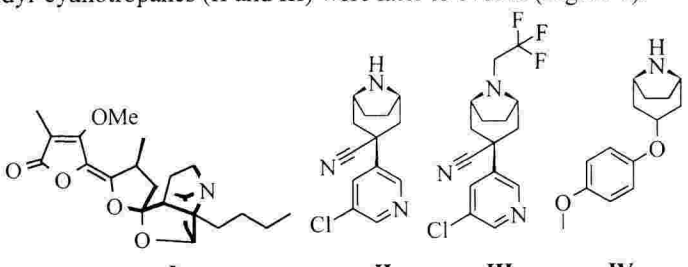
ABSTRACT

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.

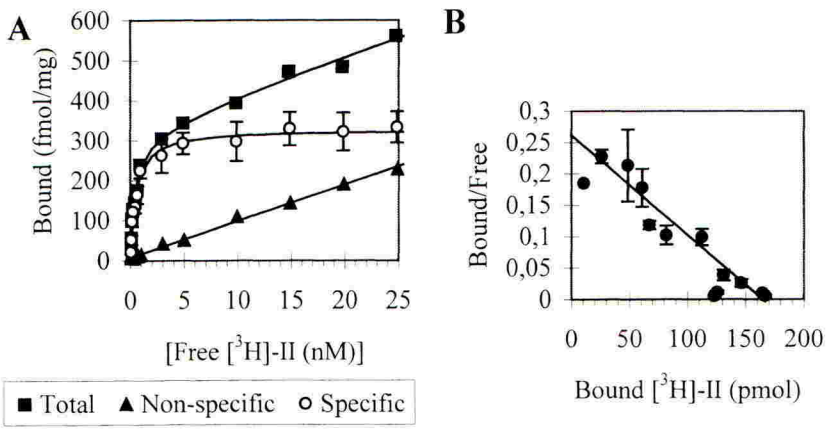


Figure 2. Saturation (A) and Scatchard (B) plot of [^{3}H]-II binding to *M. persicae* membranes. Data shown gives the mean±SEM.

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.

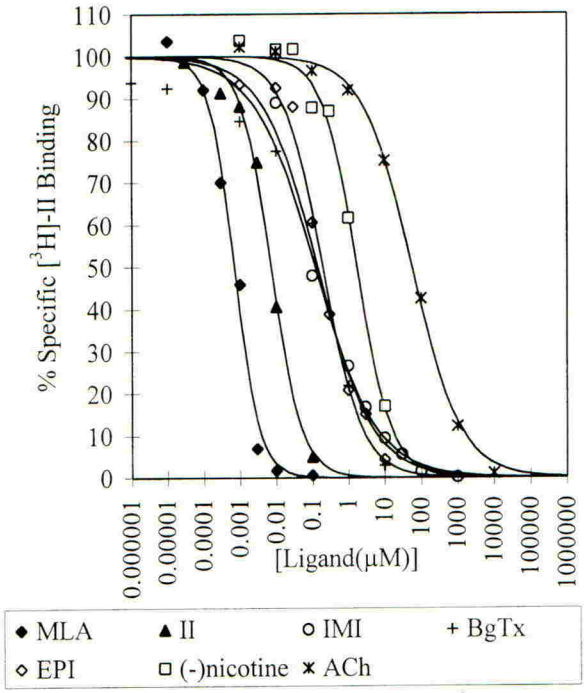


Figure 3. Displacement curves for nicotinic ligands against [^{3}H]-II binding in *M. persicae* membranes.

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.

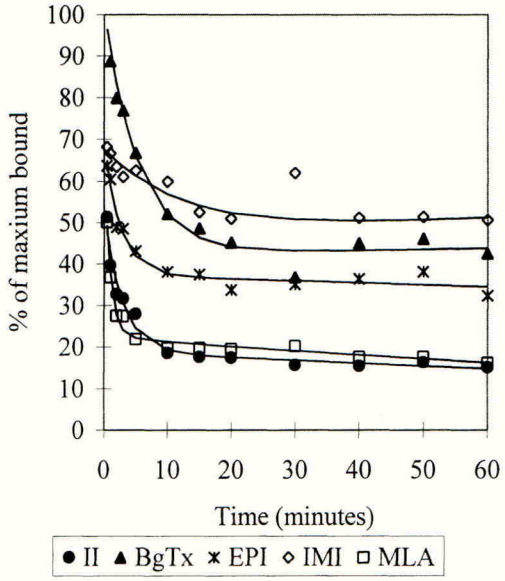


Figure 4. Isotopic dissociation curves of [^{3}H]-II binding to *M. persicae* membranes demonstrating allosteric interactions with other nicotinic ligands.

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).

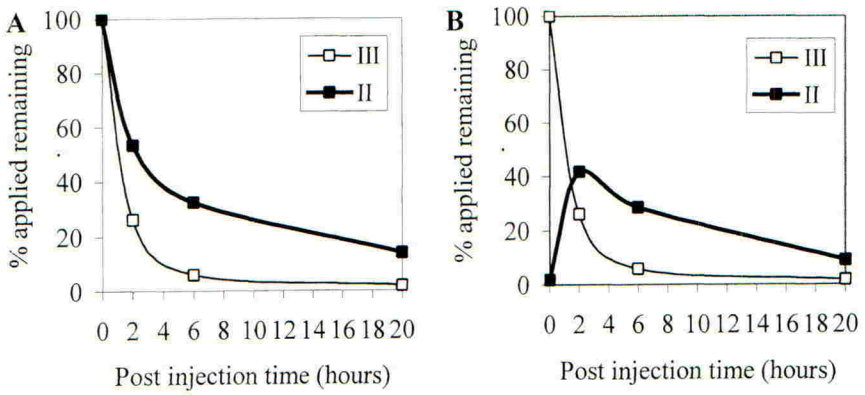


Figure 5. **A**. Relative stability of III and II in 5th instar *H. virescens*. **B**. Conversion of III to II in 5th instar *H. virescens*.

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.

ACKNOWLEDGEMENTS

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Neonicotinoid pharmacokinetics

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ABSTRACT

The pharmacokinetic profiles of the neonicotinoid insecticide imidacloprid in 6th instar (200 mg) larvae of *Spodoptera littoralis* are consistent with high metabolism, and an important role of non-target tissues in competing with the target tissue (the central nervous system) for material circulating in the haemolymph following either topical or oral administration. Penetration through the gut wall was more rapid than that observed for the cuticle, and exposure of the target tissue achieved with oral administration was twice that found with topical application. Results of studies using isolated cuticles and guts were consistent with the *in vivo* pharmacokinetics that account for the low toxicity of this neonicotinoid to lepidopteran larvae.

INTRODUCTION

Many classes of insecticides are neurotoxicants, and when encountered by an insect either by contact with the cuticle or through ingestion of treated food, the active material has to reach the target site(s) in the nervous system. For a compound with a given pharmacodynamic activity, the rate of arrival at the target site, the concentration accumulated there, and the length of exposure will determine the rate of onset and intensity of symptoms of poisoning. The relative importance of the various pharmacokinetic processes, including penetration through cuticle or gut wall, mass transport in the haemolymph, diffusion into the target tissue, and detoxification and elimination, will vary depending on the properties of the compound, and the anatomy and physiology of the insect species. General models of pharmacokinetics in insects are not available. This may be partly due to the diverse range of body form and function in insect pests compared with that found in mammals, and partly to the small size of insects that makes pharmacokinetic studies technically difficult.

Where small insects such as mustard beetles have been used as model species, simple models based on changes in the mass of insecticide remaining on the surface, and the mass of penetrated material have been used (Welling & Paterson, 1985). Where larger insects, such as cockroaches, locusts, or lepidopteran larvae, have been used, more complex, and more physiologically based models have been developed. Such models describe the changes in mass (and concentration) with time of toxicant in various body compartments such as cuticle, muscle, fat body, gut wall, gut contents, central nervous system (CNS), and the haemolymph. The latter acts as the main distributive phase, and a mammilary model similar to those applied in vertebrates, can be used where all other tissues can be regarded as capacitors linked to the haemolymph (Greenwood, *et al.*, 1990). A further advantage of using large insects is that toxicant can be administered orally (either by feeding known quantities of treated food, or by

gavage) as well as by topical application. Excretion of insecticide can take place through detoxification or through direct elimination of parent compound in the urine formed by the Malpighian tubules that empty into the hind gut, or through regurgitation of material in the mid and foregut. Urine is normally combined with the faeces for elimination, and masses of xenobiotic in the faeces will comprise material entering the gut by all possible routes.

The relative capacities of the various insect tissues for a given compound are related to the dry weight of the tissue relative to its water content. In effect this represents the total organic content, mainly lipid, carbohydrate and protein, relative to the water content. The tissue with the lowest dry weight to water ratio (0.027) in lepidopteran larvae is the haemolymph, and that with the highest (1.7) is the CNS. As a result of partitioning the lowest concentration of a non-polar compound at steady state will be in the haemolymph, and the highest in the CNS. This would be an advantage for a non-polar compound such as a pyrethroid or organochlorine that is active in the CNS (Greenwood, *et al.*, 1990).

Most of the published studies of insect pharmacokinetics have concentrated on pyrethroid insecticides, and a range of models describing their action has been published. However, less work has been done to model the pharmacokinetics of other groups of insecticides including the neonicotinoids. Since there are marked differences between the physicochemical properties of these two groups of neurotoxicants, differences between the pharmacokinetic profiles observed for the two might be expected, with concomitant differences in toxicology. The neonicotinoids are very active against homopteran species, but have relatively low toxicity to lepidopteran larvae. This contrasts with the activity spectrum of the pyrethroids that are highly toxic to lepidopteran pests. However, the same general pharmacokinetic processes will apply for both sets of compounds, and so conclusions drawn from earlier studies on other groups of insecticides should aid the interpretation of studies on neonicotinoid compounds.

Imidacloprid (IMI) is the most important commercial member of the neonicotinoid insecticides that are nicotinic acetylcholine receptor agonists. It is both a contact and a systemic insecticide with an LD_{50} by contact application that is 10,000 fold greater for larvae of *Spodoptera exigua* than for the aphid *Myzus persicae*. Since there is only a ten-fold difference between the binding affinities of IMI to the nicotinic acetylcholine receptors of the two species, the large difference in toxicity must depend little on differences in pharmacodynamic activity, and more on pharmacokinetic factors. Pharmacokinetic studies in aphids are difficult because of their small size, fragility, and liquid feeding habit. This study concentrated on the pharmacokinetics of IMI in lepidopteran larvae in order to obtain understanding of the relative importance of the factors that affect the uptake, distribution and elimination of this compound. This may in turn facilitate an understanding of the toxicity profile of IMI across the various insect species.

MATERIALS AND METHODS

Spodoptera littoralis were from a culture maintained at the University of Portsmouth reared at 25^{O}C, with a photoperiod of 16:8 light:dark, in plastic tanks (50 x 25x 25 cm) supplied with a constant air flow. The larvae were fed on Chinese cabbage leaves.

In all pharmacokinetic studies 200 mg 6th instar larvae were used, and were starved for 24 h prior to experiments in order to facilitate oral dosing using treated leaf discs. Larvae were dosed with 390 ng, (equivalent to 1.95 $\mu g\ g^{-1}$) of [methylene-^{14}C] IMI (specific activity 30.85

mCi mmol l^{-1}) dissolved in acetone/DMSO 60/40. For topical administration this was applied in a drop (1 µl) dorsally behind the head capsule, for the oral route the IMI was applied to cabbage leaf discs (4mm diameter) which were ingested. Only larvae consuming the whole leaf disc within 2 minutes were used. Larvae were kept in glass Petri dishes during the experiment.

At a range of elapsed times (nominally 0, 30 , 120, 240, 360,480, 720, and 1440 minutes) following dosing, insects were sampled. Three or four replicates were used at each time point. The external surface was washed with two aliquots (2 ml) of Analar acetone for 30 s per wash. The fourth right proleg was cut and haemolymph was collected from this wound using microcapillary tubes that were emptied into a scintillation vial containing Analar acetone (1 ml). The larvae were dissected on a Sylgard block. The gut was removed intact, and then the contents emptied into Analar acetone (1 ml). The gut wall was frozen in liquid nitrogen, ground to a fine powder in a pestle and mortar, and transferred in three aliquots (5 ml) of Analar acetone. The CNS was removed with the main lateral connectives and placed in a vial containing Analar acetone (200 µl). Muscle and fat were scraped from the cuticle using a blunt scraper and transferred to Analar acetone (2 ml). The cuticle was frozen in liquid nitrogen, ground to a fine powder in a pestle and mortar, and transferred to a scintillation vial with three aliquots (5 ml) of Analar acetone. The Sylgard block was then washed with two aliquots (2 ml) of Analar acetone which were transferred to a scintillation vial. The Petri dishes that held the insects were then washed with two aliquots (5 ml) of Analar acetone which were combined in a glass vial. All extracts were evaporated to dryness under a stream of nitrogen before the addition of Optiphase Hi-Safe 3 (Wallac) scintillation fluid (10 ml). The samples were then counted for 10 minutes in a liquid scintillation counter (Packard Tricarb).

The methods for the *in vitro* study using isolated gut and cuticle preparations were based on those of Watson, 1993. Cuticle from 6th instar larvae (600 mg) of *S. littoralis* was dissected free of other body components, and wired to a glass tube (ID 6 mm) with the epicuticular surface facing the glass tube. The tube was then held in a plastic lid so that the endocuticular face was just below the surface of a stirred receiving phase comprising haemolymph (1.5 ml) collected from *S. littoralis* larvae, and a trehalose (2% w/v) solution (1.5 ml) containing phenylthiourea (0.1% w/v). [^{14}C] IMI (390 µg) dissolved in acetone/DMSO 60/40 was applied topically (1 µl drop) to the epicuticular surface, and then aliquots (10 µl) of the receiving phase removed at a range of elapsed times up to 48 h for scintillation counting as described above.

Guts were removed from 6th instar larvae (200 mg) of *S. littoralis* by cutting through the oesophagus at the mouth, and the rectum at the anus. The [^{14}C]IMI (390 µg) dissolved in acetone/DMSO (60/40) was injected (1 µl drop) into the crop through the cut end of the oesophagus, and both ends of the gut sealed by silk ligatures. The preparation was then submerged in 3 ml of the receiving phase described above for the isolated cuticle, and aliquots (10 µl) removed for scintillation counting at a range of elapsed times up to 48 h.

RESULTS

The whole animal pharmacokinetic behaviour of imidacloprid (IMI) was investigated for both oral and topical application in 200 mg 6th instar larvae of a susceptible strain of *littoralis*. A low dose (390 ng per insect, equivalent to 1.95µg g^{-1}) that did not produce any observable

whole animal symptoms was used throughout these studies to avoid the effects of a toxic response on the pharmacokinetics (Ford, 1988). The median lethal dose that produced vomiting by oral application in 400 mg 6^{th} instar larvae was 13 $\mu g\ g^{-1}$, and by topical application in 4^{th} instar 50 mg larvae was 7.02×10^{3} $\mu g\ g^{-1}$ (Scarr, 1997).

Larvae were dosed either topically or orally and masses of labelled material available for extraction from the insect surface, and accumulating in the various tissues (haemolymph, gut wall, gut contents, fat and muscle, and central nervous system (CNS)) were then measured at a range of elapsed times up to 24 hours. There is a delay (7 minutes following topical , and 9 minutes following oral administration) due to the time for dissection, and processing the insects to produce tissue extracts, and with the latter route the time taken to ingest the treated leaf disc. This should be considered when interpreting events at early elapsed times. Material was detected in all tissues at the earliest time of sampling for both routes of administration (Figures 1 and 2). The rate of disappearance of topically applied material from the external surface was slow, and after a lag over the first 6 hours the disappearance became approximately linear. At an elapsed time of 24 h some 60% of the applied dose remained on the surface. Orally administered material was also detected in external washes. Initially this may be due to contamination of the mouth parts during feeding, and a later increase due to movement from the internal compartment. At later times the mass of material on the external surface fell as material was redistributed to internal compartments.

The haemolymph is the main distributive phase and comes into intimate contact with all other tissues. Levels in this tissue will therefore reflect the net effects of penetration from the site of application, movement into and out of the various tissues, and elimination from the insect. In insects treated by topical application (Figure 1), the level in the haemolymph remained low throughout the experiment, increasing from $1.47 \pm 0{,}12$ ng at 0.5 hours to 5.6 ± 1.42 ng at 4 hours, and then fluctuated around an average of 5 ng (between 2.54 ± 0.87 and 9.2 ± 0.98 ng) throughout the remainder of the experiment. In contrast in orally dosed insects (Figure 2) the mass in the haemolymph rose rapidly over the first 30 minutes to reach a maximum of 47.8 ± 2.98 ng, after which the level fell approximately exponentially to reach a similar level (11.3 ± 2.8 ng) to that (9.2 ± 0.98 ng) observed in topically treated insects at the end of the experiment.

Since the outer surface of the cuticle was washed with solvent, insecticide subsequently extracted from the isolated cuticle must comprise mainly material from the hydrated endocuticle. This tissue, the muscle and fat, and gut wall comprise the major non-target tissues and act as sinks for applied material during the early stages of distribution, and have the potential to buffer levels of toxicant in the haemolymph as insecticide is eliminated from the body. Although these sinks differ in capacity, their pharmacokinetic profiles are of similar form. However, these profiles differ with route of administration (Figures 1 and 2). With oral dosing the labelled material recovered from these tissues falls slowly throughout the experiment after an initial rapid rise over 1-4 h, with the cuticle taking longest to reach its maximum level. In topically treated larvae the initial rise is slower than for oral application, but again this initial phase is longer for the cuticle than for the other tissues. In contrast with oral administration, the levels in these tissues continue to rise throughout the experiment.

The CNS, the target tissue, receives a greater exposure (as measured by the area under the pharmacokinetic profile) following oral (0.015 μg h) compared with topical application

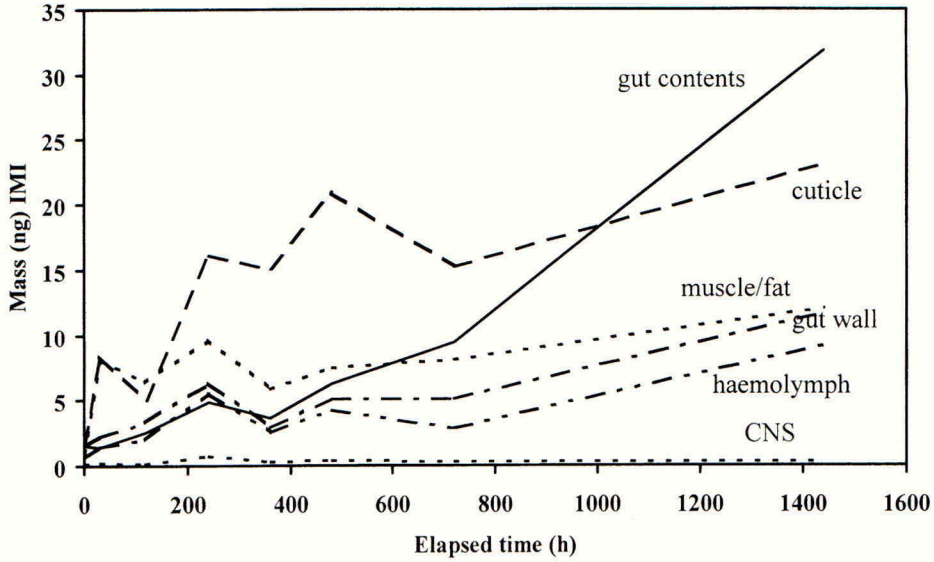

Figure 1. Mean (n=4) mass (ng) of [^{14}C] IMI recovered from the tissues of 6th instar larvae (200 mg) of *S littoralis* following topical application (390 ng per insect).

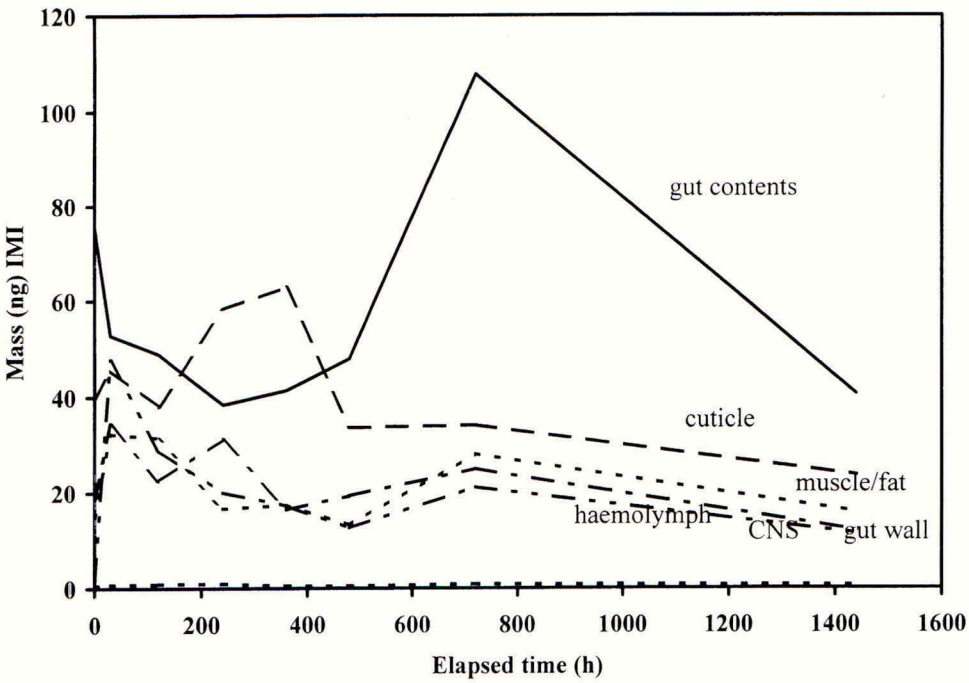

Figure 2. Mean (n=4) mass (ng) of [^{14}C] IMI recovered from the tissues of 6th instar larvae (200 mg) of *S littoralis* following oral administration (390 ng per insect).

(0.008 μg h). In both cases there is an initial rapid rise over the first 4 h followed by a slow decline.

However, the level at early times is three to four times higher in the CNS in orally than in topically treated larvae. This may explain the observed differences in speed of onset of symptoms and endpoint toxicity between the two administration methods (Lagadic *et al.*,1993)

The pharmacokinetic profile of the gut contents differs from those of the other compartments. In topically dosed larvae after the first 6 hours the rate of accumulation of IMI in the gut contents increased markedly over that in all other compartments. In orally treated insects there was an initial rapid loss from this tissue, the site of application, followed after 4 h by an increase to reach double the mass accumulated in other compartments at 12 h, and then by a fall over the last half of the experimental period. Both of these profiles are consistent with an increased opportunity for elimination from the gut lumen.

Since total radiolabel was used to determine the levels of IMI, metabolism was measured in an independent experiment. An initial rapid metabolism was observed with a loss of 33% of the dose over the first four hours. Thereafter metabolism was slower, and by an elapsed time of 24 hours only a further 22% had been lost by this mechanism in orally dosed larvae. The early rapid metabolism will aid the penetration of parent compound from the site of application.

Whilst the pharmacokinetic profiles, based on masses of compound recovered at various elapsed times following topical application help to explain the toxicological behaviour, it is useful to examine the concentrations (mean ± 95% CI) in the various body compartments (despite the bias due to metabolism) in relation to the tissue properties. The highest steady state concentration (2.59 ± 1.27 (n=4) μM) was observed in the gut contents. The other major non-target tissues (cuticle, muscle and fat, and gut wall) showed similar concentrations (1.54 ± 0.19 (n=18), 1.72 ± 0.20 (n=11) and 1.99 ± 0.10 (n=4) μM respectively). The concentration (0.63 ± 0.13 (n=4) μM) in haemolymph was similar to that (0.95 ± 0.12 (n=12) μM) found in the CNS.

The *in vivo* rate of distribution of insecticide following topical application was slow compared with that following oral administration. This was investigated *in vitro* using isolated cuticle and gut preparations. Little labelled IMI penetrated isolated cuticle into the receiving compartment during the first four hours following topical application (390 ng) to the epicuticular surface. The mass in the receiving phase fluctuated around 39 ± 4.86 ng throughout this time, and then slowly increased to reach 128 ± 22 ng by the end of the experiment (48 h). In contrast the penetration rate through isolated gut into the receiving phase started to increase after only 4 minutes. By 48 hours the mass (396 ± 39 ng) having penetrated the gut wall was three-fold greater than that for the cuticle. However, it is difficult to compare fluxes directly since the surface area of the gut preparation was not known. The mean (± 95% CI) mass of IMI extracted from the isolated cuticle (76 ± 43 ng, n=3) at the end of the experiment was twice that from the gut sac (38 ± 12 ng, n= 3).

DISCUSSION

The importance of non-target tissues as competitors for an insecticide is determined by capacity, and that depends on a combination of affinity and size. Tissue affinity relative to that

of the distributive phase, the haemolymph, will affect the rate of accumulation in a tissue. Haemolymph comprises 30%, cuticle 23%, gut contents 24%, and the remaining tissues collectively 25% of the body mass of a 200 mg larva of *S. littoralis* (Greenwood *et al.*, 1990). The CNS forms only 0.7% of the body weight, and thus if all compartments had a similar capacity per mg tissue weight, less than 1% of the applied dose would reach the target.

For a compound with a logP of zero, then the composition (organic content to water content ratio) of the tissues will have little effect on the steady state concentration of the material. In the absence of active transport mechanisms, differences in detoxification rates or binding properties, distribution will be uniform across all organ systems. The further away the logP value of a xenobiotic is from zero, the greater will be the effect of tissue composition on the distribution of that compound. This explains the small differences in concentration of IMI (logP = 0.56) observed between the various tissues of *S. littoralis* in contrast with the large differences reported (Greenwood *et al.*, 1990) for cypermethrin (logP = 6.6). The affinities of the more hydrated non-target tissues for cypermethrin are low. The lowest steady state concentration (0.0079 μM) was found in the haemolymph (dry weight to water ratio = 0.0270), and the highest (1.7 μM) in the CNS (dry weight to water ratio 0.56). The large difference in affinity between the CNS and haemolymph for cypermethrin, and the lower competition of non-target tissues for this insecticide, favour accumulation in the target tissue, giving a pharmacokinetic enhancement of activity not found with IMI. The concentration of IMI in the CNS appears to be steady over the last 18 hours of poisoning, but the observed concentration (0.95μM) is below that (3.7 μM) predicted on the basis of the relationship between the log(tissue concentration) and log(dry weight/water content) for the other tissues. This may reflect either a difference in apparent permeability or metabolism between the CNS and all other tissues, and is worthy of further investigation.

Penetration through the gut wall was more rapid than through the cuticle both *in vivo* and *in vitro*, and higher exposures of the CNS were achieved via the former route, despite increased exposure of pesticide to metabolism at earlier times. In part, the difference in penetration can be explained by the large capacity of the endocuticle for IMI compared with that of the gut wall. The *in vitro* cuticular penetration of IMI shows a long lag phase compared with those observed by Watson (1993) for pyrethroids, and this could also be due to the thick hydrated endocuticle which has a high affinity for the polar neonicotinoid, and a low affinity for the non-polar pyrethroids. Even in aphids where the cuticle is relatively thinner the LD95 by topical application is 80 fold higher than for oral administration (Elbert *et al.*, 1991). The differences between the *in vivo* and *in vitro* preparations indicate the importance of the rapid circulation of haemolymph, and the removal of material from this phase by non-target tissues. These factors maintain the diffusion gradient that drives penetration.

The profile of the gut contents for IMI is markedly different from that of the other tissues. With oral application there is an initial fall in insecticide in the gut contents as material is distributed to other tissues, but after 4 hours the level rises to approach a concentration two to three times higher than those found in the other tissues. Over the last 12 hours the concentration in the gut falls but is still approximately double that in most other tissues at the end of the experiment. Similarly with topical application the level of IMI in the gut contents rises to 3.5 times that observed in the other non-target tissues, including the gut wall. These observations are consistent with excretion in the urine, breakdown in the gut lumen and/or the paracellular movement of the compound in the water stream effected by the active transport system that replenishes the water content of the midgut in lepidopteran larvae (Reynolds &

Bellward, 1989). Since the gut is a compartment from which physical loss of toxicant to the external environment can take place through the movement of food, faeces and urine along the lumen to be voided via the anus, the high accumulation there will increase elimination of insecticide from the insect. This contrasts with the behaviour of pyrethroids which are some six orders less polar than IMI, and have much lower water solubilities. Cypermethrin, for example, is lost from the gut by regurgitation at early times, but at later elapsed times after topical application of 146 ng the profiles of gut contents and gut wall are similar, and there is no significant movement of pyrethroid into the gut.

The factors discussed above will be important in determining interspecific differences in pharmacokinetics and hence toxicity of insecticides. For instance, large differences would be expected between aphids and lepidopteran larvae since the former lack the thick cuticle associated with the use of a hydrostatic skeleton, and contain a large volume of fluid relative to body weight because of the sap feeding habit. The latter involves the processing of large volumes of plant fluids to concentrate essential nutrients and excrete excess water and sugars. This is achieved by the circulation of water through gut and haemocoel compartments that for a systemic compound such as a neonicotinoid will maintain an exposure of the CNS.

CONCLUSIONS

The low toxicity of IMI to lepidopterous larvae is due to a combination of metabolism, loss via the gut, unfavourable relative affinities of the CNS and haemolymph, the distributive phase, and the high capacities of non-target tissues, especially the endocuticle, for this compound.

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Incidence and management of insect resistance to neonicotinoids

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ABSTRACT

The development of neonicotinoid insecticides has provided growers with invaluable new tools for managing some of the world's most destructive crop pests. To date, neonicotinoids have proved relatively resilient to the development of resistance, although strong resistance has been confirmed in the whitefly, *Bemisia tabaci*, and the Colorado beetle, *Leptinotarsa decemlineata*. Resistance in *B. tabaci* appears due to enhanced oxidative detoxification of neonicotinoids. The possibility of target-site resistance in *L. decemlineata* is being investigated further. Strategies to combat neonicotinoid resistance must take account of the cross-resistance characteristics of these mechanisms, the ecology of target pests on different host plants, and the implications of increasing diversification of the neonicotinoid market due to a continuing introduction of new molecules.

INTRODUCTION

The invention and subsequent commercial development of neonicotinoid insecticides has provided agricultural producers with invaluable new tools for managing some of the world's most destructive crop pests. Insect groups targeted by neonicotinoids – primarily Hemiptera (aphids, whiteflies and planthoppers) and Coleoptera (beetles) – include species with a long history of resistance to earlier-used products. However, the speed and scale with which imidacloprid, the commercial forerunner of neonicotinoids, was incorporated into control strategies around the world prompted widespread concern over the development of imidacloprid resistance (e.g. Cahill & Denholm, 1999). To a large extent these pessimistic forecasts have not been borne out in practice. Imidacloprid has proved remarkably resilient to resistance, and cases that have been reported are still relatively manageable and/or geographically localised. The existence of strong resistance is some species has nonetheless demonstrated the potential of pests to adapt and resist field applications of neonicotinoids. The ongoing introduction of new molecules (e.g. acetamiprid, thiamethoxam, nitenpyram, thiacloprid and clothianidin), unless carefully regulated and co-ordinated, seems bound to increase exposure to neonicotinoids and to enhance conditions favouring resistant phenotypes. This paper reviews knowledge of the incidence, mechanisms and practical implications of existing cases of neonicotinoid resistance, and considers factors relevant to the design of resistance management strategies.

INCIDENCE OF RESISTANCE TO NEONICOTINOIDS

Whiteflies

Probably the best documented example of pests evolving resistance in response to field use of neonicotinoids relates to whiteflies, *Bemisia tabaci*, in the intensive horticultural production system occupying over 30,000 ha near Almeria in southern Spain. Due to a continuous production cycle and favourable climatic conditions, *B. tabaci* frequently reaches very high densities, causing direct damage through feeding and by transmitting virus diseases to tomatoes, peppers and curcubits. Over-use of insecticides, often as tank-mixes, has led to the loss of many older insecticides through resistance, and placed excessive pressure on novel products introduced to the region (Denholm, *et al.*, 1996). A number of strains collected from Almeria in 1994 and 1995, and tested using a systemic leaf-dip bioassay, showed significantly reduced mortality at a diagnostic concentration of imidacloprid (Cahill, *et al.*, 1996). At the time there was still no evidence of this impairing control efficacy in the field (Elbert & Nauen 2000). During the late 1990s, however, resistance increased in potency with more recently-collected strains exhibiting more than 100-fold resistance to imidacloprid, and comparable levels of resistance to thiamethoxam and acetamiprid (Elbert & Nauen, 2000; Nauen, *et al.*, 2002; I Denholm, *et al.*, unpublished data). As a result, the usefulness of neonicotinoids for controlling whiteflies in this region has been compromised considerably.

Continuous laboratory selection with imidacloprid of a population of *B. tabaci* collected from melons in the Imperial Valley of California resulted in >80-fold resistance after 24 generations, implying a similar genetic potential for resistance in whiteflies inhabiting southwestern USA (Prabhaker, *et al.*, 1997). However, extensive temporal monitoring of whiteflies in Arizona, despite disclosing low frequencies of resistant individuals, has not yet shown a directional increase in resistance to economically-damaging levels (Dennehy & Denholm, 1998; Williams & Dennehy, 1998; Li, *et al.*, 2000). This may reflect the adoption of strict guidelines for managing resistance to neonicotinoids and other whitefly control agents (Dennehy & Williams, 1997; Williams, *et al.*, 1998), and/or a severe fitness penalty associated with any resistance mechanisms that have arisen.

The status of *B. tabaci* resistance to neonicotinoids in other countries is less well documented. However, there is evidence of increasing resistance on cotton in Israel (A R Horowitz, unpublished data) and on horticultural crops and cotton in Australia (R Gunning, unpublished data). In contrast, bioassays with recently-collected field strains from Egypt (El-Kady, *et al.*, 2002) and from glasshouses in Europe showed almost full susceptibility to imidacloprid. The other major whitefly pest targeted by neonicotinoids is the glasshouse whitefly (*Trialeurodes vaporariorum*). A survey of several contemporary UK strains of this species showed no evidence of resistance to imidacloprid (Gorman, *et al.*, 2001). We are unaware of control problems with *T. vaporariorum* elsewhere in the world.

Aphids

Clones of the peach-potato aphid (*Myzus persicae*) exhibiting 3- to 7-fold resistance to imidacloprid in contact and ingestion bioassays were originally isolated from strains collected from several crops in Greece, Germany and Japan (Devine, *et al.*, 1996; Nauen, *et al.*, 1996). More recently, aphids with up to 18-fold resistance have been recorded from additional regions including Zimbabwe, the USA, and southern and northern Europe (Cox, *et al.*, 2002;

Foster, *et al.*, in press). Levels of resistance to imidacloprid correlate closely with those to nitenpyram and acetamiprid (Foster, *et al.*, in press), and also to nicotine (Devine, *et al.*, 1996; Nauen, *et al.*, 1996). Thus, it is possible that resistance arose originally as an adaptation to feeding on tobacco rather than from field exposure to neonicotinoids *per se*, and subsequently spread to countries such as the UK where tobacco does not occur as a cultivated or wild host. Alternatively, resistance could be selected by nicotine used as a fumigant for aphid control.

The practical implications of neonicotinoid resistance in *M. persicae* have been investigated in the laboratory and the field. Under field conditions, imidacloprid and clothianidin were applied as seed treatments to sugar beet at recommended application rates, or ones corresponding to one-half and one-sixth the recommended rate (Haylock, *et al.*, 2002). The insecticides gave comparable levels of aphid control, and even at reduced application rates did not differentiate between aphids fully susceptible to neonicotinoids and ones with up to 6-fold resistance in bioassays. In laboratory population cages, however, aphids with higher levels of resistance (up to 15-fold) showed increased survival and reproduction on cabbage and tobacco treated with lower than recommended rates of imidacloprid (Foster, *et al.*, in press; D. Cox, unpublished data). Concentrations in plants lower than those recommended for aphid control can arise from poor application techniques, treatments against pests other than aphids, or as a result of insecticide metabolism or plant growth. The ability of some aphids to survive better than others under such conditions demonstrates the potential for further selection, leading to a more marked impact on the extent and/or duration of control efficacy.

Other reports of imidacloprid resistance in *M. persicae* (Choi, *et al.*, 2001) and the cotton-melon aphid (*Aphis gossypii*) (Wang, *et al.*, 2001), result from laboratory selection pressure and their relevance to events in the field remains unclear. Two other important aphid targets of neonicotinoids in Europe are the currant-lettuce aphid (*Nasonovia ribisnigri*) and the damson-hop aphid (*Phorodon humuli*). A survey of *N. ribisnigri* strains collected in the UK and Spain between 1999 and 2001 showed no evidence of reduced susceptibility to imidacloprid (M Barber, unpublished data). Extensive monitoring of *P. humuli* on hops in Germany disclosed slight variations in susceptibility to imidacloprid during the 2001 season (Weichel, *et al.*, 2002). The extent to which these represent minor seasonal fluctuations in susceptibility or a directional increase in resistance is currently being investigated.

Colorado beetles and other pests

The Colorado potato beetle, (*Leptinotarsa decemlineata*) has a history of developing resistance to virtually all insecticides used for its control. Imidacloprid was introduced for controlling this species in North America in 1995. Concerns over resistance development were reinforced when extensive monitoring of populations from North America and Europe disclosed c. 30-fold variation in LC_{50} values from ingestion and contact bioassays against neonates (Olsen, *et al.*, 2000). Much of this variation appeared unconnected with imidacloprid use *per se*, and probably a consequence of cross-resistance from chemicals used earlier. Lowest levels of susceptibility occurred in populations from Long Island, New York, an area that has experienced the most severe resistance problems of all with *L. decemlineata*. Zhao, *et al.* (2000) and Hollingworth, *et al.* (2002) independently studied single strains collected from different areas in Long Island, both treated intensively with imidacloprid between 1995 and 1997. In the first study, grower's observations of reduced control were supported by resistance ratios for imidacloprid of 100-fold and 13-fold in adults and larvae, respectively. The second study reported 150-fold resistance from topical application bioassays against

adults. In this case the strain was also tested with thiamethoxam, which had not been used for beetle control at the time of collection. Interestingly, resistance to thiamethoxam (c. 3-fold) was far lower than to imidacloprid.

Planthoppers (Hemiptera: Delphacidae) are important targets of neonicotinoids on rice in Japan. Nine strains of the small brown planthopper (*Laodelphax striatellus*) collected from localities throughout Japan during the early 1990s were fully susceptible to imidacloprid. However, one strain maintained in the laboratory under strong selection by organophosphates and carbamates apparently acquired 18-fold resistance to imidacloprid without exposure to this insecticide (Sone, *et al.*, 1995). The lygus bug (*Lygus hesperus*), a major cotton pest in Arizona and a target for neonicotinoid use, showed remarkable (up to 100-fold) between-strain variation in response to imidacloprid (Dennehy & Russell, 1996). The most tolerant strain was again simultaneously resistant to a range of organophosphate insecticides. Strains of the western flower thrips (*Frankliniella occidentalis*) and tobacco budworm (*Heliothis virescens*) have also shown tolerance to imidacloprid as a consequence of exposure to compounds unrelated to neonicotinoids (Zhao, *et al.*, 1995; Elzen, 1997). Neither of the latter species are currently major targets of neonicotinoid use.

MECHANISMS OF RESISTANCE AND CROSS-RESISTANCE IMPLICATIONS

Resistance most commonly arises through either an increased ability to detoxify insecticides, or a modification of their target sites conferring insensitivity to the toxin. To date, the clearest evidence for detoxification as a primary mechanism of neonicotinoid resistance involves Spanish populations of the whitefly *B. tabaci* (Nauen, *et al.*, 2002; Stumpf & Nauen, 2002). Pre-exposure of whiteflies to synergists known to inhibit detoxifying enzymes increased the toxicity of imidacloprid against a resistant strain, suggesting an involvement of cytochrome P-450 dependent monooxygenases in conferring resistance. This was supported by a close correlation between resistance levels in several strains and monooxygenase activity measured using a model enzyme substrate. Ligand competition experiments using [^{3}H] imidacloprid showed no significant difference in insecticide binding to nicotinic acetylcholine receptors, and hence no evidence of target site insensitivity in resistant *B. tabaci* strains. Similar experiments also disclosed no difference in binding between strains of the aphid *M. persicae* differing in susceptibility to imidacloprid (Nauen, *et al.*, 1996).

Attempts to identify mechanisms of neonicotinoid resistance in strains of Colorado beetle from Long Island have yielded contrasting results. In one strain, synergism studies implicated detoxification by monooxygenases as the primary mechanism in adults and as a contributing factor in larvae (Zhao, *et al.*, 2000). However, pharmacokinetic experiments with other strains of *L. decemlineata* showed no significant difference in *in vivo* metabolism of radio-labelled imidacloprid (Hollingworth, *et al.*, 2002). Binding studies to investigate a possible target site-based mechanism in this strain are underway (R M Hollingworth, personal communication).

Overall, data on cross resistance threats posed to neonicotinoids are inconsistent and difficult to interpret. Work investigating baseline responses to neonicotinoids of strains already resistant to older, unrelated molecules (reviewed by Cahill & Denholm, 1999) has disclosed little or no impact of pre-existing resistance mechanisms. Some anomalous findings relating to lygus bugs (Dennehy & Russell, 1996), Colorado beetles (Olsen, *et al.*, 2000), planthoppers (Sone, *et al.*, 1995) and thrips (Zhao, *et al.*, 1995) probably do demonstrate the potential for

broad-spectrum detoxification systems encompassing neonicotinoids as well as carbamates, organophosphates and/or pyrethroids. However, it could be argued that if such cross resistance represents a significant threat to neonicotinoids, resistance to the latter would have become manifest far more rapidly than it has done in practice.

Patterns of cross-resistance within the neonicotinoid class are crucially important. They determine, for example, whether members of this class might be alternated without continuous selection for the same resistance mechanism. In both *B. tabaci* and *M. persicae*, resistance detected initially to imidacloprid has been found to affect all other neonicotinoids tested to a similar extent (Cahill, *et al.*, 1996; Foster, *et al.*, in press; Nauen, *et al.*, 2002). In contrast, a strain of *L. decemlineata* showing 150-fold resistance to imidacloprid, and postulated to contain a mechanism of target site insensitivity, exhibited only c. 3-fold resistance to thiamethoxam (Hollingworth, *et al.* 2002). This is consistent with reports of differences in the receptor-binding properties of imidacloprid and thiamethoxam (Wiesner & Kayser, 2000; Kayser *et al.*, 2002). It is conceivable that future work will disclose mechanisms showing selectivity among neonicotinoids that is sufficiently consistent to be exploited in practice. At present, however, the approach advocated by the Insecticide Resistance Action Committee (http://PlantProtection.org/IRAC) of regarding neonicotinoids as a single cross-resisted group is unquestionably the correct one to adopt from a resistance management standpoint.

RESISTANCE MANAGEMENT PERSPECTIVES

Broad principles for combating insecticide resistance apply to all chemical groups, irrespective of their structures or modes of action. Tactics based on limiting exposure to key compounds in space and/or time, or on alternating between non cross-resisted molecules have been reviewed in general terms (e.g. Roush, 1989; Denholm & Rowland, 1992) and with particular reference to neonicotinoids (Elbert, *et al.*, 1996; Cahill & Denholm, 1999). The challenge of optimising and implementing such tactics for specific pests depends on a suite of ecological, genetic, operational and socio-economic factors that are outside the scope of this paper but are also reviewed elsewhere (Denholm, *et al.*, 1998; Cahill & Denholm, 1999).

An issue of more specific relevance to neonicotinoids relates to their outstanding versatility as control agents. Members of this class can be used both as long-lasting systemic treatments or as shorter-lasting foliar sprays, generating speculation as to which mode of application is more likely to promote resistance. This versatility can be viewed as an advantage or as a drawback, according to one's perspective. It is advantageous in that neonicotinoid treatments can be matched more precisely than is usually possible to the needs of different cropping systems (Denholm, *et al.*, 1998). Thus, in cases where Hemipteran or Coleopteran pests are a persistent and predictable early season problem, use of the most systemic compounds as a seed treatment or soil application is fully justified and may relieve pressure on chemicals used later in the season. For pests that are more erratic, or only damaging for short time intervals, prophylactic systemic treatments are best avoided in favour of foliar sprays applied when insect numbers exceed defined treatment thresholds. The drawback with this versatility is that it can be perceived by growers as a 'cure-all' offering continuous control through a succession of systemic and foliar applications. Several authors (Elbert, *et al.*, 1996; Cahill & Denholm, 1999; Olsen, *et al.*, 2000) have rightly placed great emphasis on avoiding this scenario.

Opportunities for containing resistance by limiting neonicotinoids to particular crops within a regional agro-ecosystem are exemplified well by control strategies implemented in 1996 to control *B. tabaci* in the cotton-melon system in Arizona (Dennehy & Denholm, 1998). Until recently, imidacloprid use was confined largely to systemic treatment of spring and autumn vegetables, with cotton providing a 'neonicotinoid-free' summer crop dependent on other insecticide classes for whitefly control. Increasing commercial pressures to permit foliar sprays of neonicotinoids against *B. tabaci* on cotton pose a tangible threat to the sustainability of pest management in this region. Similar concerns apply to controlling *M. persicae* in the UK and elsewhere in Europe. The approval of neonicotinoids for an increasing number of crops attacked by *M. persicae* must inevitably be imposing greater pressure for resistance development (Foster, *et al.*, in press). The use on some *M. persicae* hosts of application rates lower then those normally recommended for aphid control (e.g. to control cabbage-stem flea beetle, *Psylloides chrysocephala*, on oilseed rape) demands close vigilence as this may enable genes conferring low-level tolerance to accumulate and enhance the resistance phenotype.

Over the last two decades, the agrochemical industry has contributed significantly to combating resistance through inter-company collaborations aimed at limiting exposure to insecticide groups as a whole (e.g. Leonard & Perrin, 1994). The challenge of extending this approach to neonicotinoids is a formidable one given the commercial pressures to establish newcomer molecules in a market currently dominated by imidacloprid (Denholm, *et al.*, 1998). However, it is one that should be confronted in order to protect this outstandingly effective class of insecticides from the fate encountered by many of its predecessors.

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