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Toxicity and nicotinic acetylcholine receptor interaction of imidacloprid and its metabolites in *Apis mellifera* (Hymenoptera: Apidae)

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Abstract: Acute oral and contact toxicity tests of imidacloprid, an insecticide acting agonistically on nicotinic acetylcholine receptors (nAChR), to adult honeybees, *Apis mellifera* L var *carnica*, were carried out by seven different European research facilities. Results indicated that the 48-h oral LD_{50} of imidacloprid is between 41 and >81 ng per bee, and the contact LD_{50} between 49 and 102 ng per bee. The ingested amount of imidacloprid-containing sucrose solution decreased with increasing imidacloprid concentrations and may be attributed to dose-related sub-lethal intoxication symptoms or to antifeedant responses. Some previously reported imidacloprid metabolites occuring at low levels *in planta* after seed dressing, ie olefine-, 5-OH- and 4,5-OH-imidacloprid, showed lower oral LD_{50} values (>36, >49 and 159 ng per bee, respectively) compared with the concurrently tested parent molecule (41 ng per bee). The urea metabolite and 6-chloronicotinic acid (6-CNA) exhibited LD_{50} values of >99 500 and >121 500 ng per bee, respectively.

The pharmacological profile of the [^{3}H]imidacloprid binding site in honeybee head membrane preparations is consistent with that anticipated for a nAChR. IC_{50} values for the displacement of [^{3}H]imidacloprid by several metabolites such as olefine, 5-OH-, 4,5-OH-imidacloprid, urea and 6-CNA were 0.45, 24, 6600, >100 000, and >100 000 nM, respectively. Displacement of [^{3}H]imidacloprid by imidacloprid revealed an IC_{50} value of 2.9 nM, thus correlating well with the observed acute oral toxicity of the compounds in honeybees.

Neurons isolated from the antennal lobe of *A mellifera* and subjected to whole-cell voltage clamp electrophysiology responded to the application of 100 μM acetylcholine with a fast inward current of between 30 and 1600 pA at −70 mV clamp potential. Imidacloprid and two of the metabolites (olefine- and 5-OH-imidacloprid) acted agonistically on these neurons, whereas the others did not induce currents at test conencentrations up to 3 mM. The electrophysiological data revealed Hill coefficients of approximately 1, indicating a single binding site responsible for an activation of the receptor and no direct cooperativity or allosteric interaction with a second binding site.

Keywords: *Apis mellifera*; acetylcholine receptor; nAChR; imidacloprid; neurons; neonicotinoids; toxicity

1 INTRODUCTION

The nicotinic acetylcholine receptor (nAChR) is the molecular target of the neonicotinoid insecticide imidacloprid.[1–3] Neonicotinoid insecticides such as imidacloprid, thiacloprid and acetamiprid are selective to insects in terms of toxicity, and this is attributed to their high agonistic affinity to insect nAChR preparations compared with those from vertebrates.[4,5] In order to characterize the binding site of neonicotinoid insecticides, [^{3}H]imidacloprid was synthesized and introduced as a suitable radioligand several years ago.[5,6] Since then imidacloprid binding sites have been studied in many different pest insects, eg aphids and whiteflies.[7–10] Saturable [^{3}H]imidacloprid binding was also analyzed in *Nephotettix cincticeps* (Uhl.) (Homoptera: Cicadellidae), *Periplaneta americana* (L) (Dictyoptera: Blattidae), *Lucilia sericata* (Meig) (Diptera: Calliphoridae), *Drosophila melanogaster* Meig (Diptera: Drosophiloidea), *Manduca sexta* (Joh) (Lepidoptera: Sphingidae), *Heliothis virescens* F (Lepidoptera: Noctuidae) and *Ctenocephalides felis* (Bche) (Siphonaptera: Pulicidae), and interestingly very high affinity binding sites in addition to a high-affinity binding site (K_d values of approximately 1 nM) were only found in homopteran species, such as *Myzus persicae* (Sulz) and *N cincticeps*, whereas, in all other insects studied, only one binding site was described.[5,9,11] However, a single binding site was also described in *Apis mellifera L* and *M sexta* when saturable binding of the antagonist [^{125}I]-α-bungarotoxin to nAChR was analyzed.[12–14] This biochemical knowledge (very high affinity binding sites in homo-

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pterans versus high-affinity sites in other insect orders) coincides quite well with the observation that aphids and leafhoppers are the most susceptible insects among targeted pests.

Registered use patterns of imidacloprid include foliar spray, trunk (injection) application, soil (drench) application and seed treatment.[15,16] When applied to the soil or as a seed treatment, imidacloprid is metabolized more or less completely depending on plant species and time.[17–19] It has been shown that some of the metabolites appearing *in planta* during the degradation process are also insecticidal against aphids and whiteflies but, due to their fate and very low concentration, they do not provide sufficient insect control without certain levels of the parent compound itself.[20,21] However, nAChR binding of some of the *in-planta*-occuring metabolites in housefly head membrane preparations correlated quite well with their observed toxicity to aphids in oral ingestion bioassays.[20]

The objective of the present study was to elucidate the toxicological profile of imidacloprid and its major metabolites on honeybees, *A mellifera*, an important pollinator and non-target insect. For this reason, this species was subjected to a classical toxicity assay using an internationally adopted test guideline (EPPO No 170). For hundreds of years *A mellifera* has been bred specifically for passive/peaceful behaviour, high nectar yields and pollen foraging activity. From this perspective it is to be expected that there is much lower genetic variability within this species than for many other insect species and, thus, a more homogeneous response to a toxic compound. For this reason, the toxicity of imidacloprid to honeybees from different apiaries was tested using the same testing method. Furthermore we analyzed [^{3}H]imidacloprid binding sites in bee head membrane preparations and determined the affinity of numerous ligands to this binding site by ligand competition assays. This was supplemented by whole-cell voltage clamp electrophysiological measurements of the agonistic potential of imidacloprid and selected metabolites in isolated bee neurons from antennal lobes. Finally, the risk potential of imidacloprid and its metabolites to honeybees under field-relevant exposure conditions is discussed.

2 MATERIALS AND METHODS

2.1 Chemicals

Technical imidacloprid and imidacloprid metabolites (Fig 1), acetamiprid, thiacloprid and nitenpyram were obtained in-house (Bayer AG, Leverkusen, Germany) with a purity of at least 98%. Lobeline, cytisine, α-bungarotoxin and +/−nicotine were purchased from Sigma Chemicals. All other chemicals and organic solvents used were of analytical grade. [^{3}H]imidacloprid (1.406 GBq μmol^{-1}) for receptor binding studies was synthesized and labelled as described elsewhere.[6]

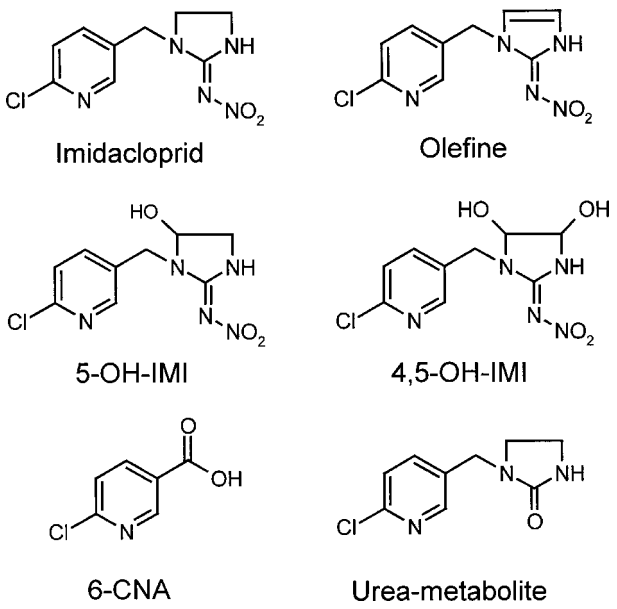

Figure 1. Chemical structures of imidacloprid and some common metabolites.

2.1.1 *Test solutions for ligand displacement studies and electrophysiology*

Stock solutions of compounds (putative ligands) used in [^{3}H]imidacloprid competition assays were prepared at 50 mM in dimethyl sulfoxide (DMSO) and immediately diluted in 100 mM potassium phosphate buffer, pH 7.4 containing 5 g litre^{-1} bovine serum albumin, yielding a concentration of 1 mM. Subsequent dilutions were prepared in potassium phosphate buffer as described above. For electrophysiological recordings, stock solutions of test compounds were prepared at 100 mM in DMSO and subsequently diluted using a Ringer solution containing sodium chloride (150 mM), potassium chloride (4 mM), magnesium chloride (2 mM), calcium chloride (2 mM), trehalose (150 mM) and Hepes (10 mM), adjusted to pH 7.4 with sodium hydroxide.

2.1.2 *Test solution for toxicity assays*

Depending on the research facility, test compounds were stored either at room temperature (maximum 30 °C) or, whenever possible, at 4 °C in the dark. Solutions of the test doses were stirred immediately prior to use and were visually homogenous when administered. Stock solutions of the test compounds were prepared and diluted as appropriate.

For topical application imidacloprid was dissolved in acetone. For oral dosing, imidacloprid was first dissolved in acetone (except the lower doses at the test facility Germany III) and then diluted in 50% aqueous sucrose (1 ml = *c.* 1.2 g) or ready-to-use syrup (Apiinvert: 30% saccharose, 31% glucose, 39% fructose). Imidacloprid dissolved in acetone at stock solution concentration (1.4 g litre^{-1}) gave a clear, colourless solution. The highest acetone concentration in the test solutions was 0.05 ml litre^{-1}. Test solutions were prepared a maximum of 2 h prior to use.

2.2 Honeybees

All tests described were carried out using adult worker honeybees, *Apis mellifera* L (Hymenoptera: Apidae) taken from a single queen-right colony owned and maintained by the respective test facility (see Table 1). These colonies were disease-free and had received no chemical treatments (eg varroacide) for at least 4 weeks prior to testing. For acute oral and contact toxicity tests, adult worker bees (mostly between 14 and 42 days old according to Diemer)[22] were collected from the hive combs (avoiding the brood nest area) or from the flight board, and any unintentionally collected drones were discarded. Before treatment, honeybees were acclimatized to the test conditions for at least 2 h.

For biochemical studies, worker honeybees were carefully collected from hive combs (collection site: Burscheid, Germany) and directly frozen using dry ice. Bee heads were then separated from other body parts by vigorous shaking and recovered by sieving. The heads were then stored frozen at −40 °C (usually not longer than 6 weeks) until use.

2.3. Honeybee toxicity bioassays

The testing procedure followed the European Plant Protection Organisation (EPPO) guidelines (1992)[23] and methods were in accordance with the draft EPA Ecological Effects Test Guidelines (OPPTS 850.3020 Honey Bee Acute Contact Toxicity) and OECD guideline 213 and 214 Honeybees, Acute Oral and Contact Toxicity (September 1998).[24,25] All doses and toxicity data for the test compounds refer to the respective active ingredient but were not adjusted for the analytical purity of the testing materials (>98%). During the test period the bees were kept in the dark (except during assessments) in an incubator at 24–29 °C and 46–84% relative humidity. Test cages were between 36 and 85 cm^2 in size and were ventilated to minimize potential accumulation of test substance vapours. Sub-lethal effects were assessed according to pre-determined categories: knock-down (ie alive, but immobile), staggering (ie moving, but in a poorly co-ordinated manner).

2.3.1 Oral bioassay

Bee allocation to the treatment groups was made impartially. Before being used for the test, bees were subjected to a starvation period of between 1 h 10 min and 2 h 15 min under test conditions. Three batches of bees (10 bees per batch) were then subjected to different doses of the test compound (nominal doses between 0.1 and 81 ng AI per bee) offered in 0.2–0.25 ml aqueous sucrose (500 g $litre^{-1}$) in a glass feeder (controls received sucrose solution only) for 3–4 h. The glass test feeders, containing any unconsumed portions of the doses, were then removed. The dose consumed was determined by comparing the remaining weight of the glass feeders with their start weight. Afterwards fresh aqueous sucrose was then supplied in feeders within the cages. Mortality and sub-lethal effects were first assessed at 4 h after dosing. Further assessments were made at 24 and 48 h.

2.3.2 Contact bioassay

The effects of contact exposure were assessed by topical application of the test substance in acetone to the ventral side of the thoracic body surface. Immediately prior to treatment bees were anaesthetized with carbon dioxide for a maximum of 1 min. Then, each bee was dosed with 1–5 µl (volume differed between test facilities) of test substance or an equivalent volume of acetone (controls) using a calibrated micropipette. A dose range of 40 to 154 ng AI per bee and a control were used, with three to five replicates of 10 bees per dose rate. After dosing, the bees were allowed to recover and kept in the incubator with a continuous supply of aqueous sucrose solution (500 g $litre^{-1}$) as a food source. Mortality and sub-lethal effects were assessed at intervals of 4, 24, and 48 h post-dose.

2.4 [^{3}H]Imidacloprid displacement studies

Bee heads weighing 10 g were homogenized in 200 ml ice-cold 0.1 M potassium phosphate buffer, pH 7.4 containing 95 mM sucrose using a motor-driven Ultra Turrax blender. The homogenate was then centrifuged for 10 min at 1200 *g* and the resulting supernatant was filtered through five layers of cheesecloth and then used without prior purification. Protein

Table 1. Origin of honeybee strains used in the acute toxicity assays

Bee strain, apiary location, and sampling procedure	*Compounds tested*
Apis mellifera L var *carnica* POLLM (The Netherlands I)	Imidacloprid
Apis mellifera L var *carnica* POLLM (Germany I), sampled from the flight board at day 0	Imidacloprid Olefine-imidacloprid 5-OH-Imidacloprid 4,5-OH-Imidacloprid Urea-metabolite 6-CNA
Apis mellifera L var *carnica* POLLM (Germany II), brushed from the combs on day 0	Imidacloprid
Apis mellifera L var *carnica* POLLM (Germany III), brushed from the combs on day 0	Imidacloprid
Apis mellifera L var *carnica* POLLM (Germany IV), brushed from the combs on day 1	Imidacloprid
Apis mellifera L var *carnica* POLLM (Germany V), sampled from a bypass in the hive roof on day 0	Imidacloprid
Apis mellifera L (strain not specified) (United Kingdom I), shaken from the combs on day 0	Imidacloprid

concentration was determined according to Bradford.[26] Unless otherwise stated the final assay volume of 250 µl in a 96-well microtitre plate with bonded GF/C filter membrane (Packard UniFilter®-96, GF/C®) consisted of 200 µl of homogenate (0.48 mg protein), 25 µl of [^{3}H]imidacloprid (576 pM) and 25 µl of competing ligand. Ligand concentrations used ranged from 0.001 to 10 000 nM and were tested at least in duplicate per competition assay. The assay was started by the addition of homogenate and incubated for 60 min at room temperature. Bound [^{3}H]imidacloprid was quantified by filtration into a second 96-well filter plate (conditioned with ice-cold 100 mM potassium phosphate buffer, pH 7.4 (including BSA 5 g litre^{-1})) using a commercial cell harvester (Brandel). After three washing steps (1 ml each) with buffer the 96-well filter plates were dried overnight. Each well was then loaded with 25 µl of scintillation cocktail (Microszint-O-Filtercount, Packard) and the plate counted in a Topcount scintillation counter (Packard). Non-specific binding was determined using a final concentration of 10 µM unlabelled imidacloprid.

2.5 Electrophysiological measurements

Whole-cell voltage-clamp electrophysiology was performed using isolated neuronal cell bodies from freshly collected worker bees. Antennal lobes of individual worker bees were dissected and placed into dissociation solution (Sigma C-1419). The ganglia were treated with 0.002 g litre^{-1} dispase, incubated for 5 min at 37 °C, centrifuged, and resuspended in culture buffer by gentle aspiration with a fire-polished pasteur pipette with slight modifications, as described elsewhere.[27] Cell somata were plated onto glass cover slips previously coated with concanavalin-A (400 µg ml^{-1}) and laminin (4 µg ml^{-1}). The cells were kept at room temperature and used for experiments on the following 2 days.

Electrophysiological recordings were made with the whole-cell voltage clamp technique.[28] The microelectrodes were pulled from borosilicate glass capillaries. The resistance of the fire-polished pipettes was 4–7 MΩ using the internal and external solutions described below. All experiments were carried out at room temperature (22–25 °C).

The cells were placed in a perfusion chamber of approximately 0.5 ml volume and superfused continuously (flow rate 3 ml min^{-1}) with external bath solution driven by gravity. The fluid in the chamber was therefore renewed every 10 s and completely washed out in less than 60 s. The external bath contained the Ringer solution detailed in Section 2.1.1. The (internal) pipette solution contained caesium fluoride (120 mM), caesium chloride (30 mM) caesium - EGTA (10 mM), calcium chloride (1 mM), trehalose (150 mM), Hepes (10 mM), adjusted to pH 7.5 with caesium hydroxide. Compounds were applied to the cells using the U-tube reversed flow technique.[29]

Currents were measured with an L/M-EPC 7 patch-clamp amplifier (List, Darmstadt, Germany). The holding potential was −70 mV. Current records were low-pass Bessel filtered at 315 Hz and digitized at 1 kHz sample rate.

2.6 Data calculation

In order to calculate LD_{50} values, mortality rates of treatment groups were corrected for control mortality according to Abbott.[30] LD_{50} values were determined by probit regression analysis,[31] non-linear regression or by the moving average method.[32–34] Confidence intervals were calculated using standard procedures described elsewhere.[35]

IC_{50} values (concentration of unlabelled ligand displacing 50% of [^{3}H]imidacloprid from its binding site) were calculated using a four-parameter logistic non-linear curve fitting routine (GRAPHPAD-PRISM, available at www.graphpad.com). Data storage and analysis of electrophysiological measurements, ie calculation of EC_{50} values and Hill coefficients (n_H), were done using the pClampV 6.03 software package (Axon Instruments, Foster City, CA) and Origin 5.0 Software (Microcal Software Inc, Northampton, MA, USA), respectively.

3 RESULTS

3.1 Toxicology

3.1.1 Toxicity of imidacloprid to honeybees from different apiaries

Seven laboratories from the United Kingdom, The

Table 2. Acute oral and contact toxicity (48 h) of imidacloprid to honeybees from different apiaries

Oral LD_{50}[a] *(ng per bee)*	*Contact LD_{50} (ng per bee)*	*Test period*	*Origin of tested honeybees*
c 41 (50%)[b]	Not determined	July 1999	Germany I (control mortality: 10%)
>20 (0%)	104[c] (83.0–130)[d]	July 1999	The Netherlands I (control mortality: 0%)
>81 (20%)	61.0 (26.0–90.0)	May 2000	Germany II (control mortality: 0%)
>81 (13%)	50.0 (9.1–71.0)	May 2000	United Kingdom I (control mortality: 3%)
>81 (17%)	42.0 (20.0–59.0)	May 2000	Germany III (control mortality: 0%)
>81 (17%)	42.9 (34.6–53.2)	May 2000	Germany IV (control mortality: 0%)
>81 (47%)	74.9 (61.8–90.9)	July 2000	Germany V (control mortality: 3%)

[a] Oral toxicity values refer to nominally applied doses.
[b] Mortality at the highest applied dose.
[c] LD_{50} at 72 h.
[d] 95% Confidence limits.

Table 3. Acute oral toxicity of imidacloprid and its metabolites in honeybees as determined in testing facility Germany I

Compound	Oral LD_{50} (ng AI per bee)	Oral NOED[a] (ng AI per bee)	Testing period
Imidacloprid	*c* 41	1.5	July 6–10, 1999
Olefine	>36	2.4	Sept 8–12, 1999
5-OH-Imidacloprid	159	1.2	Sept 8–12, 1999
Di-OH-imidacloprid	>49	49	July 6–8, 1999
Urea-metabolite	>99500	1200	July 7–9, 1999
6-CNA	>121500	121500	July 7–9, 1999

[a] No observed effect dose.

Netherlands and Germany examined the acute oral and contact toxicity of technical grade imidacloprid to honeybees between May, 1999 and July, 2000 (Table 1). There was no indication that either season or origin of honeybees (home apiary) had an influence on the measured LD_{50} values. The oral LD_{50} was between *c* 41 and > 81 ng AI per bee, with a no-observed effect dose (NOED) of ≤1.5 ng per bee (Tables 2 and 3). Mortalities (17–50%) were only observed at the higher doses (≥ 3.1 ng AI per bee). In all oral toxicity studies there was a poor fit in the dose-response relationship.

During these tests it was noted that honeybees rejected sucrose solutions containing imidacloprid at concentrations of 1 mg kg^{-1} or higher in a dose-dependent pattern. At a dose level of 81 ng per bee the sucrose ingestion was reduced by 13% to 57% with an average value of 33 (±18)%. This lowered intake may be due to a dose-related antifeedant effect of imidacloprid or to the knock-down effects as observed in some laboratories at 4, 24 and 48 h in the top dose level(s), where the bees (20–90%) were immobile and therefore unable to feed. Two research facilities extended the post-treatment observation period by a further 48 h but observed no further sub-lethal effects, indicating that intoxication persisted for a maximum of 48 h.

In contact tests, sub-lethal effects were observed after 4 h at all doses, with either recovery or death after 48 h. At the top dose levels, some mortality was observed already 24 h after treatment. The contact LD_{50} was between 42 and 104 ng imidacloprid per bee, with a good fit of the resulting dose-response curves (Table 2).

3.1.2 Acute toxicity of imidacloprid metabolites

Some imidacloprid metabolites (Fig 1) were tested along with imidacloprid for their oral toxicity to honeybees at one test facility (Germany I). From the values listed in Table 3 it is evident that metabolites which contain the nitroguanidine pharmacophore (olefine-, hydroxy- and dihydroxy-imidacloprid) still exhibit some toxicity to honeybees, whereas those metabolites not carrying this pharmacophor [urea-metabolite, 6-chloronicotinic acid (6-CNA)] are non-toxic to bees.

3.2. [^{3}H]imidacloprid displacement studies

3.2.1. Imidacloprid and metabolites

Some of the common metabolites of imidacloprid (Fig 1) occuring *in planta* were tested for their ability to displace [^{3}H]imidacloprid from its binding site using honeybee head membrane preparations. Furthermore a selection of cholinergic ligands known to interact with nAChRs were tested to demonstrate the nicotinic character of the [^{3}H]imidacloprid binding site in bee head membranes. The whole study was completed by testing the displacement-efficacy of a few other neonicotinoid insecticides, such as thiacloprid, acetamiprid and nitenpyram.

Among the metabolites tested, 6-CNA and the urea metabolite were not active in displacing [^{3}H]imidacloprid from its receptor binding site, even at concentrations as high as 0.1 mM (Table 4). Thus, these metabolites were at least five orders of magnitude less effective than imidacloprid, which showed an IC_{50} value of 2.9 nM. The affinity of the other (active) metabolites decreased in the order olefine-> 4-OH-imidacloprid ≫ 4,5-OH-imidacloprid (Fig 2). Among the active metabolites tested, 4,5-OH-imidacloprid exhibited the lowest affinity (IC_{50} 6.6 μM) for the [^{3}H]imidacloprid binding site on putative bee head membrane nAChRs. As observed with other insect head membrane preparations, the olefine metabolite showed the highest affinity to honey bee [^{3}H]imidacloprid binding sites *in vitro*. The IC_{50} values for the active metabolites determined in this study correlated

Table 4. Pharmacological profile of [^{3}H]imidacloprid binding sites in honeybee head membrane preparations (*n*=4–8)

Ligand	IC_{50}, (nM)	95% CI
Imidacloprid	2.9	1.8–4.6
Imidacloprid-olefine	0.45	0.28–0.72
5-OH-imidacloprid	24	15–37
4,5-Di-OH-imidacloprid	6600	3500–13000
Urea-metabolite	>100000	—
6-CNA	>100000	—
Acetamiprid	2.1	1.1–3.7
Thiacloprid	0.75	0.47–1.3
Nitenpyram	2.3	1.2–4.5
α-Bungarotoxin	1700	240–12000
Cytisine	1400	420–4500
Lobeline	590	340–1000
Nicotine	5200	2000–13000

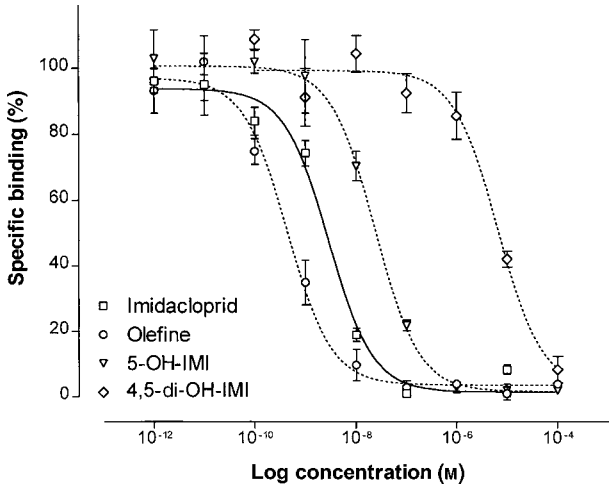

Figure 2. Displacement of [^{3}H]imidacloprid bound to honeybee head membrane preparations by imidacloprid (IMI) and its metabolites.

quite well with those published very recently for houseflies, thus allowing the conclusion that [^{3}H]imidacloprid binding sites in both membrane preparations are similar (Fig 3).

3.2.2 Other neonicotinoid insecticides

The open-chain neonicotinoids acetamiprid and nitenpyram showed essentially the same potency as imidacloprid as inhibitors of [^{3}H]imidacloprid binding in honeybee head membrane preparations (Table 4), whereas thiacloprid was slightly more potent than imidacloprid. The IC_{50} values determined for acetamiprid, nitenpyram and thiacloprid were 2.1, 2.3 and 0.75 nM, respectively.

3.2.3. Classical cholinergic ligands

A small number of known cholinergic ligands, such as lobeline, α-bungarotoxin, cytisine and nicotine were tested for their ability to inhibit [^{3}H]imidacloprid binding in honeybee head membranes (Table 4). The results indicated a pharmacological profile typical for nAChRs, and were similar to characterizations performed for other invertebrate receptors. IC_{50} values were 1.7, 1.4, 5.2 and 0.59 μM for alpha-bungarotoxin, cytisine, nicotine and lobeline, respectively.

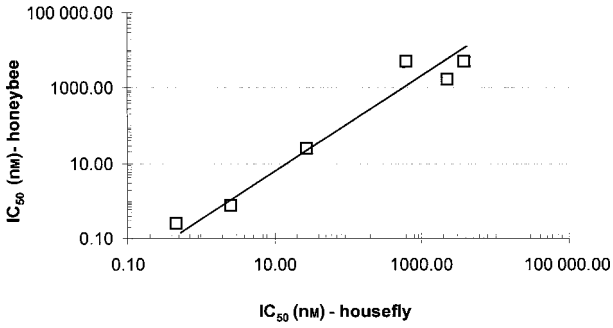

Figure 3. Correlation between ligand affinities to [^{3}H]imidacloprid binding sites in honeybee heads and houseflies.

3.3 Electrophysiology using neurons from antennal lobes

The preparation of the cell bodies from the antennal lobe as described above usually yielded, among some debris, a fair number of large, round cells. Cells were typically 30–50 μm in diameter and sometimes had a short protusion, suggesting the remnant of an axon that was cut off during the preparation. Most of these cells (32 of 38) responded to a test application of 100 μM acetylcholine (ACh) with a fast inward current between 30 and 1600 pA at −70 mV clamp potential. Due to the lack of run-down phenomena, it was possible to analyze real currents instead of relative amplitudes over a period of more than 1 h, thus allowing the measurement of full dose-response curves using a single cell without correction by frequent ACh applications. All of the neurons prepared responded to imidacloprid with a spontaneous inward current, displaying the agonistic nature of the compound. Depending on the neuron, imidacloprid either acts as a (nearly) full agonist or as a partial agonist (very small currents), suggesting different receptor populations (Fig 4). However, estimated EC_{50} values for those receptor populations in which imidacloprid acts as a partial agonist were absolutely comparable to those in which it acts as a full agonist, thus indicating no differences in binding affinity. Imidacloprid shows an EC_{50} value (which corresponds to half-maximal activation of nAChRs induced by an agonist) of 0.83 μM, whereas the olefine metabolite was more active by a factor of two (0.35 μM). All other metabolites, ie 6-CNA, urea-metabolite and 4,5-OH-imidacloprid, were not electrophysiologically active on honeybee neurons, except 5-OH-imidacloprid which exhibited a rather low affinity (7.5 μM) to nAChRs in such preparations (Fig 5). Hill coefficients were determined to be close to 1 for those compounds binding to nAChRs on honeybee neurons from antennal lobes, indicating a single binding site necessary for channel activation by imidacloprid, olefine- and 5-OH-imidacloprid.

4 DISCUSSION

The first step of the *in vivo* testing programme gave us some information as to whether honeybees from different origins may differ in their susceptibility to imidacloprid. Based on LD_{50} values determined at seven different laboratories, it appeared that the origin of honeybees was not a decisive factor for the determination of the toxicity of imidacloprid to honeybees. This conclusion is further supported by toxicity data reported from other continents. Using American honeybees and an FS 240 formulation of imidacloprid, a contact LD_{50} value of 43.9 ng AI per bee had been reported earlier.[36] Using Chinese honeybees and technical grade imidacloprid, Ruzhong *et al*[37] determined a contact LD_{50} value of 30 ng AI per bee. The same authors report an oral LD_{50} value of 151.2 ng AI per bee using a WP 20 formulation of

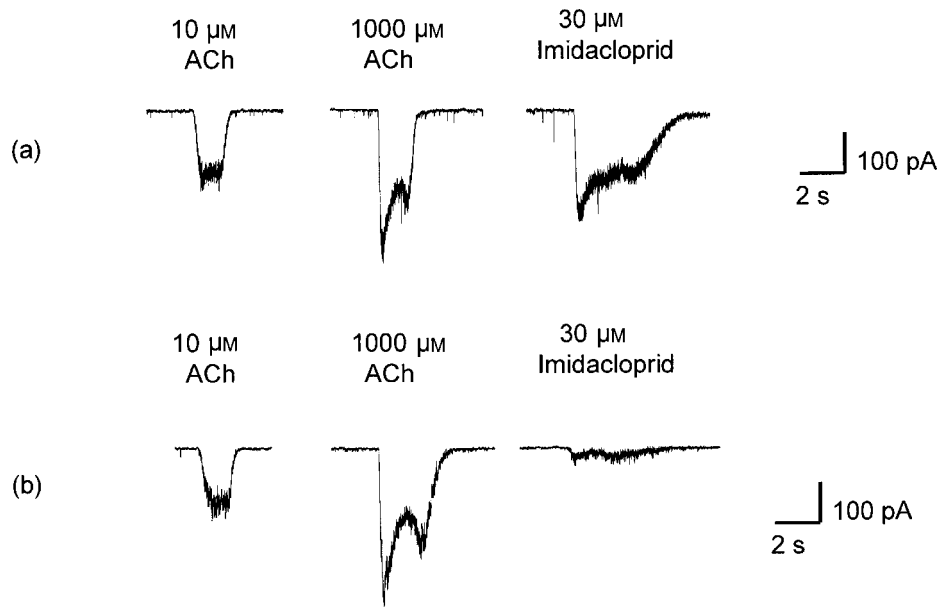

Figure 4. Whole cell responses from two subpopulations of neuron cells isolated from the antennal lobe of *Apis mellifera*: (a) imidacloprid is a full or nearly full agonist; (b) imidacloprid is a partial agonist (or open channel blocker?). ACh=acetylcholine.

imidacloprid. Contact LD_{50} values of 81.0 and 12–24 ng AI per bee published by Pflüger and Schmuck[38] and Suchail *et al*,[39] respectively, are not very different from those reported in the present paper. Even different strains of honeybees seem to provide a relatively homogeneous response, as reported by Suchail *et al*[39] for the sub-species *mellifera* and *caucasica*. In contrast to contact toxicity tests, significantly lower values for oral toxicities have been reported in some cases, eg LD_{50} values of 3.7 and *c* 5 ng AI per bee.[38,39] These larger differences in oral toxicity values between different research facilities may

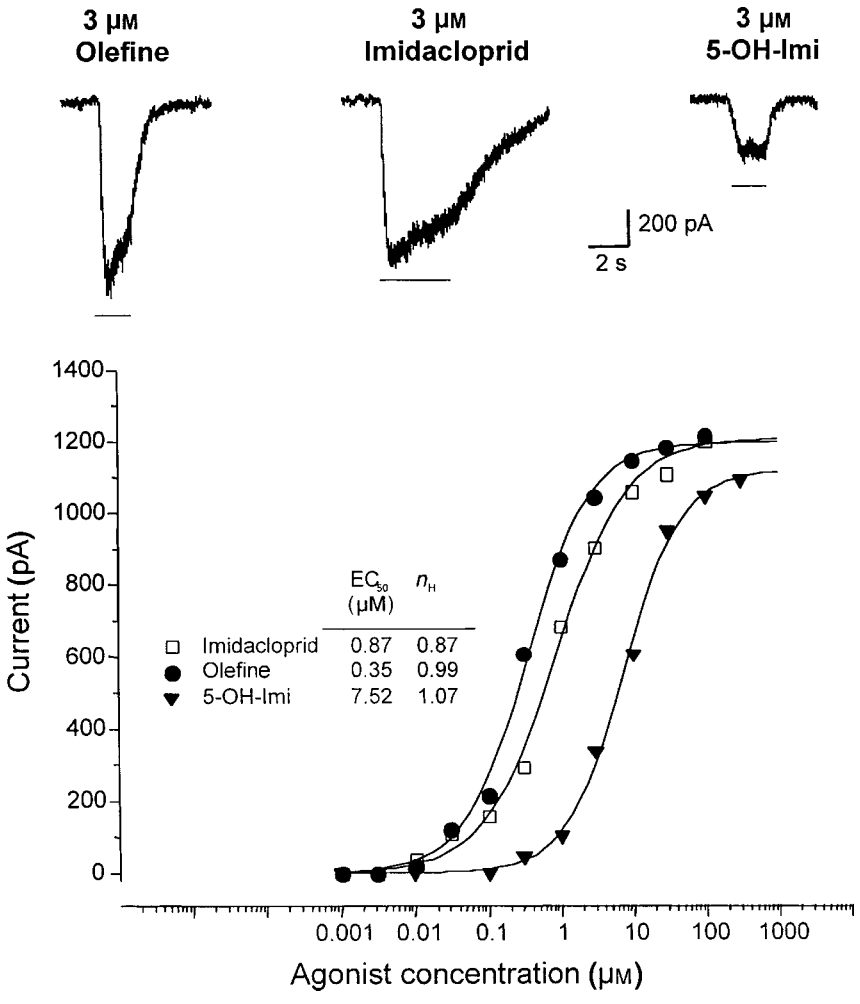

Figure 5. Whole cell current responses of a 'type (a)' neuron (see Fig 4) isolated from the antennal lobe of *Apis mellifera* after application of different neonicotiniods. The dose response curve was fitted by the Hill equation. EC_{50} values given here correspond to the half-maximal receptor activation induced by each agonist. The Hill coefficient (n_H) of all tested compound here was close to 1. The upper inset shows the corresponding responses for the neonicotinoids at 3 µM (holding potential −70 mV). All currents were obtained from the same cell.

be attributable mainly to the application technique. While the topical application of a definite volume of the test compound is relatively easy to standardize, the dose applied to an individual bee in the oral toxicity assay is difficult to determine precisely. Dosing is typically done by offering compound-containing sucrose solutions to groups of bees which share this food by trophallaxis. However, trophallaxis may be suppressed if a compound is toxic to bees, which may explain the finding of a delayed toxicity at higher imidacloprid doses ie 200 ng per bee.[39] Homogeneous solvation of test compounds might be more difficult to obtain in the large volume of aqueous solution than in a small volume of acetone needed for the contact test. This might result in the ingestion of unequal doses for the replicate cages and would explain the sometimes high variability of mortality figures between replicates. The nutritive status of the honeybees at the time of application may also be important, since toxicity could be correlated with the rate of digestion. Finally, there is an age-related difference in the acceptance of imidacloprid-containing sucrose solutions and the age of the tested bees was not well standardized in these routine toxicity tests (Table 1).[40] The poor fit of the dose-response curves frequently observed in oral toxicity tests underlines the assumption that many factors can bias the oral LD_{50} values.

Taking all these considerations into account, it appeared better to perform this comparative toxicity testing between imidacloprid and its metabolites at the same testing facility. This approach revealed that none of the tested metabolites had a significantly higher oral toxicity to honeybees than the parent compound itself, in contrast to homopterans, for example.[20,21] However, all metabolites which contained the nitroguanidine pharmacophor still exhibited a significant toxicity to honeybees, albeit low in some cases. Both findings are in agreement with those results published by other research groups.[41]

Ligand competition experiments carried out using honeybee head membrane preparations revealed that the IC_{50} values for neonicotinoids interfering with the [^{3}H]imidacloprid binding site of putative nAChRs were in the same range as those already published for *Musca domestica* L and *D melanogaster*.[3–5] The pharmacological profile of the [^{3}H]imidacloprid binding site in honeybee head membranes is clearly consistent with that anticipated for a nAChR, and this is the first time that such a profile (including other neonicotinoids) for this binding site has been published for putative honeybee nAChRs. Preliminary Scatchard analysis (data not shown) revealed a single high-affinity binding site for imidacloprid as decribed in many other non-homopteran insects.[4,5,14] This coincides with the findings of Tomizawa *et al*,[13] who reported a single high-affinity binding site for imidacloprid in honeybee head membrane preparations when using [^{125}I]α-bungarotoxin as a radioligand, which obviously also acts as a competitive inhibitor of the [^{3}H]imidacloprid binding site.[4,13] The occurrence of a single binding site for imidacloprid at nAChRs located on cell bodies isolated from the antennal lobe was also confiirmed in this study by electrophysiological whole-cell voltage clamp measurements, resulting in Hill coefficients of approximately 1. Some of the imidacloprid metabolites occuring *in planta* were also tested on nAChR in honeybee head membrane preparations and the binding affinity of those correlated quite well with the *in vivo* results obtained in oral ingestion bioassays. The olefine metabolite showed a slightly greater affinity to nAChRs in ligand competition and electrophysiological experiments on native neuron preparations. Our data are in the same range as those published for housefly receptors in previous studies.[20] It should be mentioned here that only one of the metabolites, 6-CNA, is sufficiently mobile in plant tissues to be distributed symplastically, whereas the others, due to their physicochemical properties, are in general apoplastically translocated.[21] However, 6-CNA and the urea-metabolite were not active in displacing [^{3}H]imidacloprid from its binding site in putative nAChRs in honeybee membrane preparations using concentrations up to 0.1 mM. This was additionally confirmed by electrophysiological measurements, where these metabolites and, additionally, 4,5-OH-imidacloprid evoked no channel opening on isolated neurons at concentrations up to 3mM. However, these data clearly indicate that it is unlikely that there is any biological activity from 6-CNA and the urea metabolite by interference with imidacloprid binding sites in bee nAChRs. The same conclusions have already been drawn when testing the above-mentioned imidacloprid metabolites against aphids.[20] The other neonicotinoid insecticides, ie nitenpyram and acetamiprid, tested for [^{3}H]imidacloprid competition in bee head membranes did not significantly differ from imidacloprid in their affinity to nAChR. The high binding-affinity of neonicotinoids to nAChRs is not necessarily correlated with high toxicity to bees, as very recently shown by the new neonicotinoid insecticide thiacloprid, bearing a cyanoamidine toxophor such as acetamiprid.[1,42] The compound's foliar application efficacy profile is comparable to that of imidacloprid, and it is additionally suited to the control of codling moths, for example, but honeybees are several orders of magnitude less susceptible to thiacloprid than to some other neonicotinoids.[42]

IC_{50} values obtained in receptor binding studies correlated well with EC_{50} values calculated from whole-cell voltage clamp studies using neurons isolated from antennal lobes. Whereas the receptor affinity for imidacloprid and other neonicotinoids in head membrane preparations is in general in the lower nanomolar range, that of electrophysiological recordings using isolated neurons is in the sub-micromolar range. This was also described very recently when binding data from housefly head membranes and electrophysiological data from isolated neurons from *H virescens* were analyzed.[1] Similar differences in biochemical binding and functional assay studies were

also observed for different vertebrate nAChRs.[43] It has been hypothesized that this is due to different conformational states of the nAChR which have different affinities for various ligands, eg a desensitized receptor shows a higher affinity than a receptor being in its open state.[1] Furthermore our electrophysiological studies revealed that at least two populations of nAChRs exist, differing in their agonistic response to imidacloprid, but not to acetylcholine.

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