

Effects of imidacloprid and common plant metabolites of imidacloprid in the honeybee: toxicological and biochemical considerations

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Abstract

Honeybees foraging in crop plants seed-dressed with imidacloprid may be exposed to imidacloprid and imidacloprid plant metabolites. Metabolism studies on a large variety of crop plants were reviewed to identify plant metabolites which have a potential toxicological relevance to honeybees. Three different bioassays were conducted to characterize the pharmacological and toxicological profile of imidacloprid and these potentially relevant plant metabolites in the honeybee.

The nicotinic acetylcholine receptor (nAChR) was identified as the molecular target of [³H]imidacloprid and some of the tested plant metabolites in honeybee head membrane preparations. IC₅₀-values for the displacement of [³H]imidacloprid of 2.9, 0.45, 24, 6600, >100000, and >100000 nM were recorded for imidacloprid and the plant metabolites olefine, 5-OH-imidacloprid, 4,5-OH-imidacloprid, urea metabolite and 6-chloronicotinic acid (6-CNA), respectively. These values indicate a potential toxicological relevance only for the olefine and the 5-hydroxy metabolite.

Whole cell voltage clamp electrophysiology revealed that imidacloprid, the olefine and the 5-hydroxy metabolite act agonistically on the nAChR of neurons isolated from the antennal lobe of *Apis mellifera*. All other metabolites did not induce inward currents at test concentrations up to 3 mM. As for the parent compound, the electrophysiology data of the active metabolites revealed Hill coefficients of approximately 1, thus indicating a single binding site responsible for an activation of the receptor and no direct cooperativity or allosteric interaction with a second binding site, respectively.

In standard toxicity assays following the EPPO guideline 170 LD₅₀ values between 40 and 104 ng/bee were determined for the oral and contact toxicity of imidacloprid to honeybees. No indications were found for significant differences in sensitivity to imidacloprid between honeybees from different apiaries. The acute oral toxicity of the potentially relevant plant metabolites of imidacloprid to honeybees were well correlated with their receptor binding affinity and receptor activation potential. Only the olefine and the 5-hydroxy metabolite were identified as toxicologically relevant.

The results from the current studies suggest that residue investigations aiming to define the field exposure of honeybees to imidacloprid applied as seed dressing should not only include a detection method sensitive for the parent compound but should also be sensitive to the presence of the olefine and 5-hydroxy-imidacloprid.

Key words: *Apis mellifera*, acetylcholine receptor, nAChR, imidacloprid, metabolites, neurons, neonicotinoids, toxicity.

Introduction

Registered uses of the systemic insecticide imidacloprid include foliar spray, soil (drench) and seed treatment applications (Elbert *et al.*, 1998). When applied to the soil or as a seed treatment imidacloprid is metabolized more or less completely depending on plant species and time (Araki *et al.*, 1994). Accordingly, honeybees may encounter not only the parent molecule but also plant metabolites of this compound when foraging on nectar and pollen of seed-treated crops. Since some of the metabolites appearing *in planta* revealed insecticidal activities against aphids and whiteflies (Nauen *et al.*, 1999), these metabolites may pose a risk to foraging honeybees if they enter the reproductive parts of the crop plants during blossom.

The objective of the present study was to investigate in the honeybee, *Apis mellifera* L. (Hymenoptera: Apidae), the pharmacological and toxicological profile of imidacloprid and those imidacloprid plant metabolites which were commonly detected during plant metabolism studies in reproductive parts of seed-treated crops (Sur and Stork, 2003) using three different bioassays. In the first bioassay, the molecular target of imidacloprid

and the potentially relevant plant metabolites in bees were analysed by standard ligand competition assays. In target insects, the effect of imidacloprid is based on an agonistic action at the nicotinic acetylcholine receptor (nAChR) (Bai *et al.*, 1991). In order to characterise the molecular target site of this neonicotinoid insecticide in the honeybee [³H]imidacloprid was used in receptor binding studies and performed as described for various pest insects, e.g. aphids and whiteflies (Chao *et al.*, 1997; Nauen *et al.*, 1998; Lind *et al.*, 1998; Huang *et al.*, 1999). Furthermore whole cell voltage clamp electrophysiological measurements were conducted to highlight the agonistic potential of imidacloprid and its potentially relevant plant metabolites in isolated bee neurons from antennal lobes. Finally, classical honeybee toxicity assays were conducted with imidacloprid and the potentially relevant imidacloprid plant metabolites following an internationally adopted test guideline (EPPO, 1992). Since in Europe various bee strains are used for honey yields and pollination services it was further examined whether significant differences exist in the sensitivity of honeybees from different apiaries to imidacloprid. For this purpose the same batch of imidacloprid was tested at seven different European research

facilities which all were well experienced in honeybee toxicity testing (at least 3 year testing history) and which obtained their bees from different sources.

The results of all three bioassays were used to precisely characterize the risk potential posed by an imidacloprid seed dressing to honeybees under field-relevant exposure conditions.

Material and methods

Chemicals

Technical imidacloprid and imidacloprid metabolites (figure 1) were obtained in-house (Bayer AG, Leverkusen, Germany) with a purity of at least 98%. Lobeline, cytosine, alpha-bungarotoxin and +/- nicotine were purchased from Sigma Chemicals. All other chemicals and organic solvents used were of analytical grade. [³H]imidacloprid (1.406 GBq μmol⁻¹) for receptor binding studies was synthesized and labelled as described by Latli and Casida (1992).

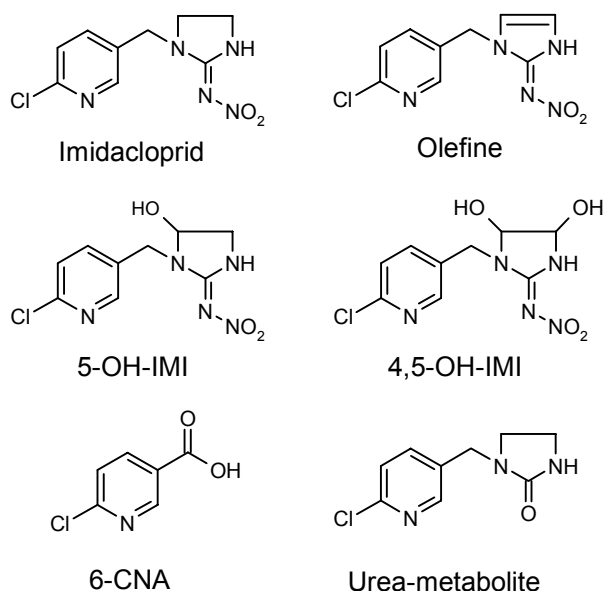


Figure 1. Chemical structures of imidacloprid and common imidacloprid plant metabolites.

[³H]imidacloprid displacement studies

Stock solutions of putative ligands 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 albumine, yielding a concentration of 1 mM. Subsequent dilutions were prepared in potassium phosphate buffer. For the biochemical studies worker honeybees were collected from hive combs (apiary: J. Gilli, Euskirchen, Germany) and immediately frozen on 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 six weeks) until use. A bee head aliquot of 10 g was homogenized in 200 ml ice-cilled 0.1 M potassium phosphate buffer, pH 7.4

containing 95 mM sucrose using a motor-driven Ultra Turrax. The homogenate was centrifuged for 10 min at 1200g and the resulting supernatant filtered through five layers of cheesecloth and then used without prior purification. Protein concentration was determined according to Bradford (1976). Unless otherwise stated the final assay volume of 250 μl in a 96-well microtiter plate with bonded GF/C filter membrane (Packard UniFilterTM-96, GF/CTM) consisted of 200 μl homogenate (0.48 mg protein), 25 μl [³H]imidacloprid (576 pM) and 25 μl competing ligand. Ligand concentrations used ranged from 0.001-10000 nM at least in duplicate per competition assay. The assay was started by the addition of the homogenate and incubated for 60 min at room temperature. Bound [³H]imidacloprid was quantified by filtration into a second 96-well filter plate (conditioned with ice-cilled 100 mM potassium phosphate buffer, pH 7.4 (incl. 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-0-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.

Electrophysiological measurements

Stock solutions of test compounds were prepared at 100 mM in DMSO and subsequently diluted using a Ringer solution, containing (in mM): 150 NaCl, 4 KCl, 2 MgCl₂, 2 CaCl₂, 150 Trehalose, 10 Hepes (pH 7.4 adjusted with NaOH). 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 (Oland *et al.*, 1996). 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 subsequent two days. Electrophysiological recordings were made with the whole-cell voltage clamp technique (Hamill *et al.*, 1981). The microelectrodes were pulled from borosilicate glass capillaries. The resistance of the fire-polished pipettes was 4 - 7 MOhm 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 thereby exchanged each 10 s and completely washed out in less than 60 s. The external bath contained (in mM): 150 NaCl, 4 KCl, 2 MgCl₂, 2 CaCl₂, 150 trehalose, 10 HEPES (pH 7.4 adjusted with NaOH). The (internal) pipette solution contained (in mM): 120 CsF, 30 CsCl, 10 Cs-EGTA, 1 CaCl₂, 150 trehalose, 10 HEPES (pH 7.4 adjusted with CsOH). Compounds were

applied to the cells using the U-tube reversed flow technique (Fenwick *et al.*, 1982). 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.

Honeybee toxicity bioassays

Stock solutions of the test compounds were prepared immediately prior to use 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). The highest acetone concentration in the test solutions was 0.005% v/v. All tests were carried out using adult worker honeybees, *A. mellifera* L. var. *carnica* Pollm., 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 (e.g. varroacide) 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, 1986) 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. The testing procedure followed the European Plant Protection Organisation guideline no. 170 (EPPO, 1992). Test bees were allocated impartially to the treatment groups. 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 cm² in size. Before being used for the oral toxicity test, bees were subjected to a starvation period between 1h 10min and 2 h 15min 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 to 81 ng a.s./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 hrs. 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.

The effects of contact exposure were assessed by topical application of the test substance in acetone to the thoracic body surface. Immediately prior to treatment bees were anaesthetised 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 a.s./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 hours post-dose. All doses and toxicity data for the test compounds refer to the respective active ingredient but were not adjusted for the analytical purity (> 98%). Sub-lethal effects were assessed according to pre-determined categories: Knock-down (i.e. alive, but immobile), staggering (i.e. moving, but in a poorly co-ordinated manner).

Data calculation

IC₅₀-values (concentration of unlabelled ligand displacing 50% of [³H]imidacloprid from its binding site) were calculated using a 4 parameter logistic non-linear curve fitting routine (GRAPHPAD-PRISM, www.graphpad.com). Data storage and analysis of electrophysiological measurements, i.e. calculation of EC₅₀-values and Hill coefficients (n_H), were done using the pClamp V 6.03 software package (Axon Instruments, Foster City, CA) and Origin 5.0 Software (Microcal Software Inc., Northampton, MA, USA), respectively. Mortality rates of treatment groups were corrected for control mortality according to Abbott (1925). LD₅₀-values were determined by probit regression analysis (Finney, 1971), non-linear regression or by the moving average method (Bennett, 1952; Harris, 1959; Bates and Watts, 1988). Confidence intervals were calculated using standard procedures described by Breitig and Tümling (1982).

Results

[³H]imidacloprid displacement studies

Standard cholinergic ligands, such as lobeline, alpha-bungarotoxin, cytosine and nicotine were tested for their ability to inhibit [³H]imidacloprid binding in honeybee head membranes (figure 2A). The results indicated a pharmacological profile typical for nAChR's, and were similar to characterizations performed for other invertebrate nACh receptors. IC₅₀-values were 1.7, 1.4, 5.2 and 0.59 µM for alpha-bungarotoxin, cytosine, nicotine and lobeline, respectively.

As in other insect species imidacloprid binds with a high affinity to the nicotinic acetylcholine receptor of honeybees with an IC₅₀-value of 2.9 nM (figure 2B). Among the plant metabolites tested 6-CNA and the urea metabolite were not active in displacing [³H]imidacloprid from its receptor binding site even at concentrations as high as 0.1 mM. Thus, these metabolites were at least 5 orders of magnitude less effective than imidacloprid. The affinity of the other (active) metabolites decreased in the order olefine > 4-OH-imidacloprid >> 4,5-OH-imidacloprid (figure 2B) with IC₅₀-values of 0.45, 24 and 6,600 nM, respectively.

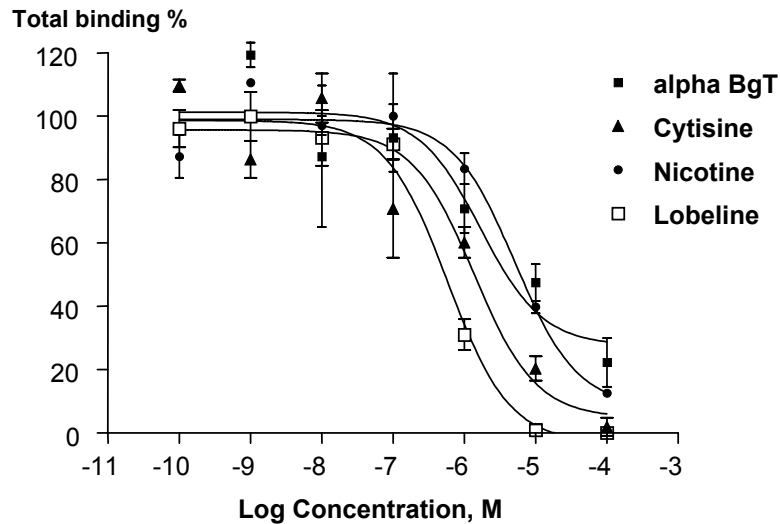


Figure 2A. Displacement of [³H]imidacloprid in honeybee head membrane by standard cholinergic ligands. BgT = Bungaro-Toxin.

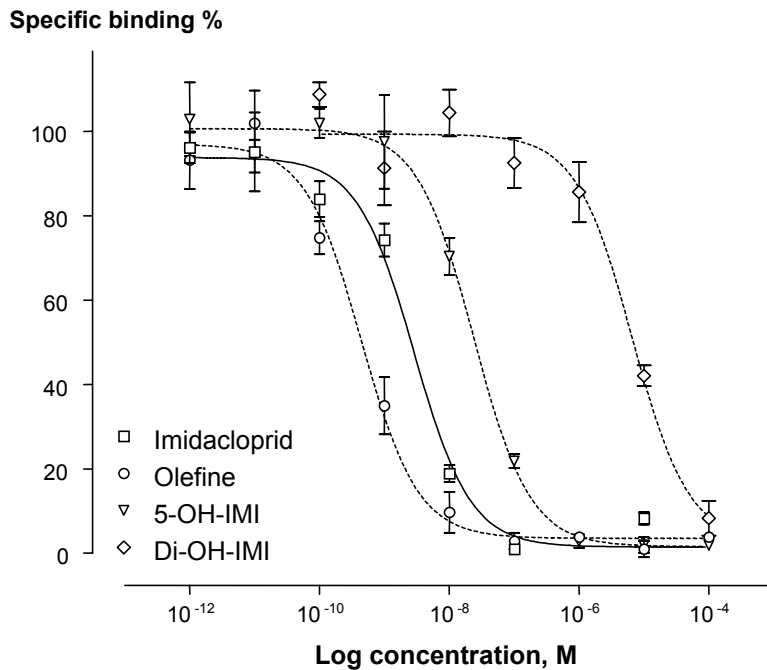


Figure 2B. Dose-related displacement of [³H]imidacloprid in honeybee head membrane by imidacloprid.

Electrophysiology using neurons from antennal lobes

The preparation of the cell bodies from the antennal lobe of honeybees usually yielded a fair number of large, round cells. Cells were typically about 30 - 50 μ m in diameter and sometimes had a short protrusion, 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 μ M acetylcholine 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 one hour, 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. However, estimated EC_{50} -values for those receptor populations where imidacloprid acts as a partial agonist were fully comparable to those where it acts as a full agonist, thus indicating no differences in binding affinity. Imidacloprid shows an EC_{50} -value (corresponds to half-maximal activation of nAChR's induced by an agonist) of 0.83 μ M, whereas the olefine metabolite was

by a factor of 2 more active (0.35 μM). All other metabolites, i.e. 6-CNA, urea-metabolite and 4,5-OH-imidacloprid, were electrophysiologically not active on honeybee neurons, except 5-OH-imidacloprid which exhibits a rather low affinity (7.5 μM) to nAChR's in such preparations (figure 3). Hill coefficients were determined close to 1 for those compounds binding to nAChR's on honeybee neurons from antennal lobes, indicating a single binding site necessary for channel activation by imidacloprid, olefine and 5-OH-imidacloprid.

Toxicity of imidacloprid and potentially relevant plant metabolites to honeybees

Between July 1999 and July 2000 seven European laboratories examined the acute oral and contact toxicity of technical grade imidacloprid to honeybees (table 1). The calculated oral LD_{50} was between 41 and > 81 ng a.s. per honeybee (table 2). At concentrations of 1 mg kg^{-1} diet or higher honeybees rejected in a dose dependent pattern the ingestion of the sucrose solution which

resulted in a poor fit in the dose response curve. In contrast, the dose response curve of the contact toxicity revealed a good fit with calculated LD_{50} values between 42 and 104 ng imidacloprid per honeybee (table 2).

At one of the testing facilities (Germany I) the potentially relevant imidacloprid plant metabolites (figure 1) were tested in addition to imidacloprid for their oral toxicity to honeybees. From table 3 it is evident that metabolites which contain the nitroguanidine pharmacophore (olefine-, hydroxy- imidacloprid) still exhibit a relevant toxicity to honeybees, whereas those metabolites not carrying this pharmacophore (urea-metabolite, 6-CNA) are non-toxic to bees. An exception from this rule was Dihydroxy-imidacloprid. Although the pharmacophore is still present in this metabolite, no honeybee mortality was recorded. Dihydroxy-imidacloprid caused a very strong antifeedant response of honeybees. At the maximum ingested dose of 49 ng/bee no adverse effects were recorded.

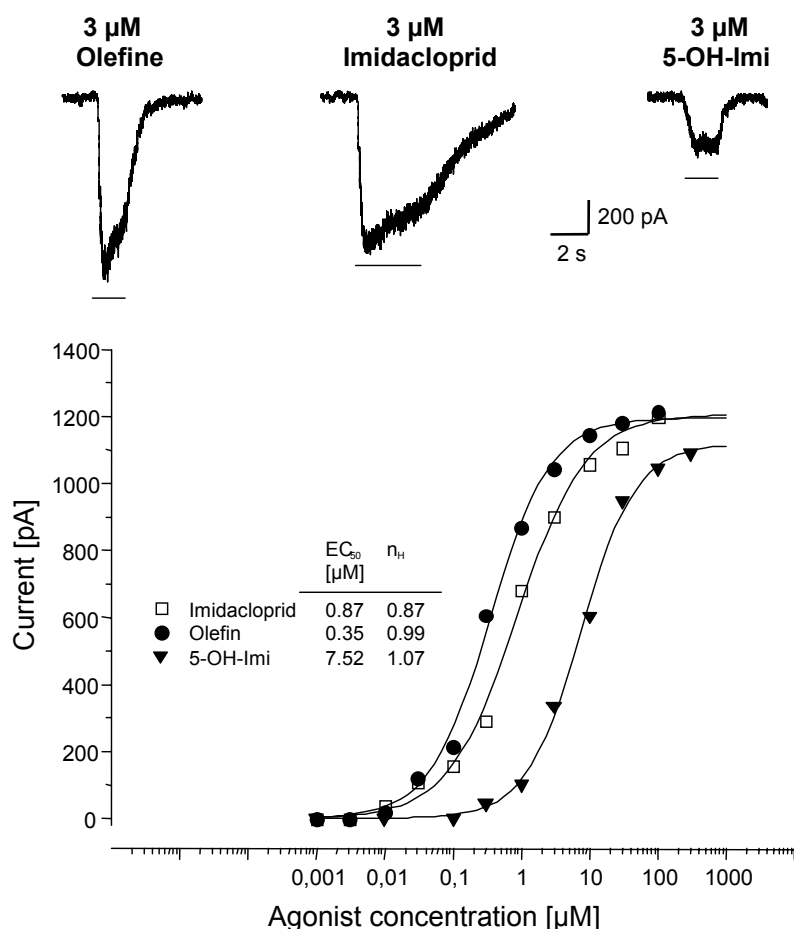


Figure 3. Whole cell current responses of a “Type (a)” neuron isolated from the antennal lobe of *Apis mellifera* after application of imidacloprid and common imidacloprid plant metabolites. The upper graphs show the corresponding responses for the active test compounds at 3 μM (holding potential -70 mV). All currents were obtained from the very same cell.

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

Bee strain, apiary location, and sampling procedure	Compounds tested
<i>Apis mellifera</i> L. var. <i>carnica</i> Pollm. [Netherland I]	Imidacloprid
<i>Apis mellifera</i> L. var. <i>carnica</i> 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
<i>Apis mellifera</i> L. var. <i>carnica</i> Pollm. [Germany II] brushed from the combs on day 0	Imidacloprid
<i>Apis mellifera</i> L. var. <i>carnica</i> Pollm. [Germany III] brushed from the combs on day 0	Imidacloprid
<i>Apis mellifera</i> L. var. <i>carnica</i> Pollm. [Germany IV] brushed from the combs on day -1	Imidacloprid
<i>Apis mellifera</i> L. var. <i>carnica</i> Pollm. [Germany V] sampled from a bypass in the hive roof on day 0	Imidacloprid
<i>Apis mellifera</i> L. (strain not specified) [United Kingdom I] shaken from the combs on day 0	Imidacloprid

Table 2. Acute oral and contact toxicity (48h) of imidacloprid to honeybees from different apiaries. Oral toxicity values refer to nominally applied doses.

Oral LD ₅₀ [ng/bee]	Contact LD ₅₀ [ng/bee]	Test period	Origin of tested honeybees
c 41 [50%] ^a	not determined	July 1999	Germany I [control mortality: 10%]
> 20 [0%]	104 ^b (83.0 – 130) ^c	July 1999	Netherland 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 Mortality at the highest applied dose; ^b LD₅₀ at 72 h; ^c 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 A.S./bee]	Oral NOED ^a [ng A.S./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	> 99,500	1200	July 7-9, 1999
6-CNA	> 121,500	121,500	July 7-9, 1999

^a No observed effect dose

Discussion

Competition experiments with standard ligands identified the nAChR as the [³H]imidacloprid binding site in honeybee head membranes. As in other non-homopteran insects (Lind *et al.*, 1999; Zhang *et al.*, 2000) imidacloprid revealed a high affinity to this binding site with an IC₅₀ value of 2.9 nM. From the imidacloprid plant metabolites which were commonly found in reproductive parts of seed-dressed crop plants (Sur and Stork, 2003), those containing the nitroguanidine pharmacophore also showed some affinity to this receptor. The olefine metabolite showed a slightly greater affinity to nAChR's in ligand competition experiments than the parent compound. The 6-CNA and the urea-metabolite were not active in displacing [³H]imidacloprid from its binding site in putative nAChR's in honeybee membrane preparations at concentrations up to 0.1 mM.

The IC₅₀-values obtained in receptor binding studies correlated well with EC₅₀-values calculated from whole cell voltage clamp studies using neurons isolated from antennal lobes. The olefine and the 5-hydroxy-metabolite revealed a clear agonistic activity on the nAChR whereas the 6-CNA and the urea-metabolite induced no currents up to concentration levels of 3 mM. The 4,5-OH-imidacloprid metabolite which showed a weak receptor affinity displayed no receptor activation potential in the electrophysiological studies indicating a low toxicological relevance for honeybees. The Hill coefficient as calculated from the electrophysiological whole cell voltage clamp measurements was close to 1 indicating the occurrence of a single binding site for imidacloprid at nAChR's located on cell bodies isolated from the antennal lobe of honeybees. A single binding site for *A. mellifera* and *Manduca sexta* (Joh.) (Lepidoptera: Sphingidae) was also described by other researchers (Tomizawa and Yamamoto, 1992; Tomozawa *et al.*, 1995; Eastham *et al.*, 1998) who analysed saturable binding of the antagonist [¹²⁵I]-alpha-bungarotoxin to nAChR. Two distinct binding sites could only be identified in neuronal tissues of homopteran species, such as *Myzus persicae* (Sulz) and *Nephotettix cincticeps* (Uhl.) (Homoptera: Cicadellidae) whereas in all other yet examined insect taxa only a single binding site is reported (Lind *et al.*, 1999; Zhang *et al.*, 2000). The effective concentrations recorded in the electrophysiology studies were about 3 orders of magnitude higher than those needed in the receptor binding studies. Similar differences in biochemical binding and functional assay studies were observed for other insect species (Nauen *et al.*, 2001b). These differences are discussed as being the result of different conformational states of the nAChR which have different affinities for various ligands, e.g. in the desensitized status the receptor shows a much higher affinity than in its sensitized conformation.

The nAChR binding data of imidacloprid and its potentially relevant plant metabolites correlated very well with their toxicological properties as recorded in standard toxicity assays (EPPO, 1992). No acute toxicity were found for 6-CNA, the urea metabolite and 4,5-dihydroxy-imidacloprid and all three metabolites did not

show significant interactions with the molecular target of imidacloprid in the honeybee. In contrast, a relevant oral toxicity was found for the olefine and the 5-hydroxy metabolite. These two metabolites acted agonistically on the nAChR indicating a common mode of action between these metabolites and the parent molecule. Accordingly, it can be concluded with a high degree of confidence that of those imidacloprid plant metabolites which were commonly detected in reproductive parts of seed-dressed crop plants, only the olefine and the 5-hydroxy-imidacloprid are of toxicological concern for honeybees.

Another important aspect of a comprehensive evaluation of the risks posed by an imidacloprid seed dressing to honeybees concerns the question as to whether honeybees from different apiaries may differ in their sensitivity to imidacloprid. For this purpose, the oral and contact toxicity of imidacloprid to honeybees was determined by seven different European laboratories. The calculated LD₅₀ values did not indicate significant differences in sensitivity between honeybees of different apiaries. This view is further supported by toxicity data reported from other researchers. Contact LD₅₀-values of 44, 81, 30, and 12-24 ng a.s./bee were reported by Mayer *et al.* (1991), Pflüger and Schmuck (1991), Ruzhong *et al.* (1999), and Suchail *et al.* (2000), respectively. A larger variability was only observed for the oral toxicity with reported LD₅₀-values of c. 4 (Pflüger and Schmuck, 1991), 151 (Ruzhong *et al.*, 1999), and c. 5 ng a.s./bee (Suchail *et al.*, 2000). These larger differences in the oral toxicity values, however, may be attributed to methodological shortcomings of the oral toxicity bioassay rather than reflect existing differences in bee sensitivity (see also Nauen *et al.*, 2001a).

From all reported findings it can be concluded that the risk posed by an imidacloprid seed dressing to honeybees is defined by the combined activity of the parent compound and the plant metabolites olefine and 5-hydroxy-imidacloprid. Due to the common mode of action an enhanced toxicity by a synergistic effect between the parent and these two plant metabolites is very unlikely and the risk to honeybees can be best evaluated by assuming an additive toxicity between the parent molecule and these two metabolites. This means that analytical investigations aiming to characterize the exposure of honeybees when foraging in seed-treated crops should not only include a detection method sensitive for the parent compound but should also be sensitive to the presence of olefine and 5-hydroxy-imidacloprid.

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