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Cycloxaprid Insecticide: Nicotinic Acetylcholine Receptor Binding Site and Metabolism

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Supporting Information

ABSTRACT: Cycloxaprid (CYC) is a novel neonicotinoid prepared from the (nitromethylene)imidazole (NMI) analogue of imidacloprid. In this study we consider whether CYC is active per se or only as a proinsecticide for NMI. The IC_{50} values (nM) for displacing [^{3}H]NMI binding are 43−49 for CYC and 2.3−3.2 for NMI in house fly and honeybee head membranes and 302 and 7.2, respectively, in mouse brain membranes, potency relationships interpreted as partial conversion of some CYC to NMI under the assay conditions. The 6−8-fold difference in toxicity of injected CYC and NMI to house flies is consistent with their relative potencies as in vivo nicotinic acetylcholine receptor (nAChR) inhibitors in brain measured with [^{3}H]NMI binding assays. CYC metabolism in mice largely involves cytochrome P450 pathways without NMI as a major intermediate. Metabolites of CYC tentatively assigned are five monohydroxy derivatives and one each of dihydroxy, nitroso, and amino modifications. CYC appears be a proinsecticide, serving as a slow-release reservoir for NMI with selective activity for insect versus mammalian nAChRs.

KEYWORDS: *cycloxaprid, house fly, honeybee, mouse, metabolism, neonicotinoid, nicotinic acetylcholine receptor*

INTRODUCTION

Cycloxaprid (CYC), one of the most effective neonicotinoid insecticides, is currently under development for agricultural pest control.[1−4] CYC is unique in having the nitro substituent in the *cis* configuration, whereas in all other commercial neonicotinoids the nitro is in the *trans* configuration.[4−6] It is especially effective against imidacloprid (IMI)-resistant pests, including the brown planthopper, and appears to activate a different site compared to IMI on the nicotinic acetylcholine receptor (nAChR).[5,6] CYC is prepared by adding succinaldehyde[6] to a highly potent insecticidal (nitromethylene)imidazole (NMI)[7] and might revert to NMI on aqueous hydrolysis (Figure 1). Other NMI adducts are proposed to act directly[8] or

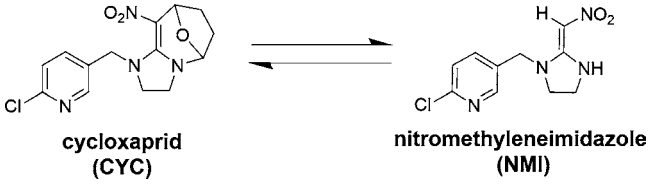

Figure 1. Structural relationship between CYC and NMI. CYC is prepared from NMI, and although photostabilized, it is easily hydrolyzed and may serve in part as a proinsecticide.

as proinsecticides by reverting to NMI for their activity at the nAChR.[9,10] It has yet to be determined whether CYC is directly active at the nAChR or if its insecticidal activity is due to conversion to NMI. NMI and similar nitromethylene neonicotinoid analogues are more potent agonists with insect than mammalian nAChRs.[11,12]

In this investigation we determine the involvement of NMI in CYC action by studying nAChR binding characteristics and metabolism. We use [^{3}H]CYC and [^{3}H]NMI as radioligands to examine selective interactions with house fly, honeybee, and mouse brain nAChRs. For studies on mice, we then compare CYC and NMI metabolism 15 and 120 min after intraperitoneal (ip) treatment to determine the extent of CYC conversion to NMI and common versus unique aspects of their metabolic pathways.

MATERIALS AND METHODS

Chemicals. [^{3}H]CYC (35.9 Ci/mmol, 98% radiochemical purity) was synthesized by Vitrax Co. (Placentia, CA) and [^{3}H]NMI (60 Ci/mmol, 98% radiochemical purity) by Shanghai Ruxu Radiochemicals Inc. (Shanghai, China).

nAChR Binding Assays. House fly and honeybee head membranes were prepared as described in the Supporting Information. Briefly, the frozen heads were homogenized in 0.32 M sucrose, 0.1 mM EDTA, and 100 mM sodium phosphate (pH 7.4), and the 500 g supernatant fraction was centrifuged at 25000*g* to obtain the membrane pellet which was used fresh or after storage at −80 °C. Mouse brains (after discarding the cerebellum) were homogenized at 10% (w/v) in 0.25 M sucrose and 10 mM Tris/HCl using a Polytron (Brinkmann Instruments) for 15 s. [13] The homogenate was centrifuged at 1000*g* for 15 min and the supernatant at 39000*g* for 30 min at 4 °C. The pellet was resuspended in assay buffer (20 mM HEPES, 1 mM magnesium chloride, 120 mM sodium chloride, 5 mM potassium chloride, 2 mM calcium chloride, and 1 mM EDTA, pH 8.0) at 20% fresh weight equivalent, washed by centrifuging at 39000*g* as above, and resuspended in assay buffer. Incubation mixtures consisted of 300−500 μg of insect or mouse brain protein[14] and 1 nM [^{3}H]CYC or [^{3}H]NMI in 50 mM sodium chloride and 10 mM sodium phosphate (pH 7.4) (500 μL) (binding buffer). Bound radioligand was determined after incubation for 60 min at 22 °C for insect or

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Table 1. CYC and NMI nAChR Binding Site Specificity and Toxicity

parameter	CYC	NMI
radioligand binding in brain membrane, fmol/mg of protein		
house fly	88 ± 7	877 ± 69
honeybee	57 ± 5	610 ± 53
mouse	6 ± 0.7	56 ± 7
IC_{50}([^{3}H]NMI), nM		
house fly	43 ± 6	2.3 ± 0.5
honeybee	49 ± 8	3.2 ± 0.9
mouse	302 ± 29	7.3 ± 1.6
LD_{50}, μg/g		
house fly	0.3	0.05
mouse	1260[a]	7−15[b]

[a]Data provided by the Shanghai Municipal Center for Disease Control and Prevention. [b]Reference 13.

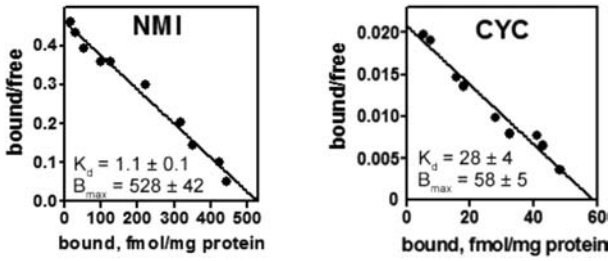

Figure 2. Scatchard plots for [^{3}H]NMI and [^{3}H]CYC binding in house fly head nAChR.

37 °C for mouse by filtration with Whatman GF/B glass-fiber filters and then two rinses each with ice cold binding buffer (pH 7.4 for insect and pH 8.0 for mouse) and liquid scintillation counting with normalization relative to protein levels. Experiments were repeated at least twice with triplicate samples. Specific binding was determined by the difference between total binding with 1 nM [^{3}H]NMI radioligand only compared with the addition of 20 μM unlabeled NMI (for nonspecific binding) and averaged 80−90% for house fly, 60−70% for honeybee, and 20−30% for mouse brain.

nAChR Inhibition. IC_{50} values (inhibitor concentrations to reduce specific binding by 50%) were determined in vitro by adding test chemicals to the membrane in binding buffer. The data from two or three separate experiments each with triplicate samples were calculated by nonlinear regression analysis for logarithm of inhibitor concentration versus probit percentage inhibition. In a modification of the in vitro procedure, the effects of CYC and NMI were determined on house fly poisoning signs and nAChR inhibition in brain 1 h after treatment by intrathoracic injection (Supporting Information). Poisoning signs for groups of 50 treated flies were individually rated for each fly as 0 for normal, 1 for impaired movement, and 2 for dead, such that all impaired was rated 50 and complete mortality was scored 100.

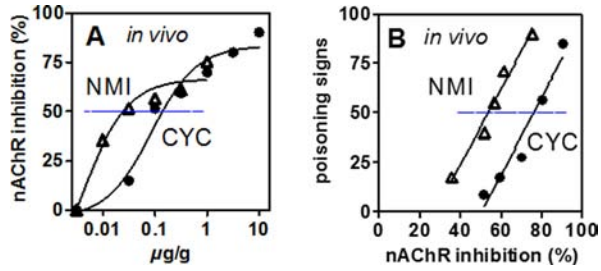

Figure 4. CYC and NMI inhibit house fly nAChR in vivo relative to the injected dose (0.01, 0.03, 0.1, 0.3, 1, 3, and 10 μg/g) (A) and corresponding to the poisoning signs (B).

Table 2. Metabolites of CYC and NMI in Mice

	LC/MS		detection sites[b]	
compd[a]	m/z	t_R[c] (min)	15 min	120 min
	CYC Treatment			
CYC	322.6	13.5	l, b, p	l, b, p
NMI	254.7	8.5	l, b, p	l, b, p
OH-CYC[d]				
a	338.6	7.0	l	
b	338.6	10.7	l, p	l
c	338.6	12.0	l, b, p	l, b, p
d	338.6	19.6	l, b, p	l, b, p
e	338.6	20.0	l	l
$(OH)_2$-CYC	354.5	6.3	l	l
NO-CYC	306.6	7.1	l	
NH_2-CYC	292.6	8.3	l	l
	NMI Treatment			
NMI	254.7	8.5	l, b, p	l, b, p
OH-NMI	270.6	5.4	l	
NO-NMI	238.7	3.3	l	l

[a]Structures are shown in Figure 5. [b]Sites of metabolite detection: l = liver, b = brain, p = plasma. [c]Average values from several experiments. [d]Multiple peaks (a−e) of m/z 338.6 refer to different OH-(R/S)-CYC isomers.

Mouse Experiments. Mice treated ip were used to analyze CYC and NMI metabolism because of the extensive comparative data for other neonicotinoids with this system.[15,16] Animal housing, maintenance, and procedures were in compliance with a University of California, Berkeley, Animal Care and Use Committtee approved protocol. Male albino Swiss Webster mice (25−35 g) (Charles River Laboratories, Wilmington, MA) were administered either CYC (ip, 20 mg/kg) or NMI (ip, 3 mg/kg) in dimethyl sulfoxide (DMSO) (1 μL/g of body mass). These treatments gave no apparent adverse effects. Tissues were collected by the procedures of Ford and Casida.[15,16] Blood was obtained by cardiac puncture 15 or 120 min after treatment, and then the mice were euthanized. The livers and brains were removed, frozen in liquid nitrogen, and stored at −80 °C. Plasma was isolated by centrifuging blood at 5000g for 10 min at 4 °C and stored in −80 °C until analysis. Other CYC- or NMI-treated mice

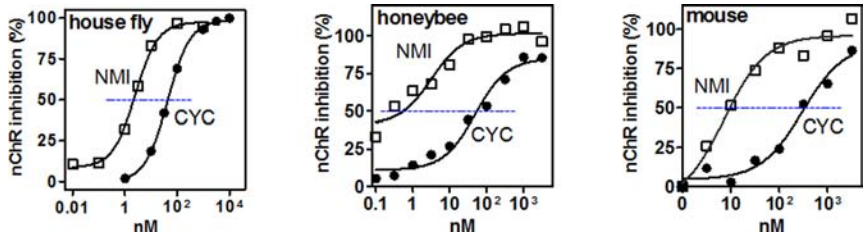

Figure 3. CYC and NMI inhibit house fly, honeybee, and mouse nAChR in vitro.

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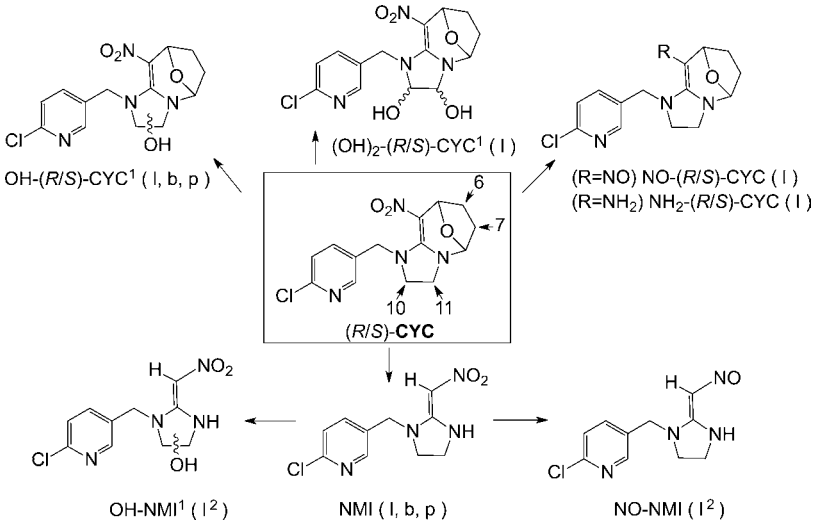

Figure 5. Metabolites of CYC and NMI in mice. Numbers in the CYC structure refer to ring positions. Mono- and dihydroxylation sites are arbitrarily shown as carbon 10 or 11 or both, although carbons 6 and 7 are also possible. Sites of metabolite detection: l = liver, b = brain, p = plasma. Notes: (1) The number of possible isomers is four for OH-NMI and eight for OH-(*R*/*S*)-CYC and $(OH)_2$-(*R*/*S*)-CYC. (2) OH-NMI and NO-NMI were detected in NMI-treated but not CYC-treated mice.

were kept in glass metabolism cages to collect urine and feces for up to 24 h. Neonicotinoids and metabolites were recovered for analysis by homogenizing the liver (500 mg), brain (350−450 mg), plasma (100 μL), urine (200 μL), or feces (100 mg) in ice-cold acetonitrile (ACN) (750 μL containing 10 nmol of thiacloprid (THI) as an internal standard) using a sonic dismembrator (Fisher Scientific, Pittsburgh, PA). The internal standard was not added to urine or feces. The homogenates were centrifuged at 900*g* for 15 min, and the clear supernatant was decanted and evaporated to dryness under N_2 at 25 °C. The residues from evaporation were resuspended in 5:95 ACN/5 mM NH_4OH (pH 9) (250 μL) and then passed through 0.2 μm nylon syringe filters (Pall Life Sciences, Ann Arbor, MI) for LC/MS analysis. The 5 mM NH_4OH (pH 9) solution used here and in further experiments was almost fully effective in preventing breakdown of CYC to NMI; i.e., untreated tissue samples spiked with CYC yielded little or no NMI on LC/MS.

Cytochrome P450 (CYP) Experiments. Microsomes from the liver of untreated mice were prepared by homogenization in phosphate-buffered saline (PBS; pH 7.4) (Invitrogen, Grand Island, NY) (2 mL/g liver) followed by differential centrifugation of the supernatant (1000*g* for 10 min, 10000*g* for 30 min, and finally 100000*g* for 1 h). The microsomal 100000*g* pellet was resuspended in PBS and the protein concentration measured.[14] Each reaction mixture consisted of 1 mg of microsomal protein in 25 μL of PBS added to CYC or NMI (100 μM final concentration) in 155 μL of 5 mM NH_4OH, pH 9. Following a 5 min preincubation in a 37 °C shaking water bath, NADPH (0 or 1 mM final concentration) in 20 μL of 5 mM NH_4OH, pH 9, was added (200 μL total volume) and the resulting mixture incubated for 30 min in a 37 °C shaking water bath. Reactions were terminated by addition of ice-cold ACN (200 μL containing 10 nmol of THI as an internal standard) and placed on ice for 5 min to precipitate protein. After centrifugation at 1000*g* for 5 min, the supernatant was evaporated to dryness under N_2 at 25 °C, resuspended in 5:95 ACN/5 mM NH_4OH (pH 9) (500 μL), and then passed through 0.2 μm nylon syringe filters for LC/MS analysis.

LC/MS Analysis. CYC and NMI metabolites were analyzed on an Agilent 1100 series liquid chromatograph with a Phenomenex Kinetex XB-C18 column (100 × 2.1 mm, 2.6 μm) and a Waters LCT Premier XE mass spectrometer. Electrospray ionization was in the positive mode with source parameters as follows: capillary voltage, 1300 V; sampling cone voltage, 54 V; source temperature, 90 °C; desolvation temperature, 200 °C. The two mobile phases were 100% ACN and 5 mM NH_4OH (pH 9) with the gradient beginning with 5% ACN for 3 min and increasing to 100% by 25 min at a flow rate of 0.18 mL/min. A final 10 min wash with 5% ACN/95% 5 mM NH_4OH (pH 9) eluted interfering materials. LC/MS standard curves for peak areas were developed using CYC or NMI standards versus THI. Neonicotinoid and metabolite levels were quantitated by comparing the peak areas for relevant *m*/*z* values with the internal standard (THI) and corrected using the standard curves (the CYC standard curve was used for CYC and CYC metabolites, and the NMI standard curve was used for NMI and NMI metabolites) with GraphPad Prism (version 6.0). CYC levels not within the standard curve limits were not quantitated. Data are presented as the mean ± standard error (SE) with $n = 3$. Metabolite differences in in vitro experiments were analyzed using Student's *t* test with $p < 0.05$ considered statistically significant.

RESULTS

nAChR Binding Site. The availability of [^{3}H]CYC and [^{3}H]NMI as high specific activity radioligands allowed direct determination of their binding site affinity in house fly, honeybee, and mouse brain membranes. Specific binding in femtomoles per milligram of protein was about 10-fold higher for [^{3}H]NMI than [^{3}H]CYC and 10-fold higher for insect than for mouse brain membranes (Table 1). In house fly head membranes, the radioligands undergo specific and saturable binding with a k_d of 1.1 nM for [^{3}H]NMI and 28 nM for [^{3}H]CYC (Figure 2). The comparative affinity was also examined by displacement curves for NMI and CYC in the [^{3}H]NMI assay with brain membranes of the three species (Figure 3, Table 1). Under these assay conditions, NMI was 19-fold more potent than CYC with house flies, 15-fold with the almost equally sensitive honeybees, and 41-fold with the less sensitive mouse. Thus, NMI formed from CYC during the assay may contribute to or account for the activity of CYC. NMI and CYC are both very toxic to house flies, allowing a comparison of their relative potency for nAChR inhibition in vivo (Figure 4). NMI is 22-fold more potent than CYC in this assay, and the in vivo inhibition at 1 h after treatment generally agrees with the poisoning signs.

Metabolite Structure Assignments. CYC and NMI metabolites are tentatively assigned but not conclusively identified since synthetic standards were not available. However, metabolites were recognized by comparing chromatograms with

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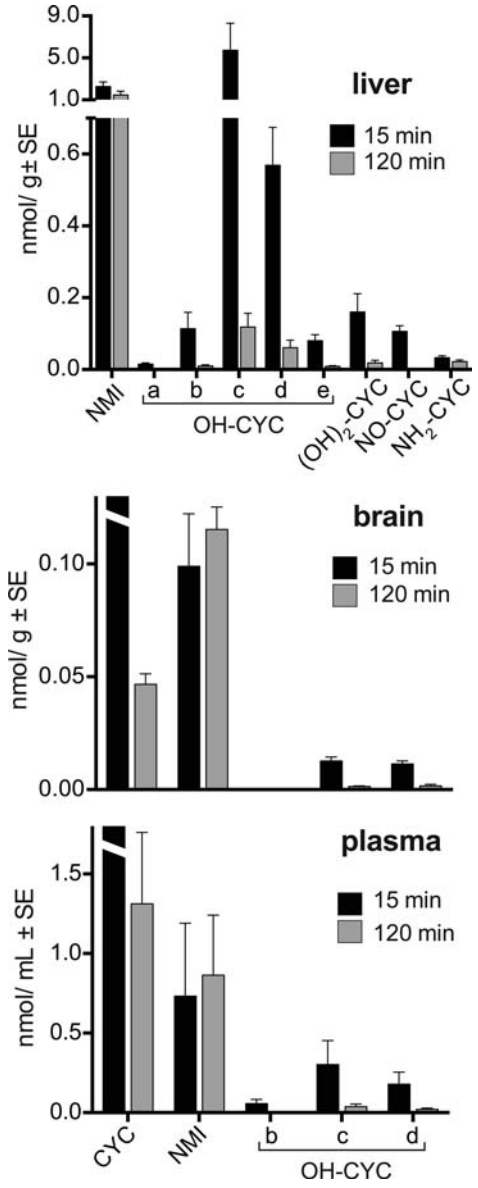

Figure 6. Levels of CYC and its metabolites in mouse liver, brain, and plasma 15 and 120 min after CYC ip administration at 20 mg/kg. The CYC level is not shown for the liver at 15 and 120 min and is not quantitated for the brain and plasma at 15 min, but was much higher than that for any of its metabolites. OH-CYC (b) was not observed in the brain.

DMSO-treated control mice and tentatively assigned on the basis of calculated m/z values and characteristic chlorine isotope patterns (Table 2). The structures are based on analogy with established neonicotinoid metabolic pathways.[15−17] The proposed metabolic pathways for (*R*/*S*)-CYC and NMI are shown in Figure 5.

Metabolism in Mice. The levels of CYC and its metabolites 15 and 120 min after CYC ip administration are shown in Figure 6. CYC metabolites detected in liver were primarily oxidation products assigned as isomers of hydroxyl addition at the 6-, 7-, 10-, or 11- position (Figures 6 and 7, Table 2). The next most abundant product was NMI followed by small amounts of the CYC-diol ($(OH)_2$-CYC), nitroso-CYC (NO-CYC), and amino-CYC (NH_2-CYC) (Figures 6 and 7, Table 2). CYC and all metabolite levels decreased from 15 to

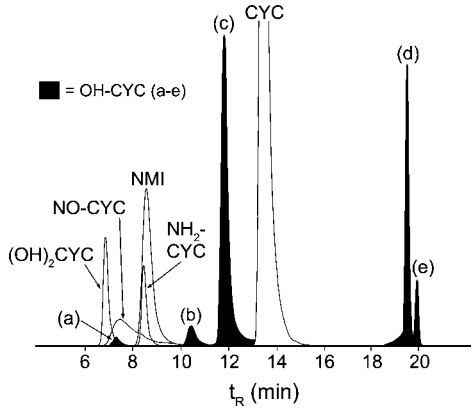

Figure 7. Representative LC/MS chromatogram of CYC metabolites in mouse liver 15 min after CYC ip administration. The peaks are for compounds shown in Table 2 with their m/z and t_R values.

120 min (Figure 6), and no metabolites were detected by 24 h with the exception of NMI (less than 2% of the level observed at 15 min). Metabolites detected in the brain of CYC-treated mice included NMI and two hydroxylation (+16) products with t_R values corresponding to OH-CYC (c) and OH-CYC (d) found in the liver (Figure 6, Table 2). NMI levels were maintained after 120 min, but hydroxylation product levels decreased. No metabolites were detected in the brain at 24 h. Major plasma metabolites included NMI and three hydroxylation products with t_R values corresponding to OH-CYC (b), OH-CYC (c), and OH-CYC (d) found in the liver (Figure 6, Table 2). NMI levels were maintained at 120 min, but hydroxylation product levels decreased. Finally, no metabolites were detected in plasma at 24 h. Urine contained CYC, NMI, four monohydroxylation products, $(OH)_2$-CYC, NO-CYC, NH_2-CYC, one NMI hydroxylation product (OH-NMI), and one peak of a mass corresponding to loss of an oxygen. Feces contained all the products identified in urine, except only two hydroxylation products were found.

When mice were administered NMI, the parent compound, OH-NMI, and one nitroso product (NO-NMI) were found in the liver (Figure 5, Table 2). Only the parent compound (NMI) was identified in the brain and plasma. All compound levels were greatly decreased by 120 min. Urine contained both NMI and OH-NMI, while feces only had NMI.

Metabolism by Mouse CYPs. CYC or NMI was incubated with mouse liver microsomes with and without NADPH for 30 min at 37 °C to determine if metabolites formed in an NADPH-dependent manner indicating CYP involvement. Although NMI levels from CYC were higher in the presence of NADPH compared to non-NADPH samples, this difference was not statistically significant (Supporting Figure 1 in the Supporting Information). CYC metabolites included five hydroxylation products and one $(OH)_2$-CYC peak, consistent with the in vivo products (m/z values and t_R). Oxidation products were not detected in incubations without NADPH. NMI incubations produced two hydroxylation products (NMI m/z + 16) that were formed only in the presence of NADPH (Supporting Figure 2 in the Supporting Information).

■ DISCUSSION

NMI was the first ultra-high-potency neonicotinoid,[7,18] but the photolability of the nitromethylene substituent[19] ruled against its use in agricultural pest control. Many Mannich adducts of

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NMI were highly effective proinsecticides, but IMI was developed instead because of its preferred overall properties.[9,10] Reconsideration of other NMI adducts led to the remarkable properties of CYC as a photostabilized derivative, but one which is hydrolytically unstable, leading to the possibility that CYC is a proinsecticide, chemically or metabolically converting to NMI. This hypothesis was tested here with comparative studies of CYC and NMI related to nAChR binding and metabolism.

NMI is much more toxic than CYC, and the in vitro binding affinity to the nAChR of the three species is 15−40-fold greater for NMI than CYC determined by competitive inhibition of [^{3}H]NMI binding. The approximately 10-fold lower B_{max} value for CYC than NMI with house fly head membranes is interpreted as about 10% conversion of CYC to NMI under the assay conditions. Insect brain nAChR is much more sensitive than mouse brain nAChR to NMI and CYC, providing target site selectivity. Neonicotinoids in house flies generally give nAChR inhibition correlating with their poisoning signs. However, although CYC is less potent in vitro than NMI, it gives higher in vivo nAChR inhibition than NMI for the same poisoning signs perhaps due to the higher CYC dose required, a portion of which may have hydrolyzed to the more potent NMI during analysis.

This is the first report on the metabolism of CYC. On considering metabolites in mice 15 and 120 min post-treatment, it is concluded that CYC is converted in part to NMI, but is mostly oxidized to multiple monohydroxylation products within 15 min which dissipate by 120 min in the liver. There are many possible isomeric CYC monohydroxylation products from hydroxylation on the 6-, 7-, 10-, or 11-position in each case with two possible stereoisomers, of which five are detected as distinct peaks by LC/MS. Other products include $(OH)_2$-CYC and metabolites from nitro reduction, i.e., NO-CYC and NH_2-CYC. As previously noted, minor amounts of NMI detected may be due to degradation of CYC rather than its in vivo metabolism (e.g., 24 h liver samples). NMI per se was not extensively metabolized. Only minor amounts of one hydroxylation and one nitroso product were evident. Dose considerations may limit observation of minor NMI metabolites since only 3 mg/kg NMI was administered compared to 20 mg/kg for CYC. In vitro findings confirm that the oxidation products of both CYC and NMI are formed by CYPs (in an NADPH-dependent manner). NMI formation from CYC, however, is probably due to hydrolysis during in vitro incubations and analysis rather than metabolism by CYPs.

The proinsecticide hypothesis is supported by acid lability and comparative nAChR potency but disfavored by metabolic findings in mouse and liver CYPs. The mouse is not efficient in converting injected CYC to NMI due to competing metabolic pathways as observed here, but,although not directly examined, the insect may be better at forming NMI with fewer competing pathways. In conclusion, CYC appears to serve as a proinsecticide and photostabilized slow-release reservoir for NMI as the likely ultimate active agent.

■ ASSOCIATED CONTENT

Supporting Information

Methods for nAChR binding assays in vitro and nAChR inhibition in vivo and figures showing CYC and NMI metabolism by mouse liver microsomes. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ACN, acetonitrile; CYC, cycloxaprid; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; IMI, imidacloprid; ip, intraperitoneal; LC/MS, liquid chromatography/mass spectrometry; nAChR, nicotinic acetylcholine receptor; NMI, (nitromethylene)imidazole; NH_2-CYC, aminocycloxaprid; NO-CYC, nitrosocycloxaprid; NO-NMI, nitroso-(nitromethylene)imidazole; $(OH)_2$-CYC, dihydroxycycloxaprid; OH-CYC, monohydroxycycloxaprid; OH-NMI, hydroxy-(nitromethylene)imidazole; PBS, phosphate-buffered saline, pH 7.4; SE, standard error; t_R, retention time; THI, thiacloprid

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