Original Article

Metabolism of Imidacloprid in Houseflies

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Metabolism of imidacloprid in the housefly *Musca domestica* was investigated. After the injection of ^{14}C -labeled imidacloprid, radioactivity decreased to approximately 20% of the initial level within 24 hr. Most of the ^{14}C in the excreta as well as that in the insect body was accounted for by an olefin derivative of imidacloprid. The pre-application of piperonyl butoxide or propargyl propyl phenylphosphonate as a synergist suppressed the degradation of imidacloprid and delayed the excretion of insecticidal components from houseflies. These results suggested that the insecticidal activity of imidacloprid was affected by oxidative metabolic processes.

Keywords: imidacloprid, neonicotinoids, metabolism, housefly, synergists.

INTRODUCTION

Imidacloprid (IMI) is a well-known insecticide that acts on the nicotinic acetylcholine receptor (nAChR).^{1,2)} It is one of the first members of the neonicotinoid insecticide family, which is becoming important agents for pest insect control. Many laboratories have performed toxicological studies on IMI, including its mode of action and the molecular mechanism of selective toxicity between insects and mammals.^{3–10)}

In our laboratory, we have been engaged in an analysis of the structure-activity relationship in order to elucidate the chemical basis for the expression of insecticidal activity by neonicotinoids. We measured the insecticidal activity in the housefly *Musca domestica* of a set of compounds structurally related to IMI, as well as their activity to bind an nAChR fraction prepared from housefly heads.^{11–14)} A positive correlation was found between these two activities, demonstrating that the interaction of a compound with nAChR plays a critical role in the expression of insecticidal activity. Furthermore, we quantitatively showed that the receptor binding did activate the ion channel function of nAChR.¹⁵⁾ After treatment with the insecticidal compounds, an inward flux was detected in *Xenopus laevis* oocytes expressing *Drosophila* SAD/chicken $\beta 2$ hybrid-nAChR using the voltage clamp method. The flux-inducing potency of the compounds was well correlated with their insecticidal activity. In insecticidal tests in our laboratory, it was observed that the pre-application of the synergist piperonyl butoxide (PBO) or propargyl propyl phenylphosphonate

(NIA16388, NIA) increased insecticidal activity against houseflies. Synergistic effects of NIA and PBO on the insecticidal activity of IMI in houseflies and cockroaches have been reported by three distinct research groups.^{16–18)} Since PBO and NIA are well-known inhibitors of xenobiotic transformation, it is evident that the insecticidal activity of IMI depends on the metabolism by insects. However, to date only a small number of studies have been conducted regarding the metabolic fate of IMI in insects.¹⁹⁾ Recently, the metabolism of thiamethoxam, another member of the neonicotinoid insecticide family, in *Spodoptera frugiperda* larvae has been examined.²⁰⁾ In this paper, we report the results of our study on the metabolism of IMI in houseflies, including identification of the major metabolites. Effects of the synergists on the metabolic conversion of IMI are also described.

MATERIALS AND METHODS

1. Chemicals

[Pyridinyl- ^{14}C -methyl]- and [4,5- $^{14}\text{C}_2$ -imidazolidine]IMI (4.11 MBq/mg and 1.00 MBq/mg, respectively) were kindly supplied by Bayer CropScience K.K. (Ibaraki, Japan). Radiochemical purities were over 98% and 96%, respectively. Non-labeled IMI (1) and its olefin derivative (2) were the same preparations used in our previous studies.^{11,15)} The hydroxylated IMI (3) was prepared using the method described below. 6-Chloronicotinic acid (4) was purchased from Tokyo Kasei Co. (Tokyo, Japan). 2-Nitroimino-imidazolidine (5) was synthesized according to a previous report.²¹⁾ The guanidine (6) and urea (7) derivatives were also kindly supplied by Bayer CropScience K.K. Technical grade PBO (>99%) was purchased from Wako (Kyoto,

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Fig. 1. Structures of test compounds used in this study.

For compound 1, the arrow and asterisks indicate the labeled sites in [pyridinyl- ^{14}C -methyl]IMI and [4,5- $^{14}\text{C}_2$ -imidazolidine]IMI, respectively.

Japan) and used without further purification. NIA was the same as that described in our previous reports.^{11–15,17,22)} Its purity was confirmed to be >99% by TLC analysis. The structures of the metabolites are shown in Fig. 1.

2. Insects

Houseflies (*Musca domestica*, L. Takatsuki strain) were reared at 25°C under a L12:D12 photoperiod. Four to seven day old female adult insects were used in this study.

3. In vivo Metabolism

Fifty female houseflies were anesthetized with carbon dioxide and 0.24 μl of a 50% aqueous methanol solution containing [pyridinyl- ^{14}C -methyl]IMI was injected into the dorsal side of the thorax, using a 10 μl microsyringe and a repeating dispenser (PB600-1, Hamilton Co., NV, USA). The dose in the absence of a synergist was 2 ng (8 Bq) per insect. In the presence of a synergist, the dose was reduced to 1 ng (4 Bq), because a considerable toxic effect was observed at 2 ng. Synergists were topically applied on the abdominal area at a dose of 2 ng per insect as 1 μl of methanol solution 1 hr before the administration of IMI. The treated insects were housed in an Erlenmeyer flask at 25°C. After a specified period (1, 2, 3, 6, 12 and 24 hr), the insects were washed with 2 ml of methanol and the washings were combined with the excreta fraction described below. The insect bodies were then homogenized in 30 ml of methanol with a mortar and pestle, and the homogenate was filtered under reduced pressure with the aid of an air pump (AP-220ZN, Iwaki Co., Ltd., Tokyo, Japan) to separate the extract and the residue. The extract was evaporated to 10 ml in a rotary evaporator at 35°C. One milliliter of the concentrated solution was transferred into a glass vial, and then 3 ml of Aquasol-2 (Packard, CT, USA) was added as a scintillation cocktail to be radioassayed by a liquid scintillation counter (LSC) ALOKA-1000 (Aloka Co. Ltd., Tokyo, Japan). The residual 9 ml of the extract fraction was further evaporated to about 500 μl , and analyzed by TLC.

The ^{14}C in the residue was converted to carbon dioxide by combustion in the sealed glassware, according to the

method of Saito *et al.*²³⁾ A known amount (50 mg) of the dried housefly residue was combusted with filter paper under the oxygen gas flow and the evolved carbon dioxide was absorbed in 10 ml of Carbo-sorb E (Packard, CA, USA). Five milliliters of the carbon dioxide containing solution were transferred to a glass vial, and radioassayed by LSC after the addition of 10 ml of Permafluor E⁺ (Packard, CA, USA) as a scintillation cocktail.

The radioactivity in the excreta was recovered by rinsing the glassware, in which the flies were housed, with 4 ml of methanol. At 1, 2, 3 and 6 hr, the rinsed methanol was combined with the housefly body washing (total, 6 ml) and the whole methanol solution was radioassayed by LSC after the addition of 3 ml of Aquasol-2. At 12 and 24 hr, a 1 ml portion of the methanol solution was quantified by LSC, and the residual 5 ml was concentrated to 500 μl to be analyzed by TLC.

[4,5- $^{14}\text{C}_2$ -Imidazolidine]IMI was also used for analyses of the metabolites in the absence of synergist. The applied dose was 4 ng (4 Bq) per insect. The analytical procedures were the same as those described above.

4. TLC

The metabolites were analyzed by two-dimensional TLC. The concentrated extract was applied to a precoated silica gel TLC plate (silica gel 60 F₂₅₄, 0.25 mm thickness, 20×20 cm, Merck Japan Ltd., Tokyo, Japan) and developed in two-dimensions, first with ethyl acetate/toluene/methanol/acetic acid (80:20:20:1, by volume) and then with ethyl acetate/2-propanol/water (65:23:12, by volume). The ^{14}C on the plate was exposed on a phosphor screen imaging plate (BAS-MS2040, Fuji Photo Film Co. Ltd., Tokyo, Japan) for 2–3 days and was detected using a BAS2000 Bio imaging analyzer (Fuji Photo Film Co. Ltd., Tokyo, Japan). IMI and its metabolites were identified by co-chromatography with non-labeled standard samples that were detected by UV irradiation (254 nm). The relative intensity of these radioactive spots was calculated using MacBAS ver 1.01 (Fuji Photo Film Co. Ltd., Tokyo, Japan).

5. Preparation of a Reference Sample of 3

The reference sample of 3 for the identification of metabolites by TLC analyses was prepared from IMI using the rat liver S-9 fraction. Thirty milliliters of phosphate buffer (pH 7.2) including 4.8% S-9 (Kikkoman Co., Chiba, Japan), 2 mM β -NADP, 2.3 mM glucose-6-phosphate, 10 mM potassium chloride, 1 mM magnesium sulfate, 4 mM HEPES and IMI (5 mg) was incubated at 37°C for 24 hr. The reaction mixture was extracted with CH_2Cl_2 , and the metabolite was purified by preparative TLC using the same solvent systems as those mentioned above. The purified compound was analyzed by LCMS with an ESI interface (HPLC, Shimadzu LC-10VP system; MS, Shimadzu LCMS-2010A). Analytical conditions were: HPLC column, Cadenza CD-C18 (Imtakt Co., Kyoto, Japan), 4.6×30 mm; solvent system,

water/acetonitrile/acetic acid (82/18/0.01); flow rate, 0.5 ml/min; temperature, 35°C, MS detection, positive mode; probe voltage, +4.5 kV; nebulizing gas flow, 4.5 l/min. The obtained MS was consistent with **3**. LCMS: m/z 272 ($M+H^+$), 226 ($M-NO_2$) and 191 ($M-NO_2-Cl$).

RESULTS

1. Metabolism of IMI in Houseflies in the Absence of Synergists

1.1. Excretion of IMI in houseflies

The distribution of the radioactivity in houseflies after

treatment with [^{14}C]IMI by injection in the absence of synergists is shown in Figs. 2(a) and (b) for [pyridinyl- ^{14}C -methyl]IMI and [4,5- $^{14}C_2$ -imidazolidine]IMI, respectively. The radioactivity in the housefly body rapidly decreased to about 20–30% of the applied ^{14}C within 24 hr. As the radioactivity in the insect body decreased, that in the excreta increased, amounting to 60–70% at 24 hr. The ^{14}C in the residue was below 15% at each sampling period. Approximately 100% total recovery was obtained throughout the experimental period, by summing the radioactivity in each fraction.

Fig. 2. Changes in the radioactivity in the extract, excreta and residue fractions of houseflies after injection of (a) [pyridinyl- ^{14}C -methyl]IMI. [^{14}C]IMI at the rate of 2 ng/insect, and (b) [4,5- $^{14}C_2$ -imidazolidine]IMI at the rate of 4 ng/insect.

Fig. 3. TLC analyses of the metabolites of (a) [pyridinyl- ^{14}C -methyl]IMI, and (b) [4,5- $^{14}C_2$ -imidazolidine]IMI in houseflies. Solvent systems: (I) ethyl acetate/toluene/methanol/acetic acid (80 : 20 : 20 : 1); (II) ethyl acetate/2-propanol/water (65 : 23 : 12).

1.2. Metabolite analysis and pharmacodynamics of IMI in houseflies

In the extract fraction from the housefly body, at least 6 spots including the origin were detected on TLC in the case of [pyridinyl-¹⁴C-methyl], whereas 4 spots were detected for [4,5-¹⁴C₂-imidazolidine]IMI (Figs. 3(a) and (b), respectively). The spots were identified by cochromatography with the authentic samples, with spot **A** being IMI, and **B** being **2**. On TLC for [pyridinyl-¹⁴C-methyl]IMI, **D** was identified as **4**, whereas **E** on TLC for [4,5-¹⁴C₂-imidazolidine]IMI was identified as **5**. Spot **C** was presumed to be the oxidative metabolite **3**, based on the literature.²⁴⁾ Since we had no authentic sample for this metabolite, we performed an *in vitro* experiment using the rat liver S-9 preparation for confirmation. When IMI was incubated with S-9 in the presence of NADPH for 24 hr, a spot whose *R_f* value agreed with that of spot **C** on the TLC chromatogram for [¹⁴C]IMI could be detected. When this purified compound was analyzed by LCMS, the obtained mass spectrum was consistent with that of **3**. The hydroxylated position is likely to be either at C-4 or C-5 of the imidazolidine ring, although we could not discriminate between them in this study. Spots **F** and **G** could not be characterized in this study. The guanidine and urea type metabolites, **6** and **7**, were not detected.

The amount of each metabolite produced within 24 hr after the administration is shown in Table 1. At 24 hr after the administration, about half of the administered radioactivity was detected as the olefin-derivative (50.4 (= 11.4 + 39.0) %) and 51.6 (= 16.5 + 35.1) % for [pyridinyl-¹⁴C-methyl]IMI and [4,5-¹⁴C₂-imidazolidine]IMI, respectively), of which about 70–80% was present in the excreta. Other metabolites were also detected more in the excreta.

2. Effect of Synergists on Metabolism

The effect of synergists was examined by treating the houseflies with 2 ng of PBO or NIA 1 hr before the application of [¹⁴C]IMI. The radioactivity in the housefly body and excreta was analyzed in the same manner as the treatment without the synergists. Total recovery was also about 100% in these experiments (data not shown). The changes in radioactivity in the housefly body and excreta are graphically shown in Fig. 4, together with the change in the level of IMI in the housefly body. In the presence of synergists, the elimination rate of total radioactivity in the insect bodies was evidently reduced (Fig. 4(a)). Accordingly, the excretion of IMI and its metabolites from the insect body was suppressed (Fig. 4(b)). TLC analyses showed that a large part of the radioactivity in the bodies of synergist-treated insects was accounted for by IMI (Figs. 4(a) and (c), and Fig. 5), due to the inhibition of metabolic conversion. Consequently, elimination of IMI was markedly delayed by the synergists, as shown in Fig. 4(c). The degree of the suppression by NIA was higher than that by PBO.

Table 1. The amount of each metabolite of imidacloprid from extract and excreta fractions within 24 hr after the administration using [pyridinyl-¹⁴C-methyl]IMI and [4,5-¹⁴C₂-imidazolidine]IMI^{a)}

	Radioactivity (% of total)													
	[Pyridinyl- ¹⁴ C-methyl]IMI							[4,5- ¹⁴ C ₂ -Imidazolidine]IMI						
	A 1	B 2	C 3	D 4	Origin	Others	Total	A 1	B 2	C 3	E 5	Origin	Others	Total
Extract														
1 hr	56.5±0.5	20.8±1.5	8.8±1.3	3.6±0.1	2.9±0.5	nd ^{b)}	92.7	53.5±0.8	21.1±0.3	8.0±0.7	2.4±0.3	nd	nd	86.8
2 hr	31.0±0.7	22.7±0.6	5.1±0.2	3.2±0.7	3.9±0.7	nd	65.9	37.3±4.4	29.1±1.6	4.7±0.6	4.0±1.0	1.6±1.1	0.3±0.2	78.0
3 hr	26.2±1.1	26.0±0.3	3.9±0.1	3.6±0.4	3.0±0.4	0.5±0.1	63.1	25.8±3.6	28.6±1.0	3.1±0.5	2.0±1.4	3.3±2.5	0.5±0.3	66.7
6 hr	10.7±2.6	18.8±5.9	3.3±0.2	4.6±1.4	6.3±2.9	1.4±1.0	45.0	16.8±0.0	26.0±0.0	2.8±0.0	4.7±0.4	2.4±0.5	0.5±0.1	53.8
12 hr	6.6±0.6	15.6±0.9	2.2±0.5	1.4±0.4	2.3±0.7	0.6±0.1	28.8	9.9±6.6	24.0±0.6	1.7±0.3	5.0±0.1	3.4±0.0	1.4±0.2	46.0
24 hr	1.9±0.0	11.4±0.1	0.9±0.2	2.2±0.1	2.9±1.9	2.3±0.8	21.6	6.5±1.2	16.5±0.6	0.9±0.1	4.1±0.7	0.4±0.1	2.8±0.3	31.6
Excreta														
12 hr	10.0±0.7	36.7±1.3	9.4±0.4	6.3±1.1	6.4±1.0	1.2±0.2	70.0	6.0±4.3	24.0±0.1	5.5±1.0	nd	6.0±0.8	1.0±0.3	43.0
24 hr	4.3±0.4	39.0±0.3	6.5±0.3	7.9±0.5	11.6±2.8	3.2±0.2	72.5	6.6±0.7	35.1±1.0	5.6±0.1	nd	8.3±1.5	1.6±0.2	58.3

^{a)} Mean values with SD (*n*=2).

^{b)} Not determined.

Fig. 4. Effects of synergists on pharmacodynamics of imidacloprid and its metabolites in houseflies.

(a) Total radioactivity in insect body. (b) Total radioactivity in excreta. (c) IMI in insect body. The data in the absence of synergists in (b) and (c) are taken from those in Fig. 2(a).

Fig. 5. Effects of synergists on the metabolic conversion of [pyridinyl- ^{14}C -methyl]IMI in houseflies after 6 hr.

The region around the spot of imidacloprid in the TLC chromatogram is focused on for convenience. No other major metabolite was observed in each analysis. TLC conditions are the same as those described for Fig. 3. Figures denote the metabolite numbers shown in Fig. 1.

DISCUSSION

The metabolism of IMI has been examined in animals, soil, and plants.²⁴⁻²⁷⁾ These studies found that the transformation of IMI could be categorized into the following four types: i) hydroxylation and desaturation of the imidazolidine moiety to give 3 and 2; ii) cleavage between pyridine and imidazolidine moieties to give 4 and 5; iii) reduction and cleavage of the nitroimino group to give 6 and 7; and iv) conjugation of the metabolites formed by i)–iii). Schulz-Jander *et al.* also reported the metabolism of IMI by human cytochrome P450, in which type i) and iii) transformations were observed to occur.^{28,29)} The present study demonstrated that the major metabolite of IMI in houseflies was 2. This was compatible with the results of a study *in vitro* using microsomal preparations from houseflies, in which the formation of hydroxylated IMI and olefin was observed to

occur in the presence of NADPH.¹⁹⁾ Small amounts of 4 and 5 were also detected by TLC, indicating that a minor amount of IMI underwent cleavage between the pyridine and imidazolidine moieties. The spot G detected around the origin on TLC in Fig. 3(a) suggests the formation of polar conjugates, although it was not characterized in the present study. Based on these findings, the metabolic pathways of IMI in houseflies are proposed as shown in Fig. 6.

It has been reported that the mixed functional oxygenase inhibitors NIA and PBO,³⁰⁾ enhance the insecticidal activity of IMI.¹⁶⁻¹⁸⁾ Those observations appear to be compatible with our result that IMI mainly underwent oxidative metabolism in houseflies. However, the relevance of metabolism to the toxicity of IMI in houseflies is difficult to explain. The major metabolite 2 has almost the same insecticidal activity as IMI. The affinity of 2 for nAChR in the heads of houseflies is also about the same between these

Fig. 6. Proposed metabolic pathways of imidacloprid in houseflies.

two compounds.¹⁵⁾ On the other hand, compound **3**, the intermediate in the conversion of IMI to **2**, has less activity to bind the receptor than IMI.²⁷⁾ Therefore, the major metabolic pathway of IMI in houseflies contains both detoxification and activation steps in a single conversion sequence. The enhancement of the insecticidal activity of IMI by synergists cannot be explained simply by the inhibition of metabolism.

In order to take into consideration the toxicity of **2**, we calculated the sum of the levels of IMI and **2** in the housefly body at each time point, and examined the time course of change. As shown in Fig. 7, it is evident that the treatment with synergists caused a significant delay in the drop in the total level of these two insecticidal components. This indicates that the excretion of IMI-related compounds is enhanced as a result of metabolism. In general, the excretion of a compound depends on its hydrophobicity, with the less hydrophobic compounds being excreted more efficiently. The hydrophobicity of IMI and **2** is almost the same, *i.e.* log *P* (*n*-octanol/water) values are 0.60 and 0.40, respectively, when determined by the flask shaking method.³¹⁾ Therefore, the excretion readiness is unlikely to be greatly different be-

Fig. 7. The effects of the synergists on the sum of radioactivity of IMI and **2** recovered from houseflies using [pyridinyl-¹⁴C-methyl]IMI.

tween these two compounds. On the other hand, **3**, whose log *P* is estimated to be -0.06 using *C* log *P* software,³²⁾ is more hydrophilic, so this intermediate metabolite could play an important role. The first oxidative conversion of IMI to **3** might destine the compound for some specific compartment for elimination in the insect body. Then, the following dehydration to **2** could occur away from the site of action during the elimination process, with its toxicity less significantly affecting the insect.

Although the precise mechanisms remain unknown, the present study has demonstrated that the synergistic effects of PBO and NIA are related to the oxidative metabolism of IMI with respect to the imidazolidine ring of IMI. In this relation, it is interesting to note that PBO and NIA also have synergistic effects on the insecticidal activity of acetamiprid, dinotefuran and clothianidin,^{33,34)} even though they lack the imidazolidine ring in their molecular structures. For these compounds, the oxidative process that causes the cleavage between the (perhydro)hetaryl methyl and the amidine or the guanidine moieties could play a major role in the detoxification. Recently, it has been shown that the cytochrome P450 gene, CYP 6G1, confers resistance to acetamiprid and nitenpyram (closely related to clothianidin in structure), in addition to IMI, in *Drosophila*.³⁵⁾

IMI shows greater selectivity toward insects than vertebrates. As demonstrated by a study *in vitro* using human enzyme preparations,^{28,29)} the metabolism of IMI by cytochrome P450 in principle occurs not only oxidatively, but also reductively to bring about the cleavage of the nitroimino group. The reductive pathway is considered favorable for the detoxification, for the metabolite of this pathway like **6**, has been shown to be less toxic to insects and to have weak affinity for nAChR.¹⁶⁾ In houseflies, however, we have now demonstrated that the oxidative transformation was the major pathway for the metabolism of IMI. This was essentially the same as in other animals. Therefore, the selective toxicity of IMI is unlikely to be attributed to the species difference in the metabolic pathway. It is generally accepted that the selectivity is achieved at the target site level, based on the observed differences in the binding affinity of IMI for nAChR among various animal species.^{5,18)} While the difference in the molecular nature of nAChR comprises the "basic" mechanism of selectivity, the metabolism of IMI does affect the toxicity, as indicated by the synergistic effects of PBO and NIA. Houseflies are classified into an insect group that is less sensitive to IMI. Such insensitivity might be accounted for by their metabolic activity, although the toxicity of major metabolites is apparently incompatible with detoxification. If the metabolism affects the toxicity of IMI to a great extent, it is also likely to be related to the resistance. In this regard, Byrne and co-workers recently investigated the metabolism of IMI in resistant whiteflies *Bemisia tabaci*, but failed to obtain evidence for a positive correlation between enhanced

metabolic activity and the resistance.¹⁹⁾ A quantitative evaluation of the metabolic activity in insects will be important for understanding in more detail the sensitivity/insensitivity to IMI.

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