Mode of action of novel acaricide pyflubumide: Effects on the mitochondrial respiratory chain

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This study aimed to determine the mode of action of pyflubumide, a novel acaricide under development by Nihon Nohyaku Co., Ltd. Because of its structural similarity to succinate-dehydrogenase inhibitor (SDHI) fungicides, its ability to inhibit mitochondrial complex II was investigated. Pyflubumide exhibited low inhibitory activity on spider mite mitochondria; conversely, its deacylated metabolite (NH-form) showed high mitochondrial inhibitory activity. Pyflubumide was quickly metabolized to its NH-form in the homogenate of spider mites. These results suggest that pyflubumide is a prodrug and that the NH-form is active. Indeed, the NH-form of pyflubumide and the OH-form of cyenopyrafen, a complex II inhibitory acaricide, act on the same enzyme; a double-inhibitor titration assay showed that the binding sites on mitochondrial complex II and/or the manners of binding of these compounds exhibit clear differences. This finding suggests that these two acaricides should be classified into different groups in terms of their mode of action. © Pesticide Science Society of Japan

Keywords: pyflubumide, NNI-0711, acaricide, mitochondrial complex II, Tetramychus urticae.

Introduction

Spider mites, mostly polyphagous species, are common and serious pests in modern agricultural production systems; their feeding disrupts the physiology of host plants. In addition, some spider mite species are known to be plant virus vectors. In spite of their relatively small size, phytophagous mites can cause considerable losses in crop yield and quality because they have short life spans and their populations quickly reach high abundance under favorable conditions. Therefore, the control of spider mites in crop production is important for maintaining a high crop yield; however, spider mites, including the two-spotted spider mite (Tetramychus urticae Koch), can easily develop resistance to new agrochemicals due to their biological properties, such as parthenogenesis, short generation time, and wide feeding habits.1) Therefore, new classes of acaricides without cross-resistance to existing chemicals are in continuous demand. Many acaricides have been developed with different modes of action,2) including mitochondrial complex I (NADH-quinone oxidoreductase) inhibition,3) mitochondrial complex III (quinone cytochrome c oxidoreductase) inhibition,4) ATP synthesis inhibition,5) oxidative phosphorylation uncoupling,6) octopamine receptor agonism,7) lipid biosynthesis inhibition,8) chitin biosynthesis inhibition,9) and so on. The most recently developed class of acaricides are mitochondrial complex II (succinate-quinone oxidoreductase) inhibitors, such as cyenopyrafen.10–12) For resistance management and effective and sustainable use of these compounds, it is important to execute a resistance-management strategy through rotating acaricides with differing modes of action.13)

Pyflubumide is a novel and potent acaricidal compound that was developed by Nihon Nohyaku Co., Ltd. (R=COCH(CH₃)₂, Fig. 1A). To date, no cross-resistance with existing acaricides has been found for this compound. Nevertheless, it is important to determine its mode of action to clarify its role in resistance-management strategies.

After applying pyflubumide to spider mites, we found that the compound had a relatively quick action, which led us to suppose that it may act on the nervous system or on energy metabolism through the mitochondria. From the structural point of view, pyflubumide can be classified as a carboxamide, which is a class of fungicides that act as complex II inhibitors (SDHI).14) Accordingly, we examined the inhibitory effects of pyflubumide and its metabolite (NH-form) on mitochondrial complex II obtained from two-spotted spider mites and investigated the selectivity...
on a variety of species. In addition, we compared the inhibitory mechanism of pyflubumide with that of an active form of cyenopyrafen (OH-form, Fig. 1B), which is known to be an inhibitor of mitochondrial complex II.10

Materials and Methods

1. Chemicals

Pyflubumide, its deacylated metabolite (NH-form), and the OH-form of cyenopyrafen were prepared using methods reported previously.15,16 2,6-Dichlorophenolindophenol (DCIP) and decylubiquinone (DB) were purchased from Sigma-Aldrich Co., LLC (MO, U.S.A.). All other chemicals were of analytical reagent grade and were commercially available.

2. Two-spotted spider mites and other organisms

The two-spotted spider mites (T. urticae) used in this study were reared at the research center of Nihon Nohyaku Co., Ltd., for over 20 years without any exposure to insecticides or acaricides. The common cutworms (Spodoptera litura) used in this study were also reared at that facility. The northern blowflies (Phormia terraenovae) were obtained from the animal pathology laboratory, Graduate School of Science, Osaka City University. The rainbow trout were obtained from a commercial hatchery (Takihata Lakeside Tourist Amago-Trout Fishing Place, Osaka, Japan). The rats (Sprague Dawley strain) were supplied by Japan SLC Inc. (Tokyo, Japan).

3. Preparation of the mitochondrial fraction

The mitochondrial fraction from two-spotted spider mites was prepared according to a previously reported method.13 In brief, spider mites were gently disrupted in 3 mM Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), and 0.1% bovine serum albumin (BSA) on ice and centrifuged at 1000 g for 10 min. The precipitate was homogenized in the same buffer using a Teflon homogenizer. After the homogenate was centrifuged at 1000 g for 10 min, the resulting supernatant was centrifuged at 10,000 g for 15 min. The pellet obtained was washed twice with a DTT- and BSA-free buffer. The resulting mitochondrial fraction was stored at −80°C until measuring mitochondrial complex II activity. Mitochondrial fractions from the common cutworm, northern blowfly, rat liver, and rainbow trout liver were prepared according to previously reported methods.3,17,18 The protein contents of the fractions were determined by the Bradford method using BSA as the standard.19

4. Mitochondrial complex II activity

The electron transport activity of mitochondrial complex II was determined by monitoring the coupled reduction of DCIP using succinate as an electron donor. The reaction medium consisted of 0.25 M sucrose, 1 mM MgCl₂, 2 mM KCN, 5 µM antimycin A, 50 µM DB, and 50 µM DCIP in 50 mM potassium phosphate buffer (pH 7.4). The reaction medium of 2 mL and appropriate amounts of the mitochondrial suspension and compounds dissolved in 2 µL of ethanol were combined and incubated for 5 min at 25°C. For double-inhibitor titration, the solutions of two compounds were combined and used as the test solution. The reaction was initiated by adding a sodium succinate solution to a final concentration of 10 mM; succinate solutions at prescribed concentrations were used for the kinetic study. The reduction of DCIP was determined by monitoring absorbance at 600 nm using a U-2910 UV-Vis spectrophotometer (Hitachi High-Technologies Corp., Tokyo, Japan).

5. Metabolism of pyflubumide in the spider mite

The spider mite homogenate was prepared with DTT- and BSA-free buffer according to the same methods used for the preparation of the mitochondrial fraction without the final centrifugation and washing and was used as the enzyme source. The reaction was started by adding 1 µL of pyflubumide solution in ethanol to 0.5 mL of the homogenate (0.5 µM final concentration). After a pre-fixed incubation time at 25°C, 7.3 mL of acetone and 0.7 mL of water were added to the reaction mixture, and then the mixture was centrifuged at 5600 g for 5 min. The supernatant was diluted with acetone and extracted with brine and n-hexane. Finally, the n-hexane layer was dried, reconstituted using 30% acetonitrile, and subjected to LC-MS/MS analysis.

A Prominence high-performance liquid chromatography (HPLC) machine (Shimadzu Corp., Kyoto, Japan) equipped with an XBridge™ column (3×5 mm, 2.5 µm, Waters Co., Ltd., MA, U.S.A.) was operated at a 0.3 mL/min flow rate with a gradient elution of increasing acetonitrile concentration in water containing 0.1% formic acid. The eluate from the HPLC column was introduced to a 3200 QTRAP® LC-MS/MS system (AB SCIEX, MA, U.S.A.) that was operated in the positive MRM mode. Pyflubumide and its NH-form were detected with the following transitions, m/z 536.2→155.1 and 466.3→137.2, respectively.

Results and Discussion

1. The inhibitory activity of pyflubumide and its NH-form on mitochondrial complex II, and the mechanism of selective acaricidal activity

1.1 The inhibitory activity of pyflubumide and its NH-form

The inhibition of complex II activity (i.e., succinate-DCIP oxidoreductase activity) in spider mite mitochondria by pyflubumide was examined. Pyflubumide did not exhibit any potent inhibitory activity (the inhibition rate was 27% at 1000 nM).
However, our previous studies of pyflubumide derivatives have shown that the non-N-acyl-substituted amide compounds of pyflubumide derivatives have potent acaricidal activity in vivo.\(^{20}\)

Therefore, the inhibitory activity of the deacylated metabolite of pyflubumide (i.e., NH-form, Fig. 1A) was also examined. The NH-form of pyflubumide strongly inhibited complex II activity (Fig. 2). The IC\(_{50}\) value of this NH-form of pyflubumide was 25±6.8 nM. These results suggest that pyflubumide is metabolized to its NH-form in the spider mite body and that its NH-form inhibited electron transport in mitochondrial complex II.

1.2 Metabolism of pyflubumide in homogenate of spider mites

As described above, the NH-form of pyflubumide exhibited more potent inhibitory activity than pyflubumide against mitochondrial complex II prepared from the two-spotted spider mite. We hypothesized that pyflubumide functions as a prodrug and, thus, is quickly converted to its active NH-form in the spider mite's body. To confirm this hypothesis, we performed an in vitro metabolism study of pyflubumide using whole-body homogenate of spider mites as the enzyme source. As shown in Fig. 3, at time zero, pyflubumide was the major component, accounting for more than 69% of the recovered material. Up to 15 hr after the reaction started, an almost linear decrease in pyflubumide was observed, and a corresponding increase in the NH-form of pyflubumide accounted for almost all of the observed pyflubumide decrease. In the latter phase, probably as a result of deactivation of the enzyme, the production of the NH-form of pyflubumide slowed down and was associated with a decrease in the degradation rate of pyflubumide. However, even with this long incubation period, the reduction in the NH-form of pyflubumide was not significant. To understand the metabolism of pyflubumide by spider mites in vivo, additional studies, e.g., employing \(^{14}\)C-labeled pyflubumide, are necessary; however, the results of this study clearly show that two-spotted spider mites can hydrolyze pyflubumide to its NH-form (Fig. 3). A comparative metabolism study using \(^{14}\)C-labeled pyflubumide is in progress.

1.3 Inhibitory activities of the NH-form of pyflubumide on mitochondria from organisms of various species

Pyflubumide exhibited high acaricidal activity,\(^{21}\) but it showed no activities against pests or useful insects.\(^{22}\) This compound also exhibited low acute toxicity against rats and rainbow trout (data not shown). To discuss the reason for the selective toxicity of pyflubumide, the inhibition of mitochondrial complex II by its NH-form was evaluated in a variety of species. The NH-form of pyflubumide showed no inhibition against mitochondria from organisms of species other than spider mites (Table 1). Accordingly, it is likely that the main reason for the selective toxicity in vivo is due to its high selectivity in the inhibition of its target enzyme, complex II.

![Fig. 2. Inhibition of complex II activity by the NH-form of pyflubumide.](image1)

![Fig. 3. Analysis of concentration of pyflubumide and its NH-form after incubation with a spider mite homogenate.](image2)

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<th>Table 1. Inhibitory activities of the NH-form of pyflubumide against mitochondrial complex II activity (µM)</th>
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2. Enzymatic studies of the NH-form of pyflubumide

2.1 Inhibitory mechanism of the NH-form of pyflubumide

To investigate the inhibitory mechanism of the NH-form of pyflubumide on mitochondrial complex II, the competition between succinate and the NH-form of pyflubumide was examined. Figure 4A shows double reciprocal plots of 1/ enzyme activity (V) versus 1/ succinate concentration (S) against mitochondrial complex II of the spider mite. (A): malonate; 0 (●), 10 (■), and 20 µM (▲). (B): NH-form of pyflubumide; 0 (●), 20 (■), and 40 nM (▲).

2.2 Comparison of the NH-form of pyflubumide with an existing non-competitive inhibitor, the OH-form of cyenopyrafen

Cyenopyrafen was recently developed as an acaricide and was introduced in the market. It has been reported that cyenopyrafen is activated to its OH-form (Fig. 1B) in the bodies of spider mites and that it inhibits the electron transport of mitochondrial complex II in a non-competitive manner with succinate. Therefore, we were interested in the similarity (or dissimilarity) of the mode of action between pyflubumide and cyenopyrafen. To clarify the differences in the modes of action between these two compounds, we performed double-inhibitor titration for the inhibition of complex II activity with a combination of the NH-form of pyflubumide and the OH-form of cyenopyrafen.

The concept of the double-inhibitor titration assay is briefly described below. If the binding sites of the two inhibitors are identical, the extent of their inhibition will be additive, and the maximum inhibition by one inhibitor will be attained at a lower concentration than that attained without the other inhibitor (Fig. 5A). However, if the binding sites are not identical, and there is no cooperativity between the two sites, the inhibition will not be additive, and the concentration that generates maximum inhibition by one inhibitor will be affected by the presence of the other inhibitor (Fig. 5B). In addition to those two typical cases, if the binding sites of the two inhibitors are different, but there is cooperativity between the sites, the titrations will become complicated. Figure 5C illustrates the case of negative cooperativity; namely, the extent of inhibition by inhibitor I will be reduced in the presence of inhibitor II.

Figure 6A shows the double-inhibitor titrations using the same compound (OH-form of cyenopyrafen vs. OH-form of cyenopyrafen). The inhibition was almost additive and similar to the pattern illustrated in Fig. 5A. Figure 6B shows the double-inhibitor titrations using the NH-form of pyflubumide and the OH-form of cyenopyrafen; partial inhibition (vertical axis) was attained with low concentrations of the NH-form of pyflubumide. The inhibition curves were crossed, similar to the pattern shown in Fig. 5C. These results suggest that the binding site and/or manner of binding of the NH-form of pyflubumide are not identical to those of the OH-form of cyenopyrafen.
Insight into the structural features of the quinone-binding pocket and computational chemistry studies, were conducted to gain the Qo site of complex III. The present results revealed that pyflubumide was metabolically deacetylated in spider mites and that its resultant NH-form was not identical to those of the OH-form of cyenopyrafen, though the binding sites may be close to each other. In this study, the mode of action of pyflubumide was revealed, and the differences between pyflubumide and cyenopyrafen were described. These results suggest that these two acaricides should be classified into different groups in terms of their modes of action, which may be useful for resistance-management strategies for spider mites.

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**References**