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Study on the structure and insecticide sensitivity of the RDL GABA receptor#

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In this study, a novel A2′N mutation that confers fipronil resistance was found in the membrane-spanning region M2 of the RDL GABA receptor from fipronil-resistant planthoppers in the heterozygous state. Furthermore, rapid monitoring methods using polymerase chain reaction-restriction fragment polymorphism were employed to detect fipronil-resistant planthoppers carrying the A2′N mutation. It was found that 3-benzamido-*N*-[4-(perfluoropropan-2-yl)phenyl]benzamides (meta-diamides) comprise a distinct class of RDL GABA receptor antagonists with high insecticidal activity. A membrane potential assay demonstrated that meta-diamides acted on A2′ mutant RDL GABA receptors at the same level as the wild-type RDL GABA receptor. In addition, meta-diamides may act on the M1–M3 intersubunit pocket, whereas cyclodienes and fipronil act on the A2′ residue in the pore formed by M2s. Thus, meta-diamides are expected to be effective against cyclodiene- and fipronil-resistant pests carrying A2′ mutations. Meta-diamides also exhibited high selectivity toward insect RDL GABA receptors.

Keywords: RDL GABA receptor, fipronil resistance, meta-diamide.

Introduction

The insect RDL GABA receptor is a major target of insecticides and its subunit was cloned from dieldrin-resistant *Drosophila melanogaster* and designated as *Rdl* (resistant to dieldrin).[1)] The RDL GABA receptor comprises a large N-terminal extracellular domain, four membrane spanning regions (M1–M4), three loops connecting the membrane spanning regions (M1–M2, M2–M3, and M3–M4), and a C-terminal extracellular domain (Fig. 1). The recombinant RDL GABA receptor functions as a pentameric homomer, and its pharmacological features are similar to those of native insect GABA receptors. However, it is still unclear whether native insect GABA receptors are homomeric RDL GABA receptors.[2)] The binding of GABA to the N-terminal extracellular region of the RDL GABA receptor triggers the opening of the channel and induces the selective permeation of chloride ions. Cyclodienes, such as dieldrin and α-endosulfan, and phenylpyrazole insecticides, such as fipronil, and lindane, are non-competitive antagonists (NCAs) against insect RDL GABA receptors (Fig. 2A). NCAs inhibit the GABA-induced influx of chloride ions into nerve cells, thereby causing hyperexcitation of the nervous system and the death of insect pests.

Lindane and cyclodienes, such as dieldrin and α-endosulfan, represent the first generation of NCAs. It has been reported that A2′S and A2′G mutations in M2 of the RDL GABA receptor confers resistance to lindane and cyclodienes (Fig. 1).[3–10)] Thus, the development of cyclodiene-resistant pests and a worldwide outbreak of fipronil-resistant pests are causing problems. Fipronil is a second generation NCA, but A2′S and A2′G mutations provide a low level of cross-resistance to fipronil.[9)] A homozygous A2′S mutation in the RDL GABA receptor subunit gene was found in fipronil-resistant *Oulema oryzae*, and it has been suggested that the GABA concentration is an important factor that affects the level of fipronil resistance.[10)]

The novel mechanisms of fipronil resistance acquired by planthoppers were investigated in this study, and methods were developed for monitoring fipronil-resistant planthoppers. Furthermore, the novel modes of action of meta-diamides were elucidated.

1. Mechanisms of fipronil resistance in planthoppers

1.1. Mechanisms of fipronil resistance in Laodelphax striatellus

L. striatellus is a major pest of rice, which causes severe damage by transmitting rice stripe virus and rice black-streaked dwarf

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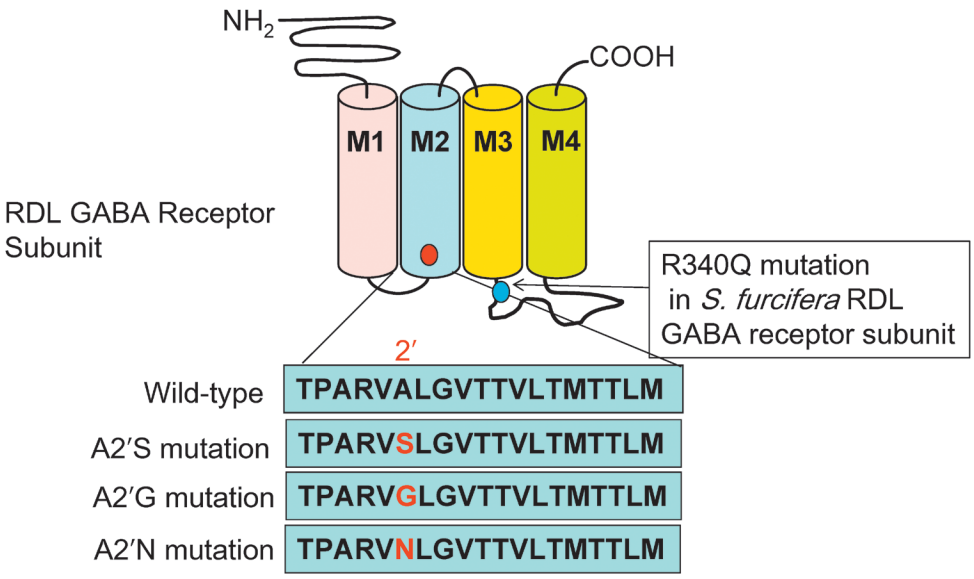

Fig. 1. RDL GABA receptor subunit and mutations conferring cyclodiene resistance or fipronil resistance.

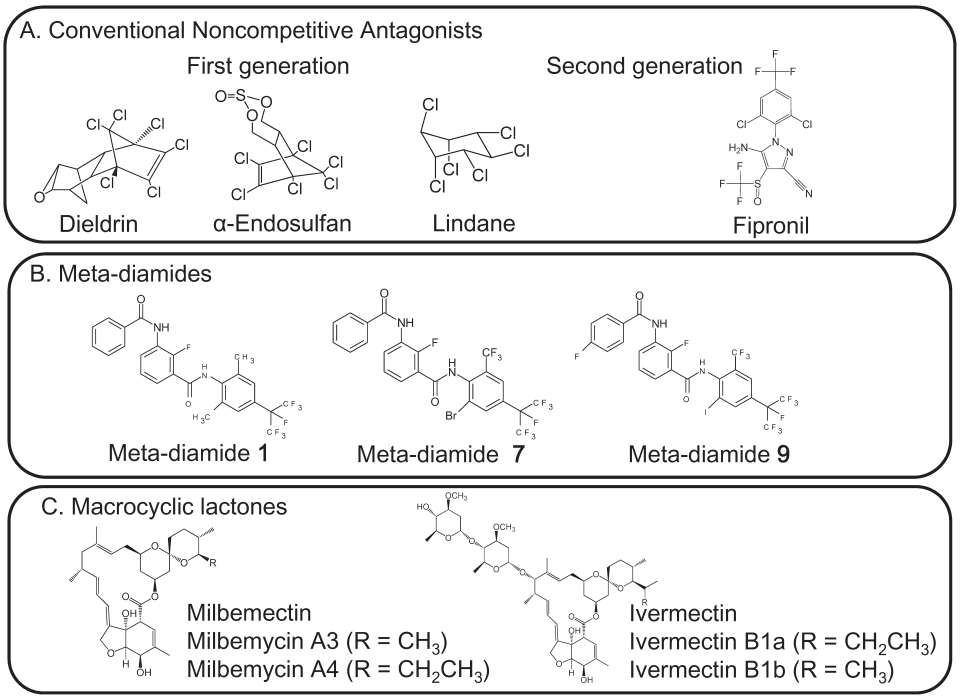

Fig. 2. Structures of conventional noncompetitive antagonists (A), meta-diamides (B), and macrocyclic lactones (C).

virus. *L. striatellus* has developed resistance to fipronil in Japan and resistance to imidacloprid in China. After the migration of imidacloprid-resistant *L. striatellus* from China to Japan, an outbreak of fipronil- and imidacloprid-resistant *L. striatellus* occurred.[11)]

Sequence analysis of RDL GABA receptor subunit genes from a fipronil-resistant *L. striatellus* population collected in Fukuoka Prefecture in 2009 identified an A2′N mutation (Fig. 1) in the heterozygous state.[12)] To confirm the role of the A2′N mutation of the RDL GABA receptor subunit, *Drosophila* S2 cells were transfected with wild-type and A2′N mutant *L. striatellus* RDL GABA receptor subunit genes, either individually or together.

A membrane potential assay showed that the half maximal inhibitory concentration (IC_{50}) value of fipronil for the wild-type *L. striatellus* RDL GABA receptor subunit homomer was 14 nM. However, the A2′N mutation abolished the inhibitory activity of fipronil in the cells transfected with A2′N mutant *L. striatellus* RDL GABA receptor subunit gene with or without the wild-

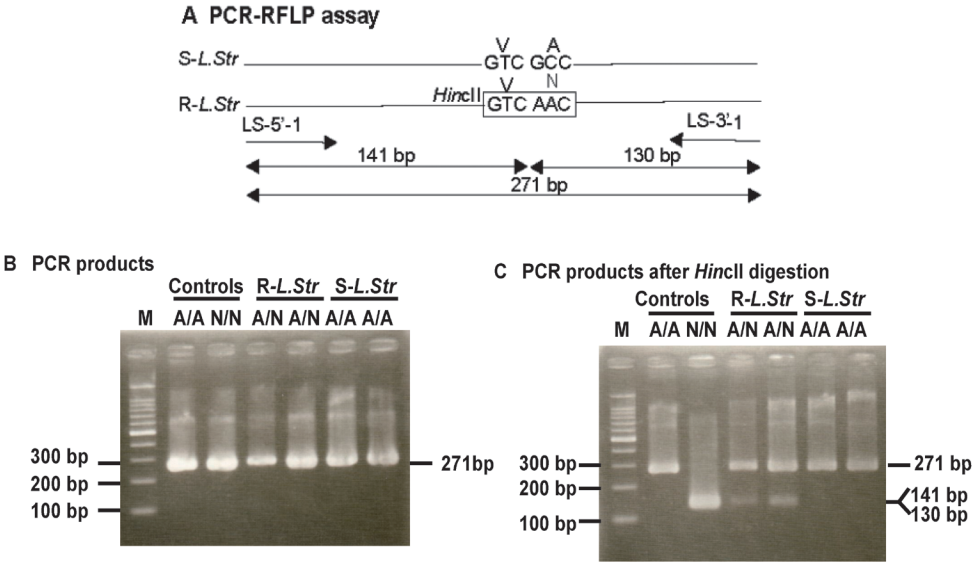

Fig. 3. PCR–RFLP assay to detect the A2′N mutation in the *L. striatellus* RDL GABA receptor subunit gene. (A) S-*L.Str* indicates the wild-type allele. R-*L.Str* indicates the A2′N mutant allele. (B and C) PCR products of fipronil-sensitive and -resistant *L. striatellus* before (B) and after digestion with *Hin*cII (C). Lane M is a 100-bp ladder. Controls A/A and N/N are PCR products amplified from plasmids containing the wild-type and A2′N mutant genes, respectively. In R-*L.Str*, A/N shows the heterozygous A2′N mutant allele. In S-*L.Str*, A/A shows the homozygous wild-type allele. (Reproduced with permission from the Pesticide Science Society of Japan).[16]

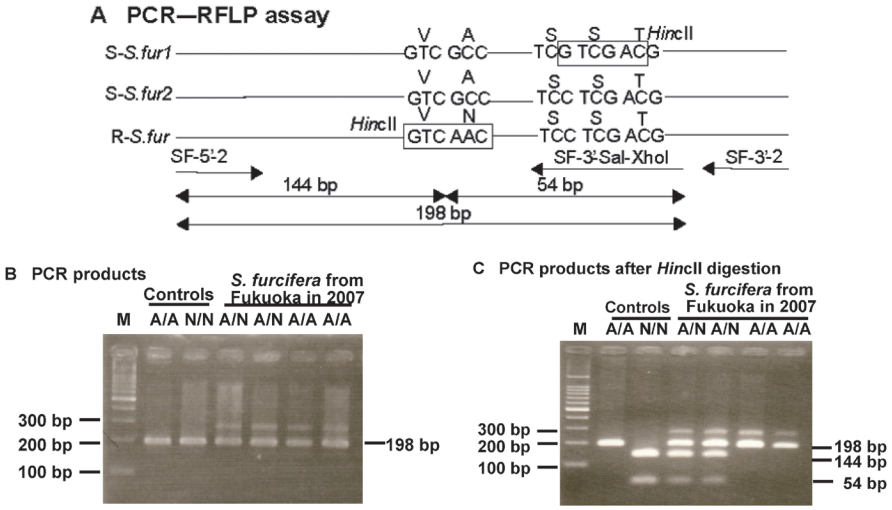

Fig. 4. PCR–RFLP assay to detect the A2′N mutation in the *S. furcifera* RDL GABA receptor subunit gene. (A) S-*S.fur1* and S-*S.fur2* indicate the wild-type alleles with the synonymous polymorphism. R-*S.fur* indicates the A2′N mutant allele. (B and C) PCR products of *S. furcifera* collected from Fukuoka Prefecture in 2007 before (B) and after digestion with *Hin*cII (C). Lane M is a 100 bp ladder. Controls A/A and N/N are PCR products amplified from plasmids containing the wild-type and A2′N mutant genes, respectively. A/N shows the heterozygous A2′N mutant allele and A/A shows the homozygous wild-type allele. (Reproduced with permission from the Pesticide Science Society of Japan).[16]

type *L. striatellus* RDL GABA receptor subunit gene.[12] Thus, the A2′N mutation in the *L. striatellus* RDL GABA receptor subunit gene appears to confer fipronil resistance.

1.2. Mechanisms of fipronil resistance in Sogatella furcifera

S. furcifera is also a major pest of rice. *S. furcifera* attacks rice plants by sucking. Recently, it was discovered that Southern rice black-streaked dwarf virus is transmitted by *S. furcifera*.[13]

Sequence analysis of RDL GABA receptor subunit genes from a fipronil-resistant *S. furcifera* population collected in Fukuoka Prefecture in 2007 identified the A2′N mutation in the heterozygous state.[14] In addition, a novel R340Q mutation was found in the cytoplasmic loop M3–M4 (Fig. 1). The R340Q mutation is always found as an A2′N·R340Q double mutation.

To confirm the role of the A2′N mutation and the A2′N·R340Q double mutation in the RDL GABA receptor subunit, *Drosophila* S2 cells were transfected with wild-type and mutant *S. furcifera* RDL GABA receptor subunit genes, either

individually or together. A membrane potential assay showed that the IC_{50} value of fipronil for the wild-type *S. furcifera* RDL GABA receptor subunit homomer was 79 nM.[15)]

RDL GABA receptors were inhibited by up to 40% with 3 μM fipronil in cells co-transfected with wild-type and A2′N mutant genes. By contrast, the RDL GABA receptors were not inhibited at all by 3 μM fipronil in cells co-transfected with wild-type and A2′N·R340Q double mutant genes.[15)] These results suggests that the A2′N·R340Q double mutation confers a higher level of resistance to fipronil than the A2′N single mutation in the heterozygous expression of the wild-type and mutant *S. furcifera* RDL GABA receptor subunit genes.

Fipronil had no inhibitory effect on cells that expressed the A2′N mutant RDL GABA receptor subunit homomer or the A2′N·R340Q double mutant RDL GABA receptor subunit homomer.[15)]

1.3. Detection of the A2′N mutation in the RDL GABA receptor subunits of fipronil-resistant planthoppers using a polymerase chain reaction-restriction fragment polymorphism assay

A rapid polymerase chain reaction-restriction fragment polymorphism (PCR-RFLP) assay was developed to detect the A2′N mutation in the RDL GABA receptor subunits from individual planthoppers, *i.e.*, *L. striatellus* and *S. furcifera*.

The A2′N mutation in the M2 region of the RDL GABA receptor subunit is caused by two nucleotide substitutions (GCC to AAC), which create a *Hinc*II restriction site. When genomic DNA was amplified from individual *L. striatellus* and *S. furcifera* carrying the A2'N mutation in the heterozygous state, *Hinc*II treatment yielded non-digested DNA and digested DNA (Figs. 3 and 4, respectively).[16)]

This assay is useful for monitoring fipronil resistance in planthoppers that carry the A2′N mutation in the RDL GABA receptor subunit and it may facilitate the management of fipronil-resistant planthoppers.

2. Mode of action of novel meta-diamide insecticides

2.1. Target of the meta-diamide insecticides

Meta-diamides (3-benzamido-*N*-[4-(perfluoropropan-2-yl)-phenyl]benzamides) (Fig. 2B) are novel insecticides produced by Mitsui Chemicals Agro, Inc.[17)]

Spodoptera litura, which is a severe pest of several crops, exhibits excitatory symptoms such as convulsions and paralysis after treatment with meta-diamides. These symptoms are similar to those induced by fipronil, so the inhibitory activities of meta-diamides against the *S. litura* RDL GABA receptor were examined.

The genotype of the *S. litura* RDA GABA receptor subunit gene was determined in *S. litura* populations from various sites in Japan, which showed that all of the *S. litura* RDL GABA receptor genes had Ser in the 2′ position.[18)] In the present study, the *S. litura* RDA GABA receptor subunit with Ser in the 2′ position is referred to as the wild-type *S. litura* RDL GABA receptor subunit.

The larvicidal activities of fipronil in *S. litura* and the inhibitory activities against *S. litura* wild-type RDL GABA receptor had a linear relationship (R^2=0.94), which suggests that the RDL GABA receptor is a toxicologically relevant target of meta-diamides.[19)]

2.2. Effects of meta-diamides and conventional NCAs on wild-type and mutant S. litura *RDL GABA receptors*

A membrane potential assay was used to determine the antagonist activities of meta-diamides and NCAs, *i.e.*, 4′-ethynyl-4-*n*-propylbicycloorthobenzoate (EBOB), picrotoxin, lindane, dieldrin, α-endosulfan, and fipronil, against *S. litura* RDL GABA receptors.

Among the conventional NCAs, fipronil had the highest antagonist activity with an IC_{50} of 105 nM.[19)] The antagonist activities of other NCAs against *S. litura* RDL GABA receptors were low, probably because the wild-type *S. litura* RDL GABA receptor has Ser in the 2′ position.[19)] By contrast, the antagonist activities of the meta-diamides against *S. litura* RDL GABA receptors were much higher than those of the NCAs, where the IC_{50} values of meta-diamides **1**, **7**, and **9** were 9.0 nM, 1.3 nM, and 3.1 nM, respectively.[19)]

The effects of S2′N, T6′V, and G319M mutations on the antagonist activities of conventional NCAs differed from those of meta-diamides **1**, **7**, and **9**.[18)] The effects of the S2′N and T6′V mutations on the antagonist activities of fipronil and α-endosulfan were great, thereby suggesting that fipronil and α-endosulfan act on the 2′ and 6′ residues in the M2 region of the RDL GABA receptor. A G319M mutation in the M3 region of the *S. litura* RDL GABA receptor had only weak effects on the inhibitory activities of fipronil and α-endosulfan. Furthermore, T6′V and S2′N mutations had no effects on the antagonist activities of the meta-diamides against *S. litura* RDL GABA receptors, whereas the G319M mutation decreased the inhibitory activities of the meta-diamides greatly. These results suggest that meta-diamides act at or near G319 in the M3 region of the *S. litura* RDL GABA receptor.

2.3. Effects of meta-diamides and conventional NCAs on the wild-type and mutant Drosophila *RDL GABA receptor subunit*

The differences between the modes of action of meta-diamides and those of conventional NCAs were clearly illustrated by studies using *Drosophila* RDL GABA receptors.

Meta-diamides **1**, **7**, and **9** inhibited the wild-type and mutant A2′S, A2′N, and A2′G *Drosophila* RDL GABA receptors (Fig. 5) with similar potency, thereby suggesting that meta-diamides will be effective against cyclodiene- and fipronil-resistant insects that carry A2′ mutations in their RDL GABA receptor.[17,19)]

These results were supported by binding studies, where [^{3}H]meta-diamide **1** was shown to have a similar level of binding potency with membranes from both dieldrin-susceptible and -resistant houseflies.[20)]

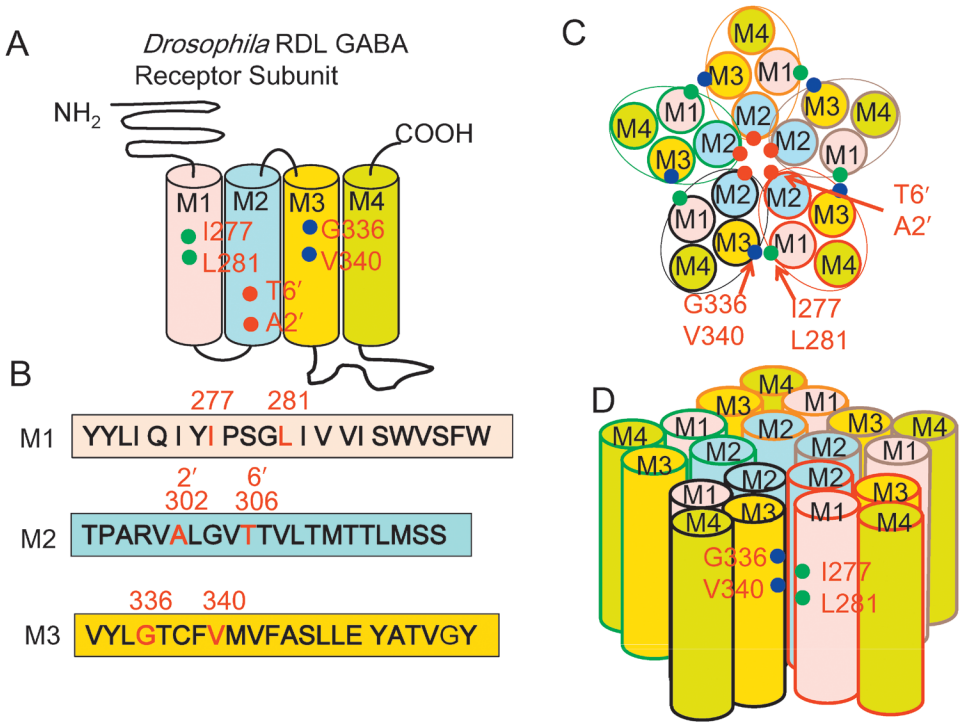

Fig. 5. *Drosophila* RDL GABA receptor subunit (A); sequences of M1, M2, and M3 (B); and top view (C) and side view (D) of the *Drosophila* RDL GABA receptor with the residues mutated in this study.

At least one of the A2′S, A2′G, or A2′N mutations decreased the inhibitory activities of each NCA greatly, while the A2′S·T6′V mutation (Fig. 5) abolished the inhibitory activities of NCAs, thereby suggesting that NCAs act on the A2′ and T6′ residues in the M2 region of the *Drosophila* RDL GABA receptor.[20]

By contrast, the A2′S·T6′V mutation had no effect on the inhibitory activities of meta-diamides, **1**, **7**, and **9**. G336 (Fig. 5) in the *Drosophila* RDL GABA receptor subunit is equivalent to G319 in the *S. litura* RDL GABA receptor subunit. The G336M mutation abolished the inhibitory activities of meta-diamides **1**, **7**, and **9**, whereas the G336M mutation had no effect on the inhibitory activities of the NCAs.[17,19] These results suggest that the binding sites of meta-diamides differ from those of NCAs.

Binding studies using houseflies showed that [^{3}H]EBOB binding was not inhibited completely by meta-diamides and that [^{3}H]meta-diamide **1** binding was facilitated by EBOB and fipronil, which suggests that allosteric interactions occur between meta-diamides and EBOB- and fipronil-binding sites.[20]

To confirm that residue 336 in the M3 region of the *Drosophila* RDL GABA receptor is important for the antagonist activities of meta-diamides, 19 of G336 mutant *Drosophila* RDL GABA receptors were created, 13 of which exhibited GABA sensitivity.[19]

All of the G336 mutations reduced the inhibitory activity of meta-diamide **7** greatly. The IC_{50} values of meta-diamide **7** for the G336A, G336S, G336C, G336H mutant homomers were $<3\,\mu$M and those of the other mutant homomers were $>3\,\mu$M.[19] By contrast, none of the G336 mutations affected the inhibitory activity of fipronil greatly,[19] which suggests that G336 is not related to the inhibitory action of fipronil. These results suggest that meta-diamide **7** acts at or near G336 in the M3 region of the *Drosophila* GABA receptor, which differs from the site of action for fipronil.

According to a homology model of the *Drosophila* GABA receptor, I277 and L281 in M1 are located close to G336 in the M3 region (Fig. 5). The inhibitory activity of meta-diamide **7** decreased by approximately six fold due to the I277F and L281C mutations. By contrast, the I277F and L281C mutations had minor effects on the inhibitory activity of fipronil. These results support the hypothesis that meta-diamide **7** binds at or near G336 in the M3 region of the RDL receptor in *Drosophila*.[19]

Meta-diamide **7** had little or no activity against G336 mutant homomers, thereby suggesting that homozygote G336 mutations confer meta-diamide resistance. Thus, do G336 mutations in the heterozygous state confer meta-diamide resistance? To answer this question, cell lines transfected with wild-type and G336 mutant *Drosophila* RDL GABA receptor subunit genes were established. Co-expression of the wild-type and G336 mutant *Drosophila* RDL GABA receptor subunit genes increased the sensitivity of GABA.[17] Excluding the cells transfected with the wild-type gene and a G336Q or G336M mutant gene, the cells transfected with the wild-type and G336 mutant genes exhibited inhibitory activities at the same level as the cells that expressed the wild-type homomers.[17] The antagonist activities of meta-diamide **7** in the cells expressing G336Q and G336M mutations in the heterozygous state decreased by approximately six fold

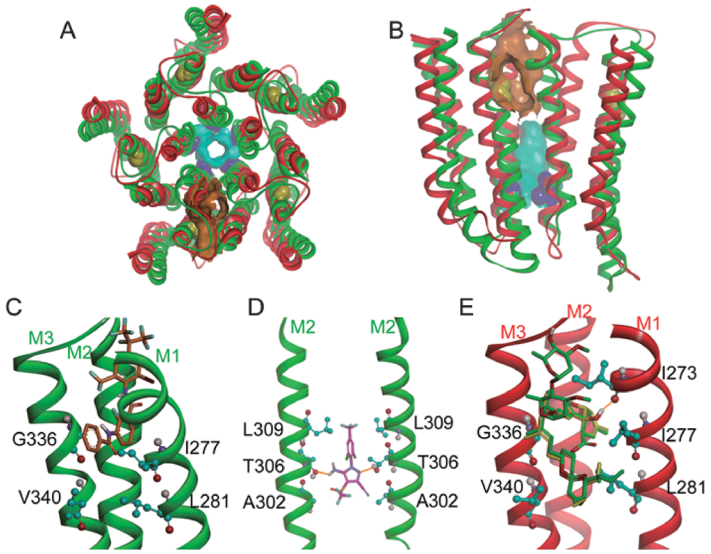

Fig. 6. Docking of meta-diamide **7**, fipronil, and macrocyclic lactones with *Drosophila* RDL GABA receptor subunit homomers. (A) Top view of the homology models of *Drosophila* RDL GABA receptor subunit homomers, where the extracellular domains have been removed for clarity. The opened and closed conformations are rendered as red and green ribbon models, respectively. G336 and A302 are shown as yellow and blue CPK models, respectively. The intersubunit cavity near G336 and the pore cavity around A302 are shown by orange and cyan surfaces, respectively. (B) Side view of the homology models of *Drosophila* RDL GABA receptor subunit homomers. For clarity, only two adjacent subunits (M1 and M3) are shown by ribbon representations. (C) The best scoring docking pose for meta-diamide **7** in the closed conformation as a stick model (carbon, orange; nitrogen, blue; fluorine, cyan; bromine, brown). (D) The best scoring docking pose for fipronil in the closed conformation as a magenta stick model. The interhydrogen bonds are shown as orange lines. (E) The best scoring docking poses for ivermectin B1a and milbemectin A4 in the open conformation. Ivermectin B1a and milbemectin A4 are shown as green and yellow stick models, respectively. (Reproduced with permission from Elsevier).[22]

and 50-fold, respectively.[17] However, these mutations require a 2- or 3-base pair mutation. Further studies are required, but these *in vitro* studies suggest that most heterozygous G336 mutations do not confer meta-diamide resistance.

2.4. *Comparison of the modes of action of meta-diamides and macrocyclic lactone insecticides on the RDL GABA receptor*

Macrocyclic lactones, such as milbemectin and ivermectin (Fig. 1C), act as agonists against glutamate-gated chloride channels (GluCl) to obtain insecticidal effects. Meta-diamide **1** had no effect on housefly GluCl, so the toxicological targets differ between macrocyclic lactones and meta-diamides.

However, binding studies showed that the binding of [^{3}H]meta-diamide **1** was inhibited completely by milbemectin and ivermectin (Fig. 1C), thereby suggesting that the binding sites of milbemectin and ivermectin overlap with those of meta-diamides in the housefly RDL GABA receptor.[20] The three-dimensional structure of *Caenorhabditis elegans* GluCl*α* with ivermectin has been resolved, which shows that ivermectin binds to the M1–M3 intersubunit pocket.[21] The M1–M3 intersubunit pocket in *C. elegans* GluCl*α* corresponds to the M1–M3 intersubunit pocket near G336 in the *Drosophila* RDL GABA receptor subunit. Therefore, the actions of meta-diamide **7** and macrocyclic lactone insecticides on the RDL GABA receptor were compared.

Membrane potential assays showed that ivermectin and milbemectin act as allosteric agonists against the wild-type *Drosophila* RDL GABA receptor.[22] Studies using multiple G336 mutations in the *Drosophila* RDL GABA receptor suggested that ivermectin and milbemectin act at or near G336 in the M3 region of the *Drosophila* RDL GABA receptor.[22] The I277F and L281C mutations are located near G336 (Fig. 5). I277F and L281C mutations decreased the inhibitory activity of meta-diamide **7** approximately six fold.[19] By contrast, the I277F mutation had only minor effects on the allosteric agonist activities of ivermectin and milbemectin, while the L281C mutation changed ivermectin and milbemectin from allosteric agonists into antagonists.[22]

In addition, V340 is located near G336 in the M3 region of the *Drosophila* RDL GABA receptor (Fig. 5). V340Q and V340N mutations changed ivermectin and milbemectin from allosteric agonists into antagonists.[22] By contrast, V340 mutations had minor effects on the inhibitory activities of meta-diamide **7**. Furthermore, the A2′ mutation changed the allosteric agonist activities of ivermectin and milbemectin into antagonist activities, although the A2′ mutation did not affect the inhibitory activity of meta-diamide **7**.[22] Thus, the effects of mutations on the activities of the macrolides differed from those of meta-diamide **7**.

2.5. *Docking studies of meta-diamide 7, fipronil, and macrocyclic lactones with* Drosophila *RDL GABA receptor subunit homomers*

Docking studies were performed using homology models of the *Drosophila* RDL GABA receptor subunit homomer in closed

and opened states, which were constructed based on the structures of the pentameric ligand-gated ion channel of *Gleobacter violaceus* (PDB ID: 4NPQ) and GluCl of *C. elegans* (PDB ID: 3RHW), respectively (Fig. 6A and 6B). G336 in the *Drosophila* RDL GABA receptor subunit homomer is important for the actions of meta-diamide **7**, ivermectin B1a, and milbemectin A4, so they were docked with these models within 20 Å of the alpha-carbon of G336. In addition, fipronil was docked with these models within 20 Å of the center of five alpha-carbons in each A2′ (A302) residue. Meta-diamide **7** docked with the M1–M3 intersubunit pocket in the closed state had the best score (Fig. 6C).[17] By contrast, fipronil docked with a pore formed by M2s in the closed state (Fig. 6D).[17]

According to the results of these docking studies, the binding site of meta-diamide **7** is separated from that of fipronil by approximately 20 Å. Thus, the binding site of meta-diamides differs from that of conventional NCAs such as fipronil.[17]

Macrocyclic lactones (ivermectin B1a and milbemectin A4) docked with the M1–M3 intersubunit pocket in the open state had the best scores (Fig. 6E), which suggests that the binding sites of milbemectin and ivermectin overlap with those of meta-diamides. However, the preferred binding conformation differs between meta-diamide **7** and macrocyclic lactones. These results suggest that the mode of action of meta-diamides differs from that of macrocyclic lactones.

3. Selectivity of meta-diamides for insect RDL GABA receptors

Compared with first-generation NCAs, such as lindane, dieldrin, and α-endosulfan, the second-generation NCA fipronil has higher selectivity for insect RDA GABA receptors than those in mammals.[23,24] Meta-diamides exhibit high antagonist activities against insect RDL GABA receptors, whereas their activities against human GABA type A ($GABA_AR$) $\alpha1\beta2\gamma2$, mammalian $GABA_AR\alpha1\beta3\gamma2$, and the human glycine receptor (GlyR) $\alpha1\beta$ are low.[25] *In vitro* studies suggest that the target site selectivity of meta-amides for insect RDL GABA receptor is higher than that of fipronil.[25]

Residue G336 in the M3 region of the *Drosophila* RDL GABA receptor subunit corresponds to residue A288 in human GlyR $\alpha1$ and M286 in human $GABA_AR$ $\beta3$, so the effects of an A288G mutation in GlyR and an M286G mutation in human $GABA_AR$ $\beta3$ on the antagonist activities of meta-diamides were studied. The inhibitory activities of meta-diamides **1**, **7**, and **9** were increased dramatically by the A288G mutation in GlyR.[25] Human $GABA_AR$ $\beta3$ is reported to be a spontaneously opened channel. Fipronil and picrotoxin blocked the spontaneous opening of $GABA_AR$ $\beta3$ in a concentration-dependent manner, whereas meta-diamides **1**, **7**, and **9** did not.[26] However, meta-diamides **1**, **7**, and **9** blocked the spontaneous opening of $GABA_AR$ $\beta3$-M286G in a concentration-dependent manner.[26] These results suggest that the residue equivalent to G336 in the insect RDL GABA receptor subunit is important for the sensitivity of meta-diamides in human GlyR $\alpha1$ and $GABA_AR$ $\beta3$.

Conclusion

This study elucidated novel mechanisms of fipronil resistance in the major rice pests, *L. striatellus* and *S. furcifera*. *L. striatellus* and *S. furcifera* have acquired fipronil-resistance by carrying an A2′N mutation. Thus, rapid monitoring methods were developed in this study using PCR-RFLP. Furthermore, the novel mode of action of meta-diamide insecticides was clarified. The site of action of meta-diamides appears to be different from that of conventional NCAs. Thus, meta-diamide insecticides are expected to be effective against fipronil-resistant pests that carry A2′ mutations. In addition, it is likely that meta-diamide insecticides are more selective against insect RDL GABA receptors than mammalian GABA receptors.

In future research, meta-diamide insecticides and novel insecticides should be tested to assess their contributions to pest control and improved agricultural production.

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