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Application of computational methods in the analysis of pesticide target-site and resistance mechanisms

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Meta-diamide insecticides including broflanilide have a high insecticidal activity by acting on RDL GABA receptors. Both membrane potential assays and docking studies suggest that the target site of *meta*-diamides is different from that of conventional noncompetitive inhibitors, such as fipronil. In fact, *meta*-diamides are effective against cyclodiene- and fipronil-resistant pests that carry target-site mutations. Dinotefuran uniquely possesses a tetrahydrofuran ring, whereas other neonicotinoids possess aromatic rings. Moreover, dinotefuran has been reported to be effective against imidacloprid-resistant strains. A docking study predicted the weak binding of dinotefuran to cytochrome P450s which are associated with imidacloprid resistance. Metabolic assays revealed that dinotefuran was not metabolized by these cytochrome P450s. These findings suggest that the lack of metabolic activity of P450s against dinotefuran causes a low level of cross-resistance.

Keywords: broflanilide, dinotefuran, GABA receptor, cytochrome P450, homology model, docking study.

Introduction

Pesticides are an essential component of food security. They help farmers grow more food on less land by protecting crops from pests, diseases, and weeds. However, the effectiveness of pesticides is threatened by the evolution of resistant strains. There are various resistance mechanisms, such as target site mutations and metabolic breakdown. The elucidation of pesticide target-site and resistant mechanisms provides fundamental knowledge about the genetics, biochemistry, and physiology of target species. In turn, these insights offer greater prospects to develop or fine-tune strategies to minimize the impact of resistance on pest management.[1)] Therefore, pesticide manufacturers must undertake extensive research to understand the action and resistance mechanism of their pesticides.

With a rapidly growing field of molecular biology and structural biology, computational science becomes a promising rational approach in the elucidation of pesticide target-site and resistant mechanisms as well as pesticide design.[2)] In addition, theoretical models using computational science have helped understand their action mechanisms.[3,4)]

In this study, a computational method was applied to elucidate the mode of actions of pesticides and their resistant mechanisms, focusing on *meta*-diamide insecticides and dinotefuran (DTF).

1. Interaction of *meta*-diamides with GABA receptors

1.1. Action site of meta-*diamides*

Broflanilide (Fig. 1), which was discovered by Mitsui Chemicals Agro Inc., has a unique chemical structure characterized as a *meta*-diamide, and it has a high activity against various pests, including Coleopteran and Thysanopteran pests.[5)] The insect ionotropic γ-aminobutyric acid (GABA) receptor is a ligand-gated chloride channel and an important target of insecticides, such as cyclodienes and fipronil. Cyclodienes and fipronil are noncompetitive antagonists (NCAs) of the GABA receptor and are classified as Insecticide Resistance Action Committee (IRAC) group 2 chemicals. *Meta*-diamides induce excitatory symptoms, such as convulsions and paralysis.[5)] Similar symptoms were observed with NCAs. A *meta*-diamide, 3-benzamido-*N*-(2-bromo-4-(perfluoropropan-2-yl)-6-(trifluoromethyl) phenyl)-2-fluorobenzamide (*meta*-diamide **7**, Fig. 1), had a higher larvicidal activity against common cutworm *Spodoptera litura* than that of fipronil.[6)] *Meta*-diamide **7** also had a higher inhibitory activity against the RDL GABA receptors of *S. litura* than that of fipronil.[6)] *Meta*-diamide derivatives show a linear

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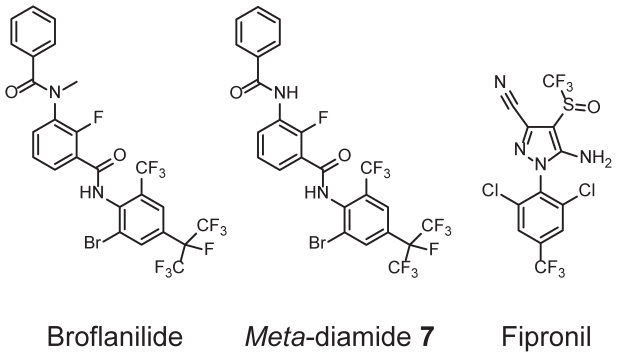

Fig. 1. Structures of broflanilide, *meta*-diamide **7**, and fipronil.

relationship (R^2=0.94) between their larvicidal activity and RDL GABA receptor inhibitory activity against *S. litura*, suggesting that the GABA receptor is the toxicologically relevant target of *meta*-diamides.[6)]

GABA receptors have five subunits; each subunit contains a large extracellular domain and four membrane-spanning regions designated M1–M4. A homology model of *Drosophila melanogaster* RDL GABA receptors (DM-RDL) was constructed to identify the binding site of *meta*-diamides.[6)] The numbering of the amino acid sequence of DM-RDL subunits is described in Fig. 4 in a study by Ffrench-Constant *et al.*[7)] NCAs are known to act on pores formed by M2s (Fig. 2A, C).[6)] A docking study suggested that *meta*-diamide **7** could act on a intersubunit pocket near the glycine residue 336 (G336) in the M3 of DM-RDL receptors (Fig. 2A, B).[6)]

Using a membrane potential assay, the effects of *meta*-diamide **7** and fipronil on mutant DM-RDLs were examined. Fipronil had a little or no inhibitory activity against both A2′N and A2′S·T6′S mutant receptors, which were reported to confer resistance to NCAs (Fig. 3A). In contrast, *meta*-diamide **7** inhibited these mutant receptors at the same level with wild-type receptors (Fig. 3B). Although G336M mutation had a little effect on the inhibitory activities of fipronil, the G336M mutation abolished the inhibitory activities of *meta*-diamide **7** (Fig. 3). Both the docking studies and the membrane potential assays suggested that the binding site of *meta*-diamides was different from that of NCAs.[6,8)]

1.2. Insecticidal activity of meta*-diamides against existing NCAs*

Pests have gained widespread resistance to NCAs *via* A2′S, A2′G, and A2′N mutations in the M2 region of GABA receptors.[9–11)] The predicted different binding sites between *meta*-diamides and NCAs suggested that *meta*-diamides will not show cross-resistance to biotypes carrying the A2′ mutations. In fact, no cross-resistance between *meta*-diamides and NCAs was observed.[12)] The resistant strains, which carry the A2′ mutations in the GABA receptor, were resistant to dieldrin and fipronil. In contrast, both *meta*-diamide **7** and broflanilide were effective against the resistant strains.[12)] Based on these findings, broflanilide was classified into a new IRAC group 30, GABA-gated chloride channel allosteric modulators.

1.3. Selectivity of meta*-diamides with mammal GABA receptors*

Although *meta*-diamides inhibited the DM-RDL receptor with a high potency, low-level inhibitory activities of *meta*-diamides have been demonstrated against the human GABA type A re-

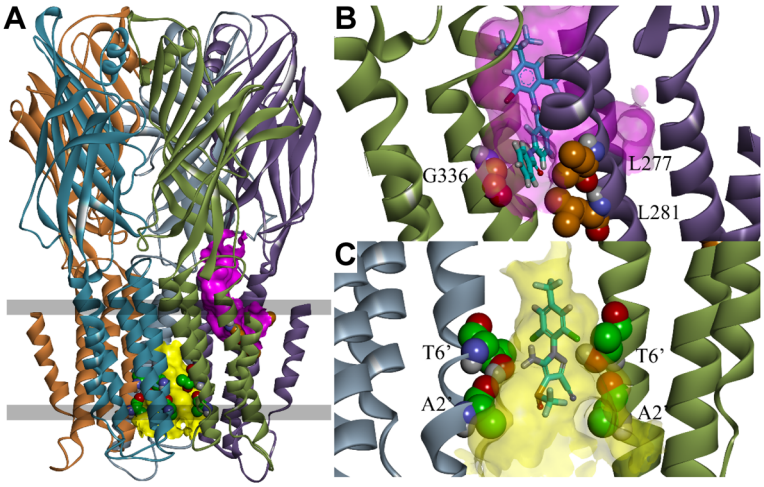

Fig. 2. Homology model of DM-RDL homomers (A) and docking of *meta*-diamide **7** (B) and fipronil (C) to the DM-RDL model. (A) View of the DM-RDL model parallel to the lipid membrane. The solvent-accessible surfaces of transmembrane intersubunit pockets and channel pores are shown in magenta and yellow, respectively. The gray horizontal bars indicate the membrane boundary. (B) The intersubunit pocket, focusing on a possible *meta*-diamide binding site. The docking pose of *meta*-diamide **7** obtained using the Glide XP mode is represented by a stick model (color code: carbon, cyan; nitrogen, blue; oxygen, red; fluorine, sky blue; bromine, brown; and hydrogen, white). For clarity, only two adjacent subunits are shown in the ribbon representation. G336, I277, and L281 are shown in the space-filling representation. (C) The channel pore, focusing on a possible NCA binding site. The docking pose of fipronil is represented by a stick model. A2′ and T6′ are shown in the space-filling representation (reproduced from Nakao *et al.*,[6)] with permission from Elsevier, Copyright 2013).

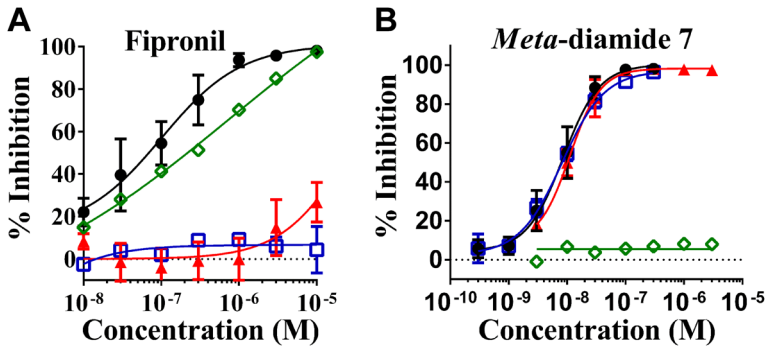

Fig. 3. Concentration-response curves of fipronil (A) and *meta*-diamide **7** (B) for wild-type and mutant DM-RDL receptors in the presence of GABA. The data are expressed as the percent inhibition of the response of wild-type (solid circle), A2′N mutant (open square), A2′S·T6′V mutant (solid triangle), and G336M mutant (open diamond) to the EC_{80} concentrations of GABA in the absence of each test compound. The vertical bars represent the standard error of the mean for three independent experiments conducted in duplicates. (modified from Nakao *et al.*,[6] with permission from Elsevier, Copyright 2013).

ceptor ($GABA_AR$) $\alpha1\beta2\gamma2S$ and $\beta3$, mammalian $GABA_AR$ $\alpha1\beta3\gamma2S$, and human glycine receptor (GlyR) $\alpha1$ and $\alpha1\beta$.[13,14] The G336 in the DM-RDL subunit is essential for the high inhibitory activity of *meta*-diamide **7**.[6] The alanine residue 288 in human GlyR $\alpha1$ and methionine residue 286 (M286) in human $GABA_AR$ $\beta3$ are the equivalent positions of G336 in DM-RDL. The equivalent glycine mutations A288G and M286G dramatically increased the inhibitory activities of *meta*-diamide **7**, indicating that the glycine residue in M3 is important for the binding of *meta*-diamides to mammalian and insect receptors (Fig. 4).[14] A homology model of human $GABA_AR$ $\beta3$ homomers in a closed state was made using human $GABA_AR$ $\alpha1\beta3\gamma2$ (PDB entry code 6HUK) as a template. As shown in Fig. 5, the M286 in human $GABA_AR$ $\beta3$ subunit is positioned at the entrance of the intersubunit pocket, and the bulky side chain of M286 reduced the size of the entrance. Based on the docking studies of *meta*-diamide **7** to human $GABA_AR$ $\beta3$ homomers using the CDOCKER module of BIOVIA Discovery Studio 2019, the interaction energies of *meta*-diamide **7** in top-scoring poses were $-42.0\,kcal\,mol^{-1}$ for the M286G mutant and $-27.4\,kcal\,mol^{-1}$ for the wild type, indicating that it was more favorable for *meta*-diamide **7** to bind to the M286G mutant than to the wild type.[14] *Meta*-diamide **7** was attached to the surface of the wild-type receptor and was not allowed to interact with the intersubunit pocket, whereas most of its parts were inserted into the pocket of the M286G mutant receptor (Fig. 5). These results suggest that the M286G mutation of human $GABA_AR$ is important to obtain a favorable interaction between *meta*-diamide **7** and the intersubunit pocket. The amino acids of human $GABA_ARs$ at positions equivalent to the G336 of DM-RDL are not glycine residues, except for $GABA_AR$ π, whose amino acids around the intersubunit pocket had a low similarity with those of insect GABA receptors.[14] Thus, *meta*-diamides are expected to be highly specific for insect GABA receptors.

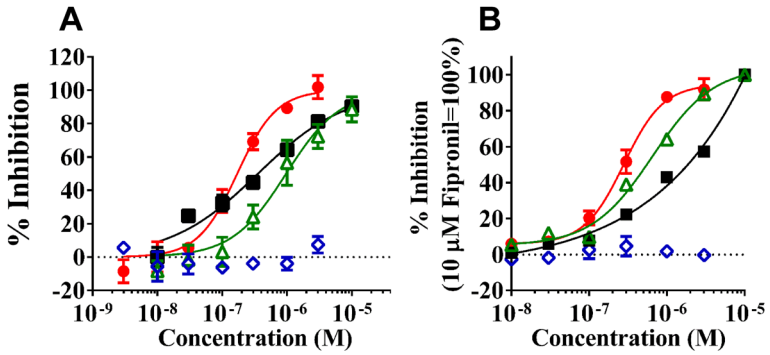

Fig. 4. Concentration-response curves of human GlyR $\alpha1$ (A) and human $GABA_AR$ $\beta3$ receptors (B) to fipronil (wild type, solid square; Gly mutant, open triangle) and *meta*-diamide **7** (wild type, open diamond; Gly mutant, solid circle). (A) The data are expressed as the percent of inhibition of the GlyR response relative to the EC_{80} values of glycine in the absence of each test compound. (B) The percent inhibition at $10\,\mu mol\,L^{-1}$ of fipronil was assumed to be 100%. The error bars represent the standard error of the mean for three independent experiments conducted in duplicates. ([A] modified from Nakao and Hirase[13] with permission from the Pesticide Science Society of Japan, Copyright 2014; [B] modified from Nakao and Banba[14] with permission from Wiley, Copyright 2020).

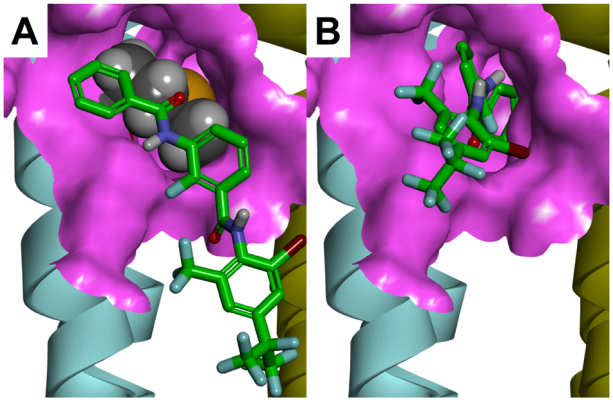

Fig. 5. Docking of *meta*-diamide **7** to human $GABA_AR$ $\beta3$ homomers (A) and $\beta3$-M286G homomers (B). The intersubunit pocket of the human $GABA_AR$ $\beta3$-M286G mutant is shown with a magenta solvent-accessible surface representation. Methionine 286 is represented by the space-filling model (color code: carbon, gray; hydrogen, white; and sulfur, orange). Only two adjacent subunits are shown in pale blue and deep yellow in the ribbon representation. The top-scoring docking poses of *meta*-diamide **7** in the human $GABA_AR$ $\beta3$ model (A) and $\beta3$-M286G model (B) are shown in the stick representation (color code: carbon, green; nitrogen, blue; oxygen, red; fluorine, pale blue; bromine, brown; and hydrogen, white).

2. Cross-resistance of other neonicotinoids to imidacloprid-resistant strains

2.1. Background of imidacloprid-resistant strains

Imidacloprid (IMI) showed an excellent efficacy against globally important crop pests, such as the peach–potato aphid *Myzus persicae*, whitefly *Bemisia tabaci*, and brown plant hopper *Nilaparvata lugens* (Stål).[15] However, the intensive use of IMI resulted in the development of IMI resistance.[15] The most common mechanism of IMI resistance is the overexpression of cytochrome P450 monooxygenase enzymes (CYPs). The overexpression of CYP6CM1 and CYP6ER1 confers IMI resistance to *B. tabaci* and *N. lungens*, respectively.[16–18] In *M. persicae*, both CYP6CY3 overexpression and target-site mutation (R81T) confer IMI resistance.[19–22] Although these pests showed cross-resistance to other neonicotinoids, DTF has been reported to be effective against IMI-resistant strains.[23–28]

2.2. Binding affinity of DTF to cytochrome P450s that confer IMI resistance

Homology models of CYP6CM1 and CYP6ER1 were built based on human CYP3A4 (PDB entry code 4D75).[29] Glide was used to estimate the binding poses and scores of IMI and DTF for the active site of these CYPs. As shown in Fig. 6, the active sites of these CYSs are hydrophobic pockets, suggesting that hydrophobic compounds are preferred for binding. The Glide scores of IMI and DTF are −4.9 and −3.5 for CYP6CM1 and −5.4 and −3.5 for CYP6ER1, respectively. According to the Glide scores, the binding of DTF to these CYPs was weaker than that of IMI, which is consistent with the previous calculations on CYP6CM1.[30] To confirm this prediction experimentally, a competition between a substrate and IMI or DTF for CYP6CM1 was evaluated using Luciferin-MultiCYP as a nonselective CYP450 bioluminescent substrate.[31] IMI at a concentration of 640 μM completely inhibited CYP6CM1-catalyzed Luciferin demethylation, whereas DTF at the same concentration only produced a weak inhibition.[31] These results suggested that the differences in resistance level between IMI and DTF may be explained by the weaker binding of DTF to the CYPs associated with IMI resistance.

2.3. Metabolism of neonicotinoids by cytochrome P450s responsible for IMI resistance

The metabolic activities of *M. persicae* CYP6CY3 and *B. tabaci* CYP6CM1 variants against DTF and other neonicotinoids (Fig. 7) were evaluated using *Drosophila* S2 cells stably expressing these CYPs.[31,32] After 2 days of incubation in S2 cells stably expressing the CYP6CM1 variants, IMI was decreased by three CYP6CM1 variants, and hydroxyl-IMI was generated.[31] Clothianidin was also metabolized by these CYP6CM1s, but DTF and thiamethoxam were not metabolized (Fig. 8A). After 4 days

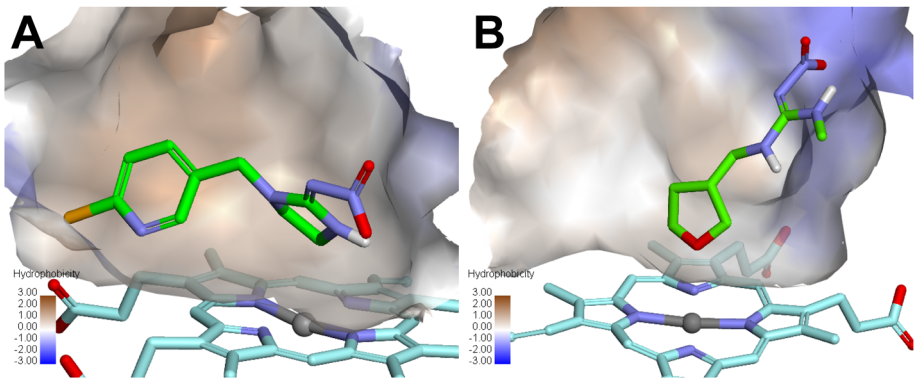

Fig. 6. Docking of IMI to the CYP6CM1-vQ model (A) and of DTF to the CYP6ER1+del3 model (B). The solvent-accessible surfaces of the active site are shown with colors based on residue hydrophobicity (color code: blue, hydrophilic; and brown, hydrophobic). For clarity, only the heavy atoms and polar hydrogen atoms in the ligands and heme are shown in the stick representation (color code: heme's carbon, pale blue; ligands' carbon, green; oxygen, red; nitrogen, blue; chlorine, orange; hydrogen, white; and iron, gray).

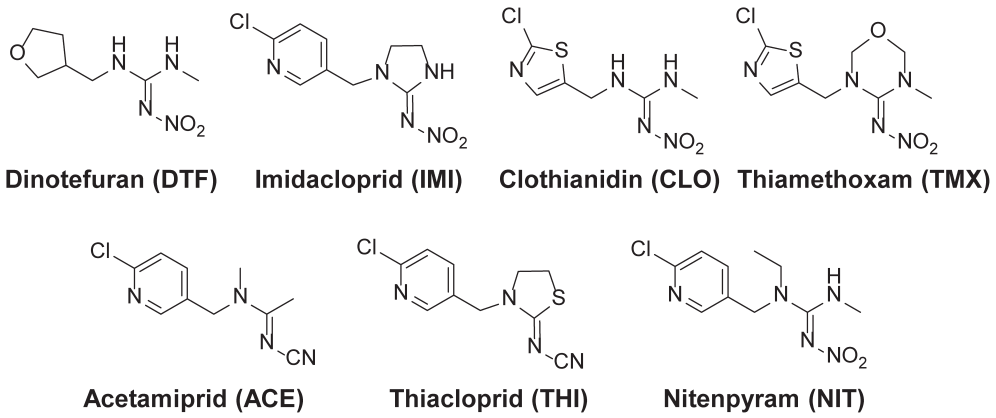

Fig. 7. Structures of neonicotinoids.

of incubation, CYP6CY3 showed a metabolic activity against IMI, acetamiprid, clothianidin, and thiacloprid, but it had no activity against DTF (Fig. 8B).[32] The metabolism of neonicotinoids was also measured using the microsomes prepared from Sf9 cells expressing the CYP6ER1 variants.[33] After 2 hr of incubation, both CYP6ER1 variants metabolized IMI, acetamiprid, and thiacloprid, but they did not metabolize DTF (Fig. 9).[33] One of the CYP6ER1 variants metabolized clothianidin and thiamethoxam. Nitenpyram was metabolized slightly by one variant. Comparing the octanol-water partition coefficient (log P) shown in Fig. 9, hydrophilic compounds are less likely to be metabolized, which is consistent with human CYPs.[34] These findings indicate that IMI-resistant biotypes carrying the overexpression of these CYPs will not have a strong resistance to DTF. However, recent studies, which used *N. lugens* selected by neonicotinoids, indicated that CYP6ER1 confers cross-resistance to most neonicotinoids, including DTF.[35–38] Our results indicated that CYP variants showed a different substrate selectivity (Figs. 8, 9). Many CYP6ER1 variants have also been reported,[18] and one of these variants might be at risk to confer high resistance to DTF. Therefore, it is important to determine which variant is associated with resistance to each neonicotinoid and to continuously monitor the susceptibility of field strains to each neonicotinoid.

Concluding remarks

In this study, the novel mode of action of *meta*-diamide insecticides was demonstrated by combining *in vitro* assays and computational methods. A glycine residue in M3 is important for the high selectivity of insect GABA receptors compared with those of mammals. It was also reported that the CYPs associated with IMI resistance do not metabolize DTF, probably because a highly hydrophilic DTF is a weak binder for these CYPs. Computational science is a promising rational tool to elucidate pesticide target-site and resistant mechanisms by combining the rapidly growing field of molecular biology and structural biology.

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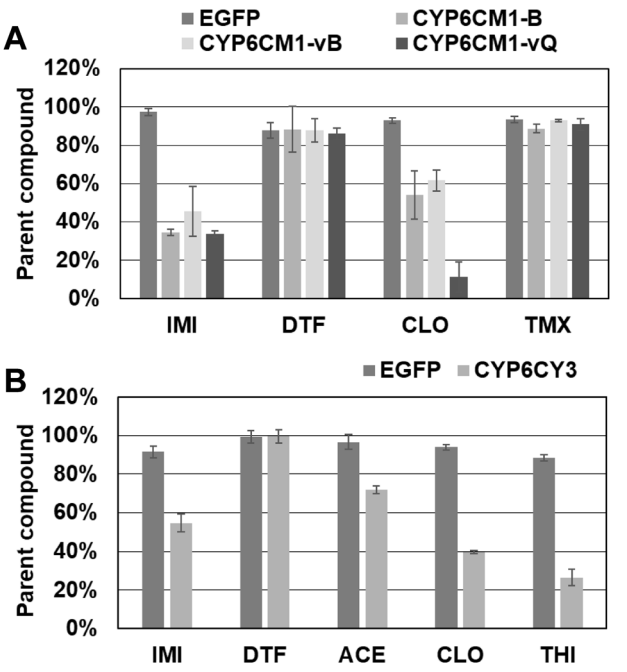

Fig. 8. Metabolism of neonicotinoids by S2 cells stably expressing EGFP, CYP6CM1 variants (A), and CYP6CY3 (B) (GenBank accession number: EGFP, U55762; CYP6CM1-B, GQ214539; CYP6CM1-vB, EU642555; CYP6CM1-vQ, EU344879; CYP6CY3, and HM009309). The experiments were performed in duplicates thrice. The data are expressed as mean±standard deviation. ([A] modified from Hamada *et al.*,[31] with permission from Elsevier, Copyright 2019; [B] modified from Nakao *et al.*,[32] with permission from the Pesticide Science Society of Japan, Copyright 2019).

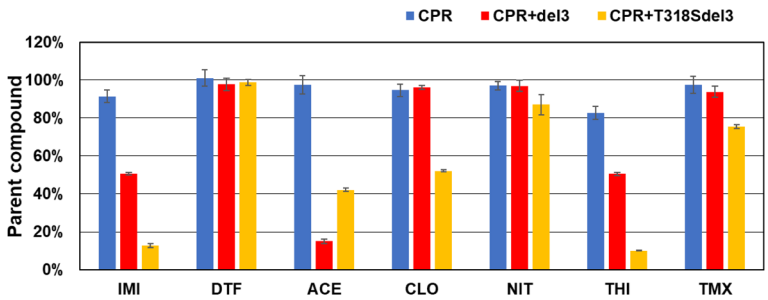

Fig. 9. Metabolism of neonicotinoids by microsomes prepared from Sf9 cells expressing NADPH cytochrome P450 reductase (CPR), CPR and CYP6ER1+del3, and CPR and CYP6ER1+T318Sdel3 (GenBank accession number: CPR, X93090; CYP6ER1+del3, JF928994). The experiments were performed at least in triplicates. The data are expressed as mean±standard deviation. The Log*P* values obtained from the Pesticide Properties Database are as follows: IMI, 0.57; DTF, −0.549; ACE, 0.8; CLO, 0.905; NIT, −0.66; THI, 1.26; and TMX, −0.13. (ver.200422, University of Hertfordshire, UK). (modified from Hamada *et al.*,[33] with permission from Elsevier, Copyright 2020).

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