Title: Glycine at the third position of TM3 determines the action of fluralaner on insect and rat GABA receptor

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BACKGROUND AND PURPOSE

Fluralaner is a novel isoxazoline insecticide with broad insect spectrum, and mainly acts on the insect GABA receptor with unique binding action, but its molecular interaction with insect GABA receptor has not been deeply identified on molecular level according to its selectivity between target (insect) and non-target (mammal) organisms.

EXPERIMENTAL APPOACH

The potential binding residues (I258T and L275I in TM1; V288I, M298N, G303N and A304S in TM2; G3'M/S, A327S, G336N, M338I and A339F in TM3; M473V and I477D in TM4) were predicted by SYBYL-X 2.1 software, and verified respectively by the site-directed mutagenesis and two-electrode voltage clamp (TEVC) technique.

KEY RESULTS

In the 11 predicted amino acids, the G3'M has the strongest ability to reduce the sensitivity of recombinant rice stem borer RDL homomeric channel to fluralaner. Compared with the wild-type (WT)-RDL, the G3'M mutation almost completely abolish the binding of fluralaner and avermectin, but not fipronil on recombinant homomeric channel of RDL from several orders of insects *in vitro*. In addition, the M3'G on rat *Mus musculus* β2 improved the sensitivity of recombinant heteromeric *Mm*α1β2-M3'G channel to fluralaner. Our results demonstrated that the glycine at the third position of TM3 determines the action of fluralaner and should be the binding site of fluralaner with RDL.

CONCLUSION AND IMPLICATIONS

These results would contribute to understanding the molecular interaction of fluralaner with RDL homomeric channel and may be used to guide future modification of isoxazolines to achieve highly selective control of pests with minimal effects on non-targeted organisms.

Abbreviations:

resistant to dieldrin (RDL);

γ-aminobutyric acid (GABA);

transmembrane (TM);

transmembrane subunit interface (TSI);

median inhibition concentration (IC_{50});

median effective concentration (EC_{50});

confidence interval (CI);

dimethyl sulfoxide (DMSO);

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Introduction

Insect γ-aminobutyric acid (GABA) receptor (GABAR) is one of the major insecticide targets from the mid to late 1900s with billions of pounds of polychlorocycloalkanes and millions of pounds of fiproles (Casida et al., 2015). However, mutation of alanine (302) at second transmembrane (TM2) of resistant to dieldrin (RDL) subunit have lead the insect generate high resistance level to fipronil (A302N) and dieldrin (A302S) (ffrench-Constant et al., 1990; Nakao et al., 2011; Casida et al., 2015). What makes people excited is that both meta-diamide and isoxazoline insecticides also act on the insect GABA receptor have been marketed in the world in recent years.

Fluralaner, as an representative isoxazoline insecticide, is first synthesized by Lahm (Lahm et al., 2011) and reported as A1443 by Professor Ozoe research team (Ozoe et al., 2010). Fluralaner acts at previously unrecognized site(s) on RDL homomeric channel without cross-resistance to other non-comparative antagonists (NCAs) including fipronil and dieldrin (Casida et al., 2015). To explore the detailed binding site(s) of fluralaner interacting with insect GABAR, Nakata et al. (2017) replaced the distinct amino acid residues in the transmembrane subunit interface (TSI) between the housefly *Musca domestica* glutamate-gated chloride channels and GABACls, and found that the amino acid (Leu315) in *Musca* glutamate-gated chloride channel plays a significant role in determining the selectivity of fluralaner (Nakata et al., 2017). After three years, Yamato et al. (2020) substituted amino acid residues in the TSI of the *M. domestica* GABAR with various amino acids, and found that four amino acids (Gln271, Ile274, Leu278, and Gly333) in the TSI are associated with the high sensitivity of GABARs to fluralaner (Yamato et al., 2020). Except those, it is worth to noting that fluralaner has been marketed as parasiticide for flea and tick control in mammals including dog and cat (Taenzler et al., 2014; Dryden et al., 2015; Zhao et al., 2015), which indicated that some important differences between insects and mammals GABA receptor determined the selective toxicity and mechanistically based safety (Ozoe et al., 2010; Casida, 2015; Nakao et al., 2016).
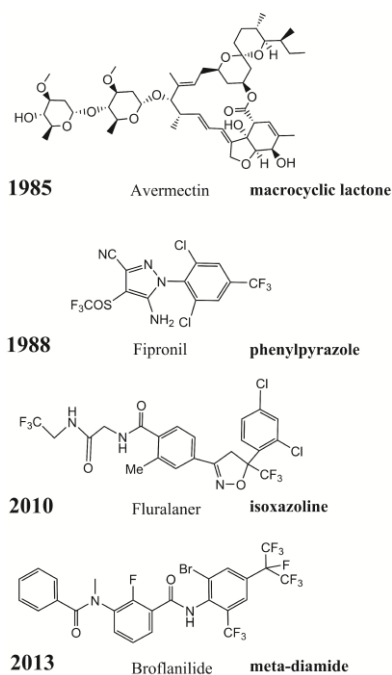
Fig. 1 Structures of GABAergic noncompetitive antagonist and allosteric modulator pesticides
Date represented the year for discovery or first introduction.

Therefore, we explored the molecular differences between the insect RDL subunit and mammal subunit of GABA receptor using molecular docking, and characterized the potential amino acid residues in rice stem borer *Chilo suppressalis* RDL homomeric channel of fluralaner binding. RDL genes from various species of insects including honeybee (*Apis mellifera* L.), *C. suppressalis*, two spotted mite (*Tetranychus urticae* Koch), fruit fly (*Drosophila melanogaster*), and small brown planthopper (*Laodelphax striatellus*) were functionally evaluated in African frog *Xenopus laevis* oocytes using site-directed mutagenesis and two-electrode voltage clamp (TEVC) electrophysiology to identify the common of mutant site(s). In contrast, the amino acid residue in the rat *Mus musculus* GABA receptor subunit was replaced with the positionally equivalent amino acid of *Chilo* GABACls to verify the potential amino acid residue. At last, we established that receptor-site-dependent residues in the insect RDL homomeric channels to fluralaner. Knowledge gained from this study may be used to broadly guide further modification of isoxazoline to achieve highly selective control of insect pests with minimal effects on mammals.

Results

1. Prediction of potential binding sites

According to the alignment of the amino acid sequences of GABA receptor from different kinds of animals, 35 amino acid residues (8 in TM1, 9 in TM2, 11 in TM3, and 7 in TM4) were selected (**Fig. S1, Table S1** and **Text S1**). Subsequently, 11 potential binding residues (2 in TM1, 4 in TM2, 4 in TM3 and 1 in TM4) and 2 negative residues (1 in TM3 and 1 in TM4) were selected based on the increase of binding energy of the fluralaner with exchange amino acid residues between mammal and arthropod GABAR subunit. To

facilitate the alignment of GABAR subunits from different species, the nomenclature, which is used for second transmembrane (TM2) of insect RDL (Chen *et al.*, 2006), was used in this study. According to this nomenclature, the first residue at the left of third transmembrane (TM3) of RDL is designated as "0" (**Fig. 2**). It is worthy to note that the glycine (G3') in TM3, which is responsible to the avermectin binding on glutamate-gated chloride channel (Nakata *et al.*, 2017), is conserved in RDL from five orders of arthropod. In contrast, at the corresponding position, the methionine (M) is found in the β subunits of mammals (**Fig. S1**). To determine the role of G3' in sensitivity to fluralaner, the G3' was substituted with M (G3'M) and serine (G3'S) in the arthropod RDL homomeric channels.
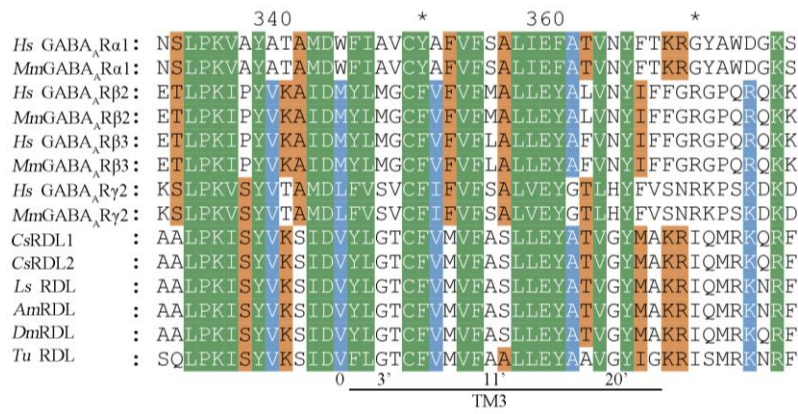

Fig. 2 Alignment of TM3 and the flanking amino acid sequences of GABAR subunit from mammals and insects

Note, the subunit-specific number is given at the left for the first residue of each aligned sequence and the index numbers for positioning in TM3 are shown at the top. The GenBank number were *Hs*GABA$_A$R α1 (NP_001121120.1), *Mm*GABA$_A$R α1 (NP_034380.1), *Hs*GABA$_A$R β2 (NP_000804.1), *Mm*GABA$_A$R β2 (NP_001334243.1), *Hs*GABA$_A$R β3 (NP_068712.1), *Mm*GABA$_A$R β3 (NP_001033790.1), *Hs*GABA$_A$Rγ2 (AAH74795.1), *Mm*GABA$_A$Rγ2 (NP_032099.1) *Cs*RDL1 (ASY91961.1), *Cs*RDL2 (ASY91962.1), *Ls*RDL (BAF31884.1), *Am*RDL (ANC68177.1), *Dm*RDL (NP_523991.2), *Tu*RDL (BAJ41377.1)

2. Electrophysiological response of WT and mutant CsRDL homomeric channel to GABA

In the 14 mutant *Cs*RDL homomeric channels expressed in *X. laevis* oocytes, the I477D cannot be elicited inward chloride current under GABA-stimulation in two-electrode voltage-clamp (TEVC) assay (**Fig. 3A**). The GABA-induced current (I_{max}) was weaker in the mutant channels except G3'M than those in WT RDL homomeric channel. Except M473V and I477D, other mutations did not alter the concentration-dependence of activation of the *Cs*RDL homomeric channel, but caused a polarizing right shift in the concentration dependence (**Fig. 3A**). The EC_{50s} values of other 11 mutant *Cs*RDL homomeric channels showed 1.48- to 63.97- fold compared with WT RDL homomeric channel (**Table S2**). The EC_{50} values of G3' mutations of G3'M and G3'S were the highest and showed 34- and 64-fold increase compared with WT in the mutant channels (**Fig. 3A**). In TEVC assay, fluralaner inhibited GABA-induced currents in WT *Cs*RDL homomeric channel (**Fig. 3B**), and yielded a dose-response curve with a median inhibition concentration (IC_{50}) of 4.2 nM (**Table S3**). Surprisingly, the G3'M substitution drastically reduced > 2389-fold sensitivity of *Cs*RDL homomeric channels to fluralaner. In contrast, the double-mutation (MA338-339IF)

increases the sensitivity of *Cs*RDL homomeric channel to fluralaner. The IC$_{50}$ values of other substitutions except G319M exhibited 0.33-5.90 folds increase than that of the WT *Cs*RDL homomeric channel (**Fig. 3B** and **Table S3**).
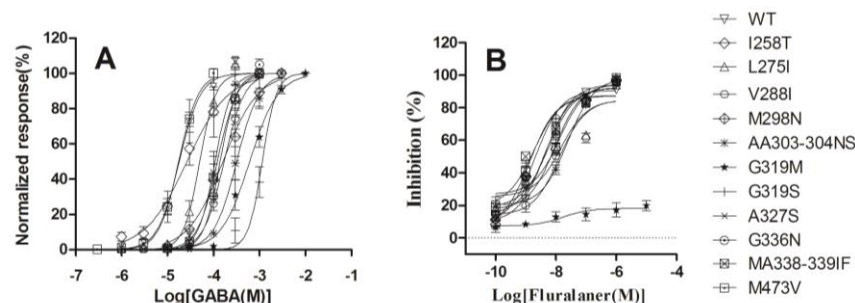

Fig. 3 Effect of mutations on the sensitivity of the *Cs*RDL channels to GABA (A) and fluralaner (B)

3. G3'M in TM3 contributes sensitivity of *Cs*RDL homomeric channel to avermectin

Both fipronil and avermectin strongly inhibited the GABA-induced in WT *Cs*RDL homomeric channel with an IC$_{50}$ of 10.02 and 69.90 nM, respectively (**Fig. 4** and **Table S4**). The WT *Cs*RDL homomeric channel showed more sensitive to fipronil, fluralaner than avermectin (**Table S4**). However, the fluralaner at 10^{-5} M cannot be able to half inhibit the GABA-induced current in G3'M RDL homomeric channel, whereas the IC$_{50}$ of fipronil is 22.42 nM (**Fig. 3** and **Table S4**).
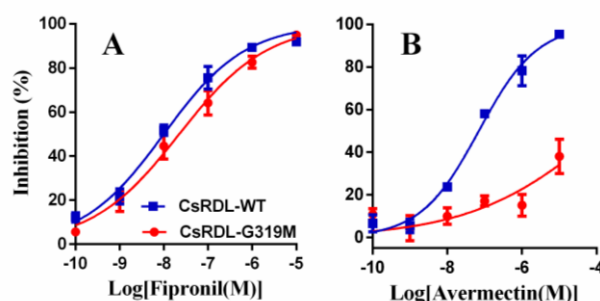

Fig. 4. Inhibition of avermectin and fipronil to GABA-induced current in *Cs*RDL-G3'M homomeric channel

4. G3'M in TM3 contributes the resistance to fluralaner in insect RDL homomeric channels

To examine whether a similar potency enhancement would be observed for the G3'M of RDL from different arthropod species, the mutation of G3'M was performed in RDL from Hymenoptera (*A. mellifera*), Hemiptera (*L. striatellus*), Arachnoidea (*T. urticae*), Lepidoptera (*C. suppressalis*), and Diptera (*D. melanogaster*). The potency of G'3M RDL homomeric channel to GABA decreased 8.0 - 34 folds compared with that in the WT RDL homomeric channel (**Fig. 5A**). The fluralaner at 10^{-5} M showed almost same and weak inhibition potency to mutant arthropod RDL (G3'M) homomeric channels (**Fig. 5B**).
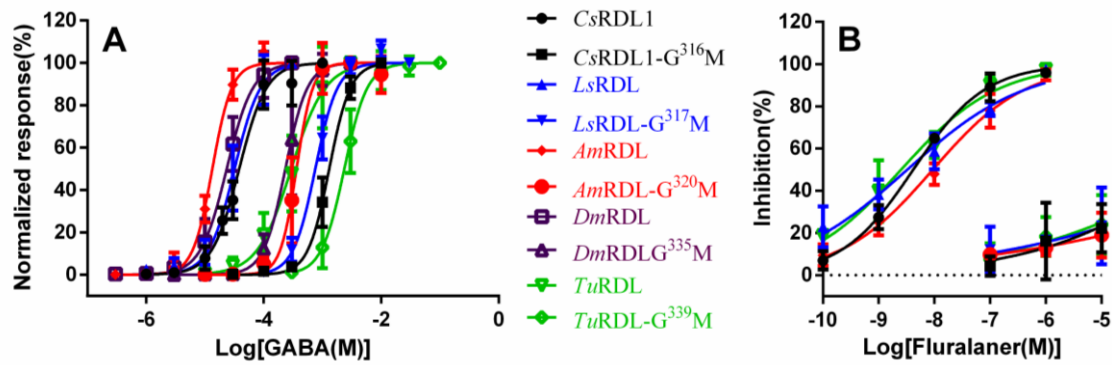
Fig. 5 Effect of the sensitivity of the arthropod G3'M-RDL channels to GABA (A) and fluralaner (B)

5. M3'G in TM3 affects the sensitive of $Mm\alpha1\beta2$ heteromeric channel to fluralaner

To verify the contribution of G3' in mammal GABA receptor to fluralaner sensitivity, the mutation of M3'G was performed in $Mm\beta2$ subunit. There is no detectable response in oocytes injected $\beta2$ or $\beta2\text{-M}^{310}\text{G}$ alone. However, the co-expression of $Mm\alpha1\beta2$ and $Mm\alpha1\beta2\text{-M}^{310}\text{G}$ could form functional GABA-gated heteromeric channel with EC_{50} values of 9.18 μM and 3.25 μM (**Fig. 6A and Table S6**), respectively. Fluralaner could inhibit the GABA-induced currents more efficiency in heteromeric $Mm\alpha1\beta2\text{-M}^{310}\text{G}$ than $Mm\alpha1\beta2$ (**Fig. 6B** and **Table S7**).
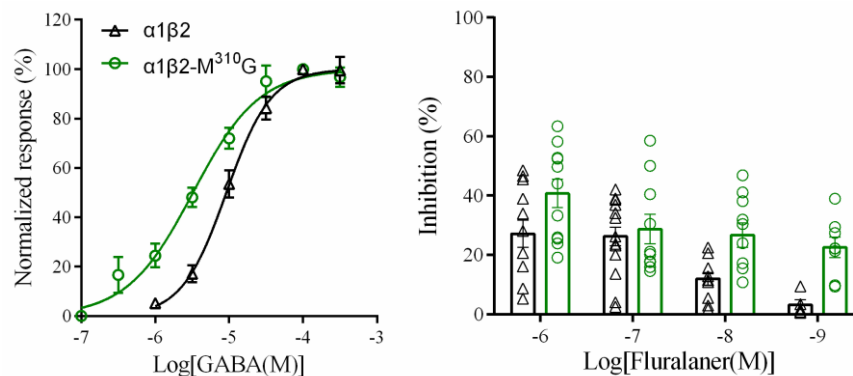
Fig. 6 Concentration-response curves of GABA (A) and inhibition of GABA-induced currents (B) in heteromeric $Mm\alpha1\beta2$ and $Mm\alpha1\beta2\text{-M}^{310}\text{G}$

Discussion

Fluralaner, as a highly selective insecticide targeting GABA receptor, exhibits excellent activity to insect but no/ low toxic to mammals (Ozoe *et al.*, 2010; Zhao *et al.*, 2014b; Jia *et al.*, 2018). Existing data demonstrate that insect RDL homomeric channel with traditional mutations of A2'S/G/N in TM2 still be inhibited by fluralaner (Ozoe *et al.*, 2010; Ozoe *et al.*, 2013; Zhao *et al.*, 2014a; Ozoe *et al.*, 2015; Nakata *et al.*, 2017). In this study, the amino acid sequences among arthropod and mammal GABA receptors were aligned in order to explore the potential binding site of fluralaner on arthropod RDL. Similar research approach has been performed on the bumblebee $Bi\text{Na}_v1$ sodium channel (Wu *et al.*, 2017). They firstly identified three amino acid residues by phylogenetic and mutational analyses with sensitivity and non-

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sensitivity voltage-gated sodium channel from bees and other insect species, and finally uncovered four additional amino acid residues by computer modeling and mutagenesis, which underlie the sensitivity of bumblebees to the majority of pyrethroids and selective resistance to tau-fluvalinate (Wu *et al.*, 2017).

In the present study, 35 different amino acid residues were identified by the alignment of mammal and insects inotropic GABAR subunits (**Table S1**). In the site-directed mutagenesis study, mutation of G3'M significantly decreased the inhibition of fluralaner and avermectin potency to GABA-induced currents of *Cs*RDL homomeric channel. Although this mutation may allosterically affect GABA binding to orthosteric site, the dose-response curve indicated that the channel functioned normally to induce currents in response to GABA. In other study, both fluralaner (Nakao *et al.*, 2013b; Ozoe *et al.*, 2013) and avermectin, which inhibit the [^{3}H]fluralaner binding on housefly membrane (Zhao *et al.*, 2014a), were also observed when the glycine (G) in TM3 was replaced by methionine (M) at the corresponding position in the *Sl*RDL and *Dm*RDL homomeric channels (Nakao *et al.*, 2013a; Nakao *et al.*, 2013c). Therefore, we could speculated that the G3'M in TM3 is a critical residue, which could determine the sensitivity of insect RDL homomeric channels to fluralaner. At the same time, the sensitivity of *Cs*RDL-G3'M homomeric channel to fipronil decrease up to 2.2-fold, which is similar to those in *Sl*G3'M-RDL and *Dm*G3'M-RDL with 3.2- and 2.5-fold, respectively (Nakao *et al.*, 2013a; Nakao *et al.*, 2013b). In Nakao et al. (2013) study, except for the G336H mutation, amino acid volumes of the wild-type and G336A, G336S, and G336C mutations are small, suggesting that the amino acid volume at position 336 determines the inhibitory activity of meta-diamide 7. In contrast, none of the G336 mutations affected the inhibitory activity of fipronil profoundly, suggesting that G336 is not related to the inhibitory action of fipronil. These results suggest that meta-diamide 7, which have a common genesis with fluralaner, has a site of action near G336 in M3 of the *Dm*RDL GABA receptor that is different from the site of fipronil action (Nakao *et al.*, 2013a).

The G'3M mutation in *Cs*RDL almost completely abolish the inhibition potency of avermectin. Avermectin belonging to macrocyclic lactone mainly act on the insect glutamate receptor, and partly on the GABA receptor (Ozoe, 2013). Both G323D in *Tu*Glu and G315E in *Px*Glu (equal position of G3'M) are associated with the resistance of insect to avermectin (Kwon *et al.*, 2010; Wang *et al.*, 2017). Mutation (G3'M) of RDL from different arthropod species could generated GABA-gated homomeric channel, and exhibited less sensitive up to 8.0 - 34 folds than that in the WT-RDL to fluralaner (**Figs. 4 &5**). Therefore, we could speculate that the glycine in the TM3 determines the activity of fluralaner to insect. Similar phenomenon exists in the A2'S or A2'N of TM2 of insect RDL. For example, the point-mutation of alanine to serine reduced the sensitive of RDL homomeric channel to cyclodiene in fruitfly *D. melanogaster*, tobacco budworm *Heliothis virescens* and diamondback moth *Plutella xylostella* (ffrench-Constant *et al.*, 1993; Wolff *et al.*, 1998; Li *et al.*, 2021). The point-mutation of alanine to asparagine reduced the sensitive of RDL homomeric channel to fiproles in small brown planthopper *Laodelphax striatellus* Fallén and white-backed planthopper *Sogatella furcifera* (Nakao *et al.*, 2011; Nakao *et al.*, 2012; Nakao *et al.*, 2013c; Sheng

et al., 2018a). Therefore, we speculated that the resistance-associated mutation of RDL to a certain type of insecticide would be common in most insects.

It is worth noting that in mammals, the G3' in β3 subunit is the binding site of etomidate (Li *et al., 2006*) and important for the activity of anesthetics (Olsen *et al., 2011*).In this study, the hetermeric channel of *Mm*α1β2-M[310]G was more sensitive to GABA than that of *Mm*α1β2, which could demonstrated that the selective of fluralaner between insect and mammal would depends on third amino acid residue of TM3 of GABA subunit.

In conclusion, our study provided a key residue in the insect RDL chloride channel that displays a striking selective resistance to some GABAR-targeted insecticides, including isoxazoline, meta-diamide and macrocyclic lactone. The hypothesis function of G3' in the TM3 in the interaction of fluralaner and RDL homomeric channel, which could encourage more effort on future knowledge-based precise modification of isoxazoline and meta-diamide to achieve highly selective control of pests with non-influence to mammals. However, the related study should be performed *in vivo* with CRISPR/Cas9 technique or UAS-Gal4 expression system to verify the existence of G3'M in insect.

Experimental procedures

Chemicals, insect strain and rat mRNA

Except fluralaner ($\geq$ 99%) purified from Bravecto[TM], other insecticides, reagents and solvents were obtained from commercial suppliers. Other insect species were cultured in our lab. The rat mRNA was kindly provided by Dr. Hui-Xing Lin (Nanjing Agricultural University).

Prediction and site-directed mutagenesis of mutation sites

As we known, the fluralaner showed high activity to insects but less toxic to mammals. Hence, 35 residues were selected according to the comparison of amino acid sequences from different animal (**Fig. S1** and **Table S1**). The WT *Cs*RDL1 homomers model was constructed by SWISS-MODEL (https://swissmodel.expasy.org/) using human GABA$_A$R-β3 homopentamer (PDB code: 4COF) as template. The constructed model was evaluated by the online programs of PROCHECK (Laskowski *et al., 1993;* Laskowski *et al., 1996*) and ProSA-web (Wiederstein *et al., 2007*). The structure of fluralaner was built using SYBYL-X 2.1 software (Tripos Inc. St. Louis, CA) running on a windows 7 workstation, and was then docked into the *Cs*RDL1 homomers model using Surflex-Dock module of SYBYL-X. The potential docking sites were generated using the residue-based mode of Surflex-Dock and the other parameters were set as default. Different single- or double-point mutations (**Table S2**) of the constructed structure were generated using Mutate Monomers module of SYBYL-X 2.1 and then the mutant models were minimized under the Tripos force field with MMFF94 charges by the Powell method with a gradient convergence criterion of 0.005 kcal/mol Å. Mutant amino acid residues, which lead to significant changes in binding affinity of fluralaner, were selected for further analysis.

Expression of heterologous in *Xenopus laevis* oocytes

All predicted binding sites were mutated using the designed specific primers (**Table S8**) and Fast Mutagenesis System (TransGen Biotech, Beijing, China) according the previous report (Sheng *et al.*, 2018a). The pGH19 was used as plasmid for expression of GABA receptor in *Xenopus laevis* oocytes. Procedure for oocyte preparation, cRNA injection into oocytes were identical to those described previously (Sheng *et al.*, 2018b). It is worth mentioning that heteromeric $Mm\alpha1\beta2$ and $Mm\alpha1\beta2$-$M^{310}G$ expression, the ratios of two injected subunits were 1:1, respectively.

Electrophysiological assays

Electrophysiological assays were performed and recorded on the Axoclamp 900A Microelectrode Amplifier platform (Molecular Devices, CA) at a holding potential of -60 mV as previously described (Sheng *et al.*, 2018b). Axon Digidata 1440A Data Acquisition System (Molecular Devices, CA) was used to record the current signals. Oocytes were placed in a recording chamber (RC-3Z, Warner Instruments, Hamden, CT) using standard oocytes saline (SOS) medium with perfusion speed at 8-10 mL/min, and electrophysiological assays were performed at 20 ℃. GABA was dissolved in a SOS medium and applied to stimulate oocytes for 5 s at interval of 85 s. Concentration-response curves of GABA were obtained by sequential applications of increasing concentrations to GABA receptor subunits. The median effective concentration (EC_{50}) was calculated with GraphPad Prism 5 (GraphPad Software, Inc.). Insecticides were first dissolved in dimethyl sulfoxide (DMSO), and diluted with a SOS medium to a final DMSO concentration less than 0.1% (v/v), which could ensure no effect on response of oocytes. Insecticide solution was added to the perfusate after successive control applications of the EC_{50} of GABA, and then applied consecutively for the remainder of experiments for 5 s at 85 s intervals during perfusion.

Statically analysis

Median inhibitory concentration (IC_{50}) value and the scatter plot were determined from the mean of 5-15 replications using standard probit analysis on GraphPad Prism 5 (GraphPad Software, Inc.). Each experiment was performed in triplicate and repeated three times.

Conflict of interest

The authors declare they have no conflict of interest with the contents of this article.

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