Activity, selection response and molecular mode of action of the isoxazoline afoxolaner in *Tetranychus urticae*

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Abstract

Background: Afoxolaner is a novel representative of the isoxazolines, a class of ectoparasiticides which has been commercialised for the control of tick and flea infestations in dogs. In this study, the biological efficacy of afoxolaner against the two-spotted spider mite *Tetranychus urticae* was evaluated. Furthermore, as isoxazolines are known inhibitors of γ-aminobutyric acid-gated chloride channels (GABACls), the molecular mode of action of afoxolaner on *T. urticae* GABACls (*Tu*Rdls) was studied using functional expression in *Xenopus* oocytes followed by two-electrode voltage-clamp (TEVC) electrophysiology and results were compared with inhibition by fluralaner, fipronil and endosulfan. To examine the influence of known GABACl resistance mutations, H301A, I305T and A350T substitutions in *Tu*Rdl1 and a S301A substitution in *Tu*Rdl2 were introduced.

Results: Bioasassays revealed excellent efficacy of afoxolaner against all developmental stages and no cross-resistance was found in a panel of strains resistant to most currently used acaricides. Laboratory selection over a period of 3 years did not result in resistance. TEVC revealed clear antagonistic activity of afoxolaner and fluralaner for all homomeric *Tu*Rdl1/2/3 channels. The introduction of single, double or triple mutations to *Tu*Rdl1 and *Tu*Rdl2 did not lower channel sensitivity. Contrastingly, both endosulfan and fipronil had minimal antagonistic activities against *Tu*Rdl1/2/3, and channels carrying single mutations while the sensitivity of double and triple *Tu*Rdl1 mutants was significantly increased.

Conclusions: Our results demonstrate that afoxolaner is a potent antagonist of GABACls of *T. urticae* and has a powerful mode of action to control spider mites.

Keywords: afoxolaner, laboratory selection, cross-resistance, γ-aminobutyric acid - gated chloride channel, *Tetranychus urticae*, two-electrode voltage-clamp electrophysiology

1. INTRODUCTION

The Rdl receptor is an invertebrate γ-aminobutyric acid (GABA) gated chloride channel expressed throughout the central nervous system (CNS) where it predominantly mediates inhibitory neurotransmission[1, 2]. As a member of the cys-loop ligand-gated ion channel (cysLGIC) superfamily, it is comprised of 5 homologous subunits forming a selective central pore which allows the influx of chloride ions upon binding the ligand GABA. The Rdl subunits, encoded by the Rdl gene, have extracellular N- and C-termini and four transmembrane regions (TM1-4), the second of which lines the ion pore[3, 4]. Based on its function, Rdl receptors are related to vertebrate $GABA_A$ receptors, but have a unique pharmacology as they differ in subunit compositions[1, 2, 5]. Therefore, GABACls have been an important target of a wide range of neurotoxic insecticidal and acaricidal compounds like lindane[6], picrotoxin[7], cyclodienes (such as dieldrin and endosulfan)[8], phenylpyrazoles (such as fipronil)[9] and macrocyclic lactones[10]. These noncompetitive antagonists (NCAs) or GABA blockers inhibit the GABA-induced influx of chloride ions into the nerve cells without any effect on the binding of GABA to the receptor, resulting in hyperexcitation of the nervous system[11]. Due to their extensive use over several decades however, many mutations of Rdl subunits associated with resistance to these GABAClI blockers have been reported in agricultural pests. The term Rdl (Resistance to Dieldrin) itself refers to the first mutation identified in a GABAClI subunit of *Drosophila melanogaster*, which was associated with high resistance to dieldrin and limited cross-resistance to fipronil [2, 8, 12, 13]. This alanine to serine (A301S) and by extension alanine to glycine (A301G), asparagine (A301N) or Leucine (A301L) mutation in TM2 has since been strongly associated with cyclodiene and, at varying levels, been linked with fipronil resistance in various insect species [3, 14-19]. Recent *in vivo* and *in vitro* studies on *Drosophila* uncovered that the glycine mutation results in higher fipronil resistance than the serine mutation[20]. In 2010 a T305L mutation in TM2 was linked with dieldrin resistance in the cattle tick *Rhipicephalus (Boophilus) microplus* by Hope et al. (2010)[21], while a T350M mutation, located at TM3, was identified in a laboratory selected *Drosophila simulans* population, highly resistant to both dieldrin and fipronil. Further functional experiments revealed that T350M contributed to fipronil resistance when it co-existed with A301S, as well when present as a single mutation[4, 22]. The multitude of emerging resistance cases combined with a ban of compounds like dieldrin and lindane due to their environmental persistence,

lowered the interest in these classic GABACl blockers[4, 23]. However, in the last decade the Rdl receptor regained popularity as a target with the emergence of two novel acting compound classes, the meta-diamides[24] and isoxazolines[23, 25], both belonging to the Insecticide Resistance Action Committee (IRAC) mode of action group 30[26]. These GABACl antagonist have the advantage of acting on different binding sites and are unaffected by Rdl receptors with subunits containing TM2 resistance mutations[4].

Here, the biological efficacy of afoxolaner, a novel isoxazoline ectoparasiticide, against the two-spotted spider mite *Tetranychus urticae* was studied as isoxazoline compounds were shown to have acaricidal activities and act as GABACl blockers[25]. Furthermore, research by Dermauw et al. (2012)[3] has shown that all three *T. urticae* Rdl orthologues contain the resistance associated serine (*Tu*Rdl2 and *Tu*Rdl3) or a histidine (*Tu*Rdl1) at position 301 (TM2). Additionally, the *Tu*Rdl1 subunits was found to contain a T305I and T350A substitution in TM2 and TM3, respectively. To examine the effect of these mutations on the potency of both classic GABACl blockers and the isoxazolines afoxolaner and fluralaner, site directed mutagenesis was applied to introduce the reversed H301A, I305T and A350T substitutions in *Tu*Rdl1 and a S301A substitution in *Tu*Rdl2 receptors.

2. MATERIALS AND METHODS

2.1. Chemicals (Fig. 1)

All chemicals, including y-aminobutyric acid (GABA; CAS number 56-12-2) and the technical standards of fluralaner (CAS number 864731-61-3), fipronil (CAS number 120068-37-3) and endosulfan (CAS number 959-98-8), were purchased from Sigma–Aldrich (Overijse, Belgium) unless stated otherwise. Afoxolaner (CAS number 1093861-60-9) was kindly provided by Sven Geibel (Bayer, Monheim, Germany)

2.2. Toxicity bioassays

To assess the baseline toxicity of afoxolaner, bioassays were conducted on a reference panel of 12 susceptible and acaricide-resistant spider mite strains previously described and listed in Table 1. All strains were mass reared on potted kidney bean plants (*Phaseolus vulgaris*) under controlled conditions (25 ± 0.5 °C, 60% RH and 16:8 h (L:D) photoperiod), fresh bean plants were offered when needed.

Female adulticidal bioassays were performed as previously described by Van Leeuwen et al. (2004)[27]. Briefly, afoxolaner was dissolved in a mixture of N,N-dimethylformamide (DMF) and emulsifier W (alkylarylpolyglycolether), 3:1 w/w, respectively, and subsequently diluted with deionized water 100-fold. About 20–30 young adult female mites were transferred to the upper (adaxial) side of a 9cm^2 square bean leaf discs on wet cotton wool and subsequently sprayed at 1 bar pressure in a custom-built spray tower (Ghent University, Ghent, Belgium) resulting in 2,00 ± 0,02 mg aqueous acaricide deposit per cm^2. The plates were subsequently placed in a climatically controlled room as described above. Mortality was scored after 24 hours. For bioassays of the different life stages, 30 adult females of the London strain were transferred to the upper side of 9 cm^2 square bean leaf discs on wet cotton wool, and permitted to lay eggs for 4-6h, after which they were removed. The plates were then placed in a climatically controlled room. Immediately after removal of the adults (egg bioassay), after hatching of the eggs (larval bioassay) or when more than 50% of the mites were developed into the desired stage (protonymph, deutonymph and adult male), the eggs or mites were sprayed with spray fluid at 1 bar pressure (2,00 ± 0,02 mg aqueous acaricide deposit per cm^2). Mortality was assessed when the next life stage appeared in the water sprayed control (egg, larva, protonymph and deutonymph bioassay) or 24h after treatment (adult male bioassay). For all bioassays, 4 replicates

per concentration with a minimum of 6 concentrations of afoxolaner plus a control (deionised water + emulsifier W + DMF) were tested. Mites were scored as dead if they did not move one body length within 10 seconds after being prodded with a fine brush. Mites that had drowned in the water barrier were excluded from the analysis (less than 5%).

LC_{50} values and their 95% confidence limits were calculated from probit regression using the PoloPlus-PC program (LeOra Software, Berkely, USA). Resistance ratios (RRs) were calculated by dividing the LC_{50} value of the resistant strain by the LC_{50} value of the susceptible London strain.

2.3. Selection for afoxolaner resistance

To select for afoxolaner resistance, both HOL 1 and MAR-AB strains were maintained on potted bean plants treated with afoxolaner, hereby establishing a continuous selection pressure. Plants were sprayed with a hand pressurised sprayer (Birchmeyer, Switzerland) until runoff with approximately 6-8 mg (corresponding to about the LC_{75}) of afoxolaner L^{-1} at the start of the experiment, which gradually increased to ± 25 mg L^{-1} as susceptibility decreased (Fig. 2). Before spraying, secondary leaf buds were removed and only the primary leaves were kept on the potted bean plants. Mite numbers were kept high by maintaining the strain on a minimum of 18 bean plants, and only replacing with fresh plants when leaves where overgrown.

2.4. Two-electrode voltage-clamp electrophysiology

2.4.1. Vector construction and cRNA synthesis

Wild type *Tu*Rdl constructs (*Tu*Rdl1,*Tu*Rdl2 and *Tu*Rdl3), translating in homomeric *Tu*GABA*C*l channels, were *in silico* generated as previously described in Xue *et al*. 2021[28]. The introduction of substitutions into the *Tu*Rdl cDNA sequences were created using site-directed mutagenesis and both *Tu*Rdl1 and *Tu*Rdl2 plasmids as a template (Table 3). All Rdl coding sequences preceded with a KOZAK sequence (‘GCCAC’) and were codon optimised for *Xenopus* expression using the OptimumGene™-Codon Optimization software of GeneScript (Piscataway, NJ,USA). cRNA synthesis was carried out as previously described[28, 29]. Quality and quantity of cRNA was evaluated via a spectrophotometer (Thermo Scientific NanoDrop 2000 or a DeNovix DS-11 (DeNovix, Willmington, DE, USA)) and agarose gel electrophoresis and cRNA was stored at −80 °C until use.

2.4.2. Oocyte injection

Mature stage V or VI, defolliculated *Xenopus laevis* oocytes were purchased from Ecocyte Bioscience (Castrop-Rauxel, Germany) and incubated for 2h at 19°C upon arrival. Next, 25 ng cRNA encoding GABAR subunits was injected into the oocytes using a Nanoject III Programmable Nanoliter Injector (Drummond Scientific Co., Broomali, PA, USA), the oocytes were incubated in sterile Barth's solution supplemented with 20 µg/mL gentamycin (Ecocyte Bioscience) for a minimum of 24h before experimentation. Optimal expression was achieved at 2-3 days post injection.

2.4.3 TEVC electrophysiology

TEVC recordings were made using the fully automated Roboocyte2 (Multi Channel Systems MCS GmbH, Reutlingen, Germany) at a holding potential of −60 mV at 19°C. Oocytes were held in a standard 96-well microtitre plate and impaled with two glass microelectrodes filled with 0.1 M KCl 1.5 M potassium acetate solution to yield a resistance of ~1MΩ.

The natural agonist GABA and the acaricide technical standards afoxolaner, fluralaner, fipronil, and endosulfan were prepared as 1 mM stock solutions in dimethyl sulfoxide (DMSO) and dissolved in Normal Frog Ringer (NFR) solution (Ecocyte Bioscience) resulting in a maximal final DMSO concentration of 1%. Concentration-response relationships for GABA were carried out by exposing the oocytes to the compound for 30 s in an ascending order of concentration with a 90 s recorded wash-out (NFR) between applications to allow the current to return to baseline. To analyse the antagonism of *Tu*GABAClS by afoxolaner, fluralaner, fipronil and endosulfan oocytes were tested as described in Xue *et al.* 2021. Oocytes were first exposed to GABA 4 times for 30 s every 1.5 min at the beginning of the experiment to test for expression and to stabilize the response. Subsequently, oocytes were pre-exposed for 75 s to the antagonist (1nM–10 µM) followed by 30 s of co-application with GABA (EC_{50}). Both compounds were washed out with NFR (non-recorded) for 30 s before increasing to the next concentration.

All experiments were replicated using at least 6 oocytes. The data are presented as the mean ± SEM. TEVC recordings were analysed using the Roboocyte 2+ V. 1.4.3.

software (Multi Channel Systems MCSGmbH), EC_{50} and IC_{50} values were determined according to the dose-response relationships by four-parameter logistic curve (Hill equation) on response data using SigmaPlot software 13.0 (Systat Software, San Jose, CA, USA).

3 RESULTS

3.1. Toxicity bioassays and selection for afoxolaner resistance

The baseline toxicity of afoxolaner (LC_{50} and slope) in 10 acaricide-(multi)resistant strains and two reference susceptible strains is presented in Table 1. GSS was by far the most susceptible strain under investigation, with an LC_{50} as low as 0.0191 mg L^{-1} , while the London susceptible strain displayed an LC_{50} of 3.18 mg L^{-1}. The LC_{50} values of all resistant strains varied between 2 and 5 mg L^{-1} except for HOL1, MAR-AB and JP-R with a slightly elevated LC_{50} value of 7.74, 6.02 and 11.3, respectively. None of the strains showed clear resistance to afoxolaner, hereby excluding cross-resistance with the acaricides listed in Table 1. Egg and larval toxicity bioassay data indicated that the eggs and larvae of the London strain are more susceptible to afoxolaner than adults, proto- and deutonymphs with an LC_{50} of only 0.651 and 1.05 mg L^{-1} for London eggs and larvae, respectively (Table 2).

HOL1 and MAR-AB strains were chosen for laboratory selection since they both showed elevated LC_{50} values and heterogeneity in afoxolaner toxicity. Both strains were maintained on potted bean plants sprayed with afoxolaner at a starting concentration of 6 and 8 mg L^{-1} for MAR-AB and HOL1, respectively (Fig 2). Selecting concentrations of afoxolaner were increased if populations were able to grow to sufficient numbers resulting in a final LC_{50} of only 21.9 mg L^{-1} for MAR-AB and 29.7 mg L^{-1} for HOL1 after 3 year of continuous selection. Resistance progressed slowly in both strains during the first 18 months and stagnated despite multiple attempts to increase selecting concentrations.

3.2 Two-electrode voltage-clamp electrophysiology

3.2.1 Responses of wild type and mutant *Tu*GABACls to GABA

To investigate the effects of (known) mutations on the potencies of GABA, afoxolaner, fluralaner, endosulfan and fipronil against *Tu*GABACls, 3 substitutions (H301A, I305T and A350T) in *Tu*Rdl1 and 1 substitution (S301A) in *Tu*Rdl2 were introduced, generating 6 mutant *Tu*GABACls (Table 3). In combination with 3 wild type *Tu*GABACls

(*Tu*Rdl1, *Tu*Rdl2 and *Tu*Rdl3), a total of 9 different *Tu*GABACls were examined. All wild type and mutant *Tu*GABACls showed robust responses to the natural agonist GABA as previously described[2], generating rapid inward currents with a slow desensitization as long as the agonist was applied, followed by a very rapid desensitization once wash-out with NFR was started (Fig. 3-5). The averaged dose-response curves for GABA are listed in Table 4. EC_{50}'s of all mutant *Tu*Rdl1 receptors fell within close range to the EC_{50} of *Tu*Rdl1 (15.5 µM), indicating that substitutions did not influence GABA sensitivity. The same was observed for *Tu*Rdl2 (68.6 µM) and *Tu*Rdl2 S301A (65.8 µM).

3.2.2 Afoxolaner and fluralaner inhibition of GABA induced currents in wild type and mutant *Tu*GABACls

To assess dose-dependent inhibitory effects of the antagonists, a cumulative exposure was used where the oocytes were pre-incubated with the antagonist for 75 s followed by co-application with GABA . Figure 3B,C and D shows the dose-dependent inhibition curves of afoxolaner and fluralaner for GABA activated currents in *Tu*GABACls. Both isoxazolines exhibited strong antagonistic activity against all *Tu*GABACls. Fluralaner turned out to be a more potent inhibitor than afoxolaner with IC_{50} values of ± 50 nM. IC_{50} values for afoxolaner ranged between 100 and 200 nM, with the exception of the triple mutant *Tu*Rdl1 H301A I305T A350T (IC_{50} = 290 nM) (Table 4).

3.2.3 Endosulfan and fipronil inhibition of GABA induced currents in wild type and mutant *Tu*GABACls

Figure 4D and 5D shows that endosulfan and fipronil failed to block GABA-induced currents in wild type (*Tu*Rdl1, *Tu*Rdl2 and *Tu*Rdl3) and single mutant *Tu*Rdls (*Tu*Rdl1 H301A, *Tu*Rdl1 I305T, *Tu*Rdl1 A350T and *Tu*Rdl2 S301A) in the nanomolar range (1-100nM). Low antagonistic activity was observed at the highest concentrations (1-10µM) with a maximum inhibition of ± 20% of the GABA-induced currents at 10µM endosulfan or fipronil. The IC_{50} values could therefore not be calculated for *Tu*Rdl1, *Tu*Rdl2, *Tu*Rdl3, *Tu*Rdl1 H301A, *Tu*Rdl1 I305T, *Tu*Rdl1 A350T and *Tu*Rdl2 S301A. Contrastingly, both endosulfan and fipronil had high inhibitory activity in double mutants *Tu*Rdl1 H301A A350T and *Tu*Rdl1 H301A I305T in the nanomolar and micromolar range with an IC_{50} of 178 nM and 71 nM respectively for endosulfan and

58 nM and 125 nM for fipronil (Table 4). The same was observed for the triple mutant *Tu*Rdl1 H301A I305T A350T with an IC_{50} value of 107 nM for endosulfan and 205 nM for fipronil. Figure 4D and 5D show no additional effect of the third mutation to the fipronil and endosulfan inhibitory effect compared to double mutants.

4. DISCUSSION

In this study, afoxolaner was shown to have strong acaricidal activity against a reference panel of resistant *T. urticae* strains, suggesting that cross-resistance with commonly used acaricides is not very likely. For other newly launched chemicals, such as cyflumetofen and cyenopyrafen, cross-resistance was detected using the same panel[30]. The LC_{50} values of adult female bioassays found in this study generally ranged from 2-5 mg L^{-1}, which coincides with results reported for other group 30 compounds like fluxametamide and broflanilide on susceptible *T. urticae* strains with LC_{50}s of 5.02 and 2.07 mg L^{-1}, respectively[31, 32]. Afoxolaner also proved effective on all developmental stages with eggs and larvae being the most sensitive. Previous studies have shown that pesticide susceptibility of *T. urticae* can vary between sexes or developmental stages due to morphological/metabolic differences such as body size or differential expression of detoxification enzymes[**33, 34**]. The increased susceptibility of eggs and larvae for afoxolaner found in this study, might be explained by the higher surface-to-volume ratio of eggs and larvae compared to larger adult stages resulting in higher exposure to the compound. The attempt to select in the laboratory for resistance using two genetically independent populations failed, even when selecting for an unusual long period of 3 consecutive years. Selection responses have been considerably high for many other compounds when selected in the laboratory prior to field application, including bifenazate[**35**], chlorfenapyr[36], pyflubumide[33] , cyenopyrafen[37] and cyflumetofen[**30**] resulting in high resistance levels generally within 10-15 or even as few as two generations of selection in the case of pyflubumide. This indicates that afoxolaner resistance is not readily selected based upon genetic variation present in the MAR-AB and HOL1 strains used for selection and further corroborates its potential for controlling spider mites in field crops. So far, afoxolaner has mainly been screened for efficacy via oral administration to dogs against parasitic flea, tick and mite species[38-41]. Both *in vivo* and *in vitro* studies revealed remarkable effectiveness of afoxolaner against these parasites. In addition, Shoop et al. (2014)[38] demonstrated the lack of cross-resistance with cyclodienes using wild type and cyclodiene-resistant strains of *Drosophila*. Other members of IRAC group 30, such as the isoxazolines fluralaner, fluxametamide and isocycloseram, and the meta-diamide broflanilide, were previously shown to exhibit excellent acaricidal activity against *T. urticae*, with in some cases even higher activity compared to commercially available acaricides[31, 32, 42-45].

Both fluralaner and broflanilide were found to act on insect GABACl's by using either binding assays with [(3)H]ethynylbicycloorthobenzoate (EBOB) and*/or* TEVC assays, respectively[23, 46]. The same was observed for fluxametamide, confirming that isoxazoline and meta-diamides have insect GABACls as a target[31]. Asahi et al. (2015) used TEVC to confirm that fluralaner blocked GABA-induced chloride currents in *Xenopus* oocytes expressing homomeric *T. urticae* Rdl1 receptors. In this study, nine different homomeric *Tu*GABACls were functionally expressed in *Xenopus* oocytes and examined for their sensitivity to fluralaner and afoxolaner, and classical GABACl blockers such as fipronil and endosulfan. Both afoxolaner and fluralaner strongly inhibited GABA-induced responses in wild type *Tu*Rdl1/2/3 channels, while antagonistic activities of endosulfan and fipronil were low. Previous studies with isoxazolines like fluralaner[25], lotilaner[47] and fluxametamide[31] have described similar strong antagonistic activity against *Tu*GABACls as observed here and linked high antagonistic activity *in vitro* with high acaricidal activity *in vivo*. Further, Asahi et al. (2015) also described that low acaricidal activity of fipronil against *T. urticae* is due to its low inhibitory activity against the *Tu*Rdl1 channel. In natural populations, the presence of A301S/H, T305I and/or T350A substitution's in *Tu*Rdl channels has been assumed to explain the reduced sensitivity of spider mites to both fipronil and endosulfan[3]. Our data confirms these previous reports as both endosulfan and fipronil had minimal antagonistic activities against wild type *Tu*Rdl1/2/3, and both compounds were found not to be toxic to the spider mite populations available in our laboratory (data not shown). Noteworthy, the above mentioned Rdl substitutions or variants thereof are also present in orthologous Rdl subunits of other spider mites, such as *Tetranychus evansi*, *Panonychus citri*, the false spider mite *Brevipalpus yothersi*, and the bulb mite *Rhizoglyphus robinii* (Fig. S1) and might, to some extent, explain why endosulfan has only moderate acaricidal activity against spider mites and the bulb mite[48-50] and has not been recommended for spider mite control [51, 52]. In contrast, endosulfan has been frequently used to control bud-and rust mites (Eriophydiae) and cyclamen/broad mites (Tarsonemidae)[52-55] and for those Eriophyidae for which transcriptomic/genomic data is available, at least one Rdl subunit with no or only favoured substitutions (i.e. A301, T305 and T350S)[56] could be identified. However, such subunits could also be identified in *Galendromus occidentalis* and *Amblyseius swirskii*, predatory mites for which endosulfan is considered as low to moderately

348 toxic[57-59], and suggests that endosulfan susceptibility is not only target-site related (Fig.
349 S1).

350 The distinctive antagonism of afoxolaner and fluralaner on *T. urticae* GABACls
351 confirms that these isoxazolines do not share binding sites with fipronil and endosulfan.
352 Cyclodienes and fenylpyrazoles have been assumed to act on binding sites which
353 reside within the channel pore (formed by the TM2s of the different subunits)[4, 25, 60],
354 while previous research[61-63] suggested that the binding site of fluralaner and meta-
355 diamides is located in the outer half of the transmembrane subunit interface of TM1
356 and/or TM3, as amino acid substitutions in this region had significant effect on the
357 potency of fluralaner[64] and meta-diamides[24, 65, 66]. They reported that a G271L
358 substitution in TM1 and G333M/S/A substitutions in TM3 of *Musca domestica*
359 GABACl's resulted in a significant reduction in the potency of fluralaner, while G336
360 substitutions in *Drosophila* Rdl or a G319M substitution in *Spodoptera* Rdl abolished
361 the inhibitory activity of metadiamides**.** Similar results were observed on honeybee
362 (*Apis mellifera* L) by Sheng et al. (2019)[67], who used docking simulations to suggest
363 that fluralaner binds TM1 and TM3. More recently, Huang et al. (2022)[68] demonstrated
364 that glycine at the third position of TM3 determines the action of fluralaner and
365 therefore should be the exact binding site of fluralaner in insect GABACl's.

In this research the effect of A301S/H, I305A (TM2) and T350A (TM3) mutations on channel sensitivity was examined by introducing reversed single, double and triple mutations in *Tu*Rdl1 and *Tu*Rdl2. None of the reversed mutations had any effect on channel inhibition by afoxolaner and fluralaner. By contrast, *Tu*Rdl1 sensitivity to fipronil and endosulfan was partially restored when double and triple reversed mutations were introduced without any additional effect of the third mutation. Similar findings were recently reported by Kobayashi (2020)[69] where single and multiple mutations (A301S/H and I305A) were introduced in TM2 of *Tu*Rdl to examine their sensitivities to picrotoxinin and fipronil and the cyclodiene dieldrin. The double (H301A and I305T) mutation significantly increased the channels sensitivity to all three compounds, but the introduction of single mutations had no effect. Additionally, our data revealed that there was no difference in restored sensitivity when both TM2 mutations where introduced in *Tu*Rdl1 compared to the combination of one TM2 (H301A) and the TM3 (T350A) mutation, indicating that the A350T substitution contributes equally to fipronil and endsulfan resistance compared to the combination of TM2 mutations (H301A and I305T).

Remarkably, the introduction of the TM2 S301A mutation in *Tu*Rdl2 had little influence on endosulfan and fipronil sensitivity even though *Tu*Rdl2 naturally carries T305, suggesting that mutations at these positions (301 and 305) are of little importance for *Tu*Rdl2 sensitivity. Ozoe et al. (2009)[70] describes that the channel pore is formed by the TM2 segments of the five subunits and amino acids at the 1', 2' (corresponding to 301 in *Tu*Rdl), 6' (corresponding to 305 in *Tu*Rdl), 9', 13' and 16' position face the channel pore. As mutations at the 2' and 6' positions influence the sensitivity of GABACl to NCAs like fipronil and endosulfan, the channel-lining region formed by 2' and 6' amino acids must contain these NCA binding sites. On the other hand, our results suggest that T350A at TM3 also influences fipronil and endosulfan sensitivity which coincides with the findings of Le Goff et al (2005)[22] for fipronil activity on *D. simulans* Rdl. However, whether or not this amino acid residue in the TM3 segment is located in the fipronil and/or endosulfan *Tu*GABACl binding site remains to be determined.

5. CONCLUSION

In conclusion, this study reveals that afoxolaner is an excellent candidate for control of *T. urticae* as it is a potent noncompetitive antagonist of *Tu*GABAChs, unaffected by resistance associated TM2 and TM3 mutations. Furthermore, *in vivo* assays have revealed that afoxolaner shows no cross-resistance with the most commonly used acaricides and despite its reputation to rapidly develop resistance, a decreased efficacy of afoxolaner against *T. urticae* could not be observed after long-time exposure to increasing concentrations.

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8. TABLES LEGENDS

Table 1. Adult female probit mortality data for afoxolaner on susceptible and characterised resistant strains of *T. urticae*.

Strain	Resistance	Maintenance†	LC_{50}‡ (95% CI) (mg L^{-1})	Slope + SE	RR§ (London)
GSS[71]	susceptible	-	0.0191 (0.0044 - 0.0491)	3.07 ± 0.31	-
London[72]	susceptible	-	3.18 (2.83 -3.52)	3.82 ± 0.21	-
SR-TK[73]	spirodiclofen	spirodiclofen (1200 mg L^{-1})	2.44 (2.18 - 2.66)	5.95 ± 0.62	0.8
MR-VP[74]	multi resistant	tebufenpyrad (500 mg L^{-1})	5.26 (4.32 - 6.04)	3.48 ± 0.37	1.3
Akita[75]	METIs (fenpyroximate)	fenpyroximate (500 mg L^{-1})	4.14 (3.81 - 4.47)	4.79 ± 0.50	1.3
MR-VL[76]	multi resistant	bifenthrin (359 mg L^{-1})	4.11 (3.54 - 4.59)	4.08 ± 0.45	1.3
JP-R[37]	cyenopyrafen. cyflumetofen. pyridaben	cyenopyrafen (200 mg L^{-1})	11.2 (9.5 - 13.0)	2.55 ± 0.22	3.5
EtoxR[77]	etoxazole. clofentezine	etoxazole (1100 mg L^{-1})	3.66 (3.23 - 4.07)	3.05 ± 0.20	1.2
TU008R[72]	Cyflumetofen	cyflumentofen (500 mg L^{-1})	0.596 (0.356 - 0.800)	0.784 ± 0.073	0.2
SR-VP[78]	spirodiclofen	spirodiclofen (1200 mg L^{-1})	2.79 (1.91 - 3.29)	5.15 ± 0.72	0.9
HOL1[71]	bifenazate	bifenazate (720 mg L^{-1})	7.74 (5.93 - 9.35)	2.68 ± 0.24	2.4
MAR-AB[74]	multi resistant	abamectin (9 mg L^{-1})	6.02 (4.97 - 6.95)	2.99 ± 0.22	1.9

† Acaricide exposure of resistant mite strains for maintenance.

‡Median lethal concentration (expressed as mg L−1), with 95% confidence interval.

§Resistance Ratio: LC_{50} relative to the LC_{50} of the susceptible population London

Table 2. Probit mortality data for afoxolaner on different life stages of London *T. urticae* strain.

Life stage	LC_{50} (95% CI) (mg L−1)	Slope + SE
egg	0.651 (0.552 - 0.759)	2.15 ± 0.11
larvae	1.05 (0.88 - 1.24)	2.39 ± 0.16
protonymph	2.53 (1.18 - 4.23)	1.14 ± 0.08
deutonymph	3.26 (2.61 - 3.97)	2.30 ± 0.14
adult male	2.42 (2.03 – 2.77)	4.46 ± 0.24
adult female	3.18 (2.83 -3.52)	3.82 ± 0.21

Table 3. Aligned amino acid sequences of transmembrane domain (TM) 2 and 3 of the coding sequence in 3 wild type and 6 mutant *Tu*Rdl plasmids. Substitutions are highlighted in red. Transmembrane domain regions were predicted using DeepTMHMM[79] and *Tu*Rdl1 as input.

		TM2		TM3		
*Tu*Rdl1	272…	PARVHLGVITVLTMTTLMSST	NSQLPKISYVK	SIDVFLGTCFVMVFAALLEYAAV	GYIG	…332
*Tu*Rdl1 A350T	272…	PARVHLGVITVLTMTTLMSST	NSQLPKISYVK	SIDVFLGTCFVMVFAALLEYA**T**V	GYIG	…332
*Tu*Rdl1 H301A	272…	PARV**A**LGVITVLTMTTLMSST	NSQLPKISYVK	SIDVFLGTCFVMVFAALLEYAAV	GYIG	…332
*Tu*Rdl1 H301A A350T	272…	PARV**A**LGVITVLTMTTLMSST	NSQLPKISYVK	SIDVFLGTCFVMVFAALLEYA**T**V	GYIG	…332
*Tu*Rdl1 H301A I305T	272…	PARV**A**LGV**T**TVLTMTTLMSST	NSQLPKISYVK	SIDVFLGTCFVMVFAALLEYAAV	GYIG	…332
*Tu*Rdl1 H301A I305T A350T	272…	PARV**A**LGV**T**TVLTMTTLMSST	NSQLPKISYVK	SIDVFLGTCFVMVFAALLEYA**T**V	GYIG	…332
*Tu*Rdl2	266…	PARVSLGVTTVLTMTTLMSST	NAQLPKISYIK	SIDVFLGTCFVMVFASLLEYATV	GYLG	…326
*Tu*Rdl2 S301A	266…	PARV**A**LGVTTVLTMTTLMSST	NAQLPKISYIK	SIDVFLGTCFVMVFASLLEYATV	GYLG	…326
*Tu*Rdl3	265…	PARVSLGVTTVLTMTTLMSST	NAQLPKISYIK	SIDVFLGTCFVMVFASLLEYATV	GYLG	…325

692 **Table 4. Responses of *Tetranychus urticae* GABACls expressed in *Xenopus* oocytes.**

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	GABA			afoxolaner			fluralaner			fipronil			endosulfan		
	EC_{50} (μM)	pEC_{50}	nH	IC_{50} (μM)	pIC_{50}	nH	IC_{50} (μM)	pIC_{50}	nH	IC_{50} (μM)	pIC_{50}	nH	IC_{50} (μM)	pIC_{50}	nH
*Tu*Rdl1	15.5	4.81 ± 0.01	5.61 ± 0.56	0.0751	7.11 ± 1.65	-0.632 ± 0.171	0.0212	7.67 ± 0.36	-0.292 ± 0.081	-	-	-	-	-	-
*Tu*Rdl1 H301A	13.1	4.88 ± 0.05	4.53 ± 1.44	0.0602	7.21 ± 0.05	-0.514 ± 0.143	0.0644	7.18 ± 0.05	-0.491 ± 0.343	-	-	-	-	-	-
*Tu*Rdl1 A350T	13.2	4.88 ± 0.01	2.87 ± 0.15	0.118	6.92 ± 0.01	-0.442 ± 0.181	0.0752	7.12 ± 0.28	-0.592 ± 0.274	-	-	-	-	-	-
*Tu*Rdl1 H301A A350T	14.1	4.85 ± 0.03	3.97 ± 1.01	0.126	6.91 ± 0.13	-0.343 ± 0.074	0.0684	7.16 ± 0.01	-0.778 ± 0.013	0.0581	7.25 ±1.31	-0.201 ± 0.341	0.178	6.74 ± 0.11	-0.321 ± 0.042
*Tu*Rdl1 H301A I305T	14.4	4.82 ± 0.02	4.41 ± 0.95	0.0664	7.17 ± 0.38	-0.454 ± 0.288	0.0415	7.39 ± 0.29	-0.473 ± 0.295	0.125	7.13 ± 0.13	-0.494-± 0.104	0.0712	7.13 ± 0.38	-0.474 ± 0.281
*Tu*Rdl1 H301A I305T A350T	18.0	4.74 ± 0.03	2.97 ± 0.51	0.290	6.53 ± 0.05	-0.434 ± 0.046	0.0387	7.41 ± 0.05	-0.532 ± 0.035	0.205	6.90 ± 0.82	-0.396 ± 0.423	0.107	6.96 ± 0.05	-0.547 ± 0.046
*Tu*Rdl2	68.6	4.16 ± 0.03	1.56 ± 0.16	0.116	6.95 ± 0.25	-0.571 ± 0.232	0.0561	7.59 ± 0.45	-0.378 ± 0.279	-	-	-	-	-	-
*Tu*Rdl2 S301A	65.8	4.18 ± 0.03	1.82 ± 0.19	0.133	6.81 ± 0.04	-0.594 ± 0.094	0.0660	7.00 ± 0.14	-0.320 ± 0.112	-	-	-	-	-	-
*Tu*Rdl3	140	3.85 ± 0.04	3.36 ± 0.65	0.188	6.92 ± 0.21	-0.824 ± 0.362	0.0616	7.21 ± 0.49	-0.484 ± 0.342	-	-	-	-	-	-

694 pEC_{50} = the negative logarithm of EC_{50}

695 pIC_{50} = the negative logarithm of IC_{50}

696 Data are the mean of 6–8 oocytes ± SEM. n_H. Hill coefficient

10. FIGURES

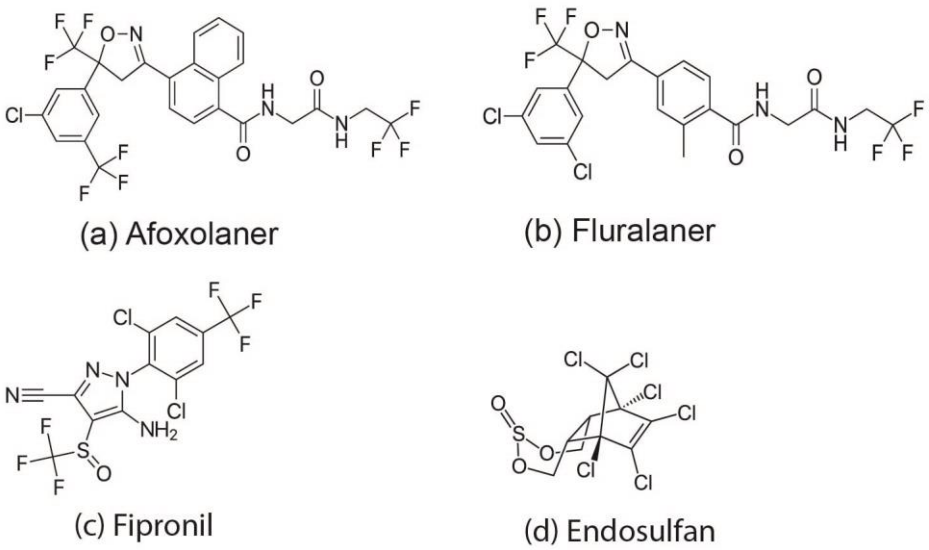


Fig. 1. Chemical structures of afoxolaner, fluralaner, firponil and endosulfan.

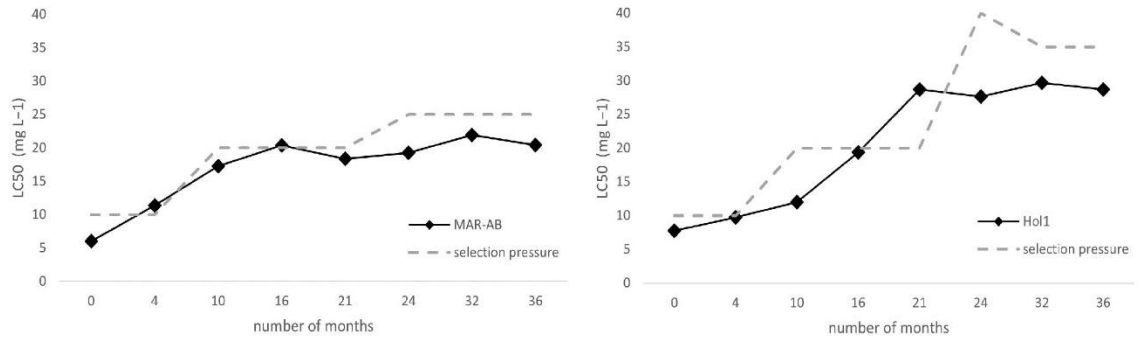
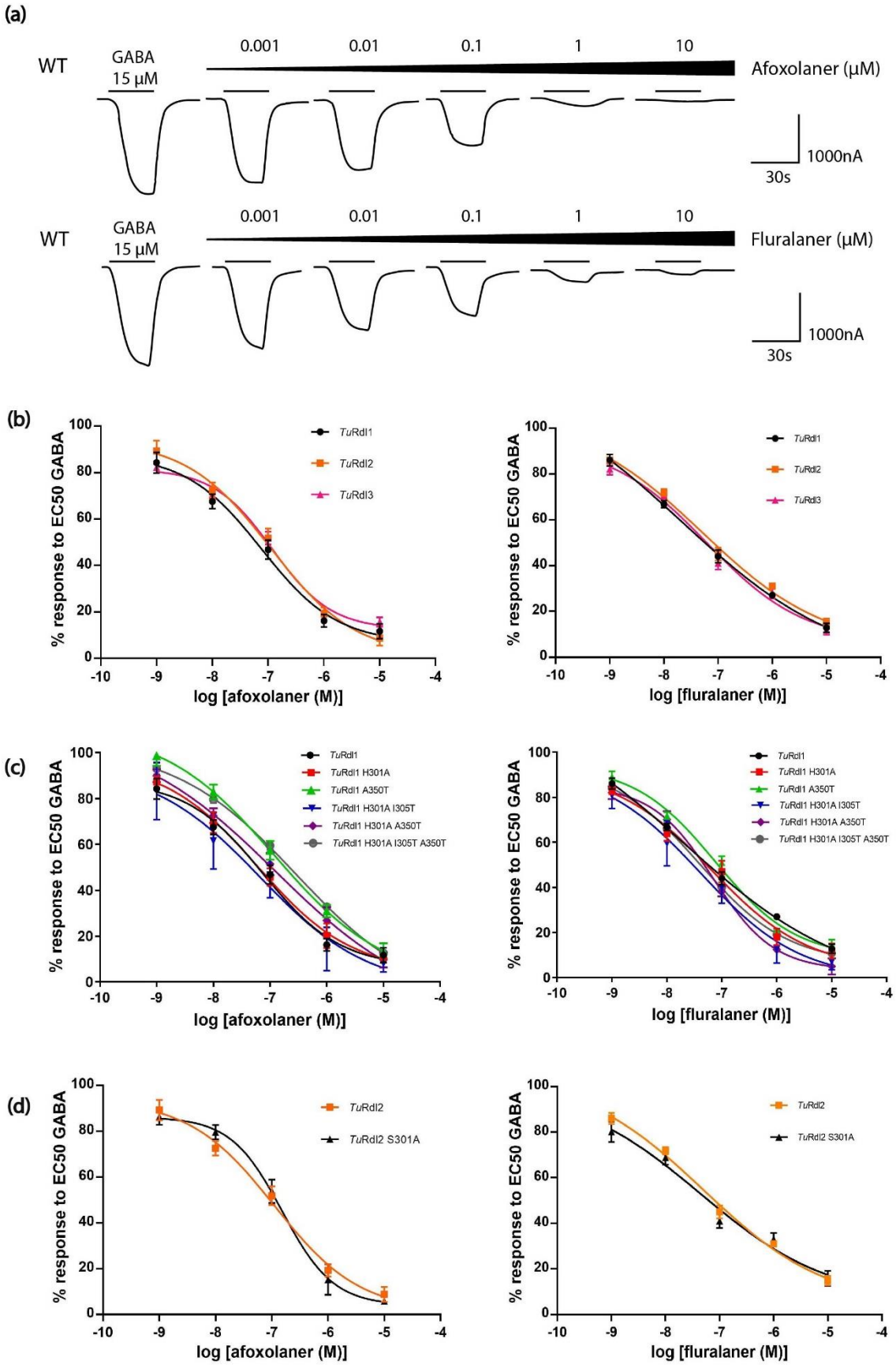


Fig. 2. Selection for afoxolaner resistance.

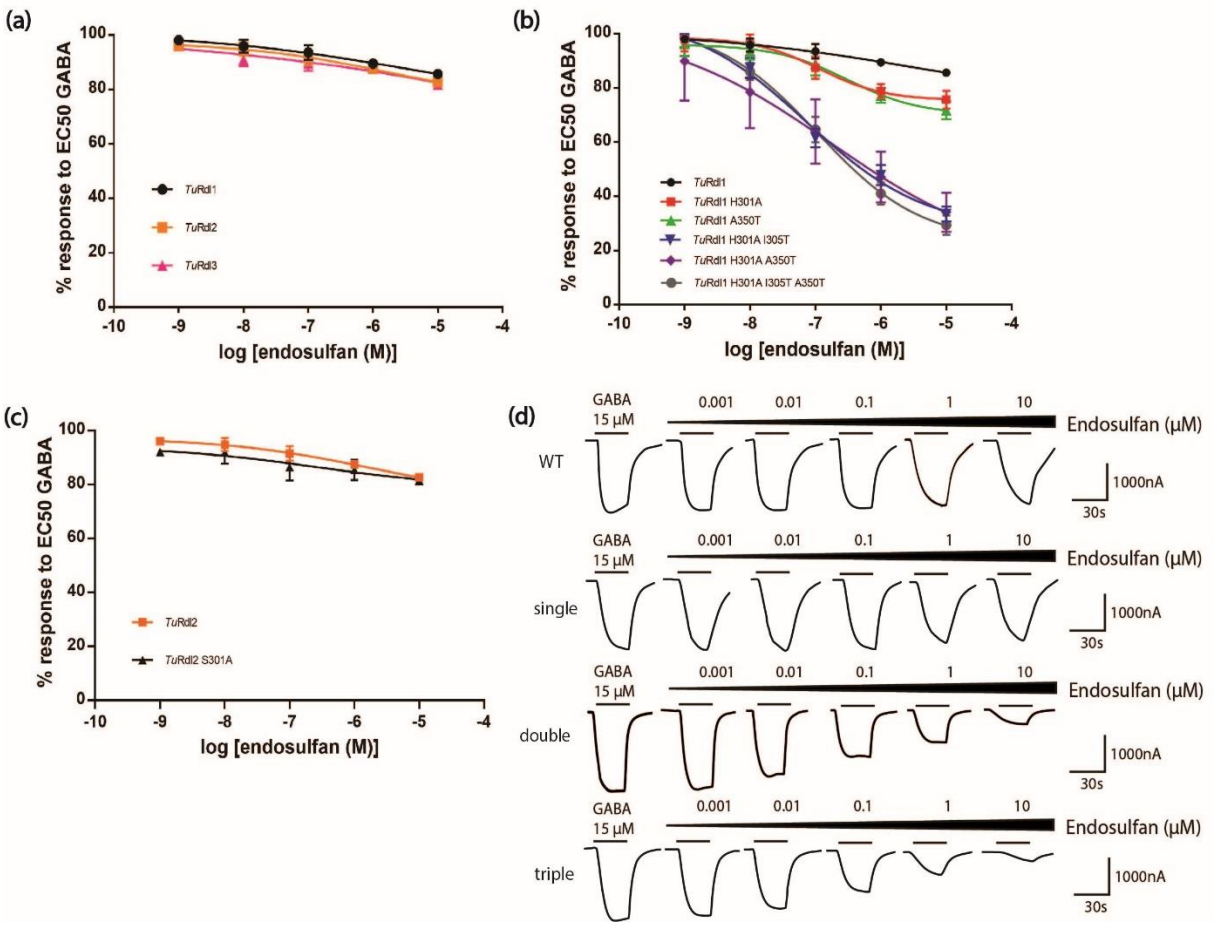
Changes in estimated LC_{50} values of afoxolaner for the HOL1 and MAR-AB *T. urticae* strains over 3 consecutive years are presented by the black squares (■). The dotted line indicates the selecting concentrations (ppm) sprayed on potted bean plants.


(a)
WT
GABA
15 µM
0.001
0.01
0.1
1
10
Afoxolaner (µM)
1000nA
30s
WT
GABA
15 µM
0.001
0.01
0.1
1
10
Fluralaner (µM)
1000nA
30s
(b)
% response to EC50 GABA
log [afoxolaner (M)]
log [fluralaner (M)]
TuRdl1
TuRdl2
TuRdl3
(c)
% response to EC50 GABA
log [afoxolaner (M)]
log [fluralaner (M)]
TuRdl1
TuRdl1 H301A
TuRdl1 A350T
TuRdl1 H301A I305T
TuRdl1 H301A A350T
TuRdl1 H301A I305T A350T
(d)
% response to EC50 GABA
log [afoxolaner (M)]
log [fluralaner (M)]
TuRdl2
TuRdl2 S301A

712

Fig. 3. Antagonistic activity of afoxolaner and fluralaner on the *Tu*GABACls expressed in *Xenopus* oocytes.

(a) Representative current traces from cumulative exposure to increasing dosage of afoxolaner and fluralaner obtained for *Xenopus* oocytes expressing *Tu*Rdl1 (WT). The bars indicate the time period of co-application of GABA (15 µM) and increasing concentrations of afoxolaner or fluralaner(1 nM–10 µM); (b) Inhibition dose–response curves measured for afoxolaner and fluralaner obtained from oocytes expressing *Tu*Rdl1. *Tu*Rdl2 or *Tu*Rdl3; (c) Inhibition dose–response curves measured for afoxolaner and fluralaner obtained from oocytes expressing single, double and triple mutant *Tu*Rdl1 receptors; (d) Inhibition dose–response curves measured for afoxolaner and fluralaner obtained from oocytes expressing *Tu*Rdl2 and *Tu*Rdl2 S301A. Error bars indicate SEM (n = 6–8).



732

733 **Fig. 4 Antagonistic activity of endosulfan on the *Tu*GABACls expressed in**
734 ***Xenopus* oocytes.**

735 (a) Inhibition dose–response curves measured for endosulfan obtained from oocytes
736 expressing *Tu*Rdl1. *Tu*Rdl2 or *Tu*Rdl3; (b) Inhibition dose–response curves measured
737 for endosulfan obtained from oocytes expressing single, double and triple mutant
738 *Tu*Rdl1 receptors; (c) Inhibition dose–response curves measured for endosulfan
739 obtained from oocytes expressing *Tu*Rdl2 and *Tu*Rdl2 S301A; (d) Representative
740 current traces from cumulative exposure to increasing dosage of endosulfan obtained
741 for *Xenopus* oocytes expressing *Tu*Rdl1 (WT), *Tu*Rdl1 A350T (single), *Tu*Rdl1 H301A
742 A350T (double) and *Tu*Rdl1 H301A I305T A350T (triple). The bars indicate the time
743 period of co-application of GABA (15 µM) and increasing concentrations of endosulfan
744 (1 nM–10 µM). Error bars indicate SEM (n = 6–8).

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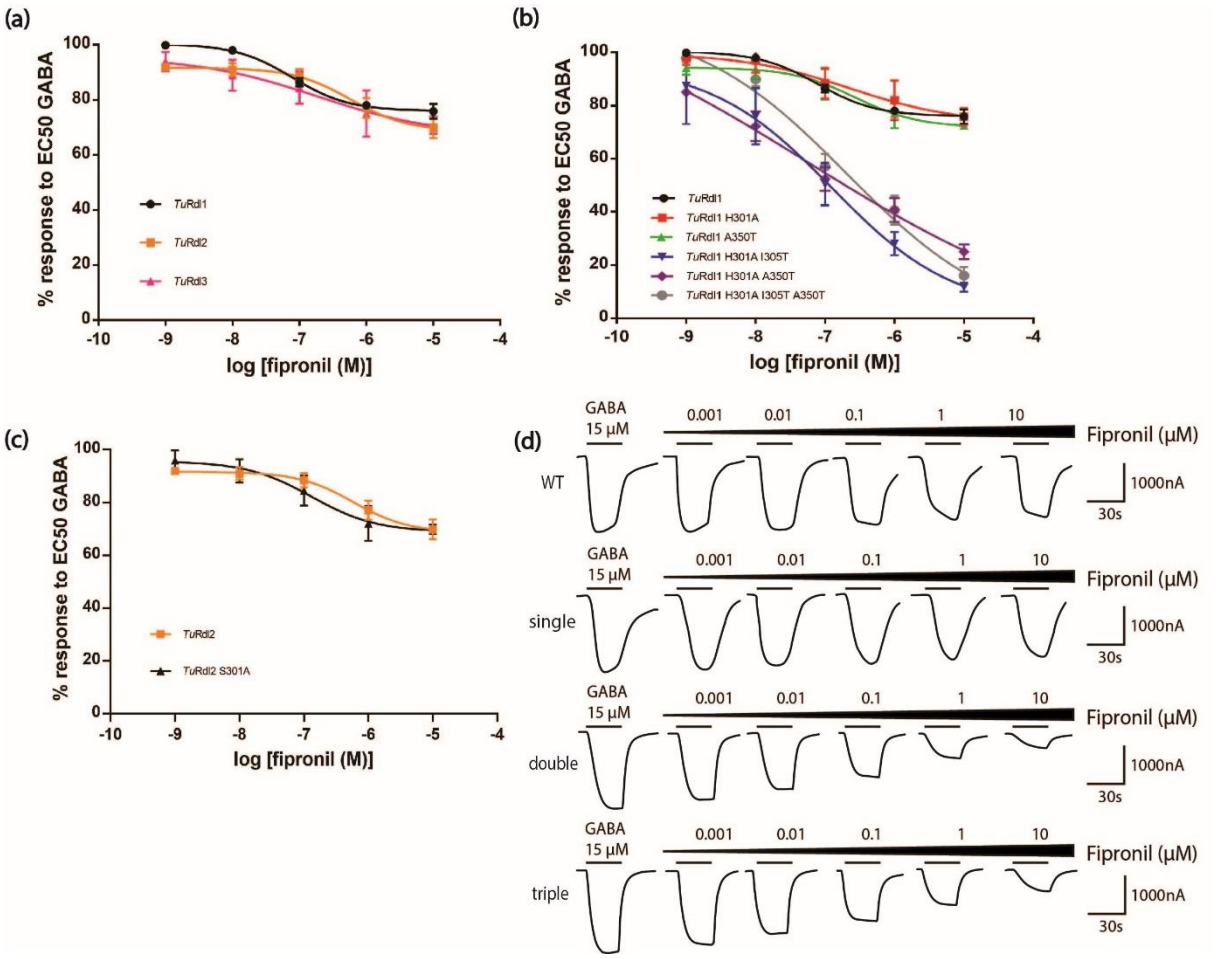

Fig. 5. Antagonistic activity of fipronil on the *Tu*GABACIs expressed in *Xenopus* oocytes.

(a) Inhibition dose–response curves measured for fipronil obtained from oocytes expressing *Tu*Rdl1. *Tu*Rdl2 or *Tu*Rdl3; (b) Inhibition dose–response curves measured for fipronil obtained from oocytes expressing single, double and triple mutant *Tu*Rdl1 receptors; (c) Inhibition dose–response curves measured for fipronil obtained from oocytes expressing *Tu*Rdl2 and *Tu*Rdl2 S301A. (d) Representative current traces from cumulative exposure to increasing dosage of fipronil obtained for *Xenopus* oocytes expressing*Tu*Rdl1 (WT), *Tu*Rdl1 A350T (single), *Tu*Rdl1 H301A A350T (double) and *Tu*Rdl1 H301A I305T A350T (triple). The bars indicate the time period of co-application of GABA (15 µM) and increasing concentrations of fipronil (1 nM–10 µM). Error bars indicate SEM (n = 6–8).