Insect Biochemistry and Molecular Biology 45 (2014) 111–124

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

The novel isoxazoline ectoparasiticide fluralaner: Selective inhibition of arthropod γ-aminobutyric acid- and L-glutamate-gated chloride channels and insecticidal/acaricidal activity

Michael Gassel [1], Christian Wolf [1], Sandra Noack, Heike Williams, Thomas Ilg*

MSD Animal Health Innovation GmbH, Zur Propstei, 55270 Schwabenheim, Germany

ARTICLE INFO

Article history:
Received 20 September 2013
Received in revised form
22 November 2013
Accepted 28 November 2013

Keywords:
GABA-gated chloride channel
Glutamate-gated chloride channel
Isoxazoline
Ectoparasiticidal target
Cat flea
Cattle tick
Fipronil
Fluralaner

ABSTRACT

Isoxazolines are a novel class of parasiticides that are potent inhibitors of γ-aminobutyric acid (GABA)-gated chloride channels (GABACls) and L-glutamate-gated chloride channels (GluCls). In this study, the effects of the isoxazoline drug fluralaner on insect and acarid GABACl (RDL) and GluCl and its parasiticidal potency were investigated. We report the identification and cDNA cloning of *Rhipicephalus* (*R.*) *microplus* RDL and GluCl genes, and their functional expression in *Xenopus laevis* oocytes. The generation of six clonal HEK293 cell lines expressing *Rhipicephalus microplus* RDL and GluCl, *Ctenocephalides felis* RDL-A_{285} and RDL-S_{285}, as well as *Drosophila melanogaster* RDLCl-A_{302} and RDL-S_{302}, combined with the development of a membrane potential fluorescence dye assay allowed the comparison of ion channel inhibition by fluralaner with that of established insecticides addressing RDL and GluCl as targets. In these assays fluralaner was several orders of magnitude more potent than picrotoxinin and dieldrin, and performed 5-236 fold better than fipronil on the arthropod RDLs, while a rat GABACl remained unaffected. Comparative studies showed that *R. microplus* RDL is 52-fold more sensitive than *R. microplus* GluCl to fluralaner inhibition, confirming that the GABA-gated chloride channel is the primary target of this new parasiticide. In agreement with the superior RDL on-target activity, fluralaner outperformed dieldrin and fipronil in insecticidal screens on cat fleas (*Ctenocephalides felis*), yellow fever mosquito larvae (*Aedes aegypti*) and sheep blowfly larvae (*Lucilia cuprina*), as well as in acaricidal screens on cattle tick (*R. microplus*) adult females, brown dog tick (*Rhipicephalus sanguineus*) adult females and *Ornithodoros moubata* nymphs. These findings highlight the potential of fluralaner as a novel ectoparasiticide.

1. Introduction

The dominant inhibitory neurotransmitter of vertebrates is the zwitterionic amino acid γ-aminobutyric acid (GABA; Krnjević, 2004, 2010). GABA released from synaptic vesicles engages either metabotropic G protein-coupled receptors (Marshall and Foord, 2010), or receptors that act as chloride-conducting ligand-gated ion channels belonging to the cystine loop superfamily (Sine and Engel, 2006). GABA-mediated chloride influx into cells leads to hyperpolarization of the membrane and generates an inhibitory postsynaptic potential, which decreases the probability of the occurrence of action potentials (Macdonald and Olsen, 1994; Hevers and Lüddens, 1998). GABA-gated chloride channels (GABACls) in vertebrates are pentamers of α (α_1–α_6), β (β_1–β_3) and γ (γ_1–γ_3) subunits, typically in the arrangement $(\alpha_x)_2(\beta_x)_2\gamma_x$. Further variation is generated by the minor subunits δ, ε, π and θ, that can replace γ in the pentamers, and by ρ_1–ρ_3-containing receptors, that are found specifically in the retina. Altogether 19 GABACl subunit genes have been identified in mammals, and additional complexity is generated by alternative mRNA splicing (Sieghart, 2006; Whiting, 2006; Olsen and Sieghart, 2009, D'Hulst et al., 2009). GABACls are ubiquitously expressed in the central nervous system (CNS) of vertebrates, and a multitude of psychoactive drugs and convulsants act at this molecular target, such as barbiturates, benzodiazepines, steroids, and picrotoxinin (Fig. 1) (Hevers and Lüddens, 1998; Glykys and Mody, 2007; Bateson, 2009; Winsky-Sommerer, 2009; Mirza and Munro, 2010; Sigel and Steinmann, 2012).

* Corresponding author. Tel.: +49 6130 948315.
E-mail addresses: thomas.ilg@msd.de, TSIlg@web.de (T. Ilg).
[1] MG and CW contributed equally to this paper and should both be considered first author.

0965-1748/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ibmb.2013.11.009

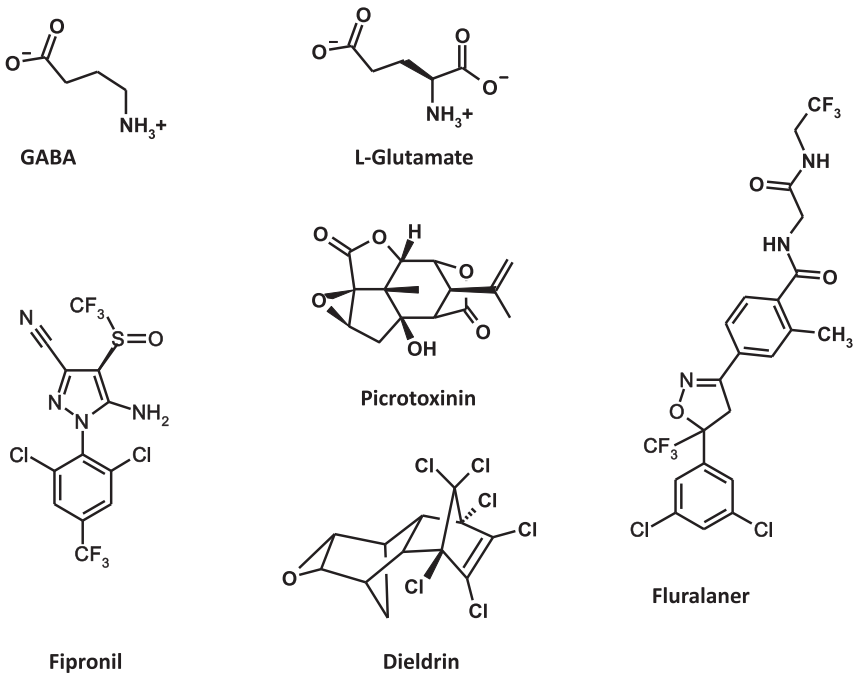

Fig. 1. Chemical structures of chloride channel agonists and antagonists.

Insects and other invertebrates also possess GABACls (Gerschenfeld, 1973). These receptors are not only present in the CNS, where they generate inhibitory potentials for the correct integration of neuronal signals, but also at peripheral neuromuscular sites, where they promote muscular relaxation (Lummis, 1990; Rauh et al., 1990; Schuske et al., 2004). About 30 years ago, it was discovered that GABACls are the molecular targets for the insecticides lindane and cyclodiene derivatives (Ghiasuddin and Matsumura, 1982), a contention confirmed by a large number of subsequent biochemical and electrophysiological studies (Sattelle, 1990; Rauh et al., 1990; Casida, 1990).

The cloning of a GABACl (RDL) subunit gene from the cyclodiene resistance locus, *rdl,* of *Drosophila melanogaster* confirmed at the molecular biology level that this ion channel is the cyclodiene target (ffrench-Constant et al., 1991). The corresponding gene, *dmrdl*, displayed in a dieldrin-resistant *D. melanogaster* isolate a single point mutation leading to an amino acid exchange A_{302} ➔ S_{302}, conferring high level resistance to the cyclodiene insecticide class (ffrench-Constant et al., 1993a, b). The *rdl* gene and the resistance mutation have been identified in several insect orders (ffrench-Constant, 1994; ffrench-Constant et al., 2000). Homozygous disruption of the *rdl* locus is lethal in *D. melanogaster* (Stilwell et al., 1995). The recombinant homomers of the *rdl* protein product, expressed either in *Xenopus laevis* oocytes or in cell culture, are considered good, albeit not perfect, models of native insect receptors (Buckingham et al., 2005). Other GABACl subunit gene candidates have been reported and may contribute *in vivo* in localized areas to insect GABA and insecticide pharmacology (Hosie et al., 1997; Raymond and Sattelle, 2002; Buckingham et al., 2005).

A large set of compound binding studies have confirmed that lindane, cyclodienes such as dieldrin (Fig. 1), picrotoxinin (Fig. 1), and other convulsants, such as 3,3-bis(trifluoromethyl)bicyclo [2.2.1]heptane-2,2-dicarbonitrile (BIDN), 4′-ethynyl-4-*n*-propylbicycloorthobenzoate (EBOB) and *tert*-butylbicyclophosphorothionate (TBPS), bind to a common allosteric site on insect GABACls (RDL) within the chloride channel lumen, and that their binding leads to a channel block (reviewed by Hosie et al., 1997; Bloomquist, 1998, 2001). The phenylpyrazole insecticides, such as fipronil (Fig. 1) also bind to native and recombinant insect RDLs, and inhibit GABA-induced chloride currents (Cole et al., 1993; Buckingham et al., 1994; Hosie et al., 1995; Gant et al., 1998). Fipronil displaces ^{3}H-EBOB and ^{3}H-BIDN or ^{3}H-TBPS from their insect brain binding sites indicating that this compound binds at or near the dieldrin binding site. This suggestion is supported by the observation that cyclodiene resistance in housefly leads to a partial resistance to fipronil (Bloomquist, 1993).

Initially, it was thought that the macrocyclic lactones of the avermectin and milbemycin classes, that are insecticidal, acaricidal and nematocidal compounds (Shoop et al., 1995; Geary, 2005), also address primarily GABACls as molecular targets (Wang and Pong, 1982; Martin and Pennington, 1989; Holden-Dye and Walker, 1990; Bermudez et al., 1991). However, further studies revealed the predominant role of glutamate-gated chloride channels (GluCls) for the macrocyclic lactone mode of action. Avermectins and milbemycins potentiate on GluCls the agonistic effect of glutamate, or activate directly these ion channels, and it is now well-established that these ligand-gated chloride channels represent a second major parasiticide target class (Cully et al., 1994, 1996; reviewed by Wolstenholme and Rogers, 2005; Wolstenholme, 2012). Interestingly, in addition to its RDL blocker action, fipronil is also acting as a potent inhibitor of insect GluCl. It has been proposed that this additional GluCl inhibition activity contributes to the insecticidal activity of this phenylpyrazole drug (Ikeda et al., 2003; Zhao et al., 2004; Zhao and Salgado, 2010; Narahashi et al., 2010).

Taken together, a large number of experimental studies and reviews have emphasized the prominent roles of RDLs and GluCls for the parasiticidal action of antiparasitic drugs and promote these ion channels as prime targets, also for the identification of novel pesticides (reviewed in Raymond and Sattelle, 2002; Bloomquist, 2003; Buckingham et al., 2005; Raymond-Delpech et al., 2005; Narahashi et al., 2010; Ozoe, 2013). The viability of this contention has recently been confirmed by the introduction of two novel

classes of pesticidal substances, the insecticidal and acaricidal isoxazolines (Ozoe et al., 2010; Lahm et al., 2013; García-Reynaga et al., 2013), and the meta-diamide insecticides (Nakao et al., 2013), which were both shown to address primarily the RDL.

Insect and acarid ectoparasites cause vast damage and distress to livestock, companion animals and humans. In the case of companion animals, the cat flea *Ctenocephalides (C.) felis* is the commercially most important ectoparasite (Rust and Dryden 1997; Rust 2005), while the cattle tick *Rhipicephalus (R.) microplus* is responsible for large economical losses to livestock operations worldwide due to direct damage and transmitted diseases (de Castro, 1997; Guerrero et al., 2012). Therefore, these two arthropod species are a major focus of veterinary research aiming at novel ectoparasiticides.

In this study, we investigate the ectoparasiticidal activity and parasite molecular target pharmacology of fluralaner (A1443, Ozoe et al., 2010), a representative of the novel isoxazoline chemical class. We report the gene identification, the gene cloning and the functional expression in *Xenopus* oocytes of *Rhipicephalus microplus* (cattle tick) RDL and GluCl subunits. Furthermore, *Ctenocephalides felis* and *D. melanogaster* RDL subunit genes were prepared as wild type and dieldrin-resistant forms. All RDL and GluCl genes were stably expressed in HEK293 cells, clonal cell lines were selected and, based on these ion channel transgenic HEK293 cell lines, fluorescent membrane potential dye assays were developed.*Xenopus* oocyte two electrode voltage clamp electrophysiology ion channel assays were employed to assess receptor functionality. Using these assays, the pharmacology of insect and tick RDL and GluCl homopentamers were established for the specific agonists GABA and glutamate, as well as for the antagonists picrotoxinin, dieldrin, and fipronil in comparison with the novel drug fluralaner. In a set of *in vivo* experiments, the ectoparasiticidal activity of dieldrin, fipronil and fluralanerwas tested and compared with imidacloprid and deltamethrin on four insect and three tick parasite species.

2. Materials and methods

2.1. Reagents and cell lines

Bacterial cultures were grown in Luria–Bertani (LB) medium modified with supplements as required by the bacterial background and the introduced resistance genes. Chemicals and salts were from Sigma–Aldrich or from Merck (Darmstadt, Germany), unless stated otherwise. The FMP-dyes RED and BLUE were from Molecular Devices. Dimethyl sulfoxide (DMSO) was from Acros Organics, fipronil from Riedel-de-Haen, dieldrin and picrotoxinin from Sigma, ivermectin (mixture of B_{1a} and B_{1b}) from Dr. Ehrenstorfer, deltamethrin from Sigma–Aldrich or Riedel-de-Haen, and imidacloprid from Riedel-de-Haen. Racemic A1443 (Ozoe et al., 2010) was obtained from Nissan Chemical Industries, Ltd. The HEK293 cell line and the WS-1 cell line (Wong et al., 1992) were from ATCC.

2.2. General DNA and RNA techniques

Total RNA was extracted from *R. microplus* larvae, *D. melanogaster* adult flies or *C. felis* adult fleas by a modification of the guanidinium thiocyanate/phenol extraction method (Chomczynski and Sacchi, 1987; Trizol, Sigma). Polymerase chain reaction (PCR) products were cloned into pCR2.1-Topo or pCRII-Topo, and introduced into *Escherichia* (*E.*) *coli* Top10 cells (Invitrogen). Subcloning was performed into the mammalian expression vector pcDNA3.1(+) using *E. coli* JM109 (Promega) as a host. Site-directed mutagenesis was performed using the QuikChange XL kit (Stratagene) with specific mutagenesis primer pairs. Other molecular biology techniques are specifically indicated or were performed essentially as described by Sambrook and Russell (2001).

2.3. Identification and isolation of GABA-gated Cl^- channel and glutamate-gated Cl^- channel subunit genes from R. microplus

In the case of the *R. microplus* RDL, reverse transcription of total RNA was performed using the Expand Reverse Transcriptase kit (Roche) with total RNA (0.9 μg/50 μl) as template and the degenerate primer TTNACRTAIGADATYTTNGG derived from the conserved RDL subunit polypeptide sequence PKISYVK. From the reaction product, 1 μl was used as a template for a polymerase chain reaction (PCR) using the Expand High Fidelity kit (Roche) (40 cycles of 30 min at 94 °C, 30 s at 45 °C and 1 min 30 s at 72 °C) and the two degenerate primer AANATHTGGGTICCNGAYAC and TTNAICCAR AAIGANACCCA, derived from the conserved GABACl subunit polypeptide sequences (N/K)IWVPDT and WVSFW(L/I)N, respectively. From the 460 bp degenerate primer PCR fragment (KF881794), the missing cDNA sequences of the *R. microplus* gene were then obtained by rapid amplification (RACE) of cDNA ends using total RNA from tick larvae as template and the SMART RACE cDNA Amplification Kit (Clontech) with generic flanking 5′- and 3′-RACE primer (Clontech), as outlined by the manufacturer. The 5′-gene region and the start codon was identified by 5′-RACE (specific primer CCGCATGAAAGTAGGCGCTCTTCTCGTTGG), and the 3′-gene region and the stop codon by 3′-RACE (specific primer-1 TCCAGATCTA CATCCCGGCCGGATTGATCG). The 5′-RACE product yielded KF881795. In the case of the 3′-RACE, another specific primer 2 (TACCGCGCTCCTGGAGTACGCCACGGTAGG) was generated based on the preliminary sequence of a product with primer1 (not shown). The resulting 3′-RACE product of primer 2 yielded KF881796. Based on the deduced start and stop codon positions in the 5′- and 3′-RACE product sequences KF881795 and KF881796, respectively, the PCR primers GGGATCCACCATGAGA CAAGCGATGGCGTTCAGTTGC and CCCGGGCTAGTCGTCGCCGA CATCGTCCGGCAGAACG were then designed for the PCR amplification of the full length genes from tick larvae cDNA. The restriction enzyme sites introduced by the primers are underlined. The PCR products were cloned into pCR2.1-Topo and sequenced, and a PCR error-free *R. microplus* RDL subunit gene (*rmrdl*) was selected (KF881797). The *rmrdl* open reading frame was subcloned into BamHI/EcoRV-cut pcDNA3.1(+) *via* the PCR-introduced BamHI/SmaI restriction enzyme sites yielding pcDNA3.1(+)-*rmrdl*.

For the identification of the *R. microplus* GluCl gene, PCR was performed using larval cDNA and the Expand High Fidelity kit (Roche) (40 cycles of 30 min at 94 °C, 30 s at 45 °C and 1 min 30 s at 72 °C) and the two degenerate primers TGYAHIAGYAAR ACIAAYACNGG and TCNARCCARAAIGANACCCA derived from the conserved GluCl subunit polypeptide sequences C(N/T)SKTNTG and WVSFWLD, respectively. The resulting 142 bp PCR fragment TGTACGAGCAACACGAATACGGGCGAGTACAGCTGCTTGCGCGTGGA CCTGGTGTTCAAGCGCGAGTTCAGCTACTACCTGATCCAGATCTACATC CCGTGCTGCATGCTGGTCATCGTGTCCTGGGTATCCTTCTGGCTCG allowed the design of nested primer pairs for 5′-RACE (primers ACACGATGACCAGCATGCAGCACGGGATG and CCAGGTCCACGCG CAAGCAGCTGTACTCG) and 3′-RACE (primers GCGAGTACAGCTGCT TGCGCGTGGACCTG and ACATCCCGTGCTGCATGCTGGTCATCGTG). By nested RACE reactions on tick larvae RNA, the missing cDNA sequences of the *R. microplus* gene was then obtained (SMART RACE cDNA Amplification Kit, Clontech) with generic flanking 5′- and 3′-RACE primer (Clontech), as outlined by the manufacturer. The nested RACE products were cloned into pCR2.1 and sequenced (5′-RACE, 890 bp, KF881798; 3′-RACE, 818 bp, KF881799). Start and stop codons were deduced from the 5′- and 3′-RACE product

sequences, and the PCR primers GATCGATGGTACCATGAGCGTA CATTCATGGCGCTTTTGTG and GTCTAGAGTCGACTTACTCGTCTTCCT CGTCTTCCCGGAAG were then designed for the PCR amplification of the full length genes from tick larvae cDNA. The restriction enzyme sites introduced by the primers are underlined. The PCR products were cloned into pCR2.1-Topo and from the sequenced clones, a PCR error-free *R. microplus* GluCl subunit gene version (*rmglucl*) was selected (KF881800). The *rmglucl* open reading frames were subcloned into KpnI/XhoI-cut pcDNA3.1(+) *via* the PCR-introduced KpnI/SalI restriction enzyme sites yielding pcDNA3.1(+)-*rmglucl*.

2.4. Isolation and site-directed mutagenesis of RDL subunit genes from C. felis and D. melanogaster

Reverse transcription of total RNA from *C. felis* and *D. melanogaster* to cDNA was performed using the Expand Reverse Transcriptase kit (Roche) with total RNA (0.9 μg/50 μl) as template. For the isolation of the *C. felis* RDL subunit gene (*cfrdl*-S_{285}), PCR was performed using cDNA and the Expand High Fidelity kit (Roche) (40 cycles of 30 min at 94 °C, 30 s at 45 °C and 1 min 30 s at 72 °C) with the forward primers ATGAAGAATCCGGGCCGTCG and GGCATGGCGGCGCTGACTCG and the corresponding reverse primer CGCCCTCTATTTGTCTTCTCCCA. The resulting PCR products was cloned into pCRII-TOPO, sequenced (KF881790) and subcloned resulting in pcDNA3.1(+)-*cfrdl*-S_{285}. To change the serine at position 285 to alanine, the coding sequence was mutagenized with the primers GCTACACCAGCTCGAGTCGCTCTCGGAGTGACCACTGTGTG and CAACACAGTGGTCACTCCGAGAGCGACTCGAGCTGGTGTAGC resulting in pcDNA3.1(+)-*cfrdl*-A_{285} (KF881791). For the identification of *D. melanogaster* RDL, PCR was performed using cDNA and the Expand High Fidelity kit (Roche) (40 cycles of 30 min at 94 °C, 30 s at 45 °C and 1 min 30 s at 72 °C) with the two primers CAC CACCATGAGTGATTCAAAAATGGAC and GCTTGTGGACGACGCCC TACTCCTC. The resulting PCR product was cloned into pCRII-TOPO, sequenced (KF881792) and subcloned resulting in pcDNA3.1(+)-*dmrdl*-A_{302}. To change the alanine at position 302 to serine, the coding sequence was mutagenized with the primers CGCCGGCGCGTGTGTCGCTCGGTGTGACAACCG and CGGTTGTCA CACCGAGCGACACACGCGCCGGCG (KF881793) resulting in pcDNA3.1(+)-*dmrdl*-S_{302}.

2.5. Bioinformatics analysis of GABACl and GluCl subunit genes

ClustalW multiple sequence alignments of the *R. microplus* RDL and GluCl subunit genes and the corresponding genes from other arthropods, the generation of phylogenetic trees and bootstrap analyses were performed with the DNAStar Lasergene software package. Bioinformatics analysis for the presence of endoplasmic reticulum import sequences and transmembrane helices were performed using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011), and TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), respectively.

2.6. Xenopus laevis oocyte expression and electrophysiology experiments with RDL and GluCl subunit genes from D. melanogaster, C. felis and R. microplus

The open reading frames of *dmrdl*-A_{302}, *dmrdl*-S_{302}, *cfrdl*-A_{285}, *cfrdl*-S_{285}, *rmrdl*, and *rmglucl* were either present in pCR2.1 downstream of a T7 promoter or were subcloned into pcDNA3.1(+) downstream of the T7 promoter. The resulting plasmids were linearized 3′ of the gene sequence by restriction enzyme digests resulting in 5′-overhangs, and *in vitro* transcription was performed to obtain 5′-capped cRNA, and subsequent polyadenylation was performed according to the manufacturer's protocol (mMESSAGE mMACHINE T7 transcription kit, Ambion). Transcripts were recovered by LiCl precipitation, dissolved in nuclease-free water at a final concentration of ~2 μg/μl, and stored at −80 °C until use.

Defolliculated *X. laevis* oocytes (states V-VI) were purchased from Ecocyte Biosciences. 50 nl cRNAs were injected using a micromanipulator (World precision instruments). The oocytes were incubated for 48–96 h at 17 °C in modified Barths solution (5 mM HEPES pH 7.2, 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 2.4 mM Na-pyruvate). Oocytes held in bath were perfused with Barths solution (5 mM Hepes pH 7.2, 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$) at a flow rate of approximately 1.8 ml/min, and were voltage clamped at −60 mV using the two electrode clamp mode of a Turbo Tec-03x amplifier (NPI electronic). Electrodes were pulled from borosilicate glass (Science products) using the Puller PC-10 (Narishige group), and filled with 3 M KCl. The electrode resistance ranged between 1 and 5 MΩ on the current-passing side. Agonist solutions, freshly prepared in Barths solution from DMSO stock solutions (100 mM–1 M), with final DMSO concentrations not exceeding 1.5% (v/v), were applied *via* bath perfusion for 30 s. The resulting inward current was recorded using CellWorksLite 5.5.1 (NPI electronic), and analysed later. An interval of 2 min was routinely maintained between agonist applications, which was elongated in some case to up to 5 min.

2.7. Heterologous expression of RDL or GluCl subunit genes in HEK293 cells

For expression of arthropod RDL and GluCl subunit genes in HEK293, pcDNA3.1(+)-*dmrdl*-A_{302}, pcDNA3.1(+)-*dmrdl*-S_{302}, pcDNA3.1(+)-*cfrdl*-A_{285}, pcDNA3.1(+)-*cfrdl*-S_{285}, pcDNA3.1(+)-*rmrdl* and pcDNA3.1(+)-*rmglucl*, respectively, were introduced into the cells by Polyfect (Qiagen) transfection. The HEK293 cells were cultured in Dulbecco's modified Eagle's medium, 10% inactivated fetal calf serum (FCS) at 37 °C and 5% CO_2, and recombinant cells were selected by the addition of 600 μg/ml G418. Single cell cloning was performed by the limiting dilution method in 96 well plates. This procedure led to the following cell lines: HEK-RmRDL, HEK-CfRDL-A_{285}, HEK-CfRDL-S_{285} HEK-DmRDL-A_{302}, HEK-DmRDL-S_{302}, and HEK-RmGluCl.

2.8. Cell culture procedure

For the RDL expressing cell-lines HEK-RmRDL, HEK-CfRDL-A_{285}, HEK-CfRDL-S_{285} HEK-DmRDL-A_{302}, HEK-DmRDL-S_{302} the long-term stability was tested for at least 50 passages. All cell lines used in tests originated from passages 5 to 20. These cells were incubated at 37 °C with 5% CO_2. Cultures were split 1:6 every 3–4 days, and additional changes of medium were done as applicable. All cell lines were cultured in the same medium (500 ml MEM, 57 mL FCS, 5.7 ml L-glutamine 200 mM, 5.7 ml NEAA (non essential amino acids), 5.7 ml Penicillin 10000 U/ml/Streptomycin 10 mg/ml, 6.9 mL G418 stock solution with 50 mg/ml (final concentration 600 μg/ml)). To detach the cells from the cell-culture flasks the cells were washed with PBS and incubated for 2–5 min with Accutase (Sigma). Afterwards, dilutions with culture medium were performed to the desired split ratio.

The long term stability of the WS-1 cell line (Wong et al., 1992) was tested for >50 passages. The cells used in this test were between passage 15 and passage 18. This cell line was incubated at 37 °C with 10% CO_2. Cultures were split 1:6 every 3–4 days, and additional changes of medium were done as applicable. This cell line was cultured in a slightly different medium (500 ml DMEM, 57 ml FCS, 11.4 ml L-glutamine 200 mM, 5.7 ml Na-pyruvate), but passaged as described above for the HEK293 cell lines.

2.9. Fluorescent membrane potential dye assays, EC_{50} and IC_{50} determinations of RDLs

For fluorescent membrane potential dye assays, 2–2.5 × 10^4 cells in 30 μl medium were seeded in poly-D-lysine-coated 384 well plates and allowed to grow for 24 h. Next, 26 μl of FMP-dye RED in VSP buffer (160 mM Na-gluconate, 4.5 mM K-gluconate, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES, pH 7.4) was added to each well in a dilution corresponding to 50–100% of the concentration recommended by the manufacturer, and incubated at room temperature for 30–60 min. In the case of inhibitor studies, 7 μl of test compounds and control compounds in VSP buffer/10% DMSO were distributed into the wells and incubated at room temperature for 30 min. To activate the ion channels, 7 μl of GABA was added leading to the following final concentrations: 30 μM GABA for the HEK-RmRDL cell line, 100 μM GABA for the HEK-CfRDL-A_{285} cell line, 100 μM GABA for the HEK-CfRDL-S_{285} cell line, 10 μM GABA for the HEK-DmRDL-A_{302} cell line, 10 μM GABA for HEK-DmRDL-S_{302} cell line and 10 μM GABA for WS-1 cell line. After a further 30 min incubation at room temperature, fluorescence was measured at 565 nm emission wavelength with 530 nm excitation wavelength using a Safire 2 (Tecan) or a FlexstationII384 (Molecular Devices).

2.10. Fluorescent membrane potential dye assays, EC_{50} and IC_{50} determinations of the R. microplus GluCl

For fluorescent membrane potential dye assays, 2.5 × 10^4 cells of the HEK-RmGluCl line in 30 μl medium (see above) were seeded in poly-D-lysine-coated 384 well plates and allowed to grow for 48 h. 20 μl of FMP-dye BLUE in VSP buffer (160 mM Na-gluconate, 4.5 mM K-gluconate, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES, pH 7.4) was added to each well in a dilution corresponding to 100% of the concentration recommended by the manufacturer, and incubated at room temperature for 30–60 min. In the case of inhibitor studies, 12.5 μl of test compounds and control compounds in VSP buffer/10% DMSO were distributed into the wells and incubated at room temperature for 30 min. To activate the ion channels, 12.5 μl glutamate was added to 100 μM final concentrations, and fluorescence was measured kinetically at 510 nm excitation and 545/565 nm emission using a FLIPR^{Tetrall} (Molecular Devices).

2.11. Analysis of data

Data were analysed using the data management system ActivityBase™ including XLfit® (IDBS: ID Business Solutions Ltd., 2 Occam Court, Surrey Research Park, Guildford, Surrey, GU2 7QB UK).

Percentage activity after GABA stimulation was calculated as follows:

$$\%\ \text{activity} = \frac{V - B}{C - B} \times \ 100\%$$

V: mean fluorescence with test compound (GABA/glutamate stimulated)

B: mean fluorescence of HI_control (without GABA/glutamate stimulation)

C: mean fluorescence of LO_control (GABA/glutamate stimulated positive control)

% inhibition is calculated as follows: % inhibition = 100% - % activity

IC_{50} values and the hill coefficients were calculated using the four parameter equation 205 ($y = A + ((B-A)/(1+((C/x)D))))$ from XLfit® (IDBS: ID Business Solutions Ltd., 2 Occam Court, Surrey Research Park, Guildford, Surrey, GU2 7QB UK) and the option unlock.

2.12. Evaluation of insecticidal and acaricidal potency of compounds

Unfed adult fleas of *C. felis* (adapted to artificial feeding, Wade and Georgi, 1988) were collected from long term *in vitro* cultures. Defibrinated blood was offered to adult fleas through a parafilm membrane for feeding and to induce oviposition. The laid eggs were reared to larvae, which were then fed on a blood meal medium until pupation. The unfed adult fleas emerging from these pupae were used in this study to assess the insecticidal activity after feeding on declining concentrations of the test compounds. Flies of *Stomoxys (S.) calcitrans* and *Lucilia (L.) cuprina,* and mosquitoes of *Aedes (A.) aegypti* were collected from *in vitro* artificial rearing systems. The adults were fed with defibrinated blood using a blood-soaked cotton swab (*Stomoxys calcitrans*), a parafilm membrane (*Aedes aegypti*), or with a protein/sugar medium (*Lucilia cuprina*). Organic vegetable medium (consisting of wheat and lucerne as major components) was offered to adult *S. calcitrans* for oviposition. Eggs were reared in the medium for development from larval hatch to pupation. The unfed adult flies emerging from these pupae were used in this study to assess the insecticidal activity after feeding on declining concentrations of the test compounds. Meat medium was offered to adult *L. cuprina* for oviposition. The freshly hatched fly larvae (L1) were harvested and used in this study to assess the insecticidal activity after exposure to declining concentrations of the test compounds. A moist filter paper was offered to adult *A. aegypti* for oviposition. The laid eggs were exposed to water until larval hatch. The freshly hatched mosquito larvae were harvested and used in this study to assess the insecticidal activity after exposure to declining concentrations of the test compounds.

Engorged females of the ixodide (hard-bodied) tick *R. microplus* were collected after dropping from cattle which had been infested with larvae approximately 3 weeks earlier, and used in this study to assess the acaricidal activity after immersion in declining concentrations of the test compounds. Each tick stage (larva, nymph, adult) of the ixodide tick *Rhipicephalus sanguineus* was reared on rabbits until full engorgement and dropping from the host. Fully engorged larvae/nymphs were incubated under controlled climate conditions until molting into the next stage (nymphs/adults). The unfed adults were used in this study to assess the acaricidal activity after immersion in declining concentrations of the test compounds. Nymphal and adult stages of the argaside (soft-bodied) tick *Ornithodoros (O.) moubata* were reared on rabbit or cattle until full engorgement and dropping from the hosts. Unfed nymphs (N3) were used in this study to assess the acaricidal activity after feeding on declining concentrations of the test compounds. All parasite cultures were maintained at MSD Animal Health Innovation GmbH, Schwabenheim, Germany.

To test for insecticidal and/or acaricidal activity, the compounds were dissolved in a premix consisting of DMSO and emulsifiers, and diluted with deionized water or blood to obtain a stock solution of 1 mg/ml (1000 parts per million, ppm). Further dilutions (100 ppm, 10 ppm, 1 ppm, etc.) were generated from this stock using either deionized water (*L. cuprina*, *A. aegypti*, *R. microplus*, *R. sanguineus*) or blood (*C. felis*, *S. calcitrans*, *Ornithodoros moubata*). Deltamethrin and imidacloprid were used as reference insecticides/acaricides. For *C. felis*, 20 adult cat fleas per compound solution were continuously fed with a medicated blood diet through a parafilm membrane until

Fig. 2. Alignment of RDL protein sequences from *D. melanogaster*, *C. felis* and *R. microplus*. *C. felis* (KF881790) and *R. microplus* RDL (KF881797) polypeptide sequences identified in this study were aligned with *D. melanogaster* RDL (M69057). The open arrow indicates the peptide sequence used for the design of the single degenerate primer used for primer extension. The peptide sequences used for RT-PCR degenerate primer pair design are marked with filled horizontal arrows. The predicted endoplasmic reticulum (ER) import signal sequences are highlighted by grey shading of black letters. The Cys loop is indicated by a half-circle connection and the four transmembrane helices (TM1-TM4) are highlighted by black bars under the consensus sequence.

assessment of mortality (after approximately 48 h). For *S. calcitrans*, 25 adult flies per compound solution were continuously fed with a medicated blood diet until assessment of mortality (after approximately 24 h). For *L. cuprina*, the compound solutions were mixed 9 + 1 (w/v) with ground beef meat. Approximately 20 larvae L1 were added per compound solution and incubated until assessment of mortality and inhibition of larval development (after approximately 48 h). For *A. aegypti*, liquid growth medium (MSD Animal Health Innovation GmbH) was used for serial dilutions with the compound in 128-well trays. Approximately 20 larvae L1 were added per compound solution and incubated until assessment of mortality and inhibition of larval development (after approximately 48 h). For *R. microplus*, 20 engorged adults were immersed in 20 ml compound solution for approximately 5 min. Thereafter, the ticks were dried off and incubated in a petri dish lined with a filter paper until assessment of mortality and oviposition (after approximately 3 weeks). For *R. sanguineus*, 20 unfed adults were immersed in 20 ml compound solution for approximately 5 min. Thereafter, the ticks were dried off and incubated in a petri dish lined with a filter paper until assessment of mortality (after approximately 48 h). For *O. moubata*, nymphs N3 were fed on a medicated blood diet through a parafilm membrane until engorgement. 10 engorged nymphs were collected and then kept under controlled climate conditions in a petri dish lined with a filter paper to enable the assessment of mortality and inhibition of molting into the next stage (after approximately 3 weeks).

3. Results

3.1. Identification of *R. microplus* genes encoding RDL and GluCl subunits and full length cDNA cloning

For the identification of the RDL subunit gene of *R. microplus* (*rmrdl*), specific cDNA was enriched by reverse transcription of larval total RNA using a degenerate primer deduced from the conserved peptide sequence PKISYVK (Fig. 2). The reaction product was then used as a template for RT-PCR using a degenerate primer pair from the conserved peptide sequences (N/K)IWVPDT and WVSFW(L/I)N) (Fig. 2). An RT-PCR product of 460 bp was cloned and sequenced (KF881794). The deduced protein sequence showed extensive sequence identities (74.5%) to the corresponding region of the *D. melanogaster* RDL gene *dmrdl* (M69057, ffrench-Constant et al., 1991) indicating that the correct gene fragment had been identified. The missing cDNA sequences of the *R. microplus* RDL gene (*rmrdl*) were obtained by 5′- and 3′-RACE using tick larval total RNA as template (KF881795 and KF881796, respectively). Based on the deduced start and stop codon positions in the 5′- and 3′-RACE product sequences of *R. microplus rmrdl*, PCR primers were then designed for the PCR amplification of the respective full length genes from tick cDNA (KF881797).

In the case of *R. microplus* GluCl, the gene was identified by degenerate primer PCR with a primer pair derived from the conserved peptide sequences C(N/T)SKTNTG and WVSFWLD (Fig. 3). The resulting PCR product of 142 bp was cloned

and sequenced. The deduced protein sequence (CTSNTNTGEY SCLRVDLVFKREFSYYLIQIYIPCCMLVIVSWVSFWL) was 89.4% identical to the corresponding region in the *D. melanogaster* GluCl gene (*dmglucl*, U58776, Cully et al., 1996), which was a strong indication that an orthologous gene fragment had been identified. The start and stop codons of the *R. microplus* GluCl gene were identified by sequencing 5′-RACE and 3′-RACE products (KF881798and KF881799, respectively). By the use of flanking primers around the start and stop codons and PCR on *R. microplus* larval cDNA, a full length gene of 1364 bp was identified (KF881800, *rmglucl*). The tick protein was 71.8% identical to the *D. melanogaster* U58776 polypeptide sequence (Cully et al., 1996), suggesting that the cloned gene *rmglucl* could be the tick ortholog of *dmglucl*. However, with respect to exon 3, *rmglucl* of this study is more similar to the *D. melanogaster glucl* splice variant AJ002232 described by Semenov and Pak (1999).

3.2. Full length cDNA cloning of *C. felis* and *D. melanogaster* RDL subunits

The gene encoding *C. felis* RDL (*cfrdl*) was isolated by (RT-)PCR using a full length primer pair derived from a published sequence (US7476728) using *C. felis* cDNA. A PCR error-free version of the *C. felis* RDL cDNA (KF881790), which corresponds to *dmrdl* splice variant *ac* (see below) was used for further studies. This form (*cfrdl*-S_{285}) represented a presumably dieldrin-resistant form of the receptor, with a serine at the position corresponding to amino acid 302 in *dmrdl* (ffrench-Constant et al., 1993a,b). Site-directed mutagenesis at this position was performed, resulting in the *C. felis* RDL cDNA *cfrdl*-A_{285}, the presumably dieldrin-sensitive form of the gene (KF881791).

For comparison, the gene encoding *D. melanogaster* RDL (*dmrdl*) was isolated by PCR using a full length primer pair derived from the database sequence M69057 and fly total cDNA. A PCR error-free version (KF881792) corresponding to splice variant *ac* was used for further studies. This *dmrdl* gene version corresponded to the dieldrin-sensitive form with an alanine at position 302 (*dmrdl*-A_{302}). To make the dieldrin-resistant form available for further studies, site-directed mutagenesis at this position was performed resulting in the gene version *dmrdl*-S_{302} (KF881793).

3.3. Bioinformatics analysis of *R. microplus*, *C. felis* RDL subunits and *R. microplus* GluCl, and their functional expression in *X. laevis* oocytes

The deduced polypeptide sequences of the putative RDL and GluCl subunit genes from *R. microplus* and *C. felis* identified in this study showed many of the elements typical of the ligand-gated ion channel superfamily: first, they all contained signal sequences for import into the endoplasmic reticulum, as indicated by high scores (RDL: 0.924 for *C. felis*, 0.874 for *R. microplus;* GluCl: 0.906 for *R. microplus*) in the SignalP 4.1 algorithm (Petersen et al., 2011). Second, the polypeptides of all three subunits possessed the conserved cysteines required for the formation of the cystine loop, the hallmark of the protein family (Figs. 2 and 3). Third, prediction of transmembrane helices with TMHMM 2.0 showed for all three polypeptides, and independent of the presence of A or S at position 285 in CfRDL, the presence of four such helices each (Figs. 2 and 3), with the arrangement of extracellular and intracellular domains in agreement with the known architecture of ligand-gated ion channels (not shown).

In the case of GluCl, comparisons of the *R. microplus* protein described in this study (Fig. 3) with the *Caenorhabditis elegans* GluClα subunit X-ray structure (Cully et al., 1994; Hibbs and Gouaux, 2011) showed, that 8 out of 9 residues described in the nematode receptor as being part of the loops A–G and being involved in L-glutamate binding are conserved in the tick sequence (Fig. 3).

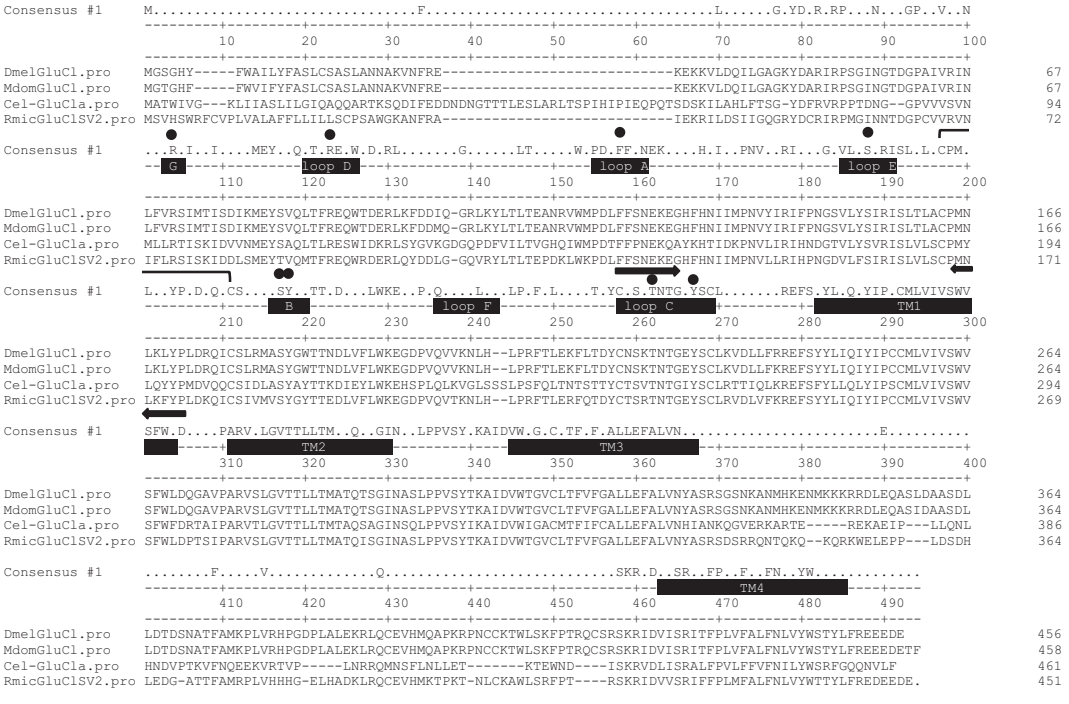

Fig. 3. Alignment of GluCl protein sequences from *D. melanogaster*, *C. felis* and *R. microplus*. The *R. microplus* GluCl polypeptide sequence identified in this study (KF881800) was aligned with those of *D. melanogaster* GluCl (U58776), *M. domestica* GluCl (AB177546), and *C. elegans* GluClα (U14524). The peptide sequences used for degenerate primer design are marked with horizontal arrows. The predicted endoplasmic reticulum (ER) import signal sequences are highlighted by grey shading of black letters. The Cys loop is indicated by a half-circle connection and the four transmembrane helices (TM1-TM4) as well as the loops A-G identified in X-ray structure analysis of *C. elegans* GluClα (Hibbs and Gouaux, 2011; Wolstenholme, 2012) are highlighted by black bars under the consensus sequence. Conserved residues involved in L-glutamate agonist binding are highlighted by black circles.

Construction of a DNASTAR/ClustalW-based molecular tree of polypeptide sequences (Fig. 4) showed, that the *R. microplus* and *C. felis* RDL subunits form a cluster with the corresponding protein family, while the same is observed for the *R. microplus* CluCl subunit in their corresponding protein family. Furthermore, the tick sequences are in their respective subclusters of acarid GluCl and RDL subunits, while the *C. felis* RDL clustered in the respective insect subfamily (Fig. 4).

The functionality of all *rdl* and *glucl* subunit genes identified and isolated in this study was investigated by *X. laevis* oocyte electrophysiology experiments after injection of *in vitro*-transcribed, capped, polyadenylated cRNA. In the case of the tick gene *rmrdl*, application of GABA resulted in strong currents of up to 12 μA (Fig. 5A), suggesting that the channel subunits were assembled into functional homomers in the oocytes. Similarly, *Xenopus* oocyte expression of *cfrdl*-A_{285} and *cfrdl*-S_{285} (Fig. 5B and C), as well as *dmrdl*-A_{285} and *dmrdl*-S_{285} (not shown) resulted in currents in the range of 5 μA. Expression of the tick *rmglucl* gene in *Xenopus* oocytes led to ion channels that were reversibly opened by glutamate with currents up to up to 7 μA (Fig. 5D), while application of ivermectin onto this receptor led to the expected permanent ion channel opening (Fig. 5E).

3.4. Generation of arthropod RDL- and GluCl-expressing HEK293 cell lines, development of a membrane potential fluorescent dye assay, and characterization of agonist and antagonist pharmacology

To investigate the effects of agonists and antagonists on the *rdl*- or *glucl*-encoded arthropod RDL and GluCl subunits in more detail, transgenic clonal HEK293 cell lines were generated, that

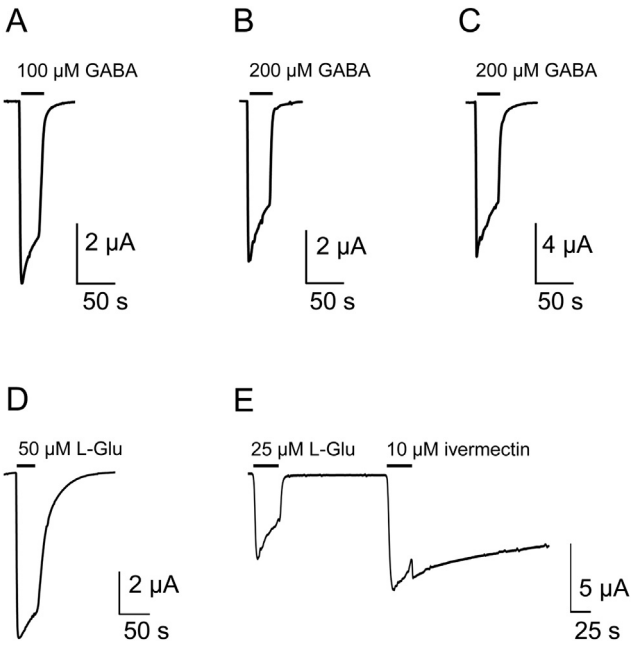

Fig. 5. Examples for electrical current responses of homomeric *C. felis* and *R. microplus* RDL and GluCl ion channels expressed in *Xenopus laevis* oocytes. (A) *R. microplus* RDL; (B) *C. felis* RDL-A_{285}; (C) *C. felis* RDL-S_{285}; (D,E) *R. microplus* GluCl. The identity, concentration, and length of application of applied agonists are indicated; L-Glu: L-glutamate.

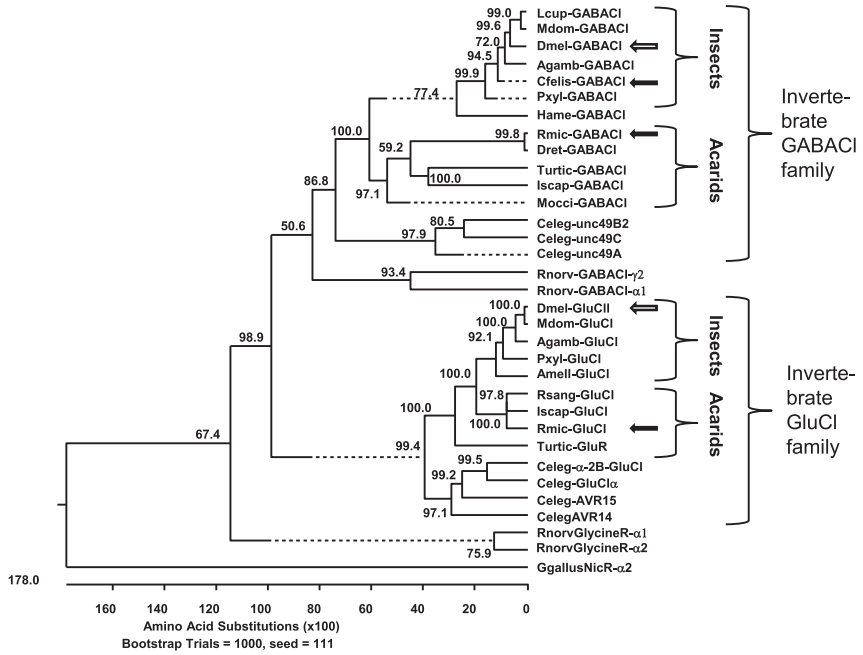

Fig. 4. Amino acid sequence identity relationships of *R. microplus* RDL, *C. felis* RDL and *R. microplus* GluCl to other RDL/GABACl and GluCl subunits. The dendrogram (DNAStar) was derived from CLUSTALW-aligned protein sequences of *R. microplus* RDL (Rmic-GABACl, KF881797), *C. felis* RDL (Cfelis-GABACl, KF881790), *R. microplus* GluCl (Rmic-GluCl, KF881800) identified in this study (highlighted by black arrows) together with the RDL subunits of *D. melanogaster* (Dmel-GABACl, M69057), *M. domestica* (Mdom-GABACl AB177547), *L. cuprina* (Lcup-GABACl, AF024647), *Anopheles gambiae* (Agamb-GABACl, XM_316071), *Plutella xylostyla* (Pxyl-GABA-Cl, EU273945), *Homarus americanus* (Hame-GABACl, AY098943), *Dermacentor variabilis* (Dvar-GABACl, BD432650), *Tetranychus urticae*, (Turtic-GABACl, AB634459), *Ixodes scapularis* (Iscap-GABACl, XM_002411520), *Metaseiulus occidentalis* (Mocci-GABACl, XM_00373780), *Caenorhabditis elegans* (unc49A, AF151640; unc49B2, AF151642; unc49C, AF151644), *Rattus norvegicus* (GABACl-α1, NM_183326; GABACl-γ2, NM_183327), and the GluCl subunits of *D. melanogaster* (Dmel-GluCl, U58776), *M. domestica* (Mdom-GluCl AB177546), *Anopheles gambiae* (Agamb-GluCl, XM_321696), *Plutella xylostyla* (Pxyl-GluCl, GQ221939), *Apis mellifera* (Amell-GluCl, NM_001077809), *Rhipicephalus sanguineus* (Rsang-GluCl, GQ215234), *Ixodes scapularis* (Iscap-GluCl, XM_002413465), *Tetranychus urticae*, (Turtic-GluCl, AB567687), *Caenorhabditis elegans* (GluClα-2B, AJ000537; GluClα, U14524; AVR15, AJ243914; AVR14, NM_001025791). Furthermore, *Rattus norvegicus* glycin-gated chloride channel sequences were included (RnorvGlycineR-1α1, NM_012568; RnorvGlycineR-α2, NM_013133), and the *Gallus gallus* nicotinic receptor α2 subunit (GgallusNicR-a2, NM_204815) served as an outgroup.

stably express either DmRDL-A_{302}, DmRDL-S_{302}, CfRDL-A_{285}, CfRDL-S_{285}, RmRDL, or RmGluCl. In addition, a rat GABACl-expressing cell line, derived from the transfection of rat GABACl α_1, β_1 and γ_2 subunit genes into HEK293 cells (Wong et al., 1992) was purchased from ATCC, and used as a reference. A membrane potential dye assay was established, that allowed the determination of dose–response curves and effective concentrations 50% (EC_{50}s) of specific agonists (Fig. 6A and C). The GABA EC_{50}s for all GABACls investigated in this study were in the low μM range, with DmRDL being the most sensitive receptor, and the Hill coefficients suggested pronounced positive cooperativity for all GABA receptors investigated (Table 1, see Fig. 6C as an example). Only little differences were seen between the GABA EC_{50}s and Hill coefficients of the DmRDL-A_{302} *versus* DmRDL-S_{302} and of the CfRDL-A_{285} *versus* CfRDL-S_{285} receptor versions. The RmGluCl showed an EC_{50} for the natural ligand L-glutamate of ~18 μM, and the Hill coefficient suggested that this tick receptor does not show any cooperativity (Table 1, Fig. 6A).

By contrast to the findings on RDL EC_{50}s for the natural agonist GABA, major differences were detected with respect to sensitivity to antagonists: when the inhibition potencies of dieldrin and picrotoxinin were analysed on the GABA receptor compendium, IC_{50}s in the low μM to high nM range could be determined on DmRDL-A_{302} and CfRDL-A_{285} for these two antagonists. However, on the corresponding S_{302}/S_{285} ion channel versions, IC_{50}s could not be determined due to very low receptor sensitivities, or, in the case of CfRDL-S_{285} and picrotoxinin, the IC_{50} was ~25 × higher than on its CfRDL-A_{285} counterpart (Table 1, and Fig. 6F). These data confirmed the expected dieldrin resistance phenotypes reported for the DmRDL A_{302} ➔ S_{302} mutation, and the corresponding RDL mutants on other insect receptors (ffrench-Constant et al., 1993a,b; ffrench-Constant, 1994). Dieldrin showed very poor antagonism on the rat GABACl investigated in this study (IC_{50} > 30 μM), while the cattle tick receptor proved to be highly sensitive to this organochlorine cyclodiene, showing an IC_{50} in the low nM range. For the convulsant picrotoxinin, both the rat GABACl and the tick RDL exhibited IC_{50}s in the low μM range.

In the case of the ectoparasiticidal market product fipronil (Fig. 1), the DmRDL-A_{302} variant showed an IC_{50} of ~36 nM, while the dieldrin-resistant S_{302} variant was ~18-fold less sensitive to this drug (Table 1). This suggested that the A_{302} ➔ S_{302} mutation has also a pronounced negative influence on fipronil activity, a result in agreement with earlier *D. melanogaster* studies (Hosie et al., 1995). By contrast, no such negative influence of the A_{285} ➔ S_{285} mutation was seen on fipronil activity towards CfRDL, as both forms had IC_{50}s around 10 nM. Also, the *R. microplus* RDL displayed a fipronil IC_{50} of ~8.5 nM, while this phenylpyrazole compound had no inhibitory activity on the rat GABACl analysed in this study. Furthermore, it was observed that RmGluCl is strongly inhibited by fipronil in the nM range (Table 1).

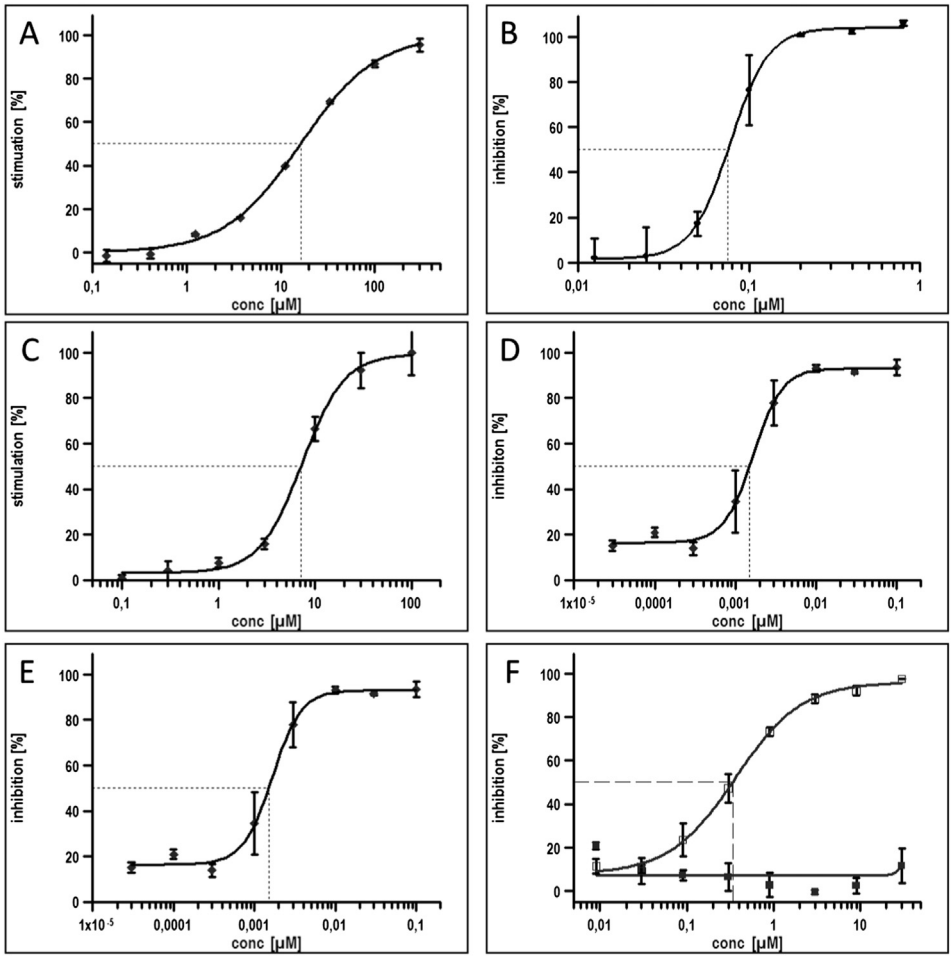

Fig. 6. Examples for membrane fluorescence dye assay agonist and antagonist responses of homomeric *C. felis* and *R. microplus* RDL and GluCl ion channels expressed in HEK293 cells. (A) RmGluCl, L-glutamate stimulation dose response; (B) RmGluCl, 100 μM L-glutamate, fluralaner inhibition dose response; (C) RmRDL, GABA stimulation dose response; (D) RmRDL, 30 μM GABA, fluralaner inhibition dose response; (E) CfRDL-S_{285}, 100 μM GABA, fluralaner inhibition dose response; (F) CfRDL-A_{285} (open squares) *versus* CfRDL-S_{285} (filled squares), 100 μM GABA, dieldrin inhibition dose response.

Table 1
GABACl and GluCl channels: EC_{50} values and Hill coefficients for specific agonists, IC_{50} values of antagonists.

	Rat GABACl ($\alpha_1\beta_1\gamma_2$; WS-1)	DmRDL-A_{302}	DmRDL-S_{302}	CfRDL-A_{285}	CfRDL-S_{285}	RmRDL	RmGluCl
EC_{50} (μM)							
GABA	1.9 ± 0.7	1.2 ± 0.3	1.4 ± 0.4	19.2 ± 2.6	17.7 ± 2.6	9.8 ± 2.7	17.8 ± 2.8 (L-glutamate)
Hill coefficient							
GABA	1.82 ± 0.28	2.16 ± 0.36	1.97 ± 0.73	2.30 ± 0.49	2.48 ± 0.38	2.01 ± 0.02	1.10 ± 0.07 (L-glutamate)
IC_{50} (nM)							
Dieldrin	>30,000	3301 ± 1154	>30,000	550 ± 189	>30,000	<9	n.d.
Picrotoxinin	4539 ± 574	773 ± 386	>30,000	346 ± 199	9980 ± 3769	2017 ± 1774	n.d.
Fipronil	>30,000	36.3 ± 8	663 ± 236	10.0 ± 6.5	10.8 ± 1.8	8.5 ± 4.1	60.7 ± 16.5
Fluralaner	>30,000	1.6 ± 0.4	2.8 ± 1.5	0.45 ± 0.4	1.7 ± 0.7	1.6 ± 0.8	82.5 ± 14.1

±Standard deviation derived from at least six measurements.

The novel isoxazoline ectoparasiticide fluralaner showed significantly higher inhibitory activity (5–236-fold) than fipronil on all five arthropod RDLs investigated in this study. The observed IC_{50}s ranged from 2.8 nM (DmRDL-S_{302}) to as low as 0.45 nM (CfRDL-A_{285}), while no inhibitory activity on rat GABACl was found (Table 1, Fig. 6E as example). Only small changes in fluralaner IC_{50}s were observed for the dieldrin-resistant *versus* the dieldrin-sensitive insect RDL forms. Fluralaner was also a potent inhibitor of RmGluCl, with an IC_{50} of ~80 nM (Fig. 6B, Table 1), which is in the same range as seen for fipronil.

3.5. Ectoparasiticidal activity of fluralaner in comparison with dieldrin, fipronil, imidacloprid, and deltamethrin

The ectoparasiticidal activity of fluralaner was investigated using *in vitro* test systems on four insect parasites, the cat flea *C. felis*, the stable fly *S. calcitrans*, the sheep blowfly *L. cuprina* and the yellow fever mosquito *A. aegypti*, as well as on three tick species, the hard-bodied *R. microplus* (cattle tick) and *R. sanguineus* (brown dog tick), and the soft-bodied tick *O. moubata*. Parasiticidal potency was assessed in comparison with dieldrin and the current market product insecticides and acaricides fipronil, imidacloprid and deltamethrin.

The cat flea colony used in our study exhibited the *cfrdl*-S_{285} genotype expected to confer dieldrin resistance. Indeed, the fleas survived up to 100 ppm dieldrin exposure, confirming that the *C. felis* RDL resistance genotype also translates into a phenotype. Fluralaner was not affected by this dieldrin resistance and killed exposed fleas at concentrations as low as 0.01 ppm. Remarkably, this isoxazoline derivative was superior to the marketed compounds fipronil and imidacloprid in its flea killing activity by one order of magnitude (Table 2). When applied to larvae from blowfly and from mosquito, fluralaner was at least one to two orders of magnitude more potent than all reference compounds for *L. cuprina*, and proved to be even about four orders of magnitude more potent against *A. aegypti*. In the case of adult engorged female hard ticks as well as soft tick nymphs, fluralaner killed these parasites at lower concentrations than the established acaricide fipronil. Furthermore, fluralaner killed engorged female *R. microplus* at concentrations as low as 1 ppm whereas deltamethrin at 1000 ppm failed to reach the 90% killing threshold on ticks of this isolate (Table 2).

Fluralaner proved to be arthropodicidal for each organism investigated and demonstrated very high potency at low test concentrations in the range of ≥1–0.0000012 ppm, generally outperforming the other test compounds. The isoxazoline derivative showed like fipronil 100% mortality at 1 ppm for *S. calcitrans* adult flies, and shared with deltamethrin a very low mortality concentration of 0.0001 ppm for *O. moubata* ticks. Only in two assays (*S. calcitrans*, *R. sanguineus*) the pyrethroid deltamethrin displayed an insecticidal/acaricidal activity at a lower test concentration than fluralaner. However, in all other test settings, fluralaner performed best as ectoparasiticide (Table 2). Taken together, fluralaner was of superior potency compared to the insecticides imidacloprid and dieldrin, and also exceeded the potency of fipronil and deltamethrin in the majority of the arthropods tested.

4. Discussion

In our study on the biochemical and parasitological properties of the RDL and GluCl antagonist fluralaner (A1443, Ozoe et al., 2010), a first step comprised the identification, full length cDNA cloning and demonstration of functionality of the putative target genes from

Table 2
Insecticidal and acaricidal activity of fluralaner and other selected ectoparasiticidal market products.

	Fluralaner	Fipronil	Dieldrin	Imidacloprid	Deltamethrin
	Threshold of mortality ≥90% (ppm)				
Ctenocephalides felis, adult fleas[a]	≥0.01	≥0.1	None at 100 ppm	≥0.1	None at 100 ppm
Stomoxys calcitrans, adult flies[a]	≥1	≥1	n. d.	n. d.	≥0.1
Lucilia cuprina, fly larvae L1[b]	≥1	≥100	≥100	≥10	≥100
Aedes aegypti, mosquito larvae L1[c]	≥0.0000012	≥0.02	≥0.08	≥0.08	≥0.01
R. microplus, engorged female ticks[c]	≥1	≥3.9	n. d.	n. d.	None at 1000 ppm
Rhipicephalus sanguineus adult ticks[c]	≥1	≥125	n. d.	n. d.	≥0.5
Ornithodoros moubata, tick nymphs N3[a]	≥0.0001	≥0.001	≥1	None at 10 ppm	≥0.0001

n. d.: not determined.
[a] Medicated blood diet.
[b] Medicated meat diet.
[c] Medicated immersion fluid.

R. microplus, *rmrdl* and *rmglucl*, to build the basis for tick on-target studies. Based on protein sequence homology analyses (Fig. 4) and other bioinformatics evaluations (Figs. 2 and 3), the *rmrdl* and *rmglucl* genes studied in this report belong to the arthropod RDL and GluCl gene families, respectively, which strongly suggests that *R. microplus* gene orthologs for these two ion channels have been identified.

Functional expression and two electrode voltage clamp studies on *rmrdl* cRNA injected into *Xenopus* oocytes demonstrated that its gene product acts as a RDL channel. The deduced *rmrdl* protein sequence is virtually identical (except for three conservative R ➔ K exchanges) to the translated ORF of the *R. microplus rdl* gene from another tick isolate reported while this study was ongoing (Hope et al., 2010). Also, the *rmrdl* translated ORF is 98.3% identical to the protein sequence of a *Dermacentor variabilis rdl* gene, whose expression product has been functionally characterized as RDL (Zheng et al., 2003). In the case of RmGluCl, the *Xenopus* oocyte electrophysiology studies presented here demonstrated its reversible channel gating by L-glutamate and the irreversible channel opening by ivermectin, consistent with the functional properties of arthropod GluCls (Wolstenholme, 2012). Comparison of RmGluCl with translated *R. sanguineus* DNA sequences (BD437104-BD43106) from a patent (Amuran et al., 2004), that were predicted to be L-glutamate-gated chloride channel genes, show 98.2% sequence identity.

To complement our molecular investigations with the most important companion animal ectoparasite, the cat flea, we cloned the gene encoding *C. felis* RDL based on DNA sequences from an earlier report (AX477445, Wisniewski and Brandt, 2002). The deduced protein sequence of the *cfrdl* cDNA isolated from our *C. felis* strain differed from the earlier AX477445 at four positions, including an A_{285} ➔ S_{285} exchange predicted to confer dieldrin resistance (Bass et al., 2004; Brunet et al., 2009). Since the earlier studies on *cfrdl* were limited to DNA methods (Daborn et al., 2004; Bass et al., 2004; Brunet et al., 2009), *Xenopus* oocyte expression combined with voltage clamp electrophysiology was performed in this study, confirming that this gene encodes for a functional CfRDL (Fig. 5C). To investigate effects of the dieldrin resistance mutation A_{285} ➔ S_{285}, the CfRDL-A_{285}-encoding version of *cfrdl* was generated, which proved to be functional in *Xenopus* oocytes (Fig. 5B). Since the vast majority of historic RDL studies in insects have been performed with the *D. melanogaster* channel (Buckingham et al., 2005), the genes encoding the best-studied *ac*-splice variant (ffrench-Constant et al., 1991; Hosie et al., 2001) in its dieldrin-sensitive and -resistant *dmrdl* forms (A_{302}, S_{302}) were included in this study and functionally characterized, to allow better comparisons and connections to results published earlier by others.

In a second step of our study, transgenic stable and clonal cell lines were generated for the three parasite RDL gene forms *rmrdl*, *cfrdl*-A_{285} and *cfrdl*-S_{285}, for the two *D. melanogaster* gene versions *dmrdl*-A_{302} and *dmrdl*-S_{302}, as well as the tick *rmglucl*. The HEK293 cell line WS-1, that had been transfected with the rat $GABA_A$ receptor subunit genes α_1, β_2 and γ_2 (Wong et al., 1992), served as a mammalian GABACl control. The development of a membrane potential dye assay for these seven cell lines was a prerequisite for performing comparative determinations of agonist EC_{50} values and antagonist IC_{50} values.

The GABA EC_{50} for the mammalian control WS-1 cell line was determined to be 1.9 μM (Table 1), which is in the same range as the value (4.7 μM) published by Joesch et al. (2008) using a membrane fluorescence dye assay set-up and a similar cell line. In the case of DmRDL, importantly, the GABA EC_{50} values did not differ significantly between the dieldrin-resistant (S_{302}) and -sensitive (A_{302}) forms (1.2 μM *versus* 1.4 μM). These GABA EC_{50} values were somewhat lower than those previously reported from *Xenopus* oocyte voltage-clamp electrophysiology on DmRDLs corresponding to the *ac* splice variant used in this study (between 9.8 μM and 50 μM; ffrench-Constant et al., 1993a; Hosie and Sattelle, 1996, Belelli et al., 1996; Edwards and Lees, 1997; McGurk et al., 1998; Pistis et al., 1999; Hosie et al., 2001, 2006). Other DmRDL splice variants have been reported with even higher GABA EC_{50} values in *Xenopus* assays (splice variant *bd*: 152 μM, Chen et al., 1994, 103 μM, Hosie and Sattelle, 1996; 152 μM, Hosie et al., 2001; splice variant *ad*: 58 μM, Hosie et al., 2001). By contrast, a more recent study, using a membrane potential dye assay similar to that employed in this study, reported a GABA EC_{50} of 1 μM for DmRDL expressed in *Drosophila D.Mel-2* cells (Nakao et al., 2010), which is a value very close to our results. There is the possibility that voltage-clamp electrophysiology readouts of *Xenopus* GABACl expression systems might generally generate higher EC_{50}s than membrane potential dye assays in cell culture.

The GABA sensitivity of cat flea CfRDL was more than 10-fold lower than that of DmRDL, but the CfRDL EC_{50} was, similar to the observations for DmRDL, largely unaffected in its specific agonist pharmacology by the dieldrin resistance mutation (19.2 μM *versus* 17.7 μM, A_{285} ➔ S_{285}). In line with the results on DmRDL in HEK293 cell membrane fluorescence dye assays, the GABA EC_{50} of CfRDLs expressed in HEK293 cells tended to be lower than those obtained by *Xenopus* oocyte electrophysiology in several other insect species (*Heliothis virescens*: 19.1 μM, Wolff and Wingate, 1998; *Drosophila simulans*: 70 μM, Le Goff et al., 2005; *Musca domestica*: 100 μM, Eguchi et al., 2006; 160 μM, Ozoe et al., 2010), and in whole cell current recording on cockroach (*Periplanata americana*) throracic ganglion neurons (52.9 μM, Zhao et al., 2003).

The *R. microplus* receptor RmRDL showed a GABA EC_{50} of 9.8 μM, again much lower than the value published previously for a *Dermacentor variabilis* RDL, which was estimated in *Xenopus* oocyte voltage-clamp electrophysiology experiments as >100 μM (Zheng et al., 2003). In the case of the novel *R. microplus* RmGluCl, the EC_{50} for L-glutamate in the membrane potential dye assay (17.8 μM) could not be compared to a tick counterpart, but its EC_{50} was also somewhat lower than those published earlier based on *Xenopus* oocyte voltage-clamp electrophysiology for *D. melanogaster* GluCl (23 μM, Cully et al., 1996) and *M. domestica* GluCl, (33.2 μM, Eguchi et al., 2006), and based on whole cell current recording on *Periplaneta americana* thoracic ganglion neurons (36.8 μM, Ikeda et al., 2003).

As a third step in this study, the panel of RDL-, mammalian GABACl-, and tick GluCl-expressing cell lines was employed to assess inhibitor actions by insecticides, including the novel isoxazoline compound fluralaner (Fig. 1). None of the dieldrin, fipronil or fluralaner insecticides investigated in this work showed a pronounced inhibitory effect on the rat GABACls expressed in the WS-1 cell line. Only the nonspecific insecticidal and convulsant drug picrotoxinin showed a moderate potency on this cell line, with an IC_{50} of approximately 4.5 μM (Table 1). The most prominent representative of the cyclodiene group of insecticides, dieldrin, showed inhibitory action on DmRDL-A_{302} and Cf-RDL-A_{285} (IC_{50}s of 3.3 μM and 0.55 μM, respectively), while, in agreement with expectation (ffrench-Constant et al., 2000), the DmRDL-S_{302} and CfRDL-S_{285} variants with the dieldrin resistance mutations were not inhibited in the concentration range investigated (Table 1, Fig. 6F). A similar loss of inhibitory activity in the A ➔ S mutant channel forms was seen with picrotoxinin, also as expected, given the shared binding site with cyclodienes (Chen et al., 2006).

Fipronil (Fig. 1) is a phenylpyrazole insecticide and acaricide introduced in 1993 and used in agriculture as well as in veterinary medicine. This compound is known to block both GABA- and L-glutamate-gated chloride channels (Hainzl et al., 1998; Bloomquist, 2003; Narahashi et al., 2010). In our study, fipronil proved to be an effective inhibitor of DmRDL-A_{302} (IC_{50} = 36.3 nM), but the dieldrin

resistance mutant DmRDL-S_{302} did lead to a significant loss of potency by a factor of 18 (IC_{50} = 663 nM). This loss of fipronil inhibitor activity was not seen for the CfRDL dieldrin resistance mutant, where this inhibitor was approximately equipotent on the A_{285} and the S_{285} variants (~10 nM and 11 nM, Table 1). This observation may provide an explanation for the fact that despite the widespread occurrence of the dieldrin resistance mutation in cat flea populations (Bass et al., 2004; Daborn et al., 2004), failure of fipronil efficacy in connection with this mutation has so far not been reported (Brunet et al., 2009). In the case of RmRDL, fipronil inhibited this tick ion channel strongly with an IC_{50} of 8.5 nM, which is in agreement with the high acaricidal potency of this compound (Davey et al., 1998, Table 2). The IC_{50} values of fipronil on parasite and fruit fly RDLs of this study compare well with earlier reports on *D. melanogaster* and *M. domestica* RDLs using the *Xenopus* voltage clamp electrophysiology readouts (3.6–31.4 nM, Le Goff et al., 2005, 24.5 nM, Ozoe et al., 2010; respectively). In addition to RDLs, GluCls of various insects have also been described as fipronil targets, where this inhibitor reaches nanomolar potency (Ikeda et al., 2003; Zhao et al., 2004; Zhao and Salgado, 2010; Narahashi et al., 2010; Ozoe et al., 2010). Our experiments on *R. microplus* GluCl show that this acarid chloride channel is also susceptible to fipronil. The IC_{50} of fipronil on RmGluCls is approximately 61 nM. However, given that RmRDL is more than 7-fold more sensitive to this pesticide, it appears that at least in the tick *R. microplus*, GluCls may be secondary fipronil targets after RDLs.

Isoxazolines have emerged recently as a novel class of parasiticides, and insect RDL and GluCl have been implicated as targets (Ozoe et al., 2010; Lahm et al., 2013; García-Reynaga et al., 2013). The experiments in this study have shown that the isoxazoline drug fluralaner (A1443, Ozoe et al., 2010) inhibits insect and acarid RDL channels from three species in the low nanomolar to high picomolar IC_{50} range. Only a minor, statistically not significant difference was seen between the CfRDL-A_{285}/S_{285} and DmRDL-A_{302}/S_{302} channel versions (Table 1), suggesting that fluralaner is not affected by the dieldrin resistance mutation in the cat flea and the fruit fly. These results are in agreement with earlier findings on *Musca* (*M.*) *domestica* (Ozoe et al., 2010) and *D. melanogaster* (Lahm et al., 2013) RDL forms. Comparison of fipronil and fluralaner RDL on-target efficacy showed, that the isoxazoline drug gave rise to a 5-fold (RmRDL) to 236-fold (DmRDL-S_{302}) lower IC_{50} values than the phenylpyrazole drug (Table 1), which is in line with Ozoe et al. (2010), who have reported a 5-fold higher inhibitor efficacy of fluralaner over fipronil on *M. domestica* RDL. Fluralaner did not show any inhibitory action on the rat GABACl expressed in WS-1 cells (Table 1).

On the *R. microplus* GluCl, fluralaner exhibits also nanomolar inhibitory activity (IC_{50} = 82.5 nM), but its on-target potency on the corresponding *R. microplus* RDL is approximately 52-fold higher (Table 1). In *M. domestica*, a direct comparison of fluralaner inhibitory action on RDL and GluCl has also shown a 15-fold higher potency on the GABA-gated ion channel (Ozoe et al., 2010). Our current data together with previous studies collectively suggest, that fluralaner is a highly potent arthropod-specific RDL channel inhibitor, with a less potent, but still significant, inhibitory activity on arthropod GluCls. Fluralaner proved to be of higher RDL on-target inhibitory activity compared to fipronil on all arthropod channels investigated so far, while on arthropod GluCls both pesticides are approximately equally active (Table 1, Ozoe et al., 2010).

It has been shown that fipronil is converted to fipronil sulfone by insect cytochrome P450 enzyme systems, and that this metabolite possesses also ectoparasiticidal activity and acts as antagonist on RDLs and GluCls (Hainzl et al., 1998; Scharf 1999, Durham et al., 2002; Zhao et al., 2005). This raises the possibility that fipronil sulfone might be of higher on-target potency than fluralaner, an aspect that remains to be tested. However, our bioscreen results demonstrate, that in an *in vivo* situation expected to lead to fipronil oxidation to fipronil sulfone in the ectoparasites, fluralaner still outperforms this phenylpyrazole drug in most test systems: In the evaluation of insecticidal and acaricidal potency of fluralaner, fipronil, dieldrin, imidacloprid and deltamethrin with four insect and three tick species (bioscreen), fluralaner showed a stronger activity compared to fipronil in all tests, except for *S. calcitrans*, where both compounds were showed 100% mortality at the lowest concentration tested (1ppm, Table 2). The parasiticidal activity of fluralaner exceeded that of fipronil by factors ranging from 3.9 (*R. microplus* adults ticks), to 10 (*C. felis* adult fleas), to 100 (*L. cuprina* larvae), to more than 16,000 (*A. aegypti* larvae). Furthermore, fluralaner was more potent in most assays than the other established insecticides/acaricides investigated here (dieldrin, imidacloprid and deltamethrin, Table 2). The bioscreen data of fluralaner were in agreement with the arthropod RDL inhibition data, suggesting that on-target potency on this ion channel may be a major contributing factor to its superior parasiticidal properties.

In summary, our study demonstrates that the novel isoxazoline parasiticide fluralaner outperforms fipronil on the primary molecular target RDL. Furthermore in a large number of bioscreens assessing insecticidal and acaricidal activity, fluralaner exhibited higher parasiticidal activity compared to the effects of fipronil, dieldrin and other marketed substances with different molecular mode of actions. This suggests that the isoxazoline fluralaner emerges as a new ectoparasiticidal drug with several superior properties. *In vivo* animal studies of ectoparasite challenge models have highlighted the therapeutic potential of this novel compound class (Heckeroth et al., 2013).

Acknowledgements

We would like to thank Mirjam Békefi, Marina Breitmann, Bernd Jürgens, Annette Schmitt, and Hartmut Zoller for expertly performing the bioscreens. Furthermore we acknowledge the expert technical assistance of Frank Fischer, Christa Grassmann, Susanne Schmalz, Sabine Thomas and Andrea Wagner in molecular sciences. We are also grateful to Dr. Michael Berger for the help with Fig. 1. Finally, we would like to thank Dr. Ralf Warrass and Dr. Holger Lehmann for critically reading the manuscript and suggestions for improvement.

References

Amuran, M.J., Yang, Y., Cully, D.F. and Warmke, J.W., 2004. DNA Molecules Encoding L-glutamate-gated Chloride Channels from Rhipicephalus Sanguineus. Patent: JP 2004500118-A 2 08-JAN-2004, Merck and Co Inc.

Belelli, D., Callachan, H., Hill-Venning, C., Peters, J.A., Lambert, J.J., 1996. Interaction of positive allosteric modulators with human and Drosophila recombinant GABA receptors expressed in Xenopus laevis oocytes. Br. J. Pharmacol. 118, 563–576.

Bermudez, I., Hawkins, C.A., Taylor, A.M., Beadle, D.J., 1991. Actions of insecticides on the insect GABA receptor complex. J. Recept. Res. 11, 221–232.

Bass, C., Schroeder, I., Turberg, A., Field, L.M., Williamson, M.S., 2004. Identification of the Rdl mutation in laboratory and field strains of the cat flea, *Ctenocephalides felis* (Siphonaptera: Pulicidae). Pest. Manag. Sci. 60, 1157–1162.

Bateson, A.N., 2009. The benzodiazepine site of the GABAA receptor: an old target with new potential? Sleep Med. 5 (Suppl 1), S9–S15.

Bloomquist, J.R., 1993. Toxicology, mode of action and target site-mediated resistance to insecticides acting on chloride channels. Comp. Biochem. Physiol. C. 106, 301–314.

Bloomquist, J.R., 1998. Chemistry and toxicology of the chlorinated cyclodienes and lindane. Rev. Toxicol. 2, 333–355.

Bloomquist, J.R., 2001. In: Ishaaya, Isaac (Ed.), GABA and Glutamate Receptors as Biochemical Sites for Insecticide Action. Springer-Verlag, Berlin, Heidelberg, pp. 17–41.

Bloomquist, J.R., 2003. Chloride channels as tools for developing selective insecticides. Arch. Insect Biochem. Physiol. 54, 145–156.

Brunet, S., Le Meter, C., Murray, M., Soll, M., Audonnet, J.C., 2009. Rdl gene polymorphism and sequence analysis and relation to in vivo fipronil susceptibility in strains of the cat flea. J. Econ. Entomol. 102, 366–372.
Buckingham, S.D., Hosie, A.M., Roush, R.L., Sattelle, D.B., 1994. Actions of agonists and convulsant antagonists on a *Drosophila melanogaster* GABA receptor (Rdl) homo-oligomer expressed in Xenopus oocytes. Neurosci. Lett. 181, 137–140.
Buckingham, S.D., Biggin, P.C., Sattelle, B.M., Brown, L.A., Sattelle, D.B., 2005. Insect GABA receptors: splicing, editing, and targeting by antiparasitics and insecticides. Mol. Pharmacol. 68, 942–951.
Casida, J.E., 1990. Insecticide action at the GABA-gated chloride channel: recognition, progress, and prospects. Arch. Insect. Biochem. Physiol. 22, 13–23.
Chen, R., Belelli, D., Lambert, J.J., Peters, J.A., Reyes, A., Lan, N.C., 1994. Cloning and functional expression of a Drosophila gamma-aminobutyric acid receptor. Proc. Natl. Acad. Sci. U. S. A 91, 6069–6073.
Chen, L., Durkin, K.A., Casida, J.E., 2006. Structural model for gamma-aminobutyric acid receptor noncompetitive antagonist binding: widely diverse structures fit the same site. Proc. Natl. Acad. Sci. U. S. A 103, 5185–5190.
Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.
Cole, L.M., Nicholson, R.A., Casida, J.E., 1993. Action of phenylpyrazole insecticides at the GABA-gated chloride channel. Pestic. Biochem. Physiol. 46, 47–54.
Cully, D.F., Vassilatis, D.K., Liu, K.K., Paress, P.S., Van der Ploeg, L.H., Schaeffer, J.M., Arena, J.P., 1994. Cloning of an avermectin-sensitive glutamate-gated chloride channel from *Caenorhabditis elegans*. Nature 371, 707–711.
Cully, D.F., Paress, P.S., Liu, K.K., Schaeffer, J.M., Arena, J.P., 1996. Identification of a *Drosophila melanogaster* glutamate-gated chloride channel sensitive to the antiparasitic agent avermectin. J. Biol. Chem. 271, 20187–20191.
Daborn, P., McCart, C., Woods, D., ffrench-Constant, R.H., 2004. Detection of insecticide resistance-associated mutations in cat flea Rdl by TaqMan-allele specific amplification. Pest. Biochem. Physiol. 79, 25–30.
Davey, R.B., Ahrens, E.H., George, J.E., Hunter 3rd, J.S., Jeannin, P., 1998. Therapeutic and persistent efficacy of fipronil against *Boophilus microplus* (Acari: Ixodidae) on cattle. Vet. Parasitol. 74, 261–276.
de Castro, J.J., 1997. Sustainable tick and tickborne disease control in livestock improvement in developing countries. Vet. Parasitol. 71, 77–97.
D'Hulst, C., Atack, J.R., Kooy, R.F., 2009. The complexity of the GABAA receptor shapes unique pharmacological profiles. Drug Discov. Today 14, 866–875.
Durham, E.W., Siegfried, B.D., Scharf, M.E., 2002. In vivo and in vitro metabolism of fipronil by larvae of the European corn borer Ostrinia nubilalis. Pest. Manag. Sci. 58, 799–804.
Edwards, M.D., Lees, G., 1997. Modulation of a recombinant invertebrate gamma-aminobutyric acid receptor-chloride channel complex by isoflurane: effects of a point mutation in the M2 domain. Br. J. Pharmacol. 122, 726–732.
Eguchi, Y., Ihara, M., Ochi, E., Shibata, Y., Matsuda, K., Fushiki, S., Sugama, H., Hamasaki, Y., Niwa, H., Wada, M., Ozoe, F., Ozoe, Y., 2006. Functional characterization of Musca glutamate- and GABA-gated chloride channels expressed independently and coexpressed in Xenopus oocytes. Insect. Mol. Biol. 15, 773–783.
ffrench-Constant, R.H., Mortlock, D.P., Shaffer, C.D., MacIntyre, R.J., Roush, R.T., 1991. Molecular cloning and transformation of cyclodiene resistance in Drosophila: an invertebrate gamma-aminobutyric acid subtype A receptor locus. Proc. Natl. Acad. Sci. U. S. A 88, 7209–7213.
ffrench-Constant, R.H., Steichen, J.C., Rocheleau, T.A., Aronstein, K., Roush, R.T., 1993a. A single-amino acid substitution in a gamma-aminobutyric acid subtype A receptor locus is associated with cyclodiene insecticide resistance in Drosophila populations. Proc. Natl. Acad. Sci. U. S. A 90, 1957–1961.
ffrench-Constant, R.H., Rocheleau, T.A., Steichen, J.C., Chalmers, A.E., 1993b. A point mutation in a Drosophila GABA receptor confers insecticide resistance. Nature 363, 449–451.
ffrench-Constant, R.H., 1994. The molecular and population genetics of cyclodiene insecticide resistance. Insect Biochem. Mol. Biol. 24, 335–345.
ffrench-Constant, R.H., Rocheleau, T., Anthony, N., Aronstein, K., Stilwell, G., 2000. Cyclodiene insecticide resistance: from molecular to population genetics. Annu. Rev. Entomol. 48, 449–466.
Gant, D., Chalmers, A., Wolff, M., Hoffman, H., Bushey, D., 1998. Fipronil: action at the GABA receptor. Rev. Toxicol. 2, 147–156.
García-Reynaga, P., Zhao, C., Sarpong, R., Casida, J.E., 2013. New GABA/glutamate receptor target for [3H]Isoxazoline insecticide. Chem. Res. Toxicol. 26, 514–516.
Geary, T.G., 2005. Ivermectin 20 years on: maturation of a wonder drug. Trends Parasitol. 21, 530–532.
Gerschenfeld, H.M., 1973. Chemical transmission in invertebrate central nervous systems and neuromuscular junctions. Physiol. Rev. 53, 1–119.
Ghiasuddin, S.M., Matsumura, F., 1982. Inhibition of gamma-aminobutyric acid (GABA)-induced chloride uptake by gamma-BHC and heptachlor epoxide. Comp. Biochem. Physiol. 73C, 141–144.
Glykys, J., Mody, I., 2007. Activation of GABAA receptors: views from outside the synaptic cleft. Neuron 56, 763–770.
Guerrero, F.D., Miller, R.J., Pérez de León, A.A., 2012. Cattle tick vaccines: many candidate antigens, but will a commercially viable product emerge? Int. J. Parasitol. 42, 421–427.
Hainzl, D., Cole, L.M., Casida, J.E., 1998. Mechanisms for selective toxicity of fipronil insecticide and its sulfone metabolite and desulfinyl photoproduct. Chem. Res. Toxicol. 11, 1529–1535.
Heckeroth, A.R., Lutz, J., Christina Mertens, Williams, H., Zoller, H., Mita, T., 2013. Isoxazoline compositions and their use as antiparasitics. EP 2190289 B1.
Hevers, W., Lüddens, H., 1998. The diversity of GABAA receptors. Pharmacological and electrophysiological properties of GABAA channel subtypes. Mol. Neurobiol. 18, 35–86.
Hibbs, R.E., Gouaux, E., 2011. Principles of activation and permeation in an anion-selective Cys-loop receptor. Nature 474, 54–60.
Holden-Dye, L., Walker, R.J., 1990. Avermectin and avermectin derivatives are antagonists at the 4-aminobutyric acid (GABA) receptor on the somatic muscle cells of Ascaris; is this the site of anthelmintic action? Parasitology 101, 265–271.
Hope, M., Menzies, M., Kemp, D., 2010. Identification of a dieldrin resistance-associated mutation in Rhipicephalus (Boophilus) microplus (Acari: Ixodidae). J. Econ. Entomol. 103, 1355–1359.
Hosie, A.M., Baylis, H.A., Buckingham, S.D., Sattelle, D.B., 1995. Actions of the insecticide fipronil, on dieldrin-sensitive and- resistant GABA receptors of *Drosophila melanogaster*. Br. J. Pharmacol. 115, 909–912.
Hosie, A.M., Sattelle, D.B., 1996. Allosteric modulation of an expressed homo-oligomeric GABA-gated chloride channel of *Drosophila melanogaster*. Br. J. Pharmacol. 117, 1229–1237.
Hosie, A.M., Aronstein, K., Sattelle, D.B., ffrench-Constant, R.H., 1997. Molecular biology of insect neuronal GABA receptors. Trends Neurosci. 20, 578–583.
Hosie, A.M., Buckingham, S.D., Presnail, J.K., Sattelle, D.B., 2001. Alternative splicing of a Drosophila GABA receptor subunit gene identifies determinants of agonist potency. Neuroscience 102, 709–714.
Hosie, A.M., Buckingham, S.D., Hamon, A., Sattelle, D.B., 2006. Replacement of asparagine with arginine at the extracellular end of the second transmembrane (M2) region of insect GABA receptors increases sensitivity to penicillin G. Invert. Neurosci. 6, 75–79.
Ikeda, T., Zhao, X., Kono, Y., Yeh, J.Z., Narahashi, T., 2003. Fipronil modulation of glutamate-induced chloride currents in cockroach thoracic ganglion neurons. Neurotoxicology 24, 807–815.
Joesch, C., Guevarra, E., Parel, S.P., Bergner, A., Zbinden, P., Konrad, D., Albrecht, H., 2008. Use of FLIPR membrane potential dyes for validation of high-throughput screening with the FLIPR and microARCS technologies: identification of ion channel modulators acting on the GABA(A) receptor. J. Biomol. Screen 13, 218–228.
Krnjević, K., 2004. How does a little acronym become a big transmitter? Biochem. Pharmacol. 68, 1549–1555.
Krnjević, K., 2010. When and why amino acids? J. Physiol. 588, 33–44.
Lahm, G.P., Cordova, D., Barry, J.D., Pahutski, T.F., Smith, B.K., Long, J.K., Benner, E.A., Holyoke, C.W., Joraski, K., Xu, M., Schroeder, M.E., Wagerle, T., Mahaffey, M.J., Smith, R.M., Tong, M.H., 2013. 4-Azolylphenyl isoxazoline insecticides acting at the GABA gated chloride channel. Bioorg. Med. Chem. Lett. 23, 3001–3006.
Le Goff, G., Hamon, A., Bergé, J.B., Amichot, M., 2005. Resistance to fipronil in Drosophila simulans: influence of two point mutations in the RDL GABA receptor subunit. J. Neurochem. 92, 1295–1305.
Lummis, S.C., 1990. GABA receptors in insects. Comp. Biochem. Physiol. C 95, 1–8.
Macdonald, R.L., Olsen, R.W., 1994. $GABA_A$ receptor channels. Annu. Rev. Neurosci. 17, 569–602.
Marshall, F.H., Foord, S.M., 2010. Heterodimerization of the GABAB receptor-implications for GPCR signaling and drug discovery. Adv. Pharmacol. 58, 63–91.
Martin, R.J., Pennington, A.J., 1989. A patch-clamp study of effects of dihydroavermectin on Ascaris muscle. Br. J. Pharmacol. 98, 747–756.
McGurk, K.A., Pistis, M., Belelli, D., Hope, A.G., Lambert, J.J., 1998. The effect of a transmembrane amino acid on etomidate sensitivity of an invertebrate GABA receptor. Br. J. Pharmacol. 124, 13–20.
Mirza, N.R., Munro, G., 2010. The role of GABA(A) receptor subtypes as analgesic targets. Drug News Perspect. 23, 351–360.
Nakao, T., Naoi, A., Kawahara, N., Hirase, K., 2010. Mutation of the GABA receptor associated with fipronil resistance in the whitebacked planthopper, Sogatella furcifera. Pestic. Biochem. Physiol. 97, 262–266.
Nakao, T., Banba, S., Nomura, M., Hirase, K., 2013. Meta-diamide insecticides acting on distinct sites of RDL GABA receptor from those for conventional noncompetitive antagonists. Insect Biochem. Mol. Biol. 43, 366–375.
Narahashi, T., Zhao, X., Ikeda, T., Salgado, V.L., Yeh, J.Z., 2010. Glutamate-activated chloride channels: unique fipronil targets present in insects but not in mammals. Pestic. Biochem. Physiol. 97, 149–152.
Olsen, R.W., Sieghart, W., 2009. GABA A receptors: subtypes provide diversity of function and pharmacology. Neuropharmacology 56, 141–148.
Ozoe, Y., Asahi, M., Ozoe, F., Nakahira, K., Mita, T., 2010. The antiparasitic isoxazoline A1443 is a potent blocker of insect ligand-gated chloride channels. Biochem. Biophys. Res. Commun. 391, 744–749.
Ozoe, Y., 2013. γ-Aminobutyrate- and glutamate-gated chloride channels as targets of insecticides. Adv. Insect. Physiol. 44, 211–286.
Petersen, T.N., Brunak, S., von Heijne, G., Nielsen, H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nature Meth. 8, 785–786.
Pistis, M., Belelli, D., McGurk, K., Peters, J.A., Lambert, J.J., 1999. Complementary regulation of anaesthetic activation of human (alpha6beta3gamma2L) and Drosophila (RDL) GABA receptors by a single amino acid residue. J. Physiol. 515, 3–18.
Rauh, J.J., Lummis, S.C., Sattelle, D.B., 1990. Pharmacological and biochemical properties of insect GABA receptors. Trends Pharmacol. Sci. 11, 325–329.
Raymond, V., Sattelle, D.B., 2002. Novel animal-health drug targets from ligand-gated chloride channels. Nat. Rev. Drug Discov. 6, 427–436.
Raymond-Delpech, V., Matsuda, K., Sattelle, B.M., Rauh, J.J., Sattelle, D.B., 2005. Ion channels: molecular targets of neuroactive insecticides. Invert. Neurosci. 5, 119–133.

Rust, M.K., 2005. Advances in the control of *Ctenocephalides felis* (cat flea) on cats and dogs. Trends Parasitol. 21, 232–236.
Rust, M.K., Dryden, M.W., 1997. The biology, ecology, and management of the cat flea. Annu. Rev. Entomol. 42, 451–473.
Sambrook, J., Russell, D.W., 2001. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
Sattelle, D.B., 1990. GABA receptors of insects. Adv. Insect Physiol. 22, 1–113.
Scharf, Michael E., Siegfried, B.D., 1999. Toxicity and neurophysiological effects of fipronil and fipronil sulfone on the Western Corn Rootworm (Coleoptera: Chrysomelidae). Arch. Insect Biochem. Physiol. 40, 150–156.
Schuske, K., Beg, A.A., Jorgensen, E.M., 2004. The GABA nervous system in *C. elegans*. Trends Neurosci. 27, 407–414.
Semenov, E.P., Pak, W.L., 1999. Diversification of Drosophila chloride channel gene by multiple posttranscriptional mRNA modifications. J. Neurochem. 72, 66–72.
Shoop, W.L., Mrozik, H., Fisher, M.H., 1995. Structure and activity of avermectins and milbemycins in animal health. Vet. Parasitol. 59, 139–156.
Sieghart, W., 2006. Structure, pharmacology, and function of GABAA receptor subtypes. Adv. Pharmacol. 54, 231–263.
Sigel, E., Steinmann, M.E., 2012. Structure, function, and modulation of GABA(A) receptors. J. Biol. Chem. 287, 40224–40231.
Sine, S.M., Engel, A.G., 2006. Recent advances in Cys-loop receptor structure and function. Nature 440, 448–455.
Stilwell, G.E., Rocheleau, T., ffrench-Constant, R.H., 1995. GABA receptor minigene rescues insecticide resistance phenotypes in Drosophila. J. Mol. Biol. 253, 223–227.
Wade, S.E., Georgi, J.R., 1988. Survival and reproduction of artificially fed cat fleas, *Ctenocephalides felis* Bouché (Siphonaptera: Pulicidae). J. Med. Entomol. 25, 186–190.
Wang, C.C., Pong, S.S., 1982. Actions of avermectin B1a on GABA nerves. Prog. Clin. Biol. Res. 97, 373–395.
Whiting, P.J., 2006. GABA-A receptors: a viable target for novel anxiolytics? Curr. Opin. Pharmacol. 6, 24–29.
Winsky-Sommerer, R., 2009. Role of GABAA receptors in the physiology and pharmacology of sleep. Eur. J. Neurosci. 29, 1779–1794.
Wisniewski, N., Brandt, K.S., 2002. Flea GABA receptor subunit nucleic acid molecules, proteins and uses thereof. World patent WO0244374-A 7. Heska Corporation (US).
Wolff, M.A., Wingate, V.P., 1998. Characterization and comparative pharmacological studies of a functional gamma-aminobutyric acid (GABA) receptor cloned from the tobacco budworm, *Heliothis virescens* (Noctuidae:Lepidoptera). Invert. Neurosci. 3, 305–315.
Wolstenholme, A.J., Rogers, A.T., 2005. Glutamate-gated chloride channels and the mode of action of the avermectin/milbemycin anthelmintics. Parasitology 131 (Suppl), S85–S95.
Wolstenholme, A.J., 2012. Glutamate-gated chloride channels. J. Biol. Chem. 287, 40232–40238.
Wong, G., Sei, Y., Skolnick, P., 1992. Stable expression of type I gamma-aminobutyric acidA/benzodiazepine receptors in a transfected cell line. Mol. Pharmacol. 42, 996–1003.
Zhao, X., Salgado, V.L., Yeh, J.Z., Narahashi, T., 2003. Differential actions of fipronil and dieldrin insecticides on GABA-gated chloride channels in cockroach neurons. J. Pharmacol. Exp. Ther. 306, 914–924.
Zhao, X., Yeh, J.Z., Salgado, V.L., Narahashi, T., 2004. Fipronil is a potent open channel blocker of glutamate-activated chloride channels in cockroach neurons. J. Pharmacol. Exp. Ther. 310, 192–201.
Zhao, X., Yeh, J.Z., Salgado, V.L., Narahashi, T., 2005. Sulfone metabolite of fipronil blocks gamma-aminobutyric acid- and glutamate-activated chloride channels in mammalian and insect neurons. J. Pharmacol. Exp. Ther. 314, 363–373.
Zhao, X., Salgado, V.L., 2010. The role of GABA and glutamate receptors in susceptibility and resistance to chloride channel blocker insecticides. Pestic. Biochem. Physiol. 97, 153–160.
Zheng, Y., Priest, B., Cully, D.F., Ludmerer, S.W., 2003. RdlDv, a novel GABA-gated chloride channel gene from the American dog tick *Dermacentor variabilis*. Insect Biochem. Mol. Biol. 33, 595–599.