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

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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).

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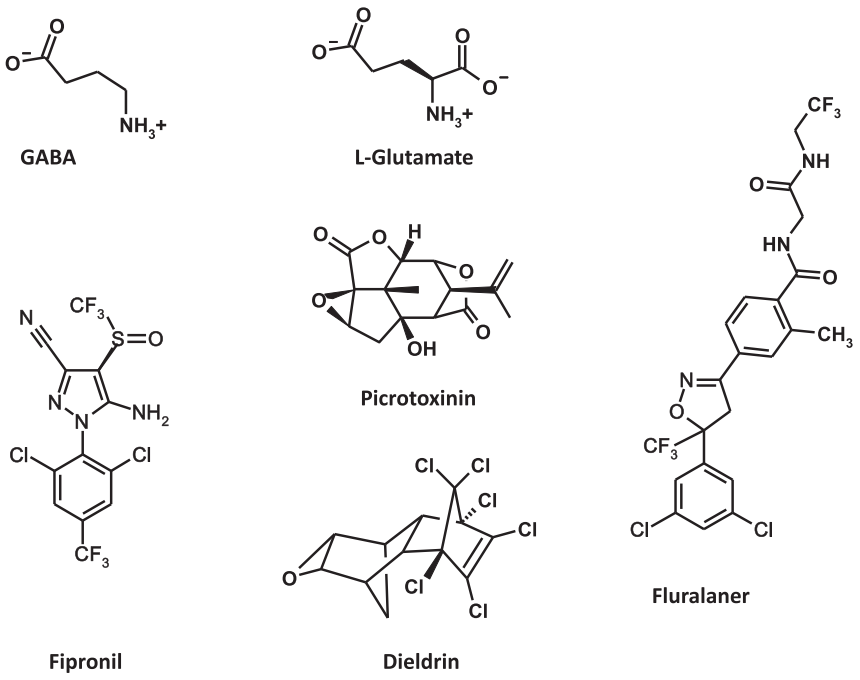

Fig. 1. Chemical structures of chloride channel agonists and antagonists.

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