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The fungal alkaloid Okaramine-B activates an L-glutamate-gated chloride channel from *Ixodes scapularis*, a tick vector of Lyme disease

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ABSTRACT

A novel L-glutamate-gated anion channel (IscaGluCl1) has been cloned from the black-legged tick, *Ixodes scapularis*, which transmits multiple pathogens including the agents of Lyme disease and human granulocytic anaplasmosis. When mRNA encoding IscaGluCl1 was expressed in *Xenopus laevis* oocytes, we detected robust 50–400 nA currents in response to 100 μM L-glutamate. Responses to L-glutamate were concentration-dependent (pEC_{50} 3.64 ± 0.11). Ibotenate was a partial agonist on IscaGluCl1. We detected no response to 100 μM aspartate, quisqualate, kainate, AMPA or NMDA. Ivermectin at 1 μM activated IscaGluCl1, whereas picrotoxinin (pIC_{50} 6.20 ± 0.04) and the phenylpyrazole fipronil (pIC_{50} 6.90 ± 0.04) showed concentration-dependent block of the L-glutamate response. The indole alkaloid okaramine B, isolated from fermentation products of *Penicillium simplicissimum* (strain AK40) grown on okara pulp, activated IscaGluCl1 in a concentration-dependent manner (pEC_{50} 5.43 ± 0.43) and may serve as a candidate lead compound for the development of new acaricides.

1. Introduction

Ticks are major ectoparasites of livestock and are also vectors of human and animal diseases worldwide (Jongejan and Uilenberg, 2004). They transmit a greater diversity of infectious agents than any other group of blood-feeding arthropods (Gulia-Nuss et al., 2016), including the Lyme disease spirochaete, *Borrelia burgdorferi* (Burgdorfer, 1984), and many other human and animal pathogens. At present, only a limited number of chemicals are available for their control (Woods and Williams, 2007; Van Leeuwen et al., 2015). Improved understanding of the molecular targets of tick control chemicals (acaricides) will enhance our ability to tackle tick-borne livestock diseases, with important implications for veterinary medicine. L-glutamate-gated chloride channels (GluCls), which belong to the di-cysteine loop-containing superfamily of ligand-gated ion channels (Cys-loop LGICs), are present in invertebrates but not vertebrates and are therefore suitable targets for antiparasitic drugs, most of which show good host-tolerance (Raymond-Delpech et al., 2005; Wolstenholme, 2012). For example, GluCls are activated by the endectocide ivermectin (22, 23-dihydro-avermectin B1a), a macrocyclic lactone isolated from the actinomycete, *Streptomyces avermitilis*, which controls both nematode endoparasites and ectoparasites such as ticks (Rugg et al., 2005). Ivermectin also targets GABA-gated chloride channels (Duce and Scott, 1985; Sattelle, 1990). First introduced in 1981, by the second half of that decade ivermectin had become the world's biggest-selling animal health product (Omura and Crump, 2014).

Other chemotypes targeting arthropod Cys-loop LGICs include the phenylpyrazole, fipronil (Cole et al., 1993; Davey et al., 1998; Denny, 2001; Zheng et al., 2003; Raymond-Delpech et al., 2005), and the isoxazolines, a group including fluralaner, afoxalaner and sarolaner (Ozoe et al., 2010; García-Reynaga et al., 2013; Gassel et al., 2014; Shoop et al., 2014; McTier et al., 2016), which block GABA-gated chloride channels and GluCls. Fluralaner is effective against multiple life stages of ticks of the Ixodidae and Argasidae families (Gassel et al., 2014;

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Rohdich et al., 2014; Wengenmayer et al., 2014; Williams et al., 2015). Okaramines are indole alkaloids isolated from fermentation products of *Penicillium simplicissimum* (strain AK40) grown on the okara pulp resulting from Soybean cake production. They are toxic to larvae of the silkworm, *Bombyx mori* (Bm) (Hayashi et al., 1989) and show strong selectivity for these lepidopteran BmGluCls (Furutani et al., 2014b). For example, they activate BmGluCls but not the silkworm GABA receptor (BmRDL). They are also ineffective on both human GABA-gated chloride channels (type A GABA receptors) and glycine-gated chloride channels (GlyCls) (Furutani et al., 2014b). Furthermore, their insecticidal profile agrees well with their LD_{50} profile on lepidopteran larvae (Furutani et al., 2017). To our knowledge, okaramine B has not been tested on tick GluCls.

Many invertebrate genomes have now been sequenced providing access to GluCls from many pests and parasites (Wolstenholme, 2012). Completion of the genomes of the medically important tick, *Ixodes scapularis* (Gulia-Nuss et al., 2016), and the agricultural pest, the two-spotted spider mite, *Tetranychus urticae* (Grbić et al., 2011), indicates that the acarine GluCl family may be quite diverse. We recently cloned and heterologously expressed in *Xenopus laevis* oocytes a member of this family from *I. scapularis* (IscaGluCl1) which formed a presumed homomeric functional GluCl responding to L-glutamate but none of the other neurotransmitters (GABA, 5-HT, ACh, dopamine, tyramine and histamine) known to activate particular invertebrate ligand-gated anion channels (Gulia-Nuss et al., 2016). This expressed GluCl was also unresponsive to glycine, which together with GABA (Olsen et al., 1999) is an important inhibitory neurotransmitter in mammalian brain.

Here we describe aspects of the pharmacology of IscaGluCl1, including the actions of ibotenate, picrotoxinin, fipronil, ivermectin and the novel indole-alkaloid, okaramine B, which activates the receptor. Okaramine B may therefore serve as a candidate lead not only for the development of novel insecticides (Furutani et al., 2014b, 2017), but also for the development of novel acaricides.

2. Materials and methods

2.1. Cloning of an Ixodes scapularis GluCl, IscaGluCl1

Unfed adult male and female *Ixodes scapularis* ticks (Wikel strain) (stored in RNAlater®) were kindly supplied by Professor Daniel Sonenshine. A mixed population of adults (ranging from 2 to 3 unfed adult ticks — mixed sex for each extraction) were stored in TRIzol® and homogenised using a Vibration Mixer Mill Retsch MM300, and total RNA was extracted according to the manufacturer's protocol. Tick (*I. scapularis*) cDNA was prepared using oligo dT(15) (Promega) and MMLV-RT RNaseH- (Promega). A partial predicted *I. scapularis* GluCl gene was identified from Vectorbase (ISCW022629). The full-length gene was obtained using degenerate primers based on the previously identified RsGluCl1 sequence (ACX33155 and US patent 7202054). The full length sequence was deposited in NCBI under accession number KR107244. The complete coding sequence of IscaGluCl1 was cloned into the p-GEM-T-Easy vector (Promega), and transcribed using SP6 Message Machine kit (Ambion) after linearisation with ApaI prior to oocyte injection.

2.2. Chemicals

L-Glutamate, D-glutamate, ivermectin and picrotoxinin (PTX) were obtained from Sigma-Aldrich (UK). Fipronil was a gift from Dr. Lance Hammerland (Merial Ltd). Kainic acid (referred to as *kainate* throughout this paper), N-methyl-D-aspartic acid (NMDA), quisqualic acid (referred to as *quisqualate* throughout this paper), L-aspartatic acid (referred to as *aspartate* throughout this paper), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) were obtained from Tocris (UK), whereas ibotenic acid (referred to as ibotenate throughout this paper) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Okaramine B was isolated from fermentation products of *P. simplicissimum* according to the original paper (Hayashi et al., 1989).

2.3. Electrophysiology on IscaGluCl1 expressed in Xenopus laevis oocytes

Ovaries were removed from adult female *Xenopus laevis* under anaesthetic (1.5 g L tricaine) according to the UK Animals (Scientific Procedures) Act 1986. Isolated oocytes were defolliculated manually following a 30 min incubation with collagenase type 1 A (2 mg ml) (Sigma) and each oocyte was injected with 50 ng of cRNA encoding IscaGluCl1. Oocytes prepared in this way were maintained in standard oocyte saline (SOS) at 16 °C (Buckingham et al., 2006). Membrane currents were recorded 24–48 h post-injection using standard two-electrode voltage clamp methods, with oocytes voltage-clamped at E_h −80 mV (Buckingham et al., 2006) unless otherwise stated. Data were only collected from oocytes which yielded stable responses to at least three control doses of 100 μM L-glutamate applied at 3 min intervals.

Agonist actions of test compounds were examined by challenging the oocyte with increasing concentrations of agonist for 5 s at a flow-rate of 7–10 ml min with at least 3 min between challenges to minimise the effects of desensitisation. Peak amplitudes of responses were normalised to the response to 1 mM L-glutamate. To evaluate allosteric or antagonist actions, test compounds were first applied alone for 1 min and then co-applied with agonists. In this case, peak amplitudes of observed responses were normalised to the response to 100 μM L-glutamate. For studies on the blocking actions of picrotoxinin and fipronil, only a single concentration of compound was tested on an individual oocyte. Picrotoxinin, ivermectin, fipronil and okaramine B were first dissolved in dimethylsulphoxide (DMSO) and then diluted in SOS to the required concentrations. Care was taken that the final concentration of DMSO did not exceed 1% (v/v) to prevent any impact of DMSO on electrophysiological recordings.

2.4. Data analysis

Data are presented as mean ± SEM of 2–6 independent experiments. Data were normalised to the peak amplitude evoked by either 1 mM or 100 μM L-glutamate as indicated in the previous section and analysed using GraphPad Prism version 5.0 (GraphPad Software Inc., USA). To calculate concentration-response relationships, normalised data were fitted to the following equation:

$$Y = I_{min} + \frac{I_{max} - I_{min}}{1 + 10^{(\log EC_{50} - X) n_H}} \quad (1)$$

where Y is the normalised response amplitude, I_{max} and I_{min} are the maximum and minimum normalised responses respectively, EC_{50} is the concentration giving half the maximum normalised response, X is log [Agonist/Antagonist (M)] and nH is the Hill coefficient. To obtain the concentration-inhibition relationship, the response after co-application of agonist and antagonist was normalised to the control response to 100 μM L-glutamate and analysed to obtain the pIC_{50} value, using above equation but in this case the pEC_{50} was replaced with pIC_{50} and I_{max} was constrained to be 1. Statistical tests were performed for comparison of pIC_{50} values using *t*-test with a significance level of $P < 0.05$.

3. Results

3.1. Sequence of IscaGluCl1

The full-length IscaGluCl1 DNA (1350 nucleotides) encodes a 449 amino acid protein which shows the characteristic features of a Cys-loop LGIC subunit including: a large extracellular N-terminal domain, a dicysteine loop (Cys-loop) with cysteines separated by 13 residues, 4 transmembrane (TM) regions and a large intracellular TM3-TM4 loop (Fig. 1). A second N-terminal loop, characteristic of ligand-gated anion

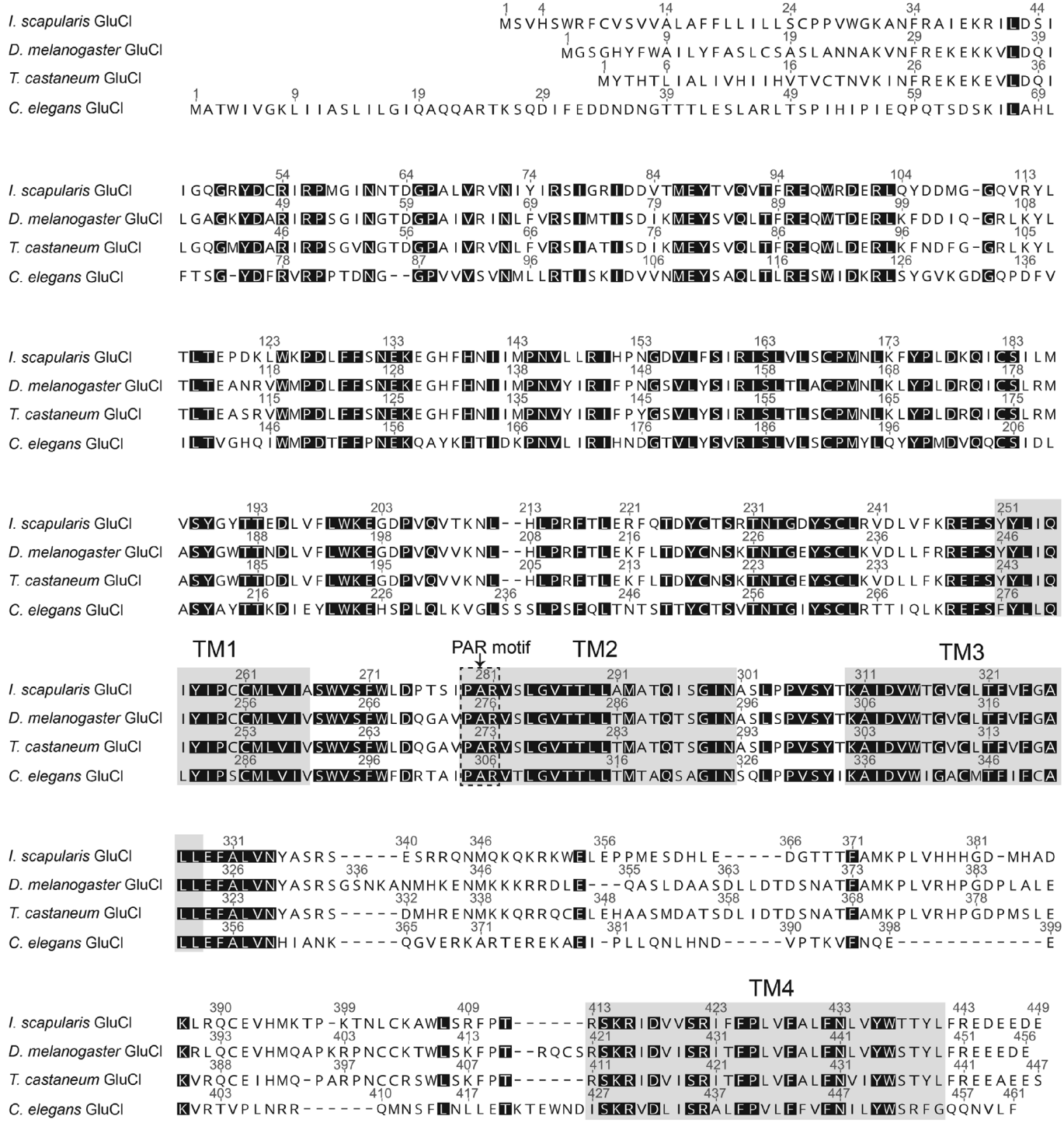


Fig. 1. Multiple sequence alignment of the *Ixodes scapularis* GluCl (IscaGluCl1) with GluCls from other arthropods and *C. elegans*. This multiple sequence alignment indicates that IscaGluCl1 shares characteristic sequence features with known GluCls. Multiple sequence alignments were performed with the MAFFT (Yamada et al., 2016) algorithm using Geneious software versiton 9 (Kearse et al., 2012), and the details were adjusted manually - gaps originally produced by MAFFT at N-/C-terminal were removed, and alignment between TM3 and TM4, a poorly conserved region, were corrected in order to reduce gaps. Accession numbers of the GluCls depicted from *C. elegans*, *D. melanogaster*, *I. scapularis* and *T. castaneum* are AAA50785, AAG40735, ALF36853 and NP_001107775, respectively.

channels (Wolstenholme, 2012), is also present. A PAR motif at the inner end of the second transmembrane segment (TM2) is typical of Cys-loop ligand-gated anion channels (Raymond and Sattelle, 2002) (Fig. 1). BLASTp analysis using the NCBInr database showed this sequence to be most similar to arthropod GluCls with highest hits to: the cattle tick, *Rhipicephalus microplus* (AHE41097), with 94% identity (E = 0.0), the common house spider, *Parasteatode tepidariorum* (XP_015920421), with 84% identity (E = 0.0), the Atlantic horseshoe crab, *Limulus polyphemus* (XP_013782904), with 78% identity (E = 0.0) and the brown marmorated stink bug, *Halyomorpha halys* (XP_014282882), with 73% identity (E = 0.0). Phylogenetic analysis with known (or predicted) insect, arachnid or helminth GluCls shows that this receptor clusters closely with the arachnid GluCls, including the previously characterised *R. microplus* and *R. sanguineus* GluCls.

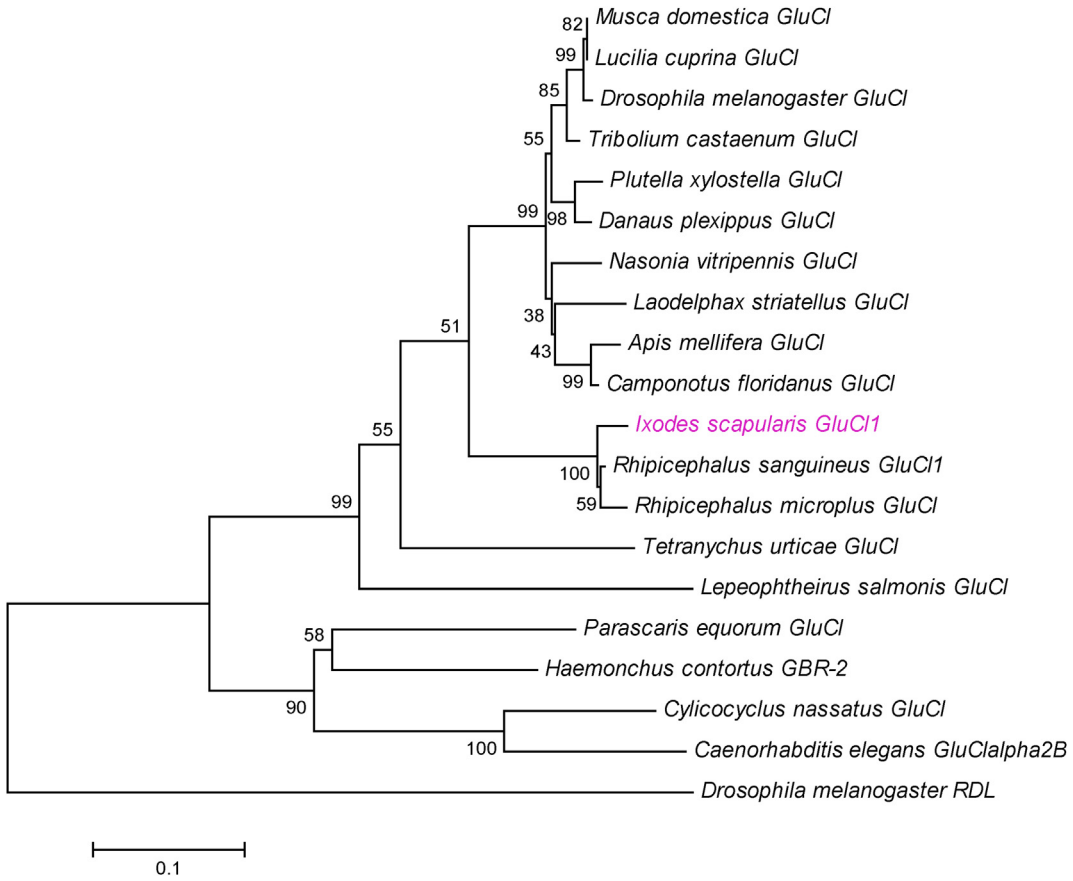


Fig. 2. Phylogenetic analysis of known arthropod and helminth GluCls. This analysis indicates that IscaGluCl clusters closely with tick GluCl1 homologues, hence the receptor has been labelled IscaGluCl1. The phylogenetic tree was constructed using Geneious software version 9 (Kearse et al., 2012) with genetic distance model of Jukes-Cantor, and with Neighbour-Joining methods. Scale bar indicates substitutions per site.

Therefore, this receptor was designated IscaGluCl1 (Fig. 2).

3.2. Actions of L-glutamate, ibotenate, kainate, quisqualate, NMDA, AMPA and aspartate on functionally-expressed, recombinant IscaGluCl1

Bath-application of L-glutamate resulted in a rapid onset, concentration-dependent, inward current in *Xenopus laevis* oocytes injected with IscaGluCl1 cRNA and clamped at E_h −80 mV (Fig. 3A). Such currents were not seen in control oocytes injected with distilled water. The response to L-glutamate reversed at a membrane potential of −27.5 ± 1.1 mV (n = 4) in SOS where the extracellular Cl^- concentration is 107.6 mM. The reversal potential is close to the chloride equilibrium potential (E_{Cl}) of −28 mV for *X. laevis* oocytes (Costa et al., 1989). By comparison, the reversal potential for potassium currents has been reported to be −84 mV (Peres et al., 1985), whilst the reversal potentials for sodium and calcium are expected to be positive. In addition, the reversal potential shifted to +4.1 ± 1.9 mV (n = 4), which is close to a predicted value of +1.6 mV, when the extracellular Cl^- concentration was changed to 37.6 mM (Fig. 3B). A pEC_{50} value of 3.64 ± 0.11 (EC_{50} 230 μM) was estimated for L-glutamate applied to IscaGluCl1 (Fig. 3C, Table 1). Ibotenate, a known activator of IscaGluCl1 (Gulia-Nuss et al., 2016), was a partial agonist with a higher affinity (pEC_{50} 4.97 ± 0.25; EC_{50} 10.8 μM) for IscaGluCl1 than L-glutamate (Fig. 3C, Table 1). We showed previously that agonists known to activate vertebrate and invertebrate chloride channels including ACh, GABA, glycine, histamine, tyramine, dopamine and 5-HT were inactive at 1 mM (Fig. 3D) (Gulia-Nuss et al., 2016). These findings together with stereo-selectivity (L-glutamate > D-glutamate) identified this LGIC subunit as a GluCl (Gulia-Nuss et al., 2016). We therefore tested other L-glutamate analogues, some of which in vertebrates help define L-glutamate receptor subtypes. These were kainate, NMDA, quisqualate, AMPA and L-aspartate. All were found to be inactive at 100 μM (data not shown).

3.3. Picrotoxinin and fipronil block IscaGluCl1

Picrotoxinin, a channel-acting blocker of many GluCls (Hibbs and Gouaux, 2011), at 100 μM blocks the response of IscaGluCl1 to 100 μM L-glutamate. This block was concentration-dependent with a pIC_{50} of 6.20 ± 0.04 (IC_{50} 638 nM) (Fig. 4, Table 2). The phenylpyrazole, fipronil, is an important antiparasitic compound used widely for the treatment of fleas and ticks with actions at both arthropod GABARs (Hosie et al., 1995) and GluCls (Furutani et al., 2014a; Kita et al., 2014). A concentration-dependent block by fipronil was observed with a pIC_{50} of 6.90 ± 0.04 (IC_{50} 125 nM) (Fig. 4, Table 2). For both antagonists, their blocking actions were irreversible at most concentrations and thus only one concentration was tested per oocyte in this series of experiments. Thus IscaGluCl1 is targeted by both picrotoxinin and fipronil, both of which suppress in a concentration-dependent manner the actions of L-glutamate.

3.4. Ivermectin and okaramine B activate IscaGluCl1

Ivermectin activated IscaGluCl1 (pEC_{50} 5.71 ± 0.05) in a concentration-dependent manner (Fig. 5) resulting in the generation of sustained inward currents recorded at E_h = −100 mV. Okaramine B at 1 μM, 3 μM and 10 μM transiently activated IscaGluCl1 in a

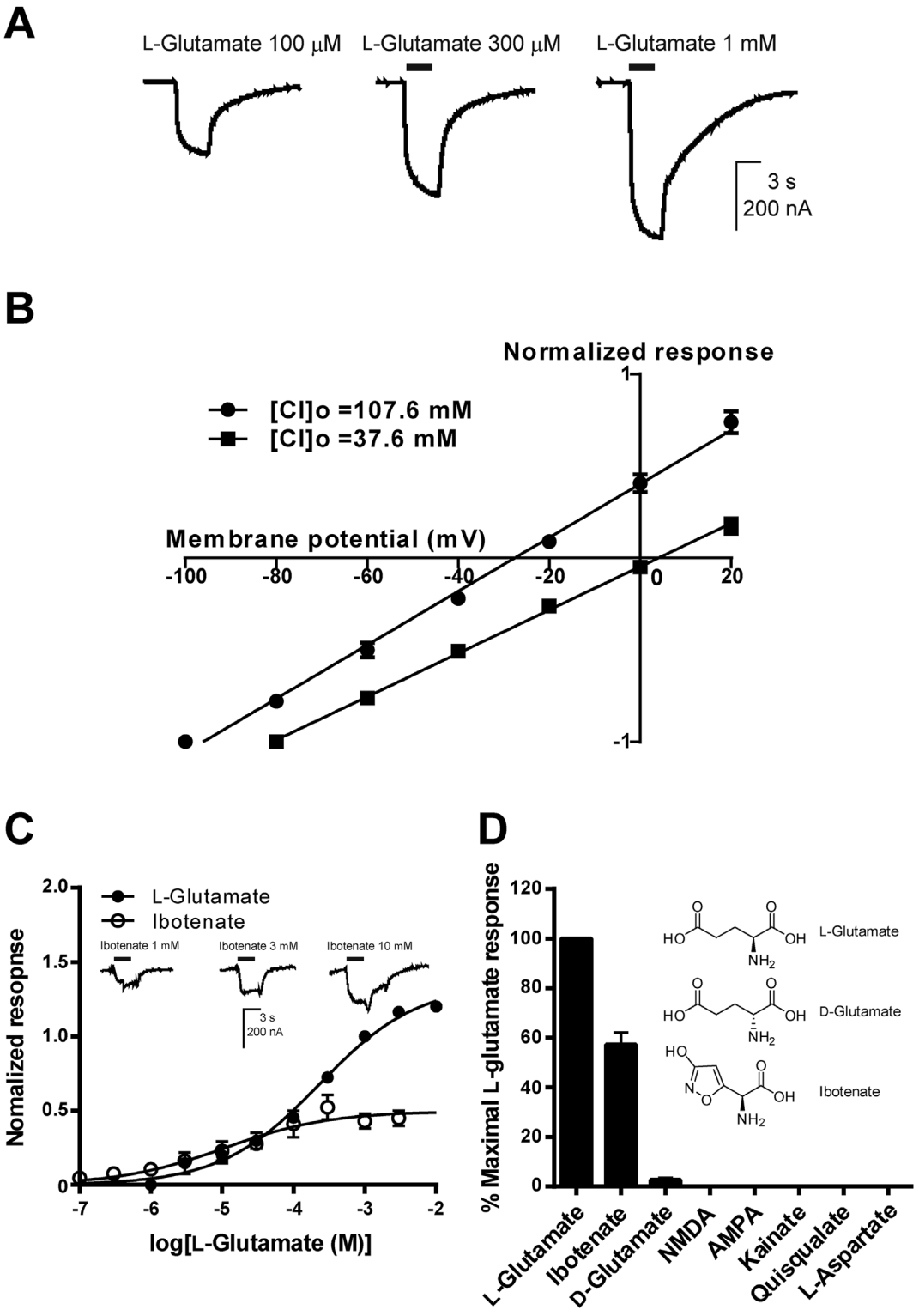


Fig. 3. Concentration-dependent responses to L-glutamate and ibotenate of recombinant homomeric IscaGluCl1 expressed in *Xenopus laevis* oocytes and the current-voltage relationship for the response to L-glutamate. A, rapidly activated and desensitized response to L-glutamate of IscaGluCl1 at three concentrations. B, peak current amplitude of the response to 1 mM L-glutamate at two external Cl^- concentrations. C, concentration-response relationship for L-glutamate and ibotenate. Ibotenate is a partial agonist but shows higher affinity for IscaGluCl1 than L-glutamate. Data were normalised to 1 mM L-glutamate. pEC_{50}s are given in Table 1. In B and C, each data plot represents mean ± standard error of the mean (n = 4). D, percent maximal L-glutamate responses to neurotransmitters applied at 1 mM of IscaGluCl1. Each bar graph represents mean ± standard error of the mean (n = 3–6).

concentration-dependent manner (Fig. 5). The resulting inward current transients recorded at E_h −100 mV for both ivermectin and okaramine B showed much slower rise times than those observed for L-glutamate. Unlike the case for ivermectin, okaramine B responses reversed quickly once the oocyte was re-bathed in saline. From the concentration-response curve constructed from responses to okaramine B over the range 10 nM–30 μM, a value for the pEC_{50} of 5.43 ± 0.43 (EC_{50} 3.70 μM) was determined (Fig. 5).

Table 1
Agonist activity of L-glutamate and ibotenate on the recombinant expressed L-glutamate gated chloride channel (IscaGluCl1) of the tick, *Ixodes scapularis*.

Compound	EC_{50} (μM) (95% Confidence Interval)	pEC_{50}	I_{max}
L-Glutamate	230 (141–377)	3.64 ± 0.11	1.35 ± 0.07
Ibotenate	10.8 (3.36–34.7)	4.97 ± 0.25	0.50 ± 0.05

Data were normalised to the response to 1 mM L-glutamate and are represented as mean ± standard error of the mean (n = 4). The pEC_{50}s for L-glutamate and ibotenate were significantly different (two-tailed *t*-test, $p < 0.05$).

Table 2
Antagonist activity of picrotoxinin and fipronil on the recombinant expressed L-glutamate-gated chloride channel (IscaGluCl1) of the tick, *Ixodes scapularis*.

Compound	IC_{50} (μM) (95% Confidence Interval)	pIC_{50}
Picrotoxinin	0.64 (0.52–0.78)	6.20 ± 0.04
Fipronil	0.13 (0.10–0.15)	6.90 ± 0.04

Data were normalised to the amplitude of the response to 100 μM L-glutamate and are represented as mean ± standard error of the mean of 4 experiments.

3.5. Okaramine allosterically potentiates L-glutamate responses of IscaGluCl1

Ivermectin is known to act as an allosteric modulator of glutamate-gated chloride channels. and we postulated that okaramine B may also act in a similar way. Hence, okaramine B was applied alone for 1 min at a low concentration (1 μM) followed by co-application with 100 μM L-glutamate. This resulted in an enhanced amplitude of the response to L-glutamate (Fig. 6A) when compared to controls, highlighting that okaramine B acts as a potent, positive allosteric modulator on IscaGluCl1 (Table 3). Thus the action of okaramine B on IscaGluCl1 differs markedly from its previously reported actions on a lepidopteran GluCl (BmGluCl of the silkworm larva *Bombyx mori*) (Furutani et al., 2014a,b, 2017), where only agonist actions have been described and its insecticidal action can be explained entirely in terms of its GluCl activation. The potency of okaramine B measured as pEC_{50} for the positive allosteric modulation of IscaGluCl1 was 5.95 ± 0.03 (1.13 μM), (Fig. 6B, Table 3). We also determined the effects of 1 μM okaramine B on the concentration-response curve for L-glutamate (Fig. 6C). Okaramine B (1 μM) had a minimal impact on the pEC_{50} value (3.62 ± 0.12 with no alkaloid present compared to 3.88 ± 0.04 in the presence of 1 μM okaramine B) (Table 3).

3.6. Ivermectin activation of IscaGluCl1 is blocked non-competitively by okaramine B

Ivermectin activated IscaGluCl1 in a concentration-dependent manner (1–100 μM). Okaramine B suppressed the amplitude of the ivermectin response while scarcely influencing pEC_{50} indicating a non-competitive type of action. The simplest explanation is that ivermectin and okaramine B act at close, but distinct, sites on IscaGluCl1 (Fig. 7, Table 4).

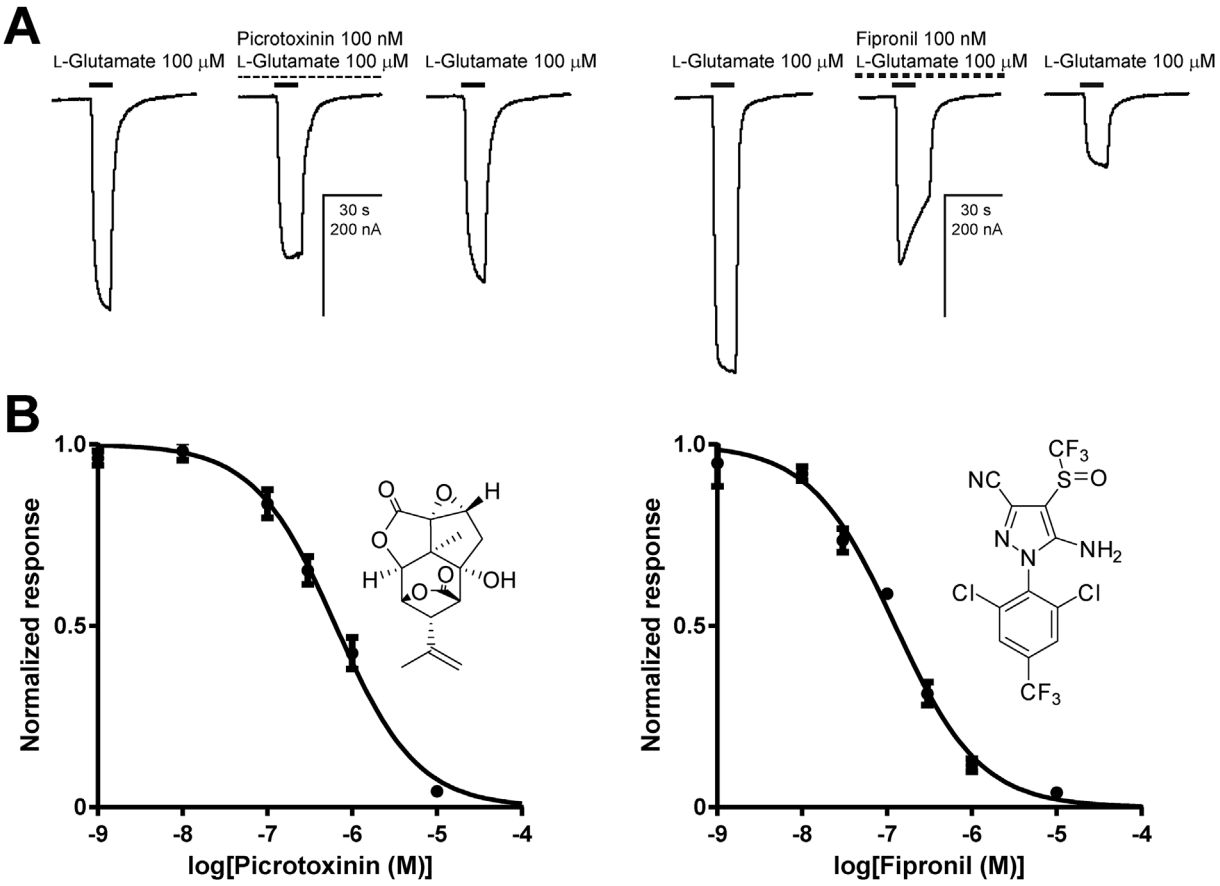


Fig. 4. Inhibition by picrotoxinin and fipronil of recombinant homomeric IscaGluCl1 expressed in *Xenopus laevis* oocytes. A. Block by picrotoxinin and fipronil (100 nM) of L-glutamate responses (100 μM) recorded from IscaGluCl1 heterologously expressed in *Xenopus laevis* oocytes. B. Concentration-inhibition relationships for the actions of picrotoxinin and fipronil on responses to L-glutamate of IscaGluCl1. Data were normalised to the response to 100 μM L-glutamate. Each data point is represented by the mean ± standard error of mean (n = 4). Curves are fits to Eq. (1) and IC_{50}s are given in Table 2.

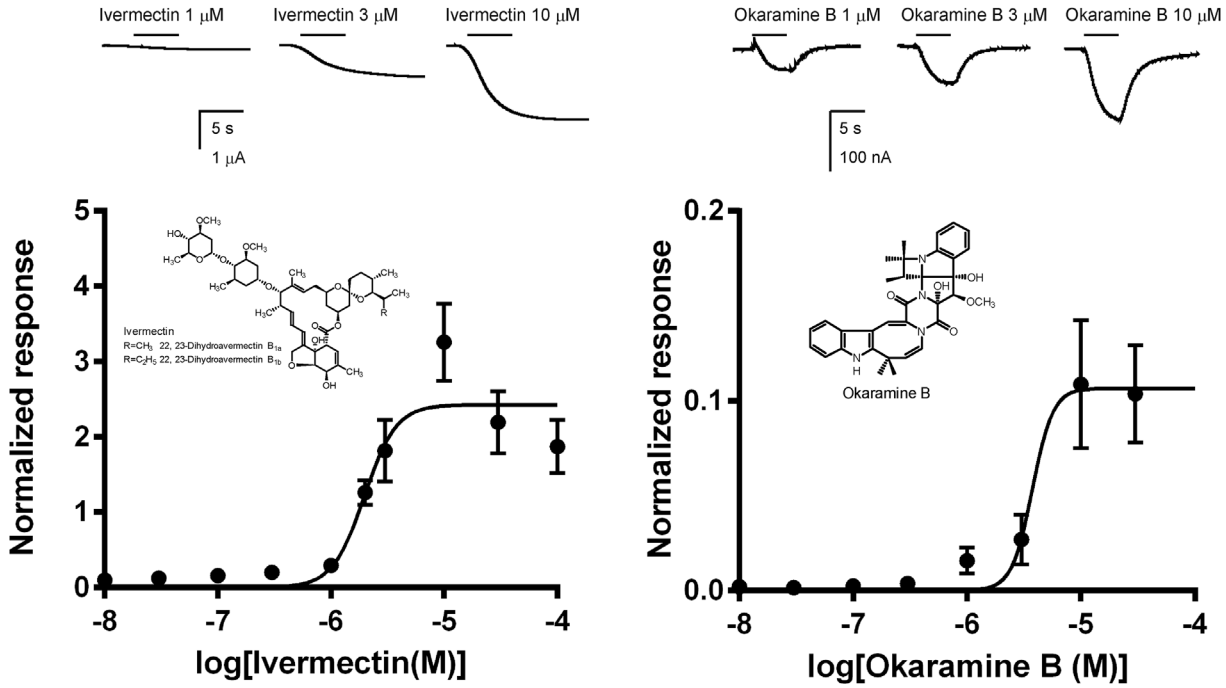


Fig. 5. Concentration-dependent activation of IscaGluCl1 by ivermectin (A) and okaramine B (B). Each data point represents the mean ± standard error of the mean (n = 4, from 2 experiments). Data were normalised to the response to 1 mM L-glutamate. Curves are fits to Eq. (1). Above the concentration-response curve, individual responses of IscaGluCl1 (recorded with oocyte voltage-clamped at E_h = −100 mV) to 1 μM, 3 μM and 10 μM ivermectin (A) or okaramine B (B) are shown.

4. Discussion

There is evidence, albeit incomplete, that L-glutamate is a neurotransmitter in ticks (Booth et al., 1985; Lucien et al., 1995). For example, orthologues of L-glutamate-gated chloride channels (GluCls) are present in all species of ticks studied to date. Here we describe the full length sequence of a tick GluCl from the Lyme disease vector, *I. scapularis*. IscaGluCl1 was designated a GluCl because only L-glutamate, of all the invertebrate neurotransmitters known to gate anion channels, was active (Gulia-Nuss et al., 2016). In addition, its pharmacological profile resembles that of an L-glutamate-gated chloride channel from the genetic model organism, *D. melanogaster* (DrosGluCl-α), where L-glutamate activates the receptor and ibotenate is a partial agonist (Cully et al., 1996). Detailed concentration-response studies presented here show that ibotenate is a partial agonist of IscaGluCl1 but, unlike the case for DrosGluClα, ibotenate has a slightly higher affinity for the receptor than L-glutamate. We have already shown that L-glutamate is more potent than D-glutamate on IscaGluCl1 confirming the anticipated stereo-selectivity for L-glutamate (Gulia-Nuss et al., 2016).

IscaGluCl1 and other putative GluCls identified from *R. sanguineus* show low homology to insect GluCls but relatively poor sequence identity is not unusual for comparisons between insect and arachnid GluCl homologues (Lees et al., 2010). Prior to the advancement of genome sequencing technologies, Cully et al. (1996) were unsuccessful in isolating GluCl transcripts from the spider mite *T. urticae* using *D. melanogaster* GluCl primers. Indeed, the recent study of the Cys-loop LGIC family from *T. urticae* has shown that all 6 identified *T. urticae* GluCls are phylogenetically divergent from insect GluCls (Dermauw et al., 2012), which could explain the cloning difficulties encountered in earlier studies. With the exception of RsGluCl1, RmGluCl and IscaGluCl1 (which most closely resemble DrosGluCl-α), most tick sequences that have been identified as candidate GluCls from *I. scapularis*(Gulia-Nuss et al., 2016), *R. sanguineus* and *Dermacentor variabilis* are phylogenetically distinct from GluCls of other arthropods (Lees and Bowman, 2007).

We have shown that IscaGluCl1 is blocked by picrotoxinin, as is the case for many, though not all, invertebrate GluCls such as GLC-3 in *C. elegans* (Horoszok et al., 2001; Wolstenholme, 2012). Another *C. elegans* GluCl (CeGluClα) is sensitive to picrotoxinin (Cleland, 1996). The pIC_{50} for picrotoxinin's action on IscaGluCl (6.2 μM) was lower than that for housefly (*Musca* domestica) GluCl (3.97 μM, Eguchi et al., 2006). Hibbs and Gouaux (2011) reported the structure of CeGluClα with picrotoxinin bound showing that the fused tricyclic rings of picrotoxinin are directed extracellularly and close to the threonine located at position 2' (based on the notation introduced by Henry Lester (1992) for the second transmembrane region (TM2) of Cys Loop LGICs). The isoprenyl tail is directed toward the cytoplasm and is proximal to the proline residues at position -2′ in TM2 of CeGluClα. In IscaGluCl1 a proline is also present at -2′ and a serine, which like threonine is an amino acid with polar but uncharged side chains, is present at position 2'. Thus key components of the picrotoxinin binding site seen in CeGluClα are also present in IscaGluCl1. However, residues other than those at the base of TM2 must account for the differences in picrotoxinin sensitivity seen between some other members of the GluCl family since the picrotoxinin-insensitive subunit from *C. elegans* (GLC-3) (Horoszok et al., 2001) also has proline at -2′ and threonine at position 2'. The residue in the 6′ position of the Varroa mite has also been shown to be important (Carpenter et al., 2013). We also show, as is the case for RmGluCl (the GluCl cloned from the cattle tick, *Rhiphicepalus microplus*) (Gassel et 2014), that fipronil and ivermectin respectively block and activate IscaGluCl1.

The endectocide ivermectin (Omura, 2008; Crump et al., 2012; Omura and Crump, 2014) is active on insect RDL GABA-gated chloride channels (Zheng et al., 2003), insect muscle GABA-gated chloride channels and insect muscle GluCls (Duce and Scott, 1985). Kaufman et al. (1986) showed that intra-haemocoelic injection of ivermectin was detrimental to salivary gland function in the female tick *A. hebraeum*. It is likely that the observed effects of ivermectin are indirect as the salivary glands are highly innervated (Binnington and Obenchain, 1982; Lees et al., 2010). Ivermectin binds at the interface of M3 on the principal subunit and M1 on the complementary subunit, with the binding site centering on a region between residues leucine 217 and isoleucine 222 on M1. In GluClα, Ser 260, which is also conserved in IscaGluCl, forms a hydrogen bond with the secondary hydroxyl group

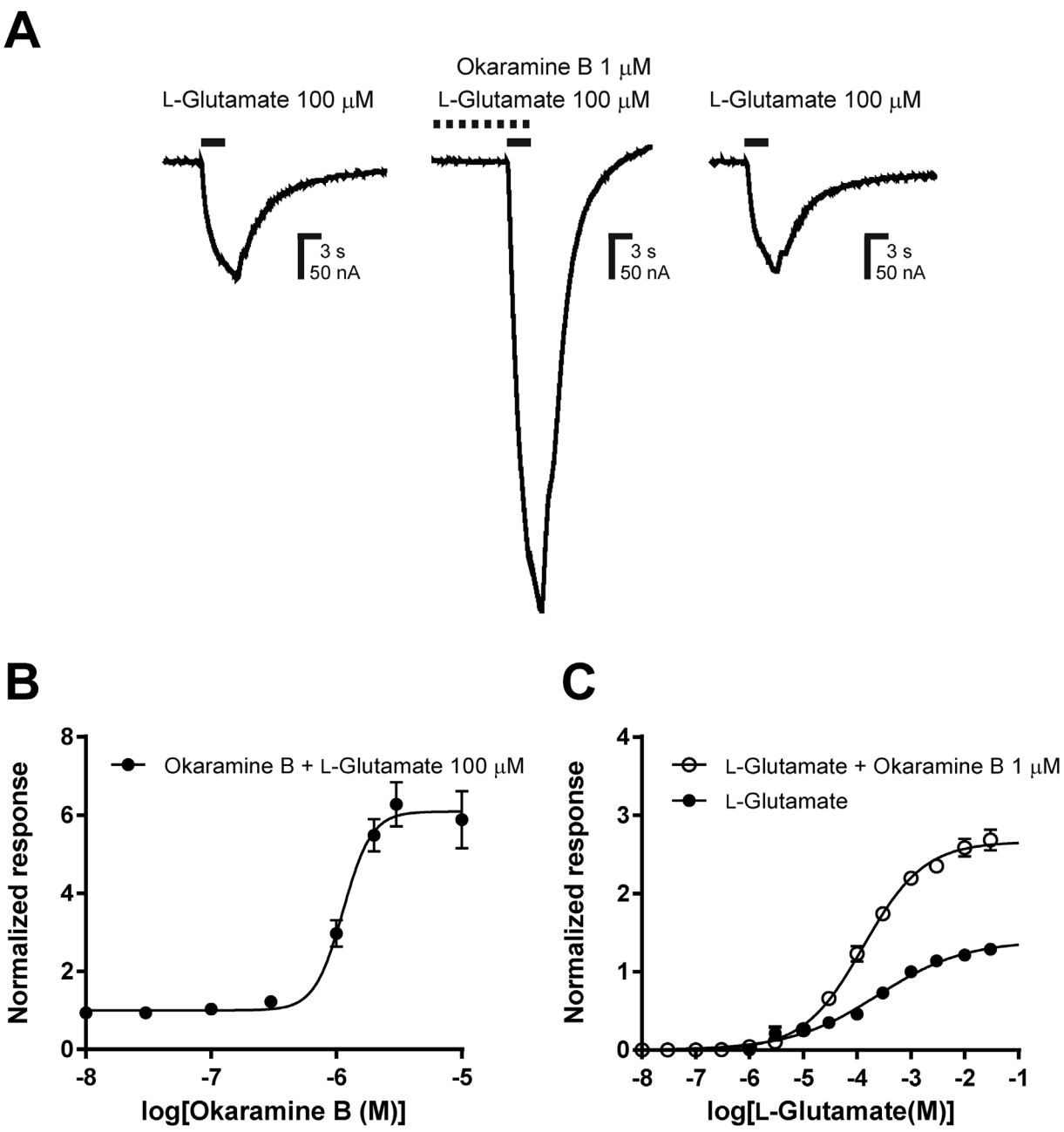


Fig. 6. Positive allosteric modulation by Okaramine B of responses to L-glutamate mediated by IscaGluCl1. A) Responses IscGluCl1 to 100 μM L-glutamate are enhanced by preincubation in 1 μM okaramine-B. B) The enhancement by okaramine-B of responses to 100 μM L-glutamate is concentration-dependent. C) Concentration-response curves for L-glutamate in the presence and absence of 1 μM okaramine-B.

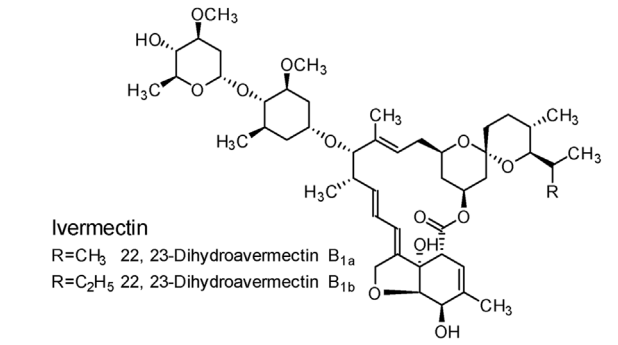
Table 3
Allosteric modulation by okaramine B of the response to L-glutamate of IscaGluCl1.

Compound	EC_{50} (μM) (95% Confidence Interval)	pEC_{50}	Imax
L-Glutamate	242 (139–421)	3.62 ± 0.12	1.40 ± 0.07
L-Glutamate + 1 μM okaramine B	132 (108–161)	3.88 ± 0.04	2.66 ± 0.05

Data were normalised to the response to 1 mM L-glutamate and are represented as mean ± standard error of the mean (n = 4).

on the cyclohexene ring of ivermectin. As noted by Hibbs and Gouaux, a serine at the equivalent position is also present in human GlyRs and both receptors are directly activated by ivermectin (Hibbs and Gouaux, 2011).

Ivermectin, picrotoxinin and fipronil also target the *D. melanogaster* GluCl (DrosGluCl-α) (Cully et al., 1996) and histamine-gated chloride channel (Iovchev et al., 2002; Yusein et al., 2008), as well as pH-sensitive chloride channels in silkworm larvae (Nakatani et al., 2016) and the glycine receptor in humans (Shan et al., 2001). Thus in insects, ivermectin can target multiple types of ligand-gated anion channels. Fipronil is a potent blocker of both GABA receptors (Zhao et al., 2003) and GluCls (Ikeda et al., 2001; Zhao et al., 2004; Eguchi et al., 2006;



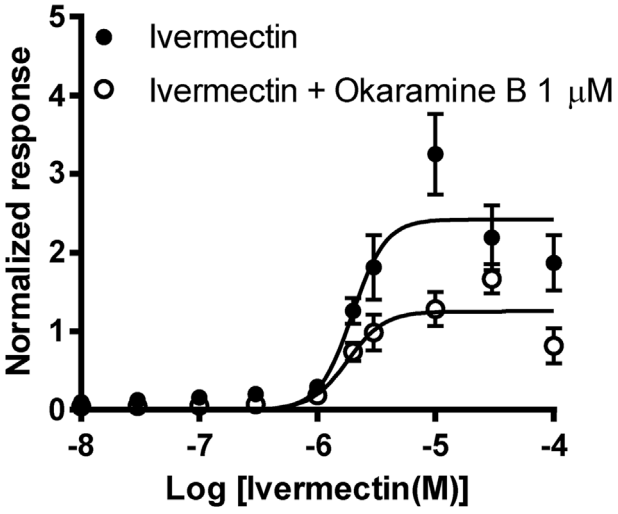


Fig. 7. Effects of okaramine B on the concentration-response to ivermectin. Each data point represents mean ± standard error of the mean (n = 4).

Table 4
Effects of co-application of okaramine B on the concentration-response curve for ivermectin.

Compound	EC_{50} (μM) (95% Confidence Interval)	pEC_{50}	I_{max}
Ivermectin	1.97 (1.55–2.51)	5.71 ± 0.05	2.42 ± 0.15
Ivermectin +1 μM okaramine B	1.80 (1.37–2.38)	5.74 ± 0.06	1.25 ± 0.08

Data were normalised to the amplitude of the response to 1 mM L-glutamateand are represented as mean ± standard error of the mean of 4 experiments.

Narahashi et al., 2007). In the case of fluralaner, comparative studies have shown that *R. microplus* RDL is 52-fold more sensitive than *R. microplus* GluCl (Gassel et al., 2014), indicating that the GABA-gated chloride channel is the primary target of this isoxazoline parasiticide. Other isoxazolines are also potent inhibitors of GABA-gated chloride channels. For example, Afoxalaner inhibits the *D. melanogaster* RDL GABA receptor (Shoop et al., 2014) and Sarolaner inhibits responses to GABA of the cat flea (*Ctenocephalides felis*) CfRDL GABA-gated chloride channel (McTier et al., 2016).

Okaramine B shows insecticidal activity on larvae of the silkworm, *Bombyx mori* (Hayashi et al., 1989). When applied to *B. mori* larval neurons at 300 nM or higher concentrations, okaramine B resulted in inward currents which reversed close to the chloride equilibrium potential and were blocked by fipronil (Furutani et al., 2014b). When tested on a silkworm GABA receptor (BmRDL) and on BmGluCl expressed separately in *X. laevis* oocytes, okaramine B activated BmGluCl, but not BmRDL (Furutani et al., 2014b). GluCl activation by 4 different members of the okaramine family correlated with their insecticidal activity, with okaramine B being the most potent (Furutani et al., 2017). Also, unlike ivermectin, okaramine B was inactive at 10 μM on human α1β2γ2 GABA gated chloride channels and human α1β glycine-gated chloride channels (Furutani et al., 2014b). Thus it offers potential promise as a new, highly-selective lead compound for the development of new insect control chemicals targeting insect GluCls.

We have begun to explore the actions of okaramine B as a possible acaricide lead and found that like picrotoxinin, fipronil and ivermectin, it is active on a tick GluCl (IscaGluCl1). As is the case with ivermectin, its actions are much slower in onset than those of L-glutamate, suggesting that it also activates IscaGluCl1 via an allosteric site rather than acting via the orthosteric (agonist) site. Okaramine B was more readily reversible than ivermectin (Fig. 5). Caution is required in interpreting findings on a single subunit when other subunits remain to be explored and the stoichiometry remains unknown for any invertebrate GluCl. However, it will be of interest to explore further the actions of okaramine B on other recombinant IscaGluCls and also on native *Ixodes scapularis* GluCls. Unlike fipronil, fluralaner and ivermectin, which in insects act on both GluCls and GABA-gated chloride channels, okaramine B shows a stronger specificity for insect GluCls (Furutani et al., 2014b), and it will be of interest to see if a similar selectivity holds in the case of ticks. We cannot be sure that GluCl-selective compounds will be of comparable efficacy to compounds active on both GABARs and GluCls but there is every likelihood that by not targeting a receptor also found in mammals they may be safer. It will be important to develop radioligand binding protocols applicable to native and expressed tick GluCls to determine whether the binding sites of okaramine B and ivermectin are distinct. If okaramine B acts at a site distinct from that of ivermectin it may offer a new, highly selective candidate lead for the development of novel acaricides. It is interesting that entomopathogenic fungi associated with the woodland habitat and even collected from nymphal *I. scapularis* can be toxic to ticks. Intriguingly, fungal species identified include *Paewcolmyces* sp., *Lecanicillium* sp. and a species of *Penicillium* (Tuininga et al., 2009). Exploring further metabolites of entomopathogenic fungi may well be fruitful therefore in the search for new acaricides.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijpddr.2018.06.001.

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