Ivermectin binding sites in human and invertebrate Cys-loop receptors

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Abstract

Ivermectin is a gold-standard antiparasitic drug that has been used successfully to treat billions of humans, livestock and pets. Until recently, the binding site on its Cys-loop receptor target had been a mystery. Recent protein crystal structures, site-directed mutagenesis data and molecular modelling now explain how ivermectin binds to these receptors and reveal why it is selective for invertebrate members of the Cys-loop receptor family. Combining this with emerging genomic information, we are now in a position to predict species sensitivity to ivermectin and better understand the molecular basis of ivermectin resistance. An understanding of the molecular structure of the ivermectin binding site, which is formed at the interface of two adjacent subunits in the transmembrane domain of the receptor, should also aid the development of new lead compounds as both anthelmintics and as therapies for a wide variety of human neurological disorders.

New insight into a wonder drug

Orally- or topically-administered ivermectin is used to eliminate parasitic nematodes and arthropods from animals and humans[1]. It is effective yet well-tolerated due to the potency with which it activates a uniquely invertebrate member of the Cys-loop receptor family of ligand-gated ion channels[2]. Although the activation of Cys-loop receptors by neurotransmitter agonists has been studied in molecular detail for 20 years, the ivermectin interaction with these receptors has received relatively little attention. This interaction requires attention, given the evidence that ivermectin resistance can occur via mutations that affect the ivermectin sensitivity of Cys-loop receptors [3,4]. In the last year, the binding sites of ivermectin in nematode and human Cys-loop receptors have been identified, revealing key molecular determinants of ivermectin sensitivity. Coinciding with these discoveries, a growing number of complete genomes have revealed the Cys-loop receptor complements of species of known ivermectin susceptibility. Combining this knowledge should allow the prediction of ivermectin sensitivity, help understand resistance mechanisms and inform the development of broader spectrum antiparasitic drugs. Recent insights in this area are reviewed here, following a brief introduction concerning the use of ivermectin and the structural basis of Cys-loop receptor function.
Origins of ivermectin

Intensive screening of soil-dwelling microbes in the 1970s led to the isolation from the bacterium, *Streptomyces avermectinius*, of a group of natural macrocyclic lactones with potent nematicidal activity, named avermectins [5]. Saturation of the avermectin 22-23 double bond produced 22,23-dihydroavermectin B1, or ivermectin. Ivermectin consists of a mixture of B1a and B1b components in a 80:20 ratio. These compounds vary in structure only at C25, a moiety that is not involved in binding. As the B1a component is more lethal to nematode, insect and acarid parasites [1], it is conventional to refer to either the mixture or the B1a component as ‘ivermectin’. Much attention has been paid to the use of ivermectin against *Onchocerca volvulus*, a mosquito-transmitted parasitic nematode that causes river blindness, but its use now extends to other human diseases including lymphatic filariasis, strongyloidiasis, scabies and head lice [1]. Despite its broad antiparasitic spectrum, ivermectin is ineffective against platyhelminths [6,7], including the blood fluke *Schistosoma mansoni* which causes bilharzia [8].

Ivermectin targets Cys-loop receptors

Early investigations into the lethal effect of ivermectin on arthropods and nematodes showed that ivermectin increased chloride conductance in neurons and thus decreased responses of neurons to excitatory input [9,10]. The search for the identity of this conductance led to the cloning of two novel subunits from the free-living nematode *Caenorhabditis elegans* that formed a recombinant glutamate-gated chloride channel (GluClR) that was activated irreversibly by nanomolar ivermectin concentrations [2]. Numerous GluClR subunits have since been cloned in various ivermectin-susceptible invertebrates [11,12]. The identification of GluClRs as the target of ivermectin was confirmed by their high ivermectin sensitivity when expressed recombinantly, their expression in tissues sensitive to low doses of ivermectin, and the development of ivermectin resistance upon their mutation [3,4,12-15]. GluClRs belong to the Cys-loop receptor family of ligand-gated channels, which includes human excitatory nicotinic acetylcholine and type-3 5-hydroxytryptamine receptors (nAChRs and 5-HT3Rs) and inhibitory GABA type-A and glycine receptors (GABA_A, Rs and GlyRs).
The Cys-loop receptor family is large, with 102 subunits in *C. elegans* and 45 in humans [16,17]. Numerous inhibitory receptors in this family are activated by ivermectin, including arthropod histamine-gated chloride channels (HisCls) and pH-gated chloride channels (pHCls) and, perhaps unexpectedly, human GABA$_A$Rs and GlyRs. A deciding factor in the combined lethality in nematode/arthropod parasites and safety in mammalian hosts is the differential potency with which ivermectin activates these receptors. α GluClRs are typically activated by low (< 20) nanomolar concentrations [2,14,18,19], whereas GABA$_A$Rs and GlyRs require concentrations in the low (1-10) micromolar range [20-23]. HisCls and pHCls respond to micromolar ivermectin, but the dose dependency has not been established [24-26]. At several other receptors, ivermectin activates no current but modulates (inhibits or potentiates) the responses to the neurotransmitter agonist. These include some invertebrate GABA$_A$Rs and the human α7 nAChR (below).

The sensitivity of human Cys-loop receptors to micromolar ivermectin, along with the preliminary findings of anti-spasticity efficacy in humans [28], begs the question as to what concentration reaches in the brain. Therapeutic doses in mice, dogs and humans produce plasma levels of 5-10 nM that persist for days, with ~2 nM in the brain [29-31], which is insufficient to activate GABA$_A$Rs and GlyRs. When α GluClRs are exogenously expressed in the mouse brain, animals respond to systemic ivermectin [32], indicating that ivermectin safety rests on the reduced sensitivity of mammalian Cys-loop receptors. P-glycoprotein, which transports avermectins out of the nervous system, is also crucial to ivermectin potency in mammals and nematodes, evidenced by the increased ivermectin toxicity that results upon its mutation [33,34].

**Structure-activity relationships in Cys-loop receptors and ivermectin**

Before describing in detail the structural features that determine ivermectin sensitivity, we briefly outline the structural basis of function in Cys-loop receptors and the determinants of efficacy within the ivermectin molecule. Functional Cys-loop receptor pentamers are formed by five of the same or, as is usually the case for native receptors, two to three different subunits (Figure 1a,b). A single subunit consists of: a large extracellular N-terminal domain (ECD) and four membrane-bound helices (M1-M4) that constitute the transmembrane domain (TMD). Two highly conserved ECD Cys residues are crosslinked within a functional receptor, forming the
eponymous Cys-loop, and the apposition of five M2 helices forms the central channel pore (Figure 1b,c). Neurotransmitter ligands bind at the extracellular interface of two adjacent subunits (red segments in Figure 1b), resulting in the closure of binding domain loop C around the agonist. This triggers structural rearrangements at the ECD-TMD interface (purple segments in Figure 1b), and ultimately the opening of the channel gate (Figure 1c,d), allowing ions to flow through the channel [35]. This chemoelectric signalling mechanism underlies most rapid synaptic transmission in the brain.

The structural basis of activity of avermectins and milbemycins (Streptomyces spp-derived compounds with similar structures and antiparasitic spectra to avermectins) has been extensively reviewed[36]. Briefly, all compounds share a macrocyclic backbone and differ at the disaccharide, spiroketal and benzofuran moieties, respectively located at the three “corners” of the molecule (Figure 2). Evidence indicates that the benzofuran is the most important potency determinant at GluClRs. For example, substitution of the hydroxyl at the C5 position on the benzofuran ring ("C5-OH") for oxime or ketone in several avermectins reduces nematicidal activity 100- and 10000-fold, respectively [37], implying that either an H-bond donating or generally hydrophilic C5-substituent is required for high potency. In contrast, changes to the spiroketal group have little effect on nematicidal potency [36,37]. Similarly, hydrogenation of double bonds C10-C11 (in the macrocyclic backbone) and 22-23 (in the spiroketal) does not reduce acaricidal potency, whereas hydrogenation of double bonds C3-C4 and C8-C9 (in or adjacent to the benzofuran) reduces both acaricidal potency and activity at mouse GABA_{A}Rs [38,39]. The sugar moiety is not essential for nematicidal potency [36,37]. However, given that epimerisation of the dissacharide decreases activity at GluClRs [40,41], polar C13 substitutions yield less biologically active avermectins than lipophilic C13 substitutions [42], and size of the sugar moiety correlates with affinity for P-glycoprotein [43], it is evident that the sugar may modulate avermectin potency in vivo.

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The binding site in anionic Cys-loop receptors

Hibbs and Gouaux recently solved the crystal structure of the C. elegans α GluClR complexed with ivermectin [44]. This gives a clear picture of the ivermectin binding site and sheds light on previous functional studies. Furthermore, with over 30 % amino acid sequence identity with the
α1 GlyR, it provides a high resolution template for the modelling of vertebrate GABA<sub>A</sub>Rs and GlyRs, which had previously relied on nAChR and prokaryotic receptor structures with much lower homologies [45-47]. Their structure shows ivermectin binding between the M3 of one subunit (the principal or (+) face) and the M1 of an adjacent subunit (the complementary or (-) face), with the benzofuran moiety contacting the M2 helix of the (+) subunit (Figure 3a). The structure implies the existence of three H-bonds: (i) between the benzofuran C5-OH and the hydroxyl sidechain of (+)M2-Ser15' (Ser260 in the α GluClR), (ii) between a spiroketal oxygen and the hydroxyl sidechain of (+)M3-Thr285, and (iii) between the benzofuran C7-OH and the backbone carbonyl oxygen of (-)M1-Leu218 (Figure 3b). Leu218, Ser260 and Thr285 are highlighted in yellow in Figure 3c, as are residues at the equivalent positions in various other Cys-loop receptors. Twelve other sidechains, highlighted in orange or red in Figure 3c, contribute potential Van der Waals interactions. One of these is (+)M3-Gly281, a position in anionic Cys-loop receptors we will henceforth refer to as "M3-Gly". M3-Gly is entirely conserved in GluClRs that show nanomolar ivermectin sensitivity (Figure 3c). In the crystal structure, the α carbon of M3-Gly is approximately 4.5 Å from each corner of the ivermectin macrocycle, such that any larger sidechain at this position - that is, any amino acid other than Gly - would hinder the access of ivermectin to its binding site or alter the binding conformation of ivermectin (Figure 3b). Hence, in the *Haemonchus contortus* α3B GluClR, the mutation of M3-Gly to Ala (the human α1 GlyR equivalent) shifts the ivermectin EC<sub>50</sub> for activation from 39 nM up to 1.2 µM - exactly that of the human α1 GlyR [48]. Indeed, the α3B GluClR and the α1 GlyR can be tuned to remarkably similar ivermectin sensitivities by introducing a common residue at the M3-Gly position [48]. Consistent with this, the Gly323Asp mutation (at the M3-Gly position) in the *Tetranychus urticae* (two-spotted spider mite) GluClR was shown to increase by 18-fold the avermectin dose required for half-maximal lethality [4]. Indeed, the equivalent Gly-Asp substitution abolished binding of a tritiated milbemycin to the α3B GluClR [49]. We have not found in the literature any example of a highly ivermectin-sensitive receptor without a Gly at the M3-Gly position.
The binding site at the α1 GlyR has also been investigated using mutagenesis and molecular modelling. Ivermectin was modeled as wedging into the same TMD interface, with the benzofuran oriented towards the (+)M2 [50]. Surprisingly, the docking of ivermectin to the Ala288Gly α1 GlyR (this is the M3-Gly position) did not differ greatly from the unmutated receptor, suggesting that the on-rate of ivermectin, rather than the ultimate binding conformation of ivermectin, is changed by this substitution [50]. Alternately, the glycine substitution may increase the gating equilibrium constant, thus facilitating receptor activation by ivermectin. Bulky substitutions at only two positions decreased both agonist and modulatory effects of ivermectin, indicative of a decisive role in binding. These positions are (+)Ala288 and (-)Pro230. Both of these positions are highlighted in red in Figure 3c, revealing the apparent requirement of a small residue at the M3-Gly position and the conservation of the "M1-Pro". M1-Pro is in close proximity to the crucial benzofuran moiety of ivermectin (Figure 3b), providing a possible steric explanation for decreased binding at this mutant. In addition, this highly conserved residue disrupts M1 helical structure thereby exposing a backbone carbonyl that is thought to form a H-bond with the ivermectin C7-OH [44]. However, the existence of this bond requires confirmation, given that C7-OH is equidistant from both the M1 backbone carbonyl and the endogenous ivermectin C1 carbonyl.

A significant role for the third putative H-bond (between O14 and T285 – see Figure 3b) can be eliminated on the basis of site-directed mutagenesis experiments in the Ala288Gly α1 GlyR, and the fact that the three GluClRs with nanomolar ivermectin affinity do not contain H-bonding sidechains at the corresponding M3 position [50]. Moving deeper into the pore, substitution of α1 GlyR (+)M3-Leu291, (+)M2-Thr264 or (-)M1-Leu233 for bulky Trp sidechains does not reduce ivermectin sensitivity but converts ivermectin from an agonist to an antagonist of glycine-activated currents [50]. Still further removed from the binding site, mutations in the conserved Cys-loop, the pre-M1 domain and the M2-M3 linker (Figure 1b) decrease ivermectin potency most likely by impairing its gating efficacy [14,18,49-51].

Native Cys-loop receptors are often heteromeric and thus do not necessarily possess the five binding sites available to ivermectin in homomers. The following evidence at the GlyR, however, suggests that the binding of two molecules of ivermectin is sufficient for either direct activation
or potentiation. First, ivermectin activates $\alpha_1\beta$ heteromeric and $\alpha_1$ homomeric GlyRs with equal potency [21], even though heteromers possess a $\beta-\alpha_1-\beta-\alpha_1-\beta$ arrangement [52] and ivermectin only binds to the $(+\alpha_1/(-)\beta$ interface [48]. Second, the $\beta$-Ile312Gly substitution (at the M3-Gly position) confers sensitivity to potentiation on the otherwise ivermectin-insensitive $\alpha_1$-Ala288Phe/$\beta$ heteromer, presumably by introducing a $(+)$/$(-)\alpha_1$ ivermectin binding site [48].

**M2 H-bonds**

The putative H-bond between the ivermectin C5-OH and the M2-15' Ser of the GluClR and GlyR seems a logical ivermectin binding interaction, as (i) it links the ivermectin molecule to the pore-lining M2 helix[44] and (ii) a H-bond donor at the C5-OH is crucial to avermectin nematicidal potency (above). However, because various GluClRs with nanomolar ivermectin sensitivities contain either an Ala or Ile at this position (Figure 3c), it is evident this H-bond is not required for high ivermectin potency [4,18,53]. In support of this, the mutant M2-Ser15'Ile substitution in the $\alpha_1$ GlyR causes no change to the agonist or modulatory effects of ivermectin [50]. However, most anionic Cys-loop receptor subunits possess several nearby residues that could act as potential H-bond acceptors in the absence of one at the M2-15' position. These include $(+)$M2-12', $(−)$M2-14', $(+)$M2-19' and also an $(−)$M1 position (Figure 4). The proximity of these sidechains to the ivermectin molecule is suggested by site-directed mutagenesis data from the $\alpha_1$ GlyR: the Thr12'Trp receptor is not activated by ivermectin, the Gln226Cys receptor has decreased ivermectin sensitivity and the Gln266Trp receptor has increased sensitivity [50]. The conservation of polarity in this domain of ivermectin-sensitive receptors is illustrated by the boxed residues in Figure 3c.

**A clear pattern?**

We now group the above ivermectin sensitivity determinants together and consider their chemical properties in several receptors with known high ivermectin sensitivity. Dividing M2-12', M2-14', M2-15', M2-19' and the M1-Gln residue into their $(+)$ and $(−)$ components gives a "+(+)Thr-Ser-$\text{Arg}/(-)\text{Gln-Gln}" arrangement, in the case of the $\alpha_1$ GlyR (Figure 4). The following five
homomeric Cys-loop receptors are particularly sensitive to ivermectin activation (i.e., activated by 1-20 nM ivermectin): Ala288Gly α1 GlyRs, *C. elegans* α, *H. contortus* α and α3B and *Drosophila* α GluClRs. Respectively, these subunits have (+)Thr-Ser-Arg/(-)Gln-Gln, (+)Thr-Ser-Asp/(-)Gln-Gln, (+)Thr-Ala-Asn/(-)Gln-Gln, (+)Thr-Ala-Asn/(-)Gln-Gln and (+)Ala-Thr-Asn/(-)Gln-Gln arrangements, and thus present an overwhelmingly polar environment to the ivermectin C5-OH moiety. Given that the α1 GlyR possesses a similar polarity in this region to GluClRs, we infer that its large M3- Gly sidechain is solely responsible for its reduced ivermectin sensitivity. UNC-49B/UNC-49C heteromeric GABA,αRs possess an M3-Gly at the (+) faces of UNC-49C, yet are not activated by ivermectin [54,55]. However, with a (+)Thr-Met-Asn/(-)Gln-Leu arrangement, they are distinct from the GluClRs and GlyRs in that both the M2-14' and M2-15' positions are occupied by larger hydrophobic residues (Figure 3c), perhaps limiting H-bonds with and channel activation by ivermectin [44]. Similarly, the *Dermacentor variabilis* (American dog tick) homomeric RDL receptor possesses an M3-Gly but is not sensitive to ivermectin (Figure 3), and its M2-14' and M2-15' positions are occupied by hydrophobic residues [56]. By contrast, MOD-1 is insensitive to ivermectin [57], despite its ostensibly suitable (+)Thr-Ser-Arg/(-)Gln-Ile arrangement; MOD-1 insensitivity is likely due to a Val residue at the M3-Gly position preventing access to the site [50]. Curiously, although mammalian GABA,π subunits possess an M3-Gly, incorporation of the π subunit actually decreased the ivermectin sensitivity of α1β2 GABA,αRs [48]. Again we suggest that the ivermectin-insensitivity results from hydrophobic residues at both M2-14' and M2-15' positions in the π subunit [58]. Finally, functional homomeric β GluClRs from *C. elegans* and *C. onchophora* are also completely insensitive to ivermectin [59], and studies with radiolabelled ivermectin show that it does not even bind to the *H. contortus* β GluClR [60]. These receptors contain a (+)Thr-Gln-Asn/(-)Gln-Met arrangement and an M3-Gly. We thus conclude that the (-)M1-Met is responsible for disrupting ivermectin sensitivity. In the above analysis, we have used the GluClR crystal structure to infer that the five polar residues are essential for high ivermectin affinity. However, it is also possible that the overwhelmingly polar environment facilitates receptor gating by ivermectin.
An alternate possibility in GABA\textsubscript{\textalpha}Rs is that ivermectin binds to a different site. For example, GAB-1/HG1A heteromers and UNC-49B homomers possess Val, Leu or Asn residues at the M3-Gly position, and these receptors are potentiated but not activated by ivermectin [54,55,61], perhaps reflecting the exclusion of ivermectin from the interfacial site and the existence of an alternate site. Moreover, HG1A and HG1E differ by only four amino acids - in the ECD and in M4 - and, whereas GAB1/HG1A heteromers are potentiated by ivermectin, GAB1/HG1E heteromers are inhibited [61]. An alternative site, consistent with HG1A/E structural differences, might be similar to that proposed for the mammalian \(\alpha\text{7} \) nAChR (see below). The possibility of distinct ivermectin binding sites in various GABA\textsubscript{\textalpha}Rs, together with the large diversity in GABA\textsubscript{\textalpha}R subunits, might underlie the differential effects of ivermectin on various native GABA\textsubscript{\textalpha}R preparations [62].

**An alternative site for potentiation of nAChRs**

Due to the differing M2-M3 domain lengths in anionic and cationic Cys-loop receptors, it is difficult to determine which amino acid occupies the M3-Gly position in the mammalian \(\alpha\text{7} \) nAChR [63]. Most alignments suggest that Ala275 in the \(\alpha\text{7} \) nAChR and Leu279 in the *Torpedo marmorata* (marbled electric ray) nAChR \(\alpha\) subunit occupy the M3-Gly position. Although the 4 Å resolution structure of the *T. marmorata* nAChR places this Leu279 in the (+)M3 helix, it faces the outside of the membrane, 1-2 helical turns above the level of (-)M1-Pro (Figure 5a). In the *T. marmorata* nAChR structure, the (+)M3 residue physically opposite (-)M1-Pro is either Met282, Ile283 or Ile286, which equate with little ambiguity to Met278, Ile279 and Gly282 in the \(\alpha\text{7} \) nAChR (Figure 5a,b). Thus, the \(\alpha\text{7} \) nAChR structural equivalent to the M3-Gly might Gly282, and the selectivity of ivermectin for \(\alpha\text{7} \) nAChRs over insensitive cationic Cys-loop receptors may be due to Val, Ile and Thr sidechains at this position (e.g., in nematode ACR-16 receptors and mammalian 5-HT\textsubscript{3}Rs [64,65]). Accordingly, the lack of H-bond propensity at M2-14', M2-15' and M2-19' positions in the \(\alpha\text{7} \) nAChR might limit the direct agonist efficacy of ivermectin. However, if the structural equivalent in \(\alpha\text{7} \) nAChR is Met278 or Ile279, access of ivermectin to the interfacial site is likely precluded, and ivermectin might bind to a distinct site.
Computer docking simulations from two separate laboratories have shown that the lowest energy docking of ivermectin to the \( \alpha_7 \) nAChR was in an intrasubunit cavity, as opposed to the interfacial site in anionic receptors [64,66]. In both studies, the ivermectin molecule wedges between M1 and M4 from the same subunit, partly in contact with the surrounding lipids, with the benzofuran moiety oriented towards either M2 or M3 of the same subunit. Substitution of \( \alpha_7 \) nAChR TMD residues for the non-conserved equivalents from the 5-HT3\(_A\)R resulted in decreased potentiation by ivermectin or conversion of potentiation to inhibition [64]. Three of the four mutations that simply decrease potentiation line a space between M1 and M4 of the one subunit, albeit two helical turns below the docked ivermectin molecule. Consistent with putative existence of this site in the \( \alpha_7 \) nAChR and not in GluClRs and GlyRs, the latter possess large sidechains that might obscure this cavity (Figure 5). Finally, the effects of ivermectin at nAChRs are reversible and are decreased by ECD-acting competitive antagonists [67], which differs from results at most anionic receptors and supports a different site or mechanism of ivermectin at nAChRs.

**Structural bases of ivermectin resistance**

There are several reports that ivermectin resistance occurs nematodes in natural settings [1], in some cases due to mutations in the coding sequences or changes in expression levels of GluClRs or GABA\(_\xi\)Rs [3,68,69]. In laboratory-induced mutant strains, single mutations in GluClRs have been clearly shown to cause avermectin resistance. For example, mutation of the M3-Gly position in the *T. urticae* GluClR causes an 18-fold increase in abamectin LD\(_{50}\) [4]. (A C22-C23 double bond in the spiroketal of abamectin is the only difference from ivermectin.) It is also possible that ivermectin insensitivity may be caused by mutations that allosterically disrupt the efficacy with which ivermectin activates the receptors, without necessarily affecting ivermectin affinity. This was first suggested by an M2-M3 linker mutation in a *Drosophila* GluClR that caused a four-fold increase in ivermectin LD\(_{100}\) [14]. Disruptions to the efficacy of GluClR gating by ivermectin most likely account for the effects of mutations identified in several resistant *H. contortus* strains [18,49]. Of course, if a GluClR mutation converts ivermectin to an inhibitor, as demonstrated at recombinant GlyRs and nAChRs (above), the usual CNS depression elicited by ivermectin could
be converted to excitation. This may underlie the resistance seen in nematodes with an up-regulation of HG1E GABA$_R$ subunits that are inhibited by ivermectin [61,68].

Predicting species susceptibility to ivermectin

The discovery of molecular determinants of ivermectin sensitivity in Cys-loop receptors provides an opportunity to investigate parasites of known ivermectin susceptibility to establish the molecular basis for this susceptibility. The complete genome of the trematode *S. mansoni* reveals 13 Cys-loop receptor subunits [70], much fewer than the 102 found in *C. elegans* and 45 in humans [16,17]. It is therefore likely that ivermectin has fewer potential Cys-loop receptor targets in *S. mansoni* than in *C. elegans* which might help explain why *S. mansoni* is insensitive to ivermectin. On the other hand, arthropods also possess few GluClRs but are ivermectin-susceptible, suggesting that there must be some feature of *S. mansoni* GluClRs (other than a small number in the genome) that renders them insensitive to ivermectin. An amino acid sequence alignment of recently available transcripts in several trematodes suggests that they indeed possess GluClRs (Figure 6). Although their functional properties remain to be investigated, these transcripts possess ~30% homology with $\alpha$ GluClRs and $\alpha1$ GlyRs, suggesting a functional relationship. Furthermore, they possess a Gly residue on the (+) face of the ECD neurotransmitter binding domain (Figure 6), at a position where small residues are correlated with Glu binding and larger residues are correlated with Gly, GABA or His binding[71]. These putative GluClRs include largely polar (+)Ile-Ser-Ala/(-)Ser-Gln arrangements (as per the scheme above), but large residues at the M3-Gly position undoubtably preclude ivermectin binding (Figure 6). Thus, large sidechains at the M3-Gly position, as opposed to a lack of GluClRs, may well underlie the ivermectin-insensitivity of trematodes. The free-living trematode *Schmidtea mediterranea* also possesses GluClR-like transcripts with large sidechains at the M3-Gly position (Figure 6). The recent sequencing of *Ascaris suum* (pig roundworm), *Anopheles gambiae* (mosquito) and *Daphnia pulex* (water flea), by contrast, reveal one or more M3-Gly GluClR transcripts, as expected for ivermectin-susceptible phyla [72-74]. This reiterates the importance of the M3-Gly position. Of course, species susceptibility also depends on if and where the relevant subunits are expressed, and the functionality with which an M3-Gly subunit heteromerises with other subunits.
Concluding remarks

Ivermectin binds to nematode GluClRs and mammalian GlyRs at the interface of adjacent (+) and (-) subunits in the TMD of the receptor. High affinity for this site is controlled by an M3 Gly residue on the (+)M3 helix that allows rapid access to the site. The C5 hydroxyl of the ivermectin benzofuran likely forms an H-bond within the subunit interface cleft, leading to potent channel activation. Potent activity requires polar sidechains at either M2-14', M2-15' or M2-19' (and perhaps other) positions. Scanning Cys-loop receptor amino acid sequences for these determinants predicts ivermectin sensitivity reasonably well, and scanning genomes for these transcripts may enable the prediction of species susceptibility to ivermectin. However, several questions are yet to be answered, including the structural basis for modulation (not activation) by ivermectin of certain GABA$_A$Rs and nAChRs, and the inability of ivermectin to activate β GluClRs. Attempts to answer these questions will be aided by the structural template provided by the GluClR crystal structure. Given the emergence of ivermectin resistance in parasitic nematode pests [75,76], the information presented here may help in the design of novel anthelmintics targeting GluClRs or other nematode Cys-loop receptors. Similarly, because ivermectin binding sites also exist in human Cys-loop receptors, understanding the ivermectin binding site structure may help identify new therapeutic pharmacophores for a wide variety of human neurological disorders.
Figure legends

Figure 1. Molecular architecture of Cys-loop receptors. (a) The *C. elegans* α GluClR crystal structure, viewed from the extracellular space (top panel) and from within the lipid membrane (lower panel) [44]. (b) Single subunit from (a). Red indicates segments that contribute to the neurotransmitter binding domain, which is formed between the (+) face of one subunit and the (-) face of an adjacent subunit; magenta indicates Loop 2, the conserved Cys-loop, the pre-M1 domain, and the M2-M3 linker, which couple neurotransmitter binding to channel movement; helices M1-M4 are indicated; M2-Leu9' sidechains that contribute to the channel gate are shown as blue spheres [35]. (c) Crystal structure of the closed bacterial channel ELIC [46] and (d) crystal structure of the open bacterial channel GLIC [45], both viewed from the extracellular space with ECDs removed for clarity; the hydrophobic M2-9' sidechain is moved from the channel axis in models of channel activation.

Figure 2. The ivermectin molecule. (a) Natta projection of ivermectin, including the C atom numbering referred to in the text. (b) 3D structure of ivermectin, as used for docking to the α1 GlyR model[50]. Certain atoms/substituents are labelled, for comparison with (a) or for their significance in the text. Image in (b) prepared with PyMOL Molecular Graphics System (Schrödinger, LLC).

Figure 3. Ivermectin binding sites and sensitivity determinants in anionic Cys-loop receptors. (a) The ivermectin binding site in the *C. elegans* α GluClR [44]. Adjacent (+) and (-) subunits are shown in grey and black, respectively, with M2 domains from the remaining three subunits shown in blue and all ECDs removed for clarity. Yellow lines indicate H-bonds with indicated residues. Orange positions indicate residues that form Van der Waals interactions with the ivermectin molecule. (b) The same site as in (a), but viewed from (+)M2-Ser260 (15°). Red lines indicate proximity of Pro230 (3.4 Å) and Gly281 (4.0 and 4.5 Å) to ivermectin; yellow lines indicate H-bonds with (-)Leu218 and (+)Thr285. (c) Amino acid sequence alignment of TMD portions of Cys-loop receptor subunits of known ivermectin sensitivity. Orange and red (Van der Waals interactions) and yellow (H-bonds) highlighting indicates residues that line the site, as in
(a) and (b); boxed residues constitute the polar binding site for the ivermectin benzofuran in ivermectin-sensitive receptors (see text and Figure 4). *Cel, Caenorhabditis elegans; Hco, Haemonchus contortus; Dme, Drosophila melanogaster; Tur, Tetanychus urticae; Hsa, Homo sapiens; Ssc, Sarcoptes scabiei; Dme, Dermacentor variabilis; Mmu, Mus musculus. "nM" and µM (nanomolar and micromolar) indicate the concentration range in which receptors of that subunit are robustly activated by ivermectin. "mod" (modulatory) means that the receptor is not activated but is potentiated or inhibited by micromolar concentrations of ivermectin. *The T. urticae α GluClR confers ivermectin susceptibility on the organism [4]; it is therefore considered highly ivermectin-sensitive although the recombinant receptor has not been tested. **The recombinant D. variabilis RDL has only been exposed to 100 nM ivermectin, which had no agonist or modulatory effect [56]. Lines joining subunits indicate that these form heteromeric receptors. References for all subunits listed are provided in the main text, except for Cel GluClR α2B [77] and Cel GLC-3 [78].

**Figure 4.** Possible H-bond partners of the ivermectin C5-hydroxyl. The ivermectin binding site in an α1 GlyR homology model [50], viewed from the (+)M3 helix, with the (+)M3 helix removed. Distances (yellow lines) are 8.1, 2.9, 4.0, 3.3 and 10.1 Å, clockwise from M2-12'.

**Figure 5.** Comparison of an intrasubunit site in nAChRs and anionic Cys-loop receptors. (a) TMD portions of adjacent (+)α and (-)γ subunits of the Torpedo marmorata ("Tma") nAChR (left; electron microscopy structure [47]) and of adjacent subunits of the C. elegans α GluClR (right, [44]), seen from within the membrane plane. For each receptor, the (+) subunit is shown in light grey, the (-) subunit in dark. nAChR α Leu279 (green) aligns with the GluClR M3-Gly position in most amino acid sequence alignments, but Met282, Ile283 (yellow) and Ile286 (red) are structurally more equivalent to GluClR M3-Gly, indicated by the proximity to M1-Pro (red in both receptors). Shown in magenta are several residues that line a space between M1 and M4 of each subunit (these are equivalent in amino acid alignments but were selected simply due to their apparent structural equivalence). Shown in cyan are residues equivalent to those whose substitution in the rat α7 nAChR decreases potentiation by ivermectin [64]; only one of these is
indicated in the GluClR structure, as it is one of the aligned residues in (b). (b) Amino acid sequence alignment. Colours indicate the residues shown in (a). Circled residues are smaller in the α7 nAChR than in other cationic receptors, providing a possible explanation for selectivity of ivermectin for the α7 nAChR over other cationic receptors. Boxed residues are smaller in the α7 nAChR than in anionic Cy-loop receptors, providing a possible explanation for the selectivity of ivermectin for the M1-M4 site in the former.

Figure 6. Putative ivermectin-insensitive receptor subunits from trematodes. An amino acid sequence alignment of characterised GluClR, GlyR, HisCl and GABA\_R (UNC-49B) subunits from vertebrates, insects and nematodes (black) and uncharacterised subunits from trematodes (purple). Sma, Schistosoma mansoni; Sja, Schistosoma japonicum; Sha, Schistosoma haematobium; Csi, Clonorchis sinensis; Sme, Schmidtea mediterranea. Loop B is part of the neurotransmitter binding domain (see Figure 1). A Glu residue at the cyan position is important to the binding of the agonists glycine, histamine and GABA [71]. Although the putative trematode subunits possess several features of the ivermectin binding site, such as the M1-Pro (first red residue) and several polar sidechains (boxed residues), they possess large hydrophobic sidechains at the M3-Gly position (second red residue), indicative of ivermectin insensitivity. Smp\_104890 and Smp\_096480 [70] and Sjp\_0079260 [79] were retrieved at GeneDB. Sha\_300716 was retrieved from the original publicaton [80]. GAA36399.1 [81] was retrieved from GenBank. mk4.001914 [82] was retrieved from SmedGD.
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(a) Leu279 Met282 Ile283 Ile286

Tma nAChR α

Cel GluCl α

(b)

Cel GluCl α

Hsa GlyR α1

Tma nAChR α

Tma nAChR γ

Rat nAChR α7

Hsa nAChR α4

Hsa nAChR β2

Mouse 5-HT3AR

(+)-M3

(-)-M1

(-)-M4

GACMTFIF

AVCLLFVF

LTMTFVI

IFVFVSL

ASTMIVG

LTMTFVT

MFTMLVT

VCMALLV

YLLQLYIPSC FEVFNILYW

YLIQMYIPSL FLIFNMFYW

FVVNVIPCL GTVSVFAGR

FIINIIAPCV GTLAIFLTG

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