Monepantel Irreversibly Binds to and Opens *Haemonchus contortus* MPTL-1 and *Caenorhabditis elegans* ACR-20 Receptors

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ABSTRACT

Monepantel is a recently developed anthelmintic with a novel mode of action. Parasitic nematodes with reduced sensitivity to monepantel have led to the identification of MPTL-1, a ligand-gated ion-channel subunit of the parasitic nematode *Haemonchus contortus*, as a potential drug target. Homomeric MPTL-1 channels reconstituted in Xenopus oocytes are gated by μM concentrations of betaine and mM concentrations of choline. Measurement of reversal potentials indicated that the channel has a similar conductance for Na⁺ and K⁺ ions and does not permeate Ca²⁺. Concentrations of monepantel (amino-acetonitrile derivative [AAD]-2225) >0.1 μM, but not its inactive enantiomer AAD-2224, induced channel opening in an irreversible manner. Currents elicited by monepantel alone were larger than the maximal current amplitudes achieved with betaine or choline, making monepantel a superagonist. Currents elicited by betaine or choline were allosterically potentiated by nM concentrations of monepantel and to a much smaller degree by AAD-2224. We have also reconstituted the *Caenorhabditis elegans* homomeric ACR-20 receptor in Xenopus oocytes. The acr-20 sequence has higher similarity to mptl-1 than acr-23, the primary target for monepantel mode of action in *C. elegans*. The ACR-20 channel is gated similarly as MPTL-1. Monepantel, but not AAD-2224, was able to induce channel opening in an irreversible manner at similar concentrations as for MPTL-1. Interestingly, the allosteric potentiation measured in the presence of betaine was much smaller than in MPTL-1 receptors. Together, these results establish the mode of action of monepantel in *H. contortus* and contribute to our understanding of the mode of action of this anthelmintic.

Introduction

Gastrointestinal nematodes of ruminant are a global threat to animal farming worldwide, causing substantial economic losses to the sheep and cattle industry (McLeod, 1995). Although alternative treatments have been developed (Wall, 2003), the use of anthelmintics has been central for control of these parasites for decades, but today their efficacies are hampered by the development of anthelmintic resistance (Wall, 2006). Fresh hope came with the launch of monepantel in 2008, a member of the amino-acetonitrile derivative (AAD) classes of compounds.

*Trichostrongylid* nematodes, such as *Haemonchus contortus*, exist in very large populations and are prolific egg layers. This, along with high mutation rates, generates a large source of resistant genotypes upon which anthelmintic selection can act (Gilleard and Beech, 2007). Unfortunately, the recent identification of monepantel-resistant *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* recovered from a goat farm in New Zealand has confirmed this fact (Scott et al., 2013). It is essential to maintain the remaining efficacy of old drugs and to manage new anthelmintics such as monepantel tightly to preserve animal productivity gains. Understanding the mode of action for new anthelmintics and the biologic role of their principal targets will help in the rational design of drug treatment strategies.

The development of nematodes resistant to monepantel has led to identification of the acr-23 gene in the nonparasitic species *Caenorhabditis elegans* and a homologous gene, mptl-1, in the parasitic nematode *H. contortus* (Kaminsky et al., 2008; Rufener et al., 2009). Both genes belong to the nematode-specific DEG-3 subfamily of ionotropic acetylcholine receptors (AChRs). ACR-23 has been localized in the body wall muscle (Rufener et al., 2013), and, in mechanosensory neurons, Peden et al. (2013) have recently shown that ACR-23 is activated by betaine and functions to maintain basal levels of locomotion. Betaine, a ubiquitous noncanonical amino acid, serves as an organic osmolyte to protect cells against osmotic stress, but it is also a methyl donor of increasingly recognized significance in biology. Farmers realized quite some time ago that applying seaweed extracts that are rich in betaine directly to plants could prevent nematode growth (Whapham et al., 1994). ACR-23 is not only activated by betaine as natural ligand, but is also the molecular target of monepantel (Rufener et al., 2013). Monepantel acts by potentiating betaine signaling during development, resulting in nematode death, similar to an excess of betaine.

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**ABBREVIATIONS:** AAD, amino-acetonitrile derivative; AChR, acetylcholine receptor; DMSO, dimethyl sulfoxide.
Recent phylogenetic studies (Rufener et al., 2010b) have shown that mptl-1 is more closely related to the C. elegans acr-20, another member of the nematode-specific deg-3 subfamily. This similarity suggests that it may function as a secondary target for monepantel. Monepantel has also been shown to potentiate the H. contortus DES-2/DEG-3 receptor at micromolar concentrations when coapplied with choline chloride (Rufener et al., 2010a). A genetic screen of C. elegans (Kaminsky et al., 2008) identified mutations in acr-23, but no other member of the deg-3 family, including acr-20, des-2, and deg-3. In fact, C. elegans acr-23 null mutants are highly resistant to monepantel despite the presence of other members of the deg-3 family. The DES-2/DEG-3 receptor is expressed solely in sensory neurones and may not affect motility of the worm (Treinien et al., 1998; Yassin et al., 2001). It may be that the ACR-20 receptor is less, or insensitive to monepantel, or it may also play a physiologic role in which activation does not affect viability of the nematodes.

To date, no direct evidence of the molecular interaction of monepantel with MPTL-1 receptors has been provided. We have recently described the functional effects of monepantel on ACR-23 (Rufener et al., 2013), and in this work we describe for the first time its effects on the MPTL-1 and ACR-20 receptors. Both proteins form homomeric betaine and choline-gated ion channels. Monepantel acts on these channels as a positive allosteric modulator at low concentrations (<1 nM) and as a direct agonist at higher concentrations (>0.1 μM). The observed allosteric modulation by monepantel of ACR-20 channels was much weaker than of MPTL-1 or ACR-23, as predicted from the observations made in vivo. Surprisingly, once MPTL-1 or ACR-20 channels are opened by monepantel, they remain open, even after extensive washing. Presumably, monepantel acts irreversibly on these ion channels, a feature that was not observed on ACR-23 channels.

Materials and Methods

Cloning of mptl-1 and acr-20 from H. contortus and C. elegans. RNA extraction, cDNA synthesis, and PCR amplification of mptl-1 were performed, as described previously in Rufener et al. (2009). In brief, total RNA was extracted from a pool of adult nematodes, and 1 μg total RNA (DNase-treated) was reverse-transcribed to cDNA using a qRT30 primer and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). The gene-specific PCRs were performed using a Phusion a (dT)30 primer and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). The gene-specific PCRs were performed using a Phusion with a Phusion polymerase (New England Biolabs, Ipswich, MA). The following primers were used to amplify the full-length sequence of mptl-1: Nehl_Hc-mptl-1_FL_F1 (ggcggctagccaaatggaaaatcctagctctgg) and Xhol_Hc-mptl-1_stop_R1 (gggatcctgatcagtcagagggcgatcttg). For acr-20, the primers were BglII_Ce-acr-20_FL_F1 (ggggatctgatcagtcagagggcgatcttg) and SpeI_Ce-acr-20_R1 (gggaattcgctgatcagtcagagggcgatcttg). The gene-specific primers were designed using the Primer3 software (available at http://frodo.wi.mit.edu)
The amplicons were applied for 20 seconds alone or in combination with the allosteric modulator, followed by incubation in modified Barth’s solution at +18°C for at least 24 hours before the measurements.

Functional Characterization. Currents were measured using a new two-electrode voltage clamp amplifier Oocyte clamp OC-725 (Warner Instruments) in combination with a XY-recorder (90% response time 0.1 second) or digitized at 100 Hz using a PowerLab 2/20 (AD Instruments) using the computer programs Chart (ADInstruments, Spechbach, Germany). Tests with a model oocyte were performed to ensure linearity in the current range. The response was linear up to 15 μA.

Electrophysiological experiments were performed by using the two-electrode voltage clamp method at a holding potential of ~80 mV. The perfusion medium contained 90 mM NaCl, 1 mM KCl, 1 mM MgCl2, 1 mM CaCl2, and 5 mM Na-HEPES (pH 7.4) and was applied by gravity flow 6 ml/min. The perfusion medium was applied through a glass capillary with an inner diameter of 1.35 mm, the mouth of which was placed about 0.4 mm from the surface of the oocyte. Cumulative concentration-response curves for betaine and choline were fitted with the equation

where is the concentration of agonist, EC50 is the concentration of agonist eliciting half-maximal current amplitude, IMAX is the maximal current amplitude, f is the current amplitude, and nH is the Hill coefficient. In experiments in which the channel was challenged several times by agonists, enough time was allowed between applications for the channel to recover from desensitization. The interval time was determined experimentally (3 minutes at low concentration; up to 10 minutes at higher concentration).

Instantaneous I-V curves were recorded in the absence and presence of agonist. The membrane potential was within 300 milliseconds, gradually changed from ~80 mV to +40 mV, and the corresponding current was recorded. The reversal potential was determined from the intersection of the two current curves.

Allosteric modulation was measured at a betaine and choline concentration of 12 μM and 0.3 mM, respectively. Betaine or choline was applied for 20 seconds alone or in combination with the allosteric modulator. Modulation of currents was expressed as [1/(I_modulator + agonist) / I_agonist + 1] × 100%. The modulatory compounds were prepared as a 10 mM stock solution in dimethyl sulfoxide (DMSO) and were dissolved in external solution, resulting in a maximal final DMSO concentration of 0.1%. The perfusion system was cleaned between drug applications by washing with DMSO to avoid contamination.

Results

Sequence Homology among MPTL-1, ACR-20, and ACR-23. Protein sequences from MPTL-1, ACR-20, and ACR-23 possess motifs typical for Cys-loop ligand-gated ion
channels, including four transmembrane domains, a Cys-loop, loops A to F, and the two adjacent cysteines in the C-loop, defining them as AChR \(\alpha\) type subunits. MPTL-1 is 564 amino acid residues long and shares 54% sequence identity with \(C.\) elegans ACR-23 and 67% with ACR-20. Those numbers increase to 64% sequence identity and 79% sequence similarity when only the ligand-binding domain and the first three transmembrane domains are aligned for ACR-23 and up to 75% sequence identity and 83% sequence for ACR-20.

**Functional Characterization of the MPTL-1 and ACR-20 Receptors.** *Xenopus* oocytes were injected with RNA coding for \(mptl-1\) receptor of *H. contortus* and \(acr-20\) of *C. elegans*. Oocytes were held at a membrane potential of \(-80\) mV and exposed to increasing concentrations of betaine or choline. This resulted for both receptors in inward currents characterized by a fast channel opening, followed by a slow desensitization at higher agonist concentration. The currents amounted to \(332 \pm 6181\) nA (\(n=5\)) and \(175 \pm 75\) nA (\(n=5\)) at a betaine or choline concentration of 1.2 mM and 20 mM, respectively, for MPTL-1. At the same concentration of agonist, the currents for ACR-20 amounted to \(328 \pm 215\) nA (\(n=4\)) and \(173 \pm 77\) nA (\(n=4\)). Figure 1, A and B, shows current traces from betaine- and choline-activated MPTL-1 receptors. Averaged concentration-response curve with betaine (closed circle) or choline (open circles) as agonist is shown in Fig. 1, C and D, for MPTL-1 and ACR-20, respectively. For MPTL-1, the betaine curve is characterized by an EC\(_{50}\) of \(41 \pm 7\) \(\mu\)M and a Hill coefficient of \(2.0 \pm 0.3\) (mean \pm S.D., \(n=4\)). The choline curve is characterized by an EC\(_{50}\) of \(1.3 \pm 0.2\) \(\mu\)M and a Hill coefficient of \(1.6 \pm 0.2\) (mean \pm S.D., \(n=4\)). The EC\(_{50}\) values obtained with ACR-20 for betaine and choline are very similar to the one of MPTL-1: the betaine curve is characterized by an EC\(_{50}\) of \(25 \pm 7\) \(\mu\)M and a Hill coefficient of \(1.3 \pm 0.1\) (mean \pm S.D., \(n=4\)). Betaine proved to be a much more potent agonist of the *H. contortus* MPTL-1 and *C. elegans* ACR-20 channels than choline with current saturation occurring at about 300 \(\mu\)M. Betaine (1 mM) elicited \(183 \pm 25\%\) (\(n=5\)) and \(207 \pm 50\%\) (\(n=4\)) of the current amplitude elicited by 40 mM choline for MPTL-1 and ACR-20, respectively. Acetylcholine (10 mM) elicited only \(2.4 \pm 1.5\%\) (\(n=4\)) of the current amplitude elicited by 10 mM choline on oocytes expressing the MPTL-1 channel (filled square, Fig. 1C). Coinjection of the \(mptl-1\) subunit with the ancillary protein \(ric-3\) did not significantly affect the measured currents (data not shown). Therefore, all the measurements were performed with \(mptl-1\) alone.

**Ion Selectivity of *H. contortus* MPTL-1 Channels.** We previously found that currents mediated by choline-activated *H. contortus* DEG-3/DES-2 channels (Rufener et al., 2010a),...
but not ACR-23 (Rufener et al., 2013) channels, permeated calcium (Ca\(^{2+}\)) in addition to monovalent cations. This was detected indirectly by activation of a Ca\(^{2+}\)-activated chloride channel endogenous to the oocyte, resulting in a shift of the reversal potential toward the chloride reversal potential. It was interesting to see whether MPTL-1 channels were permeable to Ca\(^{2+}\) ions. Instantaneous I-V curves were recorded in standard medium (1 mM CaCl\(_2\)), low calcium medium (standard medium without CaCl\(_2\), plus 0.1 mM K-EGTA), and high calcium medium (standard medium supplemented with additional 9 mM CaCl\(_2\)) in the presence or absence of 1 mM choline. An example of an I-V curve in standard medium is shown in Fig. 2. The reversal potential of the current elicited by choline was 2.0 ± 2.3 mV (n = 7) in standard medium, 1.8 ± 2.7 mV (n = 4) in low calcium medium, and −0.6 ± 3.2 mV (n = 4) in high calcium medium. The reversal potential for chloride ions was determined as −26.0 ± 1.0 mV (n = 4), monitoring current through recombinant \(\alpha_1\beta_2\gamma_2\) GABA\(_A\) receptor channels. The fact that the reversal potential does not significantly vary with the conditions indicates that the channel is not permeable to Ca\(^{2+}\) ions. The reversal potential in potassium medium, in which all Na\(^+\) was replaced by K\(^+\), was measured at 5.8 ± 2.5 mV (n = 4). Presumably, the channel is permeable to both sodium and potassium ions.

**Modulation of *H. contortus* MPTL-1 and *C. elegans* ACR-20 Channels by Monepantel.** To evaluate the effect of monepantel on MPTL-1 and ACR-20 receptors, the drug was tested as an agonist or as an allosteric modulator of betaine or choline. In the absence of agonist, monepantel showed strong agonistic effect on the MPTL-1 channel at concentrations higher than 0.1 \(\mu\)M. Figure 3A shows current traces of a concentration-response curve of monepantel. As long as the oocyte was exposed to monepantel, there was a steady increase in the current amplitude. The rate of increase was larger at higher concentrations of monepantel. After ending the exposure to the compound, the rising phase stopped and even after extensive washing the current amplitude did not recover to the initial membrane current. This indicates a very low dissociation constant and essentially irreversible binding of monepantel with MPTL-1. At concentrations >3 \(\mu\)M, monepantel elicited currents that were too large to be controlled with our amplifier. As no saturation could be reached, it was not possible to determine an EC\(_{50}\) value. A concentration of 3 \(\mu\)M monepantel behaved as a superagonist and elicited about 1.6- and 3-fold larger current amplitudes than maximal concentrations of betaine or choline in the same oocyte, respectively. We stopped experiments here, as the constant flow of large currents may have altered the intracellular ion concentrations.

We tested the effects of monepantel sulfone (AAD-4670) on the *H. contortus* MPTL-1 receptor as this compound is the major metabolite of monepantel in sheep (Karadzovska et al., 2009) that forms within hours of animal treatment, and has longer half-life than monepantel. It was also interesting to test the optical R-enantiomer of monepantel (AAD-2224), which has no nematocidal effect. Figure 3B shows the average current amplitudes measured with monepantel (filled circles),
with AAD-2224 (filled squares), or with AAD-4670 (filled diamonds). AAD-4670 opened the channel irreversibly (data not shown) to a similar extent as monepantel, and the AAD-2224 failed to elicit current alone at the concentrations used. Monepantel showed a similar, but smaller agonistic effect on ACR-20 channels (Fig. 3B, open circles, dashed line), whereas the AAD-2224 did not elicit any current at the tested concentrations (Fig. 3B, open squares, dashed line).

To investigate whether monepantel could act as a positive allosteric modulator of MPTL-1 channels, increasing concentrations of monepantel were applied using betaine as agonist (Fig. 4A). The measured current amplitudes were bigger than with the drug alone, and a modulatory effect was evident at nM concentrations of monepantel (+800% at 100 nM). At concentrations larger than 10 nM, an apparently irreversible reaction was observed, as the current traces did not revert to the baseline. Similar observations were made with choline as the agonist (data not shown). Averaged concentration-response curves for monepantel or with AAD-2224 using betaine or choline as agonist are shown in Fig. 4, B and C, respectively. The monepantel-mediated potentiation of the current was measured at a concentration of 12 µM betaine and 0.3 mM choline, respectively, which elicit only a small fraction of the maximal current amplitude in both cases. The sulfone form of monepantel was found to behave similar to its parent compound, when used in combination with betaine or choline (Fig. 4, B and C, filled diamonds). The inactive enantiomer AAD-2224 potentiated betaine and choline responses much less than monepantel (Fig. 4, B and C, filled squares). Similar experiments with ACR-20 using betaine as agonist were performed. As with MPTL-1, we observed an apparently irreversible reaction at higher concentration of monepantel (original traces not shown). These experiments are summarized in Fig. 4B.

Allosteric modulation was also determined for ACR-20 receptors with betaine as agonist. Extent of modulation by monepantel in oocytes expressing the ACR-20 receptors was about 5-fold smaller than in oocytes expressing MPTL-1 receptors (Fig. 4B, open circles, dashed line) and nearly zero with AAD-2224 (Fig. 4B, open squares, dashed line).

Oocytes injected with the *H. contortus* AAD-mutant mptl-1 sequence (exon 15 missing) failed to express any current at the tested concentrations of agonists or monepantel (data not shown).

**Discussion**

Parasitic infections by nematodes represent a serious threat to the health of humans, companion animals, and livestock. The intense use of the available anthelmintics has inevitably selected for resistant populations of parasites, with some being resistant to any treatment. Monepantel, a new anthelmintic recently made available for the treatment of gastrointestinal nematodes of sheep, may offer an attractive solution because it is able to control and kill such resistant strains. With the recent report of monepantel-resistant field isolates, there is an urgent need to better understand the mode of action of monepantel and how resistance in the field develops. Using knowledge of the monepantel-resistant field isolates, there is an urgent need to better understand the mode of action of monepantel and how resistance in the field develops. Use of knowledge of the monepantel-target pharmacological profile might allow us to prolong the life span of this new class of anthelmintic. In this respect, the target of monepantel in *H. contortus*, MPTL-1, and its closest homolog...
in *C. elegans*, ACR-20, were expressed in *Xenopus* oocytes, and their function was characterized. Previous attempts to reconstitute the MPTL-1 receptor were unsuccessful (Rufener et al., 2010a). In the present study, we have subcloned the open reading frame of MPTL-1 from pcDNA3.1 to the pT7-TS vector. This new vector allows the incorporation of the *X. laevis* β-globin untranslated DNA to the 5’ and 3’ end of the gene. This procedure was successfully able to generate a functional channel in *Xenopus* oocytes. One possible explanation could be that the 5’- and 3’-untranslated region of the β-globin gene enhances the translation of MPTL-1 or stabilizes the cRNA molecules (less degradation and/or less secondary structure).

The *C. elegans* ACR-23 and the *H. contortus* DES-2/DEG-3 receptors have recently been characterized (Rufener et al., 2010a, 2013), and we compare in the following discussion their functional properties with those of MPTL-1 and ACR-20, determined in this work. As ACR-23, both channels were preferentially gated by betaine compared with choline or acetylcholine. The EC₅₀ for betaine on MPTL-1 or ACR-20 receptors both were about 30 times lower compared with the EC₅₀ measured for choline chloride. Interestingly, no saturation up to 60 mM choline chloride was obtained with oocytes expressing the ACR-23 receptors, whereas the response of MPTL-1 or ACR-20 to choline showed saturation and was characterized by an EC₅₀ of about 1.3 mM and a current saturation about 10 mM. In contrast to the DES-2/DEG-3 channels, MPTL-1 receptors did not conduct calcium ions and did not differentiate between sodium and potassium ions.

Monepantel acted as a superagonist on oocytes expressing MPTL-1 or ACR-20 and elicited much larger currents than saturating concentrations of choline chloride or betaine. This is also true for ACR-23 (Peden et al., 2013; Rufener et al., 2013). In this case, monepantel acts as a type I allosteric modulator affecting maximal current rather than channel desensitization. The basis for this effect is likely to be a modification of the channel kinetics in the presence of monepantel. Analysis of single channel currents induced by neonicotinoid (Brown et al., 2006) or GABA_A (Mortensen et al., 2010) superagonists found increased currents were due to an increased frequency of channel opening, but increased open times could also produce the same effect.

The threshold for direct activation by monepantel for MPTL-1, ACR-20, and ACR-23 channels was at 0.1–0.3 μM. Although channel opening in ACR-23 was fully reversible after removal of monepantel, MPTL-1 and ACR-20 channels stayed open once opening had been induced. Repeated application of monepantel led to a further increase of the current amplitude. This observation suggests an exceedingly low dissociation constant of monepantel with MPTL-1. AAD-2224, the inactive enantiomer of monepantel, did not induce any currents by itself at concentrations up to 10 μM in both types of channel. Contrasting with these results, monepantel alone was not able to open the nicotinic AChR formed by the *H. contortus* DEG-3 and DES-2 subunits.

For MPTL-1 and ACR-23 channels, the response to betaine and/or choline was strongly allosterically potentiated by low concentrations of monepantel. When coapplied with betaine, the threshold for potentiation on ACR-23 channels was described at concentrations as low as 0.3 nM (Peden et al., 2013) and was determined to be 1 nM in this study on oocytes expressing MPTL-1 receptors. The difference in sensitivity between both studies is due to the fact that Peden et al. (2013) have combined monepantel with a saturating concentration of betaine (1 mM) compared with the lower concentration of 12 μM used in the present study. When we used 1 mM betaine, the threshold was 0.3 nM as well (data not shown).

For ACR-20 channels, an allosteric effect was observed at low monepantel concentrations as well (10 nM), but the extent of stimulation was much smaller. Current potentiation reached 800% with MPTL-1 channels at 100 nM monepantel and 12 μM betaine, whereas it was below 200% for ACR-20. The lower stimulation of ACR-20 by monepantel compared with ACR-23 or MPTL-1 receptors could explain why acr-23 null mutant is not sensitive to the compound while still expressing acr-20. When choline was coapplied with monepantel, the threshold of potentiation differed markedly between the different receptors. Whereas it was about 100 nM in ACR-23 channels (Rufener et al., 2013), MPTL-1 channels were about 100 times more sensitive, a difference of sensitivity toward monepantel that is also observed in vivo on worm viability: the calculated LC₅₀ for *C. elegans* is 100 nM (Rufener et al., 2010b) and 7.5 nM (data not shown) for *H. contortus*. This difference can be explained by the fact that monepantel has been optimized based on *H. contortus* efficacy in vitro and in vivo and not on *C. elegans*. Another important observation is that the concentrations of monepantel needed to obtain the allosteric modulation fit much better with the in vivo toxicity data than those required to obtain the agonistic effect. In situ, betaine will always be present at the receptor site, allowing the allosteric modulation to take place. Monepantel has also been shown to act as a positive allosteric modulator, enhancing the response to choline of the DES-2/DEG-3 receptors, but the potentiation was weaker compared with MPTL-1 or ACR-23 channel. Furthermore, the monepantel sulfone metabolite that produced equivalent effect to monepantel on the other receptors potentiated the DES-2/DEG-3 current five times more efficaciously (Rufener et al., 2010a).

Interestingly, a similar behavior between glutamate and ivermectin has been reported on glutamate-gated chloride channel receptors (Cully et al., 1994; Brownlee et al., 1997; Holden-Dye and Walker, 2006). At high concentrations, ivermectin acts as an almost irreversible, long-acting agonist of glutamate-gated chloride. However, concentrations of ivermectin that are too low to directly activate the channels would nonetheless potentiate the effects of simultaneously applied submaximal concentrations of glutamate (Cully et al., 1994; Forrester et al., 2003, 2004). It is known that glutamate and ivermectin do not compete for the same binding site and, hence, have different binding sites on the receptor (Hejmadi et al., 2000). The two sites exert complementary, and possibly additive, effects on the conformational changes needed for the channels to open. Even though this remains to be experimentally proven, a comparable complementarity between monepantel and betaine or choline is highly probable.

Whereas AAD-2224 failed to allosterically modulate ACR-23 and ACR-20 channels, it did so in MPTL-1 channels, although about 10- to 20-fold times weaker than monepantel. Both enantiomers are separated using a column fractionation strategy that provides approximately >98% purity. As a consequence, this effect may be, at least partially, due to traces of monepantel in the sample. Because the ACR-23 and ACR-20 channels are less sensitive to monepantel than...
MPTL-1, this effect was not observed with this receptor at the tested concentrations.

Oocytes injected with a mptl-1 sequence lacking the exon 15 identified in laboratory-selected monepantel-mutant H. contortus isolates did not lead to any functional channels. We can conclude that this monepantel-mutant isolate tolerates the drug because it does not produce any functional MPTL-1 channel, the primary drug target of monepantel.

We have shown that MPTL-1 receptors from the parasitic nematode H. contortus are irreversibly opened by concentrations of monepantel $>100$ nM, and that concentrations $<1$ nM strongly potentiate betaine or choline currents. We have also demonstrated on one hand that ACR-20 receptors, which share a higher homology to MPTL-1 than to ACR-23. Our results reinforce the implication of the DEG-3 subfamily channels and especially the MPTL-1 subunit in sensitivity to monepantel in the parasitic nematode H. contortus. This work enhances our understanding of monepantel mode of action in parasitic nematodes and additionally supports the development of genetic markers for the early detection of resistant genotypes in the field.

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Authorship Contributions

**Participated in research design:** Rufener, Baur, Sigel, Beech.  
**Conducted experiments:** Rufener, Baur.  
**Performed data analysis:** Rufener, Baur, Sigel.  
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