Monepantel irreversibly binds to and opens *H. contortus* MPTL-1 and *C. elegans* ACR-20 receptors

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Two channels irreversibly opened by monepantel

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NONSTANDARD ABBREVIATIONS
AAD, amino-acetonitrile derivatives; AChR, acetylcholine receptors; GluCl, glutamate-gated chloride; LBD, ligand-binding domain; TMD, transmembrane domains.
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, 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 Ca$^{2+}$. Concentrations of monepantel (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 (Waller, 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 (Waller, 2006). Fresh hope came with the launch of monepantel in 2008, a member of the Amino-Acetonitrile Derivatives (AAD) classes of compounds.

Trichostrongylid nematodes, such as *H. 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 biological 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 non-parasitic species *C. 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 (AChR). ACR-23 has been localized in the body wall muscle (Rufener et al., 2013) and in mechanosensory neurons (Peden et al., 2013). Peden et al. have recently shown that ACR-23 is activated by betaine and functions to maintain basal levels of locomotion (Peden et al., 2013). Betaine, a ubiquitous non-canonical amino acid, serves as an organic osmolyte to
protect cells against osmotic stress, but 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.

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 (Treinin et al., 1998; Yassin et al., 2001). It may be that the ACR-20 receptors is less, or insensitive to monepantel or it may also play a physiological role where 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 here 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 Haemonchus contortus and Caenorhabditis elegans

RNA extraction, cDNA synthesis, 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 of total RNA (DNase-treated) was reverse-transcribed to cDNA using a (dT)30 primer and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). The gene-specific PCRs were performed using a Phusion polymerase (NEB). The following primers were used to amplify the full-length coding sequence of mptl-1: NheI_Hc-mptl-1_FL_F1 (ggcggctagccaaatggaaaatcctagctctgg) and XhoI_Hc-mptl-1_stop_R1 (ggcgctcgagtgactagagagggcgatcttg). For acr-20, the primers were: BglII_Ce-acr-20_FL_F1 (ggcgctcgagtgactagagagggcgatcttg). For acr-20, the primers were: BglII_Ce-acr-20_FL_F1 (ggcgctcgagtgactagagagggcgatcttg). For acr-20, the primers were: BglII_Ce-acr-20_FL_F1 (ggcgctcgagtgactagagagggcgatcttg). The gene-specific primers were designed using the Primer3 software (available at http://frodo.wi.mit.edu/). The amplicons were analyzed on 1% agarose gels, excised, gel-purified using a NucleoSpin kit (Macherey Nagel), and cloned into pJET1.2 (Fermentas). Plasmid DNA was purified using the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA) and sequenced using the provided pJET1.2 forward and reverse primers at Génome Québec (Montreal, Canada). At least three clones of each construct were analyzed and compared to the sequences published on NCBI (www.ncbi.nlm.nih.gov/). The same procedure was used to obtain the mptl-1 sequence with a 127 bp deletion (removing the entire exon 15) from the H. contortus AAD-mutant nematodes. The Hco-ric-3 gene was amplified using the same primers as described in Rufener et al. 2009 (Rufener et al., 2009). The selected inserts were subcloned into a pT7-TS transcription vector (that introduces X. laevis β-globin untranslated DNA to the 5' and 3' end of the gene) via the restriction sites inserted in the primers. Plasmid DNA was purified with an EndoFree Plasmid Purification kit (Qiagen).
Expression of mptl-1 and acr-20 receptors in Xenopus oocytes

Capped cRNAs were synthesized (T7 mMessage mMACHINE kit, Ambion, Austin, TX) from the linearized vectors containing the different subunits. The concentration of the cRNA was quantified on a formaldehyde gel using Radiant Red stain (Bio-Rad) for visualization of the RNA. Known concentrations of RNA ladder (Invitrogen) were loaded as standard on the same gel. cRNAs were precipitated in ethanol/isoamylalcohol 19:1, the dried pellet dissolved in water and stored at -80°C. cRNA mixtures were prepared from these stock solutions and stored at -80°C. Xenopus laevis oocytes were prepared, injected and defolliculated as described previously (Sigel, 1987; Sigel and Minier, 2005). They were injected with 10 and 15 ng of the cRNA coding for mptl-1 and acr-20, respectively, followed by incubation in modified Barth’s solution at +18°C for at least 24 h before the measurements.

Functional characterization

Currents were measured using a modified two-electrode voltage clamp amplifier Oocyte clamp OC-725 (Warner Instruments) in combination with a XY-recorder (90% response time 0.1s) or digitized at 100 Hz using a PowerLab 2/20 (AD Instruments) using the computer programs Chart (ADInstruments GmbH, Spechbach, Germany). Tests with a model oocyte were performed to ensure linearity in the larger 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 I(c) = I_{\text{max}}/(1+(EC_{50}/c)^n), where c is the concentration of agonist, EC_{50} the concentration of agonist eliciting half maximal current amplitude, I_{\text{max}} is the maximal current amplitude, I the current amplitude and n the Hill coefficient. In experiments where 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 min at low concentration, up to 10 min at higher concentration).

Instantaneous I-V curves were recorded in the absence and presence of agonist. The membrane potential was within 300 ms gradually changed from -80 mV to +40 mV and the corresponding current 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 s alone or in combination with the allosteric compound. Modulation of currents was expressed as (I_{\text{(modulator + agonist)}} / I_{\text{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 between 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 4 transmembrane domains, a Cys-loop, loops A to F and the 2 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 (LBD) and the first three transmembrane domains (TMD) 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 ± 181 nA (n = 5) and 175 ± 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 ± 215 nA (n = 4) and 173 ± 77 nA (n = 4). Figures 1A and 1B show current traces from a betaine and choline concentration response curves, respectively, obtained from a *Xenopus* oocyte expressing MPTL-1 receptors. Averaged concentration-response curve with betaine (closed circle) or choline (open circles) as agonist are shown in Figure 1C and 1D for MPTL-1 and ACR-20, respectively. For MPTL-1, the betaine curve is characterized by an EC\textsubscript{50} of 41 ± 7 μM and a Hill coefficient of 2.0 ± 0.3 (mean ± SD, n = 4). The choline curve is characterized by an EC\textsubscript{50} of 1.3 ± 0.2 mM and a Hill coefficient of 1.6 ± 0.2 (mean ± SD, n = 4). The EC\textsubscript{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 ± 7 μM and a Hill coefficient of 1.3 ± 0.1 (mean ± SD, n = 4). The choline curve is characterized by an EC$_{50}$ of 1.2 ± 0.3 mM and a Hill coefficient of 1.7 ± 0.1 (mean ± SD, 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 μM. Betaine (1 mM) elicited 183 ± 25 % (n=5) and 207 ± 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 ± 1.5 % (n=4) of the current amplitude elicited by 10 mM choline on oocytes expressing the MPTL-1 channel (filled square, Fig. 2B). Co-injection 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 channels endogenous to the oocyte, resulting in a shift of the reversal potential towards the chloride reversal potential. It was interesting to see if 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 curves in standard medium are shown in Figure 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 α1β2γ2 GABA<sub>A</sub> receptor channels. The fact that the reversal potential does not significantly vary with the conditions indicates that the channel is not permeable to Ca<sup>2+</sup>-ions. The reversal potential in potassium medium, in which all Na<sup>+</sup> was replaced by K<sup>+</sup>, 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 μM. 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 μM 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<sub>50</sub> value. A concentration of 3 μM monepantel behaved as a superagonist and elicited about 1.6-fold 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. The 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 (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) while the AAD-2224 did not elicit any current at the tested concentrations (Fig. 3B, open squares, dashed line).

To investigate if 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 currents 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 (not shown). Averaged concentration response curves for monepantel or with AAD-2224 using betaine or choline as agonist are shown in Figures 4B 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. 4B and C, filled diamonds). The inactive enantiomer AAD-2224 potentiated betaine and choline responses much less than monepantel (Fig. 4B 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 Figure 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 anthelminitics 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 since 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-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 homologue in *C. elegans*, ACR-20, were expressed in *Xenopus* oocytes and their function 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 enhance the translation of MPTL-1 or stabilize 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; Rufener et al., 2013) and we compare in the following discussion their functional properties to those of MPTL-1 and ACR-20, determined here. As ACR-23, both channels were preferentially gated by betaine compared to choline or acetylcholine. The EC_{50} for betaine on MPTL-1 or ACR-20 receptors both were about 30 times lower compared to the EC_{50} 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 EC$_{50}$ of about 1.3 mM and a current saturation around 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. While 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 those results, monepantel alone was not able to open the nicotinic acetylcholine receptor 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. have combined monepantel with a saturating concentration of betaine (1 mM) compared to 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 to ACR-23 or MPTL-1 receptors could explain why acr-23 null mutant are 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. While 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 towards 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 fits 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 to MPTL-1 or ACR-23 channel. Furthermore, the monepantel sulfone metabolite which produced equivalent effect to monepantel on the other
receptors, potentiated the DES-2/DEG-3 current 5 times more efficaciously (Rufener et al., 2010a).

Interestingly, a similar behaviour between glutamate and ivermectin has been reported on glutamate-gated chloride (GluCl) channel receptors (Brownlee et al., 1997; Cully et al., 1994; Holden-Dye and Walker, 2006). At high concentrations, ivermectin acts as an almost irreversible, long-acting agonist of GluCl. But concentrations of ivermectin that are too low to directly activate the channels would nonetheless potentiate the effects of simultaneously applied sub-maximal concentrations of glutamate (Cully et al., 1994; Forrester et al., 2004; Forrester et al., 2003). 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 experimentaly 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 which provides approximately > 98% purity. As a consequence, this effect may be, at least partially, due to traces of monepantel in the sample. Since 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 an mptl-1 sequence lacking the exon 15 identified in a 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, could also be opened in an irreversible manner by monepantel. Furthermore, we have shown that the allosteric modulation of ACR-20 receptors was weaker than for MPTL-1 or 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

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Conducted experiments: Rufener and Baur.

Performed data analysis: Rufener, Baur, and Sigel.

Wrote or contributed to the writing of the manuscript: Rufener, Sigel, and Beech.
REFERENCES


FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Betaine concentration dependence of MPTL-1 and ACR-20 channels. A, B) Current traces from betaine (A) and choline (B) concentration response curve obtained from a Xenopus oocyte expressing MPTL-1 receptors. Oocytes were voltage-clamped at -80 mV. The bars indicate the time period of betaine or choline perfusion, respectively. Agonist concentrations are indicated above the bars. C) Averaged concentration response curves for betaine (closed circle) and choline (open circles) for MPTL-1 receptors. Individual curves were standardized to the fitted maximal current amplitude and subsequently averaged. Mean ± SD of experiments carried out with 4 oocytes from two batches each is shown. The filled square shows the average current amplitude elicited in the same oocytes by 10 mM acetylcholine. D) Averaged concentration response curves for betaine (closed circle) and choline (open circles) for ACR-20 receptors. Individual curves were standardized to the fitted maximal current amplitude and subsequently averaged. Mean ± SD of experiments carried out with 4 oocytes from two batches each is shown.

Fig. 2 I-V curves. In a Xenopus oocyte expressing MPTL-1 the membrane potential was gradually increased from -80 mV to +40 mV and the corresponding currents were recorded. The experiment was performed in the absence and presence of 1 mM choline.

Fig. 3. Monepantel is a direct agonist of MPTL-1 and ACR-20 channels. A) Current traces obtained from a Xenopus oocyte expressing MPTL-1 receptors exposed to increasing concentrations of monepantel. The bars indicate the time period of monepantel perfusion. Monepantel concentrations are indicated above the bars. B) Averaged relative current amplitudes (I_max with betaine = 100%) measured with monepantel (filled circles), with AAD-2224 (filled squares), or with AAD-4670 (filled diamonds) for MPTL-1 receptors and with
monepantel (open circles, dotted line) and with AAD-2224 (open squares, dotted line) for ACR-20 receptors. Mean ± SD of experiments carried out with 4 oocytes from two batches is shown.

**Fig. 4. Allosteric modulation of MPTL-1 and ACR-20 channels.** A) Current traces obtained from a *Xenopus* oocyte expressing MPTL-1 receptors exposed to 12 μM betaine alone or in combination with increasing concentrations of monepantel. The bars indicate the time period of betaine and monepantel perfusion. Monepantel concentrations are indicated above the bars. B, C) 12 μM betaine (B) or 0.3 mM choline (C) were applied alone or in combination with increasing concentrations of monepantel (filled circles for MPTL-1; open circles, dotted line for ACR-20), AAD-2224 (filled squares for MPTL-1; open squares, dotted line for ACR-20), or AAD-6470 (filled diamonds for MPTL-1). Current amplitudes were standardized to the current elicited by betaine or choline alone. Mean ± SEM of experiments carried out with 4-5 oocytes from two batches is shown.
Figure 1

(A) Current traces with different concentrations of betaine or choline chloride. The concentrations range from 5 μM to 160 μM.

(B) Current traces with different concentrations of betaine or choline chloride. The concentrations range from 0.16 mM to 2.5 mM.

(C) Graph showing the current amplitude (%) as a function of betaine or choline chloride concentration (M). The graph includes data points for different concentrations.

(D) Graph showing the current amplitude (%) as a function of betaine or choline chloride concentration (M). The graph includes data points for different concentrations.
Figure 2

- choline

+ choline

-80 -60 -40 -20 0 20 40 mV

500 nA
Figure 4

A

B

C