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Selective Toxicity of the Anthelmintic Emodepside Revealed by Heterologous Expression of Human KCNMA1 in *Caenorhabditis elegans*

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>'EPG', electropharyngeogram

>'emo', emodepside

**Abstract**

Emodepside is a resistance-breaking anthelmintic of a new chemical class, the cyclooctadepsipeptides. A major determinant of its anthelmintic effect is the calcium-activated potassium channel, SLO-1. SLO-1 belongs to a family of channels that are highly conserved across the animal phyla and regulate neurosecretion, hormone release, muscle contraction and neuronal network excitability. To investigate the selective toxicity of emodepside we performed transgenic experiments in which the nematode SLO-1 channel was swapped for a mammalian orthologue, human KCNMA1. Expression of either the human channel or *C. elegans slo-1* from the native *slo-1* promoter in a *C. elegans slo-1* functional null rescued behavioural deficits that otherwise resulted from loss of *slo-1* signalling. However, worms expressing the human channel were 10 to 100-fold less sensitive to emodepside than those expressing the nematode channel. Strains expressing the human KCNMA1 channel were preferentially sensitive to the mammalian channel agonists NS1619 and rottlerin. In the *C. elegans* pharyngeal nervous system *slo-1* is expressed in neurones not muscle and cell specific rescue experiments have previously shown that emodepside inhibits serotonin-stimulated feeding by interfering with SLO-1 signalling in the nervous system. Here we show that ectopic over-expression of *slo-1* in pharyngeal muscle confers sensitivity of the muscle to emodepside, consistent with a direct interaction of emodepside with the channel. Taken together these data predict an emodepside selective pharmacophore harboured by SLO-1. This has implications for the development of this drug/target interface for the treatment of helminth infections.

**Introduction**

Parasitic worms place a huge economic and health burden on society by causing disease in humans, livestock and pets. They are controlled by drugs, anthelmintics, but these are losing their effectiveness due to the emergence of drug-resistant strains of worm (Gilleard, 2006). Emodepside is a new anthelmintic drug (Figure 1) which paralyses parasitic nematode worms, including those that have developed anthelmintic-resistance (von Samson-Himmelstjerna et al., 2005). It has a broad spectrum of action against gastrointestinal nematodes including important parasites of livestock (Harder and von Samson-Himmelstjerna, 2002) and a filarial infection of humans, *Onchocerca volvulus* (Townson et al., 2005).

Studies to elucidate the mode of action of emodepside have been conducted on the parasitic nematode *Ascaris suum* and in the free-living model genetic nematode *C. elegans*. Taken together, these studies indicate that emodepside acts to inhibit neuromuscular transmission in nematodes and thus impair the vital functions of motility, feeding and reproduction (Bull et al., 2007; Willson et al., 2004; Willson et al., 2003). Mutagenesis screening for *C. elegans* resistant to the inhibitory effects of emodepside on locomotion identified SLO-1, a calcium and voltage-activated potassium channel as the major determinant of emodepside sensitivity (Guest et al., 2007; Holden-Dye et al., 2007). This is consistent with earlier *in vitro* electrophysiological experiments on *Ascaris* muscle which demonstrated a calcium and potassium dependent hyperpolarisation (Willson et al., 2003). Furthermore, in two independent genetic screens of 20,000 genomes for emodepside resistance, only loss or reduction, of function alleles of *slo-1* were recovered. As *slo-1* gain of function *C. elegans* mutants exhibit an inhibition of motility and egg-laying similar to

that of emodepside treated worms (Guest et al., 2007; Holden-Dye et al., 2007), a parsimonious explanation of emodeside's anthelmintic action is that it activates a SLO-1 dependent pathway to bring about neuromuscular inhibition and paralysis of the pathways that regulate feeding, locomotion and egg-laying in the worm.

SLO-1 belongs to a family of calcium-activated potassium channels that are highly conserved throughout the animal phyla playing key physiological roles in the regulation of muscle and neuronal excitability, in hormonal secretion and neurotransmitter release, (for review see Salkoff et al., 2006). For example, in nematodes SLO-1 regulates neurotransmitter release (Wang et al., 2001) whilst in humans there is evidence linking mutations in calcium-activated potassium channels to seizures (Du et al., 2005), Nonetheless, emodepside is well tolerated by the mammalian hosts in which it has been tested to date (Harder et al., 2003) suggesting that it may achieve its selective toxicity through pharmacological differences between the channel in the nematode and its mammalian host.

In this study we have deployed a *C. elegans slo-1* null mutant, *js379*, in order to either ectopically or heterologously express the wild-type *C. elegans* channel SLO-1 or a close mammalian orthologue, human *kcnma1*, in a genetic background devoid of native SLO-1 channel function. Ectopic over-expression of wild-type *slo-1* in the pharyngeal muscle of *C. elegans*, a tissue which does not express the native channel (Chiang et al., 2006; Wang et al., 2001), conferred sensitivity to emodepside consistent with a role for SLO-1 as an emodepside receptor. Furthermore, whilst expression of human *kcnma1* from the native *slo-1* promoter provided full rescue of the distinct and quantifiable behavioural phenotypes of *slo-1 js379* (strain NM1968;

Wang et al., 2001) it did not confer sensitivity to emodepside. Instead these strains exhibited responses to mammalian BK channel agonists (Figure 1). Thus we conclude that human KCNMA1 can functionally substitute for the nematode channel SLO-1 *in vivo* and that the two channels exhibit distinct pharmacological properties. With regard to the selective toxicity of emodepside and the potential of the new class of cyclooctodepsipeptides in tropical medicine for filariasis (Geary et al., 2010), the *C. elegans* channel is 10 to 100-fold more sensitive to emodepside than the human channel.

**Materials and Methods**

*Culturing of Caenorhabditis elegans*

*C. elegans* were grown on Nematode Growth Medium (NGM) plates (Brenner, 1974) seeded with *Escherichia coli* (*OP50* strain) at 20ºC. N2 (Bristol strain) *C. elegans* were employed as wild-type. NM1968 is a strain carrying a predicted functional null mutation, *js379*, for *slo-1* (Wang et al., 2001) and this was employed in these studies as a strain resistant to the effects of emodepside on locomotion (Guest et al., 2007). Transgenic *C. elegans* (described below) were always assayed in parallel with positive and negative controls for the emodepside sensitivity assays i.e. on the same day with N2 and *slo-1 (js379) C. elegans,* respectively.

*Sequence Analysis*

SLO-1 is encoded by a single gene on chromosome V in *C. elegans* and its human orthologue KCNMA1 is on chromosome X in the human genome. Multiple splice variants are generated by alternative splicing and post-transcriptional mechanisms (Salkoff et al., 2006). The primary sequence of *C. elegans* SLO-1 has greater than

50% amino acid identity with the mammalian channels (Butler et al., 1993; McCobb et al., 1995; Salkoff et al., 2006) and 66% identity with *Drosophila* (Adelman et al., 1992). To identify and categorize mammalian orthologues of SLO-1a (NP_001024259), and specifically to test the relationship with KCNMA1 (NP_002238), we constructed a molecular phylogeny (see Supplementary Information S2 for details).

To further compare the sequence of SLO-1a (NP_001024259) and KCNMA1 (NP_002238), we performed Basic Local Alignment (NCBI BLAST) of translated protein sequences of the channels and their individual regions. We used Specialised BLAST (NCBI) program (Johnson et al., 2008) to align two protein sequences. Algorithm parameters were set to automatic and the method used was "Compositional matrix adjust".

*Molecular biology*

A number of different splice variants of *slo-1* exist in *C. elegans* [www.wormbase.org](www.wormbase.org) and, of these, *slo-1a* is the longest variant. pBK3.1 and pBK4.1, vectors for the neuronal and body wall muscle expression of *slo-1a*, respectively, were initially provided by Lawrence Salkoff (Wang et al., 2001). The *snb-1* promoter in pBK3.1 was replaced with the promoter for *slo-1* which was amplified from *C. elegans* gDNA and cloned in front of *slo-1a*. (Primers to amplify the putative *C. elegans slo-1* promoter region were designed based on the sequence of YAC clone Y51A2D; GenBank Acc. No. AL021497. The amplified putative promoter sequence was 3084bp upstream of the start site. An analysis of the sequence indicated no recognisable promoter elements or transcription factor binding sites). This construct

gave apparently full rescue of the behavioural phenotype of the *slo-1* null mutant *js379*. The open reading frame (ORF) of *kcnma1* was amplified by polymerase chain reaction (PCR) using proof-reading polymerase PfuUltra (Invitrogen) from pCMV6-XL4 vector (OriGene Technologies, SC122094). 3' and 5' primers contained BamHI and XbaI recognition sites respectively. PCR products were separated on a 0.8% Agarose gel and the band of 3.5 kb, corresponding to *kcnma1* cDNA (ORF), was purified using QIAGEN Gel Purification kit. *Kcnma1* ORF cDNA was then ligated into pCRII-Blunt-TOPO vector (Invitrogen TOPO cloning kit) and further sub-ligated into pBK3.1, pBK4.1 and ce*pslo1*::pBK3.1 vectors.  The ORF of *kcnma1* and sites of ligation were sequenced in 3' to 5' direction using MWG Value Read sequencing service. ORF of *kcnma1* was cut from pCRII-Blunt-TOPO vector using XbaI and BamHI enzymes.  pBK3.1, pBK4.1 vectors were digested with BamHI and XbaI. *pslo1* promoter has two recognition sites for XbaI. *pslo1*::pBK3.1 vector was digested with BamHI BamHI was then inactivated by heating the digest mixture for 20 minutes at 65º C, followed by partial digest of the vector with XbaI. All plasmid DNA samples were verified for authenticity by sequencing the newly generated portions of cDNA (Eurofins MWG Operon, London, UK). A construct to drive expression of *slo-1a* in the pharyngeal muscle was constructed by ligating the *slo-1a* sequence from pBK3.1 downstream of the *myo-2* promoter sequence in plasmid pPD30.69 (a gift from Andrew Fire, Okkema et al., 1993).

*Transforming slo-1(js379) C. elegans with slo-1a and kcnma1 genes.*

*slo-1(js379) C. elegans* were injected with plasmids to drive expression of either *slo-1a* or *kcnma1* from a pan neuronal promoter (*psnb-1*) (Nonet et al., 1998; Okkema et al., 1993), a body wall muscle promoter (*pmyo-3*) (Okkema et al., 1993), a

pharyngeal muscle promoter (*pmyo-2*) (Okkema et al., 1993) or from the native *slo-1*

promoter (*pslo-1*). The plasmids were injected at 30ng µl$^{-1}$. Transformed worms were

identified by co-injecting pPD118.33 plasmid (50ng µl$^{-1}$), which drives expression of

green fluorescent protein (GFP) from the pharyngeal muscle promoter, *pmyo-2*. The

co-injected *gfp* transformation marker forms an extra-chromosomal array with the

plasmids carrying the calcium-activated potassium channel sequences and thus

worms with fluorescent green pharynxes can be identified as carrying the plasmid of

interest.  For all the experiments, at least two independently transformed lines of

transgenic *C. elegans* expressing *slo-1* or *kcnma1* behind the specified promoter

were assayed. Results between the independent lines for each construct were in

good agreement and the data presented are the pooled data from these independent

lines. Expression of the transgenes was also confirmed by reverse transcription-PCR

(RT-PCR; Supplementary Information S1).

*Locomotion assays of C. elegans on emodepside, 24 hours exposure*

NGM plates were modified with vehicle (0.5% ethanol) or emodepside as described

previously (Bull et al., 2007). Emodepside modified plates contained drug in

concentrations of 10nM, 100nM, 1 µM, and 10 µM.  The maximum calculated

concentration of ethanol is 86 mM, a concentration that does not exhibit inhibitory

effects on the locomotion of *C. elegans* (Mitchell et al., 2007). Vehicle controls were

performed for all experiments.

Experiments were performed on age synchronised worms. Larval stage 4 (L4) *C.

elegans* were grown on NGM plates modified with emodepside or vehicle for 24 h

prior to the assay. Each L4+1day worm was moved to an NGM plate (without *E. coli*)

for 30 sec to remove any adhering bacteria, followed by the transfer to a fresh NGM

plate, also without *E. coli*. After 1 minute, locomotion of *C. elegans* was quantified by

counting body bends the worm generated in 1 min. A body bend was specified as a

movement of the worm where the tip of the head or the tail of the animal makes one

full sinusoidal wave. Only completed waves were counted as one body bend. At 1

and 10 μM emodepside, some wild-type and transgenic *C. elegans* were moving

forward by protruding their static anterior via frequent shallow waves of low amplitude

generated by the rest of the body. In this case, one body bend was counted when the

tip of the head moved forward the same distance as it does in one body bend in

control animals.

*Locomotion assays of C. elegans on emodepside- and NS1619- containing plates, 3*

*hours exposure*

NS1619 is a light sensitive compound and stability was therefore an issue for long-

term exposure. Therefore an alternative approach for drug treatment was adopted in

which a 200μl dose of vehicle or drug (emodepside or NS1619) was applied to the

*OP50 E. coli* lawn on the NGM plate, and left to dry for 30 min. Parallel experiments

were conducted using emodepside with the same protocol. L4+1day *C. elegans* were

exposed to the vehicle or drug in the food source for 3 hours. Body bends were then

counted as described above.

*C. elegans reversals assay*

Wild type *C. elegans* initiate foraging behaviour in the absence of food, characterised

by forward locomotion and spontaneous reversals (Chiba and Rankin, 1990) *slo-1*

*(js379) C. elegans* have a higher frequency of reversals than wild-type (Guest et al.,

2007; Wang et al., 2001). Age synchronised (L4 plus one day old) wild-type, *slo-1 (js379)* and transgenic *C. elegans* described above were assayed on 9 cm NGM plates with no food. Each worm was first transferred to a no food plate for about 30 sec to remove any adhering bacteria and then moved to a no food plate. Following 5 min of acclimatisation on the no food plate, the number of reversals was counted for 3 min. A "reversal" was identified as a movement of *C. elegans* in which its trajectory changes from forward to reverse and in which the tip of the tail traces at least one wave of sinusoidal shape. The tail does not always return to the same position in a wave. Ten worms were assayed per plate. Each transgenic strain was assayed in parallel with *N2* and *slo-1 (js379)* as internal controls.

*C. elegans pharyngeal assays*

Well fed age synchronised  (L4 plus one day old) *C. elegans* were transferred to a 3cm petri dish containing modified Dent's Saline (in mM: 10 D-Glucose, 140 NaCl, 1 $MgCl_2$, 3 $CaCl_2$, 6 KCl, 10 HEPES; pH 7.4) supplemented with 0.01% BSA w/v. A transverse cut was made immediately posterior of the terminal bulb of the pharynx using a razor blade. The pharyngeal preparation was transferred to the recording chamber, volume approximately 1ml. EPG recordings were made using previously described methods (Dillon et al., 2009). Recordings were made in the presence of perfusion at a constant rate of 5 ml $min^{-1}$. Data were acquired using Axoscope (Axon Instruments) and recorded with a sampling rate of 2kHz. Recordings were made within 5 min of removal of the worm from the food plate and typically lasted not longer than 20 min. Each single pharyngeal  feeding cycle, or 'pump', consists of a contraction and relaxation of the radial pharyngeal muscle which is recorded as an EPG wave-form (Cook et al., 2006). EPG recordings were analysed for pump

frequency and for the pattern of pumping i.e. whether or not pumping occurred at a constant rate or consisted of bursts of activity in which pumps occurred in groups. For the latter analysis, 'pump groups' refers to the organization of individual pumps into clusters or groups. An individual pump was defined as belonging to a group if it occurred within 0.2 sec from the previous pump. Basal pumping of N2 wild-type *C. elegans* typically includes pump groups of 1, 2 or 3. In contrast, *slo-1(js379)* mutants pump mainly in groups of higher than 3, displaying "bursting" pumping (Dillon et al., 2009). To evaluate rescue of this *slo-1(js379)* pharyngeal phenotype transgenic *C. elegans* expressing either *slo-1* or *kcnma1* behind the native *pslo-1* promoter were analysed for the behaviour of pumping in groups using AutoEPG software (Dillon et al., 2009). For this, pumping in groups was analysed during the first 10 min of 20 min recordings.

For experiments involving application of emodepside the following protocol was adopted in order to permit a quantification of the inhibitory effect of emodepside on pharyngeal pumping. It is based on establishing a reproducible stimulatory response to 5-HT (Rogers et al., 2001) against which emodepside inhibition can be measured. The first 2.5 min were recorded perfusing with Dent's saline followed by 1.5 min in 5-HT, 2 min in Dent's saline, followed by 1.5 min 5-HT, followed by 5 min in either Dent's saline (control) or emodepside, followed by 1.5 min 5-HT, and finally 5 min in Dent's saline (wash step). In the absence of any emodepside the pharyngeal preparation exhibited a consistent increase in frequency of pumping in response to consecutive applications of 5-HT. The inhibitory effect of emodepside was quantified by determining the change in the 5-HT response after addition of emodepside as a % of the response obtained before addition of emodepside (control).

*Drugs*

NS1619 (1,3-Dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one) and Rottlerin were obtained from Sigma-Aldrich UK. Emodepside was provided by Bayer Monheim, Germany. Drugs were prepared in 100% ethanol at a concentration of 2mM as a stock solution. Stock solutions of NS1619 were kept at $-20^0$ C for a maximum of 2 weeks. Stock solutions of emodepside and emodepside-containing plates were kept at $4^0$ C for a maximum of 1 week. Final concentrations of 10nM, 100nM, 1µM and 10µM of the drugs in NGM or in a food source contained 0.5% ethanol. 0.5% vehicle, ethanol, was used as a control. For the electrophysiological experiments, emodepside was prepared fresh each day in 100% DMSO. Stock solution was further diluted in DMSO and Dent's saline to give a final concentration in the recording chamber of 100nM or 1µM emodepside and 0.01% DMSO. Serotonin creatinine sulfate complex (5-HT; Sigma UK) was freshly prepared each day in Dent's Saline. The stock solution was further diluted in Dent's Saline to give a final concentration of 300nM.

*Statistical analysis*

Data are presented as the mean ± SEM of 'n' experiments. Inhibition curves were fitted to the modified logistic equation using Graph Pad Prism computer software (version 4.0 San Diego California) to determine $IC_{50}$ values with 95% confidence limits. Statistical significance was determined using one way ANOVA (significance level set at $p < 0.05$) followed by Bonferroni post-tests. For the electrophysiological experiments statistical significance was determined using paired Student's t-test or one way ANOVA (statistical significance level was set at $p < 0.05$) as appropriate. The

number of corresponding pumps and the number of individual worms used to perform the statistical analysis for each strain is stated in respective figure legends.

**Results**

*Structural comparison between SLO-1 and KCNMA1*

The mammalian orthologue for SLO-1a (NP_001024259), KCNMA1 was identified using Ensembl orthologue definitions (Flicek et al.,2011) . This was confirmed by molecular phylogenetic analysis of SLO-1, KCNMA1 and the closest mammalian paralogue identified by Ensembl, KCNU1 (Supplementary Information S2). *Homo sapiens kcnma1* (ENSG00000156113) is predicted to produce 26 transcripts, with 20 transcripts coding for proteins. The protein product of Ensembl transcript KCNMA1-001 (peptide ENSP00000286627) corresponds to KCNMA1 variant 2/isoform b in the NCBI database (NP_002238). The transcript encoding this isoform (NM_002247) was available to purchase from OriGene Technologies, USA.

To further compare the sequence of SLO-1a (NP_001024259) and KCNMA1 (NP_002238), we performed Basic Local Alignment (NCBI BLAST) (Johnson et al., 2008) of translated protein sequences of the channels and their individual regions. The alignment for SLO-1a and KCNMA1 shows 55% identity and 69% similarity between the two sequences (Supplementary Information S3). Further alignments were conducted for specific regions of the protein in order to discern regions of high conservation from those that are more divergent. The most conserved and divergent regions of the channel are the calcium bowl (96% identity, 100% similarity) and the N-terminal domain (no significant similarity), respectively (Figure 2A). Other highly conserved regions are the transmembrane domains, with the exception of the first

transmembrane region S0, the channel pore and the regulatory domains, RCK1 and RCK2 (Figure 2B). Between RCK1 and RCK2 there is an additional run of 49 residues in SLO-1.

*C. elegans slo-1 and mammalian kcnma1 rescue behavioural phenotypes in slo-1(js379)*

*slo-1(js379)* mutants move with similar speed and frequency of body bends compared to wild type *C. elegans*, but their rate of reversals is significantly increased (Guest et al., 2007; Wang et al., 2001). To identify whether *slo-1* and *kcnma1* are functional orthologues we expressed cDNAs of both genes in the *slo-1(js379)* mutant background from pan-neuronal (*snb-1*), body wall muscle (*myo-3*) and the native *slo-1* promoter and determined to what extent these rescued the reversal phenotype of *slo-1(js379)*. Video analysis of s*lo-1(js379) C. elegans* confirmed that these animals stop and reverse more often than wild-type (Figure 3A; Video S1 and S2). Expression of either *slo-1* or *kcnma1* completely rescued the reversal phenotype of *slo-1(js379)* and the transgenic animals exhibited a pattern of reversals more similar to wild-type (Figure 3A; Video S3 and S4). Furthermore, expression of *slo-1* or *kcnma1* in only neurones (*psnb-1*) was also sufficient to rescue this phenotype, in agreement with the neuronal basis of the aberrant pattern of locomotion in *slo-1* mutants (Figure 3A) (Wang et al., 2001). Expression of either *slo-1* or *kcnma1* in the body wall muscle of *slo-1(js379)* also rescued the reversal phenotype (Figure 3A).

Mutations in *slo-1* also confer a pharyngeal phenotype. Thus whilst the pattern of pharyngeal pumping in wild-type worms consists of pumps that occur predominantly in groups of 1 to 3, in *slo-1(js379)* this pattern is disrupted and pumps occur in larger

groups (Dillon et al., 2009). This is consistent with the idea that SLO-1 has a role in regulating the excitability of neural networks and thus in its absence the stability of the network activity is disturbed leading to bursts of activity. We confirmed this phenotype to reinforce the functional role of SLO-1 in the pharyngeal neural circuits, and showed that worms expressing either *slo-1* or *kcnma1* from the native *slo-1* promoter were rescued for this behaviour (Figure 3B, C) thus indicating that either SLO-1 or KCNMA1 can regulate bursting activity in the pharyngeal circuit.

These studies on the functional rescue of two independent *slo-1* behavioural phenotypes with either *slo-1* or *kcnma1* suggests the mammalian orthologue of *slo-1*, *kcnma1*, can be functionally expressed in *C. elegans*, and indeed, can substitute for this channel to the extent that its expression can restore behaviour to wild-type.

*slo-1 but not kcnma1 confers sensitivity to the inhibitory effects of emodepside on locomotion*

We compared the effect of emodepside on wild-type worm locomotion with its effect on *slo-1(js379) C. elegans* that had been transformed with either *C. elegans slo-1* or its closest mammalian homologue, human *kcnma1*. We tested animals that expressed *slo-1* or *kcnma1* from three different promoters; the native *slo-1* promoter (*pslo-1*) or tissue specific promoters driving pan-neuronal expression (*psnb-1*) or body wall muscle expression (*pmyo-3*). These experiments were performed on worms that were synchronised for age (1 day old adults) to avoid variability of emodepside effects on different developmental stages of *C. elegans* (Bull et al., 2007). Worms were identified as expressing the gene of interest by visualisation of

green fluorescent protein in the pharyngeal muscle as a selection marker (Figure 4A).

The effect of emodepside on locomotion was assessed by observing the effect on body bends. Consistent with earlier reports for wild-type worms, (Bull et al., 2007; Guest et al., 2007) 100nM emodepside elicited a flaccid paralysis, particularly apparent in the anterior of the worm, and a decrease in amplitude of the sinusoidal body shape (Figure 4B, C). This effect of emodepside on body posture was not observed in *slo-1(js379)* mutants consistent with earlier reports that this mutant exhibits high level resistance to emodepside (Guest et al., 2007). However, reintroduction of a wild-type copy of *slo-1* into the *slo-1(js379)* mutant restored the ability of emodepside to affect body posture whilst expression of *kcnma1* in the *slo-1(js379)* genetic background did not (Figure 4D, E).

To more quantitatively compare the effect of emodepside in the different transgenic lines we analysed concentration-dependent effects on locomotion by assaying the frequency of body bends. Wild-type worms were sensitive to emodepside whilst *slo-1(js379)* were resistant (Figure 5A; $IC_{50}$ for wild-type worms was 16nM, 95% confidence 11nM to 24nM, n=10). Expression of *slo-1* from the native *slo-1* promoter in the *slo-1(js379)* mutant background rescued the sensitivity to emodepside to the level of wild-type ($IC_{50}$ 23nM, 95% confidence 15nM to 35nM, n=10; Figure 5A). In marked contrast, expression of *kcnma1* from the native *slo-1* promoter did not rescue sensitivity to emodepside at 10nM, 100nM and 1µM. Only at the highest concentration tested, 10µM emodepside was the mean number of body bends significantly reduced compared to control (p<0.001; Figure 5A). Closer inspection of

the behaviour of transgenic lines expressing KCNMA1 in the presence of this highest (10μM) concentration of emodepside revealed an aberrant pattern of locomotion compared to vehicle controls. They stopped moving more often and exhibited repeated reverse movements.  These periods of disorientated movement were not observed in the same transgenic animals on vehicle control and are thus best explained by an effect of emodepside at this high concentration, rather than an effect due to over-expression of KCNMA1 channel in the transgenic strains. The different effects of emodepside on locomotion in worms expressing either SLO-1 or KCNMA1 can be viewed on supplementary videos S5, S6, S7 and S8.  The repeated reversals observed for those worms expressing KCNMA1 in the presence of emodepside is reminiscent of the behaviour of the *slo-1* null mutant *js379* and a possible explanation is therefore that at high concentrations emodepside acts to inhibit signalling through the human calcium-activated potassium channel, KCNMA1.

Further studies employing different promoters to drive expression of *kcnma1* in the *slo-1(js379)* genetic background in either neurones or body wall muscle provided additional evidence that expression of *kcnma1* does not confer sensitivity to emodepside. In these strains even the highest concentration of emodepside tested, 10μM, had no effect on locomotion. This contrasts with the susceptibility of worms expressing *kcnma1* from the native *pslo-1* promoter to 10μM emodepside, and this difference is consistent with the observation that the most effective promoter for providing rescue of the reversal locomotor phenotype is also the native *pslo-1* promoter (Figure 3A).  In parallel, it was confirmed that expression of the native *C. elegans* SLO-1 channel from all the promoters tested resulted in sensitivity to emodepside in *slo-1(js379)* (Figure 5B, C).

*slo-1 but not kcnma1 confers sensitivity to the inhibitory effects of emodepside on feeding*

In the pharyngeal system of *C. elegans slo-1* is expressed in the neural circuits but not in the pharyngeal muscle (Chiang et al., 2006; Wang et al., 2001). Previously it has been shown that emodepside inhibits the fast coordinated pumping activity of the pharynx that occurs in the presence of the stimulatory neurotransmitter 5-HT (Willson et al., 2004). This effect is mediated presynaptically (Willson et al., 2004) and is highly dependent on the presence of neuronally expressed *slo-1* (Guest et al., 2007). In this study we used the pharyngeal preparation to further test the sensitivity of strains expressing either SLO-1 or KCNMA1 to emodepside by employing the transgenics we had generated that expressed either *slo-1* or *kcnma1* in the *slo-1(js379)* mutant from the native *slo-1* promoter.

In these assays the pharyngeal preparation was exposed to three consecutive applications of 5-HT separated by a 2min interval. In control experiments, each application of 5-HT elicited a robust excitatory response as reported previously (Guest et al., 2007; Willson et al., 2004). To quantify the level of emodepside inhibition we compared the pharyngeal pumping frequency observed in response to 300nM 5-HT (a sub-maximal concentration) (Rogers et al., 2001) before and 5min after addition of 100nM emodepside (Figure 6A). In wild-type controls emodepside inhibited the response to 5-HT (Figure 6A, top panel). *slo-1(js379)* were resistant to 100nM emodepside, (Figure 6A,B) whilst expression of *slo-1* but not *kcnma-1* in the *js379* mutant from the native *slo-1* promoter, *pslo-1*, restored emodepside sensitivity (Figure 6A,B). Only at the higher concentration of emodepside tested, 1μM, was a

partial, but nonetheless significant, inhibitory effect observed on pharyngeal pumping of the strains expressing *kcnma1* (Figure 6A bottom panel; Figure 6B).

The data for the pharmacological actions of emodepside on the worms expressing either *kcnma1* or *slo-1* from the native *pslo-1* promoter are summarised in Table 1 and provide evidence for a selective effect of emodepside on strains expressing *slo-1*.

*A pharmacological characterisation of transgenic lines expressing slo-1 or kcnma1 using mammalian calcium-activated potassium channel agonists*

The experiments described above show that worms expressing *kcnma1* are only weakly sensitive to emodepside despite the fact that expression of this channel from the native *slo-1* promoter completely rescued the behavioural phenotypes of *slo-1(js379)*. Therefore we tested whether or not expression of *kcnma1* in *C. elegans* conferred sensitivity to known agonists of this channel, as might be predicted from the observed functional rescue. We tested the effects of an activator of mammalian BK channels, NS1619 (Olesen et al., 1994), on locomotion of wild-type, *slo-1(js379), js379;pslo-1::slo-1* and *js379;pslo-1::kcnma1* expressing strains of *C. elegans*. NS1619 is a widely used compound for its ability to relax smooth muscle (Olesen et al., 1994) and inhibit neuronal activity (Lee et al., 1995) by a selective activation of calcium-activated BK, channels. NS1619 was chosen for these experiments as it can act at mammalian channels in the absence of accessory β subunits (Zhakarov et al., 2005). NS1619 has low stability therefore these experiments were conducted over a shorter time-frame of drug exposure, 3 rather than 24 h. Emodepside inhibited body bends of wild-type worms following this shorter incubation time but with a slightly

higher IC$_{50}$ (78nM, 95% confidence of 47 nM to 131 nM, n=10) compared to the 24 h emodepside treatment. These experiments using a shorter exposure to emodepside provided further evidence of the ability of only the high concentration to inhibit lines expressing *kcnma1* (Figure 7A*).* NS1619 at concentrations from 10nM to 10µM did not have any effect on locomotion of wild-type or *js379;pslo-1::slo-1 C. elegans* suggesting that it does not interact with *C. elegans slo-1* channels (Figure 7B). In contrast, the locomotion of worms expressing *kcnma1* (*js379;pslo-1::kcnma1)* was significantly impaired compared to vehicle control and wild type (Figure 7B; Video S9, S10, S11, S12). Worms did not appear paralysed in the presence of NS1619 but rather seemed to repeatedly be "slipping" backwards and immediately forward in the same spot on a plate without completing a complete sinusoidal body wave. This behaviour was also noted in worms expressing *kcnma1*, (*js379;pslo-1::kcnma1)*, in the presence of emodepside, but with NS1619 it was much more pronounced. These disruptions of normal co-ordinated movement became increasingly severe with increasing concentration of NS1619, which is reflected in a decreasing number of body bends (Figure 7B). NS1619 did not cause a complete inhibition of body bends and elicited a maximal inhibition of 48% at the highest concentration tested.

In a further series of experiments we tested the effect of the BK channel agonist rottlerin (Zakharov et al., 2005) on the behaviour of transgenic strains expressing either *slo-1* or *kcnma1*. Rottlerin, also known as mallotoxin, is a lipid soluble toxin isolated from *Mallotus phillippinensis* (Wu et al., 2007), which potently activates BK channels (Wu et al., 2007; Zakharov et al., 2005). It activates heterologuously expressed and mammalian muscle BK channels when applied to both extracellular (Zakharov et al., 2005) and intracellular parts of the membrane (Wu et al., 2007).

Locomotion of *C. elegans* after both short term (3 h) and long term (24 h) exposure to rottlerin was assessed. Short term, 3 h, exposure to rottlerin did not inhibit locomotion of wild-type, *slo-1 (js379)* or *js379;pslo-1::slo-1* (Figure 7C). However, movement of *slo-1 (js379)::pslo-1::kcnma1 C. elegans* exposed to 1 and 10μM rottlerin for 3 h was slowed (Figure 7C). The worms expressing *kcnma1* exposed to rottlerin also had periods when they moved backwards and forwards on the same spot with some stalls, similar to their locomotion on NS1619, suggesting that rottlerin and NS1619 are affecting locomotion in a similar manner. Longer term exposure to rottlerin, for 24 h affected locomotion of wild-type worms (Figure 8). Interestingly *slo-1(js379)* was resistant to the effect of 24 h exposure to rottlerin on locomotion, suggesting that the effect of rottlerin on wild-type worms may be mediated by SLO-1 (Figure 8). This was confirmed by the observation that the inhibitory effect of rottlerin on locomotion was restored by expressing *slo-1 f*rom the native promoter (*js379;pslo-1::slo-1* worms; Figure 8). Furthermore, worms expressing *kcnma1* were inhibited by rottlerin, 10μM, in a similar fashion to those expressing *slo-1*. This suggests that rottlerin has less selectivity than NS1619 for the human over the *C. elegans* calcium-activated potassium channel.

*Ectopic expression of slo-1 in C. elegans pharyngeal muscle*

*slo-1* is not expressed at detectable levels in pharyngeal muscle. In the pharyngeal system it is selectively expressed in neurones (Chiang et al., 2006; Wang et al., 2001) to regulate the pattern of pharyngeal pumping activity (Dillon et al., 2009). Previously it has been shown that expression of wild-type *slo-1a* from the pan-neuronal promoter *snb-1* in the *slo-1* null mutant *js379* rescues the uncoordinated pattern of bursting pharyngeal pumping activity exhibited by the *slo-1* mutant strain

(Dillon et al., 2009) consistent with its role in the nervous system. In contrast, expression of *slo-1a* in the pharyngeal muscle of *slo-1(js379)* does not rescue this behaviour (Dillon et al., 2009) consistent with the lack of native expression of, and thus physiological role for, the channel in this tissue (Dillon et al., 2009). Therefore we exploited the pharyngeal muscle for ectopic expression of *slo-1* to test its role in mediating the action of emodepside. In these studies we determined the direct effect of emodepside on the pharynx in the absence of 5-HT stimulation. In wild-type worms emodepside caused an almost complete inhibition of pumping (control $0.49 \pm 0.08$ pumps $sec^{-1}$; with 1µM emodepside $0.04 \pm 0.02$ pumps $sec^{-1}$, n=7, p<0.001; paired Student's t-test). Emodepside had no inhibitory effect on the pharyngeal muscle of the mutant *slo-1(js379)* but did inhibit the pumping activity of strains ectopically expressing *slo-1a* specifically in the pharyngeal muscle (Figure 9A,B). This effect of emodepside on strains expressing SLO-1 in the pharyngeal muscle was observed in the absence of rescue of the *slo-1(js379)* pharyngeal phenotype i.e. the bursting activity, which has previously been shown to be derived from a neuronal effect of SLO-1 (Dillon et al., 2009). These data indicate an intimate link between emodepside response and the SLO-1 channel and are consistent with the proposal that the nematode channel harbours an emodepside selective pharmacophore.

**Discussion**

Emodepside has important resistance breaking properties (Harder et al., 2003) that has stimulated interest in its molecular mechanisms of action. Two modes of action have been demonstrated. An emodepside receptor with homology to mammalian latrophilin G protein-coupled receptors has been expression cloned from *Haemonchus contortus* (Saeger et al., 2001) and its *C. elegans* orthologue, LAT-1,

confers sensitivity of the pharyngeal system to emodepside (Willson et al., 2004). However, *C. elegans* latrophilin mutants remain susceptible to the inhibitory effects of emodepside on locomotion (Guest et al., 2007) indicating another effector must also be involved. Subsequently, a chemical mutagenesis screen identified SLO-1 as a key determinant for the drug's effects on nematode development and motility (Guest et al., 2007).

The *kcnma1* channel gene is the mammalian orthologue of *slo-1* and was therefore selected as a suitable candidate to test the selective toxicity of emodepside. In order to assess the effects of emodepside on SLO-1 and KCNMA1 both genes were expressed in a *C. elegans* mutant *slo-1(js379)*, that harbours a mutation in the channel (introducing a premature stop codon), and is therefore predicted to be a functional null (Wang et al., 2001). The tractability of *C. elegans* for expression of transgenes from different tissue specific promoters was employed in order to drive expression from the native promoter, a pan-neuronal promoter and a promoter for expression in body wall muscle. Previously it has been shown that *slo-1* is widely expressed in *C. elegans* (Wang et al., 2001), in neurones and in body wall muscle, and the observation that the *slo-1* behavioural phenotypes of increased frequency of reversals (Guest et al., 2007; Wang et al., 2001) and erratic pharyngeal pumping (Dillon et al., 2009) were restored when a wild-type copy of *slo-1* was expressed from either the native promoter or a pan-neuronal promoter concurs with this. In addition, the reversal phenotype was significantly rescued by expression of *slo-1* in body wall muscle consistent with the observation that native *slo-1* is also expressed in this tissue (Carre-Pierrat et al., 2006). These assays provided a platform for functional analysis of the human channel, *kcnma1*, expressed in *C. elegans*. Expression of this

channel instead of *slo-1* was observed to robustly rescue the *slo-1* dependent locomotor and pharyngeal phenotypes. This suggests that KCNMA1 is able to substitute for SLO-1 in the neural circuits that regulate the pattern of locomotion and pharyngeal pumping and restore wild-type function in the *slo-1* null mutant.

The heterologous expression of the mammalian channel protein KCNMA1 in *C. elegans* in this study provided a highly tractable *in vivo* model for the direct comparison of the susceptibility of the nematode, versus the mammalian, channel to emodepside. It has been established that the high level resistance of the *slo-1* null mutant *js379* to the effect of emodepside on locomotion can be reversed by expression of a wild-type copy of *slo-1* in either neurones or body wall muscle (Guest et al., 2007). For these studies, to more accurately reflect the endogenous expression of *slo-1*, we employed a transgenic strain expressing *slo-1* from the native *slo-1* promoter. In these lines the susceptibility to emodepside was indistinguishable from wild-type. In parallel, experiments were performed on transgenic lines expressing human *kcnma1*, and in these lines resistance to emodepside was not alleviated. This lack of susceptibility to emodepside is unlikely to be due to low level functional expression of the human channel in the *C. elegans* biological background as these same lines exhibited a robust rescue of the behavioural phenotypes. Furthermore, RT-PCR indicated that the *kcnma1* transgene was transcribed at similar levels compared to the *slo-1* transgene (Supplementary information 1). As noted above, *kcnma* is subject to alternative splicing and we cannot rule out the possibility that one of the splice variants, other than the *kcnma1* splice isoform tested here, may be more sensitive to emodepside. Nonetheless, taken at face value these data indicate that the human channel, although functional in *C. elegans*, is not

sensitive to sub-micromolar concentrations of emodepside. However, when strains expressing *kcnma1* were tested in the presence of the highest concentration of emodepside, 10µM, an inhibitory effect on locomotion was observed. This was unlikely to be due to a non-specific effect of emodepside as this inhibitory effect was not observed in the *slo-1* null mutant. Thus we conclude that emodepside can interact with the human channel KCNMA1, but with a much lower efficacy than its interaction with SLO-1. This conclusion is further supported by the observations made on the effects of emodepside on pharyngeal pumping. In these experiments, expression of *slo-1* but not *kcnma1,* was required to confer susceptibility to low drug concentrations.

The generation of strains expressing functional human or nematode calcium-activated potassium channels enabled further comparative pharmacological analysis. Thus, in addition to providing evidence that the *C. elegans*, but not the human, channel is sensitive to nanomolar concentrations of emodepside, this study has also shown that the mammalian BK channel activator NS1619 (Lee et al., 1995) distinguishes between the two channels, in this case having higher efficacy for the human channel. NS1619 was without effect on wild-type worms, or on *slo-1(js379)*, or on any of the *slo-1* rescue lines. However, NS1619 had marked effects on strains expressing *kcnma1* at a concentration equivalent to those previously shown to activate the human channel (Zhang et al., 2003). This led to uncoordinated locomotion which reduced the mean number of body bends in a dose-dependent manner. It is interesting to note that qualitatively the behaviour of these transgenic worms expressing the human channel on either 10nM NS1619 or on a high (10µM) concentration of emodepside was similar in that they appeared to be repeatedly

"slipping" backwards and immediately forward in the same spot. These data are consistent with the suggestion that both these drugs interact with the KCNMA1 channel, but that NS1619 has an efficacy three orders of magnitude greater than emodepside at the mammalian channel.

Another activator of mammalian BK channels is rottlerin, or mallotoxin (Wu et al., 2007; Zakharov et al., 2005). In contrast to NS1619, this drug inhibited locomotion in transgenic *C. elegans* expressing either *slo-1* or *kcnma1* and indeed was observed to also inhibit locomotion of wild-type worms. This suggests that this drug is less selective for the *C. elegans* versus the human channel. The observation that *slo-1(js379)* were resistant to rottlerin suggests that at least part of its effect is due to an action mediated by the calcium-activated potassium channels, either SLO-1 or KCNMA1.

Whilst NS1619 had a significant and characteristic effect on the locomotor behaviour of worms expressing *kcnma1,* even at the highest concentration tested it did not completely inhibit locomotion. This is in contrast to the effect of emodepside on wild-type worms where a near complete inhibition of body bends was observed at micromolar concentrations. A similar observation was made with rottlerin which also elicited a sub-maximal inhibition of locomotion. One possible explanation is that emodepside has a high efficacy at the SLO-1 channel that permits a greater effect on locomotion compared to the effects observed with either rottlerin acting through SLO-1 or KCNMA1, or NS1619 acting through KCNMA1. Further experiments employing electrophysiological analysis are required to resolve this issue.

Finally, we took advantage of the lack of native *slo-1* expression in the pharyngeal muscle in order to ectopically express the channel specifically in this tissue in an otherwise *slo-1* null mutant genetic background. Emodepside had no effect on the mutant but inhibited muscle activity in strains expressing *slo-1* only in the muscle. Taken together, the results are consistent with the proposal that SLO-1 harbours a selective pharmacophore for emodepside and provides a rationale for further structure-function analysis of this therapeutically important target. In this respect, it is noteworthy that recent progress has been made in the structural characterisation of this family of channels (Wu et al., 2010).

The relatively high cost of emodepside is likely to preclude its widespread use in humans, however our study provides a platform for further drug discovery based on SLO-1 as a target. The value of the experimental approach we have adopted here is reinforced by the observation that whilst human proteins have an established capability for expression in commonly used heterologous expression systems, the expression of invertebrate receptor proteins is much less robust. Thus the method we describe, of using *C. elegans* as the expression assay, may provide a complementary approach to more conventional cell-based assays and overcome difficulties encountered in doing a comparative functional analysis across phyla.

**Authorship contribution:**

Participated in research design: Crisford, O'Connor, v Samson-Himmelstjerna, Walker, Harder and Holden-Dye

Conducted experiments: Crisford, Murray, Edwards, Kruger and Welz

Contributed new reagents or analytic tools: Harder

Performed data analysis: Crisford and Holden-Dye

Wrote or contributed to the writing of the manuscript: Crisford, O'Connor, Edwards, Walker, v Samson-Himmelstjerna and Holden-Dye

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
Footnotes:

**Legends for Figures**

**Figure 1. The structure of the calcium-activated potassium channel agonists compared to the novel cyclooctadepsipeptide anthelmintic emodepside** (Harder et al., 2003). Rottlerin (mallotoxin; 1-[6-[(3-Acetyl-2,4,6-trihydroxy-5-methylphenyl)methyl]-5,7-dihydroxy-2,2-dimethyl-2H-1-benzopyran-8-yl]-3-phenyl-2-propen-1-one) and NS16I9 (1,3-Dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one).

**Figure 2. The most divergent and most conserved regions of sequence between *C. elegans* SLO-1a and human KCNMA1 (isoform b). A.** Alignment of the calcium bowl (top) and N-terminal domain (bottom) using BLAST (NCBI). Identical amino acids are indicated by their letter symbols and conservative substitutions are indicated by "+". Gaps are indicted by "---"symbols and were introduced by the program to enable analysis of the most conserved parts of the sequences. Identity between amino acid sequences of the calcium bowl is 96%, and similarity is 100%. There is no significant similarity in the N-terminal domain. **B.** A diagram of the channel to indicate the most conserved regions of sequence.

**Figure 3. A comparison of the rescue of *C. elegans* slo-1(js379) behavioural phenotypes by *slo-1* and its human orthologue *kcnma1*. A.** A comparison of reversal frequency for wild-type, *slo-1(js379)* and transgenic lines of *slo-1(js379)* expressing either *slo-1* ▨ or *kcnma1* ▦ behind the native promoter (*pslo-1*), a pan-neuronal promoter (*psnb-1*) or in body wall muscle (*pmyo-3*). At least two stable lines for each *js379;pslo-1::slo-1* and *js379;pslo-1::kcnma1* transgenic strain were

tested. All lines for the same strain showed comparable results and the data are pooled. Data are the mean ± SEM of n ≥ 20, ***p<0.001, **p<0.01 * p<0.05, one way ANOVA with Bonferroni post-hoc test. **B.** A comparison of the pattern of pharyngeal pumping in wild-type, *slo-1(js379)* and transgenic lines of *slo-1(js379)* expressing either *slo-1* or *kcnma1* behind native promoter (*pslo-1*). Note the erratic pattern in *slo*-1(js379) which has a tendency to generate pharyngeal pumps in groups of three or more rather than as single evenly spaced pumps. This effect is ameliorated by expression of either *slo-1* or *kcnma1*, bottom two traces **C.** A summary of experiments conducted as shown in B. For each experiment ten minutes recording of basal pharyngeal pumping was acquired. This was subjected to analysis with AutoEPG (Dillon et al., 2009) which counted the number of pumps that occurred as single pumps and the number of pumps that occurred in groups of three or more and the data are expressed as the number of pumps that occurred in groups of one to three (open bars) or more than three (hatched bars) as a percentage of the total number of pumps. Data are the mean ± SEM of n = 14 to 26. Note that the pattern of pumping in both the *slo-1* and *kcnma1* rescue lines is more like wild-type than *slo-1(js379)*.

**Figure 4. Effects of emodepside on the body shape of wild-type and transgenic *C. elegans.*** The comparisons shown are between wild-type, *slo-1(js379)* and transgenic lines of *slo-1(js379)* expressing either *slo-1* or *kcnma1* behind the native promoter (*pslo-1*) or a pan-neuronal promoter (p*snb1*). Transgenic worms were identified by the appearance of green fluorescence in the pharynx due to the expression of *gfp* driven from the pharyngeal promoter *myo-2* as a positive selection marker. **A.** An example of an L4+1day old stage transgenic *slo-1 (js379) C. elegans.*

Green fluorescence in the area of pharyngeal muscle (white arrow) is driven by *pmyo-2* promoter, which is used as a marker for transformation.  **B.** Wild-type L4+1 day old worm on 0.5% ethanol vehicle control showing wild-type sinusoidal body posture. **C.** Wild-type L4+1 day old worm with 100nM emodepside (24 h exposure) showing the typical flattened wave-form, particularly in the anterior region (indicated by white arrow) and egg retention (enlarged posterior). **D**. L4+1 day old *js379;psnb-1::slo-1* on 100nM emodepside (24 h exposure) showing a similar flattened body posture to wild-type in the  anterior region (white arrow). **E.** L4+1 day old *js379;psnb-1::kcnma1* with 100nM emodepside (24 h exposure) showing a normal sinusoidal body shape. (The body shape of *js379;psnb-1::kcnma1* and *js379;psnb-1::slo-1* in the absence of emodepside was indistinguishable from wild-type worms; not shown).


**Figure 5. A comparison of the effect of emodepside on locomotor frequency in worms expressing either *slo-1* or its human orthologue *kcnma1*.**   These experiments measured the frequency of body bends of one day old adult worms to provide a quantitative read-out of the effect of 24 h exposure to emodepside on locomotor behaviour. **A.** A comparison of the effect of emodepside on the frequency of body bends on wild-type, *slo-1(js379)* and *js379* transgenic worms expressing *slo-1* (*js379;pslo-1::slo-1*) or *kcnma1* (*js379;pslo-1::kcnma1*) from the native promoter. n=10 worms for each datum point, mean ± s.e.mean. ***$p < 0.001$ with respect to vehicle control, one way ANOVA on last data points. **B.** A comparison of the effect of emodepside on the frequency of body bends on wild-type, *slo-1(js379)* and *js379* transgenic worms expressing *slo-1* (*js379;psnb-1::slo-1*) or *kcnma1* (*js379;psnb-1::kcnma1*) from a pan-neuronal promoter. n=10 worms for each datum point, mean ± s.e.mean. ***$p < 0.001$ with respect to vehicle control. **C.** A comparison of the effect

of emodepside on the frequency of body bends on wild-type, *slo-1(js379)* and *js379* transgenic worms expressing *slo-1* (*js379;pmyo-3::slo-1)* or *kcnma1* (*js379;pmyo-3::kcnma1*) from the body wall muscle promoter, *myo-3*. n=10 worms for each datum point, mean ± s.e.mean. ***p<0.001 with respect to vehicle control.

**Figure 6. A comparison of the effect of emodepside on the 5-HT stimulated pharyngeal pumping rate of wild-type and transgenic worms expressing *slo-1* or human *kcnma1*.** **A.** Extracellular recordings were made from the pharyngeal system in order to monitor the activity of the pharyngeal muscle in the presence and absence of emodepside. In these recordings each upwards and downwards deflection provides a read-out of a single pharyngeal feeding cycle or 'pump'. On a slow time-base fast pumping rate appears as a continual block of activity and individual pumps cannot be resolved. In the experiment continuous recordings were made for 20 minutes in which the preparation was first perfused with saline, followed by three to four consecutive applications of 5-HT to stimulate pumping interspersed with wash periods in saline. In the absence of emodepside, the response to consecutive applications of 5-HT were similar (not shown). The traces show the response to 300nM 5-HT before and 5 min after addition of emodepside. In the top four traces 100nM emodepside (emo) was applied and in the bottom trace 1μM emodepside was used. Scale bars indicate 2 min, 5mV. Exposure of wild-type worms expressing the transformation marker only (*pmyo-2::gfp*) to emodepside resulted in a complete inhibition of pumping (top trace). **B.** The inhibitory effect of emodepside on the 5-HT response in the different strains was compared by expressing the pumping rate in the presence of emodepside as a % change compared to the control response i.e. the pumping rate before the addition of emodepside. 1μM emodepside was also

tested in the strain expressing kcnma1 (chequered bar); (n ≥5, mean ± s.e.mean; *** p<0.001, ** p<0.01 one way ANOVA with Bonferroni post-hoc test).

**Figure 7. A comparison of the effect of emodepside and the calcium-activated potassium channel agonists NS1619 and rottlerin on locomotion of *C. elegans* expressing SLO-1 or the human channel KCNMA1.** For these experiments one day old adult worms were exposed to vehicle or drug at increasing concentrations for 3 h. **A.** The effect of a 3 h incubation with emodepside at the concentrations indicated on the frequency of body bends on wild-type, *slo-1(js379), js379;pslo-1::slo-1, js379;pslo-1::kcnma1*. n=10 worms for each datum point, mean ± s.e.mean, *** p<0.001 compared to vehicle control for the same strain. **B.** The effect of a 3 h incubation with NS1619 at the concentrations indicated on the frequency of body bends on wild-type, *slo-1(js379), js379;pslo-1::slo-1, js379;pslo-1::kcnma1*. n=10 worms for each datum point, except for *js379;pslo-1::kcnma1* for which n=20 worms for each datum point, mean ± s.e.mean, *** p<0.001 compared to vehicle control for the same strain. One way ANOVA and Bonferroni post-hoc test on last data points. **C.** The effect of a 3 h incubation with rottlerin at the concentrations indicated on the frequency of body bends on wild-type, *slo-1(js379), js379;pslo-1::slo-1, js379;pslo-1::kcnma1*. n=10 worms for each datum point, ** p<0.01, *** p<0.001 compared to vehicle control for the same strain.

**Figure 8. A comparison of the effect of rottlerin (24 hours exposure) on locomotion of *C. elegans* expressing SLO-1 or the human channel KCNMA1.** For these experiments one day old adult worms were exposed to vehicle or 10µM rottlerin for 24 h and the effect on the frequency of body bends was assayed. Data

are the mean ± s.e.mean of 10 determinations for each strain. **p<0.01, *** p<0.001

one way ANOVA with Bonferonni post-hoc test.

**Figure 9. Ectopic expression of *slo-1* in *C. elegans* pharyngeal muscle confers sensitivity to emodepside. A.** Example traces of extracellular (EPG) recordings of pharyngeal muscle. Each vertical deflection reports a single muscle contraction-relaxation cycle or 'pump'. Scale bars 9mV, 4min. Top trace is from a *slo-1* null mutant and bottom trace is a *slo-1* null mutant expressing wild-type *slo-1* from a pharyngeal muscle promoter and bottom trace is from a *slo-1* null mutant expressing *slo-1* from the pharyngeal muscle promoter *pmyo-2*. The horizontal bar indicates the duration of application of emodepside. **B.** A summary of these experiments. Data are mean ± s.e.mean, n≥8, *** p< 0.001 paired Student's t-test.

**Table 1. A comparison of the ability of emodepside to inhibit pharyngeal pumping and locomotion in _C. elegans_ expressing either _slo-1_ or _kcnma1_.** Data are a summary of the effects of 100nM and 10µM emodepside from Figures 5A and 6. [1]The values are the % of control, where 'control' is the average number of pumps (in the presence of 300nM 5-HT) or body bends in worms on vehicle plates, assessed in parallel with the drug treatment group. [2] Wild-type control are worms expressing the transformation marker _myo-2::gfp_. * indicates significant difference from control. One way ANOVA with Bonferroni post-hoc test.

|  | % of control[1] | | |
|---|---|---|---|
|  | pharyngeal pumps (100nM emo) | body bends (100nM emo) | body bends (10µM emo) |
| wild-type[2] | 8 ± 2 (n=4)* | 32 ± 4 (n=10)* | 10 ± 2 (n=10)* |
| _slo-1(js379)_ | 99 ± 9 (n=7) | 101 ± 8 (n=10) | 96 ± 9 (n=10) |
| _js379::pslo-1::slo-1_ | 10 ± 6 (n=8)* | 26 ± 5 (n=10)* | 8 ± 2 (n=10)* |
| _js379::pslo-1::kcnma1_ | 117 ± 15 (n=6) | 104 ± 6 (n=10) | 68 ± 8 (n=10)* |

# Figure 1



**Rottlerin (mallotoxin)**
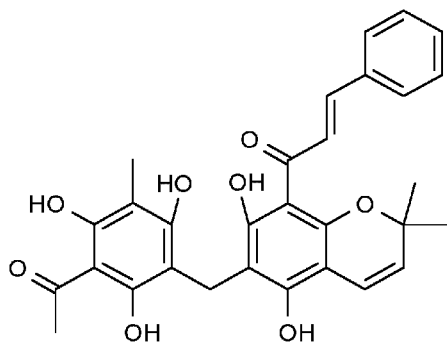
**NS1619**
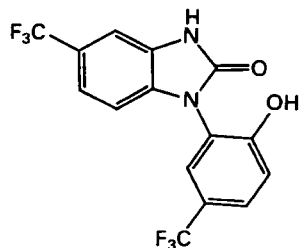
**Emodepside**
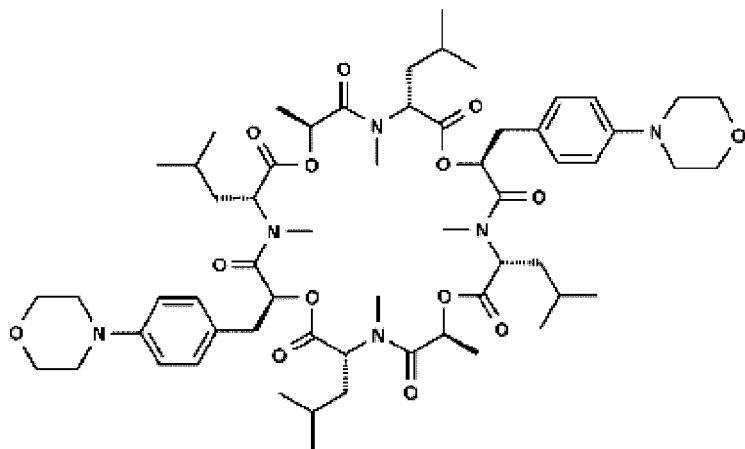
# Figure 2

**A**

**CALCIUM BOWL**

SLO-1 TELVNDSNVQFLDQDDDDDPDTELYLTQ

TELVND+NVQFLDQDDDDDPDTELYLTQ

KCNMA1 TELVNDTNVQFLDQDDDDDPDTELYLTQ

**N-TERMINAL**

SLO-1 SPSQSKGFNQPYGYPMNCNLSRVFMEMTEEDRKCLEE

S S S ++P M+ + V ME+ + R +
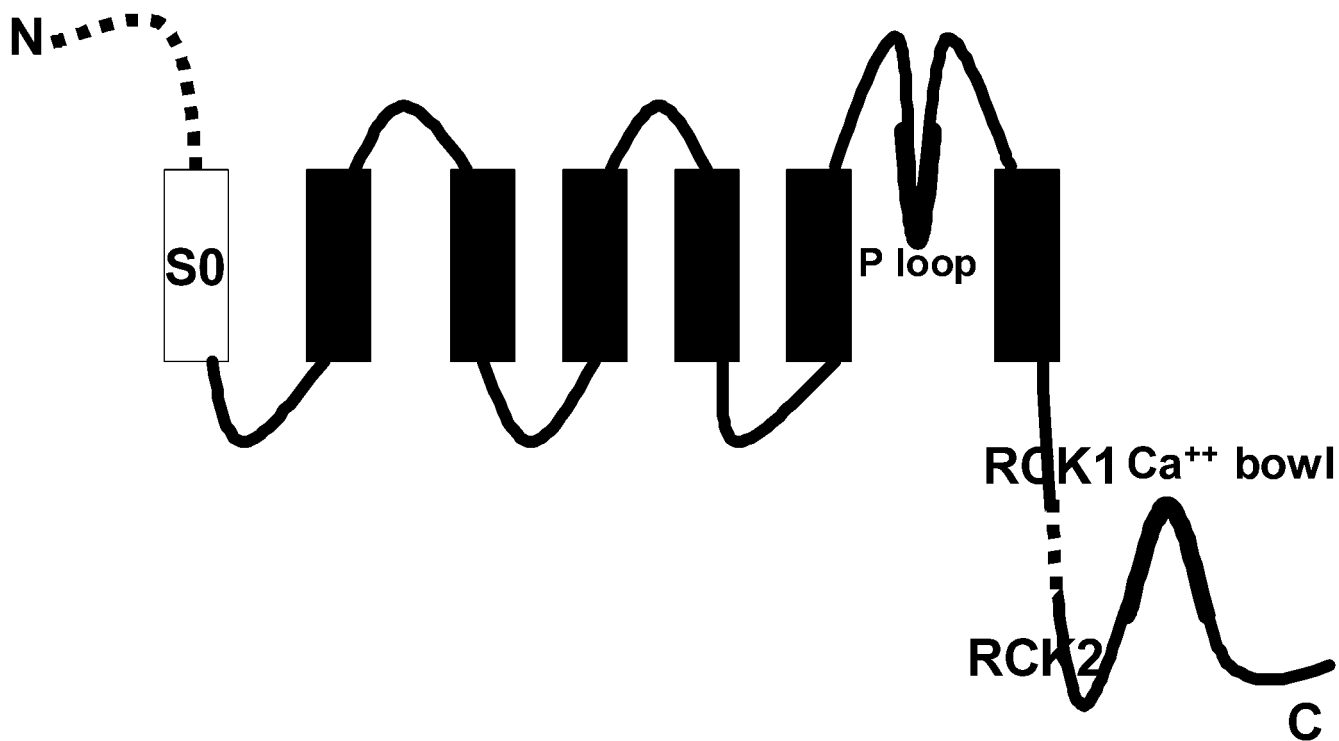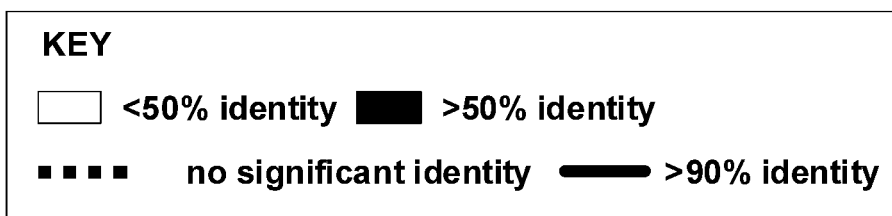
KCNMA1 SSSSSSSVHEP---KMDALIIPVTMEVPCDSRG---Q

**B**
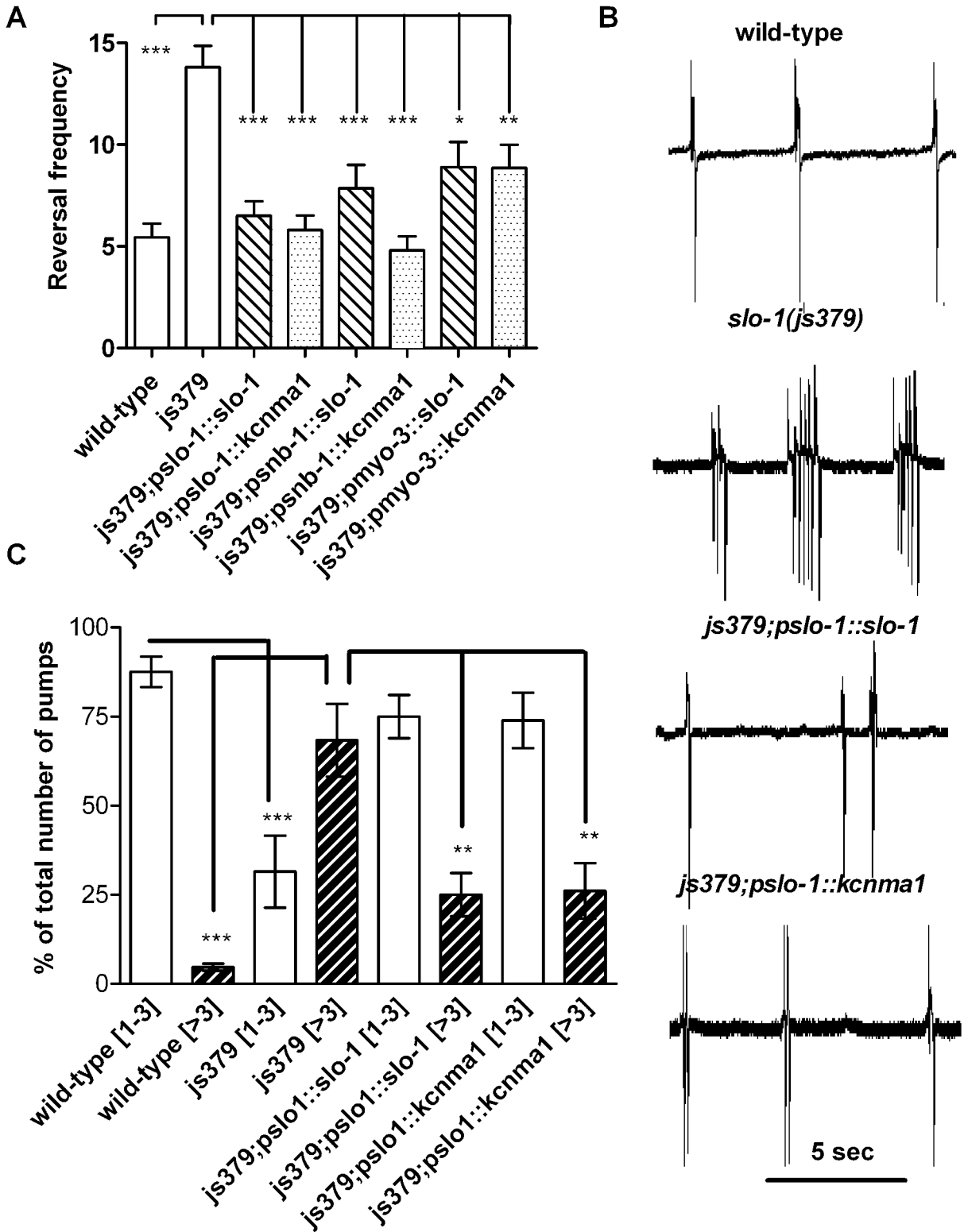


KEY

☐ <50% identity ■ >50% identity

···· no significant identity —— >90% identity

Figure 3

Figure 4

# Figure 5

# Figure 6

## A



|  | CONTROL | 100nM EMO |  |
|---|---|---|---|
| wild type | | | |
| slo-1(js379) | | | |
| js379;pslo-1::slo-1 | | | |
| js379;pslo-1::kcnma1 | | | |

|  | CONTROL | 1μM EMO |  |
|---|---|---|---|
| js379;pslo-1::kcnma1 | | | |

## B

Figure 7

# Figure 8



hatched bars = 10$\mu$M rottlerin 24 hours
open bars = 0.5% ethanol vehicle

# Figure 9

## A



*slo-1(js379)* 1μM emo

*slo-1(js379);pmyo-2::slo-1* 1μM emo

## B



pharyngeal pumps sec$^{-1}$

▨ *slo-1(js379)*
▧ *js379;pmyo-2::slo-1*

***

control    emo 1μM    control    emo 1μM