International Journal for Parasitology: Drugs and Drug Resistance 20 (2022) 108–112

Contents lists available at ScienceDirect

International Journal for Parasitology: Drugs and Drug Resistance

journal homepage: www.elsevier.com/locate/ijpddr

Diethylcarbamazine mediated potentiation of emodepside induced paralysis requires TRP-2 in adult *Brugia malayi*

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A B S T R A C T

Human and veterinary filarial nematode infections are a major health concern in tropical countries. They are transmitted by biting insects and mosquitoes. Lymphatic filariasis, a group of filarial infections caused by *Brugia* spp. and *Wucheria bancrofti* affect more than 120 million people worldwide. Infected individuals develop swollen limbs and disfigurement, leading to an inability to work and ostracization from society. Control and prophylaxis for these infections involve mass drug administration combinations of anthelmintics including diethylcarbamazine (DEC). DEC has actions on microfilariae, but its effects on adult worms are less pronounced. The SLO-1 (BK) channel activator, emodepside, kills adults of many filarial species. However, the *in vivo* efficacy of emodepside is suboptimal against *B. malayi,* possibly due to reduced bioavailability in the lymphatic system. Expressing different *slo-1* splice variants in *B. malayi* also affects sensitivity to emodepside. This study explores the potentiation of emodepside mediated paralysis by DEC in adult female *B. malayi*. Worminator motility measurements show that co-application of DEC and emodepside increases the potency of emodepside 4-fold. The potentiation of the emodepside effect persists even after the worms recover (desensitize) from the initial effects of DEC. RNAi knock-down demonstrates that the DEC-mediated potentiation of emodepside requires the presence of TRP-2 channels. Our study demonstrates that the addition of DEC could enhance the effect of emodepside where bioavailability or activity against a specific species may be low.

1. Introduction

Lymphatic filariases belong to neglected tropical diseases that affect more than 120 million people worldwide. Lymphatic filariasis is caused by adults of filarial parasites including *Brugia malayi, Brugia timori,* and *Wucheria bancrofti,* which are transmitted from infected individuals to their new hosts by biting insects. Adult filaria are found in the human lymphatic system. In some individuals, they block the lymphatic vessels, leading to severe itching and skin thickening with gross swelling of infected limbs. This severe disfigurement produces the condition known as elephantiasis. Elephantiasis is associated with an inability to work and often rejection by society. Without effective vaccines and vector control, treatment and prophylactic control of these parasitic nematode infections rely on anthelmintic chemotherapy.

The strategy for controlling filarial nematodes has been to eliminate microfilariae from host blood to prevent transmission via biting insects. Diethylcarbamazine has been used since 1947 and currently is used to treat lymphatic filariasis but not onchocerciasis. Although it is a piperazine derivative, it is not a GABA agonist and has excitatory effects on the nematode muscle (Martin, 1982), which has required further clarification. Other classes of anti-filarial anthelmintics currently used include: the benzimidazoles, like albendazole (ALB), that bind to nematode β-tubulin and inhibit microtubule formation, metabolism, and egg-laying (Lacey, 1990) and; the macrocyclic lactones, like ivermectin (IVM) and moxidectin, that act as activators or positive allosteric modulators of invertebrate glutamate-activated chloride channels (Wolstenholme and Rogers, 2005). The combination of these drugs as a cocktail is used for Mass Drug Administration (MDA) by the WHO for lymphatic filariasis. Unfortunately, while these drugs are very effective in clearing microfilariae, they do not kill all adult worms. The adult worms can survive and propagate the disease for up to 10 years.

Emodepside belongs to the cyclo-octadepsipeptide class of anthelmintics that targets a broad spectrum of parasitic nematodes. Emodepside is active *in vitro* against microfilaria and adult filaria of many species (Kulke et al., 2017). It is effective in an *in vivo* model of bovine filaria, *Onchocerca ochengi* (Bah et al., 2021). Single-dose treatment of emodepside is reported to kill some adult filariae and could prove to be a major advance over current MDA programs that are ineffective against adult filaria.

SLO-1 channels (BK/Maxi K channels) are calcium-activated and voltage-gated potassium channels that regulate neurotransmission and muscle excitability. SLO-1 channels are transmembrane proteins that conduct K^+ ions. These channels have a high single-channel conductance (250 pS). SLO-1 channels are essential for the regulation of neurotransmission in nematodes and are found in body-wall muscles and neurons (Wang et al., 2001). SLO-1 channels are activated by

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https://doi.org/10.1016/j.ijpddr.2022.10.002
Received 15 July 2022; Received in revised form 14 October 2022; Accepted 17 October 2022
Available online 29 October 2022

emodepside (Guest et al., 2007; Kulke et al., 2014a). In muscle cells of the parasitic nematode *Ascaris suum*, emodepside elicits K^+ currents inhibited by the BK channel antagonist, iberiotoxin (Buxton et al., 2011). SLO-1 channels of the filarial nematode *B. malayi* heterologously expressed in *Xenopus laevis* oocytes are also activated by emodepside (Kashyap et al., 2019). In the adult *B. malayi,* knockdown of *slo-1* transcript using dsRNA significantly reduces the emodepside induced paralysis, indicating that emodepside targets SLO-1 (Kashyap et al., 2019).

The *in vivo* efficacy of emodepside is complex. Whilst emodepside has adulticidal effects against Acanthocheilonema *vitae* in a single dose regimen, repeated doses were needed to clear *Litomosoides sigmodontis* (Zahner et al., 2001a). However, emodepside failed to kill *B. malayi* adults, although it did reduce the microfilaraemia (Zahner et al., 2001a). Various factors were attributed to this lack of effect on *B. malayi* adults, including the pharmacokinetics of emodepside limiting the distribution and concentration in the lymphatic system.

In this study, we have used motility assays using the Worminator system on adult female *B. malayi* to describe the potentiation of emodepside effects by DEC. We show that thapsigargin, a SERCA inhibitor that increases cytosolic calcium, also potentiates emodepside. We also show that the DEC potentiation of emodepside is independent of the spastic paralysis produced by DEC because it persists after the adult filariae have recovered their motility in DEC. We used RNAi with dsRNA to knock down the activation of TRP-2 channels by DEC to demonstrate that TRP-2 channels are required for the DEC potentiation of emodepside induced paralysis in *B. malayi.* These results suggest that a suitable and safe combination of DEC and emodepside could be useful to enhance the effects of emodepside against adults of recalcitrant filarial species.

2. Methods

2.1. Parasite maintenance

B. malayi adults were obtained from the NIH/NIAID Filariasis Research Reagent Resource Center (FR3; College of Veterinary Medicine, University of Georgia, Athens, GA, USA). Adult worms were maintained in non-phenol red Roswell Park Memorial Institute (RPMI) 1640 media (Life Technologies, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Fisher Scientific, USA) and 1% penicillin-streptomycin (Life Technologies, USA). The worms were stored individually in 24 well microtiter plates containing 2 mL RPMI -1640 media and placed in an incubator at 37 °C supplemented with 5% CO_2.

2.2. Brugia malayi motility analysis

Each worm was placed in a single well of the microtiter plate containing 1 mL of RPMI media containing L-glutamine. Movement of the worms in each 24-well microtiter plate was analyzed using the Worminator system, as described by Marcellino et al. (2012). Briefly, this is a visual imaging system that automates and quantifies the worm movement on each plate. The velocity of movement of the pixel units of the image of each worm is recorded to generate the motility per minute score.

For emodepside concentration-response analysis, the drugs were added, and the worms were recorded at times 0, 60-, and 120-min post-treatment using the WormAssay v1.4 software. Emodepside concentration response plots were fitted 60 min post emodepside treatment in all experiments. For dsRNA experiments, adult worms were soaked in either 30 μg/mL of *Bma trp-2*, LacZ dsRNA, or DNA/RNase free water. Motility assays were performed 72 h post dsRNA treatment. The % motility for each worm was calculated using the formula below:

$$\%\ \text{Motility} = \frac{\text{Motility per minute score of worm at 60 minutes post emodepside}}{\text{Motility per minute score of worm before drug treatment}} \times 100$$

2.3. Synthesis and delivery of dsRNA

dsRNA was synthesized as explained in (McCoy et al., 2015). Target and non-target T7 promoter labeled primers were amplified using the primers: trp2f, trp2r, trp2t7f and trp2t7r for the target Bma-*trp-2* and; LacZF, LacZR, LacZFt7 and LacZRt7 for the non-target LacZ. The sequences of these primers are shown in Table 1. Amplification was done using Techne® PRIMEG (Bibby Scientific Limited, UK) with cycling conditions: 95 °C x 5min, 35 x (95 °C x 30s, 55 °C x 30s, 72 °C x 1min), 72 °C x 10min from sequence-verified cDNA templates. dsRNA was synthesized by T7 RiboMAX™ Express RNAi kit (Promega, USA) according to the manufacturer's instructions. The concentration and purity of dsRNA were assessed using a spectrophotometer. Adult *B. malayi* were soaked in 30 μg/mL dsRNA for four days. Worms were maintained in RPMI media, as explained before. Worms were snap-frozen in liquid nitrogen and stored at −80 °C for transcript analysis.

2.4. Analysis of transcript levels

cDNA from dsRNA-treated worms was amplified using target (*Bma trp-2*) and reference gene (*Bma gapdh*) primers (Table- 1). These genes were amplified in triplicate by quantitative real-time PCR (qPCR) using the CFX96 Touch™ Real-Time PCR Detection System and SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, USA). Cycling conditions used were: 95 °C x 10min, 40 x (95 °C x 10s, 55 °C x 30s). PCR efficiencies were calculated using the CFX96 Software Suite (Bio-Rad, USA). Relative quantification of target gene knockdown was estimated by the ΔΔCt method (Pfaffl, 2001).

2.5. Drugs and solutions

Emodepside and diethylcarbamazine citrate were obtained from Millipore-Sigma (Darmstadt, Germany). They were dissolved in DMSO and water respectively. 1000x stocks were made for each concentration tested so the final concentration of the vehicle did not exceed 0.1%.

2.6. Data analysis

GraphPad Prism 7.0 (San Diego, CA) was used for data analysis. Mean ± S.E. values for each emodepside concentration data point were estimated using 6 to 11 different adult female *Brugia* worms. % Motility for was plotted against log emodepside concentrations for whole worm concentration-response relationships. Emodepside concentrations were $\log_{10}$ transformed before analysis. Concentration-response plots were constructed by fitting the data by nonlinear regression to determine the pEC_{50} values. Statistical analyses were performed on groups of values using 2-way ANOVA to determine if the group were significantly different. Bonferroni post hoc tests were used for multiple comparison tests to determine whether there were significant differences between groups. Significance levels were set to $P < 0.05$.

3. Results and discussion

3.1. DEC potentiates emodepside induced paralysis in adult female B. malayi

Emodepside has adulticidal effects on *B. malayi* (Kashyap et al., 2019). Sensitivity of emodepside varies between the sexes of adult *Brugia*: the female worms are 4.5x less sensitive than the males (Kashyap et al., 2019). DEC causes spastic paralysis of the worms, followed by

Table-1
List of primers used in this study.

Primer Name	Description	Sequence 5' - 3′
trp-2f	Bma trp-2 dsRNA PCR 5′	AAGAAGTACGTGGACCACCA
trp2r	Bma trp-2 dsRNA PCR 3′	TCGAAGTGCAACGGTACATA
trp-2fT7	Bma trp-2 dsRNA PCR with t7 promoter 5′	TAATACGACTCACTATA AAGAAGTACGTGGACCACCA
trp-2rT7	Bma trp-2 dsRNA PCR with t7 promoter 3′	TAATACGACTCACTATA TCGAAGTGCAACGGTACATA
SSK 5F	Bma GAPDH 5′ Fwd	GACGCTTCAAGGGAAGTGTTTCTG
SSK 5R	Bma GAPDH 3′ Rev	GTTTTGGCCAGCACCACGAC
LacZF	LacZ dsRNA 5′	CGTAATCATGGTCATAGCTGTTTC
LacZR	LacZR dsRNA 3′	CTTTTGCTGGCCTTTTGCTC
LacZFt7	LacZ dsRNA with t7 promoter 5′	TAATACGACTCACTATAGGGCGTAATCATGGTCATAGCTGTTTC
LacZRt7	LacZR dsRNA with t7 promoter 3′	TAATACGACTCACTATAGGGCTTTTGCTGGCCTTTTGCTC

recovery after 100 min (Verma et al., 2020). We tested the effect of 1 μM DEC on the flaccid paralytic effect of emodepside when both drugs were applied simultaneously. Fig. 1A shows concentration-dependent flaccid paralysis effect of emodepside on adult female *Brugia.* Fig. 1B&C shows that the paralysis caused by emodepside is significantly more potent in the presence of 1 μM DEC. The IC_{50} for emodepside alone was 447 nM ($pIC_{50} = 6.35 \pm 0.09$), and for the combination of emodepside + 1 μM DEC, it was 82 nM ($pIC_{50} = 7.08 \pm 0.08$) ($p < 0.001$, 2-way ANOVA).

We have previously shown that DEC activates TRP channels in *Brugia* muscle. TRP-2 channels conduct calcium, and activation of TRP-2 by DEC leads to a calcium influx into the cell (Verma et al., 2020). We hypothesized that increased cytosolic calcium due to DEC could potentiate the emodepside mediated activation of SLO-1 K channels and, therefore, worm paralysis. To test this hypothesis, we used 1 μM thapsigargin – a SERCA inhibitor that increases cytosolic calcium. Simultaneous application of emodepside and 1 μM thapsigargin significantly potentiated the emodepside mediated flaccid paralysis of adult female *B. malayi*. The IC_{50} for emodepside in this batch of worms was 418 nM ($pIC_{50} = 6.38 \pm 0.09$), and in the presence of 1 μM thapsigargin, it was 36 nM ($pIC_{50} = 3.62 \pm 0.06$), Fig. 1C ($p < 0.01$, 2-way ANOVA). Treating worms with thapsigargin alone did not produce flaccid paralysis but instead caused a transient spastic paralysis similar to DEC (data not shown).

3.2. DEC desensitization does not affect emodepside potentiation

We have previously shown that the effect of DEC on *B. malayi* motility is a transient spastic paralysis, lasting ~100 min and that the worms recover motility within 120 min even when maintained in the presence of the DEC (Verma et al., 2020). We investigated the DEC-mediated potentiation of emodepside to determine whether it was short-lasting and transient. We compared the concentration-dependent effects of emodepside immediately after the application of 1 μM DEC and at 120 min after exposure to 1 μM DEC. We observed no significant difference in emodepside potency in these two sets of worms (Fig, 2A). Thus, the potentiating effect of DEC on the action of emodepside lasts longer than the spastic paralysis that DEC produces.

3.3. TRP-2 activation by DEC required for emodepside potentiation

Using dsRNA treatment as previously described, we knocked down *trp-2* in adult female *B. malayi* (Verma et al., 2017). We observed 82.38% *trp-2* transcript knockdown in target dsRNA-treated worms and 20.75% knockdown in lacZ control worms (Fig. S1). We observed no significant change in the potency of emodepside when we compared the *trp-2* dsRNA-treated worms and control worms (Fig. 2B). Emodepside IC_{50} for *trp-2* knock-down worms was 288 nM ($pIC_{50} = 6.54 \pm 0.07$) and for *lacZ* dsRNA treated control worms, it was 395 nM ($pIC_{50} = 6.40 \pm 0.06$) ($p > 0.05$, 2-way ANOVA). These results suggest that TRP-2 channels do not interact genetically with SLO-1 channels in muscle cells.

We then treated *trp-2* knock-down worms with 1 μM DEC and emodepside simultaneously. We found that DEC did not significantly potentiate emodepside-induced paralysis in the knock-down worms (Fig. 2C). The IC_{50} values for *trp-2* dsRNA treated worms was 251 nM ($pIC_{50} = 6.60 \pm 0.8$) and was not significantly different from the *lacZ* control worms, 321 nM ($pIC_{50} = 6.54 \pm 0.07$) ($p > 0.05$, 2-way ANOVA). However, we saw significant potentiation of emodepside induced paralysis in *lacZ* dsRNA treated worms in the presence of 1 μM DEC and observed that the IC_{50} was reduced to 127 nM ($pIC_{50} = 7 \pm 0.08$), Fig. 2C ($p < 0.05$, 2-way ANOVA). The lack of potentiation in *trp-2* knock-down worms suggests that activation of the channel by DEC is required for the downstream potentiation of emodepside response.

4. Conclusions

Emodepside has been described as a potent microfilaricide in several studies (Kulke et al., 2014b; Kashyap et al., 2019; Hübner et al., 2021). In an *in vivo* study in bovines, emodepside showed potent macrofilaricidal effects against *O. ochengi* (Bah et al., 2021). In other adult filariae, like *B. malayi* and *L. sigmodontis,* the efficacy of emodepside in killing the adult worms was very modest (Zahner et al., 2001b). The sensitivity of the worm species appears to vary, as does the concentration of emodepside at the location of the worms *in vivo*. In dogs treated with an oral dose of emodepside of 1.9 mg/kg, the plasma concentration reaches 125 nM (Krüdewagen et al., 2015), although it is higher in the fat tissue. Worms residing in the lymphatic system may not receive a concentration of emodepside sufficient for the adulticide effect in the lymphatics, and this could explain the lack of effects of emodepside on adult *B. malayi.* The expression of SLO-1 isoforms also plays an important role in determining emodepside potency. In *B. malayi,* SLO-1F was described as more sensitive to emodepside than SLO-1A (Kashyap et al., 2019).

Combination therapy is used to treat several different nematode infections. Single-dose combinations of benzimidazoles with pyrantel and oxantel have been advocated against soil-transmitted helminths in recent years with relative success (Moser et al., 2019). MDA for lymphatic filariasis involves the combination of three anthelmintics: albendazole, DEC, and ivermectin. A recent trial found that a single dose of the triple therapy of ALB + DEC + IVM could clear *W. bancrofti* microfilaria more effectively than the combination of ALB + DEC (King et al., 2018). These previously described combination therapies exploit an additive effect that stems from separate drug target effects. We describe a proof of principle study here, where a combination of diethylcarbamazine and emodepside produces potentiation. The potentiation may be explained by the DEC-induced increase in cytosolic calcium and the calcium-dependent activation of the SLO-1 K channel, the target of emodepside. DEC is predicted to increase the potency of emodepside *in vivo* and kill worms in body locations where the bioavailability of emodepside is low.

There is an increasing interest in characterizing helminth TRP channels as anthelmintic targets. A recent study found a novel $TRPM_{pzq}$ receptor to be a target of praziquantel in *Schistosoma* spp. (Park et al., 2019). We have previously described that DEC activates TRPM and TRPC channels in *B. malayi* (Verma et al., 2020). Here, we observe that

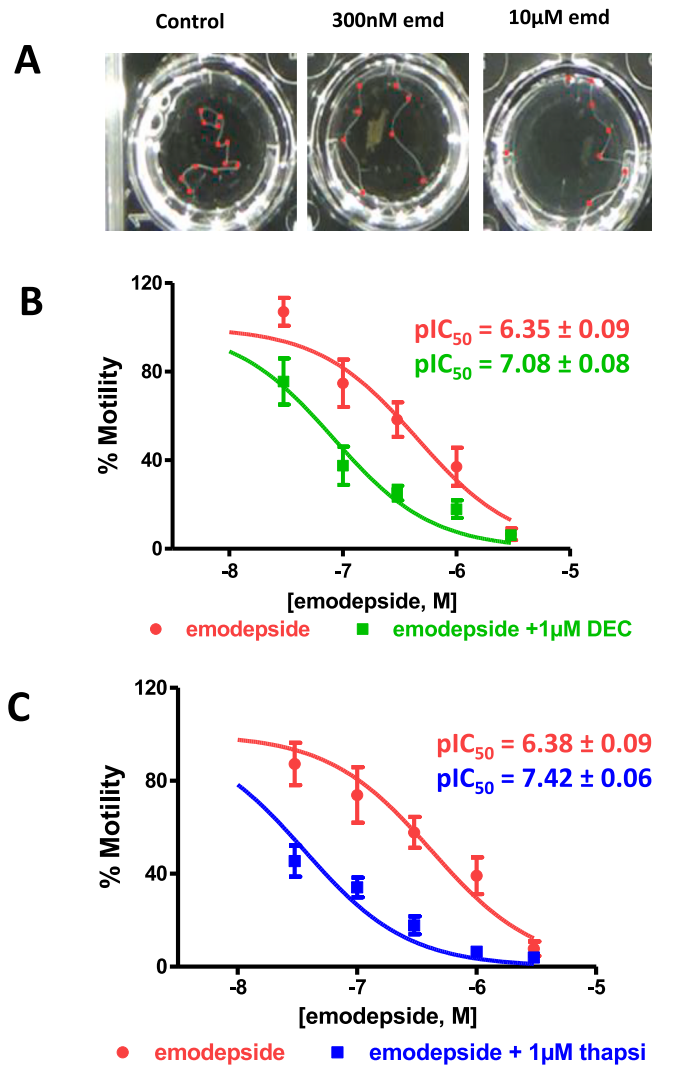

Fig. 1. Flaccid paralysis produced by emodepside and potentiation by diethylcarbamazine and thapsigargin.
A) Photographs of adult female *Brugia malayi* in a 96-well plate. A single worm is seen in each well and outlined by red dots. The control worm shows several bends as it moves, but as the emodepside concentration increases, it shows fewer bends and straightens out due to flaccid paralysis.
B) Shows potentiation of inhibitory effect of emodepside by 1 μM DEC. IC_{50} values were measured 60 min post emodepside treatment. IC_{50} - emodepside (pink): 447 nM ($pIC_{50} = 6.35 \pm 0.09$). Emodepside +1 μM DEC (green): 82 nM ($pIC_{50} = 7.08 \pm 0.08$) DEC had a significant potentiating effect on emodepside: 2-way ANOVA, <0.001. N = 7 worms.
C) (2-way ANOVA, p = 0.007). N = 8 worms for each concentration & DF = 42 for both plots
Shows 1 μM thapsigargin potentiating the effects of emodepside on the motility of adult female *B. malayi*. IC_{50}: emodepside (pink); 418 nM ($pIC_{50} = 6.38 \pm 0.09$). Emodepside + thapsigargin (blue): 36 nM ($pIC_{50} = 7.44 \pm 0.06$). 2-way ANOVA, significance of effect of thapsigargin $p < 0.001$. N = 7 worms. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

DEC potentiation requires the activation of the TRP-2 channel. RNAi of *trp-2* in adult *B. malayi* results in the loss of potentiating effects of DEC. Our result further highlights the role of TRP channels as potential anthelmintic targets.

The potentiation of emodepside by DEC needs to be evaluated in other filarial species both *in vitro* and *in vivo*. DEC is currently contraindicated in patients with onchocerciasis, but a low dose of DEC could be

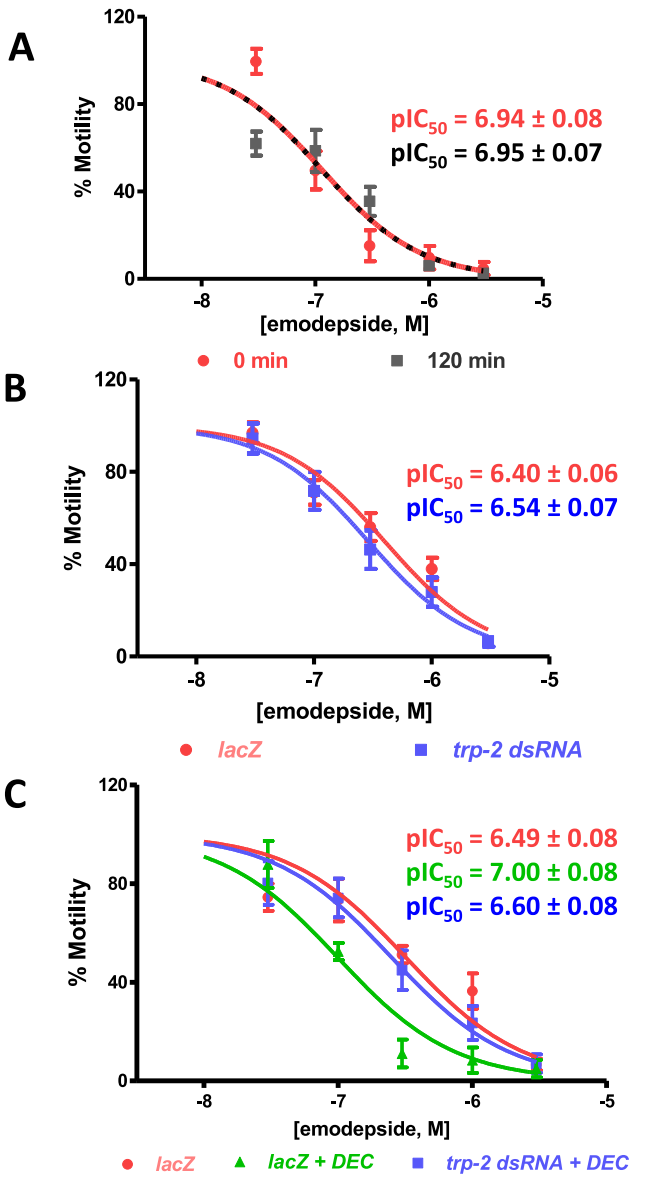

Fig. 2. Long-term effects of DEC on emodepside potentiation and effects of TRP channel knockdown.
A) Emodepside concentration-response curves on worms treated with 1 μM DEC at time 0 (pink) and 120 min (black) before emodepside treatment. IC_{50}: 114 nM ($pIC_{50} = 6.94 \pm 0.08$) and 113 nM ($pIC_{50} = 6.95 \pm 0.07$) respectively. The best fit lines superimpose.
B) Potency of emodepside is unaltered by the knockdown of *trp-2*. IC_{50}: lacZ dsRNA + emodepside (pink): 395 nM ($pIC_{50} = 6.40 \pm 0.06$). IC_{50}: *trp*-2 dsRNA + emodepside (purple): 288 nM ($pIC_{50} = 6.54 \pm 0.07$). No significant difference between lacZ and *trp-2* dsRNA: 2-way ANOVA, p = 0.44. N = 8 worms.
C) Knockdown of *trp-2* blocks the potentiation of emodepside effect caused by 1 μM DEC (blue). Non-specific dsRNA (*lacZ*) treated worms (pink) show increases sensitivity to emodepside in the presence of DEC. IC_{50}: lacZ dsRNA + emodepside (pink): 321 nM ($pIC_{50} = 6.49 \pm 0.08$) N = 7. IC_{50}: *lacZ* dsRNA + emodepside +1 μM DEC (green): 100 nM ($pIC_{50} = 7.01 \pm 0.08$). IC_{50}: *trp*-2 dsRNA + emodepside +1 μM DEC (purple): 251 nM ($pIC_{50} = 6.60 \pm 0.08$) N = 6 worms each. No significant difference was seen between the lacZ + emodepside (pink) and the trp-2 dsRNA + DEC + emodepside (blue); p = 0.57, 2-way ANOVA. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

helpful in improving the efficacy of emodepside. Emodepside is currently not being evaluated for lymphatic filariasis and its combination with DEC might prove to be very useful.

Declaration of competing interest

All authors have and declare no conflict of interest for the manuscript above for publication in IJPDDR.

Acknowledgments

We want to thank NIH/NIAID Filariasis Research Reagent Repository Center (www.filariasiscenter.org) for the regular supply of adult and microfilariae of *Brugia* malayi worms. The research was funded by: NIH NIAID grants R01AI047194, R01AI155413 and R21AI138967 to RJM and; The EA Benbrook Endowed Chair of Pathology and Parasitology. The funding agencies had no role in the design, execution or publication of this study. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2022.10.002.

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