Veterinary Parasitology 270 (2019) 40–46

Contents lists available at ScienceDirect

Veterinary Parasitology

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

Research paper

The antitrypanosomal diarylamidines, diminazene and pentamidine, show anthelmintic activity against *Haemonchus contortus in vitro*

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ARTICLE INFO

Keywords:
Anthelmintic
Drug discovery
Repurposing drugs
Nematodes
Livestock
Venom

ABSTRACT

Parasitic nematodes pose a major threat to livestock production worldwide. The blood-feeding parasite *Haemonchus contortus* is a key small-ruminant pathogen that causes anaemia, and thereby seriously impacts animal health and production. Control of this parasite relies largely upon broad-spectrum anthelmintics, but new drugs are urgently needed to combat the threat of widespread multidrug resistance. Repurposing drugs can accelerate the development pipeline by reducing costs and risks, and can be an effective way of quickly bringing new antiparasitic drugs to market. Diarylamidine compounds such as pentamidine and diminazene have been employed in the treatment of trypanosomiasis and leishmaniasis in both human and veterinary settings, but their activity against parasitic worms has not yet been reported. We screened a small panel of diarylamidine compounds against *H. contortus* to assess their potential to be repurposed as anthelmintic drugs. Pentamidine and diminazene inhibited *H. contortus* larval development at low micromolar concentrations (IC_{50} 4.9 μM and 16.1 μM, respectively, in a drug-susceptible isolate) with no existing cross-resistance in two multidrug resistant isolates and a monepantel-resistant isolate. Combinations of pentamidine with commercial anthelmintics showed additive activity, with no significant synergism detected. Pentamidine and diminazene showed different life-stage patterns of activity; both were active against early stage larvae in development assays, but only diminazene was active against the infective L3 stage in migration assays. This suggests some differences in uptake of the two drugs across the nematode cuticle, or differences in the nature and expression patterns of their molecular targets. As pentamidine and diminazene have been reported to be potent inhibitors of mammalian acid-sensing ion channels (ASIC), we tested the activity of known ASIC inhibitors against *H. contortus* to probe whether these channels may represent potential anthelmintic targets in nematodes. Remarkably, the spider-venom peptide Hi1a, a potent inhibitor of ASIC1a, inhibited *H. contortus* larval development with an IC_{50} of 22.9 ± 1.9 μM. This study highlights the potential use of diarylamidines as anthelmintics, although their activity needs to be confirmed *in vivo*. In addition, our demonstration that ASIC inhibitors have anthelmintic activity raises the possibility that this family of ion channels may represent a novel anthelmintic target.

1. Introduction

Parasitic nematodes continue to threaten livestock production around the globe. The Australian sheep industry is particularly threatened by gastrointestinal nematodes, which cost in excess of AUD $430 million annually in production losses, treatments and death (Lane et al., 2015). One of the most serious of these pathogens is *Haemonchus contortus*. *H. contortus* feeds on blood within the abomasum, causing anaemia, reduced reproductive capacity, and impairment of wool and meat production, thereby severely impacting sheep welfare and the economic viability of sheep production (Besier et al., 2016; Roeber et al., 2013). Heavy infections can drain as much as 400 ml of host blood per day and be rapidly fatal (Le Jambre, 1995).

Control of parasitic nematode infections has relied almost exclusively upon broad-spectrum anthelmintics; however, excessive use has led to widespread resistance that threatens the sustainability of production systems (Kaplan and Vidyashankar, 2012). *H. contortus* has developed resistance to all anthelmintic drug classes used against it, often within 10 years of the drug being introduced (Kotze and Prichard, 2016). The most recently introduced group of anthelmintics, the amino-acetonitrile derivatives, have been no exception with resistance to the drug monepantel reported in the Netherlands just three years after its introduction (Van den Brom et al., 2015). One strategy used to overcome resistance in Australia is combination drug therapy, however

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https://doi.org/10.1016/j.vetpar.2019.05.008
Received 4 April 2019; Received in revised form 17 May 2019; Accepted 18 May 2019

reports of multi-drug resistance further complicate managing infections (Lyndal-Murphy et al., 2014). New drugs are urgently needed to ensure sheep welfare and the continued sustainability of the Australian sheep industry.

Repurposing drugs has become a mainstay for parasitic worm drug development (Panic et al., 2014). Repurposing drugs enables accelerated development due to lower costs and the availability of existing preclinical data (Padhy and Gupta, 2011). The potential for accelerated development offered by repurposing drugs could be extremely valuable for responding to anthelmintic resistance by bridging the gap until new anthelmintics with novel modes of action can be developed and introduced.

Here we investigated the activity of a small panel of anti-trypanosomal diarylamidine compounds against *H. contortus*. Diminazene aceturate has been used for the treatment of veterinary trypanosomiasis since the 1950s and today is the most commonly used trypanocide for cattle, sheep and goats due to its relatively low toxicity and activity against both *Trypanosoma congolense* and *T. vivax* (Giordani et al., 2016). The related diarylamidine pentamidine has been used in the treatment of Human African Trypanosomiasis (HAT) since the 1940s (Babokhov et al., 2013). It is also employed against leishmaniasis, pneumocystis pneumonia in Acquired Immunodeficiency Syndrome (AIDS) patients, and has been shown to sensitise gram-negative bacteria to antibiotics (Lanteri et al., 2004; Stokes et al., 2017). Related aromatic diarylamidine compounds such as stilbamidine, furamidine and 4′,6-diamidino-2-phenylindole (DAPI) also have antitrypanosomal effects (Farahat et al., 2017; Giordani et al., 2016). However, these compounds have not been tested against parasitic worms. We report that pentamidine, diminazene and DAPI all inhibit *H. contortus* larval development at low micromolar concentrations, indicating that the diarylamidine class of compounds may provide a new avenue for anthelmintic drug discovery. Given the recent focus on the use of drug combinations as a means to counter anthelmintic resistance (Bartram et al., 2012; Leathwick, 2012), and several reports of synergistic interactions between anthelmintics (Puttachary et al., 2013; Rolfe et al., 2013), we investigated the interactions between the most active diarylamidine and the currently-used commercial anthelmintics. As diarylamidines are known to inhibit acid-sensing ion channels (ASICs) (Chen et al., 2010; Lee et al., 2018), we also investigated the effects of known ASIC inhibitors (using four venom-derived peptides, as shown in Table 1, and amiloride) to probe whether these channels might represent a potential target for anthelmintics.

2. Methods

2.1. Parasites

Four isolates of *H. contortus* were used for the study:

i) Kirby: isolated from the field at the University of New England Kirby Research Farm in New South Wales (NSW), Australia 1986; susceptible to all commercial anthelmintics (Albers and Burgess, 1988).
ii) Wallangra: isolated from the New England region of Northern NSW in 2003; resistant to benzimidazoles, closantel, levamisole (LEV), and macrocyclic lactones (Love et al., 2003).
iii) GWBII: a variant of the Wallangra isolate that has been passaged with repeated Triton® drench treatments (containing ivermectin (IVM), albendazole and LEV) and is broadly drug-resistant (Kotze et al., 2018).
iv) MPL-R: isolated from a property in southwest Queensland, Australia, in 2014, as described previously (Raza et al., 2016). Resistant to Zolvix® (active ingredient = monepantel, MPL), benzimidazoles and macrocyclic lactones (Kotze et al., 2018).

Infected sheep were housed at the Invetus animal house facility in Armidale, NSW, and at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) FD McMaster laboratory near Armidale, NSW. All animal procedures were approved by the University of New England's Animal Ethics Committee (Approval Number 2015-065), or the FD McMaster Animal Ethics Committee, CSIRO (Approval Number AEC 13/23). Faeces were collected into bags strapped to the animals, and sent by overnight courier at ambient temperature to the CSIRO laboratory in Brisbane, Queensland, for recovery of eggs. Some faecal samples were also used to establish larval cultures for recovery of the L3 life stage.

2.2. Chemicals

Diminazene, pentamidine, furamidine and amiloride were purchased from Sigma Aldrich as ≥98% pure diminazene aceturate, pentamidine diisethionate, furamidine dihydrochloride and amiloride hydrochloride hydrate, respectively. Aminostilbamidine methanosulfonate was obtained from Santa Cruz Biotechnology. Compounds were prepared in distilled water at 10 mg/ml, followed by 2-fold serial dilutions in water.

Technical grade ivermectin (IVM), thiabendazole (TBZ) and levamisole hydrochloride (LEV) were purchased from Sigma Chemical Co. Stock solutions were prepared in dimethyl sulfoxide (DMSO) at 10 mg/mL, followed by 2-fold serial dilutions in DMSO to generate multiple working solutions. The commercial drench product Zolvix® was used as a source of monepantel (MPL). The drench solution (25 mg/ml MPL) was serially diluted 2-fold in DMSO to generate a series of working solutions.

2.3. Peptides

Recombinant venom peptides Hi1a, PcTx1, and APeTx2 were generated by periplasmic expression in *E. coli* according to the general method described by Klint et al. (2013). Specific methods have been published for recombinant production of Hi1a (Chassagnon et al., 2017), PcTx1 (Saez et al., 2011), and APeTx2 (Anangi et al., 2012). Briefly, the peptides are expressed in the periplasm of *E. coli* as tobacco etch virus (TEV) protease-cleavable fusion proteins containing a maltose-binding protein (MBP) tag and an N-terminal His_6 tag to facilitate purification. Fusion proteins were purified from the soluble cell lysate over nickel affinity resin. Peptides were then liberated from the fusion protein by cleavage with TEV protease and purified by reverse-phase HPLC using a C18 column. Peptide masses were confirmed by direct injection on a Shimadzu LC/MS-2020 using electrospray ionisation mass spectrometry in positive detection mode and ASIC activity confirmed using two-electrode voltage-clamp electrophysiology as

Table 1
Venom-derived ASIC inhibitory peptides used in this study.

Toxins	Venom source	Known activity	References
Hi1a	Spider: *Hadronyche infensa*	ASIC1a inhibitor	(Chassagnon et al., 2017)
PcTx1	Spider: *Psalmopoeus cambridgei*	ASIC1a inhibitor	(Chen et al., 2005; Escoubas et al., 2000)
Mambalgin-2	Snake: *Dendroaspis polyepsis polyepsis*	ASIC1a/b inhibitor	(Diochot et al., 2012)
APeTx2	Sea anemone: *Anthopleura elegantissima*	ASIC3 inhibitor	(Diochot et al., 2004)

described previously (Chassagnon et al., 2017). Mambalgin-2 was produced by solid-phase peptide synthesis according to the method described by Schroeder et al. (2014). In brief, the peptide was synthesised in fragments: peptide thioesters corresponding to residue 1–18 and 19–36 were synthesised using *tert*-butyloxylcarbonyl (Boc) chemistry, while fragment 37–57 was synthesised using Fmoc chemistry (Fmoc = 9-fluorenylmethoxycarbonyl). Fragments were subsequently ligated using native chemical ligation (Dawson et al., 1994) in a one-pot reaction and folded in a redox buffer (100 mM Tris pH 8.0, 500 mM guanidine HCl, 1 mM oxidised glutathione and 8 mM reduced glutathione). The full-length peptide was obtained by reverse-phase HPLC purification and mass confirmed using liquid-chromatography on a QSTAR mass spectrometer coupled with an Agilent 1100 HPLC system, and by electron spray ionisation mass spectrometry on an API-2000 mass spectrometer (Applied Biosystems). Purified peptides were prepared in distilled water in two-fold serial dilutions to give a final concentration range of 0.5–200 µM.

2.4. Larval development assay (LDA)

H. contortus eggs were prepared as described by Kotze et al. (2009). In brief, faeces were filtered through 250 and 75 µm mesh filters, allowed to settle for 40 min, then the supernatant was removed by vacuum. Eggs were separated from the remaining faecal material by density centrifugation using 10 and 25% (w/v) sucrose solutions, centrifuged at 650 *g* for 7 min. Eggs were recovered from the interface of the two sucrose layers, rinsed with distilled water, sterilised with bleach, rinsed again, and diluted to 4500 eggs/ml. Tylosin tartrate (800 µg/ml) and amphotericin B (25.0 µg/ml) were added, and eggs were used immediately for LDAs.

LDAs were conducted in 96-well microtitre plates, with each well containing 50 µl of 2% agar, 20 µl of egg solution, and either 20 µl of drug solution in water or 1 µl of anthelmintic dissolved in DMSO (and 19 µl of water, final 1% v/v DMSO). Controls contained either water or DMSO (final concentration 1% v/v) depending on whether the anthelmintic agent being tested had been dissolved originally in water or DMSO. Plates were incubated at 26 °C. After 24 h, 10 µl of a nutrient solution containing *E. coli* XL1-Blue1 (grown overnight at 37 °C) and growth medium was added to each well. The growth medium consisted of yeast extract (1% w/v), Earle's salt solution (10% v/v), saline solution (0.9% NaCl, w/v), and sodium bicarbonate (1 mM) in Luria-Bertani medium (LB). Larvae were incubated for six days, then killed and stained with Lugol's iodine solution. Larvae that had developed to the infective L3 stage were counted, and the numbers in drug-treated assay wells were expressed as a percentage of the number of infective L3 stage larvae in multiple control wells.

Median inhibitory concentrations (IC_{50}) were calculated using non-linear regression analysis in GraphPad Prism 7.0. IC_{50} values were calculated from three experiments with triplicate wells for drugs, and two experiments with duplicate wells for venom-derived peptides. Cross-resistance was investigated by comparing IC_{50} values for the various diarylamidines across the three drug-resistant isolates against the value for the Kirby isolate using one-way ANOVA with Tukey's post-hoc test in GraphPad Prism 7.0.

2.5. Larval migration assay

The activities of pentamidine and diminazene were examined in a larval migration assay (LMA) as described by Kotze et al. (2006), except that the filter meshes were not overlaid with agar. L3 larvae were collected from culture flasks, and allowed to pass through a 20 µm cloth suspended in dH_2O. Larvae were collected and stored at 15 °C. The assay was conducted in 96-well assay plates. L3 stage larvae (˜3000 larvae/ml) were soaked for a period of 48 h in two-fold dilutions of drug solution. Controls for diminazene, pentamidine and amiloride were equivalent volumes of distilled water while controls for ivermectin and levamisole were exposed to 1% v/v DMSO. The larvae were then transferred to the wells of multiscreen mesh filter plates (20 µm filter) (Millipore, Australia) suspended in distilled water above 'receiver' plates. After 4 h, larvae in the receiver plate were stained and killed with Lugol's iodine solution and counted. Triplicate assays were repeated three times for concentration-response experiments. IC_{50} values were calculated for each of the three experiments, using non-linear regression analysis in GraphPad Prism 7.0.

2.6. Drug combination assays

To investigate possible synergistic effects between pentamidine and commercial anthelmintics, concentration-response LDAs were conducted with drugs combined using a constant ratio method. We compared concentration-responses derived from the experiments using combinations of drugs ('observed' responses), with the concentration-responses that would be expected if the drugs were acting in an additive fashion ('expected' responses), as described previously (Kotze et al., 2018). We did not use isobolograms for this analysis as the steep nature of the concentration-responses to several of the drugs when used alone (with only two data points between 0% and 100% larval development) resulted in fewer data points than recommended for an accurate isobologram analysis (Chou, 2010, 2014).

Initial assays of each drug alone were used to calculate IC_{50} values, as described in Section 2.3, and the subsequent relative IC_{50} values were used to approximate drug combination ratios. The final pentamidine:commercial anthelmintic ratios were: 1:10 for LEV (IC_{50} ratio 9.2); 1:100 for TBZ (IC_{50} ratio 117); 1:1000 for MPL (IC_{50} ratio 500) and 1:1000 for IVM. The indicated IC_{50} ratio for IVM was ~9000; however, we used 1:1000 for the combination experiments (approximately ten-fold lower) to avoid excessively diluting IVM in the combination to the extent (9000 fold) indicated by the IC_{50} ratio. Separate series of 2-fold dilutions were then prepared from each stock solution, and their effects were examined in LDAs. Each drug combination was examined in two separate experiments, with triplicate assay wells at each concentration.

The concentration-response data for each drug combination was then fitted using non-linear regression in GraphPad Prism 7.0 in order to derive IC_{50} values, as described in Section 2.3. This became the 'observed' response which was compared with the 'expected' response, based on the assumption that the two drugs were working in an additive fashion. The expected response for each drug combination concentration was calculated separately, based on initial assays using the drugs alone, as follows:

$$\textit{Expected response} = X + (100 - X)^* \frac{Y}{100}$$

where X is the % development observed in the presence of the commercial drug alone, Y is the % response at the corresponding concentration of pentamidine according to the ratios described above. The expected responses at each drug combination concentration were then used to generate expected concentration-response curves and IC_{50} values in GraphPad Prism 7.0. Deviations from an additive interaction, that may indicate drug antagonism or synergism, were assessed by comparing expected and observed concentration-response curves, as well as comparison of IC_{50} values using paired two-tailed parametric t-tests, with significance if $P < 0.05$.

3. Results

3.1. Diarylamidine screen against H. contortus larval development

The panel of antitrypanosomal diarylamidines was screened against larval stages of *H. contortus* in LDAs using the drug-susceptible Kirby isolate, a broadly drug-resistant isolate (GWBII), and a monepantel-resistant isolate (Fig. 1, Table 2). Pentamidine and diminazene were

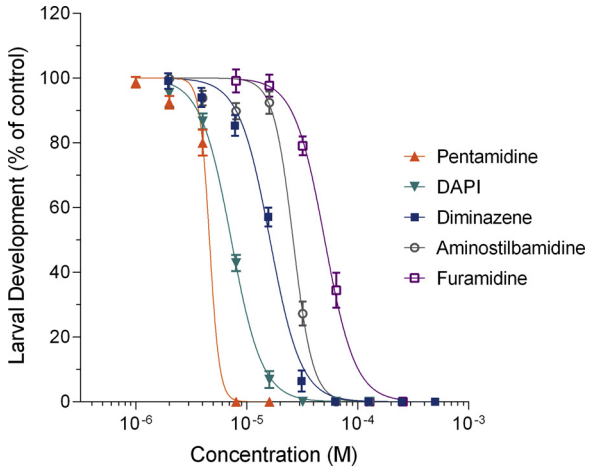


Fig. 1. Concentration-response curves for diarylamidine compounds against *H. contortus* Kirby isolate in LDAs. Each data point represents mean ± SEM, n = 9 (pooled data from three experiments, each with triplicate assay at a range of drug concentrations).

also tested against the broadly drug-resistant Wallangra isolate. Pentamidine was the most potent inhibitor, with an IC_{50} of 4.9 ± 0.7 μM against Kirby. DAPI showed similar potency, with an IC_{50} of 7.3 ± 0.1 μM against Kirby. Diminazene showed moderate efficacy, with an IC_{50} of 16.1 ± 1.9 μM. Aminostilbamidine also showed moderate activity (IC_{50} values ranging from 26.2 to 28.2 μM across the three isolates), while furamidine showed only weak activity (IC_{50} values > 50 μM). We compared the IC_{50} values for each of the strains using one-way ANOVA to investigate the presence of cross-resistance. Only furamidine showed a significantly higher IC_{50} value in the GWBII resistant isolate compared to drug-susceptible Kirby ($P = 0.02$); however, the extent of the cross-resistance was very small, with resistance ratios (ratios of IC_{50} values) of only 1.3-fold. All other drugs were equally effective across isolates, indicating no cross-resistance. The broadly-drug resistant Wallangra isolate also showed no cross-resistance towards the two drugs tested against it (Table 2).

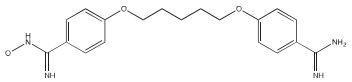
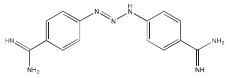
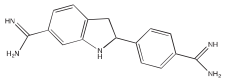
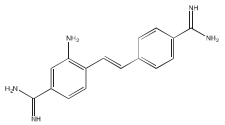
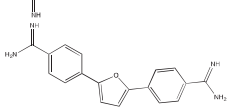
3.2. Pentamidine does not act synergistically with commercial anthelmintics

We next investigated interactions between pentamidine and four commercial anthelmintics (LEV, TBZ, IVM and MPL) using constant-ratio drug combinations in LDAs. Possible drug interactions were examined by comparing concentration-response curves and IC_{50} values for the observed versus expected responses calculated using the assumption of an additive interaction. Fig. 2 shows the concentration-response curves for pentamidine alone, as well as the expected and observed curves for pentamidine in combination with each of the commercial anthelmintics; overlay of expected and observed concentration-response curves would indicate that the two drugs act in an additive fashion, while displacement of the observed response curve to the left or right would indicate the presence of synergism or antagonism, respectively. As shown in Fig. 2, the concentration-response curves were completely overlaid for IVM and TBZ, indicating additive interactions. The observed concentration-responses for LEV and MPL were shifted slightly to the left of the expected responses, however, in both cases the IC_{50} values for the expected and observed curves were not significantly different ($P > 0.05$). Therefore, pentamidine does not act synergistically with the tested commercial anthelmintics but does show additive effects, thereby lowering the required drug concentrations at the IC_{50} for the combinations compared to the IC_{50} for each drug when used alone.

3.3. Diminazene inhibits L3 migration

We also assayed diminazene and pentamidine against the infectious L3 stage in LMAs, and compared their activity to the commercial anthelmintics LEV and IVM. Pentamidine showed no activity against L3 migration, even up to 2 mM concentration (Table 3). In contrast, diminazene showed significant anthelmintic activity, with IC_{50} values of 59.5 ± 6.7 μM and 116.0 ± 12 μM in Kirby and drug-resistant Wallangra, respectively. Diminazene was approximately 6-fold more potent than the commercial anthelmintic levamisole in the drug-susceptible isolate and three-fold more potent in the multidrug resistant isolate (IC_{50} > 300 μM for Kirby and Wallangra respectively). Diminazene was also more potent than ivermectin against Wallangra (IC_{50} > > 100 μM, the highest concentration tested).

Table 2
Activity of diarylamidine compounds against drug-susceptible Kirby, broadly-resistant GWBII and Wallangra, and monepantel-resistant (MPL-R) isolates in *H. contortus* larval development assays. Values are shown as IC_{50} ± SEM, calculated from three separate experiments each with triplicate assays at a range of drug concentrations ($n = 9$). Compounds were analysed for cross-resistance using one-way ANOVA, comparing Kirby values to drug-resistant isolates. $^{\#}$Denotes significance between the drug-susceptible *H. contortus* Kirby isolate and drug-resistant isolates ($P < 0.05$).

Compound		IC_{50} ± SEM μM in LDA			
		Kirby	GWBII	MPL-R	Wallangra
Pentamidine		4.9 ± 0.7	5.4 ± 0.3	6.4 ± 0.9	4.6 ± 0.8
Diminazene		16.1 ± 1.9	17.9 ± 2.1	21.3 ± 3.8	15.2 ± 2.7
DAPI		7.3 ± 0.1	5.6 ± 0.9	8.1 ± 0.8 $^{\#}$	—
Aminostilbamidine		26.2 ± 3.8	28.2 ± 4.1	23.9 ± 4.1	—
Furamidine		50.5 ± 3.3	67.2 ± 4.7$^{\#}$	52.5 ± 4.1	—

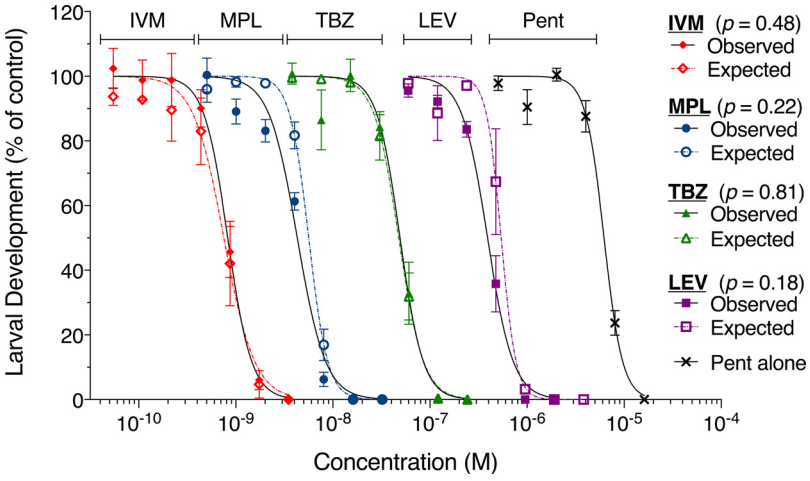


Fig. 2. Comparison of expected and observed concentration-response curves in LDAs for pentamidine (pent) in combination with commercial anthelmintics. Expected responses were calculated assuming an additive interaction between pairs of drugs. The drug combinations were based on constant ratios of IVM:pent at 1:1000; MPL:pent at 1:1000 ratio; TBZ:pent at 1:100 ratio; and LEV:pent at 1:10 ratio. The drug-susceptible *H. contortus* Kirby isolate was used for LDAs. Observed data points represent mean ± SEM, n = 6 (pooled data from two experiments, each with triplicate assays at a range of drug concentrations) and expected data points represent mean ± SEM calculated for each concentration for the two separate experiments.

Table 3
Activity of diarylamidines and commercial anthelmintics against drug-susceptible Kirby and broadly-resistant Wallangra *H. contortus* isolates in larval migration assays. Values are shown as IC_{50} ± SEM, calculated from three experiments, each with assays in triplicate over a range of drug concentrations.

Drug	Kirby (IC_{50} ± SEM μM)	Wallangra (IC_{50} ± SEM μM)
Pentamidine	> 2000	> 2000
Diminazene	59.5 ± 6.7	116 ± 12
IVM	3.8 ± 0.57	> 100
LEV	338 ± 14	367 ± 36

3.4. Assays with known inhibitors of a putative diarylamidine molecular target

Given the known potency of diminazene and pentamidine against acid-sensing ion channels (ASIC) (Chen et al., 2010) we investigated their mode of action against *H. contortus* by screening known ASIC inhibitors for anthelmintic activity. We tested four venom-derived toxins with known activity on the ASIC1a/b and ASIC3 subtypes, and amiloride, which is a non-selective blocker of channels within the epithelial sodium channel (ENaC) family, including ASICs. We found that the Australian funnel-web spider venom-derived peptide Hi1a inhibited larval development with an IC_{50} 22.9 ± 1.9 μM (Fig.3, Table 4). However, the other ASIC1a inhibitor tested, PcTx1, showed only very weak activity at high micromolar concentrations (IC_{50} ˜250 μM). Amiloride showed weak anthelmintic activity (IC_{50} 142.7 ± 6.1 μM). No activity was observed from the other ASIC-modulating toxins.

4. Discussion

In this study we provided the first demonstration of *in vitro* anthelmintic activity for the antitrypanosomal drugs diminazene and pentamidine, along with several related compounds, against *H. contortus*. We show that pentamidine and diminazene inhibit *H. contortus* larval development at low micromolar concentrations, with no existing cross-resistance to two broadly drug-resistant isolates (GWBII and Wallangra) and a monepantel-resistant isolate (MPL-R).

DAPI also inhibited larval development, but its reported cytotoxicity raises concerns about its suitability for veterinary use; for example, it is toxic to rat myoblasts with an EC_{50} of 0.86 μM (Farahat et al., 2017). In future work it would be interesting to screen analogues of DAPI with reduced cytotoxicity such as those described by Farahat et al. (2017) which were administered *in vivo* for treatment of trypanosomiasis with 100% cure rates. Other diarylamidine derivatives, such as DB75 and the related prodrug DB289 (in phase IIB clinical trials for HAT) (Lanteri et al., 2004), may also be useful to screen.

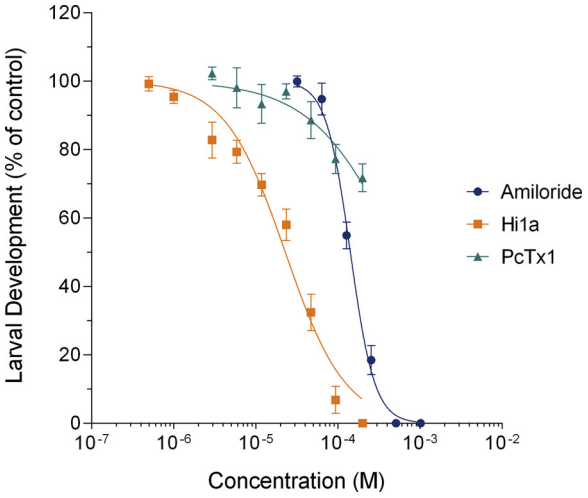


Fig. 3. Concentration-response curves for effects of Hi1a, PcTx1 and amiloride on larval development of the *H. contortus* Kirby isolate. Due to limited compound availability, the data points for Hi1a and PcTx1 represent mean ± SEM, n = 4 (pooled data from two experiments of duplicate assays). For amiloride, each data point represents mean ± SEM, n = 9 (pooled data from three experiments of triplicate assays).

Table 4
Activity of venom-derived ASIC inhibitors and amiloride against drug-susceptible *H. contortus* Kirby in LDAs. Values are shown as IC_{50} ± SEM. Due to limited compound availability, IC_{50} values for toxins were calculated from two experiments, each with duplicate assays over a range of concentrations (n = 4). For amiloride, IC_{50} values were calculated from three experiments, each with triplicate assays over a range of concentrations (n = 9).

Toxin/compound	IC_{50} ± SEM μM
Hi1a	22.9 ± 1.9
PcTx1	> 200
Mambalgin-2	> > 200
APeTx2	> > 200
Amiloride	142.7 ± 6.1

Pentamidine looked to be a promising anthelmintic candidate as its IC_{50} of 4.9 μM in the LDA against drug-susceptible *H. contortus* is only about 10-fold higher than the commercial anthelmintic levamisole. However, it did not inhibit *H. contortus* L3 larval migration. The LMA is considerably less sensitive than the LDA for some anthelmintic compounds– for example levamisole was over 400-fold less potent in the

LMA relative to the LDA. We tested pentamidine up to 2 mM (approximately 500-fold higher than the IC_{50} in the LDA) but observed no activity. In contrast, diminazene had a low micromolar IC_{50} in the LDA and it retained activity in the LMA compared to pentamidine (IC_{50} against Kirby of 59.5 μM and > 2000 μM, respectively, in the LMA). Hence, the life-stage patterns of activity were quite different for the two compounds, although the reason for this remains unclear. Possible explanations may lie firstly in differences in uptake of the two compounds across the cuticle in the non-feeding life-stage examined in the LMA (as the L3 has a non-functioning pharynx), or secondly, differences in the nature of the targets for the two compounds, and in the life-stage expression patterns of these targets.

These differences in the life-stage patterns of activity highlight the importance of using multiple life-stages and phenotypes in drug screening studies and for selecting *in vivo* candidates. In the current study, using the L3 migration assay alone would not have highlighted the anthelmintic potential of pentamidine. Similarly, benzimidazole anthelmintics are potent inhibitors of larval development, but show very little activity in the migration assay used in the present study (Kotze, unpublished data). Measuring the activity of diminazene and pentamidine against adult life-stages of *H. contortus in vivo* will be required to confirm their potential as anthelmintics.

Combination drug therapy is one of the key strategies for slowing the development of anthelmintic resistance (Bartram et al., 2012; Leathwick, 2012). Such drug combinations may have greater benefit in terms of efficacy if the drugs act synergistically, as has been reported for abamectin and derquantel *in vitro* (Puttachary et al., 2013), and abamectin and MPL *in vivo* (Rolfe et al., 2013). We therefore investigated whether pentamidine showed any interactions with the key commercial anthelmintics LEV, TBZ, IVM and MPL, but found no evidence of synergy. However, we found that each of the drug combinations worked in an additive manner, with no indication of antagonism, so they may be useful in the field as a combination therapy for slowing the development of resistance.

The mechanism of action of pentamidine and diminazene against trypanosomes remains to be fully elucidated. A number of activities have been described for these compounds; for example, diminazene has been shown to modulate the host immune response to *T. congolense* infection in mice (Kuriakose et al., 2012), while pentamidine has been found to modify ubiquitin in *Leishmania infantum* (Nguewa et al., 2005). Pentamidine and other diarylamidines interfere with mitochondrial function in yeast (Lanteri et al., 2004; Zhang et al., 2000). Most notably, the diarylamidine compounds are known to bind to the minor groove of DNA and thereby interfere with kinetoplast replication in trypanosomes (Wilson et al., 2008). DAPI, diminazene and pentamidine are also moderately potent inhibitors of ASICs (Chen et al., 2010). Although ASICs are chordate-specific sodium channels that are activated by acidic pH (Lingueglia and Lazdunski, 2013), they are closely related to the nematode-specific degenerin channels which are speculated to be anthelmintic targets (Wolstenholme, 2011). Jospin and Allard (2004) demonstrated a pH-sensitive sodium current in *C. elegans* muscle that is sensitive to amiloride, the prototypical inhibitor of the degenerin/epithelial sodium channel (ENaC) family of ion channels, suggesting that an ASIC-like channel may be present in nematodes.

To investigate whether inhibition of ASIC-like channels may be involved in the anthelmintic action of diarylamidines, we examined whether known ASIC-inhibitors (venom toxins and the small molecule amiloride) have anthelmintic activity. We found that venom-peptide Hi1a, the most potent described inhibitor of mammalian ASIC1a (Chassagnon et al., 2017; Cristofori-Armstrong and Rash, 2017), has moderate anthelmintic activity, inhibiting larval development of *H. contortus* with an IC_{50} 22.9 ± 1.9 μM. This is the first report of a spider-venom peptide showing anthelmintic activity. This finding, along with the identification of a pH-sensitive sodium channel in *C. elegans* (Jospin and Allard, 2004), raises the possibility of ASIC-like channels in nematodes that could be exploited as potential anthelmintic targets.

Interestingly, Hi1a was the only ASIC inhibitor to show significant anthelmintic activity. An NCBI BLAST search of human ASIC protein sequences against the *H. contortus* genome/transcriptome (Laing et al., 2013) revealed a number of putative ENaC/degenerin family channels, but with low sequence identity (20–30%) (Supplementary Table 1), indicating that if such channels exist in nematodes they may have quite different function and pharmacology to their mammalian counterparts. Differences in pharmacology might explain the observed differences in anthelmintic activity of PcTx1 and Hi1a, which both inhibit ASIC1a but via different mechanisms. PcTx1 promotes and stabilises the desensitised, non-conducting state of ASIC1a (Chen et al., 2005; Dawson et al., 2012). In contrast, Chassagnon et al. (2017) whereas Hi1a inhibits channel activation. Alternatively, the anthelmintic action of Hi1a may be via a currently unknown target in nematodes as the selectivity of the peptide against other channels (e.g. ENaC) has not been fully explored.

The present study suggests that the antitrypanosomal compounds pentamidine and diminazene may be a useful starting point for development of new anthelmintics directed against gastrointestinal nematodes. Diminazene is well tolerated in sheep and cattle for treatment of babesiosis and trypanosomiasis (Peregrine and Mamman, 1993), so it may be useful for treating *Haemonchus* species in sheep and cattle although this remains to be confirmed *in vivo*. One challenge in developing diminazene as an anthelmintic for broad use beyond sheep and cattle is the toxicity that has been observed in some animals, notably camels and dogs (Joubert et al., 2003; Riou and Benard, 1980). New research developing structural analogues of various diarylamidines may overcome these toxicity issues and improve potency against parasites. The surprising anthelmintic activity of the spider toxin Hi1a, and the possibility of ASIC-like channel involvement in the anthelmintic activity of diarylamidines, also warrants further investigation.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by a Westpac Bicentennial Foundation Future Leaders Scholarship awarded to Samantha Nixon and an Australian National Health & Medical Research Council Principal Research Fellowship awarded to G.F.K. We thank Mr Ben Cristofori-Armstrong, Dr Irene Chassagnon, Dr Ravi Anangi and Dr Thomas Durek from the Institute for Molecular Bioscience, The University of Queensland for kindly sharing venom peptides for this study.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2019.05.008.

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