

Arylpyrrole and fipronil analogues that inhibit the motility and/or development of *Haemonchus contortus* *in vitro*

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ABSTRACT

Due to widespread drug resistance in parasitic nematodes, there is a need to develop new anthelmintics. Given the cost and time involved in developing a new drug, the repurposing of known chemicals can be a promising, alternative approach. In this context, we tested a library (n = 600) of natural product-inspired pesticide analogues against exsheathed third stage-larvae (xL3s) of *Haemonchus contortus* (barber's pole worm) using a whole-organism, phenotypic screening technique that measures the inhibition of motility and development in treated larvae. In the primary screen, we identified 32 active analogues derived from chemical scaffolds of arylpyrrole or fipronil. The seven most promising compounds, selected based on their anthelmintic activity and/or limited cytotoxicity, are arylpyrroles that reduced the motility of fourth-stage larvae (L4s) with significant potency (IC₅₀ values ranged from 0.04 ± 0.01 μM to 4.25 ± 0.82 μM, and selectivity indices ranged from 10.6 to 412.5). Since the parent structures of the active compounds are uncouplers of oxidative phosphorylation, we tested the effect of selected analogues on oxygen consumption in xL3s using the Seahorse XF24 flux analyser. Larvae treated with the test compounds showed a significant increase in oxygen consumption compared with the untreated control, demonstrating their uncoupling activity. Overall, the results of the present study have identified natural product-derived molecules that are worth considering for chemical optimisation as anthelmintic drug leads.

1. Introduction

The control of gastrointestinal nematodes of livestock has relied largely on the use of a limited number of anti-parasitic drugs (Besier et al., 2016; Harder, 2016). However, drug resistance is now very widespread (Kaplan and Vidyashankar, 2012; Kotze and Prichard, 2016) and no vaccines are available for the vast majority of these worms, such that the development of new drugs is crucial to ensure effective and sustained nematode control into the future. Although the development of the compound monepantel (Kaminsky et al., 2008; Prichard and Geary, 2008) provided hope for the design of new classes of nematocides, there has been relatively little success in discovering

new drugs using conventional and high throughput screening approaches (Geary et al., 2015).

Given the massive cost and time required to develop and commercialise a new anthelmintic (Geary et al., 2015), the repurposing of known drugs or bioactive chemicals (Corsello et al., 2017) offers a pragmatic and sound option. The repurposing of chemicals with known safety profiles and modes of action has the potential to proceed to clinical trials more rapidly than newly discovered chemotypes (Oprea and Mestres, 2012; Andrews et al., 2014; Panic et al., 2014; Corsello et al., 2017).

Recently, in partnership with philanthropic, academic and industry partners, we screened compound libraries of bioactive compounds for

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inhibitory activity against *Haemonchus contortus* (barber's pole worm), in an attempt to repurpose synthetic and natural compounds against parasitic nematodes (Preston et al., 2016, 2017; Herath et al., 2017). For instance, we have shown that pesticides such as tolfenpyrad (Preston et al., 2016) and other pyrazole-5-carboxamides (e.g., a-15 and a-17; Jiao et al., 2017) have potent inhibitory activity on the motility and/or development of exsheathed third-stage (xL3s) and/or fourth-stage (L4s) larvae of *H. contortus* *in vitro* and that these chemicals are relatively selective for the parasite compared with a mammalian cell line (see Preston et al., 2016; Jiao et al., 2017). Using medicinal chemical methods, the potency and selectivity of some of these chemotypes have been significantly increased (unpublished findings). Taken together, this information indicates that there is merit in taking a repurposing route, provided that medicinal chemistry follows the screening effort, in order to optimise potency and safety *in vitro* and *in vivo*, pharmacokinetics and efficacy.

Here, we extend previous studies (Preston et al., 2016; Jiao et al., 2017) to screen a library of compounds ($n = 600$) obtained from the Research Institute of Elemento-Organic Chemistry, Nankai University, China. This library contains novel analogues of various pesticides, including antofine (Gao et al., 2012), arylpyrroles (Kuhn, 1997), diflubenzuron (Post and Vincent, 1973; Cohen and Casida, 1980), etoxazole (Nauen and Smaghe, 2006), fipronil (Cheng et al., 2009) and spirodiclofen (Van Pottelberge et al., 2009). Most of these analogues had been tested previously at Nankai University, and exhibited insecticidal and/or acaricidal properties (Li et al., 2012; Liu et al., 2014; Ma et al., 2014), suggesting that these compounds might act on other ecdysozoans, including nematodes of veterinary importance. Therefore, we screened the library against larval stages of *H. contortus* - one of the most important parasitic nematodes of livestock animals (Gasser and von Samson-Himmelstjerna, 2016). We employed a semi-automated phenotypic screening technique (Preston et al., 2015) to identify active ('hit') compounds and to assess their potency against larval stages of *H. contortus*, followed by an evaluation of the cytotoxicity of these hits on a mammary epithelial cell line *in vitro*, with the aim of identifying candidate compounds.

2. Materials and methods

2.1. Parasite production and maintenance

Haemonchus contortus (Haecon-5 strain) was maintained in experimental sheep as described previously (Schwarz et al., 2013; Preston et al., 2015), in accord with institutional animal ethics guidelines (permit no. 1413429; The University of Melbourne, Australia). Third-stage larvae (L3s) were cultured and exsheathed using established methods (Preston et al., 2015). In brief, L3s were exsheathed by incubation in 0.15% v/v sodium hypochlorite (NaClO) for 20 min at 37 °C and then washed five times in sterile, physiological saline (pH 7.0, 37 °C). The exsheathed L3s (xL3s) were then suspended in Luria Bertani (LB) medium supplemented with 100 IU/ml of penicillin, 100 mg/ml of streptomycin and 2.5 mg/ml of amphotericin (Fungizone, antibiotic-antimycotic; cat. no. 15240-062; Gibco, USA) (Preston et al., 2015). The supplemented LB was designated as LB*. L4s were produced by incubating xL3s in LB* at 10% v/v CO₂ and 38 °C for 7 days (Preston et al., 2015).

2.2. Screening for anthelmintic activity

The library of chemicals from the Research Institute of Elemento-Organic Chemistry, Nankai University, China, contained pure chemicals

derived from natural (120) and synthetic (480) products with known activities against arthropods (flies and/or mites) (Supplementary file 1). All 600 compounds were individually screened (in triplicate) in 96-well plates using a previously described protocol (Preston et al., 2015; cf. Fig. 1). In brief, the chemicals were each dissolved in 100% DMSO to prepare 20 mM stocks, diluted into 40 μM in 50 μl of LB* (containing 1% DMSO) and arrayed in triplicate in flat-bottom 96-well microplates (cat. no. 3635; Corning 3650, Life Sciences, USA). The xL3s (~300 in 50 μl per well) were added to wells and exposed to a final compound concentration of 20 μM (with 0.5% DMSO). The two commercially available drugs monepantel (Zolvix, Novartis Animal Health, Switzerland) and moxidectin (Cydectin, Virbac, France) were included as positive controls, and LB* + 0.5% DMSO as the negative control (six wells). Plates were placed in an incubator for 72 h (10% v/v CO₂; 38 °C). For data acquisition, plates were agitated for 20 min using an orbital shaker (126 rotations per min) (model KOM5, Ratek, Australia), and a 5 s video was recorded from each well. Digital recordings were processed using a customised script in Image J (imageJ.nih.gov/ij/), and changes in light intensity transformed into a motility index (Mi; Preston et al., 2015). The raw data were normalised with respect to positive and negative controls, in order to remove plate-to-plate variation, and the percentage of motility reduction was calculated for each well using the program Prism (v.7.02 GraphPad Software). A compound was recorded as being active if it reduced xL3 motility by ≥ 70% and/or induced phenotypic alterations in comparison with the untreated wild-type control worms (at 72 h).

2.3. Dose-response curves for active compounds (xL3 motility, L4 motility and L4 development)

All active compounds were tested as a two-fold dilution series (18 points; starting at 100 μM) to assess a reduction in xL3 motility and an inhibition of L4 development (Preston et al., 2015, Fig. 1). To measure motility, video recordings of individual wells were taken at 24 h, 48 h and 72 h. To assess development, plates were incubated further under the same conditions for four more days; worms in each well were then fixed with 50 μl of 1% iodine and the development to L4 determined by examining 30 worms from each well under a microscope at 20x magnification. L4s were differentiated from xL3s based on the presence of well-developed mouth and pharynx.

The half-maximum inhibitory concentration (IC₅₀) of each compound was determined by transforming the compound concentrations to log₁₀, and fitting the data using a variable slope four-parameter equation (v.7.02 GraphPad Software) (cf. Preston et al., 2015). IC₅₀ values were obtained from results from two independent biological assays with three technical replicates for each compound. Compounds were ranked based on the level of xL3 motility reduction, L4 development inhibition and minimal cytotoxicity. A subset of compounds was tested for their ability to inhibit L4 motility, again using a two-fold dilution series and in two biological assays with three technical replicates.

2.4. Cytotoxicity assay

The cytotoxicity of individual compounds was tested as described previously (Kumarasingha et al., 2016; Fig. 1) at the Victorian Centre for Functional Genomics in Cancer, Melbourne, Australia, with minor modifications. In brief, normal breast epithelial (MCF10A) cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; ThermoFisher, USA) containing 5% v/v horse serum (Life Technologies, Australia), 100 ng/ml of cholera toxin (Sigma, Australia),

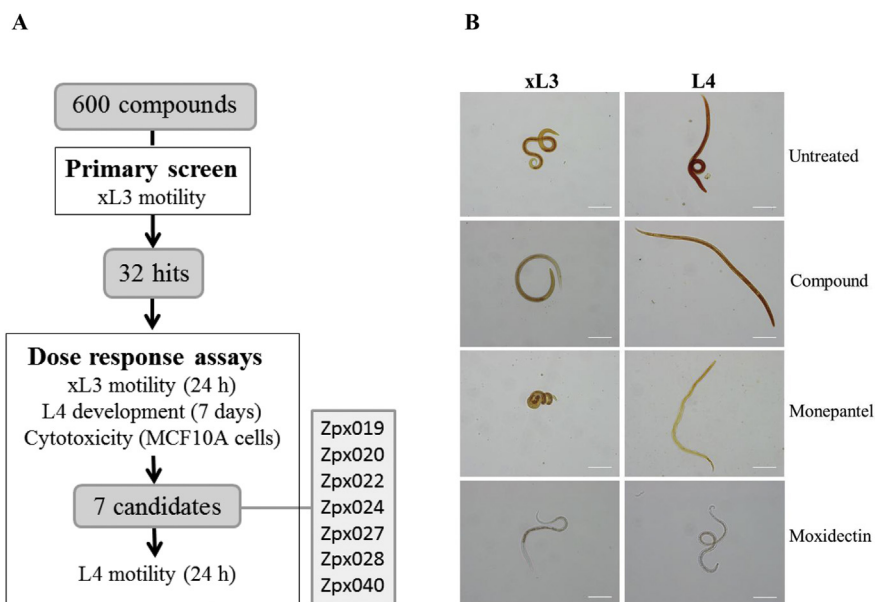


Fig. 1. Workflow used in the current study. We screened 600 chemicals against third-stage larvae (xL3s) of *H. contortus* and identified 32 active compounds (hits), which were then tested in dose response assays for the inhibition of xL3 motility and L4 development as well as toxicity to a normal breast epithelial cell line (MCF10A); seven compounds (Zpx019, Zpx020, Zpx022, Zpx024, Zpx027, Zpx028 and Zpx040) were selected for subsequent testing for their ability to inhibit L4 motility (panel A). Representative images of xL3s and L4s treated with active compound showing the phenotypic alterations by comparison with untreated (negative) and monepantel and moxidectin (positive) controls; white scale bar = 100 μ m; 20-times magnification (panel B).

20 ng/ml of human epidermal growth factor (Life Technologies, Australia), 0.5 mg/ml hydrocortisone (Sigma, Australia) and 10 mg/ml of insulin (human; Novo Nordisk Pharmaceuticals Pty Ltd, Denmark) in black-walled, flat bottom 384-well plates (Corning, USA) at a density of 700 cells per well in 40 μ l. The cells were incubated at 37 °C and 5% v/v CO₂ for 24 h. The growth medium was then aspirated, and the cells were treated with individual compounds (in quadruplicate) or with control compounds, including negative controls in every run, and then incubated for 48 h. Compounds were tested as a 5-point series (50 μ M, 25 μ M, 12.5 μ M, 6.25 μ M and 3.125 μ M) to obtain dose-response curves using an automated liquid handling robot (SciClone ALH3000 Lab Automation Liquid Handler, Caliper Lifesciences, USA), using four replicates for each compound. Doxorubicin (highest concentration of 10 μ M) and monepantel (Zolvix, Novartis Animal Health, Switzerland; highest concentration of 50 μ M) were used as positive (active) control compounds to assess cell toxicity or activity; medium without compound was used as the negative control. Following incubation, the cells in individual wells were fixed, stained with 4',6-diamidino-2-phenylindole (DAPI; 1:1000) and imaged at a fixed exposure time of 0.12 sec using a high content imager (Cellomics CellInsight Personal Cell Imager, ThermoFisher Scientific, USA) which captured ~ 90% of the well. Stained nuclei of the viable cells were counted using the Target Activation BioApplication within Cellomics Scan software (v.6.5.0, Thermo Scientific, USA). The cell density was normalised to the negative control, and IC₅₀ values were determined using the same method as described in section 2.3. The selectivity index (SI) was calculated by dividing IC₅₀ for MCF10A cells by IC₅₀ for *H. contortus* (see Preston et al., 2015).

2.5. Larval oxygen consumption assay

Selected compounds with activity on *H. contortus* were tested for their effects on mitochondrial respiration of xL3s by measuring changes in oxygen consumption using the Seahorse XF24 flux analyser (Seahorse Biosciences, USA) essentially as described previously (McGee et al., 2011). In brief, 5000 xL3s in 500 μ l of XF medium (Seahorse

Biosciences, USA) supplemented with 4.5 g/l of glucose, 0.5 mM of sodium pyruvate and 2 mM of glutamine (Sigma-Adrich, USA) were dispensed into XF24 cell culture microplates (Seahorse Biosciences, USA). The compounds dissolved in XF base medium (final concentration of 100 μ M in 1% DMSO) were then loaded individually into the injection ports and automatically dispensed into the wells after recording six initial measurements of respiration at 6 min intervals. The oxygen consumption was measured (protocol: 2 min - mix, 2 min - pause and 4 min - measure) for 120 min more at 6 min intervals (cf. McGee et al., 2011). The XF medium plus 1% DMSO (500 μ l) was dispensed into four wells as the negative control, and four wells containing 100 μ M monepantel and carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) were included as positive controls. FCCP is known as a mitochondrial uncoupler - such uncouplers are chemicals that inhibit the coupling between the electron transport chain and phosphorylation reaction, thus reducing ATP synthesis without affecting the electron transport chain and enhancing oxygen consumption through increased production of NADH (Terada, 1990). In these experiments, four technical and three biological replicates were performed. The data acquired were normalised to the basal oxygen consumption, and the graphs of oxygen consumption rates (OCR) were produced using v.7.02 GraphPad Software. The total oxygen consumption following each compound treatment was measured by calculating the 'area under the curve' (AUC) in these graphs. The statistical significance between treatments was calculated using the nonparametric (Kruskal-Wallis) one-way ANOVA and a Dunnett's multiple comparison test in the same GraphPad software.

3. Results

The primary screen of the library (Fig. 1) identified 21 compounds that reduced the motility of *H. contortus* xL3s by \geq 70% and induced a non-wildtype ("circular") phenotype as well as 11 compounds that induced a 'circular' phenotype but did not reduce the xL3 motility by \geq 70% (Supplementary file 1). All of these 32 compounds were categorised as 'hits' (Fig. 1); 30 of them were pyrrole analogues (the

Table 1

The effects of seven active analogues on xL3 and L4 motility (24 h, 48 h and 72 h) and L4 development (7 days) of *Haemonchus contortus* *in vitro*. A comparison of half of the maximum inhibitory concentration (IC₅₀) values of compounds with those of monepantel or moxidectin, expressed as mean IC₅₀ ± standard error of mean or a range.

Compound control	xL3 motility (IC ₅₀ in μM) ^a			L4 motility (IC ₅₀ in μM)			L4 development (IC ₅₀ in μM)
	24 h	48 h	72 h	24 h	48 h	72 h	7 days
Zpx019	nd	11.81 ± 0.19	7.40 ± 1.22	13.45 ± 3.66	3.13 to 6.25	3.7 ^b	4.28 ± 0.38
Zpx020	nd	nd	3.86 ± 0.28	3.47 ± 2.36	3.22 ± 1.64	2.28 ± 0.67	7.24 ± 0.97
Zpx022	nd	10.62 ± 4.07	9.14 ± 3.32	17.68 ± 0.11	4.3 ^b	4.25 ± 0.82	13.98 ± 0.98
Zpx024	25.26 ± 8.50	9.17 ± 2.60	3.2 ^b	2.45 ± 1.22	1.07 ± 0.15	1.30 ± 0.15	1.99 ± 0.02
Zpx027	9.79 ± 2.54	3.10 ± 0.01	1.22 ± 0.09	0.31 ± 0.29	0.14 ± 0.12	0.09 ± 0.04	1.18 ± 0.13
Zpx028	8.63 ± 0.90	2.9 ^b	1.25 ± 0.36	0.16 ± 0.13	0.10 ± 0.03	0.04 ± 0.01	0.57 ± 0.04
Zpx040	nd	3.1 ^b	1.68 ± 0.66	12.92 ± 4.00	2.6 ^b	3.36 ± 0.86	1.58 ± 0.09
Monepantel	2.12 ± 0.55	0.76 ± 0.32	0.59 ± 0.01	6.52 ± 2.18	0.7 ^b	0.1 ^b	0.43 ± 0.01
Moxidectin	0.50 ± 0.42	0.19 ± 0.04	0.08 ± 0.02	0.92 ± 0.90	0.002 ± 0.04	0.003 ± 0.01	not applicable

^a IC₅₀ values that could not be accurately determined using the log (inhibitor) vs. response-variable slope four parameters model are indicated as not determined (nd).

^b Estimated from the graphs in Fig. 2.

Table 2

Toxicity assessment of seven arylpyrrole analogues on MCF10A cells. A comparison of half of the maximum inhibitory concentration (IC₅₀) values of compounds with monepantel and doxorubicin; expressed as mean IC₅₀ ± standard error of mean, and selectivity indices (SI) of the compounds on motility (xL3 and L4) and development (L4) of *Haemonchus contortus* at different time points compared with monepantel.

Compound Control	IC ₅₀ (in μM) for MCF10A cells	Selectivity index (SI) for <i>H. contortus</i>						
		xL3 motility ^c			L4 motility			L4 development
		24 h	48 h	72 h	24 h	48 h	72 h	7 days
Zpx019	> 50	nd	> 4.5	> 6.8	> 3.7	> 8.0	> 13.6	> 11.7
Zpx020	> 50	nd	nd	> 12.9	> 14.4	> 15.7	> 21.9	> 7.0
Zpx022	> 50	nd	> 4.7	> 5.5	> 2.8	> 11.7	> 11.7	> 3.6
Zpx024	26.11 ± 3.92	1.0	2.9	8.1	10.7	24.4	20.1	13.1
Zpx027	6.36 ± 3.29	0.7	2.1	5.2	20.5	45.6	70.9	5.4
Zpx028	16.50 ± 2.58	1.9	5.7	13.2	103.1	165	412.5	28.9
Zpx040	35.7 ^b	n/d	11.5	21.3	2.8	13.9	10.6	22.6
Monepantel	32.8 ^b	15.5	43.3	55.7	5.0	47.4	328.4	76.4
Doxorubicin ^a	2.17 ± 0.02	na	na	na	na	na	na	na

^a Not applicable (na).

^b Estimated from the graphs of percentage MCF10A cell density vs. log (concentration).

^c If IC₅₀ could not be established, SI was not determined (nd).

Mqq and Zpx series) and two were fipronil analogues (Zqq series) (Supplementary file 1).

In the dose response assays (cf. Fig. 1), 13 of the 32 hits induced a dose-dependent inhibition of xL3 motility, with an IC₅₀ of ≤ 50 μM at 24 h (Supplementary file 2). The IC₅₀ values of these 13 compounds ranged from 8.63 ± 0.90 μM to 48.13 ± 2.71 μM (Supplementary file 2). Compound Zpx028, a pyrrole analogue, had the highest potency at 24 h, with an IC₅₀ of 8.63 ± 0.90 μM. Compound Zpx027 had the second highest potency at inhibiting xL3 motility at 24 h (IC₅₀ = 9.79 ± 2.54 μM), and was most potent at inhibiting xL3 motility at 72 h (IC₅₀ = 1.22 ± 0.09 μM) (Table 1; Supplementary file 2).

All 32 hits, except Zpx021 and Zpx90, elicited a dose-dependent inhibition of L4 development; six compounds achieved an IC₅₀ of ≤ 2 μM (Supplementary file 2). The two compounds, Zpx028 and Zpx027, with the highest potency at inhibiting xL3 motility, were also the most potent inhibitors of L4 development (Fig. 2; Table 1) - the IC₅₀

values (0.57 ± 0.04 μM for Zpx028; 1.18 ± 0.10 μM for Zpx027) are comparable to those of the commercial anthelmintic monepantel *in vitro* (IC₅₀ = 0.43 ± 0.01 μM) (Fig. 2; Table 1).

All 32 hits were tested for toxicity to MCF10A breast epithelial cells *in vitro*. The IC₅₀ values obtained ranged from 2.16 μM to 50 μM, and selectivity indices (at 24 h) varied from 0.04 to 1.91 for xL3 motility, and from 0.26 to 28.95 for L4 development (at 7 days; Supplementary file 3). Eight compounds, Zpx028, Zpx030, Zpx023, Zpx026, Zpx024, Zpx040, Zpx090 (pyrrole analogues) and Zqq1341 (fipronil analogue), exhibited moderate toxicity (IC₅₀ of 16.50–44.98 μM; Supplementary file 2). Four pyrrole analogues, Zpx019, Zpx020, Zpx021 and Zpx022, had limited toxicity to the cells, with IC₅₀ values of ≥ 50 μM (Table 2; Supplementary file 2).

On the basis of the dose-dependent inhibition of xL3 motility (at 24 h) and L4 development (7 days) and/or the degree of cytotoxicity *in vitro* (Supplementary file 2), seven compounds (Zpx019, Zpx020,

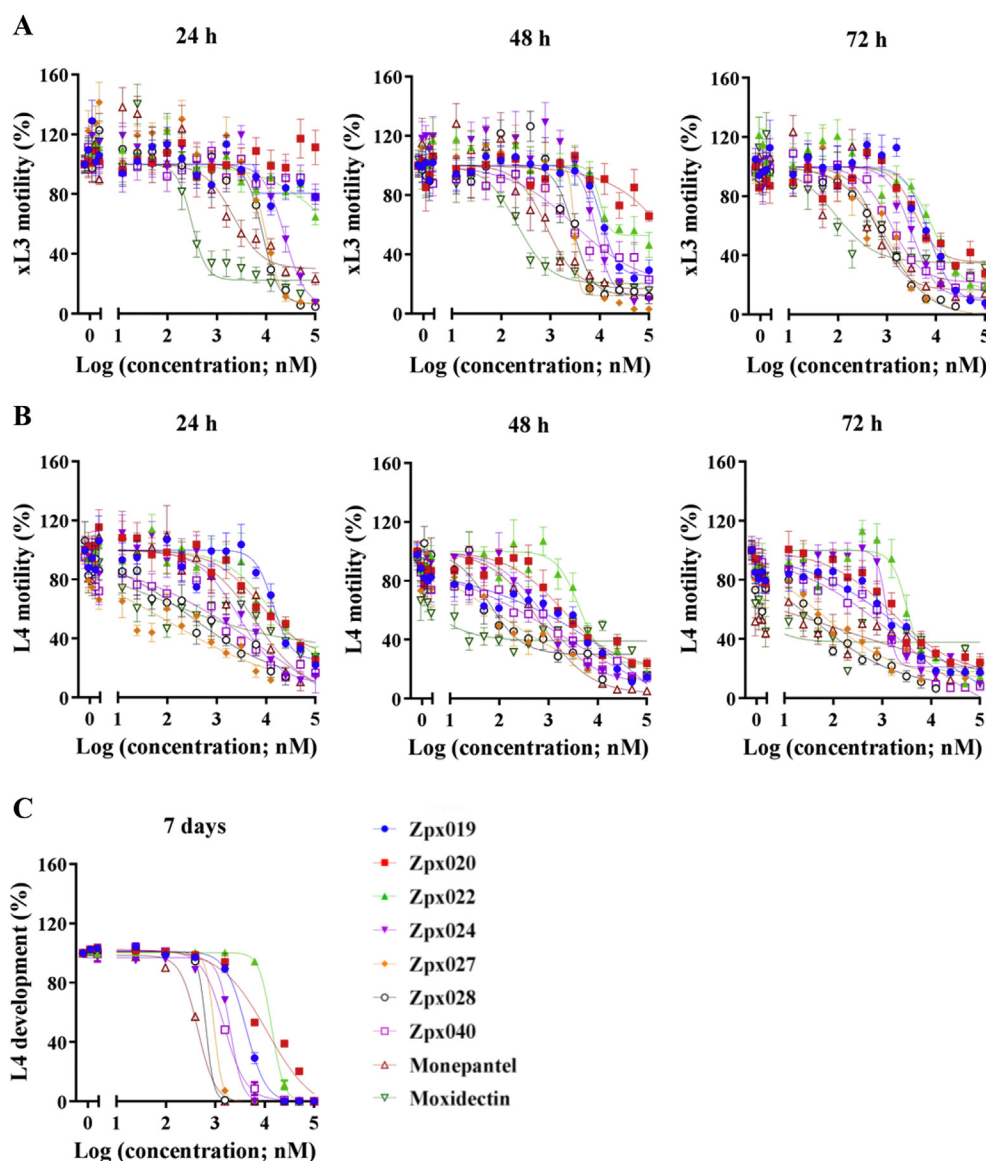


Fig. 2. Dose response curves for seven compounds (Zpx019, Zpx020, Zpx022, Zpx024, Zpx027, Zpx028 and Zpx040) with activity against larval stages of *Haemonchus contortus* in vitro. Inhibition of motility of exsheathed third-stage larvae (xL3s) (panel A) and fourth-stage larvae (L4s) (panel B) following incubation with each compound (two-fold dilution series with 18 points; starting at 100 μ M) for 24 h, 48 h and 72 h, and the inhibition of development of L4s following incubation with the same compounds for 7 days (panel C). Monepantel and moxidectin, tested at matched concentrations, were included as reference controls.

Zpx022, Zpx027, Zpx024, Zpx028 and Zpx040; all pyrrole analogues) were assessed for their ability to inhibit the motility of L4s (Fig. 1). All of these seven compounds exhibited a dose-dependent inhibition of L4 motility at 24 h, with IC_{50} values ranging from $17.68 \pm 0.11 \mu$ M to $0.16 \pm 0.13 \mu$ M (Fig. 2; Table 1). Interestingly, compounds Zpx019, Zpx020, Zpx022 and Zpx040, which did not inhibit xL3 motility in a dose-dependent manner at 24 h, did inhibit L4 motility at the same time point (Fig. 2; Table 2). The two most potent inhibitors of both xL3 motility and L4 development also exhibited the highest potency ($IC_{50} = 0.16 \pm 0.13 \mu$ M for Zpx028 and $IC_{50} = 0.31 \pm 0.29 \mu$ M for Zpx027) and selectivity (SI = 20.5 for Zpx027 and SI = 103.1 for Zpx028) for inhibiting L4 motility at 24 h (Fig. 2; Tables 1 and 2).

As pyrroles act as insecticides via respiratory uncoupling (Black

et al., 1994; Liu et al., 2014), we assessed compounds Zpx027 and Zpx028 for their effects on respiration in *H. contortus*. In response to each of these compounds, the oxygen consumption rate (OCR) of the xL3 stage increased (Fig. 3). Neither the negative (no-compound) nor the positive (monepantel) control showed a significant difference from the basal OCR in xL3s throughout the experimental period (Fig. 3). The OCR patterns for compounds Zpx028 and Zpx027 were similar to that of FCCP, a standard mitochondrial uncoupler (Fig. 3), and there was a significant difference ($P < 0.001$ for Zpx028 and $P < 0.01$ for Zpx027) in total oxygen consumption (cf. Fig. 3) between larvae treated with each test compound and the untreated and monepantel-treated controls (Fig. 3).

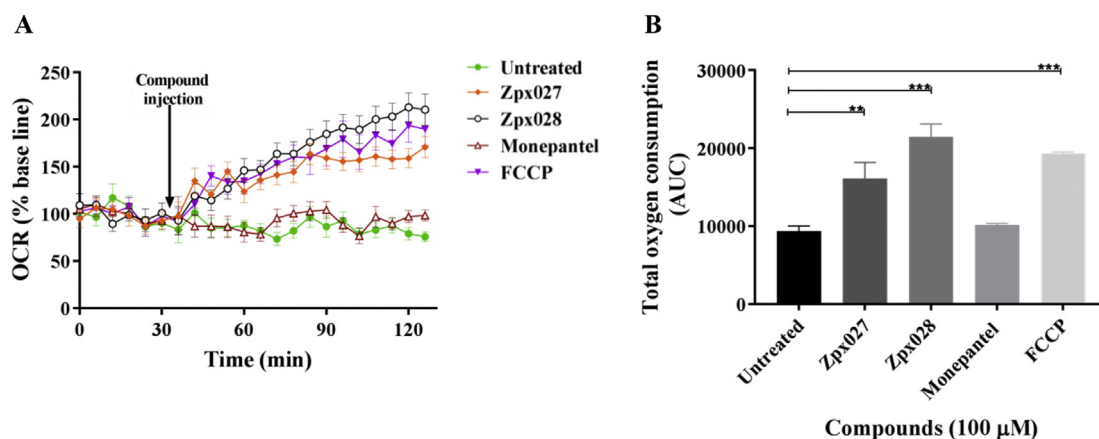


Fig. 3. The effect of selected compounds on oxygen consumption of exsheathed third-stage larvae (xL3s) of *Haemonchus contortus* in vitro. The oxygen consumption rates (OCRs) of exsheathed third-stage larvae (xL3s) before and following exposure to compound Zpx027 or Zpx028 as well as the controls XF medium only, monepantel and carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (panel A). The graphs show total oxygen consumption, calculated from the area under the curve (AUC) and expressed as mean AUC \pm standard error of mean (SEM). Asterisks indicate the values that are significantly different from one another (** $P < 0.01$, *** $P < 0.001$) (panel B).

4. Discussion

The screening of a series of pesticide analogues identified 32 compounds with activity against *H. contortus* xL3s. Seven compounds were chosen for further evaluation based on their potency against *H. contortus* larvae (motility and development) and mostly limited toxicity on a human cell line (MCF10A). Selected compounds ($n = 7$) were found to be rapid inhibitors of L4 motility, and the two most potent ones in all worm assays (Zpx027 and Zpx028) were shown to increase oxygen consumption in xL3s. This increase mirrored the behaviour of a recognised mitochondrial uncoupler, FCCP (cf. Fig. 3), inferring that heightened respiration rates stemmed from an increased electron flow through the mitochondrial electron transport chain, rather than from a non-specific removal of dissolved oxygen. Therefore, the compounds tested seem to have a mode of action that is the same or similar to that of the arylpyrrole parent structure representing 30 of the 32 compounds identified in the primary screen, with the other two being derivatives of fipronil (cf. Supplementary file 1).

The founding member of insecticidal and acaricidal arylpyrroles was a natural product, dioxapyrrolomycin, first identified as a metabolite from *Streptomyces fumanus* (see Carter et al., 1987). The structure of dioxapyrrolomycin was modified to yield the commercial product chlorfenapyr (Addor et al., 1992), a compound that is effective against a range of parasitic arthropods of plants, including the southern armyworm (*Persectantia ewingii*), tobacco budworm (*Heliothis virescens*-Fabricius), western potato leafhopper (*Empoasca abrupta*) and red spider mite (*Tetranychus urticae*) (Kuhn, 1997). Chlorfenapyr is a pro-drug that is metabolically converted to the active compound (CL303268) by N-dealkylation inside the pathogen (Black et al., 1994). The biological effects of CL303268 and dioxapyrrolomycin seem to be based on their activity as uncouplers of oxidative phosphorylation (Black et al., 1994), and it is reasonable to propose that this is also the mode of anthelmintic action of the present arylpyrrole hit compounds, given the uncoupling activity exhibited by the two members tested (i.e. Zpx027 and Zpx028). Once again, natural product-inspired s have proven to be suitable starting points to identify potential anti-infective leads.

The reason for the rapid and dose-dependent activity of arylpyrrole analogues Zpx019, Zpx020, Zpx022 and Zpx040 against L4s, with no apparent activity against xL3s at 24 h, is not yet clear. However, we propose that it relates to differences in oral drug uptake between the two larval stages of *H. contortus*. Uptake in L4s may be greater than in xL3s due to a well-developed pharynx in this stage. At this point, it

cannot be excluded that this difference might be due to variation in target function and/or in drug metabolism between the two development stages; however, given the high structural similarities among the four test compounds, this latter explanation is considered less likely, as they are expected to share the same target/mode of action and degradation pathways.

The two fipronil derivatives (Zqq-1726 and Zqq-1341) received limited experimental priority, because of their relatively low potency on *H. contortus* and higher cytotoxicities relative to the selected arylpyrrole analogues. Nonetheless, their structure-activity relationship could be explored in future work. The parent compound of these structures is a broad spectrum, highly selective synthetic phenylpyrazole insecticide that exerts its biological activity by blocking GABA-gated chloride channels (Zhao et al., 2003) and glutamate-gated chloride (GluCl) channels (Gant et al., 1998; Zhao et al., 2004), so we presume that this will also be the mode of action for the analogues studied here, although we have not yet collected the evidence to support this proposal.

In conclusion, some of the analogues with favourable activity and toxicity profiles identified in this study warrant critical evaluation as anthelmintic candidates. Medicinal chemical optimisation, supported by iterative structure-activity relationship, *in vitro* and *in vivo* toxicity and pharmacokinetic assessments, should allow the potential of lead candidates to be established. Overall, the findings of the present study encourage the screening of natural product-derived libraries for compounds with anthelmintic activity.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ijpddr.2018.06.002>.

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