Screening of a small, well-curated ‘natural compound’ library identifies two rotenoids with potent nematocidal activity against *Haemonchus contortus*

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ABSTRACT

The control of parasitic roundworms (nematodes) is heavily reliant on the use of a limited number of anthelmintic drugs. However, drug resistance is now very widespread and no vaccines are available, such that the discovery of new chemical entities is crucial. Within this context, we screened a library of pure natural products ($n = 400$) against exsheathed third-stage (xL3) larvae of the parasitic nematode *Haemonchus contortus* using a whole-organism screening method. We identified two plant-derived rotenoids, namely deguelin and rotenone, with inhibitory activity on xL3 motility. Whereas rotenone was not investigated further because of its toxicity to mammals/vertebrates, dose response and cytotoxicity studies of deguelin showed potent and selective inhibitory activity on motility of xL3 larvae of *H. contortus*. Detailed future work needs to be conducted to explore the mode of action of this compound on *H. contortus* and related nematodes and to assess its potential as an anthelmintic candidate.

Keywords:

Natural product

Rotenoid

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Deguelin

Haemonchus contortus

1. Introduction

Parasitic worms cause substantial mortality and morbidity in animals, and also major losses to animal and food production globally. Importantly, parasitic roundworms of the order Strongylida cause some of the most important diseases of livestock in Australia and worldwide, affecting hundreds of millions of food animals (including sheep, goats, cattle and pigs), with economic losses estimated at tens of billions of dollars per annum globally (Roeber et al., 2013; Lane et al., 2015). These pathogens cause gastritis, enteritis, anaemia and/or associated complications, and death in severely affected animals. All of these parasites are transmitted orally from contaminated pasture to the host through a direct life cycle (cf. Graem et al., 2014): eggs are excreted in host faeces; the first-stage larva (L1) develops inside the egg to then hatch (within 1 day) and develop through to the second- and third-stage larval stages (L2s and L3s) in about a week; the infective L3s are then ingested by the host, exsheath (xL3) and develop through fourth-stage larvae (Graem et al., 2014) to dioecious adults (within 3 weeks) in the gut of the animal.

Currently, the control of parasitic nematodes of livestock animals relies largely on the use of a limited number of anti-parasitic drugs. However, drug resistance is now very widespread (e.g., Kaplan and Vidyashankar, 2012) and no vaccines are available for the vast majority of these worms, such that the discovery of new drugs is critical, to ensure sustained and effective control into the future. Although the development of the compound monepantel (Kaminsky et al., 2008; Prichard and Geary, 2008) has provided fresh hope for the design of new classes of nematocides, success in identifying new drugs and drug targets using conventional screening approaches has been limited. Recent investigations of the genomes, transcriptomes and biology of a number of strongylid nematodes, including the barber's pole worm (*Haemonchus contortus*; Schwarz et al., 2013; Laing et al., 2013), hookworms (*Necator americanus* and *Ancylostoma ceylanicum*; Tang et al., 2014; Schwarz et al., 2015) and a nodule worm (*Oesophagostomum dentatum*; Tyagi et al. 2015), are revolutionizing the way we study these parasites, and can now support the discovery of new interventions. In collaboration with *Compounds Australia* (www.griffith.edu.au/science-aviation/compounds-australia), in the present study, we screened compounds from a small, well-curated library against parasitic stages of *H. contortus* (of ruminants), to identify any hit compounds, and then characterise and assess them as nematocide candidates.

2. Materials and methods

Third-stage (L3s) larvae of *H. contortus* (Haecon-5 strain; Schwarz et al., 2013) were cultured from the faeces from mono-specifically infected sheep as described previously (Preston et al., 2015, 2016). Approval to infect sheep was given by the University of Melbourne (animal ethics permit no. 1413429).

The natural product library was purchased from *Compounds Australia* (www.griffith.edu.au/science-aviation/compounds-australia). This open-access library was established in 2010 by R.A.D. and contained 400 distinct small molecules, the majority (~55%) of which had been purified from plants (Levrier et al., 2013), fungi (Davis, 2005) and marine invertebrates (Barnes et al., 2010). Approximately 30% of this library contained semi-synthetic natural product analogues (Barnes et al., 2016), while ~15% were known drugs or synthetic compounds inspired by natural products. All compounds (purity: >95%) were supplied at a concentration of 5 mM in dimethyl sulfoxide (DMSO; cat no. 2225; Ajax Finechem, Australia) and diluted to a concentration of 20 μ M of compound and 0.5% of DMSO in Luria Bertani (LB) medium, supplemented with 1% antibiotics-antimycotic mix (cat no. 15240-062; Gibco); this supplemented medium was designated LB* (cf. Preston et al., 2015). Test compounds were transferred using a multi-channel pipette (Finnpipette, Thermo Scientific, USA) into 96-well flat bottomed microplates (cat no. 3635; Corning 3650, Life Sciences, USA) in a volume of 50 μ l. Test and the positive control compounds

monepantel (Zolvix®, Novartis Animal Health, Switzerland) and moxidectin (Cydectin®, Virbac, France) were tested in triplicate on exsheathed L3s (xL3s) using established published methods (Preston et al., 2015, 2016). Negative controls, LB* and LB* + 0.5% DMSO were tested in replicates of six. A volume of 50 µl containing ~ 300 exsheathed L3 (xL3s) was then transferred into each well using a multi-channel pipette (Finnpipette, Thermo Scientific). Plates were incubated for 72 h at 38 °C and 10% CO₂, and monitored for movement as described previously (Preston et al., 2015, 2016). A compound was recorded as having anti-xL3 activity if it reduced motility by $\geq 70\%$ at 72 h. Compounds with anti-xL3 activity were screened twice at 20 µM to verify their inhibitory properties on motility.

To determine the 50% inhibitory concentration (IC₅₀ values of (-)-deguelin (catalogue code: LX-350-118-M005, Enzo Life Sciences, Switzerland; purity: $\geq 95\%$) on larval motility, this chemical was serially diluted two-fold in LB*, starting at a concentration of 100 µM, as described previously (Preston et al., 2015, 2016). The motility of xL3s was recorded 24 h, 48 h and 72 h following exposure to the chemical (Preston et al., 2015, 2016). To establish IC₅₀ values, compound concentrations were log₁₀-transformed, and a variable slope four-parameter equation was used, constraining the top to 100% (Graphpad Prism, v7.01, USA). A two-way analysis of variance (ANOVA) and the Dunnett's multiple comparison test were used to compare the effects of compounds on motility at different concentrations and time points. All experiments were performed in triplicate on three separate days.

Human cell toxicity was assessed as described by Fisher et al. (2014). In brief, neonatal foreskin fibroblast cells (NFF) were cultured in RPMI 1640 medium (Life Technologies, Inc., Rockville, MD, USA) supplemented with 10% foetal calf serum (CSL Biosciences, Victoria, Australia) and 1% streptomycin (Life Technologies Inc., USA). The inhibition of growth (%) was compared with matched DMSO controls. IC₅₀ values were calculated using linear interpolation of inhibition curves. The mean IC₅₀ value ($\pm$ standard deviation, SD) was recorded in three independent experiments, each carried out in triplicate wells. The selectivity index (SI) was calculated as IC₅₀ for NFF divided by IC₅₀ for *H. contortus*.

3. Results and discussion

From the *in vitro* primary screening of the 400 compounds against *H. contortus*, only rotenone and deguelin were found to reduce xL3 motility by $\geq 70\%$. Both compounds were re-screened at 20 µM and shown to consistently inhibit xL3 motility. IC₅₀ values for deguelin as well as those of monepantel and moxidectin (positive controls) on xL3 motility were determined at 24 h, 48 h and 72 h and then compared. The monepantel and moxidectin control compounds were more effective, under the current conditions, than deguelin at inhibiting xL3s after 72 h, with IC₅₀ values of 0.13 ± 0.10 µM, 0.04 ± 0.01 µM and 19.13 ± 5.71 µM respectively (Fig. 1; Table 1). In addition, deguelin was found to have low toxicity against human NFFs using a cell proliferation assay (IC₅₀ > 50 µM) with a 2.6-fold selectivity for *H. contortus*.

Given the substantial problems linked to anthelmintic resistance in parasitic worms of livestock animals, a continued effort is needed to search for new and effective chemicals for development and commercialisation, particularly if they have new modes of action compared with existing drugs (Epe and Kaminsky, 2013). There has been a renewed interest in pursuing natural products for drug discovery (Shen, 2015) because of the biologically relevant chemical diversity of such products and because they are aligned with Nature's need to develop a "chemical warfare" arsenal (Baell, 2016).

Here, we screened a small set of natural compounds ($n = 400$) against *Haemonchus contortus*, the barber's pole worm, and identified rotenone and deguelin to have an activity against this worm *in vitro*. These two flavonoids originate from plants of the family Leguminosae (e.g., Wenjie et al., 2009; Chen et al., 2014; Vats and Kamal, 2014) and have been shown to possess drug-like properties. For instance, deguelin has been reported to affect

some cancers (of lung, stomach and prostate) (Gerhauser et al., 1997; Udeani et al., 1997; Lee et al., 2005; Arulampalam et al., 2011; Boreddy and Srivastava, 2013; Farmer et al., 2013; Shang et al., 2014), and has anti-inflammatory, anti-angiogenic and apoptotic effects (Lee, 2004; Paulus et al., 2012). In addition, rotenoids extracted from plants such as *Derris elliptica* (Tuba plant), *D. involuta* (Jewel vine), *Lonchocarpus utilis* (Cub) or *L. urucu* (Barbasco) have been used as natural acaricides or insecticides (e.g., Ashack et al., 1980; Embeya et al., 2014). As rotenone is lethal to fishes but not to mammals to the same extent (Isman, 2006), there would be considerable concerns about its use as a parasiticide. Due to toxicity concerns, we did not pursue this chemical further in the present study. As deguelin is chemically related to rotenone, there was some initial concern about the safety of deguelin in vertebrates (Caboni et al., 2004). In contrast, although Parkinson's disease-like signs have been reported in mice upon chronic administration of deguelin (6 mg/kg/day) (Caboni et al., 2004), there is no evidence that deguelin exhibits toxic effects in mice or rats at 2-5 mg/kg (therapeutic doses; Udeani et al., 2001; Lee et al., 2005; Boreddy and Srivastava, 2013; Mehta et al., 2013). Moreover, deguelin and analogues thereof have been explored and assessed as anti-cancer drugs (Udeani et al., 1997; Murillo et al., 2002; Lee et al., 2016), but further work needs to be conducted to verify toxicity of various analogues to mammalian cells.

Evidence indicates that deguelin exerts its effect(s) on rapidly replicating cells (e.g., cancer cells) via extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)/Akt (Bortul et al., 2005; Mehta et al., 2013; Wu et al., 2016), insulin-like growth factor receptor (Kim et al., 2008; Suh et al., 2013; Lee et al., 2015) and/or the glycogen synthase kinase-3 β/β -catenin (Thamilselvan et al., 2011) signaling pathways. Deguelin has been reported to decrease tumor growth and metastasis in rodents via the induction of apoptosis and the inhibition of epithelial-to-mesenchymal transition by targeting the nuclear factor-kappa beta (NF- κ B) and tumour growth factor- β 1 (TGF- β 1) pathways (Boreddy and Srivastava, 2013; Liu et al., 2016). Biochemically, deguelin has been shown to reduce oxygen consumption via a down-regulation of mitochondrial electron transport and/or phorbol ester-induced ornithine decarboxylase (Gerhauser et al., 1997; Fang and Casida, 1998; Garcia et al., 2012; Vrana et al., 2013). In contrast, nothing is known about how deguelin affects parasitic nematodes. Therefore, we plan to explore the molecular differences between deguelin-treated and untreated *H. contortus* using transcriptomic and/or biochemical approaches, aiming at a better understanding of how this chemical affects biological pathways in this parasitic nematode. The relative safety of deguelin in mammals at therapeutic doses (Udeani et al., 2001; Lee et al., 2005; Boreddy and Srivastava, 2013; Mehta et al., 2013), the amenability of its chemical scaffold to chemical synthesis in order to generate new analogs and assess toxic properties (Kim et al., 2008; Chang et al., 2012; Lee et al., 2016) and the potency of deguelin against *H. contortus* observed in the present study set the scene for future investigations of deguelin and its derivatives in nematodes.

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Fig. 1. Dose response curves showing the activity of deguelin (triangle) on the exsheathed third-stage larvae (xL3) of *Haemonchus contortus* compared with reference compounds monepantel (circle) and moxidectin (square) following 24 h, 48 h and 72 of incubation with the compound.

ah: the plots might be easier to read if different colours were used for the three different compounds (symbols and fitted lines). The x-axis title should be “log₁₀ concentration” (no units). Is the scaling on the x-axis correct? If monepantel has an IC₅₀ of 0.13 μM = 0.13 · 10⁻⁶ M, then the log value should be log₁₀ [0.13 · 10⁻⁶ M / (1 M)] = -6.9. In the graph it looks more like -0.5 at 24 h..

Table 1. *In vitro*-activity of deguelin compared with that of monepantel or moxidectin on inhibition of xL3 motility after 72 h and cytotoxicity data.

Compound	Lipinski rule-of-five ^a						xL3 M _i IC ₅₀ (μM)
	1	2	3	4	5	6	
Deguelin	394.4	3.57	63.2	6	0	0	19.13±5.71

Monepantel	473.1	6.35	111.2	5	1	1	0.13 ± 0.10

Moxidectin	639.8	5.03	116.0	9	2	1	0.04 ± 0.01

^a Lipinski rule-of-five: 1 = molecular weight (g/ mol); 2 = lipophilicity;
 3 = polar surface area; 4 = H-bond acceptors; 5 = H-bond donors;
 6 = number of rule of five violations; na = not applicable.

ah: perhaps one could highlight the violating features in red. Moxidectin has only 1 Ro5 violation; Lipinski allows up to 10 H-bond acceptors.