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Low cost whole-organism screening of compounds for anthelmintic activity

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ABSTRACT

Due to major problems with drug resistance in parasitic nematodes of animals, there is a substantial need and excellent opportunities to develop new anthelmintics via genomic-guided and/or repurposing approaches. In the present study, we established a practical and cost-effective whole-organism assay for the in vitro-screening of compounds for activity against parasitic stages of the nematode *Haemonchus contortus* (barber's pole worm). The assay is based on the use of exsheathed L3 (xL3) and L4 stages of *H. contortus* of small ruminants (sheep and goats). Using this assay, we screened a panel of 522 well-curated kinase inhibitors (GlaxoSmithKline, USA; code: PKIS2) for activity against *H. contortus* by measuring the inhibition of larval motility using an automated image analysis system. We identified two chemicals within the compound classes biphenyl amides and pyrazolo[1,5-α]pyridines, which reproducibly inhibit both xL3 and L4 motility and development, with IC_{50}s of 14–47 μM. Given that these inhibitors were designed as anti-inflammatory drugs for use in humans and fit the Lipinski rule-of-five (including bioavailability), they show promise for hit-to-lead optimisation and repurposing for use against parasitic nematodes. The screening assay established here has significant advantages over conventional methods, particularly in terms of ease of use, throughput, time and cost. Although not yet fully automated, the current assay is readily suited to the screening of hundreds to thousands of compounds for subsequent hit-to-lead optimisation. The current assay is highly adaptable to many parasites of socioeconomic importance, including those causing neglected tropical diseases. This aspect is of major relevance, given the urgent need to deliver the goals of the London Declaration (http://unitingtocombat-ntds.org/resource/london-declaration) through the rapid and efficient repurposing of compounds in public–private partnerships.

1. Introduction

Infectious diseases cause substantial morbidity and mortality in animals and people worldwide, and major losses to the global food production annually. For instance, haemonchosis is one of the most important parasitic diseases of livestock worldwide, affecting hundreds of millions of small ruminants (including sheep and goats), and causing substantial production losses (Waller and Chandrawathani, 2005; Roeber et al., 2013) estimated at tens of billions of dollars per annum. The causative agent, *Haemonchus contortus* (barber's pole worm; Nematoda: Strongylida), feeds on blood in the stomach and causes gastritis, anaemia and associated complications, leading to serious production losses and death in severely affected animals. This nematode is transmitted orally from contaminated pasture to the host through a complex life cycle (Veglia, 1915): eggs are excreted in host faeces; individual L1s develop inside eggs, to then hatch (usually within 1 day) and develop through to L2s and L3s in approximately 1 week; infective L3s are then ingested by the host, exsheath (xL3) and, after a histotrophic phase, develop through L4 to dioecious adults (within 3 weeks) in the abomasum.

Although a vaccine (Barbervax®, Wormvax, Australia) has been released (October 2014) to support treatment programs against

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haemonchosis, the control of *H. contortus* and numerous other related nematodes has largely relied on the use of anthelmintic drugs. The excessive use of such drugs has led to widespread resistance in these nematodes to most classes of anthelmintic drugs (Kaplan, 2004; Wolstenholme et al., 2004; Jabbar et al., 2006; Besier, 2007; Kaplan and Vidyashankar, 2012; Wolstenholme and Kaplan, 2012; Scott et al., 2013), seriously compromising the control of parasites in many countries. Although the development of the compounds derquantel (patent WO1997/03988; Lee et al., 2001), emodepside (patent WO 1997/02256; Harder and von Samson-Himmelstjerna, 2001) and monepantel (patent WO2005/44784; Kaminsky et al., 2008) has provided hope for the design of new classes of nematocides (Prichard and Geary, 2008; Epe and Kaminsky, 2013), recent success in discovering new drugs using conventional screening (such as larval development, motility and migration inhibition methods; cf. Le Jambre, 1976; Martin and Le Jambre, 1979; Dobson et al., 1986; Kotze et al., 2006; Demeler et al., 2010) has been limited; most assays are not suitable for the efficient and effective screening of chemical libraries, mainly due to the subjective and manual recording of nematocidal activity (e.g., motility or larval development) as well as restrictions on throughput capacity (Geary et al., 2012; Paveley and Bickle, 2013; Buckingham et al., 2014). Nonetheless, there have been some recent improvements in the development of screening assays for parasitic nematodes (Smout et al., 2010; Marcellino et al., 2012; Paveley and Bickle, 2013; Storey et al., 2014), but often the financial costs of setting up automated assays can be quite high.

Recent investigations of the genome and transcriptomes of *H. contortus* (see Laing et al., 2013; Schwarz et al., 2013), which are considerably improving our understanding of the molecular biology and biochemistry of this and related strongyloid nematodes, might now support the search for new drugs through target prioritisation in silico (e.g., Crowther et al., 2010; Doyle et al., 2010; Jex et al., 2011; Schwarz et al., 2013; Taylor et al., 2013). For instance, based on gene essentiality predictions, Schwarz et al. (2013) predicted and prioritised various ion channels, G-protein coupled receptors, GTPases, peptidases, phosphatases and kinases as drug target candidates. Complemented by such genomic-guided prioritisation, the repurposing (repositioning or re-profiling) of existing and/or curated drugs (Emig et al., 2013; Panic et al., 2014) is also likely to accelerate/assist the drug development process, particularly through an availability of preclinical data sets for such drugs and the reduced cost, risk and time for translation to market (Panic et al., 2014). To overcome limitations of some conventional drug screening methods, we established here a new and inexpensive whole-organism assay for the rapid screening of compounds predicted to bind prioritised drug targets (specifically kinases) and destined for repurposing through a public–private partnership. This assay, which relies on video capture to measure the motility inhibitory properties of compounds on the parasitic larval stages (xL3s and L4s) of *H. contortus* and subsequent morphological assessment of larval development, was employed specifically to identify kinase inhibitors with activity against *H. contortus*.

2. Materials and methods

2.1. Production of parasite and storage

Haemonchus contortus (Haecon-5 strain; cf. Schwarz et al., 2013) was maintained in experimental sheep as described previously (Schwarz et al., 2013) and in accordance with the institutional animal ethics guidelines (permit no. 1111938; The University of Melbourne, Australia). In brief, helminth-free Merino sheep (8 weeks of age) were inoculated intra-ruminally with 5,000 L3s of *H. contortus*. Four weeks after infection, faecal samples were collected each day. L3s were produced from eggs by incubating faeces at 27 °C for 1 week (Schwarz et al., 2013). Then, L3s were sieved through two layers of nylon mesh (pore size: 20 μm; Rowe Scientific, Australia) to remove debris or dead larvae and stored at 10 °C for up to 3 months.

2.2. Exsheathment of L3s

L3s were exsheathed and sterilised by incubation in 0.15% v/v sodium hypochlorite (NaClO) at 37 °C for 20 min (Nikolaou et al., 2002). Following this incubation, xL3s were immediately washed five times in sterile physiological saline by centrifugation at 600*g* (5 min) at room temperature (22–24 °C). After the last wash, xL3s were immediately suspended in Luria Bertani medium [LB: 10 g of tryptone (cat no. LP0042; Oxoid, England), 5 g of yeast extract (cat no. LP0042; Oxoid) and 5 g of NaCl (cat no. K43208004210; Merck, Denmark)] in 1 L of reverse-osmosis deionised water). LB was autoclaved and supplemented with final concentrations of 100 IU/ml of penicillin, 100 μg/ml of streptomycin and 2.5 μg/ml of amphotericin (Fungizone®, antibiotic–antimycotic; cat no. 15240-062; Gibco, USA); this supplemented LB was designated LB*. For experiments involving L4s, xL3s were resuspended in 50 ml of LB* and transferred into a T175 tissue culture flask (DB Falcon, USA). The larvae were then incubated in a water-jacketed CO_2 incubator (model no. 2406 Shellab, USA) for 7 days at 38 °C and 20% v/v CO_2 to promote development into L4s.

2.3. Preparation of compounds for screening and assay plate preparation

The compound library (designated PKIS2) containing 522 kinase inhibitors was donated by GlaxoSmithKline (GSK), USA. Compounds were supplied at a concentration of 10 mM in DMSO (cat no. 2225; Ajax Finechem, Australia). Individual compounds were diluted to 40 μM in LB* containing 1% DMSO and 50 μl dispensed into the wells of sterile 96-well flat bottomed microplates (cat no. 3635; Corning 3650, Life Sciences, USA) using a liquid handler (JANUS, Perkin Elmer, USA). The anthelmintics moxidectin (Cydectin®, Virbac, France) and monepantel (Zolvix®, Novartis Animal Health, Switzerland) were used as positive-control compounds.

2.4. Screening assay to test the effect of compounds on xL3 motility

On each 96-well plate, test compounds, the positive-control compounds (moxidectin and/or monepantel) and the DMSO control in LB* were arrayed in triplicate. Six wells were used for the negative control (LB* + 0.5% DMSO). Following dispensing into the plates, 300 xL3s in 50 μl of LB* were transferred to each well of each plate (with the exception of perimeter wells) using a multi-channel pipette (Finnpipette, Thermo Scientific, USA). During dispensing, xL3s were kept in a homogenous suspension by bubbling air through the solution using an air pump (Airpump-S100; Aquatrade, Australia). Thus, following the addition of xL3s to individual wells, the final concentrations were 20 μM (compound) and 0.5% (DMSO).

Plates were incubated in a water-jacketed CO_2 incubator (model no. 2406, Shellab, USA) at 38 °C and 10% v/v CO_2. After 72 h, the plates were agitated (126 rotations per min) using an orbital shaker (model EOM5, Ratek, Australia) for 30 min at 38 °C. In order to capture the motility of xL3s at 72 h, a 10 s video recording was taken of each well on each plate using an eyepiece camera (Dino-eye, ANMO Electronic Corporation, Taiwan) attached to a stereo dissecting microscope (Olympus, Japan). After 3 min of imaging, plates were re-agitated for 5 min. The motility of xL3s was recorded in each well on each plate (Fig. 1). Each 10 s video capture of each well on each plate was processed using a custom macro in the program Image J (1.47v, imagej.nih.gov/ij). In each