Veterinary Parasitology 265 (2019) 15–18

Contents lists available at ScienceDirect

Veterinary Parasitology

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

Research paper

# Screening of a drug repurposing library with a nematode motility assay identifies promising anthelmintic hits against *Cooperia oncophora* and other ruminant parasites

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ARTICLE INFO

*Keywords:*
Anthelmintic
*Cooperia oncophora*
Repurposing library
Motility assay
EPV4593

ABSTRACT

Parasitic nematodes continue to cause significant economic losses in livestock globally. Given the limited number of anthelmintic drugs on the market and the currently increasing drug resistance, there is an urgent need for novel anthelmintics. Most motility assays of anthelmintic activity for parasitic nematodes are laborious and low throughput, and therefore not suitable for screening large compound libraries. *Cooperia oncophora* accounts for a large proportion of reports on the drug-resistance development of parasites globally. Therefore, using a WMicroTracker instrument, we established a practical, automated and low-cost whole-organism motility assay against exsheathed L3 stages (xL3s) of the ruminant parasite *Cooperia oncophora*, and screened a repurposing library comprising 2745 molecules. Fourteen known anthelmintics contained in this library were picked up in this blind screen, as well as four novel hits: thonzonium bromide, NH125, physostigmine sulfate, and EVP4593. The four hits were also active against xL3s of *Ostertagia ostertagi*, *Haemonchus contortus* and *Teladorsagia circumcincta* using the same assay. Cytotoxicity testing showed that thonzonium bromide and NH125 (1-Benzyl-3-cetyl-2-methylimidazolium iodide) have significant cytotoxicity. EVP4593 (N(4)-(2-(4-phenoxyphenyl)ethyl)-4,6-quinazolinediamine) demonstrated a potent and broad anthelmintic activity, and a high selectivity index. Moreover, given its novel and unexplored chemical scaffold for anthelmintic activity, EVP4593 is an interesting anthelmintic hit for further optimization.

## 1. Introduction

Parasitic nematodes are of major economic importance in livestock. The annual economic losses caused by parasitic nematodes in agricultural animals run into the billions of dollars worldwide (Preston et al., 2017). In the absence of vaccines for these gastrointestinal nematodes, the treatment of infections predominantly relies on a small number of anthelmintics. However, anthelmintic resistance has developed rapidly and become a serious problem in livestock (Gasbarre, 2014). Thus, there is an urgent need for novel anthelmintics (Vercruysse et al., 2018).

Drug repurposing is a promising strategy to accelerate the drug discovery and development process, offering lower costs, decreased risk and shortened time to market due to the availability of preclinical and clinical data (*e.g.* pharmacokinetics, safety, mode of action). Recently, a variety of drug repurposing efforts have been directed against a range of helminth infections and might deliver new potential drugs in the next years (Panic et al., 2014). Although *Cooperia* spp. account for a large proportion of reports on the drug-resistance development of parasites globally (Craig, 2018; Verschave et al., 2016), a drug repurposing project for the harmful cattle parasite, *Cooperia oncophora* (*C. oncophora*), has not yet been carried out.

The assessment of motility is considered to be the current gold standard for measuring drug effectiveness for helminth parasites *in vitro* (Smout et al., 2010). Moreover, the automated measurement of movement of parasites in liquid media is well-suited for the readily-scorable phenotypic readout required for high-throughput screening (Buckingham et al., 2014). Although some recent success on the

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https://doi.org/10.1016/j.vetpar.2018.11.014
Received 29 June 2018; Received in revised form 27 November 2018; Accepted 28 November 2018

development of motility screening assays for parasitic nematodes have been achieved, most assays are not suitable for the efficient screening of chemical libraries, mainly due to low throughput capacity, high cost and their time-consuming nature (Partridge et al., 2018; Preston et al., 2015). Therefore, we established an automated whole-organism motility assay with high throughput potential using parasitic exsheathed L3 stages (xL3s) of *C. oncophora,* and screened a repurposing library to discover novel candidate anthelmintics.

## 2. Material and methods

### 2.1. Chemicals and reagents

The repurposing library consists of a selection of compounds from the Pharmakon library (from MicroSource Discovery Systems Inc, http://www.msdiscovery.com/pharmakon.html) and the Selleckchem Bioactive compound library (from Selleck Chemicals LLC, http://www.selleckchem.com/screening/chemical-library.html). The targets of the majority of the compounds are known. The library consists of 2745 molecules of which ~1100 are FDA-approved, ~50 have been launched, and ~230 are in clinical development; the others are still in a preclinical stage. EVP4593 was purchased from Sigma-Aldrich, thonzonium bromide was from Medchemexpress, physostigmine sulfate and NH125 were from Tocris Bioscience.

### 2.2. Anthelmintic activity test

#### 2.2.1. Preparation of parasitic xL3 larvae

L3 larvae of *C. oncophora* were obtained by culturing the faeces of calves, artificially infected with *C. oncophora*, and maintained in water at 10 °C as described by (Heizer et al., 2013). xL3s were used in this anthelmintic assay, since large stocks of ensheathed L3s can be stored for extended periods of time (at least 3 months at 10 °C), with no significant impact on the motility of xL3s, which has major advantages over some other assays that rely on fresh materials (*e.g.*, eggs) from infected animals (Preston et al., 2015). The assessment of motility on xL3s can be considered to be a reliable standard for measuring the anthelmintic activity for parasites *in vitro*. The xL3s parasites were obtained by adding 3% sodium hypochlorite. After 15 min of incubation, the xL3s were washed five times with Milli-Q® water over a paper filter (Whatman) using a Buchner funnel, and then collected in RPMI-1640 medium. L3 larvae of *Ostertagia ostertagi* (*O. ostertagi*) were obtained from infected calves, while *Haemonchus contortus* (*H. contortus*) and *Teladorsagia circumcincta* (*T. circumcincta*) were obtained from infected sheep. The xL3s of *O. ostertagi*, *H. contortus* and *T. circumcincta* were prepared in a same manner as those of *C. oncophora*.

#### 2.2.2. Screening of compounds for their effect on parasite motility

The anthelmintic assays were performed in a sterile 96-well flat-bottom microplates. The repurposing library was screened using a blinded screening approach. One µL of individual compound stock solution (10 mM in DMSO) from the library was added into 99 µL of RPMI-1640 in the 96-well plate and arrayed in duplicate. The collected xL3s of *C. oncophora* in RPMI-1640 were adjusted approximately to 800 larvae/mL. Then 80 xL3s in 100 µL of RPMI-1640 were transferred to each well using an electronic Eppendorf Multipette® M4. The final compound concentration was 50 µM containing 0.5% DMSO (v/v). Thus, 0.5% DMSO (four replicates) was used as a solvent control, while 50 µM levamisole (four replicates) was used a positive control.

The plate was agitated (300 r/min) using an orbital shaker (Thermostar, Austria) for 10 min and incubated at 37 °C in a humidified incubator with a 5% $CO_2$ atmosphere for 8 h. Then the media in the wells were gently pipetted up and down 5 times using a multichannel pipette to stimulate the worms. Subsequently, the plate was placed into an automated tracking apparatus: WMicroTracker (Phylumtech, Argentina). Then the worms were allowed to habituate for 5 min in the (dark) chamber of the WMicroTracker, followed by incubation and motility monitoring for 3 h at 20 °C. The motility of worms in each well was measured every 30 min and recorded by the WMicroTracker through an infrared microbeam, which is interrupted when a worm passes by (each microtiter well is crossed by at least one infrared microbeam, scanned more than 10 times per second). A darkness/light (1 h/1 h) cycle in the WMicroTracker was used as a stimulus during tracking (worms kept in continuous darkness gradually decreased their spontaneous motility, making it hard to detect motility inhibition). The percentage of the average movement over 3 h of exposure to test compounds, compared with the DMSO control, was used to estimate the relative anthelmintic activity. The $Z'$ factor of each plate was calculated for assessing the quality of the screening assay (Iversen et al., 2006) as follows: $Z' = 1 - (3\sigma_p - 3\sigma_n)/|\mu_p - \mu_n|$, where $\sigma_p$ and $\sigma_n$ are the standard deviation (SD) of positive control and negative (solvent) control signals, $\mu_p$ and $\mu_n$ are the mean of positive control and negative control signals, respectively. A $Z'$ factor $\geq 0.5$ indicates a reliable assay. After primary screening, compounds with $\geq 70\%$ inhibition of motility, relative to the controls, were selected for secondary confirmation assays. The selected compounds were first retested at 50 µM to verify their inhibitory effects on motility. Compounds that consistently exerted $\geq 70\%$ inhibition were recorded as hits, and their concentration–response curves were measured to establish their $EC_{50}$ values. Compounds were tested in a similar xL3 system at 5 different concentrations. The tested concentrations were log10-transformed, and a variable slope four-parameter equation was used to calculate $EC_{50}$.

The same protocol was used to test the activity of *C. oncophora* hits on xL3s of *O. ostertagi*, *H. contortus* and *T. circumcincta*.

### 2.3. Cytotoxicity test

Understanding the cellular toxicity is important in drug discovery, and eukaryotic cell cultures are accepted as the model system of choice to obtain a first approximation of toxicity (Atterwill and Steele, 1987). To examine the potential toxicity of active compounds that were identified, their cytotoxicity was determined using a MTT assay (Gerlier and Thomasset, 1986). Two non-tumoural cell lines, HEK 293 and RAW 264.7 (ATCC, USA), were maintained in DMEM (Gibco), supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL) and 10% FBS (Sigma, lot number: 025M3355), at 37 °C and in a humidified incubator with a 5% $CO_2$ atmosphere. The cells were plated at a density of 7500 cells per well (containing 100 µL medium) in a 96-well plate, and cultured for 24 h. Subsequently, the cells were exposed to various concentrations of compounds. The plates were incubated for 24 h and cell viability was measured by adding 20 µL of MTT dye (5 mg/mL) per well. The plates were incubated for a further 3.5 h, followed by the addition of 150 µL of DMSO to dissolve formosan crystals. The absorbance was read at 590 nm with a reference filter of 620 nm, and the values were expressed as the cell viability (%) compared with the DMSO control. β-lapachone (40 µM) was used as a positive (cytotoxic) control in this assay.

### 2.4. Data analyses

Data from dose-response experiments are represented as the percentage of inhibition, and were analyzed with GraphPad Prism 6 software (San Diego, USA). A log (inhibitor) *versus* response non-linear fit was used to estimate the $EC_{50}$ and $CC_{50}$.

## 3. Results and discussion

An efficient and effective (fast and robust) screening assay is vital for the screening of compound libraries. Most motility assays for parasitic nematodes are laborious and low-throughput, which impedes the screening of large compound libraries. Inspired by a recently developed low-cost imaging-based high-throughput screening assay by

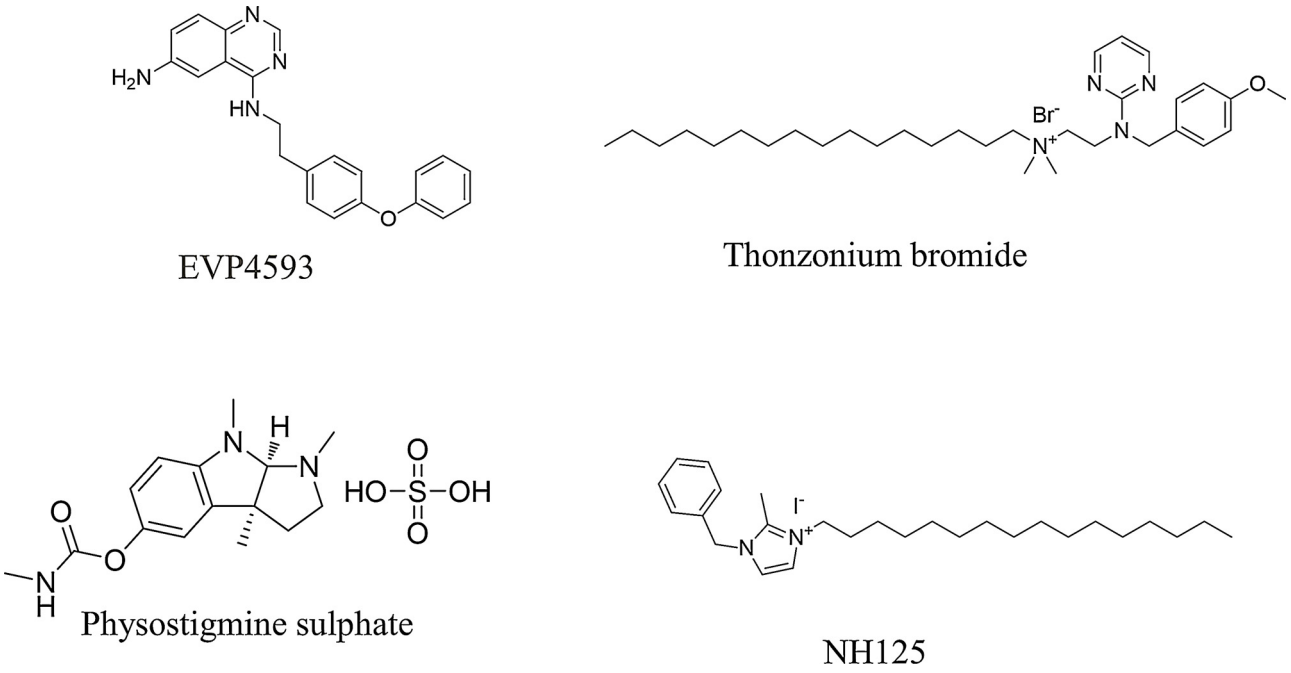


**Fig. 1.** Chemical structures of four hits.

**Table 1**
Anthelmintic activity and cytotoxicity of hits.

| Hits | $EC_{50}$ (μM) on parasites[a] | | | | $CC_{50}$ (μM) on cell lines[a] | |
|---|---|---|---|---|---|---|
| | *Cooperia oncophora* | *Ostertagia ostertagi* | *Haemonchus contortus* | *Teladorsagia circumcincta* | HEK 293 | RAW 264.7 |
| Thonzonium bromide | 4.5 ± 0.7 | 10.8 ± 2.6 | 5.1 ± 1.6 | 6.7 ± 0.8 | 9.2 ± 1.8 | 5.7 ± 1.2 |
| NH125 | 10.2 ± 1.6 | 16.8 ± 4.9 | 7.9 ± 1.3 | 9.6 ± 1.5 | 9.7 ± 2.3 | 5.9 ± 2.0 |
| Physostigmine sulfate | 14.4 ± 2.8 | 97.3 ± 17.2 | 78.4 ± 11.6 | 69.3 ± 9.4 | > 380 | > 380 |
| EVP4593 | 1.9 ± 0.3 | 3.4 ± 1.0 | 2.3 ± 0.7 | 2.7 ± 0.6 | 90.3 ± 8.5 | > 150 |

[a] Mean ± SD, n ≥ 2.

Preston et al. (2015), we aimed to establish an automated high-throughput assay for xL3s using the WMicroTracker. This approach relies on quantification of movement-related light scattering to assess the motility of worms. At first, the plate containing xL3s incubated with compounds for 8 h was agitated by an orbital shaker at a high speed (400 r/min) to stimulate the worms, and then was measured by the WMicroTracker at 37 °C. However, the motility of worms *in vitro* was not constant: the motility decreased gradually and became undetectable after 30 min. Although we increased the density of worms (200/well), changed the incubation temperature (20 °C, 28 °C) during measurement, and employed a light/darkness cycle, the detected motility failed to increase significantly, and the $Z'$ factor over a 3 h period was < 0.5, and thus not suitable for screening. Inspired by a recent assay by (Keiser et al., 2016), we tried to pipette the media up and down manually using a multichannel pipette to stimulate the worms. The results showed that the $Z'$ factor over 3 h was improved to > 0.6 reproducibly. We also tested whether the same approach could be applied to xL3s of other ruminant parasites, namely: *O. ostertagi*, *H. contortus* and *T. circumcincta*. The protocol indeed also worked well for these other ruminant parasites ($Z'$ factor > 0.5), suggesting that our assay can be highly adaptable to many other parasites.

Since the screening assay was satisfactory for our purposes, we did not systematically optimize other parameters of the assay and proceeded to use it for screening the repurposing compound library. Conditions that could be further optimized include: the concentration of DMSO, the exsheathment condition for L3s, the culture media for xL3s and the density of xL3s in wells, although some parameters in our assay were based on a well-established assay for *H. contortus* (Preston et al., 2015) (*e.g.* the concentration of DMSO control, the exsheathment condition for L3s). Moreover, an automated more standardized way to stimulate worms can be developed in the future to eliminate the potential bias and variability from manually pipetting the media up and down.

Of all 2735 compounds in the repurposing library, 18 reduced worms motility by ≥ 70% in both the primary and secondary screen, and were considered as hits. After their structures were revealed (up to that point the screening was blind), 14 turned out to be known anthelmintic (Table S1), and were not considered for further studies. These 14 compounds correspond to all known anthelmintics in the repurposing library, which further validates the reliability of our newly established assay. The other four hits are not known as anthelmintic agents (Fig. 1): thonzonium bromide, NH125, physostigmine sulfate and EVP4593. Their $EC_{50}$ values were determined, and EVP4593 showed the most potent activity (Table 1, Fig. S1). The hit rate in our assay (0.6%) is fairly low. It may be because the criteria we set for hits are strict: 70% motility inhibition as a cutoff for worms incubated with 50 μM compounds for 8 h. However, in most published assays, the incubation time of parasites with compounds is 72 h (Jiao et al., 2017; Panic et al., 2014; Preston et al., 2015). For most of our active compounds, however, a stable inhibition had been reached by the end of the assay, and clinically used anthelmintics cause inhibition rapidly, although we cannot exclude that we have missed some slow-acting compounds.

The four novel hits were further screened on xL3s of other ruminant

parasites and tested for cytotoxicity. The four hits were also active against xL3s of three other ruminant parasites (Table 1). Physostigmine sulfate was less active against the other three parasites compared to *C. oncophora*, while EVP4593, thonzonium bromide and NH125 demonstrated a comparable potency against all four parasites. Thonzonium bromide and NH125 demonstrated significant cytotoxicity on two non-tumoral cell lines with selectivity indexes < 1. EVP4593 and physostigmine sulfate did not show significant cytotoxicity with selectivity indices > 40.

Thonzonium bromide is expected to be quite toxic to cells since it is a monocationic detergent. Thonzonium bromide is an antimicrobial agent for topical use only, although injection in mice subcutaneously at 5 mg/kg and did not exert any toxic effects, even with repeated dosing (Zhu et al., 2016). NH125 is being developed as an eukaryotic translation elongation factor 2 inhibitor (EEF-2) kinase inhibitor at the preclinical stage (https://clue.io/repurposing-app). Given that it is a close analogue of thonzonium bromide and exhibited a similar anthelmintic activity and cytotoxicity profile, the two compounds may well act similarly, namely as detergents.

Physostigmine sulfate is a reversible acetylcholinesterase inhibitor used to treat glaucoma and anticholinergic poisoning in the clinic (Arens et al., 2018). Acetylcholinesterase inhibitors have been used as anthelmintics, *e.g.* haloxon, dichlorvos, aldicarb and trichlorphon (Holden-Dye and Walker, 2014). One concern for these compounds is that they may be toxic to almost all organisms that use acetylcholine as a neurotransmitter. Very recently, polypyridylruthenium(II) complexes with strong anti-cholinesterase activity have been shown to exert *in vitro* and *in vivo* nematicidal activity in a mouse trichuriasis model (Sundaraneedi et al., 2018). Therefore, although physostigmine did not show very potent anthelmintic activity in our study, it may be a starting point to design more potent and selective anthelmintic compounds targeting nematode acetylcholinesterase.

EVP4593 is the most promising hit discovered from our repurposing library owing to its potent anthelmintic activity and favorable cytotoxicity. It is an NF-kB pathway inhibitor in preclinical development (https://clue.io/repurposing-app). In addition, EVP4593 was regarded as a lead compound for treating Huntington's disease (Nekrasov et al., 2016). It was also shown to be a highly potent and specific inhibitor of mitochondrial complex I (Krishnathas et al., 2017). Such information can suggest points of departure for investigating its underlying mechanisms of anthelmintic activity. No *in vivo* data on EVP4593 are available from the literature. Considering its novel and unexplored chemical scaffold for anthelmintic activity, the observed broad anthelmintic profile, and its relatively high selectivity index, EVP4593 may be an interesting starting point for further optimization.

## 4. Conclusions

We established a practical, automated and low-cost high-throughput whole-organism motility assay for xL3s parasites using the WMicroTracker instrument. Screening a repurposing compound library led to four novel hits. One of these (EVP4593) demonstrated promising properties as an anthelmintic.

## Conflicts of interest

None.

## Acknowledgements

Maoxuan Liu was supported by a Chinese Scholarship Council doctoral fellowship. Walter Luyten largely supported himself. We thank the Centre for Drug Design and Discovery (CD3) from the KU Leuven for providing access to the repurposing library, Dr. Arnaud Marchand and Dr. Patrick Chaltin from CD3 for the fruitful discussion and detailed revision on the manuscript. We acknowledge Dr. Dave Bartley from the Moredun Research Institute in the UK for providing us *Teladorsagia circumcincta* and *Haemonchus contortus* worms.

## 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.2018.11.014.

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