An insect-tapeworm model as a proxy for anthelminthic effects in the mammalian host

Woolsey, Ian David; Fredensborg, Brian Lund; Jensen, Per Moestrup; Kapel, Christian Moliin Outzen; Meyling, Nicolai Vitt

Published in:
Parasitology Research

DOI:
10.1007/s00436-015-4477-0

Publication date:
2015

Document version
Peer reviewed version

Parasitology Research

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--Manuscript Draft--

Manuscript Number:	PARE-D-15-00225R1
Full Title:	An insect-tapeworm model as a proxy for anthelminthic effects in the mammalian host.
Article Type:	Short Communication
Funding Information:	
Abstract:	Invertebrate models provide several important advantages over their vertebrate counterparts including fewer legislative stipulations and faster, more cost-effective experimental procedures. Furthermore, various similarities between insect and mammalian systems have been highlighted. To obtain maximum use of invertebrate models in pharmacology, their fidelity as analogues of vertebrate systems requires verification. We utilised a flour beetle (Tenebrio molitor) - tapeworm (Hymenolepis diminuta) model to evaluate the efficacy of known anthelmintic compounds, praziquantel, mebendazole and levamisole against H. diminuta cysticercoid larvae in vitro. Inhibition of cysticercoid activity during the excystation procedure was used as a proxy for worm removal. The effects of the three compounds mirrored their relative efficacy in treatment against adult worms in mammalian systems; however, further study is required to determine the fidelity of this model in relation to dose administered. The model precludes comparison of consecutive daily administration of pharmaceuticals in mammals due to cysticercoids not surviving outside of the host for multiple days. Treatment of beetles in vivo, followed by excystation of cysticercoids post dissection could potentially allow for such comparisons. Further model validation will include analysis of pharmaceutical efficacy in varying H. diminuta isolates and pharmaceutical dilution in solvents other than water. Notwithstanding, our results demonstrate that this model holds promise as a method to efficiently identify promising new cestocidal candidates.
Corresponding Author:	Ian David Woolsey, MSc DENMARK
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	
Corresponding Author's Secondary Institution:	
First Author:	Ian David Woolsey, MSc
First Author Secondary Information:	
Order of Authors:	Ian David Woolsey, MSc
	Brian L Fredensborg, Ph.D
	Per M Jensen, Ph.D
	Christian M. O. Kapel, Ph.D
	Nicolai V Meyling, Ph.D
Order of Authors Secondary Information:	
Author Comments:	

Dear Editor,

More details have now been added to the abstract. I have incorporated some of reviewer #1 comments into the discussion and have checked that all species and genus names are italicised.

The old name of the journal in the references has now been amended to Parasitol Res.

Regards,

Ian Woolsey.

An insect-tapeworm model as a proxy for anthelminthic effects in the mammalian host.

Ian David Woolsey, Brian L. Fredensborg, Per M. Jensen, Christian M.O. Kapel, Nicolai V. Meyling

Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C.

Word count:

Abstract: 213

Article: 1587

Corresponding author: Ian Woolsey, e-mail: ianwoolsey@plen.ku.dk,

Phone: +45 26 70 60 96.

Abstract

Invertebrate models provide several important advantages over their vertebrate counterparts including fewer legislative stipulations and faster, more cost-effective experimental procedures. Furthermore, various similarities between insect and mammalian systems have been highlighted. To obtain maximum use of invertebrate models in pharmacology, their fidelity as analogues of vertebrate systems requires verification. We utilised a flour beetle (*Tenebrio molitor*) – tapeworm (*Hymenolepis diminuta*) model to evaluate the efficacy of known anthelmintic compounds, praziquantel, mebendazole and levamisole against *H. diminuta* cysticercoid larvae *in vitro*. Inhibition of cysticercoid activity during the excystation procedure was used as a proxy for worm removal. The effects of the three compounds mirrored their relative efficacy in treatment against adult worms in mammalian systems; however, further study is required to determine the fidelity of this model in relation to dose administered. The model precludes comparison of consecutive daily administration of pharmaceuticals in mammals due to cysticercoids not surviving outside of the host for multiple days. Treatment of beetles *in vivo*, followed by excystation of cysticercoids post dissection could potentially allow for such comparisons. Further model validation will include analysis of pharmaceutical efficacy in varying *H. diminuta* isolates and pharmaceutical dilution in solvents other than water. Notwithstanding, our results demonstrate that this model holds promise as a method to efficiently identify promising new cestocidal candidates.

Key words

Cestode, anthelminthic, *Hymenolepis diminuta*, host-parasite model, *Tenebrio molitor*

Introduction

Mammal testing remains the gold standard to screen potential drug compounds for various toxicological and pharmacological effects in humans (Baumans 2004). The general public and the scientific community have however increasingly opposed the use of experimental animals and sought to Reduce, Refine and Replace (the 3R's) vertebrate experimentation (Schuppli et al. 2004). The use of invertebrate models would constitute one method of Reduction or potentially Replacement (nc3rs 2014). The invertebrate innate immune response to infection resembles that in humans (Hoffmann et al. 1999; Kavanagh and Reeves 2007; Kimbrell and Beutler 2001), and insects have been used as

immunological research models (Pursall and Rolff 2011). As invertebrates are presently not subject to experimental animal legislation that applies to vertebrates, less administrative efforts are required to ensure appropriate standards of welfare (Kemp and Massey 2007). Furthermore, in many invertebrate species, particularly insects, experimental processes are often quicker and their high rate of reproduction means they can be produced in large quantities in a relatively short time resulting in faster dissemination of results (Scully and Bidochka 2006). Finally, laboratory space required is minimal due to much smaller housing stipulations involved in using insects (Steinert et al. 2003).

The cosmopolitan rat tapeworm *Hymenolepis diminuta* Rudolphi, 1819 has been used as an experimental model for decades. Whilst it very rarely infects humans and is easily treatable (Tanowitz et al. 2001), it is often used as a teaching model for two reasons: i) the ease of maintenance of both stages of the parasitic life cycle (cysticercoid larvae in flour beetles and adult worm in rats), and ii) the helminth represents the same basic sequence of development as most other cestodes. Thus, the study of *H. diminuta* has made substantial contributions to our understanding of cestodes in general including their survival, reproduction and development strategies (Mansur et al. 2014). The use of an invertebrate intermediate host of this parasite enables the possibility of anthelmintic screening with limited use of vertebrates. Indeed, cysticercoids of *H. diminuta* isolated from infected beetles have been used to deduce mechanisms of cestocidal activity of plant derived cysteine proteinases (found in papaya and pineapple etc.) (Mansur et al. 2014) and condensed tannins (Dhakal et al. 2015). In addition, Novak and Evans (1978) demonstrated that mebendazole can retard development of *H. diminuta* cysticercoids when fed to *Tribolium confusum* du Val, 1863 beetles suggesting a similar mode of action of the drug towards the cysticercoid stage inside insects to the adult worm inside the mammalian host.

If the pharmacologic and toxicological parameters of potential cestocidal compounds, including plant extracts, can be achieved using an insect – tapeworm model it could significantly increase the speed at which such compounds are available as alternatives to current anthelmintics as well as reducing the amount of vertebrates necessary for experiments.

In this study, we assessed the usefulness of the *Tenebrio molitor* (Linneaus, 1758) -*H. diminuta* model as a proxy for the mammalian system by evaluating: 1) if the effects of the three commonly used

anthelmintics, praziquantel, mebendazole, and levamisole on cysticercoids of *H. diminuta* resemble the effects known from adult worms in the rat host (qualitative similarity), and 2) if varying concentrations of the pharmaceutical alter the inhibition of larval cestodes from insects as they do in mammalian systems (quantitative similarity). We assessed anticestodal effects as the rate of inhibition of *in vitro* excystation of cysticercoids when exposed to the physico-chemical conditions mimicking the digestive processes in the gastrointestinal tract of the rat.

Praziquantel is a highly effective drug against adult cestodes in mammals (McKellar and Jackson 2004; Thomas and Grönnert 1977), and we therefore hypothesized a strong inhibitory effect of praziquantel on cysticercoid excystation. In contrast, mebendazole shows a moderate effect on adult cestodes (McCracken, Lipkowitz et al. 1992), but it is widely used to treat nematode infections in humans (Bennett and Guyatt 2000). Inhibitory effects of mebendazole on cysticercoid excystation are therefore only expected at relatively high concentrations. Levamisole is a nematicidal drug showing no effect on adult cestodes in mammals (Bennett, Behm et al. 1978), and it therefore served as a negative control in this study.

Materials and methods

Pharmaceutical formulation and application

Stock solutions of praziquantel (Droncit®), mebendazole (Vermox®) and levamisole hydrochloride were mixed with Mill-Q® water in order to create the desired concentrations of 0.05, 0.005, 0.0005 and 0.00005%.

Infected *T. molitor* beetles (infection protocol as in Dhakal et al. 2015) were dissected with 0.9% physiological saline in a petri dish. Cysticercoid cysts were counted then removed with a Pasture Pipette and placed in watch glasses (33 mm diameter, 7 mm deep). Ten cysts were placed in each of 15 watch glasses containing either one of the 3 drugs at the 4 different concentrations or saline control. Three controls were prepared, one for each drug. No more than 4 cysticercoids from a single beetle were used per watch glass. Once all cysts had been positioned, saline was removed from the glass with a pipette and 0.5ml of the desired anthelmintic or saline was applied. Watch glasses were then placed on a Centromat® rotating table for 60 minutes at 50 rpm at room temperature. The experiment was

repeated at three separate occasions, and these repeats were pooled for final analysis leading to 30 observations for each pharmaceutical at each concentration.

Excystation

Cysticercoid excystation was achieved utilizing a modified existing protocol (Goodchild and Davis 1972). Briefly, anthelmintic was removed with a Pasteur Pipette and 2 ml HCl-Pepsin solution [2 ml 37 % HCl, 20 ml 37°C 0.9 % saline, 0.8 g pepsin powder from porcine gastric mucosa (1:2500, Sigma Life Science)] was added to each watch glass and placed in an incubator at 37°C for 10 minutes. The HCl-Pepsin solution was then removed by pipette and the cysticercoids were washed 3 times with saline (approx. 2 ml), to remove any remaining acid, after which 1 ml of trypsin-taurocholate solution [0.1 g sodium taurocholate hydrate powder, 0.1 g trypsin powder from porcine pancreas, (97 %, Sigma Life Science), 10 ml warm phosphate-buffered saline (PBS) was added. The cysticercoids were then placed in the incubator at 37°C for 180 minutes.

Cysticercoid observations

Cysticercoids were graded on a 2-point scale; 0 – no visible sign of activity; or 1 – movement within the cysticercoid or full excystation, that is, protrusion of the scolex from the cysticercoid capsule. To differentiate between type 0 and 1, each cysticercoid was observed at 100 × magnification for 10 seconds.

Statistical analysis

Paired comparisons, across all pharmaceuticals and concentrations (including their respective controls), were executed under a generalised (binomial) linear model, which provided information on statistical differences for each comparison (total pairs = 35, each having 30 observations). The analyses were conducted under PROC GENMOD in SAS 9.1 for Windows (SAS Institute, Cary NC, USA), which also provided information on the effect of concentrations for each pharmaceutical.

Results and Discussion

Across all concentrations, praziquantel was significantly more inhibitory than mebendazole (0.05% p=0.0128, 0.005% p=0.0128, 0.0005% p=0.0168 and 0.00005% p=0.0012) and levamisole (0.05%

p=0.0002, 0.005% *p*=0.0001, 0.0005% *p*=0.0002, 0.00005% *p*=0.0001). Mebendazole was only significantly more inhibitory against levamisole at the highest two concentrations (0.05% *p*=0.0114, 0.005% *p*=0.0026, 0.0005% *p*=0.0960 and 0.00005% *p*=0.2274). The inhibitory effect on excystation was dose-dependent both for praziquantel (*p*=0.0001) and mebendazole (*p*=0.0001) but not for levamisole where no significant reduction in excystation was observed (*p*=0.0633) (Fig. 1).

Thus, the greatest inhibitory effect of anthelmintic on cysticercoid activity was observed for praziquantel, followed by mebendazole whereas no significant inhibition could be demonstrated for levamisole. This trend was observed across all concentrations and indeed, qualitatively the drugs appear to be mirroring their comparative levels of efficacy in the mammalian system against adult cestodes (Bennett et al. 1978; Dayan 2003; McCracken et al. 1992; Thomas and Grönnert 1977). Furthermore, differing concentrations of the drugs *in vitro* have a significant affect on cysticercoid inhibition. This corresponds to mammalian models in which differing doses of pharmaceuticals administered result in varying worm expulsion efficacy. In rats, 100% reduction in *H. diminuta* worm burden has been demonstrated with 5 mg/kg praziquantel application, while 0.5 mg/kg resulted in a 53% worm burden reduction (Thomas and Gönnert 1977). In this study both praziquantel and mebendazole were observed to be significantly less inhibitory at lower concentrations. Due to this varying cysticercoid inhibition as a function of concentration, future studies could focus on calibrating concentrations *in vitro* with worm expulsion in mammals in an effort to enhance quantitative similarity of this model. Furthermore, the variability (or lack thereof) in pharmaceutical efficacy in different *H. diminuta* isolates should be considered as well as pharmaceutical dilutions in solvents other than water.

When utilizing mebendazole against cestode infections a number of studies employ consecutive daily treatment of the drug (Maki and Yanagisawa 1985; McCracken et al. 1992; Varma et al. 1989). As cysticercoids will not survive outside of the host for multiple days, comparisons of such studies with this model are precluded. Treatment of beetles *in vivo,* followed by excystation of cysticercoids post dissection could potentially allow for such comparisons.

Although the quantitative properties in relation to concentration administered need further exploration in the present model, our study demonstrates that the relative effects of the three anthelmintics in

mammalian systems were mirrored by the cysticercoid excystation assay. Therefore, this model has the potential to function as a rapid and cost-effective screening technique of anthelminthic candidates for further testing. Additionally, the *T. molitor* - *H. diminuta* model may significantly reduce the number of vertebrates needed for initial drug screening complying with the increasing demand for a reduction in the number of vertebrates used in biomedical research.

Funding: This research was supported by a starting grant from the programme "Synergy in Human and Animal Research (SHARE)" at the University of Copenhagen and a research grant (no. 00007457) from the Villum Foundation.

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (Danish Experimental Animal Inspectorate permission no. 2010/561-1914 –section C10).

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Fig. 1.: The effect of three anthelmintics at each of four concentrations and controls (0%) on the *in vitro* activation of larval *Hymenolepis diminuta*. The data represent the mean percentage of activated cysticercoids across three repeats. Error bars (S.E.M.).

Figure 1
Click here to download Figure: Figure 1.docx