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HELMINTHOLOGY - ORIGINAL PAPER

Cysticidal effect of a pure naphthoquinone on *Taenia crassiceps* cysticerci

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Abstract

Cysticercosis is a disease caused by the metacestode of the parasite *Taenia solium* (*T. solium*). In humans, the most severe complication of the disease is neurocysticercosis. The drug of choice to treat this disease is albendazole; however, the bioavailability and efficacy of the drug are variable. Therefore, new molecules with therapeutic effects against this and other parasitic infections caused by helminths must be developed. Naphthoquinones are naphthalene-derived compounds that possess antibacterial, antifungal, antitumoral, and antiparasitic properties. The aim of this work was to evaluate the in vitro anti-helminthic effect of 2-[(3-chlorophenylamino)phenylmethyl]-3-hydroxy-1,4-naphthoquinone, isolated from a natural source and then synthesized (naphthoquinone 4a), using an experimental model of murine cysticercosis caused by *Taenia crassiceps* (*T. crassiceps*). This compound causes paralysis in the cysticerci membrane from day 3 of the in vitro treatment. Additionally, it induces changes in the shape, size, and appearance of the cysticerci and a decrease in the reproduction rate. In conclusion, naphthoquinone 4a has in vitro cysticidal activity on *T. crassiceps* cysticerci depending on the duration of the treatment and the concentration of the compound. Therefore, it is a promising drug candidate to be used in *T. crassiceps* and possibly *T. solium* infections.

Keywords Naphthoquinone · Anti-helminthic · Taeniasis · Cysticercosis · *Taenia crassiceps* · Drug

Introduction

Human neurocysticercosis and porcine cysticercosis are caused by the metacestode stage of the cestode parasite *Taenia solium* (*T. solium*). Neurocysticercosis is a serious

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human health problem, while porcine cysticercosis is a veterinary problem in many developing and mainly in underdeveloped countries. Neurocysticercosis affects approximately 50 million people worldwide (White 1997; Flisser et al. 2003; Garcia and Del Brutto 2005; Nash et al. 2006). Currently in the USA, human neurocysticercosis is considered as an emergent disease (White 1997). Infection in pigs leads to major economic losses and causes the continuity of the infectious cycle in humans (Gonzalez et al. 2001). Due to the natural difficulties to work with *T. solium* (intermediate host is the pig and definitive host is the human), an alternative experimental strategy has been proposed and widely used worldwide. One of the most important aspects during infection by *T. solium* is the evagination process of *T. solium* cysticerci carried out in the human gut. The evagination will originate the adult worm that starts producing thousands of eggs. The infective eggs released with the stools can contaminate the environment infecting pigs (rapidly differentiating into cysticerci, mainly in the muscle) and humans (where most severe symptoms are observed due to the presence of cysticerci in the brain) (Pawlowski et al. 2005; Nash et al. 2006).

Murine cysticercosis caused by *Taenia crassiceps* (*T. crassiceps*) is an alternative experimental strategy to study the relationship between the host and the parasite. In addition, the host's physiological factors associated with porcine cysticercosis and, to some extent, human neurocysticercosis (NCC) are also analyzed using this model (Willms and Zurabian 2010). Murine cysticercosis can be induced in male and female mice by direct injection of *T. crassiceps* metacestode in the peritoneal cavity.

Such strategy has been implemented given that working with definitive hosts is difficult and *T. solium* cysticerci can only be obtained from infected pigs. Studies using infected pigs are challenging due to the number of animals needed. In addition, individual harvesting is elaborate and time-consuming and the maintenance of the cysticerci in the laboratory is difficult (Mahanty et al. 2011).

In contrast, the cysticerci of *T. crassiceps* ORF strain proliferate asexually by budding within the intermediary host, the mice in this case, remaining indefinitely in the intraperitoneal cavity of the animal through serial passages (Willms and Zurabian 2010). However, it cannot thrive in adult tapeworms inside the definitive host because of the loss of its scolex. In addition, *T. crassiceps* ORF shares antigens and shows a high DNA homology with other cestodes as *T. solium* and *Taenia saginata* (*T. saginata*). This experimental model has been considered useful in the design of studies applicable to *T. solium* cysticerci. It has been effective to prove drug and hormone activity and evaluate the prospect of porcine potential (Palomares et al. 2004; Trejo-Chávez et al. 2011; Márquez-Navarro et al. 2013; Sciutto et al. 2013; Reynoso-Ducoing et al. 2014; Ambrosio et al. 2015; Palomares-Alonso et al. 2015).

Albendazole, praziquantel, and niclosamide are therapeutic alternatives to treat taeniasis and cysticercosis (Dayan 2003; Gongora-Rivera et al. 2006; Fleury et al. 2008). However, niclosamide is not available in the Mexican market. Both albendazole and praziquantel are frequently used in NCC treatment together with corticosteroids and other anti-inflammatory agents. Still, corticosteroids usually increase or decrease plasmatic levels of these drugs (Romo et al. 2014).

Natural sources as plants are the best opportunity to find new molecules with pharmacological activity. Quinones are a kind of organic compound whose chemical properties allow them to interact as electron transfer agents with biological targets through the formation of covalent bindings and their action in redox reactions (Kumagai et al. 2012). Within this kind of compounds are naphthoquinones, compounds derived from naphthalene that are characterized by having two carbonyl functional groups in one of the aromatic rings at positions 1 and 4 (1,4-naphthoquinones) and at positions 1 and 2 (1,2-naphthoquinones) (Cantú González et al. 2012).

Other naphthoquinones, as menaquinones, naphtharazine, spinochromes, and equinochromes, are synthesized by bacteria, fungi, and sea urchins, respectively (Cantú González et al. 2012). Particularly, 1,4-naphthoquinones are studied in organic synthesis, medicinal chemistry, and natural product chemistry; they have been demonstrated to have antibacterial, antifungal, antitumoral, and antiparasitic properties (Kumagai et al. 2012; Kumar et al. 2013; Aires et al. 2014). The antiprotozoal activity of some 1,4-naphthoquinones has been widely reported (Aires et al. 2014). For instance, lapachol, an alkyl 1,4-naphthoquinone of natural origin, was the first naphthoquinone found to counter *Plasmodium falciparum* (*P. falciparum*), the infectious agent causing malaria due to the toxicity caused by its interaction with the respiratory chain of the mitochondrion (Sharma et al. 2013; Rezende et al. 2013). Although lapachol has not been used to treat malaria, its molecular structure inspired the synthesis of atovaquone, currently used together with proguanil as a therapeutic alternative against malaria (Sharma et al. 2013). The antiparasitic activity of atovaquone is also related to its selective action on the mitochondrial cytochrome bc1 complex, inhibiting electron transportation and collapsing the mitochondrial membrane potential. Electron transportation in *P. falciparum* mostly regenerates ubiquinone, the electron acceptor of the parasite's dihydroorotate dehydrogenase, an essential enzyme to pyrimidine biosynthesis (Nepveu and Turrini 2013). Other action mechanisms of naphthoquinones are related to the production of reactive oxygen species, enzymatic inhibition (glutathione reductase, glyceraldehyde-3-phosphate

dehydrogenase), alkylation of biomolecules, and glutathione depletion, among others (Rezende et al. 2013). Nevertheless, some naphthoquinones as ubiquinone, plastoquinone, and vitamin K group take part in relevant physiological processes as cell respiration and coagulation.

Since there are no studies on naphthoquinone activity in human and animal helminths, the aim of this work was to evaluate the in vitro and in vivo effects of 2-[(3-chlorophenylamino)phenylmethyl]-3-hydroxy-1,4-naphthoquinone (4a) (López-López et al., 2017) on *T. crassiceps*. Our results show a cysticidal activity of 4a in vitro and in vivo, as well as a marked reduction of infectivity, making the molecule a new promising therapeutic agent against other types of cysticercosis.

Materials and methods

Ethics statement

Animal care and experiments at the Instituto de Investigaciones Biomédicas are constantly evaluated and approved by the Experimental Animal Care and Use Committee of the Institute (Comité Interno para el Cuidado y Uso de Animales de Laboratorio, CICUAL, permit number 2016–00,123) adhering to the official Mexican standards (NOM-062-ZOO-1999). Mexican regulations are in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH) of the USA, to ensure compliance with established international regulations and guidelines. Mice were euthanized using anesthesia overdose (Sevorane®).

Infection and collection of cysticerci of Taenia crassiceps ORF strain

Female Balb/C mice aged 6 weeks were infected intraperitoneally with 10 cysticerci of *T. crassiceps* (ORF strain) that were motile and translucent, with no buds and measuring 2 mm, approximately. After 4–6 weeks of infection, the mice were humanely sacrificed using anesthesia overdose (Sevorane®). Extraction of cysticerci from the peritoneal cavity was performed under sterile conditions. The recovered cysticerci were washed five times in sterile PBS supplemented with 250 U/mL penicillin and 250 µg/mL streptomycin (Invitrogen). For the assays involving naphthoquinone 4a activity, the cysticerci were selected according to the following criteria: They showed no buds, were motile and translucent, and measured 2 mm approximately.

Compounds derived from naphthalene: naphthoquinones

Naphthoquinone 4a used in the experiments was obtained from a natural source. The source and tissue of the plants used were *Jatropha dioica* stem and root. The plants were transported to the Department of Food Research at Universidad Autónoma de Coahuila, in black plastic bags. Immediately the samples were dried for 24–48 h at 60 °C in an oven (LABNET International, Inc.). The dry samples were ground in a mechanical mill and screened at 0.6–0.8-mm size particle. The fine and dried powder was stored in black plastic bags sealed hermetically and at room temperature under darkness, and was later purified and synthesized and chemically evaluated (Itzel López-López et al. 2017). This naphthoquinone is soluble in organic solvents as DMSO and has a molecular mass of 391.39 g/mol. For the time curve, a naphthoquinone 4a in DMSO solution was prepared at a final concentration of 20 mg/mL. The solution was sterilized using a 0.2-µm filter and kept in dark at 4 °C. From this, a 500 µg/mL stock solution (solution A) was prepared in an RPMI-supplemented medium and was then used to prepare different concentrations of naphthoquinone 4a used to evaluate the effect in the dose–response curve.

Time-response curves to evaluate the effect of naphthoquinone 4a

The effect of naphthoquinone 4a at a maximum concentration of 100 µg/mL was evaluated. RPMI 1640 medium (In Vitro SA) was supplemented with 10% fetal bovine serum (FBS) and a mix of 250 U/mL penicillin and 250 µg/mL streptomycin (Invitrogen) was used for time-response effect (1–5 days). The 4a stock solution (500 µg/mL) was added to the culture medium for a final 100 µg/mL concentration. Three different assays were performed, using 10 cysticerci and 2 mL of the 100 µg/mL solution, that were placed per well in a 6-well culture plate (Corning®). The culture plate was placed in an incubator (Revco Technologies) at 37 °C, 5% CO_2, and 90% humidity. The culture was carried out for 10 days and the medium was changed every 2 days. We included the vehicle control, in which the cysticerci were incubated in the presence of 0.5% DMSO. All data were recorded as individual measures of every parasite (10 by well) inside every culture well (5 wells/plate), at least 3 different experiments. So, the total final analysis is a $n = 150$ parasites.

Dose–response curve: *effect of naphthoquinone.*

The medium, RPMI 1640 (In Vitro), was supplemented with 10% FBS and a mix of 250 U/mL penicillin and 250 µg/

mL streptomycin (Invitrogen). The volume corresponding to the 500 µg/mL 4a stock solution was added to the culture medium to obtain different concentrations (4a at 25, 50, 75, and 100 µg/mL). The necessary volume of each concentration was prepared fresh. In every assay, 10 cysticerci and 2 mL of each solution were placed per well in a 24-well culture plate (Corning®). The culture plate was placed in the incubator (Revco Technologies) at 37 °C, 5% CO_2, and 90% humidity. The culture was carried out for 5 days and the culture medium was changed every 2 days. Two controls were included: an intact control in which cysticerci were only incubated in a supplemented RPMI 1640 medium, and the vehicle control in which cysticerci were incubated in 0.25% DMSO. At the end of the incubation period corresponding to both methods, the cysticerci were washed three times using 1 mL sterile PBS, and microphotographed.

Microscopic evaluation of the effect of naphthoquinone 4a on Taenia crassiceps ORF strain cysticerci

Daily, during the time the in vitro cultures of the time-curve and dose–response assays were kept, the parasites were observed under a stereoscopic microscope (Carl Zeiss). We evaluated and registered changes in motility, size, number of buds, and appearance of the *T. crassiceps* ORF strain cysticerci. The following scale of categories was used to evaluate the motility of the cysticerci: (+ + + completely motile), (+ + mildly motile), (+ slightly motile), and (0 non-motile). To keep a record of the changes observed in the parasites, daily photographs were taken using a 5-megapixel camera (Olympus, model MO21,) manually attached to the stereoscopic microscope (Carl Zeiss).

Taenia crassiceps cell isolation

T. crassiceps cells were extracted by tissue disruption according to the following protocol. Parasites were macerated using a sterilized nylon mesh "sandwich" (150 mm, Small Parts™) and a syringe plunger in 1 mL of RPMI medium. Meshes were washed with media and cell suspension was centrifuged at 300 × *g* for 5 min and cells were recovered in PBS. All materials were sterilized and procedure was performed in a culture room sterilized by UV in a laminar air flow hood (Thermo Fisher Corp).

Analysis of Taenia crassiceps cells by flow cytometry

T. crassiceps cells were stained with the following antibodies for 10 min at 4 °C: anti-mouse CD3-FITC, anti-mouse CD4 FITC, anti-mouse CD8-PE-Cy5, anti-mouse CD19-PE, anti-mouse Mac-1, and anti-mouse Mac-3 (all from BD Biosciences), and washed with 500 µL of staining buffer (PBS, pH 7.4, 2% fetal bovine serum, 0.02% NaN2). Cells were fixed in 2% paraformaldehyde solution and stored until analysis protected from light. *T. crassiceps* cysticerci–derived cells were stained with anti-human MHC-I-biotin antibody, washed once, and stained with APC-coupled streptavidin (BD Biosciences). All samples were analyzed by flow cytometry using a FACSCalibur (BD Biosciences) and data analyzed using the FlowJo© software (Treestar Inc.). *T. crassiceps*–derived cells were fixed in 2% paraformaldehyde solution for 10 min at 37 °C and centrifuged at 300 × *g* for 5 min. Afterwards, they were incubated in absolute methanol for 30 min at 4 °C and centrifuged at 300 × *g* for 5 min, and then washed twice with 500 µL of staining buffer (PBS, pH 7.4, 2% fetal bovine serum, 0.02% NaN2) and resuspended in the same buffer (100 µL/test). *T. crassiceps* cells were incubated in the presence of mouse anti-paramyosin (1 µg/test) (kindly provided by Dr. Pedro Ostoa, Instituto de Investigaciones Biomédicas, UNAM). Cell pellets were resuspended separately in the presence of the secondary antibody FITC or Alexa488-conjugated goat anti-mouse (Zymed) and incubated at 4 °C for 30 min in the dark. After this second incubation, cells were washed twice in staining buffer and centrifuged at 300 × *g* for 5 min. Cell pellets were resuspended in 500 µL of staining buffer in the absence of light and analyzed by flow cytometry using a FACSCalibur (BD Biosciences). Data was analyzed with FlowJo software (Treestar Inc.).

Nuclei staining with diamidino-phenylindole (DAPI) of isolated cells

For diamidino-phenylindole (DAPI) fluorescent staining, in vitro cultivated primary cells were collected, fixed in paraformaldehyde (Sigma) for 10 min, and permeabilized with 0.2% Triton X-100 (Merck) for 5 min. DAPI at a concentration of 0.5 µg/mL was added to the fixed cells on the slide, followed by an incubation for 20 min in the dark. Slides were then rinsed in PBS, Fluoprep (bioMerieux) was added, and a cover slip was applied. The stained biological substrates were visualized using an optical microscope (Nikon Corp.). For light microscopy applications, we used a Nikon Eclipse E600 microscope equipped with a Nikon DXM1200F CCD (Nikon Corp.). Immunofluorescence staining experiments were carried out using a Nikon Eclipse 80i microscope and a Nikon DXM1200C CCD (Nikon Corp.). For observation of DAPI, filter was utilized for staining technique (Nikon Corp.). Image processing and analysis were carried out using Adobe Photoshop CS3 (Adobe Systems Inc.) and Image Pro Plus 6.2 (Media Cybernetics).

Taenia crassiceps cell proliferation assay

To quantify *T. crassiceps* cell proliferation, we determined the carboxyfluorescein succinimidyl ester (CFSE) incorporation during cell mitosis. This assay was performed in cells maintained in the same culture conditions that the ones for cell viability assay, isolated from 4a-treated parasites cultured 5 days using the dose of 100 mg/mL. Flow cytometer data were analyzed with FlowJo software (Treestar Inc.). Compensation was assessed in BD FACSCalibur™ and FlowJo software with unstained samples.

In vivo *assays using naphthoquinone 4a*

Female and male Balb/C mice aged 6 weeks were infected intraperitoneally with 10 cysticerci of *T. crassiceps* (ORF strain) that were motile and translucent, with no buds and measuring 2 mm, approximately. After 4 weeks of infection, mice were intraperitoneally treated with a single dose of 100 µg/mL. After another 4 weeks (8 weeks of total infection), the mice were humanely sacrificed using anesthesia overdose (Sevorane®). Extraction of cysticerci from the peritoneal cavity was performed under sterile conditions. The recovered cysticerci were washed five times in sterile PBS supplemented with 250 U/mL penicillin and 250 µg/mL streptomycin (Invitrogen), and counted.

Infectivity assay

In Balb/C AnN female mice (the most susceptible host), the parasites cultured (control, vehicle, and 4a) treated as mentioned above (ten parasites per mice) were intraperitoneally injected into mice using a 0.25-gauge needle. Mice were euthanized by sevoflurane inhalation after 4 weeks of infection. Peritoneal cysticerci were collected and counted after rinsing the peritoneal cavity with PBS.

Statistical analysis

Time-response and dose–response curves for naphthoquinone 4a were obtained in three independent experiments, each with three wells per concentration and 10 cysticerci per well. Data obtained from the number of buds in each experiment were collected and expressed as mean ± standard deviation (SD). Data corresponding to the time-response curve were analyzed using a paired sample *t*-test while the data from the dose–response curve were analyzed with one-way ANOVA and post hoc Tukey test. Data obtained for motility were collected and expressed as mean ± standard deviation. A two-tailed *t*-test and a post hoc Mann–Whitney test were used to analyze the data from the time-response curve. The data from the dose–response curve were analyzed using Kruskal–Wallis and post hoc Dunn's tests. In all cases, differences were considered statistically significant when $P < 0.05$. All the analyses were conducted using GraphPad Prism 5. For in vivo experiment, the dependent variable was the number of parasites. Statistical analysis of two-way ANOVA and a Bonferroni post hoc test was performed with the GraphPad Prism software, version 5.0b for MacOSX.

Results

Microscopic observations of the effect produced by naphthoquinone 4a on Taenia crassiceps ORF strain cysticerci

Figure 1 shows a representative and comparative composition of the morphological changes suffered by the cysticerci treated with 4a at different concentrations, compared against those shown by intact controls and vehicles during the 5 days of culture, based on time curves. The first column of the table corresponds to intact cysticerci, which maintained their characteristic morphology and even increased in size by day 5 with respect to day 0, when all were small oval vesicles. The second column corresponds to cysticerci that were treated only with vehicle (0.25% DMSO) and increased in size while the membrane was slightly more opaque. The third column corresponds to those treated with 25 µg/mL 4a and showed a larger size and a subtle increase in the opacity of the membrane. The fourth, fifth, and sixth columns show the individuals treated with 50, 75, and 100 µg/mL 4a, respectively. From day 4, cysticerci treated with 50 µg/mL showed a slightly decreased size and were more opaque than controls at the end of the period. Those treated with 75 µg/mL decreased in size from day 3 of the treatment and, by day 4, their tegument was more opaque than that of controls and smaller concentration groups; at day 5, the decrease in size was considerable. Similarly, the cysticerci treated with 100 µg/mL 4a exhibited a greater opacity by day 3 of the treatment and considerably decreased in size, compared against controls and 25 and 50 µg/mL concentrations. However, they were only slightly smaller with respect to those individuals that received a 75 µg/mL treatment and, by day 5, the change in opacity was significant (Fig. 1).

Based on these results, the dose used for further experimentation was 100 µg/mL.

Effect of naphthoquinone 4a on the motility of Taenia crassiceps ORF strain cysticerci

Figure 2a shows the changes in motility exhibited by the cysticerci using the best dose (100 µg/mL) and the best time of incubation (5 days). It should be noted that motility was completely inhibited by the end of the

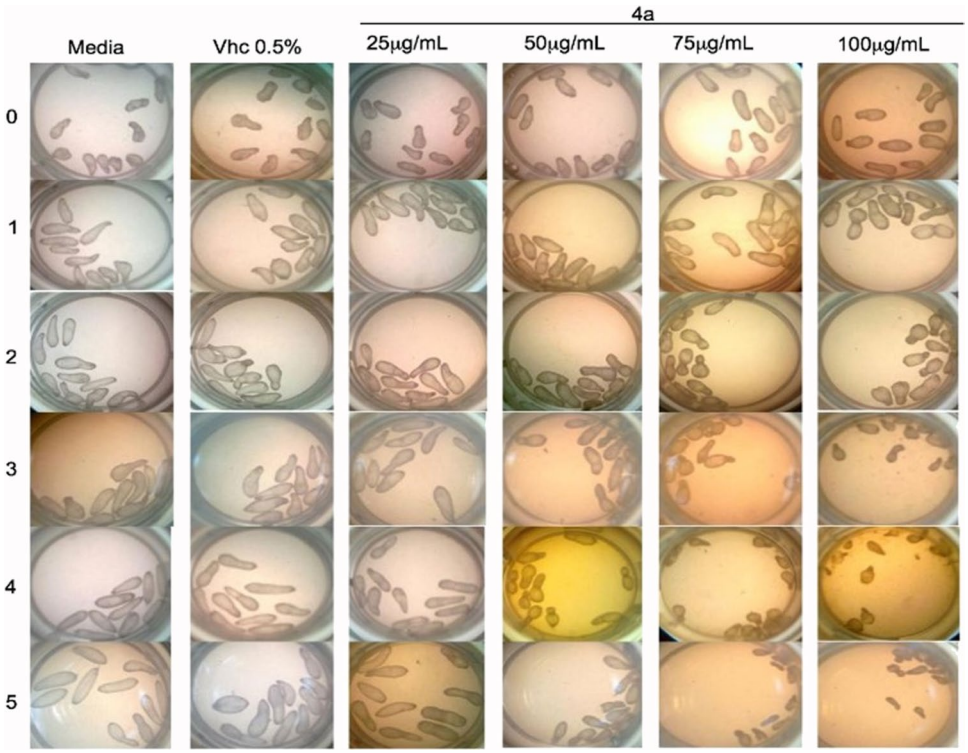

Fig. 1 In in vitro culture, naphthoquinone 4a modifies the size and appearance of *Taenia crassiceps* ORF strain cysticerci. Dose–response curve to evaluate in vitro effect of naphthoquinone 4a during a 5-day culture is shown together with progressive change of untreated and treated cysticerci with 4a at different concentrations

treatment. Intact individuals and vehicles maintained a similar behavior during the 5 days of culture. By the end of the treatment (Fig. 2a), cysticerci showed no motility at all.

Effect of naphthoquinone 4a on the reproduction of Taenia crassiceps ORF strain cysticerci

The changes induced by naphthoquinone 4a on the reproduction of the cysticerci are shown in Fig. 2b. The parasites started the asexual reproductive process at day 1. During the first days of culture, all the groups showed a similar behavior during the gemmation process, which was entirely inhibited in cysticerci treated with 100 µg/mL by days 4 and 5. There were significant differences in the number of buds between controls and the 100 µg/mL group from day 3 of the treatment. Still, larger numbers of buds were expected in the intact and vehicle individuals, as observed in previous results obtained from the time curve experiment (Fig. 2b).

Effect of naphthoquinone 4a on the mortality of Taenia crassiceps ORF strain cysticerci

The mortality of *T. crassiceps* cysticerci during the 5 days of culture in vitro are presented in Fig. 2c. The criteria established were based on the partial or total loss of motility and the decrease in size (at least over 50% with respect to the initial value), likely due to the loss of vesicular fluid. It must be noted that the size of the cysticerci was not measured, yet the images clearly show their increase or decrease in size. In addition, 100% of the parasites died by day 3 after treatment with 100 µg/mL 4a and by day 4 when treated with 75 µg/mL dosage, while treatment with 50 µg/mL caused the death of 74% of the cysticerci by day 5 (Fig. 2c).

Effect of naphthoquinone 4a on the size of Taenia crassiceps ORF strain cysticerci

In Table 1, the effect of 4a on the reduction of the size of *T. crassiceps* is shown. The starting point was 2 mm (control untreated cysticerci and vehicle). Those parasites used in the control remained unaltered during the 5 days of culture in their size, and kept around 2 mm (Table 1). However, as concentration and time increased, the reduction of cysticerci size was very clear, and, also dose and time dependent. After 5 days, using 100 ng/mL, the size of the cysts was reduced by 80% (Table 1). As demonstrated in all figures, not only size reduction was found, but also a clear decrease in reproduction, motility, survival, and, as shown in Table 1, size of parasites.

Isolation of Taenia crassiceps cells and size and complexity of Taenia crassiceps cells

In Fig. 3, it is clear that we were able to obtain isolated single cells of the cestode *T. crassiceps*. The composition shown in the first photograph of every row shows the complete parasite before the isolation process (Fig. 3a). In the second picture of the row it is shown the first step to

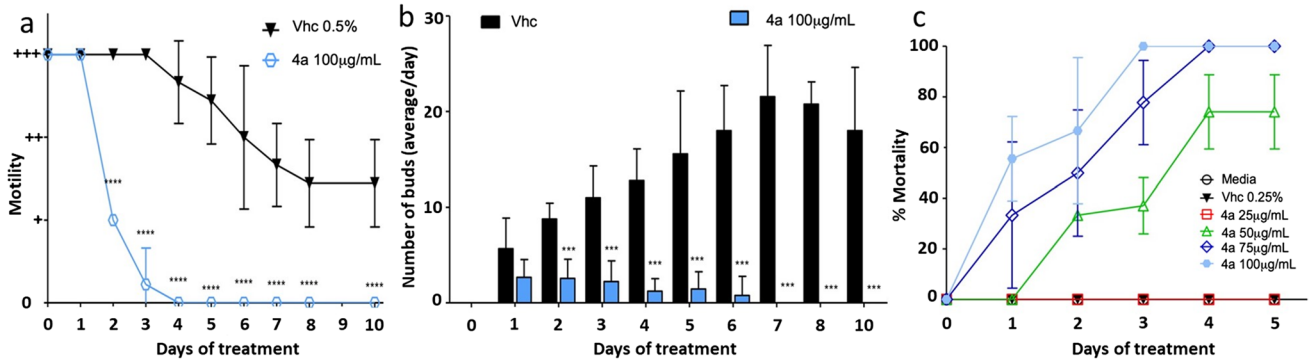

Fig. 2 In vitro effect of naphthoquinone 4a on motility, reproduction, and mortality of *T. crassiceps* ORF strain. (**a**) Naphthoquinone 4a reduces the motility of *T. crassiceps* cysticerci in a concentration-dependent manner. Each point represents the average percentage of mortality in cysticerci in three different experiments ± SD. (**b**) Dose–response curves of in vitro effect of naphthoquinone 4a on reproduction of *T. crassiceps* cysticerci at different days of treatment. (**c**) Time curve of *T. crassiceps* cysticerci mortality after exposure to naphthoquinone at different concentrations. Each point represents the average percentage of mortality in cysticerci in three different experiments ± SD. According to previous results, the following criteria were established to determine mortality of *Taenia crassiceps* cysticerci treated with 4a: loss of motility, loss of vesicular fluid, and over 50% decrease in vesicle size. Differences were considered significant when $p < 0.05$. (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$)

obtain parasite cells, by disrupting the parasite and, showing clumps of parasites, and, debris of pieces of the same (Fig. 3b). Finally, in the third picture of every row, the isolated single cells of the parasite are depicted. It is interesting to note that *T. crassiceps* cells are multinucleated and, due to the size (10 µm), it cannot be a complete parasite (Fig. 3c). Often, one may wish to monitor the presence or expression of several different molecules in cell culture. One component that is almost always monitored is the cell nucleus, which is stained with DAPI, a molecular probe characterized by λex = 358 nm and λem = 461 nm. DAPI binds to the inner groove of DNA present in cell nuclei and results in a blue emission that can be seen in the fluorescent microscope.

Table 1 Dose–response and time-response effect of in vitro treatment of naphthoquinone 4a on the size of *Taenia crassiceps* cysticerci

Days in culture					
Group	1	2	3	4	5
Control	2.2 ± 0.01	2.0 ± 0.8	2.3 ± 0.7	2.2 ± 0.1	2.3 ± 0.07
Vehicle	2.16 ± 0.24	2.0 ± 34.9	2.1 ± 0.9	2.2 ± 0.2	2.4 ± 0.19
25 mg/mL	2.1 ± 0.02	1.8 ± 8.8	1.5 ± 2.1	1.2 ± 0.2	0.6 ± 0.10
50 mg/mL	2.0 ± 0.03	1.7 ± 11.7	1.0 ± 0.3	0.8 ± 0.3	0.5 ± 0.23
75 mg/mL	2.0 ± 0.03	1.4 ± 11.7	0.9 ± 0.3	0.6 ± 0.2	0.2 ± 0.15
100 mg/mL	2.0 ± 0.01	1.2 ± 1.6	0.7 ± 0.2	0.4 ± 0.6	0.1 ± 0.01

For the assays, the cysticerci were selected according to the following criteria: they showed no buds, were motile and translucent, and measured 2 mm. Results were the average ± SD of the size of every parasite (10/well), in each well (6 per treatment) using a vernier calibrator. A total of 60 cysticerci were measured by treatment (group column), each day in culture (1–5 rows). Numbers represent the average of the size of parasites per well in millimeters. One-way ANOVA followed by the Dunnett's test. *$p < 0.05$ vs control and vehicle groups

Staining of DAPI in these parasite-isolated cells not only allows us to visualize the cell nuclei, but also permits facile quantification of the number of cells in a given field of view. A representative example of the image processing steps and subsequent phase contrast counting of cell nuclei stained with DAPI and the overlay using both techniques are shown in Fig. 3d, e and f, where *T. crassiceps* cells are depicted. Flow cytometry analysis firstly showed that *T. crassiceps* cells were different in size and complexity from mouse spleen cells. In fact, parasite cells were approximately threefold smaller and exhibited less complexity (Fig. 3g) than the mouse spleen cells. In addition, parasite cells showed no expression of the membrane markers CD3, CD4, and CD8 which are typically present in some types of mammalian leukocytes or MHC I, marker of all types of mammalian cells (not shown). On the other hand, the FACS analysis showed that *T. crassiceps*–isolated cells expressed paramyosin (Ag-B), an exclusive component of the cytoskeleton of cestodes, nematodes, and insects, while the host cells did not show expression of this molecule (Fig. 3h).

Effect of naphthoquinone 4a on the cell viability of Taenia crassiceps ORF strain cysticerci

We assessed the effect of naphthoquinone 4a on the viability of the *T. crassiceps*–isolated cells in order to compare the results found in the untreated and vehicle-obtained cells with the effects of 4a (Fig. 4d, e, f). The data showed that after 5 days of cysticerci treatment, 4a significantly decrease the cell viability by 95% (Fig. 4g). The control and vehicle-treated cells have a 90% viability as shown in Fig. 4a, b, and c (***$p \leq 0.01$).

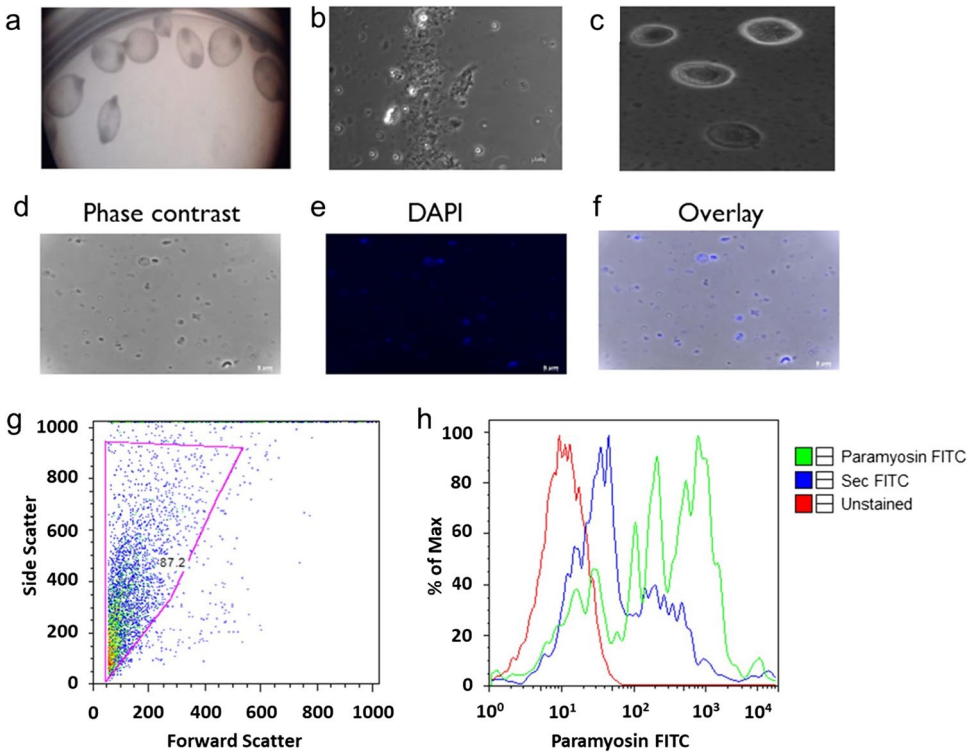

Fig. 3 Imaging of the process of getting single cells from *T. crassiceps*–treated cysticerci with 4a after mechanical disaggregation. (**a**) *T. crassiceps* complete parasite, (**b**) pieces of *T. crassiceps* after disaggregation, and (**c**) *T. crassiceps*–isolated cells. Pictures were taken using an inverted microscope (Olympus, MO21, Tokyo) at 10× and 100× magnification. (**d**) The disruption of parasite tissue and staining of primary cells. After isolation, primary cells were fixed and nuclei were stained with DAPI (**e**). Composed figure depicts stained cells with DAPI (produces blue color) of *Taenia crassiceps* cells (**f**). Also, forward/side scatter of parasite-isolated cells (**g**). *T. crassiceps* cells were disaggregated by tissue disruption, washed twice with FC buffer, and fixed using Lyse/Fix buffer (BD Biosciences). (**h**) A dot plot of *T. crassiceps* cells stained with anti-paramyosin antibodies. That positive staining (green line to the right) demonstrates that cells are from *T. crassiceps* and not cell contamination, since paramyosin is only present in cestodes and insect cells, not in mammals

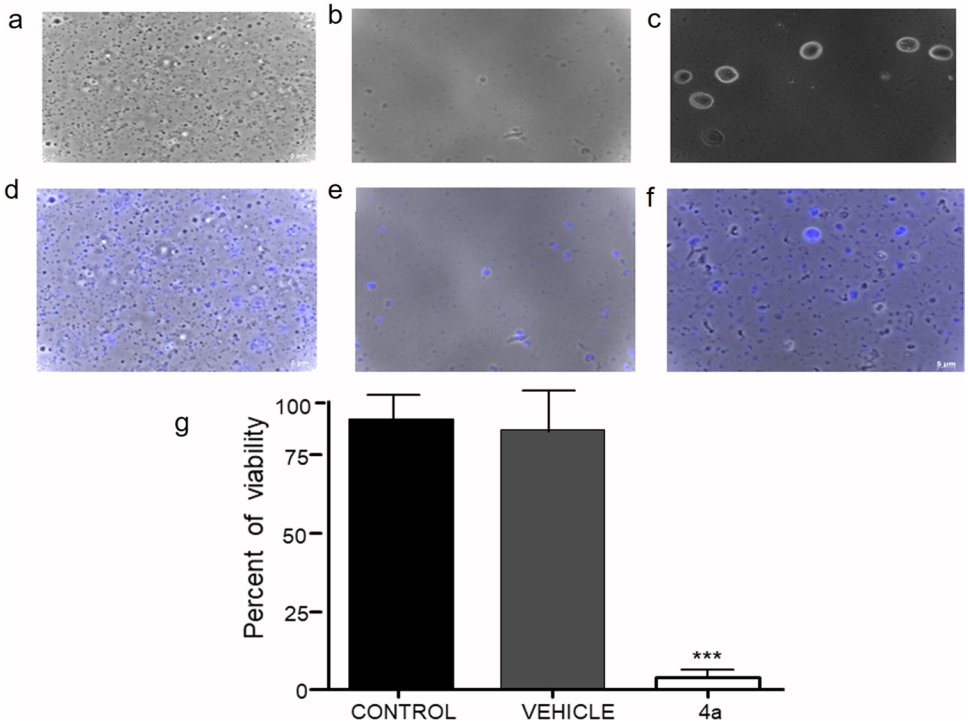

Fig. 4 Cells of *T. crassiceps* from control (**a**, **b**, **c**) and 4a-treated (**d**, **e**, **f**) cysticerci. (**a**) Total of live of control cells is seen, with no staining with trypan blue. (**b**) A closer view of the cells. (**c**) A much closer view of live cells. (**d**, **e**, **f**) The effect of 4a on parasite cell viability at different levels. (**g**) Quantification of the effect, where a clear decrease on cell viability as effect of 4a treatment is seen. Pictures were taken using an inverted microscope (Olympus, MO21, Tokyo) at 10× and 100× magnification. (***$p < 0.01$)

Effect of naphthoquinone 4a on the cell proliferation of Taenia crassiceps ORF strain cysticerci

In order to assess the 4a anti-proliferative effect, we measure CFSE incorporation in *T. crassiceps* cysticerci. Cells belonging to parasites treated with 4a divide much less than the control, as depicted in Fig. 5a, which it is the dot plot of the control, vehicle, and 4a-treated parasites. Figure 5a and b demonstrate that as mitosis decreases, there is much more colorant (red) in the cells (***$p \leq 0.01$), compared with control and vehicle-treated cells which is indicative of a decrease in proliferation index.

Infectivity assays of Taenia crassiceps cultured parasites

In order to be sure that 4a treatment has a true cysticidal activity, an infectivity assay was performed. The cultured parasites, by group, were injected into female Balb/C AnN mice, which is the most susceptible host to the infection. As seen in Fig. 6, only control and vehicle parasites were able to reproduce and grow in the host, maintaining infectivity,

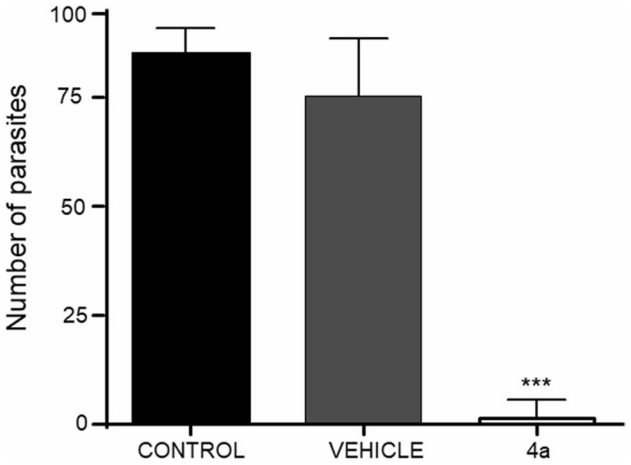

Fig. 6 Infectivity assays in mice naphthoquinone 4a reduces infectivity of *T. crassiceps*. Six-week-old female mice were intraperitoneally infected with parasites recovered from the different experimental groups in culture (control, vehicle, and 4a treated). Each bar represents the media ± SD of parasite loads in 5 infected animals (***$p < 0.001$)

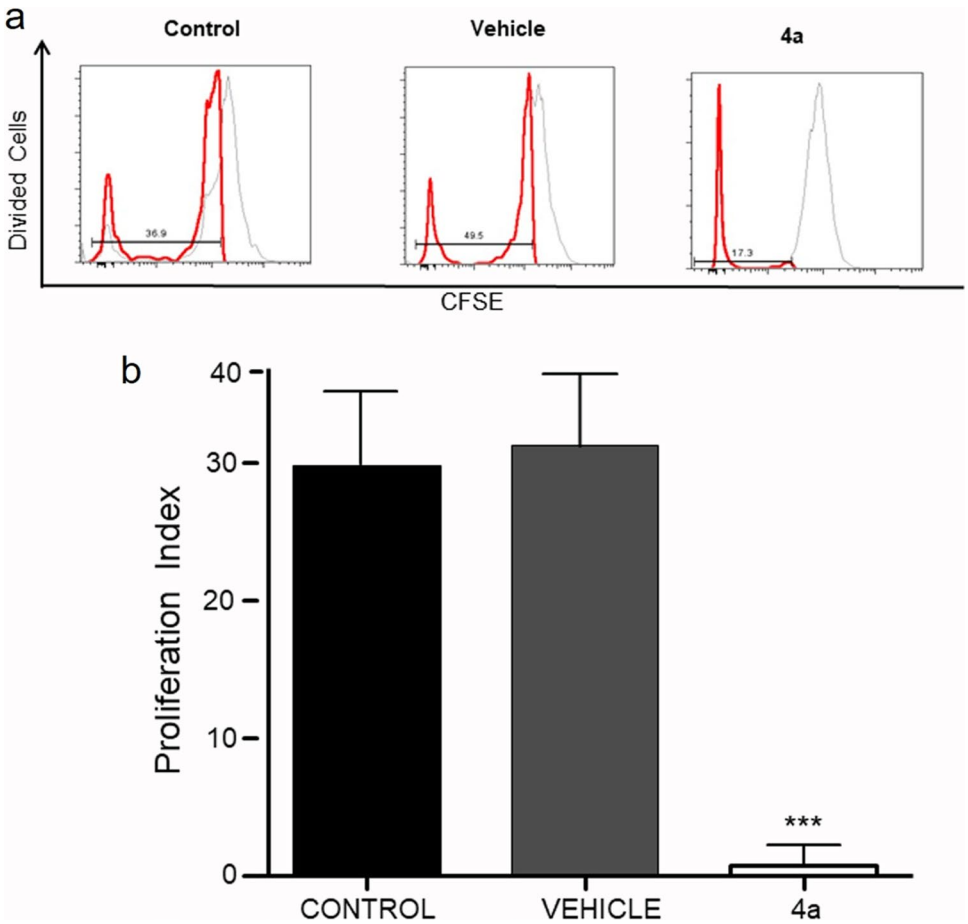

Fig. 5 Effect of 4a on the proliferation of *T. crassiceps*–isolated and cultured cells. Parasites used to isolate cells were from 5 days of cultured cysticerci (control), parasites treated with vehicle to dissolve 4a (vehicle), and treated with 100 mg/mL of 4a. Then, cells were isolated as described and culture in the presence of CFSE to measure cell division (proliferation) (***$p < 0.01$)

Table 2 Effect of in vivo 4a treatment on parasite loads in both sexes of mice

Sex	Parasite loads		
	Control	Vehicle	4a
Male	450 ± 25	480 ± 34	98 ± 17**
Female	676 ± 43	701 ± 28	134 ± 29**

For the assays, the cysticerci used for infection were selected according to the following criteria: they showed no buds, were motile and translucent, and measured 2 mm. They were injected into 6-week-old mice of both sexes. Mice were humanely sacrificed after 8 weeks of infection. The 4a dose used was 100 mg/kg of body weight and was intraperitoneally injected 4 weeks after infection. Data collected from animals with 8 weeks of infection at the time of sacrifice. Results were the average ± SD of the number of parasites in every mice ($n = 7$ per group). One-way ANOVA followed by *t*-test. $^{**}p < 0.01$ vs control and vehicle groups

while the 4a-treated parasites did not grow or reproduce, having no infectivity all ($^{**}p < 0.001$).

Naphthoquinone 4a has in vivo *cysticidal activity*

Table 2 is a summary of the data obtained of in vivo treatment with naphthoquinone 4a in infected mice of both sexes with *T. crassiceps*. First of all, as usual, we confirm and extend the fact that *T. crassiceps* infection is dimorphic, always harboring more parasite in females than males (Table 2). The vehicle in which 4a was injected has no effect, while 4a, it is able to decrease parasite loads 4.5-folds in both sexes. So, 4a is a new cysticidal compound.

Discussion

Conventional treatments are not entirely effective against some infections caused by bacteria, viruses, fungi, and/or parasites. This might be the result of the chemical characteristics of the molecule, the idiosyncrasy of each individual, or the indiscriminate use of some drugs, allowing microorganisms to develop drug resistance against treatments. Albendazole, for instance, is a wide-spectrum anti-helminthic often used against infections caused by soil-transmitted parasites as *Ascaris lumbricoides*, *Trichuris trichiura*, *Necator americanus*, and *Ancylostoma duodenale* as well as those generated by *T. solium* and *T. saginata*. In some countries, massive deworming actions are implemented, leading to resistant phenotypes, according to the WHO (World Health Organization 2016).

In addition, two benzimidazole resistance mechanisms have been identified in nematodes as *Haemonchus contortus*: One involves the loss of a susceptible gene in β-tubulin, followed by the emergence of a mutated resistance isotope coding tyrosine instead of phenylalanine at site 200 of β-tubulin (Barrère et al. 2012). Therefore, the search for natural and/or synthetic compounds to use as new treatments or joint therapy with other existing drugs is still relevant to the treatment of diseases that affect both humans and animals. Considering this, in this work, we evaluated the in vitro and in vivo anti-helminthic activity of naphthoquinone 4a in the experimental model of *T. crassiceps* ORF strain. This organism has been used as an alternative model to develop vaccines as S3PVAC, targeted against porcine cysticercosis caused by *T. solium* (Sciutto et al. 2013). It has also been used to evaluate the effect of molecules derived from benzimidazoles, as albendazole sulfoxide (ABZSO) and RCB20 (Márquez-Navarro et al. 2013), and that of the activity of plant extracts, like those from *Teloxys graveolens*, known as epazote (Palomares-Alonso et al. 2015), widely used in traditional Mexican cuisine. In addition, this model has been used to evaluate the activity of hormones as estradiol, progesterone, testosterone, and dihydrotestosterone (Ambrosio et al. 2014, 2015).

There are studies in which the in vitro and in vivo antiparasitic activity of several natural and synthetic naphthoquinones is aimed to counter protozoa such as *Plasmodium falciparum*, *Trypanosoma cruzi*, and *Leishmania*. In contrast, the information related to the activity of naphthoquinones against helminths is scarce. Some studies report the in vitro activity of naphthoquinone plumbagin against *Schistosoma mansoni* and *Fasciola gigantica* (Lorsuwannarat et al. 2013, 2014). Others evaluate the in vitro and in vivo activity of naphthoquinone β-lapachone on *Schistosoma mansoni* BH strain (Aires et al. 2014). Nowadays, there are no available studies evaluating the activity of any naphthoquinone on *T. solium* or alternative models as that of *T. crassiceps* ORF strain.

The time-response curve was a first approach to determine the in vitro activity of 4a and allowed us to establish the time of the maximum effect of 4a against *T. crassiceps* cysticerci. From these results, we determined that the maximum effect of 4a on *T. crassiceps* cysticerci occurred at day 5 of the treatment. Later, we evaluated its activity at different concentrations on *T. crassiceps* cysticerci and determined the best dose to be used in future experiments (100 μg/mL).

As mentioned before, the treatment with naphthoquinone 4a makes cysticerci decrease in size and change shape. This effect is similar to the one presented by *T. crassiceps* cysticerci treated with ABZSO in vitro. However, the concentrations at which these effects with ABZSO have been observed are lower than those we evaluated for naphthoquinone 4a. The data published for the pharmacological parameter of the effective concentration 50 (EC_{50}) for ABZSO are 0.068 μg/mL (0.041–0.0112) and 0.8 μg/mL (0.05–0.12) (Palomares et al., 2004). Nevertheless, the time to be effective is longer than 4a. They exert a maximum effect at

11 days of treatment in vitro. 4a only needs 3 days to have a strong cysticidal effect, different from ABZO. Of remark is the fact that even if there is strong evidence about sexual dimorphism in *Taenia crassiceps* cysticercosis, as well as in porcine cysticercosis and human neurocysticercosis, we found that 4a concentration used here in acute infections in immunocompetent mice exhibited an important parasiticidal effect in both sexes. In a previous study, it was suggested that estradiol promotes both pathogenicity and infection in Balb-c mice of both sexes infected with cysticerci of *Taenia crassiceps* (Morales-Montor et al., 2007); however, we did not observe this effect only in female mice treated with 4a, since parasite load in males was similar with respect to control females. Taking together the previous results and those presented here, we could hypothesize that 4a promotes the activation of the immune system leading to an inhibition in intraperitoneal replication, consequently reducing the parasite loads.

Conclusion

The data of *T. crassiceps* cell viability and proliferation demonstrates the cysticidal capability of 4a. In order to demonstrate that 4a reduces parasite infectivity, we infect female mice with in vitro treated parasites. Our results confirm and extend the 4a in vitro effect, since parasites in all mice infected with in vitro 4a-treated cysticerci did not infect, grow, or reproduce in the host, while the control and vehicle-treated mice did develop parasites, confirming its infectivity.

Finally, 4a can be proposed as a new treatment by itself or in a combined scheme with conventional treatments; however, more experiments should be achieved in order to investigate its role as a cysticidal drug in *Taenia* infections and the possible role of the immune system in the 4a response in both acute and chronic infections. Thus, 4a seems to be an interesting future candidate to be tested in porcine *Taenia solium* cysticercosis and clinical trials in human neurocysticercosis.

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Declarations

Ethics approval Animal care and experiments at the Instituto de Investigaciones Biomédicas are constantly evaluated and approved by the Experimental Animal Care and Use Committee of the Institute (Comité Interno para el Cuidado y Uso de Animales de Laboratorio, CICUAL, permit number 2016–00123) adhering to the official Mexican regulations (NOM-062-ZOO-1999). Mexican regulations are in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH) of the USA, to ensure compliance with established international regulations and guidelines.

Conflict of interest The authors declare no competing interests.

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