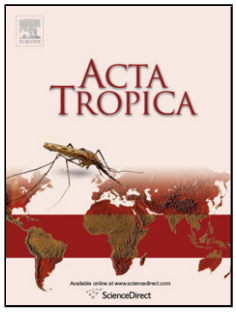

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Short Communication

In Vitro Effects of Amino Alcohols on *Echinococcus granulosus*

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ABSTRACT

Cystic echinococcosis is a globally distributed zoonotic disease, which is caused by the larval stage of *Echinococcosus granulosus sensu lato*. The chemotherapy of the disease is limited to the use of benzimidazoles. Recently, mefloquine and its analogues, aminoalcohol-carbazole, and some amino alcohol derivatives were reported to display inhibitory effects on parasites. Here, the activities of 130 amino alcohol compounds against *E. granulosus* were tested on protoscoleces and germinal cells at a concentration of 20 μg/ml over a period of three days. As a result, sixteen compounds totally were effective against both protoscoleces and germinal cells, and their IC_{50} and LC_{50} were also calculated respectively. Then effects of the most active compounds were observed on metacestodes over 14 days *in vitro*. Although the structure of active compounds were variable, hydroxyl and amino groups connected by two carbon atoms are held in common as the key feature of these compounds. The further investigation on metacestodes incubated with these active compounds revealed that the effects of JF16 and BTB4 were comparable to that of mefloquine and mebendazole. In addition, the ultrastructure alternations induced by these compounds on *E. granulosus* were confirmed by scanning electron microscopy and transmission electron microscopy observations. In conclusion, amino alcohols were a class of compounds with efficacy against *E. granulosus.* The most effective compounds JF16 and

BTB4 indicated that their basic structure would be useful in the synthesis of new compound for the treatment of echinococcosis. However, their *in vivo* efficacy and toxicity need to be carefully evaluated in the future.

Keywords: Amino alcohols; *Echinococcus granulosus*; Protoscoleces; Germinal cells; Metacestodes.

1. Introduction

Cystic echinococcosis (CE) caused by larva stage of *Echinococcus granulosus sansu lato* is globally distributed. This disease affects humans and animals, and is very important in the aspect of health and economy (Brunetti *et al.*, 2010;Moro and Schantz, 2009). For most of patients, CE is always asymptomatic for many years until the parasites grow to an extent that triggers clinical signs, which can cause serious morbidity and death. And patients' life quality significantly reduces though treatment options such as surgery and chemotherapy that is necessary when patients with multiple cysts in two or more organs that not suitable for surgical removal, or is used as prophylaxis to secondary echinococcosis after surgery (Pawlowski *et al.*, 2001). In clinics, mebendazole and albendazole are the only drugs recommended by WHO for the treatment of cystic echinococcosis. However, both of these benzimidazoles are poorly absorbed by patients, resulting in the cure rate only about 30% (Moro and Schantz, 2009). For good clinical efficacy, the alternatives are needed.

Mefloquine, developed in 1971, is a synthetic analogue of quinine and is commonly used in malaria prophylaxis and treatment of chloroquine-resistant falciparum malaria (Leed *et al.*, 2002). It also has promising inhibitory activities against other parasites (Van Nassauw *et al.*, 2008;Walter *et al.*, 1987). Recently, mefloquine was shown to be effective against *E. multilocularis metacestodes* (Kuster *et al.*, 2011) and led to the death of *E. granulosus* protoscoleces and germinal cells *in vitro* (Liu *et al.*, 2015). Furthermore, several authors have reported the antiparasitic activity of different mefloquine analogues with amino group and alcohols group. In a previous study, an aminoalcohol-carbazole series with antimalarial properties was reported and a compound showed good efficacy in *P. burgher* when orally administrated in the mouse model (Molette *et al.*, 2013). And amino alcohol derivatives also displayed inhibitory effects on promastigote forms of *Leishmania chagasi* and *Leishmania amazonensis* (Coimbra *et al.*, 2010;Del Olmo *et al.*, 2002), *Trichomonas vaginalis* trophozoites (Giordani *et al.*, 2009), and trypomastigotes forms of *Trypanosoma cruzi* (Junior *et al.*, 2010). Moreover, Fernandes Fde

S *et al* reported the *in vitro* schistosomicidal activity of amino alcohol compounds (Fernandes Fde *et al.*, 2013).

Regarding the structure characterization and anti-parasitic activity of these aforementioned compounds, 130 selected amino alcohols' effects were tested on *E. granulosus in vitro* in the hope to find the potential active compounds as the indication for treatment of CE.

2. Materials and Methods

2.1. Chemicals and reagents

A total of 130 Amino-alcohol compounds were purchased from Maybridge (UK) with purity >90% and mefloquine hydrochloride was provided by Libang Pharmaceutical Co., Ltd. (Xi'an, China). All the compounds were dissolved in DMSO at 10 mg/ml as a stock solution. Dilutions of the stocking solution to the working concentration were prepared freshly with DMSO on the day of treatment according to the test concentration. The DMSO concentration was adjusted to 0.5% in all samples, which had no significant effects on treated parasites and cells. All culture media and reagents were purchased from Gibco-BRL (Zurich, Switzerland) while mebendazole and other reagents were from Sigma (St. Louis, MO, USA).

2.2 Parasites, animals and infection

Protoscoleces were collected from hydatid cysts in fresh sheep liver and rinsed 5–8 times with Hanks' Balanced Salt Solution containing penicillin G (500 U/ml) and streptomycin (500 U/ml) under sterile conditions, and protoscoleces with viability higher than 95% were used in the experiments. About 2,000 protoscoleces were inoculated intraperitoneally into each Kunming strain mouse which were purchased from SLAC Laboratory Animal Center (Shanghai, China). The genotypes of protoscoleces from sheep and germinal cells from secondary infected mice were reported to be G1 strain (Liu, 2015).

2.3. Ethics statement

Animal care and all animal procedures were carried out in compliance with the Guidelines for the Care and Use of Laboratory Animals produced by the Shanghai Veterinary Research Institute. The study was approved by the Ethics Committee of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention. The license number was IPD-2014-2.

2.4. In vitro culture of parasites

The rinsed protoscoleces were maintained as previously described (Liu, 2015) with minor modification. In brief, protoscoleces were cultured in RPMI 1640 medium supplemented with 10 % FBS, 10 % hydatid fluid, reducing agents (1×10^{-5} M L-Dithiothreitol and 100 μM L-cysteine), 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin G and 100 μg/ml streptomycin at 37 °C in a 5 % CO_2 atmosphere. The culture medium was changed every 4-5 days. Mice secondary infected with *E. granulosus* for more than 10 months were sacrificed by cervical dislocation, and the cysts were carefully removed from the cavity of mice. After being washed several times with sterile PBS, the cysts with diameter <1 cm were cultured as protoscoleces. Then the germinal cells were prepared from larger cysts and cultured as previously described (Liu, 2015).

2.5. In vitro drug treatment of parasites

The drug treatment *in vitro* was carried out in 96-well microtiter plates (Costar, USA) for protoscoleces and germinal cells. In this study, all the compounds were initially tested at 20 μg/ml and the active ones were further studied at 0-20 μg/ml. After cultivating for 1 week, protoscoleces were seeded at about 50 per well and germinal cells were plated with a density of about 5×10^4 cells/ml per well in 200 μl medium. Then the solution of compounds was added to the each well, using mefloquine and mebendazole as positive controls and RPMI 1640 medium and RPMI 1640 with 0.5 % DMSO as negative controls. Treatments were carried out at 37 °C in a 5 % CO_2 atmosphere for 72 h. Methylene blue exclusion method was used to test the viability of protoscoleces, in brief, 100 μl 0.1% methylene blue was added to each well and observed under inverted microscope after 2 mins. The dead protoscoleces were stained blue and the surviving ones remained colorless. The viability of the germinal cells was determined by Cell Counting Kit-8 (Dojindo, Japan). The efficacy of active amino alcohols on metacestodes was carried out in 6-well microtiter plates (Costar, USA). Six metacestodes per well were collected with 6 ml medium. The tested final concentrations were 1, 5 and 10 μg/ml and metacestodes were observed for 14 days. In parallel, germinal cells treated by active compounds at 10 μg/ml were viewed by scanning electron microscopy (SEM), and the treated metacestodes were observed by SEM and transmission electron microscopy (TEM) to morphologically assess potential drug-induced damages. All experiments were carried out in triplicate and repeated at least twice.

2.6.Cytotoxicity test

Cytotoxicity was determined using three noncarcinogenic cells, L929 cells in RPMI1640, HK-2 cells in DMEM/F12 and Chang liver cells in DMEM medium, with 10% FBS; and 3 cancer cell lines, A172 cells in DMEM, A2058 cells in RPMI1640 and HCT-8 cells in DMEM medium, with 10% FBS. These cells were seeded in 96-well microtiter plates at a cell density of 5×10^4 cells/well and allowed to attach for 24 h before treatment. Drug was added, and plates were incubated for 72 h. Cell viability was also determined by CCK-8 kit.

2.7. Ultrastructural investigations of drug-treated parasites

Samples of germinal cells and metacestodes treated by amino-alcohol compounds were processed for scanning electron microscopy and transmission electron microscopy (SEM and TEM), respectively. For investigating the ultrastructure of germinal cells, these cells were grown on glass coverslips before the drug treatment. Then freshly harvested parasites were fixed overnight at 4 °C in 2.5 glutaraldehyde in 0.1 M PBS (pH 7.4) and were post-fixed for more than 2h at 4 °C in 2% osmium tetroxide. Then these samples were proceeded for SEM and TEM observation (Liu *et al.*, 2016).

2.8. Data analysis

The values of LC_{50} (the concentration that induced 50% protoscoleces death), IC_{50} (the concentration that inhibited 50% germinal cell grow) and Tox_{50} (the concentration that induced 50% cell toxicity)were calculated by probability unit method with SPSS version 17.0.

3. Results

3.1. Effectivities of 130 amino-alcohol compounds against protoscoleces and germinal cells in vitro

Firstly, A library of 130 amino alcohol compounds was screened against *E. granulosus* protoscoleces and germinal cells at 20 μg/ml over 3 days. Then the effective compounds were selected to determine the values of IC_{50} and LC_{50} (Table 1). Results from Table 1 showed that the active compounds had varied structures, but they had one thing in common that all of them contained hydroxyl group and amino group and these two groups were connected by two carbon atoms. In addition, the amino group in these compounds was nitrogen heterocyclic ring, such as carbazolyl, indole, piperidine and

piperazine. The replacement of trifluoromethyl moiety (AW6 vs AW13) and removal of halogens (AW17 vs AW19) decreased activity on parasites. However, in carbozaole and benzhydrylpiperazino moiety, the removal of one or two halogens did not effect compounds' antiparasitic activity. Moreover, most of the amino-alcohol series showed better effects on germinal cells than protoscoleces at the same concentration, indicating the germinal cells was more sensitive to these compounds. For mefloquine and mebendazole, the values of calculated IC_{50} of germinal cells were very close to that of LC_{50} of protoscoleces (Table1).

3.2. The activity of selected active amino-alcohols against E. granulosus metacestodes in vitro

Compounds with prominent effects on *E. granulosus* protoscoleces and germinal cells *in vitro* were selected and observed the effects on cultured metacestodes. And diverse effects were induced by different compounds. After 14 days incubated with 0.5% DMSO, metacestodes did not show any morphological alterations (Table S1 in Supplementary File) and exhibited an intact germinal layer (Fig. 1). However, amino-alcohols at 10 μg/ml resulted in morphological changes of the cysts, including the softening of the cysts and detachment of GL from LL. In addition, with the decline of chemical concentration, compounds of AW16, AW19, SC7, HT3 and HT24 had subtle effects on metacestodes, while other compounds (BTB3 (Liu, 2016), BTB4, JF16, MEF and MBZ) were still effective (Table S1 in Supplementary File).

3.3. Ultrastructural alterations of E. granulosus induced by effective compounds

Under the SEM, control germinal cells were round and adherent to the plastic culture matrix by abundant of cell substrate. During the incubation period with 0.5% DMSO, no ultrastructural alterations were observed (Fig. 2, a1). In contrast, the germinal cells showed dramatical damages after incubating with JF16, BTB4, MEF and MBZ at 10 μg/ml. In general, all the compounds induced the detachment of cells from the culture matrix along with alternations of cell morphology, including the loss of cell integrity, the generation of cavities in the cell surface and cell shrinkage. Moreover, most of the cell adhesion morphological changed or disappeared after the *in vitro* treatment.

For all the tested compounds, the most significant alternation caused by these effective compounds was the detachment of the inner GL from the LL confirmed by SEM and TEM. Normally, GL contains different cell types and is the only tissue responsible for the proliferation of the parasites. The outer

surface of carbohydrate-rich LL stays close to host tissue and the inner surface connects to the GL. In the control treatment, the GL was closely connected to LL by numerous microtriches, and the cells in GL were arranged in order. In contrast, some or all of the GL were separated from the LL after being incubated with compounds and no normal cell could be found connecting to LL (Fig. 2, b2-b5). Under TEM, the GL of metacestodes treated with JF16 and MBZ became vacuolized and no intact cell could be found (Fig. 2, c2, c5). In addition, the microtriches were also morphological changed by these compounds (Fig. 2, c2-c5).

(a1-a5) SEM of germinal cells after incubation for 3 days; (b1-b2) SEM of metacestodes after incubation for 14 days. LL. Laminated layer; GL, germinal layer; (c1-c5) TEM of metacestodes after incubation for 14 days. Teg, tegument; v, vacuoles; Arrows point towards microtriches that protrude into the laminated layer.

3.4. Cytotoxicity in noncarcinogenic mammalian cells and potential anti-cancer effects

The cytotoxicity of selected amino alcohols was assessed in mammalian cells using CCK-8 method. For three noncarcinogenic cells, L929, HK-2 and Chang liver, the Tox_{50} of all tested compounds were higher than 20 μM, while the cancer cells (A172, A2058 and HCT-8) were more sensitive to these compounds with the values of Tox_{50} lower than 10 μM (except HT24, HT3, SC7 and MBZ). The details can be seen in Table. S2 in Supplementary File.

4. Discussion

An appropriate screening method is crucial for high-throughput screening of drugs. In the present study, three models were used to evaluate the treatment effects of chemical compounds against *E. granulosus in vitro*. Among these models, protoscoleces and metacestodes are widely used in many anti-echinococcus drug studies (Albani and Elissondo, 2014;Verma *et al.*, 2014), but the reports about the use of *E. granulosus sensu lato* germinal cells were limited (Albani, 2010). As the most important cells in germinal layer, germinal cells are the only proliferative part of the parasite responsible for the formation of capsules or protoscoleces asexually (Eckert *et al.*, 2001), thus they are usually considered as the main target for chemotherapy. Based on the successful experiences of maintaining germinal cells from secondary infected cysts (Liu, 2015) and the use of stable cell counting method, germinal cells

were used for large-scale drug screening in this study. Compare to protoscoleces and metacestodes, the germinal cells are more suitable for the initial drug screening because of the abundant cells can be easily acquired form several infected mice and the cell activity can be rapidly and accurately determined by microplate reader. In contrast, so far, the activity of *E. granulosus* protoscoleces and metacestodes was still depend on the time-consuming manual judgment. In the present study, by the use of *E. granulosus* protoscoleces, germinal cells and cysts, some amino alcohols were indicated to have potential use in the finding of novel compounds for combating echinococcosis. For all the experiment, in order to absolutely dissolve the tested compounds, the final concentration of 0.5% DMSO was necessary, and the 0.5% DMSO was set as the negative control. Then the final results indicated the negative control did not influenced the activity of parasites during the observation period.

This study was inspired by the anthelmintic effects of mefloquine on *E. multilocularis* and *E. granulosus*. This antimalarial drug was initially discovered the promising antischistosomal properties (Van Nassauw, 2008;Zhang *et al.*, 2009) in mice and effects on *E. multilocularis in vitro* (Kuster, 2011), as well as its anthelmintic effects on *E. granulosus in vitro* in our previous study (Liu, 2015). After analyzing the structure characterization of this compound, the amino and hydroxyl groups were deduced to be the key factors responsible for its function. In order to verified our deduction, 130 compounds containing amino and alcohol groups were selected and purchased from the commercial chemical database, then tested effects on *E. granulosus* protoscoleces and germinal cells *in vitro*. The results showed that more than 20% compounds showed good effects (both of the percentage of dead protoscoleces and inhibition rate of germinal cells >80%) on the parasites (data not shown), indicating the potential anti-echinococcus properties of amino-alcohols. Then LC_{50} and IC_{50} of these candidate compounds against *E. granulosus* protoscoleces and germinal cell *in vitro* were determined, respectively. Most of compounds shower more effective on germinal cells than protoscoleces, and some compounds' effects on germinal cells were comparable to MBZ and MEF. Then these compounds were further tested on *E. granulosus* cysts, which present the parasitic status of these parasites in host. Among the candidate amino alcohols, JF16, BTB3 and BTB4 with similar structures were proved to be the most effective compounds on metacestodes in the present study. These three compounds all contained aminoalcohol-carbazole structure, which could improve their membrane permeability to potentially increase the chances of the drug passing through membrane, including the plasma membrane and the LL (the key parasitic structure for the transportation of drug into the GL) of

the *E. granulosus* metacetodes. This hypothesis is consistent with their CLogP values (JF16, 5.43; BTB3, 6.47 and BTB4, 5.98). Furthermore, the structures of BTB4 and JF16 are very similar to the reported most promising compound (compound 12) against *Plasmodium falciparum* K1 (Molette, 2013), indicating the potential activity of the three compounds on other parasites.

Besides the efficacy against *E. granulosus*, these candidate amino alcohols were also shown to be active against tumor cells. It can be attributed to the common characteristics between malignant tumors and parasites (Koppenol *et al.*, 2011;Mukhopadhyay *et al.*, 2002;Pierce *et al.*, 2012), such as the intense cell division out of host control, the interaction with the host immune response, and the high level of metabolic activity dependent on aerobic glycolysis, etc. (Lancelot *et al.*, 2013). In addition, some anti-parasitic and anti-cancer agents are proved to be effective on both of *Echinococcus spp.* and cancer cells. As a classical antihelmintic drug, mebendazole showed prospective therapeutic effects for many tumors (Bodhinayake *et al.*, 2015;Larsen *et al.*, 2015). Mefloquine and mefloquine–oxazolidine derivatives are considered to be new useful antitumor agents with rational designs. Therefore, the effects of the candidate amino alcohols against cancer cell lines were also evaluated in the present study. And higher toxicities of these compounds for A172 cells, A2058 cells and HCT-8 cells than normal host cell lines were observed. These results re-confirmed the similarities o f *E. granulosus* with cancer cells and indicated that these amino alcohol compounds could be used as prospective therapeutic agents for tumors.

In order to test the cytotoxicity of the candidate amino alcohols, their effects against mammalian cells were further evaluated *in vitro*. Most of them displayed the Tox_{50} of 25~50 μM for three mammalian normal cells. These concentrations are 5-fold in excess of the measured IC_{50} values observed for germinal cells. This selectivity of activity indicated the amino alcohols' inherent specificity for *E. granulosus* germinal cells compared to mammalian cells. The safety profile of amino alcohols was also taken into account in many studies. The compound 12 was reported to be negative in micronucleus and AMES *in vitro* assay, but showed high inhibition of the hERG (Molette, 2013). Mefloquine inhibited the hERG K+ channels in a concentration and time-dependent manner (Lopez-Izquierdo *et al.*, 2011;Traebert *et al.*, 2004). Considering the toxicity of amino alcohols reported, the further study should put emphasis on structure modification to enhance the specificity of amino alcohols on parasites rather than host tissues. At the same time, the proper treatment regime for these compounds in the use for the treatment of echinococcosis needs to be carefully explored.

In conclusion, several amino alcohols with the hydroxyl group and amino group connected by carbon atoms were effective against *E. granulosus* protoscoleces and germinal cells. The further investigation on metacestodes showed that JF16 and BTB4 may exhibit their *in vitro* activity similar with MEF but not as good as MBZ. Amino alcohols showed prominent efficacy against *E. granulosus in vitro*, but their possible toxicity should be paid more attention. In the future, the active compounds' *in vivo* efficacy against experimentally infected animals, the bioavailability, pharmacokinetics, *in vivo* toxicity, and potential side effects need to be carefully evaluated.

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Appendix A. Supplementary data

The following is Supplementary data to this article.

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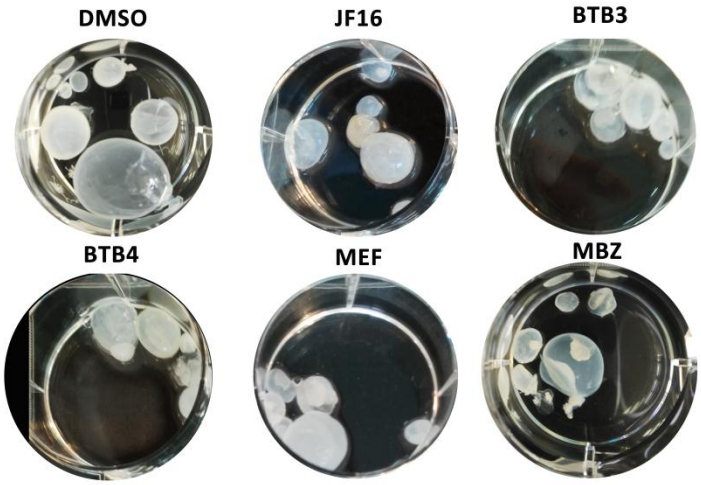


Figure 1. The morphology changes of *E. granulosus* murine metacestodes after incubation with 0.5% DMSO, JF16, BTB3, BTB4, MEF and MBZ at 10 μg/ml for 14 days *in vitro*

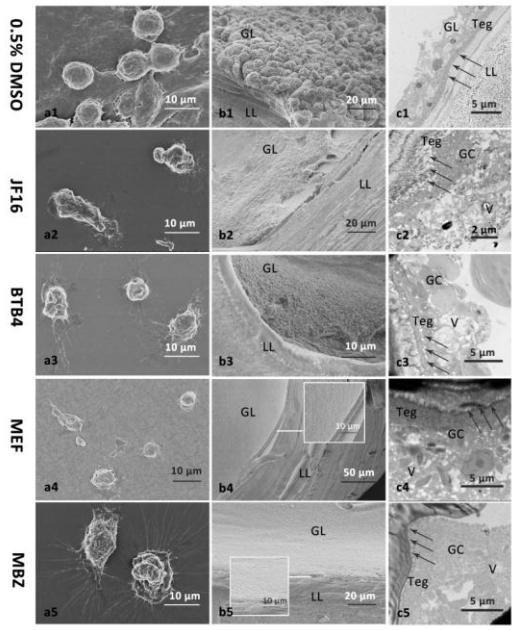
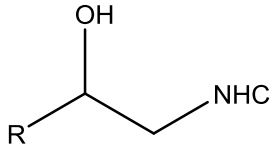
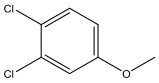
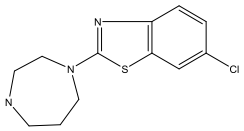
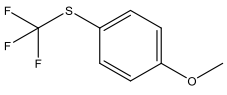
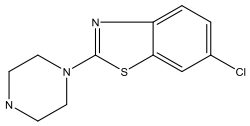
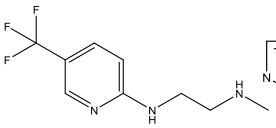
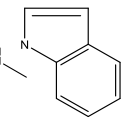
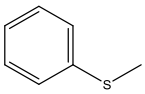
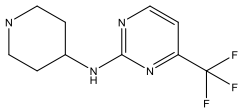
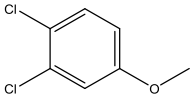
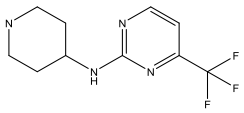
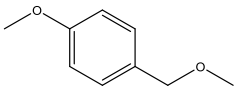
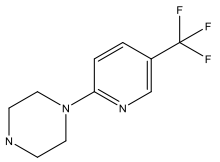
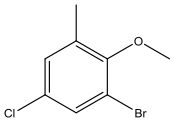
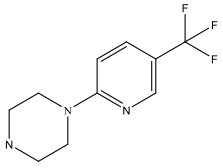
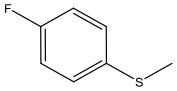
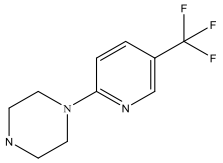
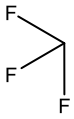
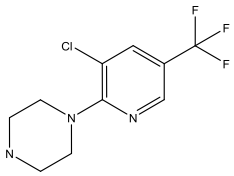
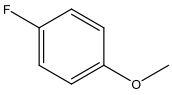
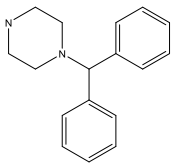
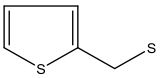
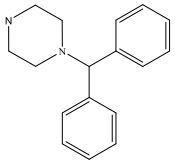
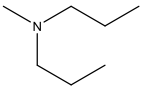
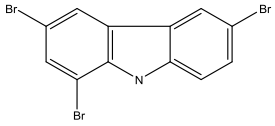
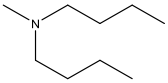
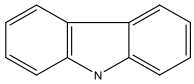
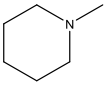
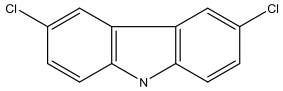
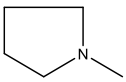
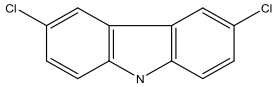
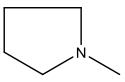
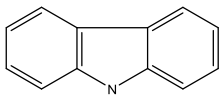
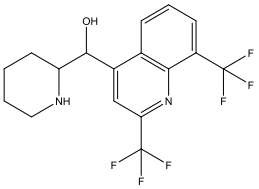
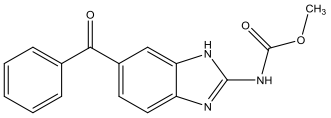


Figure 2. TEM and SEM observations of *E. granulosus* germinal cells and metacestodes incubated *in vitro* with active amino-alcohols at 10 μg/ml.

Table 1. *In vitro* effects of amino-alcohols against *E. granulosus* protoscoleces and germinal cells



Compound	R	NHC	Protoscoleces, LC50, μM	Germinal cells, IC50, μM
AW6			18.38 ± 2.51	11.34 ± 0.74
AW13			16.09 ± 1.92	5.18 ± 0.42
AW16			11.55 ± 1.93	4.81 ± 0.32
AW17			21.70 ± 2.55	5.45 ± 0.63
AW19			14.08 ± 1.38	3.01 ± 0.19
HT10			21.04 ± 1.86	6.11 ± 0.42
HT20			17.59 ± 1.61	12.23 ± 1.06
HT22			21.54 ± 2.72	5.37 ± 0.65

SC7			11.70 ± 0.64	7.20 ± 0.82
HT3			8.97 ± 0.74	4.23 ± 0.52
HT24			6.43 ± 0.82	4.95 ± 0.33
BTB2			16.39 ± 1.70	4.40 ± 0.95
BTB3			12.82 ± 1.05	6.86 ± 1.25
BTB4			10.87 ± 1.48	6.28 ± 0.66
JF16			18.42 ± 1.79	5.04 ± 0.44
JF17			30.40 ± 3.36	20.04 ± 1.12
MEF			5.77 ± 1.19	4.47 ± 0.64
MBZ			7.88 ± 0.60	6.61 ± 0.70