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In vitro effect of 5-fluorouracil and paclitaxel on *Echinococcus granulosus* larvae and cells

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ABSTRACT

Human cystic echinococcosis is a zoonosis caused by the metacestode stage of the tapeworm *Echinococcus granulosus*. Although benzimidazole compounds such as albendazole and mebendazole have been the cornerstone of chemotherapy for the disease, there is often no complete recovery after treatment. Hence, in searching for novel treatment options, we examined the *in vitro* efficacies of 5-fluorouracil (5-FU) and paclitaxel (PTX) against *E. granulosus* germinal cells, protoscoleces and cysts. 5-FU or PTX inhibited the growth of *E. granulosus* cells in a time dependent manner. Although both treatments had a protoscolicidal effect, 5-FU had a considerably stronger effect than PTX. 5-FU produced a dose- and time-dependent effect, provoking the complete loss of viability after 24 days of incubation. Moreover, cysts did not develop following the inoculation of treated protoscoleces into mice. The loss of viability was slower in PTX treated protoscoleces, reaching to approximately 60% after 30 days. The results of the *in vitro* treatment with 5-FU and PTX were similar in secondary murine cysts. The employment of SEM and TEM allowed us to examine, at an ultrastructural level, the effects induced by 5-FU and PTX on *E. granulosus* germinal cells, protoscoleces and murine cysts. In conclusion, the data obtained clearly demonstrated that 5-FU and PTX at clinically achievable concentrations inhibit the survival of larval cells, protoscoleces and metacestodes. *In vivo* studies to test the antiparasitic activities of 5-FU and PTX are currently being undertaken on the murine model of cystic echinococcosis.

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1. Introduction

Cystic echinococcosis (CE) caused by the larval stage of the tapeworm *Echinococcus granulosus* is a chronic, complex, and still neglected parasitic infection disease (Brunetti et al., 2011). Human or ungulate intermediate hosts (sheep, goats, pigs, cattle, horses or camels) become infected after ingestion of eggs passed into the environment with faeces from definitive hosts (dogs and wolves). The outcome of infection is the development of fluid-filled cysts mainly in the liver and the lungs, although other organs can also be affected (Budke et al., 2013).

The WHO-IWGE classification provides the basis for choosing basically four treatment and management options for CE: surgery, percutaneous sterilization, chemotherapy with benzimidazoles and observation (watch and wait) for inactive, clinically silent cysts (Brunetti et al., 2011). Each of these therapeutic tools has limitations depending on the individual case. The evidence supporting any of these modalities from carefully designed clinical studies is insufficient and the choice of treatment options remains controversial (Stojkovic et al., 2009).

Medical treatment is indicated when surgical removal is not appropriate for patients with multiple cysts in two or more organs, for prevention of secondary echinococcosis after surgery and in some cases for presurgical treatment (Pawlowski et al., 2001). The benzimidazole (BZ) compounds – albendazole (ABZ) and mebendazole (MBZ) – have been the cornerstone of chemotherapy for CE (McManus et al., 2012).

Several factors affect the response to medical treatment, such as size and age of the cysts, thickness of host derived connective tissue, calcification, cyst complication with multiple compartments or daughter cysts, ability of the drug to penetrate the cyst wall and persistence of adequate level of drug or its active metabolite at the site of parasite location (Ceballos et al., 2008; Hemphill and Müller, 2009). Approximately a third of patients treated with BZ

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drugs have been cured, 30–50% develop some evidence of a therapeutic response while between 20 and 40% of cases do not respond favorably (Moro and Schantz, 2009). Moreover, BZ sometimes acts exclusively as a parasitostatic agent, and relapses after chemotherapy have been reported (Stamatakos et al., 2009).

Praziquantel (PZQ), a heterocyclic pyrazinoisoquinoline derivative, has been proposed to be used alongside BZ in CE-patients. Nevertheless, further studies are required to determine whether there are significant benefits from a combination therapy with ABZ and PZQ over monotherapy with ABZ (Bygott and Chiodini, 2009). With regard to these difficulties, the development of a new therapeutic drug for human treatment of cystic echinococcosis is necessary.

There are a number of similarities between cancer cells and some parasites (Klinkert and Heussler, 2006). Particularly, *Echinococcus* metacestodes exhibit tumor-like properties, as reflected by their seemingly unlimited growth and proliferation potential, and their abilities to modulate the immune response and to form metastases (Hemphill et al., 2007). Consequently, several drugs inhibiting proliferation of cancer cells have been assayed on *Echinococcus* metacestodes and protoscoleces (Hemer and Brehm, 2012; Hübner et al., 2010; Küster et al., 2012; Liance et al., 1993; Naguleswaran et al., 2006; Spicher et al., 2008a,b). Doxorubicin, a drug commonly used in the treatment of a wide range of cancers, showed *in vivo* parasiticidal properties against *E. multilocularis* after binding to polyisohexylcyanoacrylate nanoparticles (Liance et al., 1993). The *in vitro* and *in vivo* effects of 2-methoxyestradiol, artemisinin and artemisinin derivatives against *E. granulosus* and *E. multilocularis* larval stages were evaluated (Spicher et al., 2008a,b). Furthermore, the isoflavone genistein and the genistein derivative Rm6423 exhibited profound *in vitro* activities against the mentioned parasites (Naguleswaran et al., 2006).

Fluoropyrimidine were developed in the 1950s following the observation that rat hepatomas used exogenous uracil more rapidly than normal tissues, indicating that uracil metabolism was a potential target for antimetabolite chemotherapy (Rutman et al., 1954). Heidelberger and colleagues synthesized 5-fluorouracil (5-FU), which differs from uracil by virtue of a flourine atom in place of hydrogen at the carbon-5 position of the pyrimidine ring (Heidelberger et al., 1957). This drug is widely used in the treatment of a range of cancers including breast cancer and cancers of the aerodigestive tract, but has had the greatest impact in colorectal tumors (Longley et al., 2003). 5-FU has *in vitro* and *in vivo* antiproliferative effect on endometriotic cells (Ngô et al., 2010). Furthermore, anti-malarial activity of 5-FU against *Plasmodium falciparum* has been demonstrated (Rathod et al., 1989).

Another anticancer agent is a diterpene plant product derived from the western yew *Taxus brevifolia*, namely paclitaxel (PTX) (Wani et al., 1971). PTX has antineoplastic properties and is used to treat certain human malignancies (Rowinsky and Donerhower, 1995; Huizing et al., 1995). *In vitro* studies have shown that PTX inhibits the growth of the apicomplexan parasites *P. falciparum* and *Toxoplasma gondii* (Pouvelle et al., 1994; Estes et al., 1998).

The aim of the present work was to determine the *in vitro* effect of 5-FU and PTX against *E. granulosus* germinal cells, protoscoleces and cyst.

2. Materials and methods

2.1. Drug treatments

Paclitaxel (Indena, Milan, Italy) and 5-FU (Roche Laboratories, Neuilly-sur-Seine, France) were dissolved in dimethyl sulphoxide (DMSO) at a drug concentration of 10 mg/ml. 5-FU and PTX were added to the medium 199 (Gibco BRL) resulting in final concentrations of 10, 5 and 1 μg/ml (5-FU: 75, 37.5, 7.5 μM; PTX: 12, 6, 1.2 μM). The final volume of drug solution added to the medium was 0.1%. Control culture contained 0.1% DMSO.

2.2. Parasite material and cell culture

Hydatid cysts from liver and lungs of naturally infected cattle were obtained from a slaughterhouse located in the southeast of the Buenos Aires province, Argentina. *E. granulosus* cell culture was obtained using previously reported methods (Albani et al., 2010). In brief, cells were cultured at 37 °C in medium 199 supplemented with 10% FBS, 10% hydatid fluid, reducing agents (5×10^{-5} M 2-mercaptoethanol and 100 μM L-cysteine), 2 mM L-glutamine (Bio-Rad, USA), 40 μg/ml glucose (Sigma, USA), 1 mM sodium pyruvate (Sigma, USA) and antibiotics (penicillin, streptomycin and gentamicin 100 μg/ml). *E. granulosus* cells were cultivated for at least 4 weeks. The culture medium was changed weekly and cells were subcultured once a week at a split ratio 1:2 (Albani et al., 2010).

2.3. Growth inhibitory assay on isolated cells

E. granulosus cells were seeded in 24-well microplates (5×10^5 cells/well in 1 ml medium). For this experiment cell cultures after 24 h of subculture were used. 5-FU and PTX were added in serial concentrations (10, 5 and 1 μg/ml) and cultures were incubated for 7 days. At days 0, 2, 5 and 7 viability was assessed by trypan blue dye (Sigma, USA) exclusion using a hemocytometer. Cultures were followed microscopically to determine the appearance of morphological alterations. At days 2, 5 and 7 of treatment, samples were taken for scanning electron microscopy (SEM).

2.4. Protoscoleces collection and in vitro incubation procedures

Protoscoleces were removed from cysts under aseptic conditions and washed several times with phosphate-buffered saline (PBS, pH 7.2). Viability was assessed as previously described (Elissondo et al., 2006). Viable and free protoscoleces (2000 per Leightont tube) were cultured in 10 ml of medium 199, containing 60 μg/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin and 4 mg/ml glucose. *In vitro* incubations were performed at 37 °C without changes of medium. 5-FU and PTX were added to the medium resulting in final concentrations of 10, 5 and 1 μg/ml. Culture tubes were followed microscopically every day. Samples of protoscoleces (approximately 90–100 protoscoleces in 180 μl of incubation medium) from each treatment and the controls were taken every 5–6 days for viability assessment. All experiments were performed in triplicate and were repeated three times. Additionally, ultrastructural studies with SEM and transmission electron microscopy (TEM) were performed.

2.5. Ethic statement

Animal procedures and management protocols were carried out in accordance with the 2011 revised form of *The Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institute of Health. Unnecessary animal suffering was avoided throughout the study. CF-1 mice (body weight 25 ± 5 g) were used. The animals were housed in a temperature-controlled (22 ± 1 °C), light-cycled (12-h light/dark cycle) room. Food and water were given *ad libitum*.

2.6. Determination of infectivity to mice

Viable and free protoscoleces (2000 per Leightont tube) were cultured in 10 ml of medium. 5-FU was added to the medium at

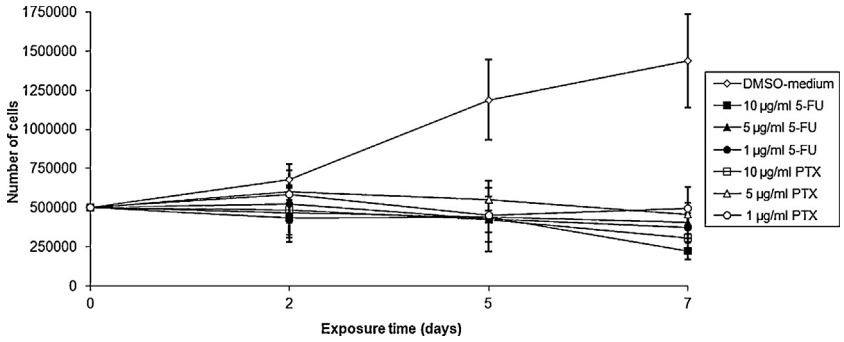


Fig. 1. Survival of *E. granulosus* larval cells after treatment with 5-FU and PTX. Each point represents the mean percentage of vital cells from three different experiments (DMSO: dimethyl sulphoxide).

a final concentration of 10 μg/ml. Protoscoleces incubated in culture medium containing DMSO served as controls. At 24 days post incubation (p.i.), protoscoleces of either 5-FU or control groups were rinsed in medium 199. After sedimentation, they were resuspended in medium 199 supplemented with 60 μg/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamicin and the concentration was adjusted to 3000 protoscoleces/ml. Each experiment was performed in quadruplicate.

Eight CF-1 mice were allocated into two groups ($n = 4$): The control and 5-FU groups were infected by intraperitoneal injection with 0.5 ml of protoscoleces suspension from *in vitro* DMSO and 5-FU treatments, respectively. At 7 months post infection, mice were necropsied and examined for larval growth.

2.7. Mouse infection and collection of cysts

Female CF-1 mice were infected by intraperitoneal inoculation with 1500 *E. granulosus* protoscoleces/animal, suspended in 0.5 ml of medium 199 (Gibco). At 8 months post-infection, mice with experimental secondary CE were euthanized, and necropsy was carried out immediately thereafter. At necropsy, the peritoneal cavity was opened and the hydatid cysts were carefully removed (Elissondo et al., 2009).

2.8. Procedures for in vitro incubation of cysts

Groups of 10 cysts (2–5 mm) were placed in Leighton tubes containing 10 ml of medium 199 with 5-FU or PTX at the following final concentrations of 10, 5 and 1 μg/ml. Cultures were maintained at 37 °C without changes of medium during the entire drug incubation period (Elissondo et al., 2007). Culture tubes were followed macro- and microscopically every day. Samples of cysts from each of the dosing groups and the controls were taken and then fixed for electron microscopy. The criteria for cysts vitality was assessed on the basis of structural vesicle integrity and the ultrastructural observation of the germinal layer as described by Elissondo et al. (2007). All experiments were performed in triplicate and were repeated three times.

2.9. Electron microscopy

E. granulosus cell samples were processed for SEM (scanning electron microscopy) using the protocol described in Albani et al. (2010). Samples of protoscoleces and cysts cultured *in vitro* were processed for SEM and TEM as described by Elissondo et al. (2006, 2007).

2.10. Statistical analysis

Log-rank test was used to assess the survival differences of larval cells and protoscoleces after exposure to 5-FU and PTX. All statistical analyses were performed using the BioEstat 5.0 software (Ayres et al., 2007). P values less than 0.05 were considered to be statistically significant.

3. Results

3.1. In vitro E. granulosus cell culture

The effect of 5-FU and PTX on the growth of *E. granulosus* cells is shown in Fig. 1. Control treatment always showed an increase in cell number reaching an average of 1.5×10^6 total cells at day 7.

In contrast, 5-FU and PTX inhibited the growth of *E. granulosus* cells in a time dependent manner. At 5 days p.i., the number of treated-cells was 64% lower than that observed in untreated-cells. Besides, 5-FU and PTX at a concentration of 10 μg/ml not only inhibited the growth of cells but also provoked a considerable reduction in the cell number after 7 days.

Studies by SEM revealed that structural damage was produced in drug-treated cells (Fig. 2). At 7 days p.i., cells incubated with 10 μg/ml of 5-FU or PTX showed morphological alteration as loss of turgidity, cellular contraction and presence of blebs on the surface.

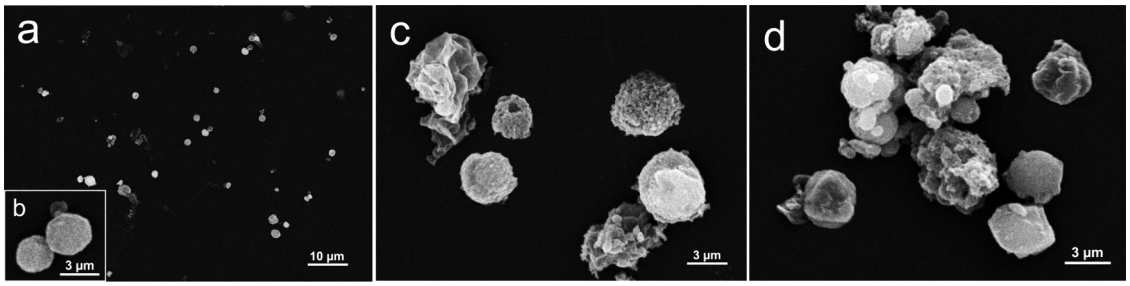


Fig. 2. Scanning electron microscopy of *E. granulosus* isolated larval cells incubated *in vitro* with 5-FU and PTX during 7 days. (a) Control (1400×); (b) detail of control cells (10,000×); (c) cells treated with 10 μg/ml 5-FU (10,000×); (d) cells treated with 10 μg/ml PTX (10,000×).

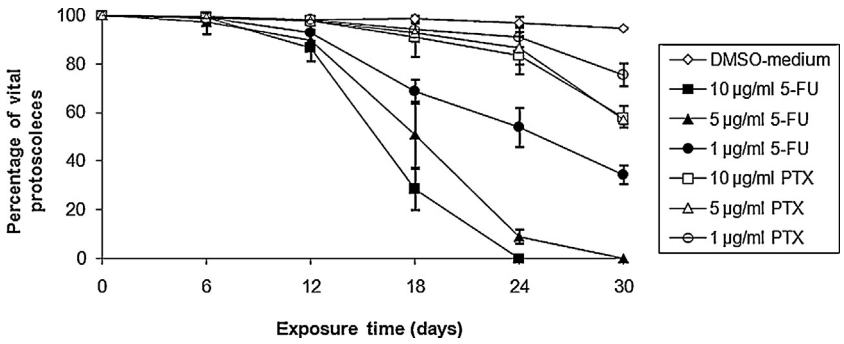


Fig. 3. Survival of *E. granulosus* protoscoleces after exposure to 5-FU and PTX. Each point represents the mean percentage of vital protoscoleces from three different experiments (DMSO: dimethyl sulphoxide).

3.2. In vitro protoscoleces incubation

The survival of *E. granulosus* protoscoleces incubated with different concentrations of of 5-FU and PTX is shown in Fig. 3. Although both treatments had a protoscolicidal effect, 5-FU had a considerably stronger effect than did PTX.

Control protoscoleces cultured in medium 199 + DMSO remained viable (94.7 ± 0.2%) after 30 days of incubation (Fig. 3). No changes in structure and ultrastructure were observed throughout the experimental period (Figs. 4a, 5a, 5b and 6a).

Treatment with 5-FU produced dose- and time-dependent effects (Fig. 3). The maximum protoscolicidal effect was found with 10 μg/ml of 5-FU. Viability was reduced to 28.3 ± 8.4% after 18 days of incubation and reached 0% after 24 days. Loss of protoscolecex viability in 5 μg/ml treated cultures became clearer after 18 days, where the percentage value was 50.9 ± 13.6%. At day 24, viability was 8.7 ± 2.9% and reached 0% after 30 days. A delayed protoscolicidal effect was observed with 1 μg/ml of 5-FU. In this case, the viability diminished to near 50% after 24 days of incubation. Viability was 0% at day 48 (data not shown).

The effect of PTX was also dose and time-dependent (Fig. 3). At 30 days p.i. with 10 and 5 μg/ml PTX, viability of protoscoleces decreased to approximately 60% and reached 0% after 72 days (data not shown). Treatment with 1 μg/ml PTX also showed protoscolicidal effect, with 75.3% of parasites remaining viable in culture after 30 days of incubation. As it was mentioned, 5-FU had a stronger effect than did PTX. For this reason, experiments of infectivity to mice were developed only with 5-FU.

The results of viability test coincide with the tegumental alterations observed daily by optical microscope (Fig. 4). Between 2 and 4 days p.i., the presence of numerous blebs in the tegument of protoscoleces treated with 10, 5 and 1 μg/ml of 5-FU was observed.

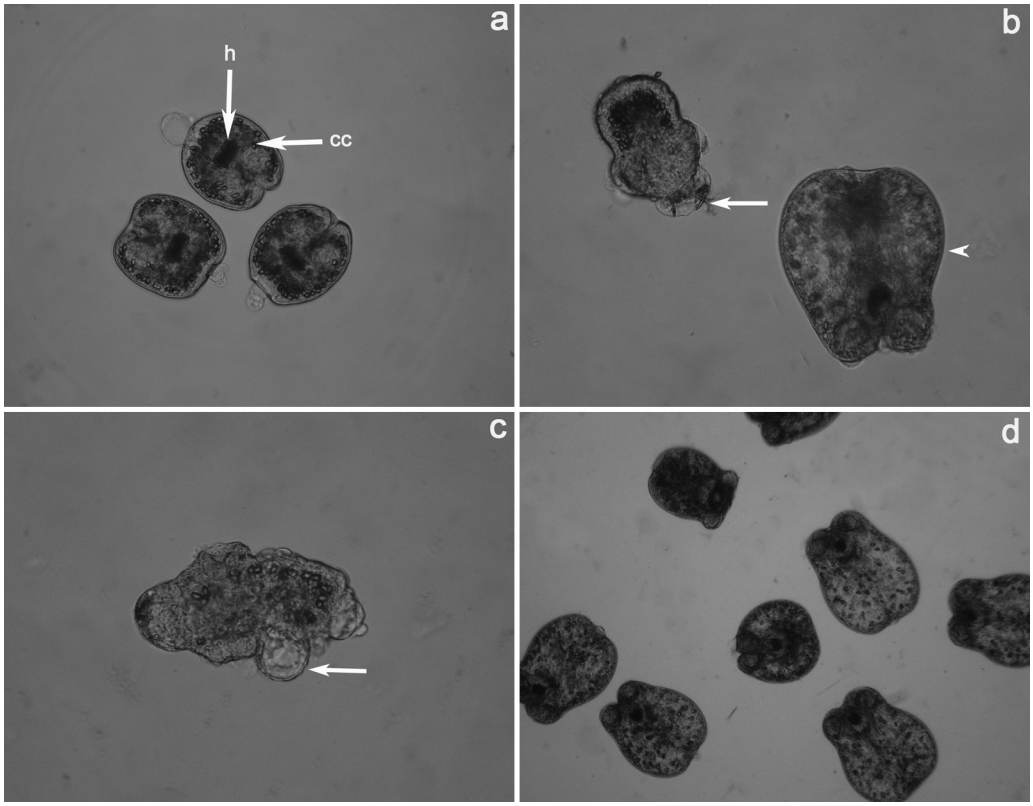


Fig. 4. Light microscopy of *E. granulosus* protoscoleces incubated *in vitro* with 5-FU and PTX. (a) Control protoscoleces (cc, calcareus corpuscles; h, hooks; 4 days p.i.; 300×); (b) protoscoleces incubated with 5 μg/ml 5-FU (3 days p.i.; 400×). Note the loss of hooks (arrow). Vesiculated protoscolex (arrowhead); (c) altered protoscolex after 3 days of incubation with 10 μg/ml 5-FU. Note the presence of numerous blebs (arrow) and the lost of hooks (300×), (d) vesiculated protoscoleces after 5 days with 10 μg/ml PTX (300×).

Other alterations such as rostellar disorganization and contraction of posterior region could be seen after 3 days (Fig. 4b and c). After 4 days of incubation with different concentrations of PTX, vesiculated protoscoleces were observed (Fig. 4d). Tegumental alterations in PTX-treated protoscoleces could be observed later, between 5 and 7 days p.i.

These results were confirmed on the ultrastructural level by SEM and TEM (Figs. 5 and 6). Control cultures exhibited no ultrastructural alterations in parasite tissue during the whole incubation period (Figs. 5a and b and 6a). In contrast, morphological and ultrastructural damages were detected in treated protoscoleces. The primary site of damage was the tegument of the parasite.

The ultrastructural effects observed after 5-FU incubation were greater than those caused by PTX (Figs. 5c–f and 6b and c). At 12 days p.i., SEM analysis of protoscoleces incubated with 5-FU showed the presence of tegumental alterations, rostellar disorganization, loss of hooks, and shedding of microtriches of the scolex region (Fig. 5c and d). Loss of morphology was evident with 5-FU at concentration of 10 μg/ml (Fig. 5e). PTX produced rostellar disorganisation and loss of microtriches of the rostellar region after 12 days (Fig. 5f).

At 12 days p.i., observations by TEM of 5-FU treated protoscoleces revealed the internal tissue altered with the presence of numerous vacuoles and lipid droplets (Fig. 6b). Upon treatment with PTX for 12 days, the internal tissue of protoscoleces was slightly vacuolated (Fig. 6c).

3.3. Determination of infectivity to mice

Control protoscoleces developed an average of 6 ± 2.3 g of cysts, similar to the inoculation of freshly isolated protoscoleces. This result showed that the infectivity of control protoscoleces was not

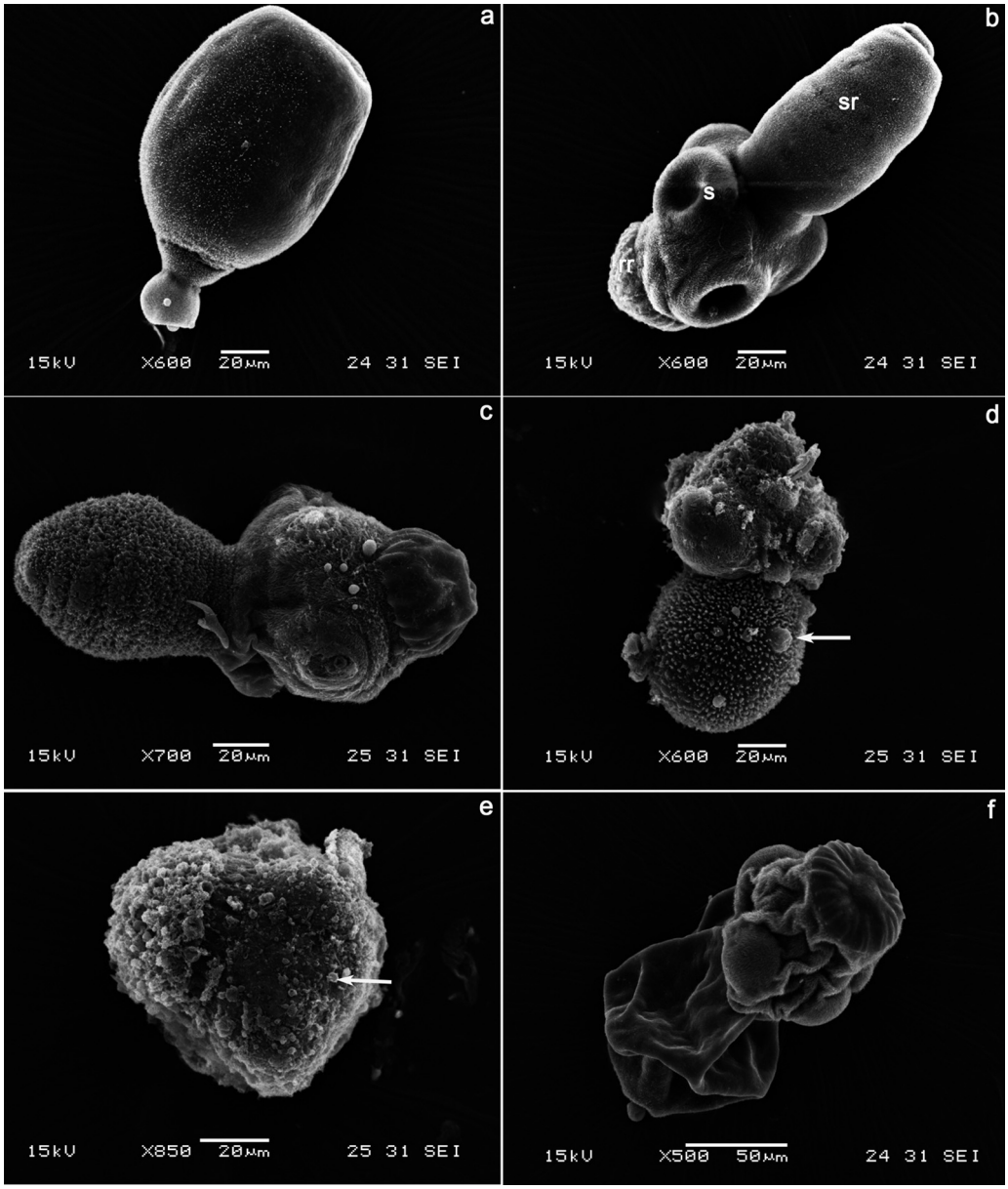


Fig. 5. Scanning electron microscopy of *E. granulosus* protoscoleces incubated *in vitro* with 5-FU and PTX. (a) Invaginated control protoscolex (18 days p.i.; 600×); (b) evaginated control protoscolex (rr, rostelar region; s, suckers; sr, soma region; 18 days p.i.; 600×); (c) altered evaginated protoscolex incubated with 1 μg/ml 5-FU during 12 days. The shedding of microtriches can be observed in the scolex region (700×); (d) Loss of hooks and microtriches and presence of numerous blebs (arrow) (5 μg/ml 5-FU; 12 days p.i.; 600×); (e) complete loss of morphology. Note the presence of numerous blebs (arrow) (10 μg/ml 5-FU; 12 days p.i.; 850×); (f) altered protoscolex after 18 days with 10 μg/ml PTX (500×).

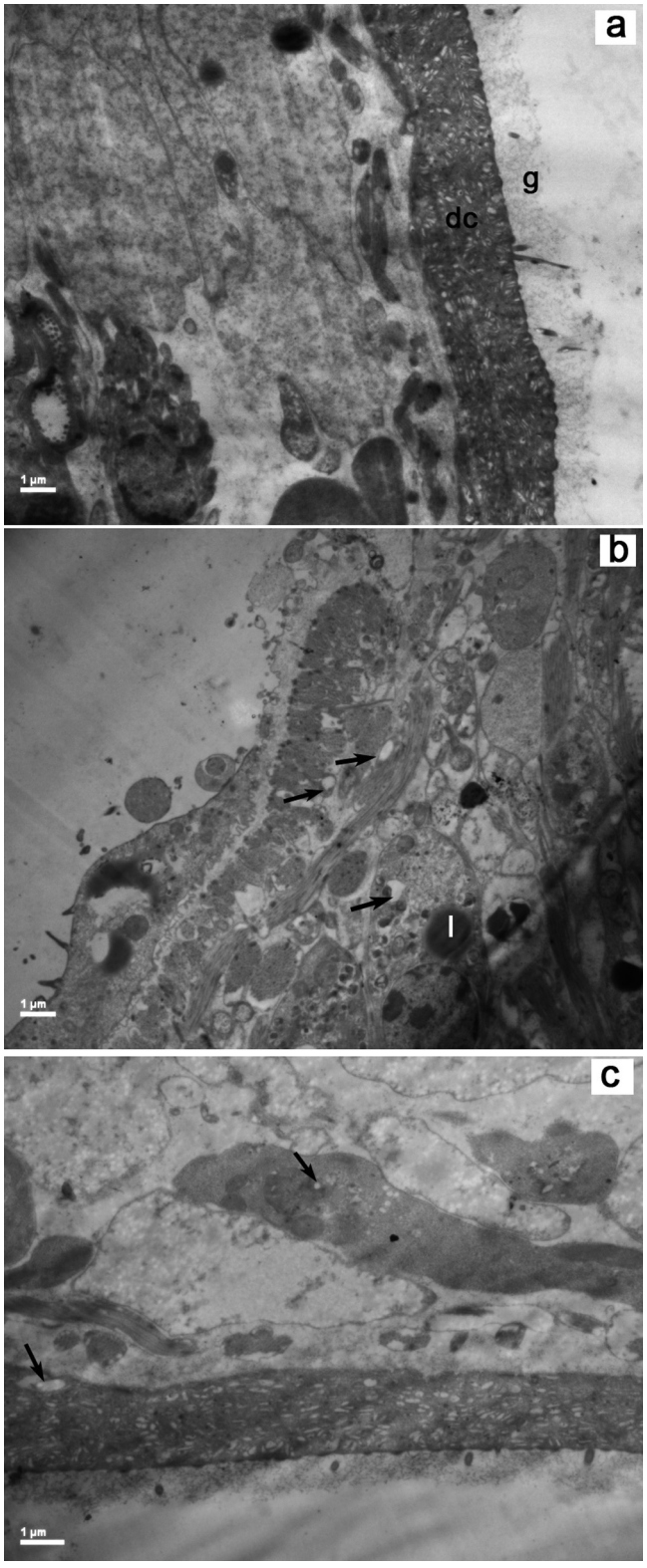


Fig. 6. TEM of *E. granulosus* protoscoleces incubated *in vitro* with 5-FU and PTX. (a) Soma region of a control protoscolex (12 days p.i.; g, glycocalix; dc, distal cytoplasm; 12,000×); (b) Treated protoscolex (12 days p.i., 10 μg/ml 5-FU). Internal tissue was altered with the presence of vacuoles (arrows) and lipid droplets (l) (12,000×); (c) protoscolex incubated with 10 μg/ml PTX (12 days p.i.). Note the vacuolation of de distal citoplasm and internal tissue (arrows) (15,000×).

affected during *in vitro* incubation for 24 days. Loss of infectivity in protoscoleces incubated with 5-FU after 24 days was observed, since all of the protoscoleces failed to develop into cysts following their inoculation into mice.

3.4. In vitro cysts incubation

Survival of cysts incubated with the different concentrations of 5-FU and PTX are shown in Fig. 7. The effect was more rapidly detected in cysts treated with 5-FU than when PTX were used.

As can be seen in Fig. 8, incubation of *E. granulosus* cysts in the presence of 5-FU and PTX resulted in dramatic alterations within 3 to 5 days after initiation of treatment. At lower concentrations, similar morphological changes occurred, although at a lower rate (data not shown). Already visible macroscopically, the parasite tissue detached from the interior lining of the laminated layer and formed a densely packed aggregate inside the vesicles. The laminated layer became translucent, mostly because large parts of the parasite tissue had been redistributed to another site.

Inspection of control cysts by SEM and TEM revealed that they exhibited typical features of *E. granulosus* metacestodes, with a distinct acellular outer laminated layer and an intact germinal layer comprised of a multitude of different, morphologically intact cell types (Fig. 9a and b).

The ultrastructural effect observed after 5-FU-incubation was greater than that caused by PTX. Clearly, after 3 days with 5-FU (10 μg/ml) the germinal layer lost the multicellular structure feature (Fig. 9c). At 10 days p.i., studies by TEM revealed complete destruction of the parasite tissue with the presence of lamellar bodies (Fig. 9d). On the other hand, less cells were observed in the germinal layer of cysts incubated with 10 μg/ml of PTX (Fig. 9e). After 10 days, the presence of numerous vacuoles was detected (Fig. 9f).

4. Discussion

Many similarities exist between cancer cells and parasites. Therefore, a promising starting point for the discovery of novel drugs to combat parasites is to examine available compounds developed against cancer for antiparasitic properties (Klinkert & Heussler, 2006). Several drugs inhibiting proliferation of cancer cells have been assayed on *Echinococcus* metacestodes and protoscoleces. This work describes for the first time the *in vitro* anthelmintic effect of the anti-cancer drugs 5-FU and PTX on *E. granulosus* larval cells, protoscoleces and cyst.

There are numerous routes and schedules of administration available for 5-FU. The weekly bolus administration of 5-FU at 15 mg/kg in clinic produces initial peak plasma levels of approximately 50 to 100 μg/ml which rapidly decline by several orders of magnitude (Calabro-Jones et al., 1982). A recommended starting dose of single-agent 5-FU given by protracted infusion is 300 mg/m^2/day (Harris et al., 1990). Continuous infusion of 2300 mg/m^2/day produces 5-FU serum concentrations on the order of 1 μg/ml (Grem et al., 1993). In addition, the exposure to 1.3 μg/ml 5-FU does not produce cytotoxic effect on primary endometrial cell cultures (epithelial and stromal cells) (Ngô et al., 2010).

At the maximum tolerated and recommended Phase II doses for PTX administered as a 6-h infusion, plasma levels have approached 10 μM for 2–4 h, and have exceeded 1 μM for 24 h, and 0.1 μM for 48 h (Rowinsky et al., 1988). Besides, PTX did not alter the growth of fibroblasts at a concentration of 8.54 μg/ml (Estes et al., 1998).

On the other hand, clear effects on *Plasmodium falciparum* were demonstrated using 5-FU at 1.6 μg/ml concentration (Rathod et al., 1989). The activity against apicomplexan parasites was determined using PTX at concentrations between 0.25 to 10 μg/ml (Estes et al.,

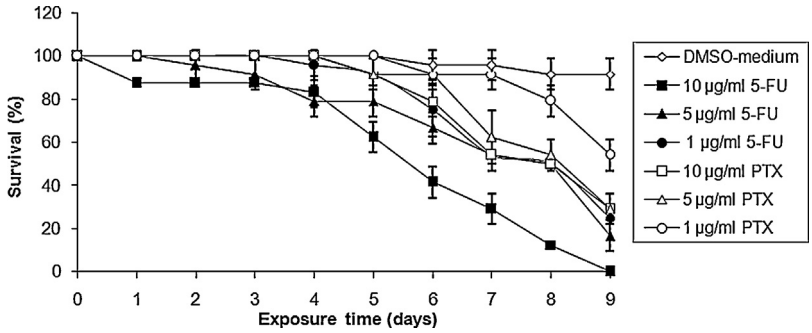


Fig. 7. Survival of *E. granulosus* cysts after treatment with 5-FU and PTX. Viability was measured on the basis of vesicle integrity (DMSO: dimethyl sulphoxide).

1998). It was therefore decided to test 5-FU and PTX at concentrations 1, 5 and 10 μg/ml.

No differences between the effect of 5-FU and PTX on the viability of germinal cells were found. 5-FU or PTX inhibited the growth of *E. granulosus* cells in a time dependent manner. Moreover, 5-FU or PTX at a concentration of 10 μg/ml not only inhibited the growth of cells but also provoked a considerable reduction in the cell number. Cells exposed to 5-FU or PTX showed morphological alteration as loss of turgidity, cellular contraction and presence of blebs on cell surface.

Interesting, larval cells were more sensitive to the drugs than protoscoleces and cysts. Isolated cells of germinal layer are more exposed to the action of the drugs due to they are detached from their normal environment and could thus be more sensitive. Protoscoleces and cysts are multicellular organisms surrounded by tegument that acts as barrier against the drugs (Galindo et al., 2008; Urrea-Paris et al., 1999). Another possible explanation could be that as drugs primarily target cells in mitosis, post-mitotic cells of entire larvae could survive longer.

The efficacy of 5-FU and PTX was also demonstrated *in vitro* on *E. granulosus* protoscoleces, and markedly differences on the effectiveness of both drugs were detected. 5-FU had a considerably stronger protoscolicidal effect than did PTX. Besides, its effect was dose- and time-dependent, provoking the complete loss of viability after 24 days of incubation. In contrast, the loss of viability was slower in PTX treated protoscoleces, reaching to approximately 60% after 30 days.

Moreover, 5-FU was able to kill *E. granulosus* protoscoleces, since following inoculation of treated parasites in mice no cysts could be recovered after 4 months post infection in all mice infected with 5-FU-treated parasites.

The morphological and ultrastructural alterations included contraction of the soma region, formation of blebs on the tegument, rostellar disorganization, loss of hooks, shedding of microtriches, and the presence of vacuoles and lipid droplets. The same alterations have also been reported by other authors working with other drugs like benzimidazoles (Pérez-Serrano et al., 1994; Elissondo et al., 2006), praziquantel (Urrea-Paris et al., 2000), ivermectin (Casado et al., 2002; Elissondo et al., 2009), and nitasoxanide (Walker et al., 2004).

Likewise, 5-FU produced a greater effect on *E: granulosus* murine cyst than that observed with PTX. A correlation between the intensity of damage and the concentration of the drug was found. The ultrastructural changes observed by SEM and TEM included the loss of the characteristic multicellular appearance of the germinal membrane. Furthermore, the same ultrastuctural alterations in cell cultures after treatment with 5-FU and PTX could be observed. These changes have been also observed in *E. granulosus* cysts following *in vitro* treatment with others anticancer drugs such as 2-methoxyestradiol, genestein and other isoflavones (Naguleswaran et al., 2006; Spicher et al., 2008a). These ultrastructural changes were similar to those reported by others authors (Elissondo et al., 2007; Walker et al., 2004).

The different mechanism of action could explain the disparate efficacy of these drugs against *E. granulosus*. 5-FU is an analogue of uracil with a fluorine atom at the C-5 position instead of hydrogen. It rapidly enters the cell using the same facilitated transport mechanism as uracil. The drug is converted intracellularly to several active metabolites: fluoro-deoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP). The active metabolites of 5-FU disrupt RNA synthesis (FUTP), inhibit the action of thymidylate synthase (TS) – a nucleotide synthetic enzyme (FdUMP) – and can also be directly misincorporated into DNA (FdUTP) (Longley et al., 2003). *E. granulosus* genome encodes a TS (EgrG_000260100) (Zheng et al., 2013) with considerable homology with de human enzyme.

By the other hand, PTX promotes the assembly of microtubules and stabilizes tubulin polymers by preventing their depolymerization. This unusual stability results in the inhibition of the normal dynamic reorganization of the microtubule network (Blagosklonny and Fojo, 1999). Since microtubules are important for mitosis and interphase functions such as maintenance of cell shape, cell

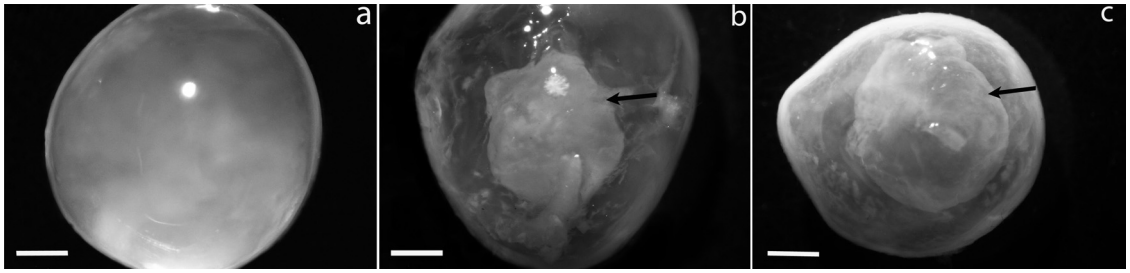


Fig. 8. Morphological effects of 5-FU and PTX treatments on *E. granulosus* cysts. (a) Control cyst (3 days p.i.); (b) cyst incubated with 10 μg/ml of 5-FU (arrow, germinal layer; 3 days p.i.); (c) cyst incubated with 10 μg/ml of PTX (arrow, germinal layer; 5 days p.i.). Bar = 300 μm.

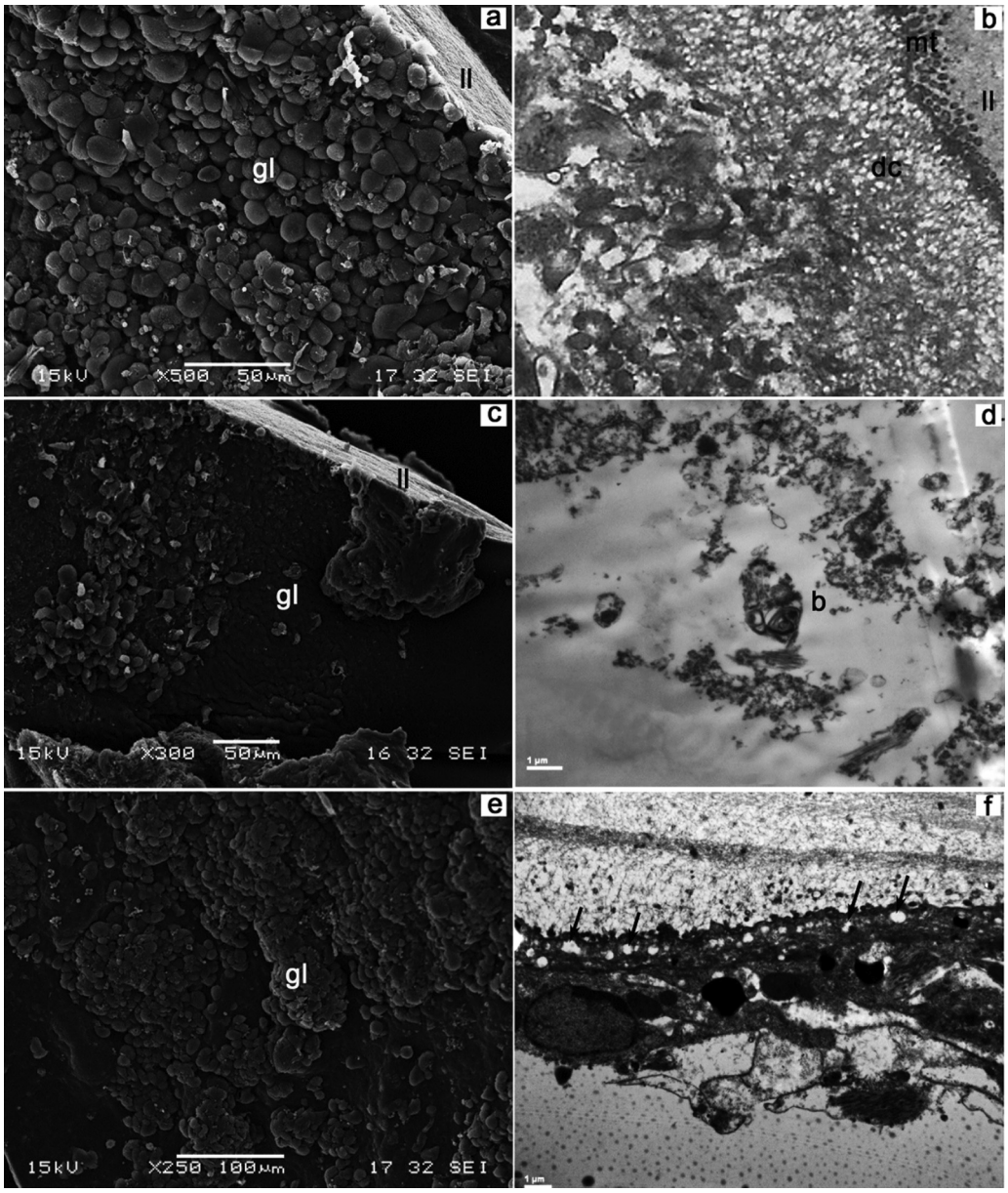


Fig. 9. Representative images of SEM and TEM of murine cysts incubated *in vitro* with 5-FU and PTX. ((a) and (b)) Control cysts (a) control cyst with an intact germinal layer (gl, germinal layer; ll, laminar layer; 3 days p.i.; SEM 500×); (b) Control cyst (ll, laminar layer; mt, microtriches; dc, distal citoplasm; 10 days p.i.; TEM 12,000×); ((c) and (d)) cyst incubated with 10 μg/ml 5-FU. (c) Note the extensive damage of the germinal layer (3 days p.i.; SEM 300×) (d) The internal tissue was severely affected ((b) residual lamillar bodies; 10 days p.i.; TEM 15,000×); ((d) and (e)) cysts incubated with 10 μg/ml PTX. (d) The germinal layer is altered (3 days p.i.; SEM 250×); (e) note the presence of vacuoles (arrows) (10 days p.i.; TEM 12,000×).

motility, and intracellular transport, PTX might be expected to affect cells during both interphase and mitotic cell cycle phases (Rowinsky et al., 1988).

In the search for an effective drug against *E. granulosus*, we showed that 5-FU and PTX, at clinically achievable concentrations, inhibited the survival of larval cells, protoscoleces and metacestodes. *In vivo* studies to test the antiparasitic activities of 5-FU and PTX are currently being undertaken on the murine model of cystic echinococcosis.

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