In vitro and in vivo effects of tamoxifen against Echinococcus granulosus larval stage

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Running Head: Tamoxifen clinical efficacy on Echinococcus granulosus

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Abstract

Cystic echinococcosis is a zoonotic infection caused by the larval stage of the cestode *Echinococcus granulosus*. Chemotherapy currently employs benzimidazoles, however 40% of cases do not respond favorably. With regard to these difficulties, novel therapeutical tools are needed to optimize treatment in humans. The aim of this work was to explore the in vitro and in vivo effect of tamoxifen (TAM) against *E. granulosus*. In addition, possible mechanisms for susceptibility of TAM are discussed in relation to calcium homeostasis, Pgp inhibition and antagonist effects upon a putative steroid receptor. After 24 h of treatment, TAM inhibits the survival of *E. granulosus* protoscoleces and metacestodes, in a low micromolar concentration range (10-50 µM). Moreover, we demonstrated the chemotherapeutic and chemopreventive pharmacological effects the drug. At the dose rate of 20 mg/kg, TAM induces protection against the infection in mice. In the clinical efficacy studies, a reduction on cyst weight was observed after the administration of 20 mg/kg in mice with cysts developed during three or six months, compared to those collected from control mice. Since collateral effects of high tamoxifen doses have been largely documented in clinical trials, the use of low doses of this drug as a short-term therapy may be a novel alternative approach for human cystic echinococcosis treatment.
INTRODUCTION

Human cystic echinococcosis (CE) or hydatidosis is a zoonotic infection caused by the larval stage of the cestode *Echinococcus granulosus*. The metacestode develops as unilocular cyst that consists of an inner germinal layer with totipotent cells which produce brood capsules with multiple protoscoleces by asexual division, supported by a laminated acellular membrane, named laminar layer. The treatment options for CE are surgery and/or chemotherapy, depending on the location, state and number of the cysts. The only two drugs licensed to date are the benzimidazole carbamate derivatives albendazole and mebendazole (1). Therefore, the search for novel candidates for the treatment of hydatidosis may be proposed. The performances of potential therapeutic compounds have been investigated applying antimicrobial and anticancer agents in *in vitro* and *in vivo* assays (2).

Several drugs inhibiting proliferation of cancer cells were assayed on *Echinococcus* metacestodes and protoscoleces (3-5). Spicher et al. (4) demonstrated the *in vitro* and *in vivo* effects of an endogenous metabolite of estrogen, 2-methoxyestradiol, either alone or combined with albendazole, against *E. multilocularis* and *E. granulosus*. Furthermore, the isoflavone genistein and the genistein derivative Rm6423 exhibited profound *in vitro* activities against the mentioned parasites (3). The cytostatic drug, imatinib, had drastic effects on the *in vitro* development and survival of *E. multilocularis* (5).

Tamoxifen (TAM) is a competitive antagonist of the estrogen receptor alpha (ERα) classified as a non-steroidal selective estrogen receptor modulator (SERM), widely used for treating primary breast cancer in premenopausal women and gynaecomastia in men receiving hormonal therapy for prostatic carcinoma (6). TAM, and their bioactive metabolites 4-hydroxy-tamoxifen (4-OH-TAM) and N-desmethyl-tamoxifen (ND-TAM), inhibit proliferation and induce apoptosis in several types of ER-positive and ER-negative breast cancer cells, rat
mammary tumors and other cancer types (7,8). Interestingly, the use of this antiestrogenic drug has also proved to be effective against several protozoan parasites, including Leishmania major, L. braziliensis, L. chagasi, L. amazonensis, and Trypanosoma cruzi (9-11). Nevertheless, TAM effect upon cestode parasites has been exclusively studied in Taenia crassiceps and T. solium (12,13). Actually, TAM inhibits T. crassiceps and T. solium proliferation and in vitro viability, whereas it induces protection against the infection in murine and hamster models, respectively (12,13). Antiestrogenic drugs have never been studied in E. granulosus.

Estrogen receptors have an evolutionary conserved modular structure. It consists in a DNA-binding domain (DBD), a nuclear localization signal NLS and a ligand-binding domain (LBD) at C-terminal. The LBD of ERα has been crystallized with 17β-estradiol (E2), diethylstilbestrol (DES) and the SERMs, TAM and raloxifene (14). The bulky side chain of these SERMs prevents from sealing the LBD and this produces anti-estrogenic action (15,16). Typical nuclear receptors (NR) have been identified in Echinococcus sp. (ortholog of SmFTZ-F1) with highest homologies (approx. 26% identical amino acids) of all identified cestode NRs to the LBDs of vertebrate ERs (17).

TAM also acts via an estrogen receptor-independent mechanism. It has a wide range of effects on mammalian cell physiology, such as induction of intracellular calcium release, apoptosis and autophagy. Moreover it possesses antioxidant and antiangiogenesis properties (18). Consistent with this wide range of cellular effects, TAM has been shown to target mammalian proteins such as calmodulin (CaM), protein kinase C, phospholipase C, phosphoinositide kinase, P-glycoprotein (Pgp) and swell-induced chloride channels (119).

Here we report data showing the in vitro activity of TAM against protoscoleces and metacestodes and the in silico analysis of a possible interaction with a putative steroid receptor. Moreover, we investigated the chemoprophylactic and clinical efficacy of TAM on mice infected with E. granulosus.
MATERIALS AND METHODS

Chemicals

Culture grade tamoxifen citrate was obtained from Sigma-Aldrich (USA). TAM was kept as a 100 mM stock in dimethyl sulfoxide (DMSO) at 20°C for in vitro assays. Dilutions were prepared in medium at concentrations of 5 to 200 μM. For in vivo experiments, oily solutions of TAM were prepared by taking aliquots from freshly alcoholic stock solutions, adding the required volume of corn oil (Sigma) and maintained under refrigeration (3–5 °C).

In vitro culture of E. granulosus protoscoleces and drug treatment

Protoscoleces were removed under aseptic conditions from hydatid cysts of infected cattle slaughtered in two abattoirs located in the southeast of Buenos Aires province, Argentina. The area where the cattle came from is known to include only the G1 strain of E. granulosus. Viable, motile, and morphological intact protoscoleces (n=3,000) were cultured using medium 199 (Gibco) supplemented with antibiotics (19). Protoscoleces were incubated in 24-well culture dishes, in presence of increasing concentrations (5-100 μM) of TAM until 7 days. Viability assays were performed daily until the death of all parasites (20). Five experiments were performed in triplicate and results were expressed as the mean percentage reduction of parasite numbers compared with untreated control wells. For scanning electron microscopy (SEM), samples were taken every 12 h and processed (20).

Detection of the cytosolic calcium levels into E. granulosus protoscoleces
Changes in cytosolic-free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_i\)]) were fluorometrically monitored using Fluo3 acetoxymethylester (Fluo-AM) probe (20). Experiments were carried out with 5 x 10\(^3\) protoscoleces and incubated with 10, 25 and 50 µM TAM for 2 h. Pretreatments with 1 mM EGTA or 100µM BAPTA-AM or both calcium chelators were carried out during 15 min. Then, fluorescence was registered with a spectrofluorimeter (model F-4500; Hitachi). Excitation was provided by the 488 nm line of a krypton-argon laser and the emitted fluorescence was collected using band pass filters: 505–530 nm. At the end of registration, 1 mM EGTA plus 100µM BAPTA-AM were added to chelate Ca\(^{2+}\). After 5 min fluorescence was measured again each 1 min by 30 min (F\(_{min}\), total fluorescence of dye in the absence of free Ca\(^{2+}\)). Each experiment was individually corrected for autofluorescence, and untreated controls were included in each replication.

**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 Institutes of Health. Unnecessary animal suffering was avoided throughout the study.

**Mouse infection and procedures for in vitro incubation of cysts**

Mouse infection and obtention of cysts were carried out as previously described (21). Briefly, female CF1 mice (body weight 25 ± 5 g) were infected by intraperitoneal infection of 1500 protoscoleces in 0.5 ml of medium 199 to produce experimental secondary hydatid disease. Animals were housed in a temperature-controlled (22 ± 1 °C), light-cycled (12-h light/dark cycle) room. Food and water were provided ad libitum. After 6 months post-infection (p.i.), mice
were euthanized, and necropsy was carried out immediately thereafter. At necropsy, the peritoneal cavity was opened, and the hydatid cysts were carefully removed.

Groups of 10-15 *E. granulosus* murine cysts with diameters between 3 and 5 mm were incubated in Leighton tubes in presence of increasing concentrations (5-100 μM) of TAM. Metacestodes viability was assessed daily through an inverted light microscope (until viability control was lower than 90%, approximately 6 days). The criteria for cysts viability assessment included the loss of turgidity and the collapse of the germinal layers. Each experiment was assayed for three replicates and repeated three times.

**Analysis of Eg-Pgp and tegumental gamma-glutamyl-transpeptidase activities of in vitro TAM-treated protoscoleces and metacestodes**

P-glycoprotein transport activity was measured in the absence and presence of 25 μM TAM from fresh samples of protoscoleces (n=1,000) and intact metacestodes (n=10) on 96 well plates using a calcein-AM assay (Molecular Probes, USA; Nicolao et al., 2014). Each experiment was assayed for three replicates and repeated three times. Following, protoscoleces were also imaged with an inverted confocal laser scanning microscope (Nikon, Confocal Microscope C1).

Gamma-glutamyl-transpeptidase (GGT), associated with damage of the internal tegument, was determined following the Szasz method from protoscoleces and metacestodes (22).

**Chemoprophylactic efficacy study**

Experimental animals were infected and housed as described above. At the time point of infection, 20 CF-1 mice were allocated into 2 experimental groups (10 animals/group) and treated as follows: a) unmedicated control group, animals receiving corn oil as a placebo; b)
treated group, TAM dissolved in corn oil. In the chemoprophylactic efficacy study, treatments were performed every 48 h (40 doses) by intragastric administration (0.3 ml/animal) at the dose rate of 20 mg/kg. Six months after infection, mice were euthanized, and necropsy was carried out immediately thereafter.

Clinical efficacy study

Two different experimental designs were performed:

Experiment number 1 (E1)

At 6 months p.i., mice were allocated into the following experimental groups (10 animals/group) and treated as follows: a) unmedicated control group, animals receiving corn oil as placebo; b) TAM suspension treated group (E1), dose rate was 20 mg/kg every 48 h (40 doses).

Experiment number 2 (E2)

At 3 months p.i., mice were allocated into the following experimental groups (10 animals/group) and treated as follows: a) unmedicated control group, animals receiving corn oil as placebo; b) TAM suspension treated group (E2a), 20 mg/kg every 48 h (40 doses); and, c) TAM suspension treated group (E2b), 100 mg/kg/day every 48 h (16 doses). In all cases the treatments were performed by intragastric administration (0.3 ml/animal). The final dose received by mice from group E2b was the double of the final dose administered to groups E1 and E2a. At the end of the treatment period, animals were euthanized, and necropsy was carried out immediately thereafter.

Determination of efficacy of in vivo treatments

At necropsy in both the prophylactic and efficacy studies, the peritoneal cavity was opened, and the hydatid cysts were carefully removed. The weight of the cysts collected from
each individual animal was recorded using an analytical balance. The efficacy of treatments (based on the weight of cysts from infected mice), was calculated using the following formula: (mean weight of control group - mean cysts weight of treated group)/ mean cysts weight of control group x 100. Samples of cysts recovered from each mouse were processed for SEM or TEM as described by Elissondo et al. (21).

**Analysis of TAM and its metabolites**

A spectrophotometric method, based on the formation of chloroform soluble ion-association complex between TAM and the dye alizarin red-S in acidic medium, was used for the determination of intracystic TAM. Cystic samples were obtained from sacrificed animals. Cystic liquids were extracted and removed the precipitated protein by centrifugation at 2000×g for 15 min. The supernatants were transferred to sample vials, capped, and stored at −20 °C until analysis by colorimetric method (23). The colored complexes between the dye, TAM and its metabolites were determined at 440 nm.

**Expression and sequences analysis of Echinococcus steroid receptor**

BLASTp search for ER homologs in the *E. granulosus* genome database http://www.sanger.ac.uk/Projects/Echinococcus) was carried out using *Homo sapiens* ERα (Hs-Er) -NP_000116- and ERβ -AAC05985- as queries. This data allowed the identification of an ortholog of EmuJ_000814300 and SmFTZ-F1 whose predicted open reading frame encode a putative *E. granulosus* steroid receptor (EgrG_000814300 included in the EgG_scaffold_0001 at position 7377598-7382189) named Eg-SR (17). Specific primers for the putative Eg-sr1 gene were designed (srFw 5’-ATTTTCGGGGACAGATATCGCGTTATCAT-3’ and sr-Rv 5’-CAATTTTGAAGCAATATCTGATCATCTGT-3’) for its amplification. Total RNA
extractions from *E. granulosus* protoscoleces and metacestodes were realized (22). The partial-length amplified cDNAs were obtained through direct sequencing of a PCR fragment (897 bp).

Sequence alignments were generated with the CLUSTALX software program and modeling of Eg-SR tertiary structure was obtained from the deduced primary structure using the Gen-THREADER (http://bioinf.cs.ucl.ac.uk/psipred/psiform.html). Analyzes of prediction of nuclear localization signals and hydrophobic cores were realized with NLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) and ProtScale program (http://web.expasy.org/protscale).

**Statistics**

Data within experiments were compared and significance was determined using the student’s *t* test and non-parametric Mann-Whitney test. All data were shown as arithmetic mean ± SEM and *p* values are indicated in each assay.

**RESULTS**

*TAM treatment increases intracellular calcium in *E. granulosus* protoscoleces*

TAM exposure (20 µM) showed an increase of seven-fold in free [Ca$^{2+}$], over a 2 h observation period, compared with the control (Fig. 1A). To determine whether TAM involved an increase from either intracellular stores or the extracellular space, we pretreated parasites with BAPTA-AM (membrane-permeable calcium chelator) and EGTA (extracellular chelator) immediately prior to addition of the drug. Fluorescence decreased to about control values only with both chelators in the medium, indicating that these drugs were able to release [Ca$^{2+}$], from
extracellular and intracellular stores in *E. granulosus*. Normalization of cytosolic calcium was also consistently delayed in TAM-treated cultures compared with controls (data not shown).

**TAM inhibits Eg-Pgp activity in protoscoleces and metacestodes**

P-glycoprotein activity is inversely proportional to the accumulation of intracellular fluorescent calcein. Control protoscoleces and metacestodes (sections and intact cysts) showed basal and stable Eg-Pgp activity, because the Ca-AM was extruded from the cells (typically 75 \( \pm 5 \) fluorescent units -FU- and 125 \( \pm 15 \) FU, respectively, Fig. 1B). In the presence of 20 \( \mu \)M of TAM, protoscoleces and metacestodes increased ten and three fold in relative fluorescent units over controls, respectively (Fig. 1B). P-glycoprotein activity was detected by confocal microscopy particularly in suckers and tegument (Fig. 1C).

**In vitro activity of tamoxifen against *E. granulosus* protoscoleces and metacestodes**

Viability decreased as a function of drug concentration in both protoscoleces and metacestodes (Fig. 2 and 3). TAM had effect from 10 \( \mu \)M and reduced the viability of treated protoscoleces with 50 \( \mu \)M to 45 \( \pm 2.3\% \) after 24 h (Fig. 2A). Protopscolex mortality was 100% after 20 \( \mu \)M TAM incubation for 7 days (Fig. 2B). Untreated protoscoleces remained at least 99 \( \pm 1.0\% \) viable during the complete experiment. After 24 h of treatment, the ultrastructure of protoscoleces incubated with 20 \( \mu \)M TAM showed significant differences compared with control samples (Fig. 2C). Protopscoleces showed the tegument completely altered, shedding of microtriches, loss of hooks, and contraction of soma region. After 24 h or 72 h, metacestodes incubated with 50 and 20 \( \mu \)M TAM presented the detachment of the germinal layer in the 100\% of cysts, respectively (Fig. 3). The calculated IC\(_{50}\) against metacestodes was 18 \( \pm 3 \) \( \mu \)M (Fig. 3A inset).
The ultrastructural alterations were accompanied by release of Eg-GGT into the medium supernatant in a dose-dependent manner (data not shown). An increase on the Eg-GGT activity in culture supernatant of cysts and protoscoleces incubated in vitro with 20 μM TAM was detected. After 24 h of incubation, total Eg-GGT activity in the protoscolex culture supernatants was measured (42 ± 5 nmol min⁻¹ mg⁻¹). This value was two times higher than in metacestodes (18 ± 5 nmol min⁻¹ mg⁻¹). Eg-GGT activity was barely detectable on control supernatants.

Chemoprophylactic efficacy study

All the infected mice from the untreated control group (10/10) developed metacestodes in the abdominal cavity, whereas in 4 out of the 10 treated mice the infection did not progress. Statistical significant differences (p<0.05) were observed in the weight of the cyst recovered from untreated mice (7.2 ± 3.4 g) compared with TAM treated group (0.64 ± 0.35 g, Fig. 4A).

All cysts removed from control mice appeared turgid, showing no observable collapse of the germinal layer and no changes in ultrastructure by SEM were detected (Fig. 4Ba). TEM analysis of cysts recovered from the untreated control group revealed typical features of E. granulosus metacestodes, with a distinct acellular outer laminated layer and a germinal layer without alterations (Fig. 4Bc). In contrast, the ultrastructural study of cysts developed in mice treated with TAM suspension revealed changes in the germinal layer. Regarding with the ultrastructural study at SEM, only debris of cells could be observed (Fig. 4Bb). TEM analysis of cysts from treated mice revealed a completely damaged germinal layer with internal tissue extensively distorted, vacuolated areas and numerous lipid droplets (Fig. 4Bd).

Clinical efficacy study
All infected animals involved in the efficacy study developed cysts in their abdominal cavity. All treatments resulted in a statistically significant reduction on the cyst weights compared with those obtained from unmedicated mice (E1 and E2, Fig. 5A).

When the treatment was initiated after 6 month p.i. (E1), the efficacy of TAM was 61%. TAM treatment resulted in a reduction on the cyst weights (4.9 ± 1 g) compared with those obtained from unmedicated mice (12.5 ± 6.2 g). This reduction was statistical significant (p<0.01). On the other hand, during E2, TAM treatment was realized on cyst developed for three months. Two different therapeutic schemes were developed: 20 mg/kg every 48 h (E2a) and, 100 mg/kg every 48 h (E2b). This 5-fold increment on the dose improved the efficacy of the treatment (83% vs 68%), but no statistically significant difference were found between the weights of cysts recovered from both groups (p> 0.05, Fig. 5A). There were statistical differences (p<0.05) between cyst weights recovered from the unmedicated group (6.3 ± 2 g) and those from E2a and E2b treated groups (2.0 ± 0.92 g and 1.1 ± 0.6 g, respectively).

All cysts removed from unmedicated control mice, three or six months p.i., appeared turgid, showing no observable collapse of the germinal layer, in which no alteration in ultrastructure by SEM was observed (Fig. 5Ba). On the other hand, the ultrastructural study of cysts developed in mice treated with TAM revealed alterations in the germinal layer. However, the damage extension appears to be broader when the treatment was initiated after 3 month p.i. (E2, Figs. 5Bc-d). Moreover, a complete loss of cells from the germinal layer of cysts was observed with the highest dose (100 mg/kg, Fig. 5Bd).

TAM and its metabolites were detected in cysts obtained from treated mice (Fig. 5C). A dose-concentration relationship was detected between E2b (100 mg/kg, 20±3 μg/ml) versus E2a (20 mg/kg, 12±2 μg/ml).

In silico analysis of putative Echinococcus steroid receptor
RT-PCR analysis showed that Eg-sr1 is expressed in control conditions from protoscoleces and metacestodes (data not shown). Eg-SR is a typical nuclear-receptor (Fig. 6). It contains a DBD (residues 340-550) with two highly conserved zinc finger motifs (CI and CII, called the P- and D-boxes), responsible of the sequence-specific DNA recognition and the dimerization (Fig. 6A). The hinge region between DBD and LBD is a poorly conserved sequence in Eg-SR as in all NR, due to functions as a flexible region. In Hs-ER, this region is involved in CaM binding (residues 248–317), and it showed 38% similar compared with Eg-SR (residues 430-500). The C-terminal LBD of Eg-SR (residues 580-770 compared with 340-530 of Hs-ER) is the highest hydrophobic portion of the protein in agreement with orthologs (Fig. 6B). This region contains two partial conserved motifs (LXXLL, named NR box) and key polar amino acids which could be involved in hydrogen bonds with 17β-estradiol and estrogen derivates.

DISCUSSION

*Echinococcus granulosus* and *E. multilocularis* 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 (4). In this study we demonstrate that TAM exhibits promising *in vitro* and *in vivo* activity against *E. granulosus*.

Tamoxifen treatment of protoscoleces (50 μM) and metacestodes (20 μM) resulted in the killing of 50% parasites after 24 h compared with the controls (Fig. 2-3). As assessed by Eg-GGT activity, the protoscolex tegument was completely contracted (Fig. 2C) and the cyst germinal layer detached (Fig 3B). Taken together, all the above results showed that 20 μM TAM has severe effects on the *in vitro* survival of *E. granulosus* larval stage in a time-dependent
manner, demonstrating the potent effect of the parental drug. While TAM exerts its toxic effects within a short time (24-48 h), other previously characterized drugs such as benzimidazoles (21,24) and praziquantel (24) presented pharmacological effects only after 5 to 12 days of treatment. In future studies, we will assay the effect of TAM at shorter times (minutes) in order to evaluate its possible application as a scolicidal agent during surgery or PAIR.

As it was mentioned, TAM induces protection against the in vivo establishment of *T. solium* and *T. crassiceps* (12,13). Although the conditions used in our experiments are different since *E. granulosus* is a tissue parasite, our results are coincident. A protection against the infection was observed during the chemoprophylactic efficacy study. Furthermore, this study describes for the first time the effect of TAM upon the clinical efficacy for a cestode parasite.

After the oral administration of TAM to mice, the drug demonstrated a preventive chemoprophylactic effect. Firstly, 20% of TAM treated mice did not develop any cyst while in all unmedicated mice the infection progressed. A deleterious drug effect on *E. granulosus* protoscoleces at the time of infection may help to explain the lack of cyst development. Additionally, statistically significant differences in cyst weight were detected between treated and control groups. Therefore, TAM may not only reduce the number of cysts that develop but may also inhibit the development of secondary hydatidosis in mice. It is important to highlight that the obtained results coincides with other treatment schedules previously assayed (25,26).

SEM and TEM evaluations revealed that TAM induces a number of characteristic alterations on the cyst germinal layer. The ultrastructural changes induced by TAM were similar to those described for other compounds such as albendazole, praziquantel, and flubendazole (25,26), including distorted internal tissue, vacuolated areas and presence of abundant lipid droplets. Hydatid cysts developed in all the infected animals involved in the clinical efficacy studies. A clear reduction on cyst weight was observed after the administration of TAM in mice with cysts developed during three or six months, compared to the unmedicated control mice.
(Fig. 5A). Not surprisingly, a greater effectiveness of the treatment was observed when TAM was administered after 3 months p.i. This coincides with the results obtained by Urrea-Paris et al. (27) and a possible explanation could be that the most pronounced development of cystic layers could protect the cyst against the drug. The germinal layer of cysts recovered from TAM treated mice was markedly altered. Nevertheless, the ultrastructural damage extension appears to be greater when the drug was administrated earlier (3 months p.i.). Furthermore, when the dose was increased (100 mg/kg) a complete destruction of the germinal layer was observed. Once again, the ultrastructural changes induced by TAM were similar to those described for other drugs, such as albendazole and flubendazole (21,28,29).

Despite the promising results, TAM has a parasitostatic rather than parasitocidal effect as it is the case for benzimidazoles (4). Future studies will have to focus on adjusting the dose of TAM to obtain a parasitocidal effect.

Tamoxifen, due to high lipid partition coefficient, has been shown to be strongly incorporated in biomembranes (30). This TAM incorporation was increased when cholesterol concentration is low. Accurately, *E. granulosus* do not synthesize cholesterol, their cell membranes are limited in this lipid, and thus this aspect may favor accumulation of TAM in the cells. Besides, TAM is a potent inhibitor of Pgp efflux transporter (31,32). Our results in *E. granulosus*, correlate well with this findings (Fig. 1B-C). Thus, this TAM retention in *Echinococcus* cells and cysts could enhance the pharmacological effects (Fig 1B-C, 4-5).

Moreover, we demonstrated that TAM and their metabolites were accumulated in the cysts in adequate quantities after *in vivo* treatment of infected mice. Our results coincide with the concentrations reported by Robinson et al. (33) in mouse tissues.

Signaling pathways involving sterol-responsive nuclear receptors are conserved from invertebrates to mammals and regulate metabolism and development (34). Recent evidence exposed that estrogens can affect *Taenia* physiology (13,35). In this line of evidence, the
efficiency of chemoprotective TAM treatment suggests the possible role of the estrogens in *Echinococcus* development. Interestingly, all nuclear receptors have been recently identified in *E. multilocularis* and *E. granulosus* genomes, and one of them showed the highest homology to the LBDs of vertebrate ER (17). In this work we verified the constitutively expression of this putative steroid receptor in *E. granulosus* larval stage, Eg-SR, an EmuJ_000814300 ortholog, which displayed structural similarities to *H. sapiens* ER and an invertebrate prototype ER (Fig. 6).

Furthermore, it has been proposed that during estrogen receptor evolution, the specificity has been determined by the early requirement of the aromatized A-ring, the final step in a conserved estrogen synthesis pathway beginning with cholesterol (36). Therein, a promiscuous ancestral steroid receptor would be activated by nonsteroidal ER agonists as diethylstilbestrol and genistein and competitively inhibited by the ER antagonists as SERMs (36). Tapeworms, like flukes, lack the ability to synthesize cholesterol de novo, and until now there is no conclusive research related with the biosynthesis of estrogens, progesterone and androgens in cestodes (17). Metabolic and transcriptomic studies have demonstrated that sterol synthesis in *E. granulosus* is interrupted at the level of farnesyl or nerolidol pyrophosphate, that the cholesterol derives from of the host, and for signalling actions, the host sterols may be modified by the parasite (37). But it is highly likely that, if *Echinococcus* sp. would have sensitivity to estrogens, the candidate receptor could be Eg-SR.

Calcium-dependent activation of Hs-ER is mediated by CaM and, CaM antagonists prevent that 17-β-estradiol could stimulate the transcription (38,39). CaM binds to Hs-ER in the hinge domain (residues 248–317), and contributes to the ER dimerization and to the ER transcriptional activity in a combination with 17-β-estradiol and calcium (38,39). Eg-SR conserves this hydrophobic region in the LBD involved in association CaM-ER, (Zhang et al., 2012, Fig. 6). On the other hand, in the attempt to understand the TAM mechanism in *E.*
granulosus, several findings suggest that TAM interfere with calcium homeostasis in eukaryotic cells (40). Different researches studying mammalian cells and yeast, clearly showed that TAM targets endoplasmic reticulum calcium pumps, protein kinase C and/or CaM. Consequently, this induces an increase in intracellular calcium as non-estrogen receptor-dependent effect (18). Our in vitro results upon the TAM effects suggest one calcium cell deregulation in treated protoscoleces (Fig. 1A) through independent or ER-indirectly dependent mechanisms which must be confirmed by further studies in the Echinococcus larval stage.

In the search for an effective drug against E. granulosus, we showed that TAM, at low micromolar concentrations, inhibits the survival of protoscoleces and metacestodes. Moreover, we demonstrated the chemotherapeutic and chemopreventive pharmacological effects of TAM. Since collateral effects of high TAM doses have been largely documented in clinical trials, the use of low doses of this drug as a short-term therapy may be a novel alternative approach for human CE treatment. Nevertheless, this is a preliminary study performed in a murine model and, for this reason, more exhaustive evaluation should be carried out in humans to fully demonstrate its therapeutical usefulness.

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REFERENCES


Figure Legends

Figure 1. *In vitro* effect of TAM treatment in *Echinococcus granulosus* protoscoleces and metacestodes (A) Determination of changes in cytosolic-free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)_i]) from control protoscoleces incubated with buffer (C, control) or with 20 µM TAM for 2 h and then loaded with Fluo3-AM (grey bars). Pretreatments with 1 mM EGTA plus 100 µM BAPTA-AM (open bars) for 15 min were carried out before of the Fluo3-AM incubations. Data are the mean ± SD of five independent experiments. (B) Relative fluorescence levels in protoscoleces (grey bars, PTS) and dissected metacestodes (black bars, MTC) in presence of 20 µM TAM, compared with control (C). Treated parasites showed a significant increase in fluorescence (p < 0.005, asterisk) respect to control, indicating that TAM is a Pgp inhibitor. The values represent means ± standard deviations. (C) Confocal imaging revealed calcein accumulation in tegument and suckers. Photomicroscopy of light field (a and c) and of fluorescence field (b and d). Control (a-b); protoscoleces treated with TAM (c-d). Bars indicate: 50 µm.

Figure 2. *In vitro* TAM treatment of *Echinococcus granulosus* protoscoleces. (A) Protoscolicidal activity of TAM from cultures maintained for 24 h. Bars show means ± standard error of five independent experiments. (B) Long-term effect of 20 µM TAM on *E. granulosus* protoscolex viability over 7 days. Each point represents the mean percentage of vital protoscoleces from three different experiments. Asterisks indicate a statistically significant difference (p < 0.05) from the corresponding control. (C) Ultrastructural changes of protoscoleces detected by SEM after incubation with TAM (20 µM, 48 h). Control (a, invaginated protoscolex; b, evaginated protoscolex) and TAM treated protoscoleces (c-d). Note the extensive drug-induced damage with contraction of soma region, alterations of tegument,
and scolex region showing loss of rostelar hooks and microtriches. Bars indicate: 20 µm in (a),
(b) and (d), and 50 µm in (e). rc, rostellar cone; su, suckers; and bo, body.

Figure 3. In vitro TAM-treatment of *Echinococcus granulosus* metacestodes. (A)
Metacestode viability measured on the basis of vesicle integrity. IC\textsubscript{50} is shown as inset graphic.
(B) Macroscopically pictures showing the detached germinal layer from the laminar layer of
metacestodes (20 µM, 48 h). Control (C, without morphological changes) and treated
metacestodes (TAM) showing increased permeability (culture medium inside cysts) and
collapsed germinal membrane (circles).

Figure 4. Chemoprophylactic activity of TAM during *Echinococcus granulosus* cyst
development. (A) Box plot showing the comparative distribution of the weight (g) of cysts
recovered from untreated and TAM treated mice (20 mg/kg). A significant cyst weight reduction
(p<0.005) was achieved in treated animals. (B) Representative SEM (a-b) and TEM (c-d) of
hydatid cysts recovered from untreated control mice (a-c) compared with TAM-treated mice (b-
d). LL, laminar layer; MT, microtriches; DC, distal cytoplasm; GL, germinal layer.

Figure 5. In vivo treatment with TAM of *Echinococcus granulosus* infected mice. (A) Box
plots indicate the distribution of cyst weights (g) in the different treatment groups. Significant
reductions of recovered parasite weights in relation to the control groups were achieved. (B)
Representative images obtained by SEM of cysts from TAM treated mice, (a) Control after 6
months p.i., (b) TAM-treated mice from E1, (c) E2a and (d) E2b (C) Intracystic concentrations
of TAM and its metabolites from experiments indicated in (B).
Figure 6. Sequence analysis of *Echinococcus granulosus* steroid receptor (Eg-SR). Multiple sequence alignment of DBD (A) and LBD (B) among invertebrate and vertebrate species: *Aplysia californica* (Ac-ER, NP_001191648, mollusca) and *Homo sapiens* (Hs-ER, NP_000116, vertebrata). Consensus is indicated in the last line, total (capital letters), partial (lowercase letters), conservative changes (asterisks), and non-conservative substitutions (dots). Nuclear localization signals (NLS) are indicated with gray shading boxes. In N-terminal DBD, the alignments of highly conserved zinc finger motifs (CI, C-X_2-C-X_13-C-X_2-C- and CII, C-X_5/10-C-X_9-C-X_2-C, dashed-line boxes) and the cystein residues (arrows) are indicated. CaM binding region is underlined and three lysine residues involved in direct association with CaM-ER are indicated with arrowheads. In C-terminal LBD the hydrophobic portion involved in ligand interaction is boxed, the conserved NR box motifs (residues with “#”) and the key polar amino acids (residues with “!”) in Hs-ER corresponds to E_353, R_394 and H_524) are indicated.
**Figure 1. In vitro effect of TAM treatment in Echinococcus granulosus protoscoleces and metacestodes.** (A) Determination of changes in [Ca$^{2+}$]$_i$ from control protoscoleces incubated with buffer (C, control) or with 20 μM TAM for 2 h and then loaded with Fluor3-AM (grey bars). Pretreatments with 1 mM EGTA plus 100 μM BAPTA-AM (open bars) for 15 min were carried out before of the Fluor3-AM incubations. Data are the mean ± SD of five independent experiments. (B) Relative fluorescence levels in protoscoleces (grey bars, PTS) and dissected metacestodes (black bars, MTC) in presence of 20μM TAM, compared with control (C). Treated parasites showed a significant increase in fluorescence (p <0.005, asterisk) respect to control, indicating that TAM is a Pgp inhibitor. The values represent means ± standard deviations. (C) Confocal imaging revealed calcein accumulation in tegument and suckers. Photomicroscopy of light field (a and c) and of fluorescence field (b and d). Control (a-b); protoscoleces treated with TAM (c-d). Bars indicate: 50 μm.
Figure 2. In vitro TAM treatment of *Echinococcus granulosus* protoscoleces. (A) Protoscolicidal activity of TAM from cultures maintained for 24h. Bars show means ± standard error of five independent experiments. (B) Long-term effect of 20 µM TAM on *E. granulosus* protoscolex viability over 7 days. Each point represents the mean percentage of vital protoscoleces from three different experiments. Asterisks indicate a statistically significant difference (p < 0.05) from the corresponding control. (C) Ultrastructural changes of protoscoleces detected by SEM after incubation with TAM (20 µM, 48 h). Control (a, invaginated protoscolex; b, evaginated protoscolex) and TAM treated protoscoleces (c-d). Note the extensive drug-induced damage with contraction of soma region, alterations of tegument, and scolex region showing loss of rostellar hooks and microtriches. Bars indicate: 20 µm in (a), (b) and (d), and 50 µm in (c). re, rostellar cone; su, suckers; and bo, body.
Figure 3. In vitro TAM-treatment of Echinococcus granulosus metacestodes. (A) Metacestode viability measured on the basis of vesicle integrity. IC$_{50}$ is shown as inset graphic. (B) Macroscopically pictures showing the detached germinal layer from the laminar layer of metacestodes (20 µM, 48 h). Control (C, without morphological changes) and treated metacestodes (TAM) showing increased permeability (culture medium inside cysts) and collapsed germinal membrane (circles).
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