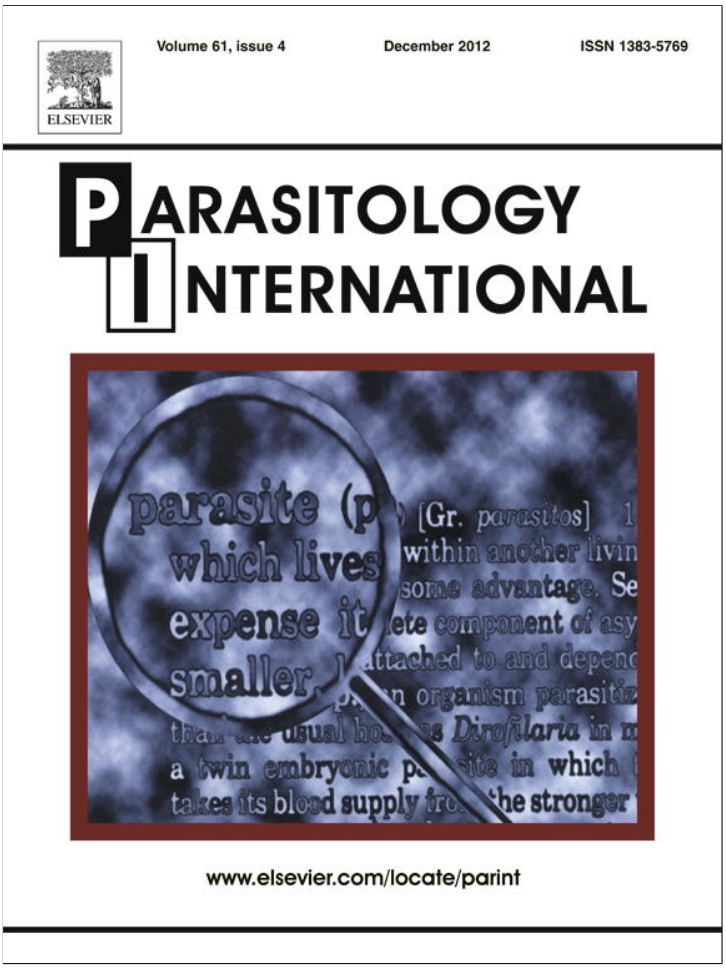

Parasitology International 61 (2012) 579–585

Contents lists available at SciVerse ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Echinococcus granulosus tegumental enzymes as *in vitro* markers of pharmacological damage: A biochemical and molecular approach ☆

Andrea C. Cumino [a,b,*], M. Celeste Nicolao [a,b], Julia A. Loos [a,c], Guillermo Denegri [a,b], M. Celina Elissondo [a,b]

[a] Laboratorio de Zoonosis Parasitarias, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata (UNMdP), Funes 3250, 7600 Mar del Plata, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina
[c] Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC), Argentina

ARTICLE INFO

Article history:
Received 17 September 2011
Received in revised form 3 May 2012
Accepted 11 May 2012
Available online 18 May 2012

Keywords:
Echinococcus granulosus
Hydatid disease
In vitro culture
Chemotherapy
Tegumental enzymes
Gene expression

ABSTRACT

Cystic echinococcosis is a chronic, complex, and neglected disease. Novel therapeutical tools are needed to optimize human treatment. A number of compounds have been investigated, either using *in vitro* cultured parasites and/or applying *in vivo* rodent models. Although some of these compounds showed promising activities *in vitro*, and to some extent also in the rodent models, they have not been translated into clinical applications. Membrane enzyme activities in culture supernatants of treated protoscoleces with calcium modulator drugs and anthelmintic drugs were measured and provided an indication of compound efficacy. This work describes for the first time the detection of alkaline phosphatase, gamma-glutamyl-transpeptidase and acetylcholinesterase activities in supernatants of *in vitro* treated *Echinococcus granulosus* protoscoleces. Marked differences on the enzymatic activities in supernatants from drug treated cultures were detected. We demonstrated that those genes that show the highest degree of conservation when compared to orthologs, are constitutively and highly expressed in protoscoleces and metacestodes. Due to high sensibility and the lack of activity in supernatants of intact protoscoleces, gamma-glutamyl-transpeptidase is proposed as the ideal viability marker during *in vitro* pharmacological studies against *E. granulosus* protoscoleces.

1. Introduction

Cystic echinococcosis (CE), an infection with the larval form of the dog tapeworm *Echinococcus granulosus*, still causes serious lung and liver disease worldwide. This parasitic infection is a chronic, complex, and still neglected disease [1]. Currently four treatment modalities are in use: Surgery, PAIR (puncture, aspiration, injection of protoscolicidal agent, reaspiration), chemotherapy with benzimidazoles, and watch and wait for inactive, clinically silent cysts [2]. Over the past 30 years, benzimidazoles (albendazole and mebendazole) have increasingly been used to treat CE. Unfortunately, 20%–40% of cases do not respond favorably to such chemotherapy [3] and these drugs produce stabilization, rather than cure in the majority of patients [4]. However, the overall efficacy of benzimidazoles has been overstated in the past. With regard to these difficulties, novel therapeutical tools are needed to optimize treatment of CE.

A number of compounds have been investigated, either using *in vitro* cultured parasites and/or applying *in vivo* rodent models. Tested compounds include benzimidazole derivatives such as flubendazole, and oxfendazole, as well as other anti-infective agents like praziquantel, ivermectin, nitazoxanide, genistein, artemisinin, timol, rapamycin, and anticancer agents such as 2-methoxyestradiol and cyclosporine A [5-13]. Although some of these compounds showed promising activities *in vitro*, and to some extent also in the rodent models, they have not been translated into clinical applications.

In our laboratory, the standard approach to identify novel chemotherapeutical compounds against *E. granulosus* has been to perform drug screenings employing *in vitro* cultured protoscoleces. The second stage would be to test the effective compounds *in vitro* on murine cysts [6]. The *in vitro* culture on a large scale of *E. granulosus* metacestodes is expensive, slow, and a low percentage of cysts are obtained after several weeks of culture [14]. The effects of *in vitro* drug treatment can be assessed mainly by light microscopy determining viability by the methylene blue exclusion test. Moreover, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have also been used to investigate ultrastructural damage. However, visual inspection relies on subjective observations, and other techniques such as SEM and TEM, are intrinsically time consuming and expensive. For this reason, there is an urgent need for a reliable, but also easy-to-handle and rapid assay for the identification of pharmacological damage during the screening of drugs against *E. granulosus*.

☆ Nucleotide sequence data reported in this paper available in the GenBank database under GenBank accession nos.: JN662938, JN662939 and JN662940, corresponds to alkaline phosphatase (AP), acetylcholinesterase (AChE) and gamma-glutamyl-transpeptidase (GGT) of *Echinococcus granulosus*.

* Corresponding author at: Laboratorio de Zoonosis Parasitarias, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata (UNMdP), Funes 3250, 7600 Mar del Plata, Argentina. Tel.: +54 223 475 2426; fax: +54 223 475 3150.
E-mail address: acumino@gmail.com (A.C. Cumino).

1383-5769/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.parint.2012.05.007

Tegument is known to be a dynamic cellular structure that plays a vital role in the physiology of cestodes, being involved in nutrient absorption, defense against enzymatic and immunological attack by the host, in excretion and ionic exchange. A large number of important enzymes are anchored in the tegument, such as glutathione S-transferase, ATP diphosphorylase, alkaline and acid phosphatases, β-glucuronidase, amino peptidase, acetylcholine esterase, phosphofructokinase, glucose transporters, serine hydrolases and several glycolytic enzymes [15]. Membrane enzymes such as alkaline and acid phosphatases, 5′ nucleotidases and maltase are proteins predominantly external surface membrane bound, on the other hand, phosphodiesterase, adenosine triphosphatase, leucine aminopeptidase and gamma-glutamyl-transpeptidase were found apparently associated with the internal membrane [15,16].

The detection of alkaline phosphatase (AP) activity in medium supernatants of drug-treated *E. multilocularis* metacestode cultures has been proposed as a method to screen for active drugs [17]. AP activity has been found to be increased in culture supernatants treated with nitazoxanide [18], 2-methoxyestradiol and artemisinin derivatives [10], but the sensitivity of this assay is not always satisfactory [19]. Stadelmann et al. [20] presented an assay based on the detection of phosphoglucose isomerase (PGI) activity. Interestingly, PGI activity was detected not only in cells, but also in the vesicle fluid of intact *E. multilocularis* metacestodes, and not in medium supernatants of intact vesicles.

The aim of this work was to develop a simple and sensitive biochemical method, based on the release of tegumental enzymes, to detect pharmacological damage during the screening of drugs against *E. granulosus*. In addition, the gene occurrence in *Echinococcus* of homologs to the studied tegumental enzymes of platyhelminths was analyzed.

2. Materials and methods

2.1. Protoscoleces collection and in vitro culture procedures

Protoscoleces of *E. granulosus* were collected aseptically from liver and lung hydatid cysts of infected cattle slaughtered in an abattoir located in the southeast of the Buenos Aires province, Argentina. Protoscoleces were washed several times in phosphate-buffered saline (PBS, pH 7.2) and viable, motile and morphological intact protoscoleces ($n = 3000$) were cultured under aseptic conditions in 0.2 ml per well of medium 199 (Gibco) supplemented with antibiotics (penicillin, streptomycin and gentamicin 100 μg/ml) and glucose (4 mg/ml) in a sterile tissue culture plate (Nunc, 96 well). *In vitro* incubations were performed at 37 °C without changes of medium. All assayed drugs were dissolved in dimethyl sulphoxide (DMSO).

Calcium compounds and calmodulin antagonists (EGTA — ethylene glycol tetraacetic acid, diltiazem, W-13 — naphthalenesulfonamide and calmidazolium; CalBiochem, USA) were assayed at 15, 30, 45 and 60 μM as final concentrations. Flubendazole (FLBZ; Janssen-Cilag Laboratories, Argentina), albendazole (ABZ; Sigma-Aldrich, USA) and ivermectin (IVM; Sigma-Aldrich, USA) were assayed at 30 μM as final concentration. Protoscoleces incubated in culture medium containing 1:1000 DMSO were used as control. Each experiment was assayed for three replicates and repeated four times. During the experiment, different samples were taken for ultrastructural studies by SEM and biochemical experiments at 1, 3 and 6 days as described below.

2.2. Electron microscopy

Samples of protoscoleces cultured *in vitro* were processed for SEM as described by Elissondo et al. [21]. Briefly, samples were fixed with 3% glutaraldehyde in sodium cacodylate buffer for 24 h at 4 °C. Then several washes in cacodylate buffer were made and the specimens were dehydrated by sequential incubations in increasing concentrations of ethanol (50–100%) and were finally immersed in hexamethyldisilazane for 5 min, 1 h and then overnight. They were then sputtercoated with gold (100 Å thick) and inspected on a JEOL JSM-6460 LV scanning electron microscope operating at 15 kV.

2.3. Mouse infection and obtention of cysts

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. After 4–5 months, the animals were sacrificed by cervical dislocation under ether anesthesia and their peritoneal cavity was opened to remove the hydatid cysts.

2.4. Enzyme assays

After two rinses with ice-cold 50 mM Tris–HCl, 0.1–0.2 g of protoscoleces was homogenized in medium containing 250 mM sucrose, 50 mM Tris–HCl, 6 mM β-mercaptoethanol, 0.3% (v/v) Triton X-100 and 1.5 mM EDTA (pH 7.5 at 4 °C) with periodic shaking. The suspensions of protoscoleces were frozen in liquid nitrogen and thawed at 37 °C for three cycles. Then, cells were lysed with a homogenizer with Teflon pestle at 0 °C and finally sonicated in an ice bath. The homogenate was centrifuged at 100,000 ×*g* for 15 min and then desalted through Sephadex G-50 columns before the enzyme activity assays. Protein concentrations were quantified by Bio-Rad protein assay kit.

Enzyme activities were determined from protoscolex protein extracts and from culture protoscolex supernatants following the procedure described below. In all cases, the activities were measured at 37 °C in a recording Shimadzu model UV–vis spectrophotometer, the volume of the reaction mixture was 1 ml with 50 μl of enzyme samples and measurements made after 30 min of incubation.

AP activity was measured using a p-nitrophenyl phosphate (p-NPP, 10 mmol/L) substrate in 50 mM NH4OH–NH4C1 buffer (pH 10.4) with 0.67 mM 2-amino-2-methyl-1-propanol and 1.6 mM MgCl2. The absorbance was read at 405 nm against appropriate blanks and the enzyme activity calculated using the molar extinction coefficient of 18.5 for p-NPP [22]. Gamma-glutamyl-transpeptidase (GGT) was determined following the Szasz method [23], whereas the rate of increase in absorbance is due to release of p-nitroanaline. The substrate solution is an aqueous buffered solution containing 2.9 mM L-gamma-glutamyl-p-nitroanilide, 100 mM glycylglycine and 5 mM $MgCl_2$. Acetylcholinesterase (AChE) was determined using conversion of acetylthiocholine to thiocholine [24]; interception of the thiol by 5,5′-dithiobis(2-nitrobenzoic acid)-DTNB- releases the yellow colored anion TNB, which can be colorimetrically detected at 412 nm.

Data within experiments were compared and their significance was determined using the Student's t-test. All data were shown as arithmetic means ± SD.

2.5. Genomic identification, expression and sequences analysis of Echinococcus AP, AChE and GGT

In order to obtain information on genomic and expressed *Echinococcus* AP, AChE and GGT sequences, the *E. multilocularis* genomic database, *E. granulosus* assembled genomic contigs (both accessible versions at SEPT-1-2011, verified at FEB-1-2012, under the same address, http://www.sanger.ac.uk/Projects/*Echinococcus*) and NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were searched with tblastn (EST others option) and blastp, using the sequences of *Schistosoma mansoni*, *Homo sapiens* and *Mus musculus* AP (EU040139, NP_000469 and AAA39928, respectively), GGT (XP_002579947, AAA35899 and AAA97395, respectively), and AChE (AAQ14321, AAA68151, and AF312033, respectively) as queries. Possible orthologs were selected based on reciprocal best hits in

blast searches, using an E-value cut-off of 10^{-25}. A list containing all the contigs that were hit was analyzed both manually and by blastp (after joining the conceptual translation of exons) against the GenBank nr database.

Newly identified genes from *E. granulosus* were analyzed using sequence information of the genome projects for specific primer construction. The full-length amplified cDNAs were obtained through direct sequencing of four overlapping PCR fragments for each gene, using the primers listed in Table 1. PCR products were purified using PCR Purification Kits (Qiagen, USA). Following direct sequencing and comparison of the obtained sequences using blast analysis were carried out. For protoscoleces studies, total RNA extractions and RT-PCR assays were realized as previously described by Cumino et al. [12]. In the case of metacestodes, the hydatid fluid was first aspirated from the cysts, and then TRIZOL reagent (Invitrogen Corp.) was injected and reaspirated several times to obtain parasite RNA free from host RNA (C. Fernández, personal communication). cDNA was generated from 10 μg of total RNA using Superscript II reverse transcriptase (Invitrogen) and Pfu (Fermentas) DNA polymerase, respectively. Equal amounts of cDNA from protoscoleces and metacestodes were amplified with gene-specific primer pairs *AP-A-Fw2-AP-A-Rv2*, *AP-B-Fw2-AP-B-Rv2*, *GGT-Fw2-GGT-Rv2* and *AChE-Fw2-AChE-Rv2* (see above) in PCRs of 30 cycles of 1 min at 94 °C, 45 s at 50 °C, 45 s at 72 °C, plus a single step at 72 °C for 10 min, resulting amplification fragments of 478, 474, 405 and 523 nucleotides, respectively. Additionally, *E. granulosus* actin expression (*act*I gene, L07773), a constitutively expressed gene in protoscoleces and metacestodes, was used as RT-PCR positive control employed a pair of specific primers (*act-Fw*: 5′-GCGATGTATGTAGCTATCCAGGCAGTGCTCTCGCT-3′ and *act-Rv*: 5′-CAATCCAGACAGAGTATTTGCGTTCCGGAGGA-3′). PCR reactions were run for 20 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C, amplifying a fragment of 633 nucleotides. PCR products were separated on a 1.5% agarose gel and stained with SYBR gold.

Sequence alignments were generated with the CLUSTALX software program. Analyses of prediction of transmembrane regions were realized with TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM) and SACS HMMTOP program, which is a combined membrane topology and signal peptide predictor. The evidence for the hydrophobic anchor to surface membranes via a covalently attached glycophosphatidylinisitol (GPI) anchor was analyzed through Big-PI; http://mendel.imp.univie.ac.at/sat/gpi/gpi_server.html) and GPI-SOM; http://gpi.unibe.ch/) programs, available for computational prediction of C-terminal GPI-anchoring signals.

3. Results

3.1. Membrane enzyme activities in culture supernatants of protoscoleces treated with calcium modulator drugs

Extracellular calcium chelator (EGTA), calcium channel blocker (diltiazem) and calmodulin antagonists (calmidazolium and W13) induced important ultrastructural changes to the tegument at 50 μM for 48 h, preceding the decline in viability. The viability of protoscoleces after 5 days incubation was estimated to be 50–80% (unpublished observation). In order to investigate the release of tegumental enzymes into the medium supernatant, protoscoleces were treated with calcium modulator at different concentrations for 24 h. AP, GGT and AChE activities increased in the presence of 15 μM of the drug, before structural alterations could be observed (Fig. 1A). After 48 h incubation, the first tegumental alterations could be observed by SEM on approximately 85% of protoscoleces incubated with calcium antagonists (Fig. 1B). The tegument was markedly altered. Moreover, the contraction of the soma region, rostellar disorganization, loss of hooks and microtriches, and, the presence of numerous blebs were observed.

In order to determine whether the maintenance of protoscoleces in *in vitro* systems leads to the release of membrane enzymes to the culture media, the enzyme activities of AP, GGT and AChE were measured on culture supernatants every two days during two weeks. Fig. 2 shows the results obtained at day 14 post-incubation. GGT and AChE released from control protoscoleces were barely detectable. However, AP release was clear. Furthermore, to determine total enzyme activities on cultured prostoscoleces, they were lysed mechanically and AP, GGT and AChE activities were measured (Fig. 2). For GGT and AP, high total enzyme activities were obtained.

3.2. Determination of membrane enzymatic activities of protoscoleces treated with anthelmintic drugs

Since AP and GGT were the most abundant enzymes determined on protoscoleces extracts (Fig. 2), only these enzyme activities were measured on culture supernatants of protoscoleces treated with IVM, ABZ, FLBZ and DMSO for 6 days. At various time points, total

Table 1
Primers used to amplify encoding genes for tegumental enzymes in *Echinococcus granulosus*.

Gene	Name	Sequence 5′-3′	Product size (bp)
Eg-apA	*AP-A-Fw1*	5′-ATGTTTAAGTCGGCATTCATAT-3′	–
Eg-apA	*AP-A-Fw2*	5´-GAAAAATGGTAAAAGAGCAAAGAATGTCATC-3´	–
Eg-apA	*AP-A-Rv1*	5′-TTAGTAAAAAAGACACCACATGATCAGTCC-3′	1599[a]
Eg-apA	*AP-A-Rv2*	5′-CAGGTCCTGACATTTCATGTTTTTATATTC-3′	478[b]
Eg-apB	*AP-B-Fw1*	5′-ATGAAAAATGGTAAAAGAGCAAAGAATG-3′	–
Eg-apB	*AP-B-Fw2*	5′-CTAAAAATGTCATTCTCTTCCTTGGTGATG-3′	–
Eg-apB	*AP-B-Rv1*	5′-TTATTCACAAGGTGTCTTTTTCTGGTCA-3′	1359[c]
Eg-apB	*AP-B-Rv2*	5′-GATGTTCAGTTACCAGTTGATAGGCTAAATC-3′	474[d]
Eg-ggt	*GGT-Fw1*	5′-ATGGCACACAGAGGTGATCGCAAGTTTC-3′	–
Eg-ggt	*GGT-Fw2*	5′-GTTTGCTCAACATTTCTTAAAGTCCGTGCAG-3′	–
Eg-ggt	*GGT-Rv1*	5′-TCACACAAGTAACGCACACAATCTCACC-3′	1698[e]
Eg-ggt	*GGT-Rv2*	5′-GTTAACTCAATAAGACGATGATAGATGAGAG-3′	405[f]
Eg-ache	*AChE-Fw1*	5′-ATGGGTCACTGGGAGGTTGTTATGTTTATC-3′	–
Eg-ache	*AChE-Fw2*	5′-CTTTCTATAGCGGTTCTTCGGTATTAGATG-3′	–
Eg-ache	*AChE-Rv1*	5′-CTAAGCTAACAATCTTTTACAAACC-3′	2214[g]
Eg-ache	*AChE-Rv2*	5′-GGTGGAAAGGGAATAGAGAAAAAGACGTTG-3′	523[h]

[a] With *AP-A-Fw1* as forward primer.
[b] With *AP-A-Fw2* as forward primer.
[c] With *AP-B-Fw1* as forward primer.
[d] With *AP-B-Fw2* as forward primer.
[e] With *GGT-Fw1* as forward primer.
[f] With *GGT-Fw2* as forward primer.
[g] With *AChE-Fw1* as forward primer.
[h] With *AChE-Fw2* as forward primer.

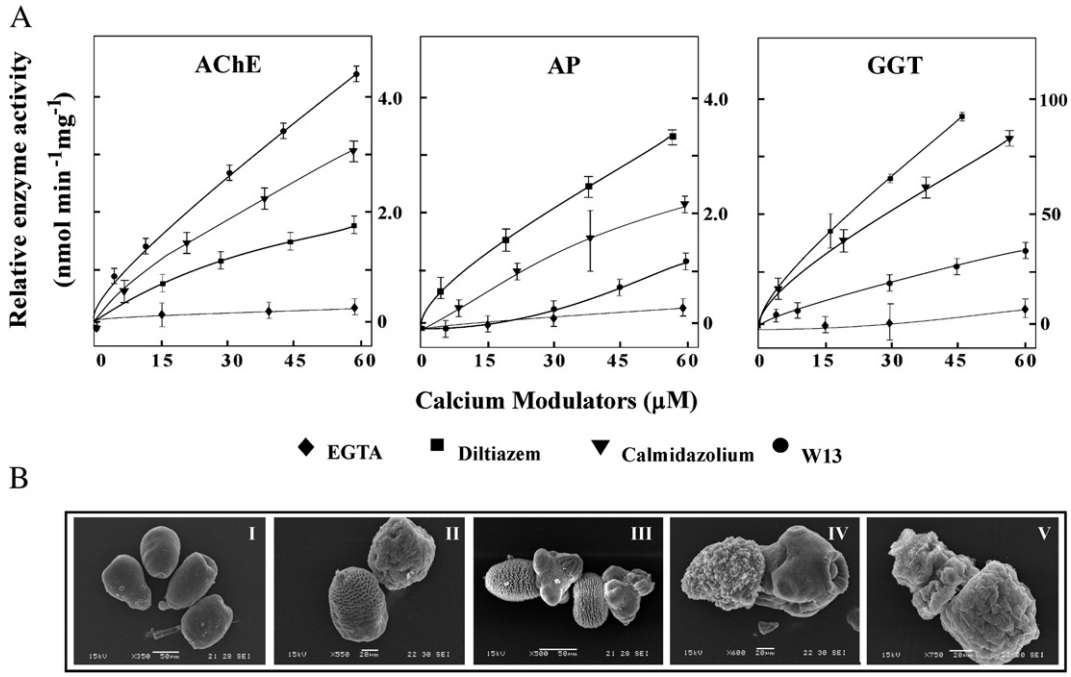

Fig. 1. Detection of membrane enzyme activities after incubation of *E. granulosus* protoscoleces with calcium antagonist. A. Dose-dependent effect of EGTA, diltiazem, calmidazolium and W13 on the release of membrane enzymes into culture supernatants. The efficiency of enzymatic release is expressed related to control. Results are average and S.E. of three different experiments. B. Scanning electron microscopy of *E. granulosus* protoscoleces incubated with 50 μM of different drugs for 48 h. I. Control invaginated protoscoleces (×350). II. EGTA-treated protoscoleces. The tegument was markedly altered (×550). III. Diltiazem-treated protoscoleces. Note the contraction of the soma region, the tegumental alterations and the rostellar disorganization (×500). IV. Calmidazolium-treated protoscolece. The soma region is altered with the presence of numerous blebs (×600) V. W13-treated protoscoleces. Loss of hooks and microtriches of the scolex region (×750).

AP and GGT activities in the culture supernatants were measured. DMSO did not lead to an increase of total AP and GGT activities. However, the activity of both enzymes began to increase after 24 h of incubation (Fig. 3A, B). Enhancement of enzyme activity upon drug treatment was time-dependent. AP activity was detected also on supernatants of control protoscoleces (Fig. 3A), but the level remained constant during the entire experiment. On the other hand, GGT activity was barely detectable on control supernatants (Fig. 3B). Interestingly, the activity of GGT on the treated group was three times higher than AP activity (Fig. 3).

SEM demonstrated the drug-induced ultrastructural damage imposed upon anthelmintic drugs treated protoscoleces (Fig. 3C). Before 6 days of incubation, no changes in ultrastructure were observed.

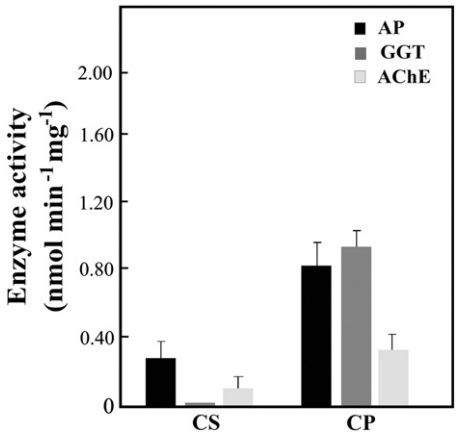

Fig. 2. Enzymatic activities of control cultured protoscoleces. The release of AP, GGT and AChE activity into the culture supernatant of control protoscoleces (14 days post-incubation) was measured and compared with the activity obtained from mechanically lysed protoscoleces (CS, supernatant of control cultured protoscoleces; CP, protein total extract of protoscoleces).

After 6 days incubation, the tegument was completely altered in approximately 70% of protoscoleces. Moreover, other signs of ultrastructural alteration were evident: the soma region was contracted, numerous blebs were observed on the tegument surface of the scolex and soma region, and, loss of hooks and microtriches was evident (Fig. 3C). The increase of enzyme activities was detected before the appearance of ultrastructural changes on the tegument of parasites (Fig. 3C). These tegumental alterations are commonly detected after 6 days post-incubation with the assayed anthelminthic drugs [6]. Furthermore, the viabilities of protoscoleces treated with IVM, ABZ and FLBZ after 6 days post-incubation were between 80 and 90% [6,25].

An increase on the activity of AP in culture supernatant of *E. granulosus* cysts incubated *in vitro* with FLBZ was detected. After 6 days incubation, total AP activity in the culture supernatants was measured (14 nmol min^{-1} mg^{-1}). This value was similar to that found in protoscoleces (Fig. 3A).

3.3. Gene identification, expression and sequence analysis of membrane enzymes from Echinococcus protoscoleces and metacestodes

In the genome of both *Echinococcus* species, blast searches revealed four, three and one homologous protein-coding genes for AP, AChE and GGT, respectively. The predicted open reading frames (ORFs) for AP included two in the Eg-NODE 61174 (*Eg-apA*, position 51381–49279 and *Eg-apB*, position 54903–53180) from *E. granulosus* and in Em-Scaffold007300 (*Em-apA*, positions 543446–541366 and *Em-apB*, position 547168–545191) from *E. multilocularis*; and the other two in Eg-NODE 287790 (*Eg-apC*, position 1276063–1281001 and *Eg-apD*, position 1271023–1268880) and in Em-Scaffold 007768 (*Em-apC*, positions 1517695–1512954 and *Em-apD* 1520 403–1522341), respectively. The predicted ORFs for AChE were included in Eg-NODE_147654 (*Eg-acheA*, position 50900–46497), Eg-NODE_280547 (*Eg-acheB*, position 169625–178414) and Eg-NODE_94165 (*Eg-acheC*, position 53036–57376) for *E. granulosus* and, with similar E-value cutoff, in EMU_scaffolds 007767, 007780

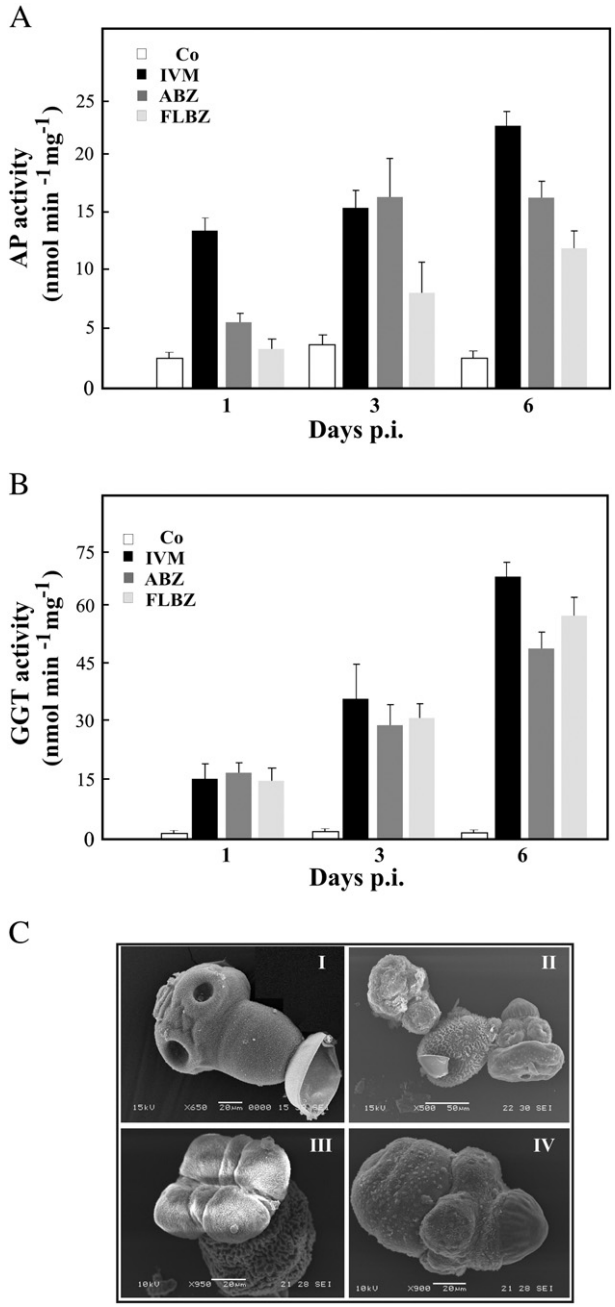

Fig. 3. Determination of tegumental enzyme activities after incubation of *E. granulosus* protoscoleces with protoscolicidal drugs. A–B. Three different anthelminthic drugs, ivermectin (IVM), albendazole (ABZ) and flubendazole (FLBZ), were assayed and the release of membrane enzymes was determined on culture supernatants. A. Alkaline phosphatase (AP) activity. B. Gamma-glutamyl-transpeptidase (GGT) activity. C. Scanning electron microscopy of *E. granulosus* protoscoleces incubated *in vitro* with 30 μM of protoscolicidal drugs during 6 days. I. Control evaginated protoscolex (×650). II: IVM-treated protoscoleces. Note the altered tegument and the contraction of the soma region (×500). III. ABZ-treated protoscolex. The tegument of the soma region was markedly altered (×950). IV. FLBZ-treated protoscolex. Loss of microtriches and presence of numerous blebs were evident (×900).

and 006004 (*Em-acheA-C*) for *E. multilocularis*. Finally, the unique predicted ORF for GGT was included in Eg-NODE_229982 (*Eg-ggtA*, position 188056–183503) and EMU_scaffold_007768 (*Em-ggtA*, position 2567515–257220) for *E. granulosus* and *E. multilocularis*, respectively. For all tegumental enzyme types no ESTs were reported in the Public DataBases.

Using sequence information of the *E. multilocularis* genome and of conserved regions for primer construction, the entire cDNAs from of those homologous genes (*Eg-apA, Eg-apB, Eg-acheA and Eg-ggt*) with other species were amplified and sequenced. It comprised 1599, 1359, 2214 and 1698 bp, and contained ORF that coded for Eg-AP-A (532 amino acids; 59 kDa), Eg-AP-B (452 amino acids; 54 kDa), Eg-AChE-A (737 amino acids; 84 kDa) and Eg-GGT (565 amino acids; 60 kDa), respectively. Finally, amplicons of smaller size (see Materials and methods) were generated, and the expression of these genes in protoscoleces and metacestodes was observed (Fig. 4A).

Alignments of these predicted proteins with other-related members revealed several conserved motifs. Both isoforms of Eg-AP exhibited a high sequence identity with mammal alkaline phosphatases (38–37% *versus H. sapiens* and *B. taurus* orthologs, respectively) as shown in Fig. 4B. In Eg-AP-A and Eg-AP-B, active site residues (Supplementary Figure S1A, S^{124} and R^{199} and S^{66} and R^{141}, respectively) and important residues involved in the metal binding (Fig. S1A, D^{77}, T^{185}, E^{342}, D^{347}, N^{350}, H^{351}, D^{388}, and H^{389} and D^{16}, T^{127}, E^{284}, D^{289}, N^{292}, H^{293}, D^{330}, and H^{331}) were highly conserved. Only in Eg-AP-A the four cysteine residues probably involved in the protein dimerization were conserved (Supplementary Figure S1A). On the other hand, in Eg-AP-A, a potential signal peptide (^{1}MFKSAFICVILLQVALANVEL^{21}) at the N-terminal end and a single predicted transmembrane domain at the C-terminus (^{513}CVACGLKVSLFAMIGLIMWCL^{529}) was identified (Fig. 4B and Supplementary Figure S1A). The amino acid sequence of Eg-AChE-A is 56% and 34% identical compared with orthologs from helminths and mammals, respectively (Fig. 4B) and it fit in esterase-lipase superfamily. In Eg-AChE, the 24 amino acid, N-terminal, sequence (^{1}MGHWE VVMFILALEILPTLTHVPI^{24}) is a potential signal peptide and it has also a single predicted transmembrane domain (^{59}TTQVDIYYGIRYAQPPT GSL^{78}, Fig. 4B). As in AChE of *S. mansoni*, Eg-AChE-A shows conserved aromatic residues important in the active site: W^{54} and F^{330}. On the other hand, potential sites for GPI-linked modification are found in C-terminal region of both Eg-AP and Eg-AChE (corresponding to residue 482, data not shown). Finally, Eg-GGT belongs to GGT superfamily and the Pfam PF01019. The degree of identity of the parasite protein to the human enzyme is 27% (Fig. 4B). The similarity between human and helminth rose to 55 and 60%, respectively, when conservative amino acids substitutions were taken into account. Eg-GGT shows the two conserved regions comprising the putative large (1–380) and small (380–565) subunits as the prototype *Escherichia coli* enzyme (PDB 2dg5), characteristic heterodimeric enzyme that is generated from the precursor protein through post-translational processing. Notably, the residues essential for GGT activity are conserved in Eg-GGT (A^{116}, D^{422}, S^{465}, and S^{468}), all involved in the binding of the gamma-glutamyl moiety (indicated by arrows in Supplementary Figure S1B). This protein shows, unlike Eg-APs and Eg-AChE, two predicted membrane regions between 58–83 and 430–494 amino acids, ^{58}LSLIYVSIAACLAAISVALLITIFF^{83} and ^{430}LPCSLEILS-(n50)-VVSGTGI^{494}, (Fig. 4 and Supplementary Figure S1). Cys-^{454} was thought to bind the gamma-glutamyl moiety (Supplementary Figure S1B).

4. Discussion

AP, AChE and GGT are enzymes broadly distributed in many species, including helminthes. They are tegumental components exposed on the surface of the parasites used in the evaluation of the effectiveness of antihelminthics [16,26-28]. The rationale for the use of enzymatic tests of parasite damage was that damaged parasites would likely release cellular contents through their tegument once the integrity of the barrier was breached. Such approaches have been demonstrated to be sensitive and specific to drug effects on *E. multilocularis*, in which total AP activity has, indeed, been shown to increase in the supernatants of drug-treated metacestodes [17,18]. Moreover, PGI has been shown to be equally or more sensitive to the effects of drugs on *E. multilocularis* metacestodes [20]. However, Naguleswaran et al. [9]

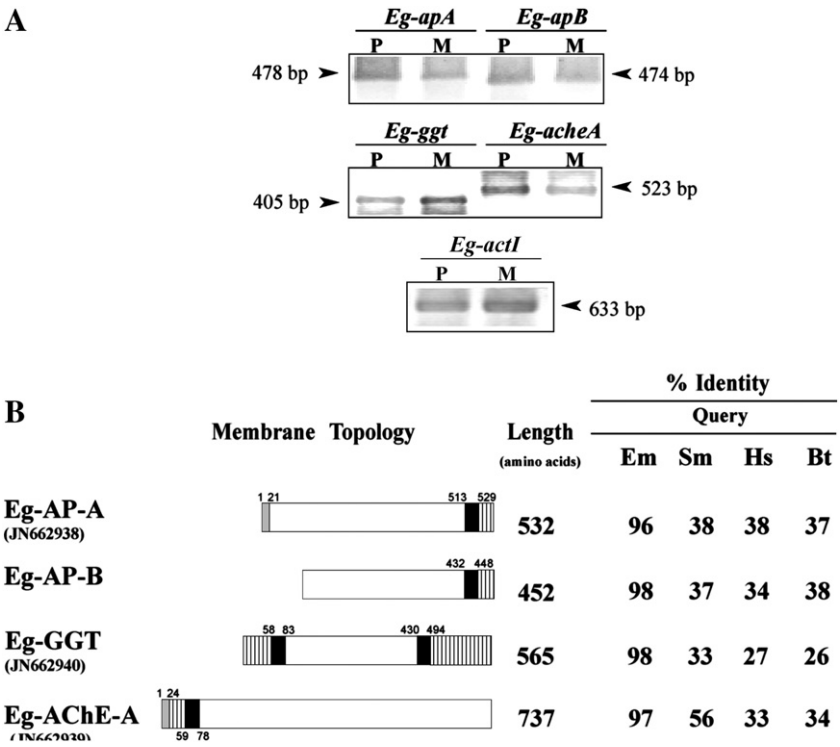

Fig. 4. Expression and sequence analysis of tegumental enzymes of *E. granulosus*. A RT-PCR analysis from 10 μg of total RNA from protoscoleces and metacestodes. Amplification of *E. granulosus actI* was used as loading control. B. Membrane topology representation after bioinformatic prediction analyses (in the left, signal peptide, gray box; extracellular protein portion, white box; cytoplasmic protein portion, lined box; membrane interaction portion, black box) and comparison of identity percentages with different queries (in the right). *E. granulosus* (Eg), *E. multilocularis* (Em), *S. mansoni* (Sm), *H. sapiens* (Hs) and *B. taurus* (Bt) The column indicate the full length in amino acids of *Echinococcus* tegumental proteins.

reported that no rise in AP activity could be detected in culture supernatant of *E. granulosus* metacestodes incubated *in vitro* with genistein. The author suggested that a possible explanation for the lack of activity could be that the enzyme is trapped within the laminated layer of *E. granulosus* metacestodes, which is much thicker compared to *E. multilocularis*. In contrast, in this work, an increase on the activity of AP in culture supernatant of *E. granulosus* cysts incubated *in vitro* with FLBZ was detected.

This work describes for the first time the detection of AP, AChE and GGT activities on supernatants of *in vitro* treated protoscoleces of *E. granulosus* (Figs. 1 and 3). Protoscoleces were incubated with calcium modulators and anthelmintic drugs and, marked differences on the enzyme activities in treated-supernatants in relation to control-supernatant were detected. These results, regardless of the enzyme or drug assayed, demonstrate the high sensitivity and accuracy of the enzymatic method in relation to ultrastructural studies and determination of viability by the methylene blue exclusion test. The release of the enzyme precedes the tegumental damage. During mass screening of new anti-infective compounds, the high sensitivity of the enzymatic method could contribute to the adjustment of the concentrations of the newly tested molecules.

Interestingly, Eg-AP and Eg-AChE activities were detected on supernatants of control cultures. A possible explanation for these activities could be the easy release to the culture medium due to the type of interaction that these enzymes have with the cell membrane, an unique attachment portion (Fig. 4B). On the other hand, Eg-GGT is also an abundant component of protoscolex tegument, but it is not secreted into the medium by intact parasites. Once damage occurs and the protoscoleces suffer from structural impairment, GGT activity is readily detected into the culture supernatant. These results were consistent with the prediction topologies of transmembrane regions of Eg-GGT. Two attachment portions in the membrane were estimated (Fig. 4 and Supplementary Figure S1). Anchoring of proteins to the extracytosolic leaflet of membranes via C-terminal attachment of GPI is ubiquitous and essential in eukaryotes. The signal for GPI-anchoring is confined to the C-terminus of the AP and AChE of certain ectoenzymes into tegumental structures of helminthes, including *Echinococcus* sp. [29,30].

The identification of genes encoding the *Echinococcus* tegumental enzymes was another contribution of this work. In this context, we included the description of the putative full set of encoding genes for AP, AChE and GGT, and determined the gene expression of two AP isoforms (*Eg-apA* and *Eg-apB*) and each of one AChE (*Eg-acheA*) and GGT (*Eg-ggt*) that had the most conserved residue compared with the highest conservation of amino acid sequences compared to metazoa orthologs (greater than 30% identity). In addition, the constitutive and high expression of these genes was demonstrated in protoscoleces and metacestodes (Fig. 4A). It is clear that the tegumental surface contains molecules that perform vital functions such as nutrient uptake, and elicit protective immunity against infection and defense mechanisms.

In this report, we also characterized *in silico* the Eg-APs, Eg-GGT and Eg-AChE (Fig. 4B and Supplementary Figure S1). APs are metalloenzymes that exist as a dimer, each monomer binding metal ions, with preference to zinc and magnesium. Our observation indicates that enzyme active site residues and metal ion binding motif are highly conserved in the predicted Eg-APs. Additionally, Eg-AChE lacks WAT and PRAD motifs (proline-rich region, and responsible of the quaternary organization and heteromeric associations) in the C-terminal portion, and functional domains which are much better conserved throughout vertebrates than the rest of the AChE [31]. On the other hand, GGT is an enzyme that consists of two polypeptide chains, a heavy and a light subunit, processed from a single chain precursor by an autocatalytic cleavage. The active site of GGT is known to be located in the light subunit. As an autocatalytic peptidase, Eg-GGT

belongs to MEROPS peptidase family T3, and it shows similar folds at GGT superfamily. The active site shows essential residues for gamma-glutamyl moiety binding allowing GGT activity [32].

The assay presented in this study allows a relatively easy, fast and specific for *in vitro* screening of a multitude of chemotherapeutical agents. Due to the higher sensibility and the lack of activity in supernatants of intact protoscoleces, GGT is proposed as the ideal viability marker during *in vitro* pharmacological studies against *E. granulosus* protoscoleces.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.parint.2012.05.007.

Acknowledgments

The authors gratefully acknowledge Dr. Melendez G. and Sr. Chasma O. (SENASA, Argentina), Lic. Oppedisano M. (Universidad Nacional de Mar del Plata) for technical assistance with microscopy, Wiener Lab Foundation (Rosario, Argentina), CIC (Comisión de Investigaciones Científicas de la Provincia de Buenos Aires), Dra. Santisteban M.A. and Dra. Elissondo N. This work was supported by ANPCyT (grant PICT 06 no. 1017 and, PICT 07 no. 1644, Universidad Nacional de Mar del Plata (grants EXA 483/10 and EXA 493/10), Argentina. The *E. multilocularis* and *E. granulosus* genome sequence data mentioned was produced by the Pathogen Sequencing Group of the Wellcome Trust Sanger Institute (Program of Helminth Sequencing; project manager: Dr. Matt Berriman).

References

[1] Brunetti E, Garcia HH, Junghanss T, On behalf of the members of the International CE Workshop in Lima, Peru, 2009. Cystic echinococcosis: chronic, complex, and still neglected. PLoS Neglected Tropical Diseases 2011;5:e1146, doi: 10.1371/journal.pntd.0001146.
[2] Stojkovic M, Zwahlen M, Teggi A, Vutova K, Cretu CM, Virdone R, et al. Treatment response of cystic echinococcosis to benzimidazoles: a systematic review. PLoS Neglected Tropical Diseases 2009;3:e524, doi:10.1371/journal.pntd.0000524.
[3] Hemphill A, Müller J. Alveolar and cystic echinococcosis: towards novel chemotherapeutical treatment options. Journal of Helminthology 2009;83: 99–111.
[4] Horton J. Human gastrointestinal helminth infections: are they now neglected diseases? Trends in Parasitology 2003;19:527–31.
[5] Ceballos L, Elissondo M, Sánchez-Bruni S, Denegri G, Alvarez L, Lanusse C. Flubendazole in cystc echinococcosis therapy: pharmaco-parasitological evaluation in mice. Parasitology International 2009;58:354–8.
[6] Elissondo M, Ceballos L, Alvarez L, Sánchez Bruni S, Lanusse C, Denegri G, et al. Flubendazole and ivermectin in vitro combination therapy produces a marked effect on *Echinococcus granulosus* protoscoleces and metacestodes. Parasitology Research 2009;105:835–42.
[7] Gavidia CM, Gonzalez AE, Lopera L, Jayashi C, Angelats R, Barron EA, et al. Evaluation of nitazoxanide and oxfendazole efficacy against cystic echinococcosis in naturally infected sheep. The American Journal of Tropical Medicine and Hygiene 2009;80:367–72.
[8] Walker M, Rossignol JF, Torgerson P, Hemphill A. *In vivo* effects of nitazoxanide on *Echinococcus granulosus* protoscoleces and metacestodes. The Journal of Antimicrobial Chemotherapy 2004;54:609–16.
[9] Naguleswaran A, Spicher M, Vonlaufen N, Ortega-Mora LM, Torgerson P, Gottstein B, et al. In vitro metacestodicidal activities of genistein and other isoflavones against *Echinococcus multilocularis* and *Echinococcus granulosus*. Antimicrobial Agents and Chemotherapy 2006;50:3770–8.
[10] Spicher M, Roethlisberger C, Lany C, Stadelmann B, Keiser J, Ortega-Mora LM, et al. *In vitro* and *in vivo* treatments of *Echinococcus* protoscoleces and metacestodes with artemisinin and artemisinin derivatives. Antimicrobial Agents and Chemotherapy 2008;52:3447–50.
[11] Elissondo MC, Albani CM, Gende L, Eguaras M, Denegri G. Efficacy of thymol against *Echinococcus granulosus* protoscoleces. Parasitology International 2008;57:185–90.
[12] Cumino AC, Lamenza P, Denegri GM. Identification of functional FKB Protein in *Echinococcus granulosus*: its involvement in the protoscolicidal action of rapamycin derivates and in calcium homeostasis. International Journal for Parasitology 2010;60:6151–61.
[13] Colebrook AL, Jenkins DJ, Jones MK, Tatarczuch L, Lightowlers MW. Effect of cyclosporin A on the survival and ultrastructure of *Echinococcus granulosus* protoscoleces *in vitro*. Parasitol 2004;129:497–504.
[14] Elissondo MC, Dopchiz MC, Brasesco M, Denegri G. *Echinococcus granulosus*: first report of microcysts formation from protoscoleces of cattle origin using the *in vitro* vesicular culture technique. Parasite 2004;11:415–8.
[15] McManus DP, Barret NJ. Isolation, fractionation and partial characterization of the tegumental surface from protoscoleces of the hydatid organism, *Echinococcus granulosus*. Parasitology 1985;90:111–29.
[16] McManus DP. Reflections on the biochemistry of *Echinococcus*: past, present and future. Parasitology 2009;136:1643–52.
[17] Stettler M, Siles-Lucas M, Sarciron E, Lawton P, Gottstein B, Hemphill A. Echinococcus *multilocularis* alkaline phosphatase as a marker for metacestode damage induced by *in vivo* drug treatment with albendazole sulfoxide and albendazole sulfone. Antimicrobial Agents and Chemotherapy 2001;45:2256–62.
[18] Stettler M, Fink R, Walker M, Gottstein B, Geary TG, Rossignol JF, et al. *In vitro* parasiticidal effect of nitazoxanide against *Echinococcus multilocularis* metacestodes. Antimicrobial Agents and Chemotherapy 2003;47:467–74.
[19] Gelmedin V, Caballero-Gamiz R, Brehm K. Characterization and inhibition of a p38-like mitogen-activated protein kinase (MAPK) from *Echinococcus multilocularis*: antiparasitic activities of p38 MAPK inhibitors. Biochemical Pharmacology 2008;76: 1068–81.
[20] Stadelmann B, Scholl S, Müller J, Hemphill A. Application of an *in vitro* drug screening assay based on the release of phosphoglucose isomerase to determine the structure–activity relationship of thiazolides against *Echinococcus multilocularis* metacestodes. The Journal of Antimicrobial Chemotherapy 2010;65:512–9.
[21] Elissondo M, Dopchiz M, Ceballos L, Alvarez L, Sánchez-Bruni S, Lanusse C, et al. *In vitro* effects of flubendazole on *Echinococcus granulosus* protoscoleces. Parasitology Research 2006;98:317–23.
[22] Bessey OA, Lowry OH, Brock MJ. A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. The Journal of Biological Chemistry 1946;164:321–9.
[23] Szasz G. A kinetic photometric method for serum gamma-glutamyl transpeptidase. Journal of Clinical Chemistry 1969;15:124–36.
[24] Ellman GL, Courtney KD, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 1961;7:88–95.
[25] Casado N, Pérez-Serrano J, Denegri G, Rodríguez-Caabeiro F. Development of chemotherapeutic model for the *in vitro* screening of drugs against *Echinococcus granulosus* cysts: the effects of an albendazole–albendazole sulphoxide combination. International Journal for Parasitology 1996;26:59–65.
[26] Lawton P, Sarciron ME, Petavy AF. *Echinococcus granulosus, E. multilocularis* and mammalian liver-type alkaline phosphatases: a comparative study. Comparative Biochemistry and Physiology Part B, Biochemistry & Molecular Biology 1995;112:295–301.
[27] Lobos E, Zahn R, Weiss N, Nutman TB. A major allergen of lymphatic filarial nematodes is a parasite homolog of the gamma-glutamyl transpeptidase. Molecular Medicine 1996;2:712–24.
[28] Mahanty S, Paredes A, Marzal M, Gonzalez E, Rodriguez S, Dorny P, et al. Sensitive *in vitro* system to assess morphological and biochemical effects of praziquantel and albendazole on *Taenia solium* cysts. Antimicrobial Agents and Chemotherapy 2011;55:211–7.
[29] Arnon R, Silman I, Tarrab-Hazdai R. Acetylcholinesterase of *Schistosoma mansoni* functional correlates. Protein Science 1999;8:2553–61.
[30] Bhardwaj R, Skelly PJ. Characterization of *Schistosome* tegumental alkaline phosphatase (SmAP). PLoS Neglected Tropical Diseases 2011;5:e1011 PMID: 21483710.
[31] Simon S, Krejci E, Massoulié J. A four-to-one association between peptide motifs: four C-terminal domains from cholinesterase assemble with one proline-rich attachment domain (PRAD) in the secretory pathway. The EMBO Journal 1998;17: 6178–87.
[32] Okada T, Suzuki H, Wada K, Kumagai H, Fukuyama K. Crystal structures of gamma-glutamyltranspeptidase from *Escherichia coli*, a key enzyme in glutathione metabolism, and its reaction intermediate. Proceedings of the National Academy of Sciences of the United States of America 2006;103:6471–6.