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P-glycoprotein expression and pharmacological modulation in larval stages of *Echinococcus granulosus*☆

María Celeste Nicolao [a,b], Guillermo M. Denegri [a,b], Juan Guillermo Cárcamo [c], Andrea C. Cumino [a,b,d,*]

[a] Laboratorio de Zoonosis Parasitarias, Departamento de Biología, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata (UNMdP), Funes 3350, Nivel Cero, 7600 Mar del Plata, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina
[c] Laboratorio de Bioquímica Farmacológica, Instituto de Bioquímica y Microbiología, Facultad de Ciencias, Universidad Austral de Chile, Campus Isla Teja, Valdivia, Chile
[d] Departamento de Química, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata (UNMdP), Funes 3350, Nivel 2, 7600 Mar del Plata, Argentina

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ABSTRACT

P-glycoprotein (Pgp) is an ATP-dependent transporter involved in the efflux of a wide variety of lipophilic substrates, such as toxins and xenobiotics, out of cells. Pgp expression level is associated with the ineffective therapeutic treatment of cancer cells and microbial pathogens which gives it high clinical importance. Research on these transporters in helminths is limited. This work describes for the first time the *Echinococcus granulosus* Pgp (Eg-Pgp) expression, in a model cestode parasite and an important human pathogen. Based on calcein efflux assays in the presence of common Pgp modulators, we demonstrated the occurrence of active Eg-Pgp in protoscoleces and metacestodes. Eg-Pgp, which showed a molecular mass of ~130 kDa in western blots, is localized in the suckers and the tegument of control protoscoleces as well as in the subtegument or all parenchymatous cells of protoscoleces treated with Pgp-interfering agents. We also identified five genes encoding Pgp which are constitutively expressed in protoscoleces and metacestodes. We showed that the Eg-*pgp*1 and Eg-*pgp*2 transcripts were up-regulated in response to *in vitro* drug treatment with amiodarone and loperamide, in agreement with the increased polypeptide levels. Finally, *in vitro* treatment of protoscoleces and metacestodes with trifluoperazine and loperamide was lethal to the parasites. This indicates that both drugs as well as cyclosporine A negatively modulate the *E. granulosus* Pgp efflux activity, favoring the retention of these drugs in the larval tissue. These events could be associated with the reduction in protoscolex and metacestode viability.

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1. Introduction

Cystic echinococcosis is a widely endemic zoonosis caused by larval stages of *Echinococcus granulosus*. In humans, metacestodes develop as large cysts, especially in the liver and lungs, causing organ dysfunction and disease [1]. Treatment of cystic echinococcosis principally consists in surgery and/or chemotherapy, being benzimidazoles (albendazole and mebendazole) the drugs currently used [2]. Novel and improved therapeutic tools are thus needed to optimize the treatment, including the study of cellular systems for excretion of drugs in this parasite.

Pgp, also named MDR1 (multidrug resistance 1), an evolutionarily well-conserved pump, is included in sub-family B of ATP binding cassette (ABC) transporters. Due to its flippase-like activity, Pgp translocates not only drugs but also different hydrophobic substrates across the plasma membrane, decreasing their intracellular concentration. Massive up-regulation of Pgp expression was identified as a multidrug resistance (MDR) mechanism, evidenced during cancer and infectious disease treatment in human patients [3]. The MDR phenomenon is ubiquitous in nature and has also been observed in prokaryotes, protozoa and helminths [4–6].

Pgps have been identified in few platyhelminths and nematodes, whose genomes have several Pgp-encoding genes and different protein isoforms involved in drug resistance, but not yet in cestodes [4,7–11]. Therefore, the co-administration of anthelmintics with MDR-reversing agents potentiates the pharmacological treatment [4].

Pgp consists of two homologous halves separated by a linker region. Each half contains a transmembrane domain (TMD) involved in drug efflux and a cytosolic nucleotide-binding domain (NBD) responsible for ATP binding and hydrolysis [12]. Its expression can be modulated *in vitro* by different agents such as verapamil, cyclosporine and trifluoperazine, which compete for drug binding and have been used as reference molecules in several investigations [13]. *In vivo*, these modulators act as MDR-reversing agents and appear to have similar effects on cancer cells and helminths [4]. In this work, we evidenced *E. granulosus* Pgp expression and its pharmacological modulation from protoscoleces

☆ Nucleotide sequence data reported in this paper are available in the GenBank database under GenBank Accession Number HM590698 (new ADM22304).

* Corresponding author at: Laboratorio de Zoonosis Parasitarias, Departamento de Biología, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata (UNMdP), Funes 3350, Nivel Cero, 7600 Mar del Plata, Argentina. Tel.: +54 223 4752426x450; fax: +54 223 4753150.
E-mail addresses: acumino@gmail.com, acumino@mdp.edu.ar (A.C. Cumino).

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and metacestodes. Furthermore, we showed that five putative Pgp genes were expressed from *E. granulosus* larval stages. Two of them were overexpressed during the pharmacological treatment with Pgp inhibitors.

2. Materials and methods

2.1. In vitro drug treatment of protoscoleces and metacestodes

E. granulosus protoscoleces were removed under aseptic conditions from hydatid cysts of infected cows presented for routine slaughter at the abattoir in the Buenos Aires province in Argentina. The area where the cattle came from is known to include only the G1 strain of *E. granulosus*. The samples were genotyped using the *cox*1 primers JB3 and JB4.5 according to the technique described by Bowles et al. [14]. Protoscolex culture ($n = 3000$), vitality assays and scanning electron microscopy (SEM) were performed as described by Cumino et al. [15]. The treatments of protoscoleces were initiated within 24 h of *in vitro* culture. Cyclosporin A (CsA), loperamide (Lp) and trifluoperazine (TFP) were assayed at 10, 50 and 100 μM and verapamil (Vp), amiodarone (Am), mebendazole (MBZ) and albendazole (ABZ) at 10, 50 and 500 μM. All these drugs are considered potential Pgp modulators. The incubation times were different for each experiment and they are indicated below. The same experimental design was performed using *E. granulosus* murine cysts (20–30 cyst with diameters between 3 and 8 mm for each drug treatment), which were obtained from the peritoneal cavities of CF-1 mice 6 months after intraperitoneal infection with protoscoleces (1500 protoscoleces/animal, dissolved in 0.5 ml of medium 199). Metacestode viability was assessed daily through an inverted light microscope on the basis of germinal membrane integrity during 6 days (until viability control was lower than 90%). Protoscoleces and metacestodes were washed with sterile and RNAse free PBS for molecular experiments and they were conserved at −80 °C until experimental use. Each experiment was assayed for three replicates and repeated three times.

2.2. Pgp functional assay by calcein accumulation

Pgp transport activity was measured using calcein-AM (Ca-AM, Molecular Probes, USA) assay as previously described by Leitch et al. [16] in the absence and presence of model inhibitors. Ca-AM is a non-fluorescent lipophilic ester that easily enters the cells, where it is cleaved by nonspecific esterases generating a highly fluorescent product calcein which is intracellularly retained. Ca-AM is a substrate of both Pgp and MRP1 (multidrug resistance proteins), while calcein is only substrate of MRP1. Therefore, Pgp extrudes Ca-AM from the plasma membrane, reducing accumulation of calcein in the cytosol. To measure the effect of modulator drugs on Pgp activity, the fresh samples of protoscoleces ($n = 1000$), small tissue pieces or intact metacestodes ($n = 10$) were incubated in PBS with or without drugs on 96 well plates for 4 h at 37 °C. Then, they were incubated with 10 μM Ca-AM for 45 min at 37 °C in the dark. The fluorescence in protoscoleces and cysts was measured in a microplate fluorometer (Thermo Scientific Fluoroskan Ascent FL) after 30 min of excitation (excitation: 480 nm, emission: 530 nm) and expressed as fluorescent units (FU). Each experiment was assayed for three replicates and repeated five times. The protoscoleces were also imaged with an inverted confocal laser scanning microscope (Nikon, Confocal Microscope C1). Pgp modulation was quantified according to Rautio et al. [17], using the following equation: % Pgp activity = 100 − [(FU compounds − FU control) / (FU TFP − FU control) × 100] where FU TFP, is fluorescence in the presence of TFP corresponding to the maximum inhibition and FU control represent the fluorescence in the absence of the drug. A Student's t-test was performed to determine the significance of the differences in % Pgp activity values between control and different compounds and to assign the categories of possible substrates or inhibitors [17].

2.3. Western blot analysis and immunohistochemistry

Polypeptides were separated by SDS-PAGE on 10% polyacrylamide gels and electroblotted onto a nitrocellulose membrane (HyBond C; Amersham) as described [15]. The membranes were incubated with primary monoclonal antibody directed against residues 1040–1280 of human MDR1 (Santa Cruz sc-55510, 1:500 dilution) or with primary monoclonal antibody of human actin (JLA-20, Developmental Studies Hybridoma Bank-DSHB, 1:2000 dilution) as a control for protein loading (both diluted in blocking solution).

Additionally, control and pharmacologically treated protoscoleces were fixed with Karnovsky's solution (0.05 M cacodylate pH 7.2, 2% paraformaldehyde and 2.5% glutaraldehyde) for 24 h and washed with buffer cacodylate 0.1 M. Then, they were dehydrated and finally embedded in Histoplast® Plus at 60 °C. The blocks were cut with a microtome (Leica, Mod. RM2125RT) and slices of 5 μm were obtained and mounted. The sections were deparaffinized in xylene and rehydrated through 100, 75, 50 and 25% ethanol, and PBS-Tween. After inactivation of endogenous phosphatase activity (through incubation at 65 °C in a humid chamber for 10 min), they were treated with blocking solution (10% FBS and TBS) for 45 min, and incubated overnight at 4 °C with the same primary anti-MDR1 (Santa Cruz sc-55510, 1:500 dilution). Sections were washed three times with 10 mM TBS, incubated in humid chamber for 1.5 h at 37 °C with goat anti-mouse IgG conjugated with alkaline phosphatase (Bio-Rad 170-6520, 1:500 dilution), then reveled with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium and observed under a light microscope. In addition, sections blocked and incubated with the primary antibody were incubated with goat anti-mouse IgG conjugated with FITC (Santa Cruz 91-SC-2010, 1:2000 dilution) and observed under a fluorescence microscope (Zeiss LSM510, Jena, Germany). Negative controls consisted of omission of primary antibody. Parallel, *in toto* immunohistochemistry was carried out as described by Fairweather et al. [18]. Control and pharmacologically treated protoscoleces were incubated for 3 days at 4 °C with the same primary antibody (1:100 dilution), and washed with PBS for 24 h at 4 °C. Finally, protoscoleces were incubated with goat anti-mouse IgG conjugated with FITC for 2 h at room temperature, washed and counterstained with 2 μg ml^{-1} propidium iodide (Molecular Probes P-3566, to observe all cell nuclei in optimal contrast conditions). They were observed with an inverted confocal laser scanning microscope (Nikon, Confocal Microscope C1).

2.4. Gene identification, reverse transcription (RT)-PCR and quantitative PCR

In order to obtain information on expressed *Echinococcus* Pgp sequences, the *Echinococcus multilocularis* genomic database, *E. granulosus* assembled genomic contigs (http://www.sanger.ac.uk/Projects/Echinococcus) and available sequences of EST (Expressed Sequence Tag) database of *E. granulosus* (http://www.nematodes.org/NeglectedGenomes/Lopho.php) were searched with blastp and tblastn, using the sequences of *Schistosoma mansoni* (AAA66477) and *Homo sapiens* (P08183) Pgps as queries. One EST sequence was obtained from *E. granulosus Lopho DB* (gb/CV679218 or, Eg_CWSLB_18F04) and was identified in *E. granulosus* genome (EgG_scaffold_0008). Also, we identified four putative Pgp genes (three in EgG_scaffold_0001 and one in EgG_scaffold 0018) which were selected based on *E. multilocularis* genome annotation. Specific primers for the five putative Eg-*pgp* genes listed in Supplementary Table 1 were designed.

Total RNA extractions, RT-PCR and qPCR analysis from *E. granulosus* protoscoleces were realized as previously described by Cumino et al. [19]. In the case of metacestodes, the hydatid fluid was first aspirated from the cysts and then TRIZOL reagent was injected and reaspirated several times to obtain parasite RNA free from host RNA (C. Fernández, personal communication). Extractions were performed from control and treated samples with 50 μM of Vp, Am and Lp during 24 h. cDNA

was generated from 10 μg of total RNA using Superscript II reverse transcriptase (Invitrogen) and Pfu (Fermentas) DNA polymerase. PCR products were analyzed by conventional electrophoresis, purified using a Qiaquick PCR Purification Kit (Qiagen No. 28104) and sequenced by external services (Unidad Genómica INTA — Castelar). The partial-length amplified cDNAs were obtained through direct sequencing of a PCR fragment for each gene. PCR reactions use the *Eg-pgp1-Fw* and *Eg-pgp1-Rv* amplifying a fragment of 457 and 591 nucleotides in larval stages of *E. granulosus*, encoding the same gene (Eg-*pgp*1), which corresponds to gb/CV679218 EST confirmed by sequencing. A pair of *E. granulosus* actin I (*actI* gene, GenBank accession number L07773) primers (*act*-Fw: 5′-GCGATGTATGTAGCTATCCAGGCAGTGCTCTCGCT-3′ and *act-Rv*: 5′-CAATCCAGACAGAGTATTTGCGTTCCGGAGGA-3′) was used to amplify a control fragment of 633 nucleotide. Equal amounts of cDNA from protoscoleces and metacestodes were amplified in PCRs of 30 cycles of 94 °C (30 seg), 50 °C (1 min), and 72 °C (1 min) plus a single step at 72 °C for 10 min. The relative abundance of transcripts from Eg-*pgp*1-5 gene was estimated by comparison of the ratios to actin. In addition, real-time reverse transcriptase-PCR (qPCR) experiments from protoscoleces were carried out in a Corbett Research cycler. The first reaction mixture (25 μl final volume) contained 50 pmol reverse specific primer (see below), 10 μg RNA, 0.5 mM dNTP mix, 1× first strand buffer, 5 mM DTT, 1 μl ribonuclease inhibitor, and 0.5 μl SuperScriptTM III reverse transcriptase (Invitrogen). The RT program was at 42 °C (60 min) and 70 °C (15 min). The second step mixture contained 3 μl cDNA, 12.5 μl of real mix (contain 0.1 μl Taq DNA polymerase, Taq DNA polymerase buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs and SYBR® Green™) and 50 pmol of each primer for each gene. The PCR program was at 95 °C (30 s), 30 cycles at 95 °C for 15 s, 49 °C for 20 s, and 72 °C for 30 s. Product identification was confirmed by a melting curve analysis and visualized on agarose gels. The relative rate of each cDNA was normalized using *actI* (see above). Data analysis for a relative quantification of gene expression was performed by the comparative Ct (threshold cycle) method.

2.5. Sequence analysis

Orthologs were selected based on reciprocal best BLAST hits and as condition, the presence of the four characteristic Pgp modules into the putative ortholog. A list containing all the *E. granulosus* contigs that were hit was analyzed both manually and by BLASTp (after joining the conceptual translation of exons) against the GenBank nr database. Sequence alignments were generated with the CLUSTALX software program. Modeling of Eg-Pgp tertiary structures were obtained from the deduced primary structure using the Gen-THREADER (SWISS-PROT) and Phyre programs. 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.

2.6. Statistics

Data within experiments were compared and significance was determined using the student's t test and $p<0.05$ was considered statistically significant. All data were shown as arithmetic mean ± SEM.

3. Results

3.1. Detection of P-glycoprotein activity in E. granulosus protoscoleces and metacestodes

Control cells showed minimal calcein fluorescence (typically 60 ± 5 FU and 125 ± 15 FU for protoscoleces and metacestodes respectively, Fig. 1A) in accordance with the presence of active Pgp. The diffusion of Ca-AM into germinal cells occurred in both cystic sections and intact metacestodes, indicating its incorporation across the laminar layer (data not shown). In the presence of 50 μM of CsA, Lp and TFP, a significant three-, six- and ten-fold increase was respectively observed in the relative fluorescent units over controls. Conversely, MBZ, Vp and Am slightly increased the fluorescence level (two fold over control) in protoscoleces and metacestodes (Fig. 1A–B and data not shown). ABZ showed the same fluorescence level as the control. Pgp activity was detected particularly in suckers and tegument as a consequence of its inhibition (Fig. 1B).

3.2. Expression of E. granulosus Pgp in protoscoleces after exposure to Pgp modulators

A polypeptide of ~130 kDa from protoscolex proteic extracts was immunodetected (Fig. 2A, in concordance with the theoretical molecular masses of *E. granulosus* predicted Pgps of approximately 1200 amino acids). Exposure of protoscoleces to 50 μM Vp or Lp for 24 h resulted in an increase in the relative levels of the Pgp-immunoreactive band (Fig. 2A). In addition, immunohistochemical labeling showed that anti-Pgp reactivity was localized in tegumental cells from control protoscoleces (Fig. 2Ba), in subtegumental regions from protoscoleces treated with Vp (Fig. 2B, b and e) and in the complete cellular territory (particularly in suckers and rostellum) from larvae treated with Lp (Fig. 2B, c and f). In the rostellar hooks, non-specific fluorescent signal was detected (Fig. 2B, a and d). These results were concordant with *in toto* immunolocalization of the transporter (Fig. 2B, g–i). No immunoreactivity was detected in samples lacking the primary antibody.

3.3. Occurrence and expression of Eg-pgp genes from E. granulosus larval stages

Extensive BLASTp searches on the available *E. multilocularis* genome and *E. granulosus* incomplete assembly genome revealed five and four genes coding for putative Pgps, respectively (Supplementary Fig. S1A). The predicted open reading frames for *E. granulosus* Pgps were included in the EgG_scaffold_0001 (Eg-Pgp2, positions 4,485,767–4,500,144; Eg-Pgp3, positions 14,668,516–14,678,025; and Eg-Pgp4, positions 14,689,163–14,696,498) and in the EgG_scaffold_0018 (Eg-Pgp5, positions 801,130–809,973). Using tBLASTn, *H. sapiens* (P08183) and *S. mansoni* (AAA66477) Pgps as queries and the single gb/CV679218 EST from *E. granulosus* metacestodes (457-bp encoding 152 residues corresponding to an NBD1 region plus a linker peptide of a putative Pgp), other gene coding for an additional *E. granulosus* Pgp named Eg-*pgp*1 was identified in EgG_scaffold_0008 (incomplete assembly). In addition, tBLASTx analysis from *E. multilocularis* genome allowed verifying the presence of the ortholog of Eg-*pgp*1 in the EMU_contig_62302 (in positions 702,552–701,962), coincidental with the predicted EmuJ_000160300 Pgp (Supplementary Fig. S1A–B).

RT-PCR analysis indicated expression of the five Eg-*pgp* genes in protoscoleces and metacestodes (Fig. 3). Designed primers for gb/CV679218 EST (*Eg-pgp1Fw* and *Eg-pgp1Rv*) amplified a 591-bp mRNA from protoscoleces and Am- and Lp-treated metacestodes (identified as ADM22304 in this work) and the expected 457-bp mRNA from control and Vp-treated metacestodes (Fig. 3A–C). Both transcript sequences were identical, except for an additional 134-bp fragment which was identified in the longer mRNA and might encode the amino acids corresponding to the linker peptide of a putative Eg-Pgp1-A isoform. Consequently, the two identified mRNAs could correspond to alternative splicing of the Eg-*pgp*1 gene, designated as Eg-*pgp*1 variant A (Eg-*pgp*1-a, corresponding to Eg-Pgp1-A) and Eg-*pgp*1 variant B (Eg-*pgp*1-b, corresponding Eg-Pgp1-B; Fig. 3A–C and Supplementary Fig. S1C). In protoscoleces, only Eg-*pgp*1-a was expressed in the control and all treatments. qPCR showed higher mRNA expression levels with Am (three-fold) and Lp (four-fold, Fig. 3A–B), and no changes with Vp regarding the control. Similar expression pattern for Eg-*pgp*2 was demonstrated from protoscoleces (Fig. 3A and B). Eg-*pgp*1-b and Eg-*pgp*2 showed no changes when compared with control and Vp-treated

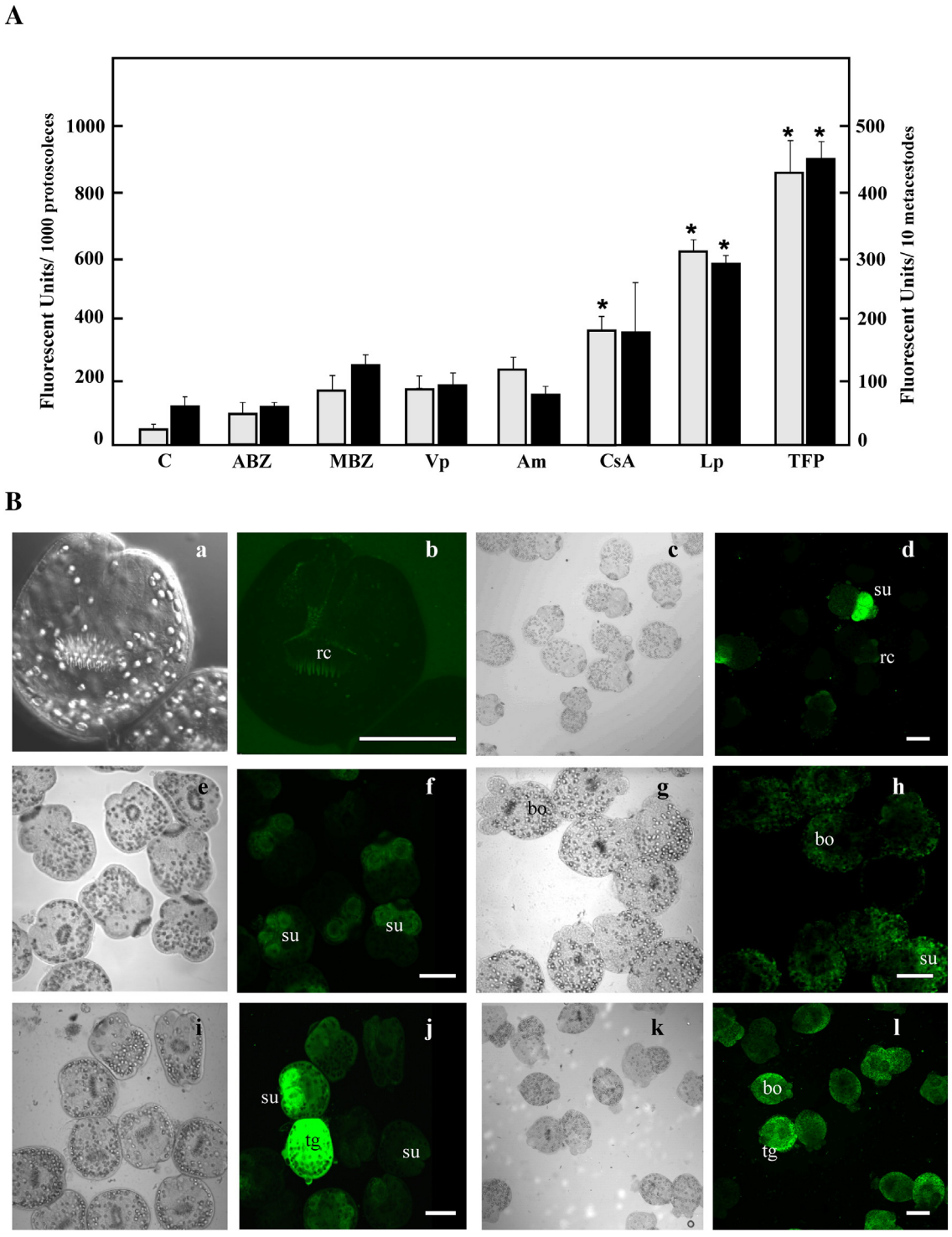

Fig. 1. Functional calcein-AM assay on Pgp transport activity in *E. granulosus* protoscoleces and metacestodes treated with different drugs. (A) Determination of relative fluorescence levels in protoscoleces (open bars) and dissected metacestodes (black bars) in the presence of 50 μM drugs, compared with the control (C). Protoscoleces treated with trifluoperazine (TFP), loperamide (Lp) and cyclosporin A (CsA) showed a significant increase in fluorescence ($p < 0.005$, asterisk) respect to control, suggesting them as possible Pgp inhibitors. Amiodarone (Am), verapamil (Vp) and mebendazole (MBZ) exhibited a weakly rise in fluorescence proposing them as potential Pgp substrates, according to Rautio et al. [17]. The values represent means ± standard deviations ($n = 5$). (B) Confocal imaging revealed calcein accumulation in structures adapted for absorption–excretion functions, as tegument and suckers. Photomicroscopy of light field (a, c, e, g, i and k) and of fluorescence field (b, d, f, h, j and l). Control (a–b); protoscoleces treated with MBZ (c–d), CsA (e–f), Am (g–h), Lp (i–j) and TFP (k–l). rc, rostellar cone; su, suckers; bo, body; tg, tegument. Bars indicate: 100 μm.

metacestodes. Metacestode treatment with 50 μM Am and Lp induced overexpression of Eg-*pgp*1-a and Eg-*pgp*2 (Fig. 3C), while Eg-*pgp*3, Eg-*pgp*4 and E-*pgp*5 showed no changes in the transcriptional expression level with drug-treatment in both larval stages (Fig. 3D and data not shown). Sequenced Eg-*pgp* regions from *E. granulosus* protoscoleces and metacestodes, contain all conserved domains required for ATP hydrolysis as characteristic motifs including the walker B motif, common to many nucleotide binding proteins, ABC-signature motif (MSGGQ, also called the C-loop), which implies an important role in catalysis, and stacking aromatic D and H loops, which are unique and more highly conserved in the MDR family (Supplementary Figs. S1C, and S2 [20]). A high degree of sequence conservation was observed between

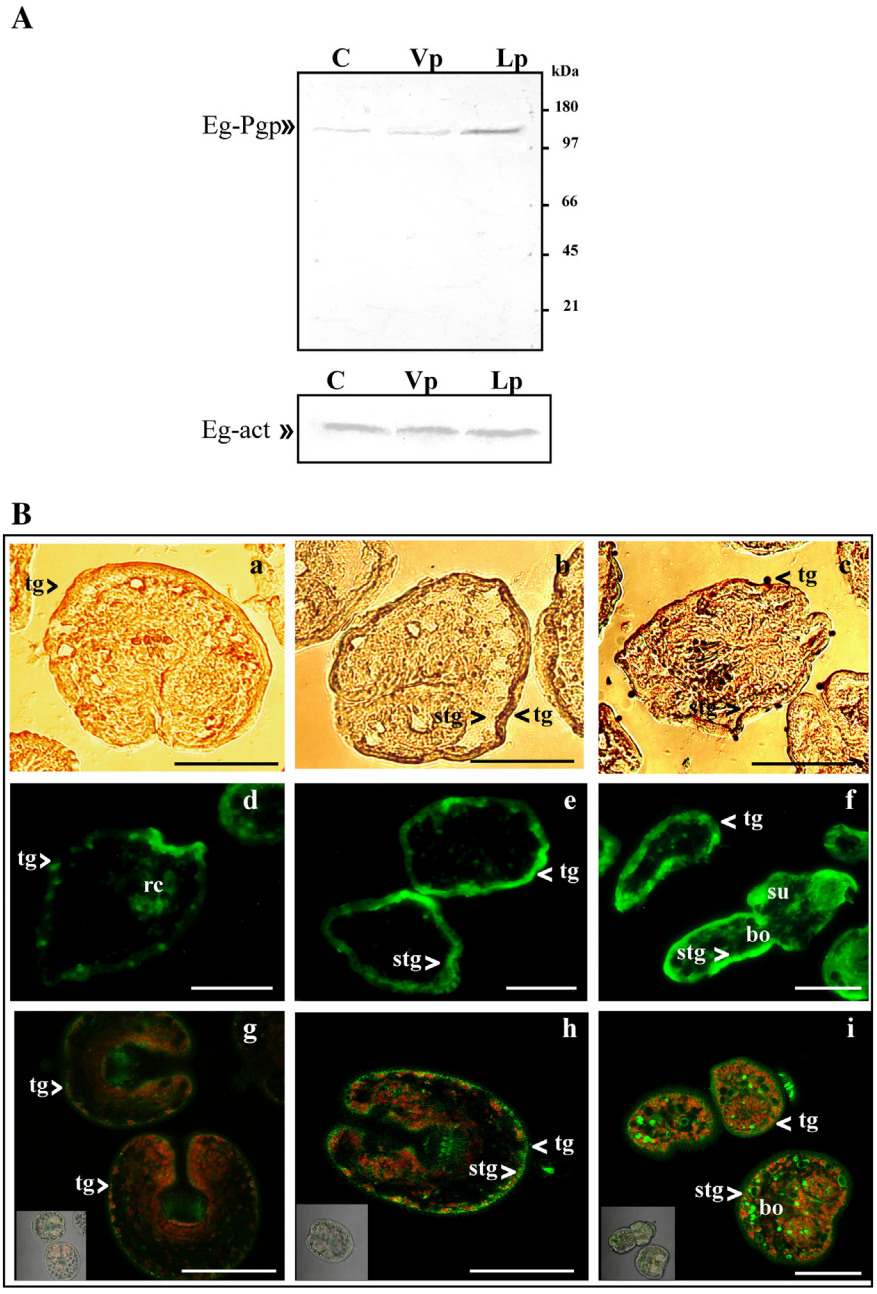

Fig. 2. Expression of Pgp and *in situ* immunolocalization in *E. granulosus* protoscoleces. (A) Immunodetection of Eg-Pgp polypeptide level revealed with a human antibody. Control protoscoleces (C, line 1) and treated with 50 μM verapamil (Vp, line 2) and loperamide (Lp, line 3), loaded with 80 μg total protein/lane. Arrows indicate position of polypeptides at 130 kDa (Pgp, top) and 42 kDa (actin, used as a loading control, bottom). (B) *In situ* localization of Pgp (s) detected by immunohistochemistry with secondary antibody conjugated with alkaline phosphatase (a–c) or conjugated with FITC (d–i) from histological sections (a–f) and *in toto* assays (g–i, propidium iodide was used to stain nuclei—red fluorescence; merge images on the left bottom). Control (a, d, g), Vp (b, e, h), and Lp (c, f, i). rc, rostellar cone; su, suckers; bo, body; tg, tegument; stg, subtegument. Bars indicate: 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Eg-Pgps and eukaryotic orthologs, annotated as pfam0005 in the Pfam database (http://pfam.sanger.ac.uk/search). The predicted Eg-Pgp sequences aligned with 39–41% and 40–42% identity with *H. sapiens* – P08183 – and *S. mansoni* – AAA66477 – orthologs, respectively (Supplementary Figs. S1B, and S2). Prediction of the topological structure of all deduced full-length Eg-Pgps, indicates that they contain 12 transmembrane alpha helices as part of their TMDs with the two NBDs, and conserve all classic motifs of prototype Pgps (Supplementary Fig. S2, and data not shown). The linker peptides, with non-conserved amino acids that separate the N- and C-terminal halves, allow conserving the secondary structure of the second predicted hemiprotein in each Eg-Pgps.

Finally, using BLASTp and the epitope of the assayed anti-MDR1 (amino acids 1040–1280 of protein P08183 included into NBD2) as query allowed revealing, with high identity score, that all predicted Eg-Pgps could be immunodetected in this work (Fig. 2 and Supplementary Fig. S2), in particular Eg-Pgp1 and Eg-Pgp-2 whose transcriptional induction with Lp-treatments were demonstrated.

3.4. Susceptibility of *E. granulosus* larval stages to common Pgp modulators

We investigated the potential *in vitro* anti-echinococcal activity of the Pgp modulators from protoscoleces and metacestodes maintained for 2 weeks in culture. CsA was checked in parallel as an anti-echinococcal positive control. Susceptibility of protoscoleces to Vp, Am, TFP and Lp was dose-dependent (Fig. 4A). These drugs had an effect on parasites at 10 μM 24 h after the treatment, reducing the vitality of

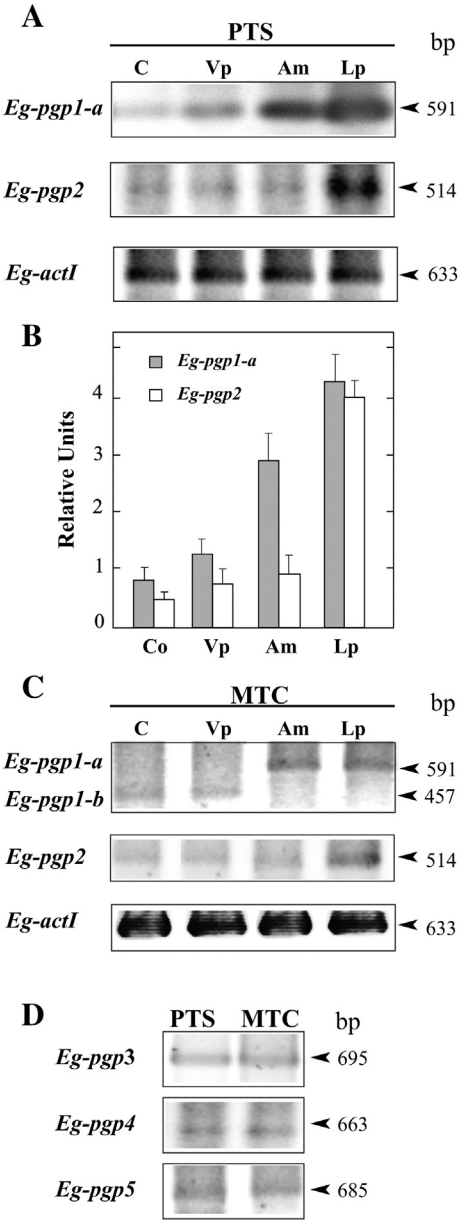

Fig. 3. Expression of the *E. granulosus pgp* genes in protoscoleces and metacestodes. (A, B and D) RT-PCR analysis from total RNA of protoscoleces (PTS) and metacestodes (MTC), in control (C) and treated with 50 μM verapamil (Vp), amiodarone (Am) and loperamide (Lp) for 24 h. Amplification of Eg-*actI* was used as a loading control. Molecular sizes of amplicons are indicated on the right and gene identity on the left. (B) Quantitative PCR (qPCR) from RNA of protoscoleces under identical conditions as in (A). Values are means ± SD of three independent experiments.

protoscoleces treated with 50 μM of Lp, TFP, Vp and Am to 31 ± 2.1%, 43 ± 2.3%, 80 ± 4.0% and 92 ± 5.1%, respectively, compared with 99 ± 0.5% survival in the control group. At 50 μM after 48 h in culture, 100% of protoscoleces died following treatment with Lp and TFP (data not shown). At the same concentration and 24 h post-treatment, *E. granulosus* larvae ultrastructure showed significant differences compared with control samples (Fig. 4B and data not shown). Treated protoscoleces presented their tegumental regions completely altered, showing destruction of microtriches, loss of rostellar hooks, and full soma and scolex retrenchment. Also, as shown in Fig. 4C, metacestode treatment with 25 μM of TFP and Lp resulted in the killing of 75% and 100% cysts after 5 days, respectively, producing a dose-dependent injury (data not shown). In addition, metacestodes treated with 50 μM of TFP and Lp showed collapsed germinal layers (evidenced as a white precipitate inside the cyst) after 2 days (Fig. 4D).

4. Discussion

In order to improve the antiparasitic control and the chemotherapeutic guidelines, understanding of pharmacokinetic interactions between anthelmintic drugs and ABC efflux transporters is required. In this context, Pgps have been identified in nematodes and trematodes as important factors associated with anthelmintic susceptibility and resistance [21]. In this study, we demonstrated *E. granulosus* Pgp activity for the first time in Cestoda, and analyzed the expression of five candidate genes (Eg-*pgp*1-5) in protoscoleces and metacestodes. Additionally, considering that most antiparasitic agents are also Pgp modulators [13,22], we showed that the assayed Pgp inhibitors have a potent *in vitro* anti-echinococcal activity. These results enable us to suggest a possible clinical interest of these drugs.

The occurrence of Eg-Pgp was inferred due to the minimal accumulation of calcein in protoscolex cells and cystic germinal cells (Fig. 1A). We also demonstrated that CsA, TFP and Lp negatively modulated the total Eg-Pgp activity, suggesting that they behaved as Pgp true inhibitors, whereas ABZ at concentrations of up to 100 μM showed no effect, in agreement with other cell types [17].

Previous studies have demonstrated the correlation between intracellular calcein signal and the Pgp expression level [23]. The exposure of protoscoleces and metacestodes to 50 μM of Pgp modulators, particularly Am and Lp, resulted in an increased expression of a ~130-kDa polypeptide which was revealed by a human monoclonal antibody against the NBD2 conserved between human and helminthic Pgps (Fig. 2A). Based in the deduced protein sequences of Eg-Pgps (with theoretical molecular weights between ~130 and 136 kDa), their predicted molecular masses coincide with the experimentally detected weight. Similar results were shown in *S. mansoni* adults treated with praziquantel, where the increased Pgp expression was evidenced as an overexpressed single polypeptide of ~140-kDa [24]. As expected, Eg-Pgp localization was detected in the larval structures adapted for the absorption or excretion (protoscolex tegument and subtegument) and in cyst germinal layer, as described in other tissues with the same specialized cell types (Fig. 2B). Probably, the Eg-Pgp overexpression may help to clear the drug from cells in the presence of Pgp-inhibitors as a cellular response to compensate the total drug efflux. On the other hand, overexpression of Pgps has been linked to drug resistance in cancerous tumors, malaria and infections caused by yeasts, HIV and helminths [6]. In *Echinococcus* sp., Pgp expression should be taken into account because it may modify the susceptibility and the bioavailability of an anti-echinococcal drug.

We also identified five genes encoding Pgps from *E. granulosus*, constitutively expressed in protoscoleces and metacestodes (Fig. 3). While humans have two genes encoding Pgps; *Caenorhabditis elegans*, *Haemonchus contortus* and *Teladorsagia circumcincta* may have at least 15, 12 and 11 Pgp genes, respectively. Thereby complete functional studies must be performed in helminths [7,8]. It is generally assumed that the transcriptional activation, the transcript stability and the translational initial control are involved as regulatory mechanisms of the Pgp gene expression [20]. Based on these assumptions, we showed that the Eg-*pgp*1 and Eg-*pgp*2 transcripts from protoscoleces and metacestodes, homologs of S*mdr*2, were expressed at significantly higher levels in parasites treated with Pgp modulators, accounting for the inhibition and up-regulation of their expression (Figs. 2 and 3A–C). These results let us to suggest a possible transcriptional regulation of both Eg-*pgp* genes, as described in different cell types [25]. Besides, we demonstrated that Eg-*pgp*1-a was transcriptionally induced and expressed in Vp-, Am- and Lp-treated protoscoleces and also in Am- and Lp-treated metacestodes, while Eg-*pgp*1-b was expressed in control and Vp-treated metacestodes (Fig. 3A–C). The occurrence of *mdr*1 mRNA alternative splicing, which has already been described [26], could allow the expression of isoforms with different substrate specificities or transporting properties.

Consensus sequences of the two NBDs with the characteristic Walker A (GXXXGKT/S) and B (XLXXDEAT) motifs and Q, D and H-loops and

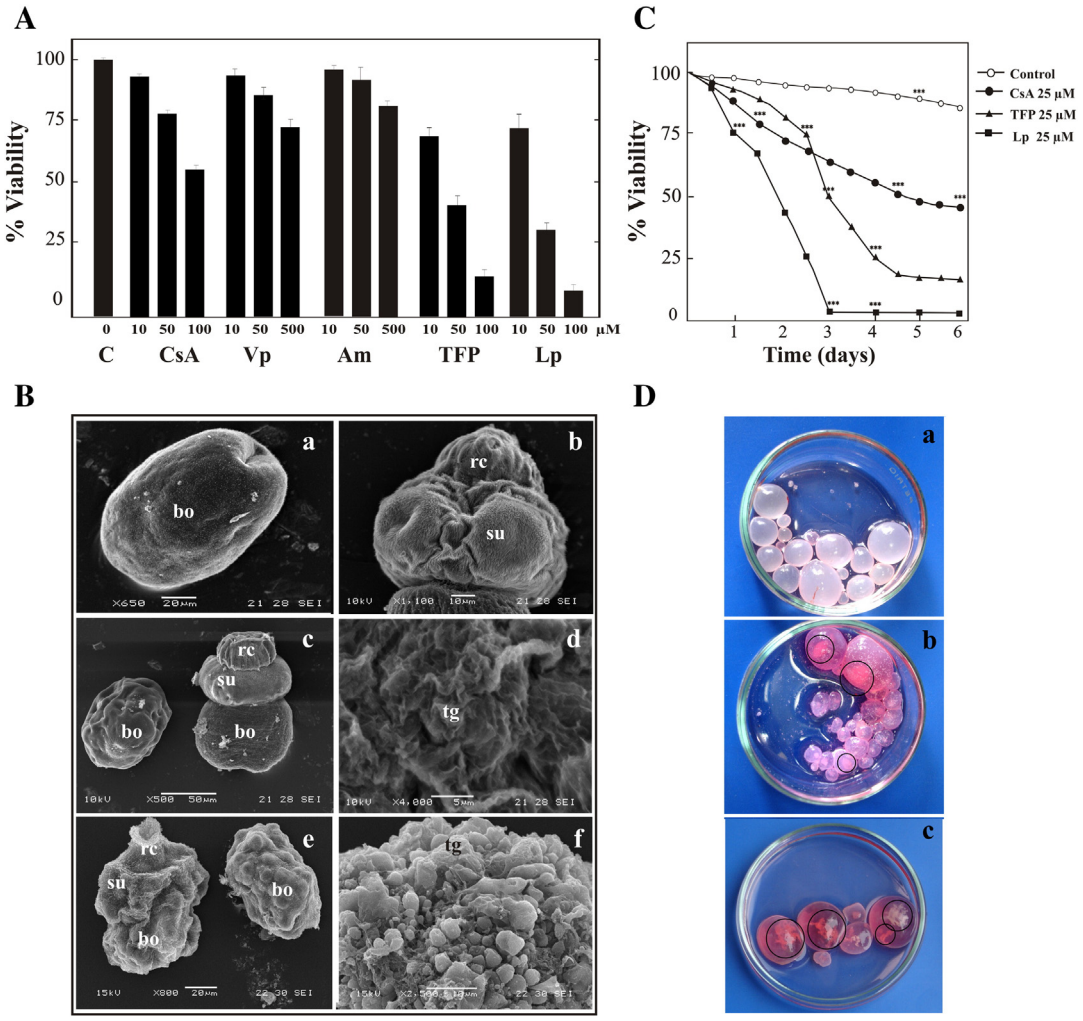

Fig. 4. Effect of Pgp modulator drugs in *E. granulosus* larval stages during *in vitro* treatments. (A) Protoscolicidal activity of cyclosporin A (CsA), verapamil (Vp), amiodarone (Am), trifluoperazine (TFP) and loperamide (Lp) from *in vitro* protoscolex cultures maintained for 24 h. These drugs were assayed at 10–50–100 μM for CsA, TFP and Lp and 10–50–500 μM for Vp and Am. Bars show means ± standard error of five independent experiments. (B) Ultrastructural changes of protoscoleces detected by SEM after pharmacological treatment for 24 h. Control (a, invaginated protoscolex; b, evaginated protoscolex), 50 μM of Lp (c–d) and TFP (e–f). Treated protoscoleces presented contraction of soma region, alteration of tegument, and scolex region showing loss of hooks and destruction of microtriches. Bars indicate: 5 μm in (d), 10 μm in (b) and (f), 20 μm in (a) and (e) and 50 μm in (c). rc, rostellar cone; su, suckers; bo, body; tg, tegument. (C) Viability of metacestode (measured on the basis of vesicle integrity) incubated with 25 μM of CsA, TFP and Lp for up to 6 days. ***Significant ($p < 0.05$). (D) Macroscopical damage of metacestodes from *in vitro* pharmacological assay at 48 h post-treatment. Control (a, without morphological changes), 50 μM of Lp (b) and TFP (c). Both treatments showed increased permeability (culture medium inside cysts) and collapsed germinal membrane (circles).

linker regions appeared to be highly conserved and they were used to confirm the identification of Eg-Pgps. TMD2 is the region where mammal and helminthic Pgps, including that of *Echinococcus* sp., have low sequence homology (Supplementary Fig. S2). Therefore, these amino acid differences could modify the binding properties of the drug recognition pocket and thus the effects of this binding on Pgp activity [13]. Interestingly, serine residues in the predicted amino acid sequences were identified as possible phosphorylation target sites into the linker region (between NBD1 and TMD2, indicated in Fig. S1C) of both mRNAs (variants a and b) of the Eg-*pgp*1 gene.

Finally, we demonstrated the dose-dependent susceptibility of *E. granulosus* larval stages to Vp, Am, TFP and Lp (Fig. 4A and C) and observed ultrastructural changes in the protoscolex tegument and in the cystic germinative membrane (Fig. 4B and D). These data allow us to report an important *in vitro* anti-echinococcal effect using calcium-Pgp modulators. We found increased mortality rates with Lp and TFP, both of which are not only Pgp inhibitors, but are also considered calcium channel blockers and calmodulin inhibitors in different cell types [27]. The mortality rates shown by these drugs were significantly higher than those produced by other anti-echinococcal compounds such as benzimidazoles, macrolides, avermectin and praziquantel (Fig. 4, [2]). On the other hand, we inferred the presence of L-type voltage-gated Ca^{2+} channels (VOCC) and store-operated calcium channels (SOCC) due to the pharmacological effects of Vp and Am and Lp and TFP respectively, in *E. granulosus* larval stages. Interestingly, several authors have focused on whether antagonizing the Pgp function and controlling the intracellular calcium-regulatory pathways are related [28,29]. Besides, calcium signal abnormalities have been observed in cells overexpressing Pgp [30]. Thus, these FDA authorized drugs may be proposed as alternative therapeutic agents in the treatment for hydatidosis due to their potential as dual blockers of the calcium signals plus the Pgp transport. The *in vivo* co-administration of anthelmintic and Pgp modulator agents has been shown to enhance both the drug systemic availability and the antiparasitic efficacy in different animal species [31]. So, the modulation of both the activity and the expression of Eg-Pgp transporters with Lp allows us to propose this drug as a potential Pgp modulator molecule with the additional advantage that it also shows anthelmintic effects. However, *in vitro* and *in vivo* additional experiments are needed before drawing conclusions on the application field of our results. The *in vitro* approach of identification and characterization of all Eg-Pgps constitutes one part of the global advance to define the role of molecular actors in the modulation of bioavailability of

anti-echinococcal drugs and will help to optimize the efficacy in human treatments.

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