Preventive and clinical efficacies of metformin against experimental cyst echinococcosis

Julia A Loos, Lic.[1,2]; Andrea C Cumino, PhD[1,2]
[1]National University of Mar del Plata, [2]National Council of Scientific and Technical Research

ABSTRACT

Metformin (Met) is an anti-hyperglycemic and potential anti-cancer agent which may exert its anti-proliferative effects via the induction of energetic stress. In this study we investigated the *in vitro* and *in vivo* efficacy of Met against *Echinococcus granulosus* larval stage. Metformin showed significant dose- and time-dependent killing effects on cultured protoscoleces and metacestodes. Notably, the combination of Met and the minimum effective concentration of ABZSO had a synergistic effect from day 4 and 12 in metacestodes and protoscoleces, respectively. Oral administration of Met (50 mg/kg/day) in *E. granulosus*-infected mice showed to be highly effective in achieving reduction of parasite weight, whereas its combination with the lowest recommended dose of ABZ (5 mg/kg/day) was even more effective. Coincidentally, intracystic Met accumulation was higher in animals treated with both drugs compared with those receiving Met alone. The drug incorporation into cyst was attributed to overexpression of *E. granulosus* organic cation transporters on metacestode cells. Furthermore, this safe plant-derived drug exhibited considerable chemopreventive properties in infected mice compared with untreated animals. In conclusion, based on our experimental data, Met emerges as a promising anti-echinococcal drug because it can efficiently inhibit the *E. granulosus* larval stage development and its combination with ABZ may improve the anti-parasitic therapy.

CONTACT

Dr. Andrea Cumino
National University of Mar del Plata
Email: acumino@mdp.edu.ar
Phone: -
Website: -

INTRODUCTION

Cystic echinococcosis (CE) is a worldwide zoonosis of public health and economic significance caused by infection with the larval stage of the cestode parasite *Echinococcus granulosus* [1]. Currently, chemotherapy used in patients (albendazole, ABZ) has not been fully effective for this disease [2], being a dire need to find new drugs for its treatment.

Metformin is an antihyperglycemic and a potential anticancer agent which may exert its anti-proliferative effects, both indirectly through the systemic reduction of insulin levels and directly, via the induction of energetic stress, involving the inhibition of ATP production, the activation of AMP activated protein kinase (AMPK) and the inhibition of the target of rapamycin complex (TORC1) patients [3-5]. The drug shows good oral bioavailability (50-60%), is stable and not metabolized [6] and its pharmacokinetics is regulated by transporters of the major facilitator superfamily (MFS) [4, 7].

The aim of this study is to test the *in vitro* effects of Met alone or in combination with low concentration of albendazole sulphoxide (ABZSO) on *E. granulosus* larval stage (metacestode and protoscolex), and to report on the *in vivo* effects of Met employing the murine CE infection model.

METHODS AND MATERIALS

In vitro culture of protoscoleces and metacestodes

Viable protoscoleces (N = 3000) and metacestodes (N= 10-20) were cultured *in vitro* in 199 medium with different concentrations of Met and ABZSO (the main active metabolite of ABZ) and mortality was calculated daily by the methylene blue exclusion assay.

Preventive and clinical efficacy of Met on mice infected with E. granulosus

CF-1 mice were inoculated intraperitoneally with viable protoscoleces and allocated into different experimental groups (10 animals/group). In the *chemoprophylactic efficacy study*, the treatment (Met 50 mg kg-1 day-1) was initiated at the time of infection, whereas in the *clinical efficacy study*, the treatments (Met 50 mg kg-1 day-1; ABZ 5 mg kg-1 day-1; Met at 50 mg kg-1 day-1 plus ABZ at 50 mg kg-1 day-1) were initiated at 4 months post-infection, when cysts have already been developed. All treatments were performed by intragastric administration every 24 h for 60 consecutive days. Four or six months after infection, mice were euthanized, and necropsy was carried out immediately thereafter, respectively. The hydatid cysts collected from the peritoneal cavity of the animals were weighed and analyzed by scanning and transmission electron microscopy.

Determination of Metformin

Metformin concentration was estimated in cysts obtained from Met- or Met plus ABZ-treated mice by two spectrophotometric methods [8, 9]

Expression and sequences analysis of Echinococcus SLC-like transporters

After *in silico* identification of solute carrier transporter family 22 (SLC22) homologs in *E. granulosus* genome, specific primers were designed and used for RT-PCR and qPCR analysis from total RNA of protoscoleces and metacestodes incubated under different experimental conditions. Amplification of Eg-*actin* I or Eg-*etif4A* III was used as an internal control.

RESULTS

Metformin exhibits in vitro anti-echinococcocal effect and it improves the efficacy of low concentrations of albendazole sulphoxide

Administration of Met to cultured metacestodes and protoscoleces showed significant dose- and time-dependent killing effects. In addition, a synergistic effect between Met and ABZSO was observed from day 4 and 12 in metacestodes and protoscoleces, respectively (Fig.1).

Metformin shows therapeutic effectiveness against fully-developed cyst

The weight of cysts was significantly decreased upon all treatments (ABZ, Met, ABZ+Met) compared with the control (C) (***$p < 0.01$), but the decrease was more prominent in the group receiving the combined treatment than that with either drug alone (**$p < 0.05$). In turn, weight reduction was greater with Met than with ABZ (*$p < 0.2$) (Fig.2A). Moreover, the cysts developed in the treated mice showed severe damage to their ultrastructure (Fig.2B).

Metformin efficiently prevents hydatid cyst growth

All the infected mice from the untreated group (10/10) developed metacestodes in the abdominal cavity, whereas in 2 out of the 10 Met-treated mice the infection did not progress. Significant differences (*$p < 0.01$) were registered in the weights of the cysts obtained from untreated mice in comparison with those recovered from Met-medicated mice (Fig.3A). The ultrastructural analysis of metacestodes from Met-treated mice showed alterations in the germinal layer surface detected by SEM and TEM (Fig.3B).

Metformin and albendazole sulphoxide increase expression of the putative organic cation transporters in E. granulosus

The five encoding genes for *Echinococcus* transporters were expressed in protoscoleces and metacestodes. The transcriptional expression of Eg-*oct* genes was higher in metacestodes treated with Met, ABZSO and Met plus ABZSO than in control (Fig.4).

1

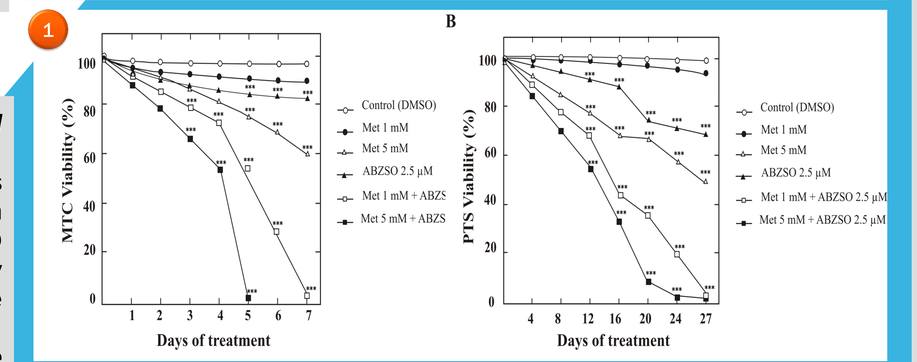

Fig 1. *In vitro* effect of metformin (Met) and its combination with low concentration of albendazole sulphoxide (ABZSO) on viability of metacestodes (MTC) and protoscoleces (PTS) of *E. granulosus*.

2

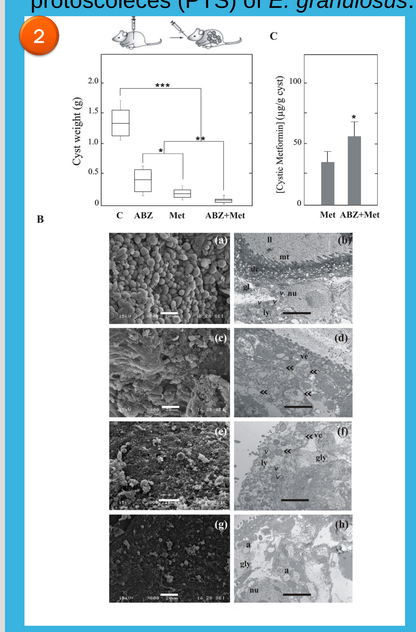

Fig 2. Therapeutic efficacy study in *E. granulosus* infected mice.

3

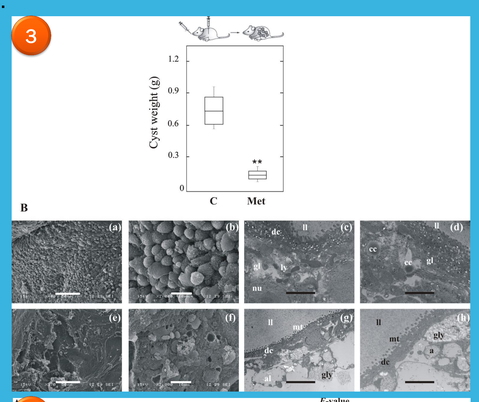

Fig 3. Chemoprophylactic activity of metformin during *E. granulosus* cyst development.

4

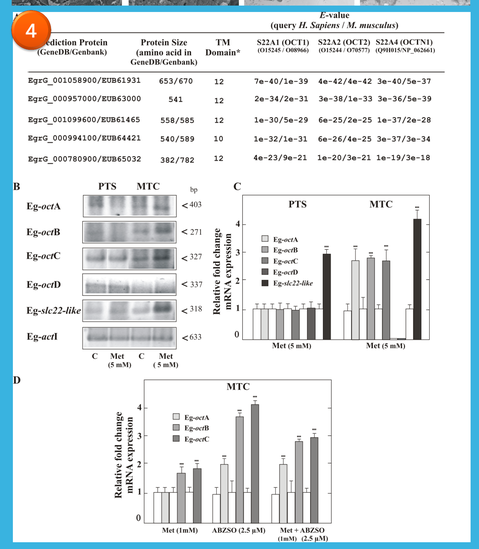

Fig 4. Identification and expression analysis of the genes encoding solute carrier family 22 (SLC22) orthologs in *E. granulosus*.

DISCUSSION

In the present study, we observed for the first time that Met either by itself or in association with ABZ has potential therapeutic effect and confers protection against the *E. granulosus* infection in an *in vivo* experimental model.

Regarding our *clinical efficacy study*, the different cellular targets between ABZ [10, 11] and Met [12] would lead to the pronounced therapeutic effects observed after combined treatment with the two agents (Fig. 2). In addition, Met and ABZSO have shown to induce the transcriptional expression of Eg-*oct* genes (Fig. 4), which could explain the higher concentration of Met detected in cysts from mice treated with both drugs (Fig. 2C), justifying the observed synergistic effect.

On the other hand, in our chemoprophylactic efficacy study Met not only reduced the weight of the cysts but also affected the proliferation of germinal layer cells (Fig. 3). The mechanistic rationale for an anti-proliferative effect of Met is convincing, by activating LKB1/AMPK pathway and subsequently inhibiting TORC1 pathway, which is crucial for the control of protein synthesis and cellular proliferation [13, 14]. We have previously identified TORC1 in the parasite [15], suggesting that the effects of Met against *E. granulosus* could be, at least partly, mediated by the AMPK-TORC1 pathway. Additionally, activating AMPK, Met may oppose to the Warburg effect [16], a strategy of the *Echinococcus* germinal cells to cope with the high demand of both energy and intermediate metabolites under limited oxygen supply. This metabolic switch from oxidative metabolism to glycolysis could confer the germinal cells susceptibility to Met, as it has been described for tumor cells [17]. Besides, it has been shown that low doses of Met selectively kill cancer stem cells in different types of breast cancer [18]. Therefore, the drug could specifically affect germinal cells of the cyst and, since BZMs have limited efficacy against undifferentiated germinal cells of this parasite, the combination of Met and ABZ may kill both stem cells and differentiated cells in the experimental echinococcosis model.

CONCLUSIONS

In this report we provide evidence into the potential benefits of Met as a new treatment option for CE, and these observations provide the impetus to evaluate the role of Met in the regulation of proliferation in other helminths. These findings enhance the importance of carrying out further studies to determine the significance of the use of the Met in relation to hydatidosis in humans.

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