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***In vitro* effects of isoprinosine and a dipeptide methyl ester on *Echinococcus multilocularis* protoscoleces**

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Abstract

A protoscoleces/vesicles *in vitro* maintenance test with assessment of viability by eosin exclusion was used to evaluate the quantitative and qualitative activities of isoprinosine, its active component inosine and the dipeptide methylester L-Phe-Phe-OMe on isolated protoscoleces of *Echinococcus multilocularis* for 24 and 48 h. Isoprinosine and inosine showed dose- and time-dependent activity, the latter displaying a more rapid effect than the former. A high activity was shown with L-Phe-Phe-OMe, when compared to praziquantel. Ultrastructural alterations were much more striking with L-Phe-Phe-OMe, with an effect similar to that of praziquantel, whereas the chemotherapeutic activity of inosine and isoprinosine appeared to be directed against a metabolic target, with a lethal effect not immediately visible at the ultrastructural level. Thus, the previously reported *in vivo* activities of these drugs result largely from a direct effect on the parasite.

Introduction

Alveolar echinococcosis, a disease caused by the larval stage of the cestode *Echinococcus multilocularis*, is still a major health problem due to the moderate efficacy of existing drugs. Long term chemotherapy usually involves benzimidazole anthelmintics such as mebendazole and albendazole, which are rather parasitostatic (Eckert & Amman, 1995). In the search for more active therapies, we previously reported the *in vivo* efficacy of a combination of mebendazole and the immunostimulant isoprinosine, or isoprinosine alone against the metacystodes of *E. multilocularis* (Sarciron *et al.*, 1991) as well as *E. granulosus* (Sarciron *et al.*, 1993) and of the dipeptide methylester L-Phe-Phe-OMe (Walchshofer *et al.*, 1993). Although treatment with these drugs had a marked effect on the tegument of the cestode and triggered a decrease in the number of protoscoleces and in alkaline phosphatase activity, it was difficult to assess whether the loss of protoscoleces was due to direct action of the chemicals or as a result of damage caused to the germinal and

laminated layers. Furthermore, an immunological effect might be considered in the case of isoprinosine. Thus, using *in vitro* techniques, it was necessary to determine if these drugs acted directly on the parasite and if inosine was an active component of isoprinosine.

Materials and methods

Parasites

Three-month-old metacystodes of *E. multilocularis* (Savoie strain) were obtained from jirds (*Meriones unguiculatus*) infected by the intraperitoneal route with 50 mg cyst tissue (Delabre-Defayolle *et al.*, 1989). The parasite material was rinsed aseptically in cold (4°C) saline and minced through a stainless steel sieve fitted with a nylon gauze. The protoscoleces and small vesicles were washed once in the same solution and resuspended in prewarmed (37°C) Hanks solution (Sigma, L'Isle d'Abeau, France) to approximately 1000 protoscoleces per ml.

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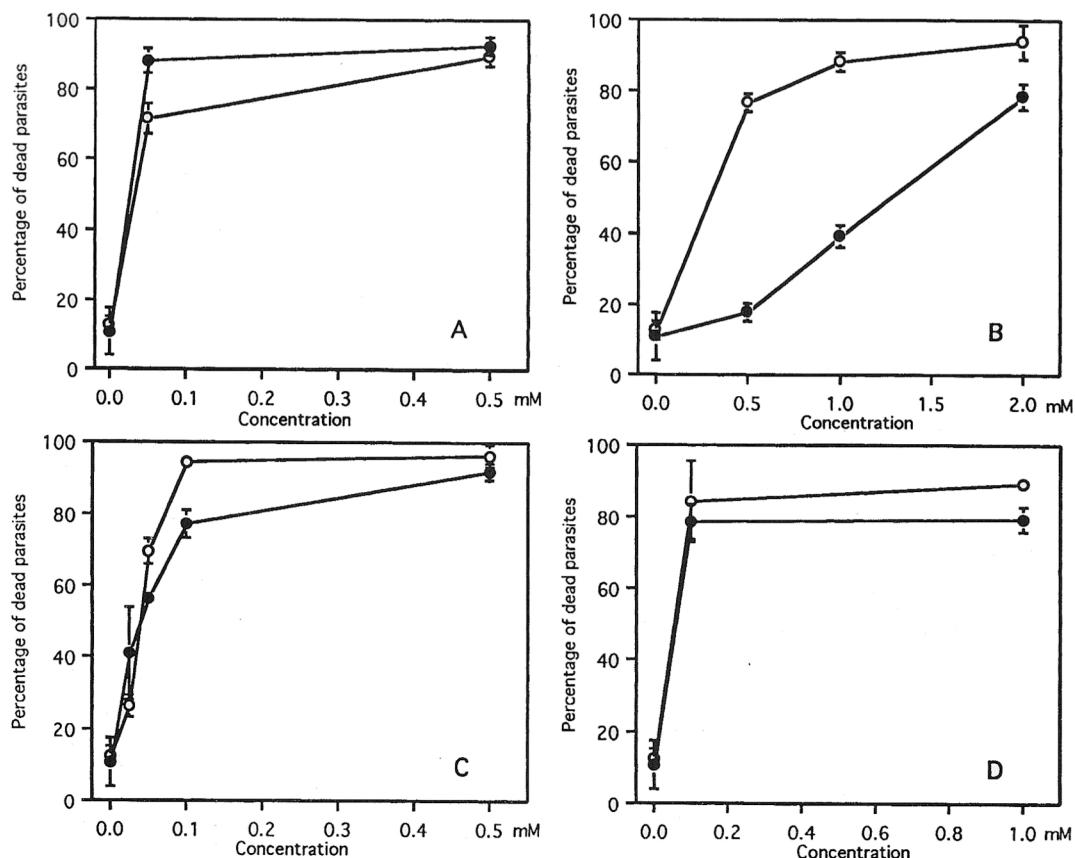


Fig. 1. Quantitative effects of the drugs on isolated *Echinococcus multilocularis* protoscoleces maintained *in vitro* and incubated for 24 h (●) and 48 h (○). A, inosine; B, isoprinosine; C, L-Phe-Phe-OMe; D, ivermectin. Results from three different experiments.

Drugs

The following drugs were used and dissolved in DMSO at a stock concentration of 200 mM: (i) isoprinosine (Synthelabo, Meudon, France): 69.5 mg ml⁻¹; (ii) the adjuvant DIP-PAcBA (dimepranol-acedoben): 69.4 mg ml⁻¹; (iii) inosine (Sigma, L'Isle d'Abeau, France): 53.6 mg ml⁻¹. Stock solutions of 100 mM ivermectin (Sigma) and 100 mg ml⁻¹ (320 mM) praziquantel (Sigma), were prepared as positive controls. The dipeptide L-Phe-Phe-OMe was synthesized as previously described (Walchshofer *et al.*, 1993) and also dissolved in DMSO at 200 mM (72.5 mg ml⁻¹).

In vitro tests

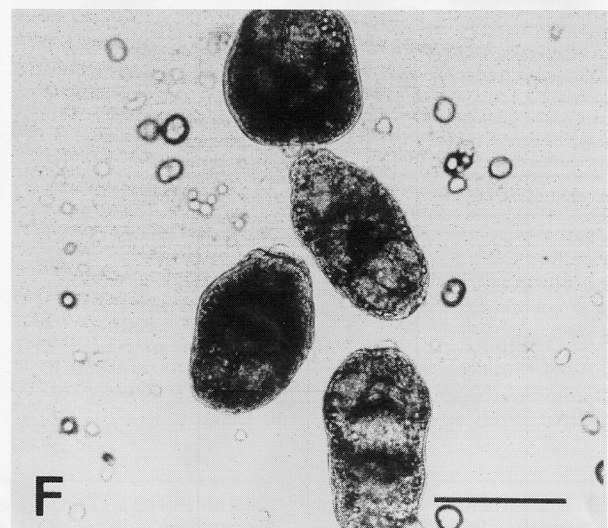
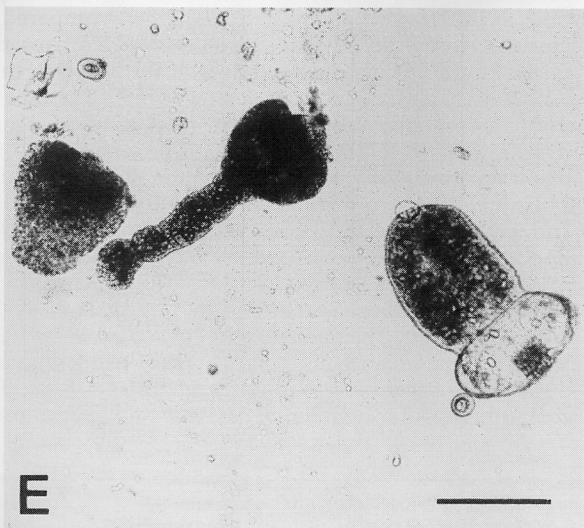
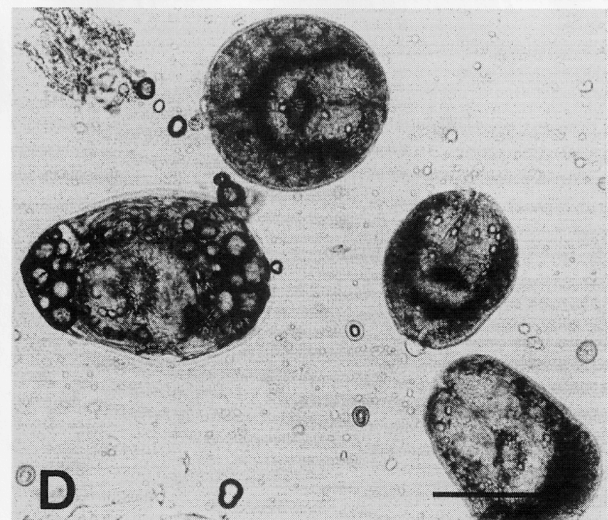
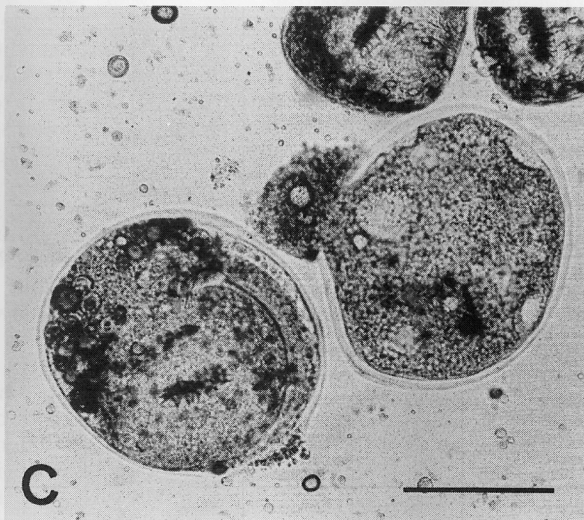
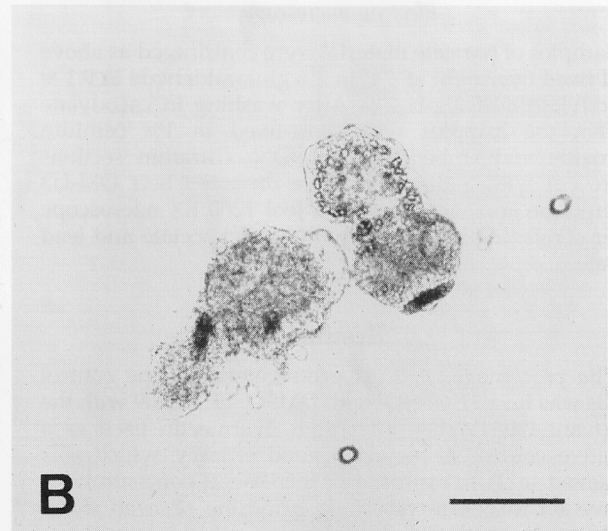
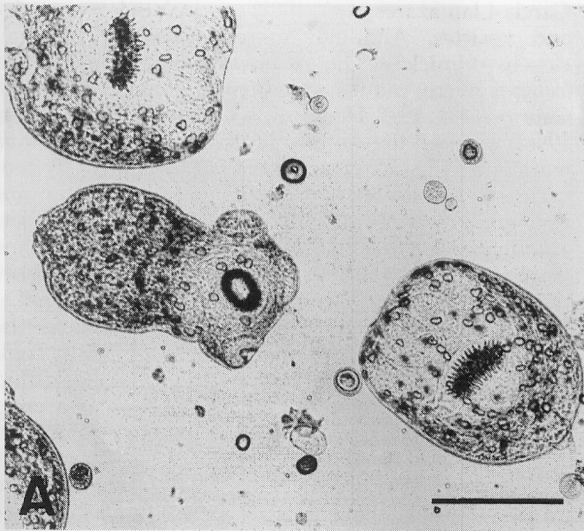
DMSO (final concentration 2% v/v) was used as negative control. The final concentrations of control chemicals were chosen to give clear-cut results. The

drugs were serially diluted at twice the required concentrations in prewarmed Hanks solution and aliquoted in triplicates (250 µl each) in 24-well culture plates (Nunc, Denmark). The protoscoleces-vesicles suspension (approx. 1000 ml⁻¹; 250 µl) was then added and the plates were incubated at 37°C in a 5% CO₂/air incubator.

Light microscopy

The percentage of dead parasites was determined using the eosin exclusion test. After 24 and 48 h, 100 µl aliquots were dispatched into microtubes, centrifuged in a Beckman Avanti 30 at 10 000 rpm for 30 s, then the pellets were mixed with 10 µl of 0.1% (w/v) eosin in Hanks solution and examined in phase contrast under a Leitz Dialux 20 microscope. The parasites were counted and the percentage of dead protoscoleces recorded.

Fig. 2. Light microscopy of isolated *Echinococcus multilocularis* protoscoleces incubated with the drugs for 24 h. A, control; B, praziquantel 1.6 mM; C, isoprinosine 2 mM – overall appearance of the protoscoleces is conserved, some are swollen, others shrink; D, isoprinosine 1 mM – protoscoleces absorb the dye and shrink; E, inosine 0.05 mM; F, L-Phe-Phe-OMe 0.1 mM – internalization of the dye and a clumping of protoscoleces are visible. Bar: 100 µm.



Electron microscopy

Samples of parasite material were centrifuged as above and fixed overnight at 4°C in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.6. After washing in cacodylate buffer, the samples were post-fixed in 1% osmium tetroxide and embedded in EPON. Ultrathin sections were cut with a diamond knife on a Reichert OM-U3 microtome and viewed with a Jeol 1200 EX microscope after conventional staining with uranyl acetate and lead citrate.

Results

The percentage of dead protoscoleces in the control wells was low: 12.6 ± 2.7 with DMSO, 11.2 ± 2.9 with the adjuvant DIP-PACBA, after 48 h. Ivermectin used as a positive control displayed a good efficacy, which was achieved in 24 h, but did not increase at concentrations above 0.1 mM. Conversely, isoprinosine showed dose- and time-dependent activity. Inosine, which appeared as the active component, displayed a comparable effect at a concentration 4 times lower (0.05 mM). The dose-dependent parasitocidal effect of L-Phe-Phe-OMe was achieved more rapidly than isoprinosine, and at lower doses. At the chosen concentration (500 µg ml⁻¹; 1.6 mM) praziquantel displayed an effect comparable to 0.025 mM L-Phe-Phe-OMe: the percentage of dead parasites were 21.6 ± 3.8 at 24 h and 37.7 ± 6.2 at 48 h, respectively (fig. 1).

Light microscopy showed evidence of shrinking of the protoscoleces which took up eosin, in the presence of inosine and isoprinosine (fig. 2). In the presence of 0.5 mM L-Phe-Phe-OMe, the majority of parasites were dead after 6 h (not shown).

Electron microscope investigations showed that isoprinosine-treated protoscoleces were not altered except for a reduction in the length of the microtriches. Damage was much more evident with L-Phe-Phe-OMe, with vacuolization, disorganization of structures beneath the syncytium together with a loss of muscular cells and a thinner syncytium. These effects are similar to those caused by praziquantel, the latter also triggering a decrease in the number and length of the microtriches (fig. 3).

Discussion

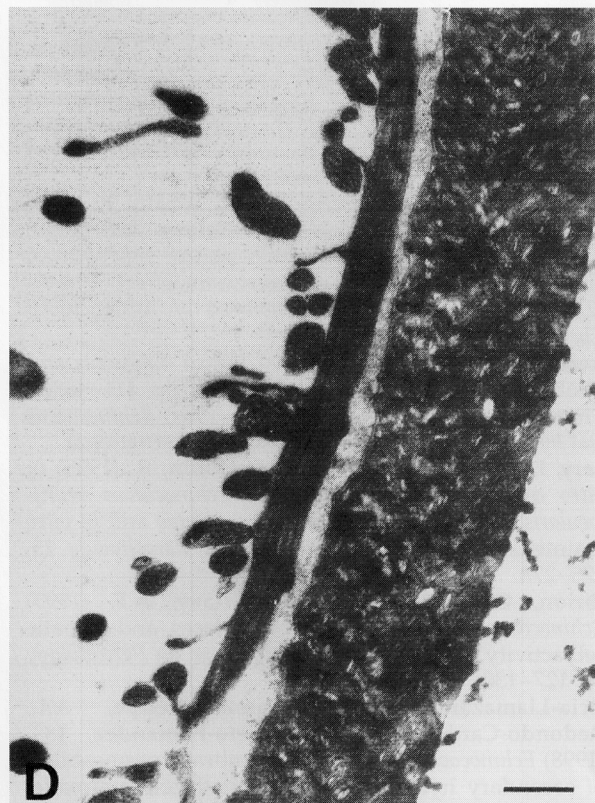
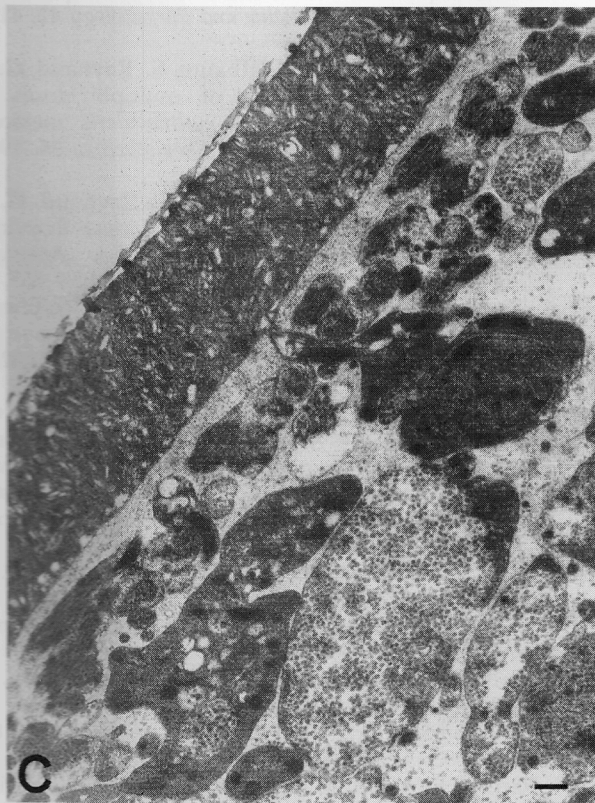
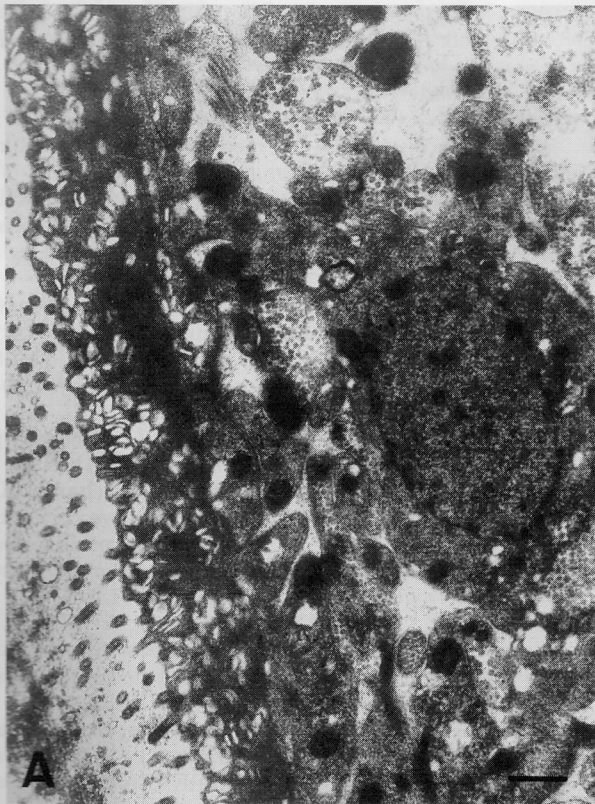
Assessing and monitoring drug activity in the case of alveolar echinococcosis is difficult because the parasites live in their secondary host within a protected environment where the laminated layer plays an important role. Hence, screening of potential drugs is impaired by the lack of symmetry between *in vivo* and *in vitro* procedures. The most widespread systems, most of them adapted to *E. granulosus* (Richards *et al.*, 1988; Casado & Rodriguez-Caabeiro, 1989; Taylor *et al.*, 1989; Casado *et al.*, 1996;

Garcia-Llamazares *et al.*, 1998) use isolated protoscoleces and vesicles. Although more specialized techniques closely mimicking the *in vivo* behaviour of *E. multilocularis* were published recently (Emery *et al.*, 1995; Gabrion *et al.*, 1995; Hemphill & Gottstein, 1995; Jura *et al.*, 1996), we used the simpler protoscoleces/vesicle *in vitro* maintenance with assessment of viability by eosin exclusion (Lorenzini & Ruggieri, 1990). However, many previous *in vivo* tests had been undertaken and positive efficacy of the tested substances had already been shown (Sarciron *et al.*, 1991, 1992, 1993, 1995, 1997a). We wished to find out if a direct activity could be demonstrated and what parasite structures might be the targets of these chemicals. Ivermectin was selected as a positive control for its known efficacy on accessible parasites (Martin *et al.*, 1997). Praziquantel was also chosen as a control because of its anticestodal activity *in vivo* (Marchiondo *et al.*, 1994) and *in vitro* (Richards *et al.*, 1988), and for its chemical structure. This is why albendazole and albendazole sulphoxide, the most widespread molecules, were not used as positive controls.

The high efficacy of the dipeptide L-Phe-Phe-OMe *in vitro* is consistent with our previous results *in vivo* where it induced a reduction of the laminated layer (Walchshofer *et al.*, 1993) and a potentialization of albendazole therapy (Sarciron *et al.*, 1997a). In aqueous solutions at neutral pH and 37°C, L-Phe-Phe-OMe undergoes a rapid cyclization to 2,6-dibenzyl-2,5-dioxopiperazine (Walchshofer *et al.*, 1997), which is structurally related to praziquantel. This could explain its powerful efficacy against protoscoleces. In our tests, L-Phe-Phe-OMe displayed at 0.025 mM, an efficacy comparable to 1.6 mM praziquantel. The latter molecule acted slowly *in vitro*, a result consistent with those of Morris *et al.* (1987) who used much lower doses, but for 28 days. In contrast, L-Phe-Phe-OMe had a visible effect after 6 h.

The adjuvant DIP-PACBA, present in the commercial drug isoprinosine, was ineffective, providing evidence that the active drug is actually inosine. Isoprinosine showed a good dose-dependent efficacy against the protoscoleces of *E. multilocularis*, but needed a longer time to be efficient at lower doses, when compared to inosine, ivermectin or to L-Phe-Phe-OMe. Perhaps this might be due to the pharmacological form of this drug which could induce not only a slower rate of uptake by the parasites, but also some kind of protection against transformation in the medium. Conversely, inosine displayed a slightly lower effect after 48 h than after 24 h, which was not statistically significant ($P > 0.05$), and might be due to the absence of the stabilizer DIP-PACBA. The damage caused to the parasites by isoprinosine was essentially restricted to the occurrence of shorter microtriches, as reported by Casado *et al.* (1994) with ivermectin. The vacuolization observed *in vivo* and with *E. granulosus* (Sarciron *et al.*, 1995) using a longer duration of treatment was not seen in our tests. The chemotherapeutic activity of inosine and isoprinosine

Fig. 3. Ultrastructural effects of the drugs on isolated *Echinococcus multilocularis* protoscoleces after 48 h incubation. A, control; B, praziquantel – almost all the internal structures have disappeared, and microtriches are poorly visible; C, isoprinosine 2 mM – overall structure is conserved, but the length of the microtriches is reduced; D, L-Phe-Phe-OMe mM – effects are similar to praziquantel with a deep internal disorganization. Bar: 500 nm.



could thus be directed against a metabolic target, with a lethal effect not immediately visible at the ultrastructural level. As for many parasites, the salvage pathway could be essential to *E. multilocularis* for its purine synthesis. We recently showed that treatment of infected jirds with isoprinosine induced an increase in the activity of the enzyme adenosine deaminase (Sarciron *et al.*, 1992) and that this enzyme as well as the activity of adenine-hypoxanthine-guanine-phosphoribosyltransferase (AHGPRT) were at a high level in the metacestodes (Suchail *et al.*, 1998). Interestingly, the active purines do not seem to be the same as those active against protozoans, since the inosine analogue 2',3'-dideoxyinosine effective *in vitro* and *in vivo* against *Toxoplasma gondii* (Sarciron *et al.*, 1997b, 1998) was ineffective in preliminary experiments. This metabolic pathway thus seems a good candidate in the search for efficient drugs against alveolar and cystic echinococcosis. This study provides evidence that the previously observed *in vivo* effects largely originate from direct interactions of the tested molecules with *Echinococcus*.

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