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Biological effects of a dipeptide methyl ester on *Echinococcus multilocularis* metacestodes in vivo

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Summary

The biological effects of Phe-Phe-OMe (**1**) were evaluated in gerbils against the causative agent of alveolar echinococcosis, *Echinococcus multilocularis*. Morphological damage to the parasite was examined by transmission electron microscopy. Administration of (**1**) led to a considerable alteration of the laminated layer. Although a histoenzymatic study showed a decrease in alkaline phosphatase activity in the parasite, a precise mode of action for (**1**) cannot be proposed.

Alveolar echinococcosis in man is caused by the larval stage of the cestode *Echinococcus multilocularis*. This damaging cestodiasis, with primarily liver injury, seems to occur only in the northern hemisphere: Alaska, Canada (Rausch, 1986), China (Graig et al., 1991), centre and east of France, Switzerland, Austria and Germany (Gottstein, 1992). Surgery is the only curative treatment but only a few cases are resectable (Bresson-Hadni et al., 1991; Todorov et al., 1992). Inoperable echinococcosis is a chronic, slowly progressive disease leading to death within 10 years in about 93% of untreated patients (Schantz et al., 1982; Amman et al., 1988). The most active compounds, benzimidazoles such as mebendazole and albendazole, have not been proved to be definitively effective in man (Davis et al., 1986). Long-term treatment and high oral doses are required to reduce the growth of the *E. multilocularis* metacestode, although the parasite is not eliminated (Amman et al., 1990).

We are engaged in a program towards the determination of biochemical targets for drugs active against *E. multilocularis*. Among enzymes which represent a potential target for chemotherapy of alveolar echinococcosis, alkaline phosphatases have been interesting to study, since they are localized at the surface of the metacestode and related to the penetration of nutri-

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ments, especially carbohydrates, the main energy reserve of helminths (Fujins et al., 1983).

We previously reported an important enzymatic activity of alkaline phosphatase in *E. multilocularis* metacestodes with biochemical characteristics different from those of the gerbil liver enzyme (Sarciron et al., 1991).

In our quest to discover novel or more active compounds (Walchshofer et al., 1990, 1991; Audin et al., 1992) for echinococcosis therapy, we investigated the effect of Phe-Phe-OMe (**1**) against *E. multilocularis* larvae as a better absorbed derivative of L-phenylalanine (Bodor et al., 1977), a well-known inhibitor of alkaline phosphatases (Onica et al., 1990).

Morphological damage to the parasite was studied after drug treatment by transmission electron microscopy and a histoenzymatic study was carried out in order to detect a possible effect of (**1**) on alkaline phosphatase activity of *E. multilocularis* metacestodes.

All our studies were performed with a daily dose of 50 mg/kg body weight during a short-term treatment in order to note the first histological and ultrastructural damage.

Coupling procedure: To a stirred suspension of L-phenylalanine methyl ester hydrochloride (2.5 g, 11.59 mmol) in 100 ml of dichloromethane was added an equivalent of triethylamine (1.17 g). The mixture was kept for 15 min at room temperature, then Boc-L-phenylalanine (3.08 g, 11.59 mmol), dicyclohexylcarbodiimide DCC (2.4 g, 11.6 mmol) and 1-hydroxybenzotriazole (HOBt; 1.57 g, 11.6 mmol) were added. After 8 h at room temperature, dicyclohexylurea (DCU) was filtered. The filtrate was washed consecutively with 10% HCl and brine, dried over Na_2SO_4 and concentrated. After a second filtration of DCU, the solvent was removed under reduced pressure; yield, 95%.

Deprotection procedure: The residue obtained above was treated with dry HCl in methanol for 24 h at room temperature under nitrogen. After removal of the solvent, the solid obtained was washed several times with ether and dried under vacuum over P_2O_5; yield, 80%. HPLC (Vydac C18 column 25 mm × 4.6 mm, detector 432 Kontron): (solvent A) 0.1% aqueous TFA; (solvent B) 60% aqueous CH_3CN/0.1% TFA, gradient from 20 to 80% B in 30 min; detection, 214 nm; flow rate, 1 ml/min; single peak, $k = 17.39$ min. m.p. (Kofler): 192°C. FT-IR (KBr, Perkin-Elmer 1600): ν 3362, 3208, 1733, 1671 cm^{-1}; ^{1}H-NMR (d_6-Me_2SO_4/Me_4Si, Brücker BZH 200/52, 250 MHz): δ (ppm) 3.05 (d, 4H, $J = 3$ Hz, CH_2-Ar; 3.6 (s, 3H, OCH_3); 4.1 (m, 1H, $\underline{CH}NH_2$); 4.5 (q, 1H, $J = 3$ Hz, $\underline{CH}COOCH_3$); 7.25 (m, 10H, ArH); 8.4 (s, 2H, NH_2); 9.4 (s, 1H, NH).

Analysis – Calc. for $C_{19}H_{22}N_2O_3 \cdot HCl$: C, 62.88; H, 6.34; N 7.72; Found: C, 63.22; H, 6.49; N, 7.84 (performed by analytical department of CNRS, Solaize, France).

$[\alpha]_D^{21} = +9°$ ($c = 2$, C_2H_5OH) (electronic digital Roussel-Jouan micropolarimeter).

3-month old male and female gerbils were used. The animals were reared in our laboratory.

Two groups of 10 animals weighing about 50–55 g were infected via the intraperitoneal route with 50 mg of *E. multilocularis* metacestode from a 3-month infected gerbil (Delabre-Defayolle et al., 1989).

The drug treatment was started on 10 animals 3 months after infestation. This first group of animals was gavaged daily with 50 mg/kg body weight of (**1**) suspended in 1% aqueous arabic gum. Each animal received the solution under a volume of 0.2 ml. The second group represented the controls (infected untreated animals). The weight of all animals was recorded daily and the autopsy was carried out on day 19 after the beginning of the treatment.

The metacestodes were collected from the peritoneal cavities and carefully separated from host tissue; random samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer at pH 7.3 for 24 h at 4°C.

The samples were washed for about 1 h in phosphate buffer, then post-fixed in 2% (w/v) osmium tetroxide buffered in 0.1 M sodium phosphate for 1 h at 4°C. After three washes, the samples were dehydrated by graded ethanol and embedded in epon (Epox 812). The sections were cut on a Reichert OMU_3 ultramicrotome, placed on 300 mesh copper grids, stained with uranyl acetate and lead citrate and examined with a Jeol 100 CX electron microscope.

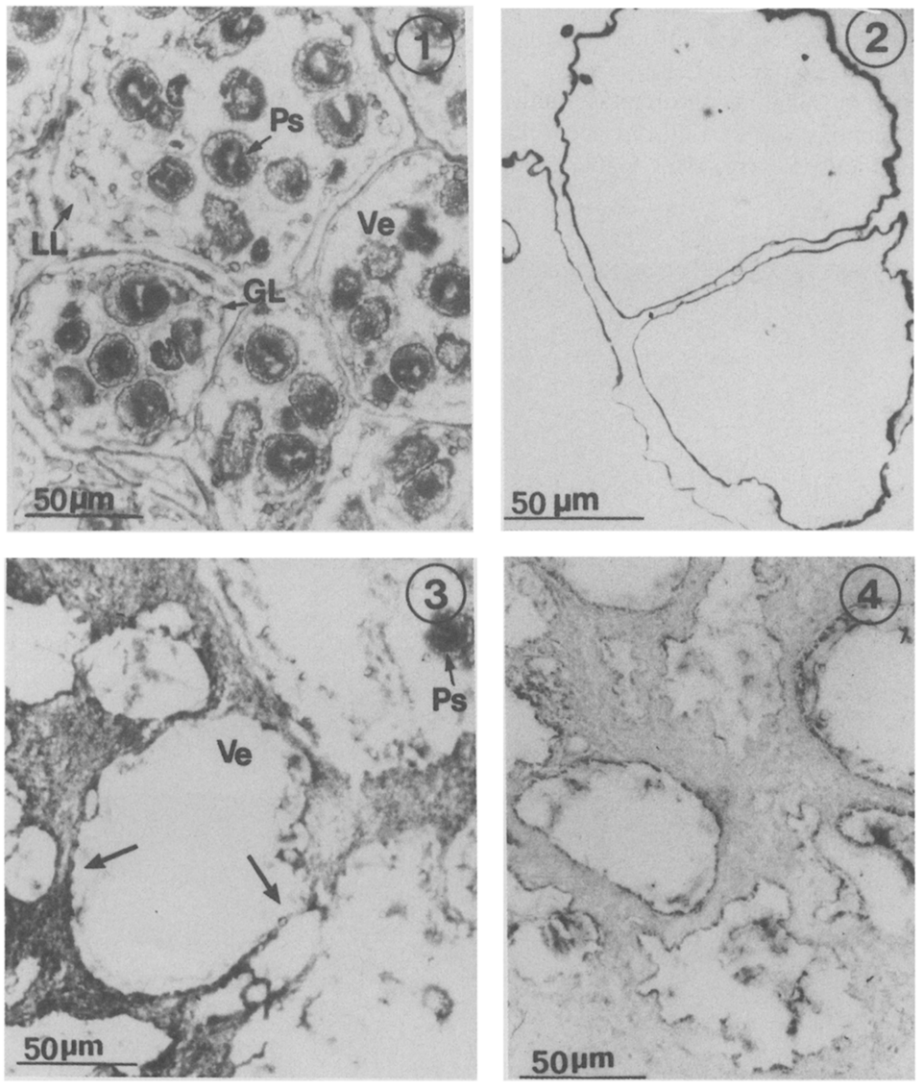


Fig. 1. Photomicrograph of the in vivo effect of compound (1) on *Echinococcus multilocularis* metacestode compared to untreated control. Histological study and phosphatase alkaline detection. Section of a control metacestode at 3 months post-infection showing a multivesicular aspect. Vesicles (Ve) contain numerous protoscoleces (Ps). The laminated layer (LL) and germinal layer (GL) are undamaged.

Fig. 2. Photomicrograph of the in vivo effect of compound (1) on *Echinococcus multilocularis* metacestode compared to untreated control. Histological study and phosphatase alkaline detection. An intense alkaline phosphatase activity is localized on the germinal layer of a control metacestode.

Fig. 3. Photomicrograph of the in vivo effect of compound (1) on *Echinococcus multilocularis* metacestode compared to untreated control. Histological study and phosphatase alkaline detection. Section of a treated metacestode at 3 months post-infection; protoscoleces are less numerous (Ps). Note the partially altered tegument (→) of vesicles (Ve).

Fig. 4. Photomicrograph of the in vivo effect of compound (1) on *Echinococcus multilocularis* metacestode compared to untreated control. Histological study and phosphatase alkaline detection. Compared to control, the alkaline phosphatase activity is weaker on the germinal layer of a treated metacestode.

Random samples of metacestodes were cryopreserved in liquid nitrogen and further sectioned in 8 mm slices with a Cryostat Leitz.

The detection of alkaline phosphatase activities on tissue sections was carried out according to literature methods (Pearse, 1960; Gabe, 1968). Briefly, incubation of sections was performed for 20 min at room temperature with the liquid medium containing α-naphthyl phosphate disodium salt (Sigma N-7255), sodium borate, magnesium sulfate and fast Blue BB (Sigma F-3378) as substrate.

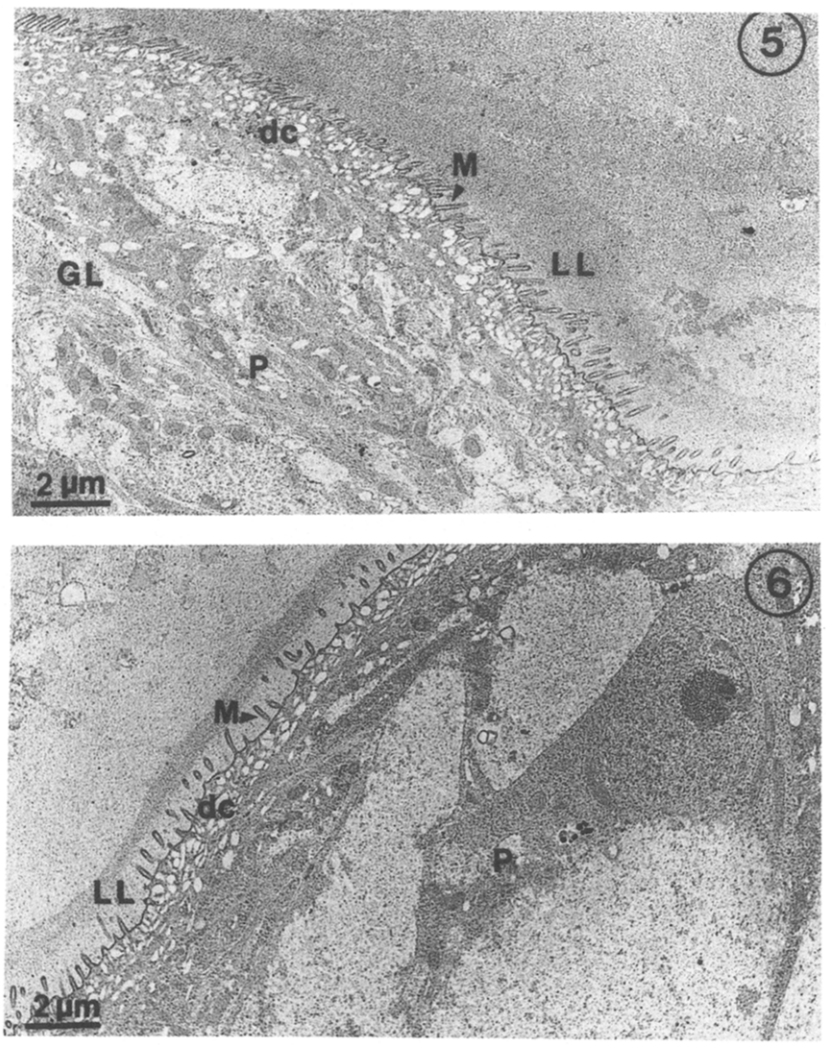


Fig. 5. Ultrastructural in vivo effect of compound (1) on *Echinococcus multilocularis* metacestode at 3 months post-infection compared to untreated control: Control metacestode. Note the thick, electron-dense laminated layer (LL) covering the distal cytoplasm (dc) of the germinal layer (GL) with numerous microvillies (M). Numerous cellular elements are visible in parenchyma (P).

Fig. 6. Ultrastructural in vivo effect of compound (1) on *Echinococcus multilocularis* metacestode at 3 months post-infection compared to untreated control: Treated metacestode with a thin, electron-lucent laminated layer (LL); short, less numerous microvilli (M) line a hypervesiculated distal cytoplasm (dc). Cellular elements are rare in parenchyma (P).

A deep purple to black coloration indicated the presence of alkaline phosphatase activity, which was observed with an optical photomicroscope (Wild Leitz MP52). Control sections were stained with hemalun-eosin.

L-Phenylalanyl-L-phenylalanine methyl ester hydrochloride (**1**) was prepared according to the method outlined in Scheme 1. *N-t*-Butyloxycarbonyl-L-phenylalanine was coupled to L-phenylalanine methyl ester using dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt). The deprotection step was performed using dry HCl in methanol to afford (**1**).

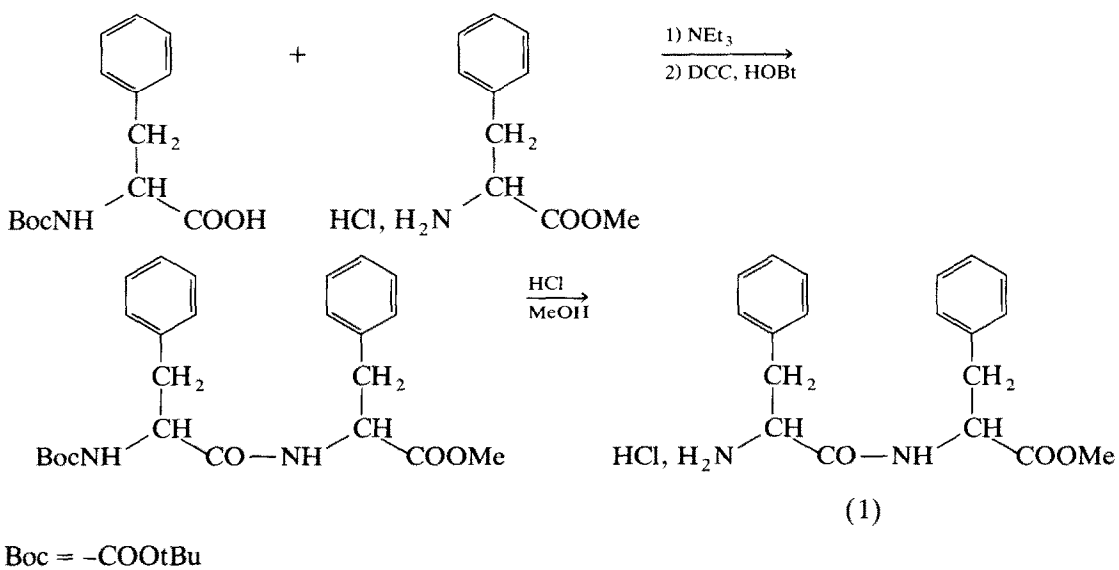
All the gerbils were still alive at the end of the treatment with (**1**). The mean weight of the treated metacestodes was 2.2 ± 0.15 g and the weight of untreated parasite 2.95 ± 0.15 g, therefore a decrease of about 25% was noted.

Vanparijs (1990) observed a reduction of *E. multilocularis* metacestode tissue weight of about 90% after a 35 day period of treatment with mebendazole started 7 days post-infection. In our chemotherapeutic experiments, we preferred to perform drug administration to animals after a 3-month delay post-infection when the parasite is well developed. This choice could explain the less important reduction of parasite weight compared to the loss of weight achieved after mebendazole treatment observed by this author.

The macroscopic aspect of the treated larvae differed from control tissue: the vesicles were smaller, very dense, yellowish, difficult to cut and frequently infected. Compared to controls, the histoenzymological studies showed a weaker alkaline phosphatase activity in treated metacestodes; the distribution of this enzymatic activity in the germinal layer of the metacestode appeared homogeneous (Figs 1–4). This decrease in enzymatic activity could lead to a decrease in nutriment absorption, thus involving glycogen depletion and energy loss for the parasite as in mebendazole treatment (Van den Bossche, 1976).

Other effects of (**1**) treatment were also apparent on the tissue sections stained by hematoxylin-eosin. A decrease in the number of brood-capsules and protoscoleces was observed; the laminated layer was thinner or absent, with host cells in close contact with the germinal layer.

Ultrastructural studies showed a considerable alteration of the parasite with a reduction of the number and length of microvilli at the surface of the hypervesiculated distal cytoplasm of the germinal layer. The inner germinal layer contained a few cellular elements. The laminated layer was



Scheme 1. Procedure for preparation of (**1**).

thinner and more electron-lucent near the microvilli (Figs 5 and 6).

Compound (**1**) leads to considerable damage of the laminated layer. This observation was of great importance, since this membrane is usually a barrier for drug penetration into the parasite, as for mebendazole, for which the degree of penetration into the metacestode is very low (Eckert, 1986). Stoitsova et al. (1992) studied the effect of three anthelminthics on the tegument of *Hymenolepis fraterna* (cestoda). The authors hypothesized that the tegumental fractures produced by drugs were due to unhindered penetration of substances and solutions from the environment. In such areas, the tegument no longer appears to be a fully functional permeability barrier. Similarly, Phe-Phe-OMe · HCl (**1**) alters the laminated layer and disrupts the permeability and osmoregularity properties of the membrane. These effects are different from those observed for another alkaline phosphatase inhibitor, isatin (2,3-indolinedione) (Delabre-Defayolle et al., 1989); the precise mode of action of (**1**) has not yet been elucidated.

We shall continue this study in combining (**1**) with albendazole. Albendazole seems more efficient in chemotherapy of echinococcosis than mebendazole but it is also more toxic (Levin et al., 1983; Steiger et al., 1990). This combination might permit the utilization of albendazole at lower doses during a shorter time period. We hope to achieve an increase in its penetration into the larvae and thus decrease adverse effects. Such work might also be extended to hydatid disease, caused by *E. granulosus* which has a more important economic impact than *E. multilocularis*.

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