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Dose-response inhibition of proteolytic activity by a cysteine protease inhibitor in a murine model of fasciolosis

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Abstract Using the film in situ zymography (FIZ) technique, it has been demonstrated that *N*-[*N*-(*L*-3-*trans*-carboxyoxirane-2-carbonyl)-*L*-leucyl]-agmatine (E-64) inhibits *Fasciola hepatica* proteolytic activity in vivo. The aim of this study was to establish the dose-response relationship of the inhibition of proteolysis as assessed by FIZ with E-64 at different dosages in a murine model of fasciolosis. Maximum effective inhibition of proteolysis was achieved at 50 mg/kg/day (87%). Mice treated with this dose survived for a mean of 10.92 days more than untreated controls, and their ova counts and egg viability were significantly ($P < 0.05$) lower than this latter group. These results indicate that intraperitoneal administration of E-64 not only inhibited liver proteolytic activity in a dose-dependent manner but also produced anti-fecundity and anti-embryonation effects, delaying the progression of fasciolosis. Yet, residual proteolysis may suggest that other E-64-non-sensitive enzymes are involved, or that E-64-enzyme chemical interactions are only capable of a partial agonistic-like effect.

Introduction

Fasciola hepatica infection in cattle and sheep causes significant economical losses (Berasain et al. 2000). At present, a number of drugs for the treatment of fasciolosis are available; yet, resistant strains are emerging. For example, reduced efficacy has been identified for closantel and triclabendazole (Mulcahy and Dalton 2001). Hence, there is always a need for new chemotherapeutic agents against this trematode species. The cysteine proteases of *F.*

hepatica represent attractive targets for chemotherapy development, as these enzymes play key roles in the life cycle of the parasite, such as evasion of host-defense mechanisms (Carmona et al. 1993), tissue invasion (Dalton and Heffernan 1989; Berasain et al. 1997), feeding (Yamasaki et al. 1989; Smith et al. 1993), and egg output (Dalton et al. 1996). Incubation of *F. hepatica* metabolic products with low-molecular-weight inhibitors abolished -thiol protease activity (Dalton and Heffernan 1989). Furthermore, a more recent evaluation of *F. hepatica*-proteolytic activity in vivo using film in situ zymography (FIZ) demonstrated that 85% of the liver fluke cysteine proteases activity was inhibited by intraperitoneal administration of a dose of 50 mg/kg (M. Bogyo, personal communication) of *N*-[*N*-(*L*-3-*trans*-carboxyoxirane-2-carbonyl)-*L*-leucyl]-agmatine (E-64) (Alcalá-Canto et al. 2005). Nonetheless, highest possible efficacy to inhibit *F. hepatica* proteolytic activity and anti-*F. hepatica* effects, and recognition of side effects of E-64, other than its teratogenic effect on early rat embryogenesis (Tachikura 1990), remain to be established in an animal model. Therefore, a dose-response relationship of E-64 assessing such variables was established.

Materials and methods

Evaluation of the in vivo anti-*F. hepatica* effects of E-64

To evaluate the anti-*F. hepatica* efficacy of an intraperitoneally administered cysteine protease inhibitor, a murine model of fasciolosis was used. Wild-type mice of 18–21 g body weight, free from *F. hepatica* infection, as confirmed by coproparasitoscopical studies, were used for this experiment. The mice were housed in cages in a temperature-controlled animal room (20–22°C) and allowed free access to water and fed a standard rodent diet (Rodent Lab Chow 5001, Purina). Metacercariae of *F. hepatica* were obtained by infecting *Lymnaea cubensis* snails, as described by Vera (1994). The cysteine protease inhibitor *N*-[*N*-(*L*-3-*trans*-carboxyoxirane-2-carbonyl)-*L*-leucyl]-agmatine (E-64) (Sigma) was dis-

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solved in dimethyl sulfoxide (DMSO) and the final concentration was adjusted with distilled sterile water. Each one of the mice belonging to infected groups received orally 15 metacercariae in suspension.

All in all, 14 groups of six mice each were established. Seven groups were infected with *F. hepatica* and six of them were treated once a day with E-64 intraperitoneally, in volumes of 100 μ l, as follows: 12.5, 25, 50, 100, 200, and 500 mg/kg. The seventh group was regarded as control and was treated only with a similar volume of the vehicle. The remaining seven groups were not infected, but equally treated, also having a control group treated with the vehicle as well. In the infected and E-64-treated groups, the inhibitor was administered on the first day 2 h before the experimental infection and 4 h after it. Thereafter, re-dosing was carried out daily for 7 weeks.

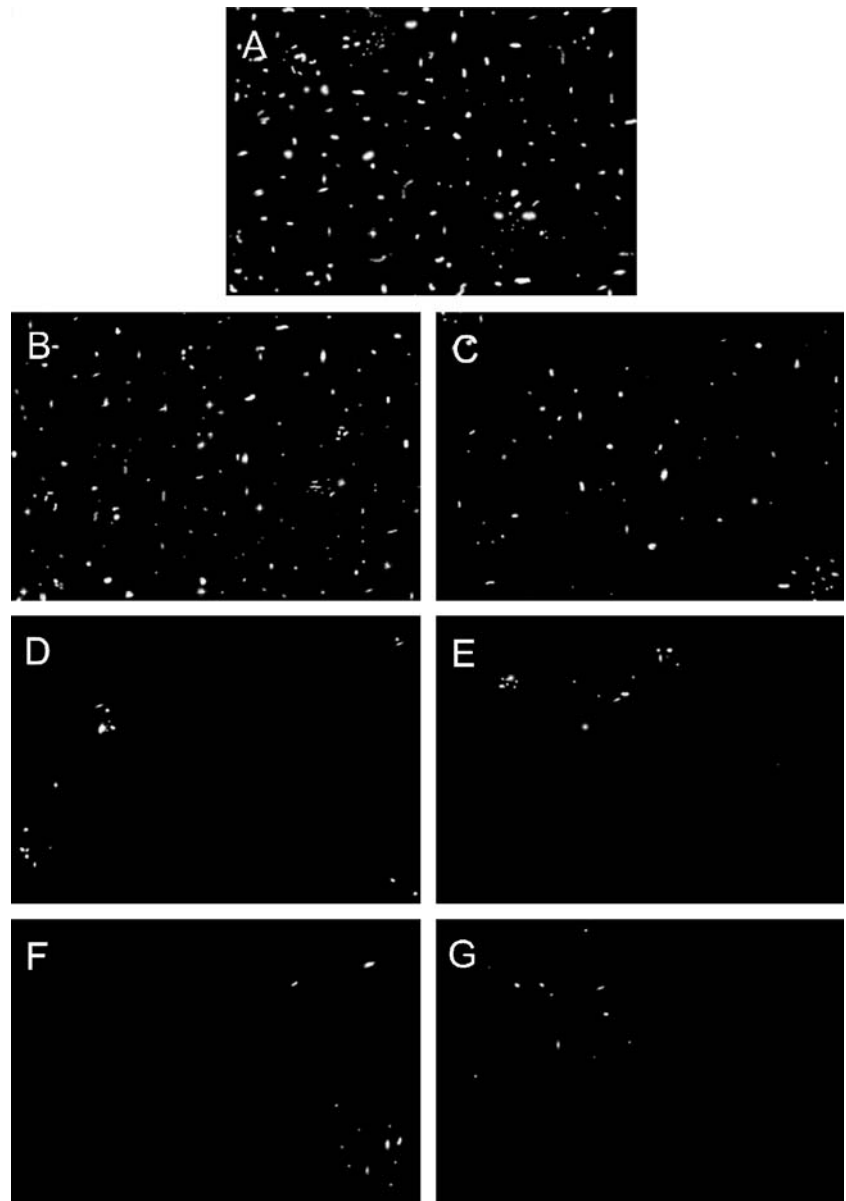
All animals were monitored for visually evident changes for 49 days at which time survivors were euthanized. In

order to estimate possible side effects of E-64, mouse liver samples from all animals were embedded in paraffin using a standard technique and sections were stained with haematoxylin–eosin. E-64-treated liver sections were compared with untreated controls.

Film in situ zymography (FIZ)

To assess the proteolytic activity of *F. hepatica* cathepsins, three liver sections of each mouse were analyzed by FIZ as previously described (Alcalá-Canto et al. 2005). In brief, 4- μ m sections of fresh mice liver tissue were embedded in cryomold O.C.T. (Tissue-Tek, Miles, Elkhart, IN) and frozen in dry ice. The frozen sections were then dipped into NTB 2 photographic emulsion (Eastman Kodak, NY) with gelatin as substrate. The sections were incubated into humidified chambers at 37°C for 12 Fh. After incubation,

Fig. 1 Dose-response inhibition of in situ gelatinolytic activity of *F. hepatica* cysteine proteases WT mice exerted by the intraperitoneal injection of E-64. Proteolytic activity is observed as *white spots* on a dark background. **a** Proteolysis in liver sections of *F. hepatica*-infected mice treated with vehicle alone. **b** A 31% reduction of gelatinolytic activity in infected mice treated with 12.5 mg/kg E-64. **c** A 46% decrease in gelatinolytic activity in liver of infected mice treated with 25 mg/kg of E-64. **d** An 88% reduction of gelatinolytic activity in liver of *F. hepatica*-infected mice treated with 50 mg/kg of E-64. **e** An 86% inhibition of gelatin degradation in hepatic tissue of mice infected and treated with 100 mg/kg of E-64. **f** An 87% decrease in the proteolytic activity of *F. hepatica* proteases in liver of mice treated with 200 mg/kg of E-64. **g** An 87% inhibition of proteolysis in hepatic sections of infected mice that were treated with 500 mg/kg of E-64



the slides were air-dried and the development of the photographic process was carried out. Proteolytic activity was assessed using light microscopy (Olympus BX40 with camera PM 10 AK, Japan) as white spots on a dark background. The photographs were scanned in a Hewlett-Packard Scanjet 4070 Photosmart scanner and in situ gelatinolysis images were analyzed using Image J software 1.33u (National Institutes of Health, USA). FIZ images were statistically analyzed by means of a chi-square test performed in Microsoft Office Excel 2003 (Microsoft).

Assessment of worm fecundity and egg viability

Gall bladders from all mice were dissected and eggs were recovered and counted from decanted bile after five washes. The eggs were incubated at 22°C for 14 days (Dalton et al. 1996). After this period, the eggs were observed in order to estimate the percentage of miracidial development. The anti-fecundity efficacy of E-64 was assessed by the reduction of egg counts after treatment, according to Vera et al. (2003).

Data analysis

Data are reported as mean±standard deviation of *n* separate experiments. Differences between groups were determined by an analysis of variance (ANOVA), the Student *t* test (Bland 2000) and the Bonferroni's multiple comparison test (Perneger 1998). ANOVA was conducted by using the SAS program (release 6.11; SAS). The Student *t* test and the Bonferroni test were tabulated in Microsoft Office Excel 2003 (Microsoft) using Windows XP operating system. The dose-response relationship of the inhibition of proteolysis was plotted by fitting the data to a sigmoidal dose-response curve (Origin 6.1, OriginLab).

Results

Dose-response inhibition of *F. hepatica* proteolytic activity revealed by FIZ

Proteolysis was detected in hepatic tissue from untreated and DMSO-treated mice infected with *F. hepatica*, but it was inhibited in liver from mice treated with doses as low as 12.5 (31%) and 25 mg/kg (46%) of E-64. Gelatin degradation in the 50-, 100-, 200-, and 500-mg/kg treatment groups was significantly decreased (88, 86, 87, and 87%, respectively), when compared to non-treated groups ($P<0.05$) (Fig. 1). Response to the 50-mg/kg dose was statistically similar to that of 100, 200, and 500 mg/kg, as higher doses did not produce further inhibition. Best fit ($r=0.99$) was obtained with a sigmoid regression analysis, from where a dose of 40 mg/kg/day of E-64 emerges as the lowest dose with highest efficacy (87%) (Fig. 2).

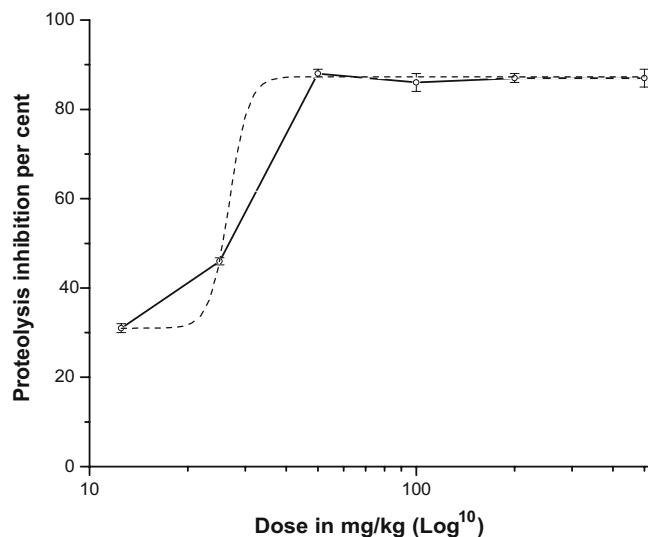


Fig. 2 Dose-response relationship of E-64 inhibiting proteolysis of *F. hepatica* cysteine proteases in vivo in a murine model. Highest inhibitory efficacy (87%) was achieved at 40 mg/kg

Evaluation of the in vivo anti-*F. hepatica* effects of E-64

Survival times of the various groups can be presented with a pattern: animals treated intraperitoneally daily with 50 mg/kg 100 mg/kg, 200 mg/kg, and 500 mg/kg of E-64 survived for a mean of 47.75±0.32 days after infection, whereas mice treated with 12.5 mg/kg and 25 mg/kg survived for a mean of 40.75±1.52 days. A statistically significant difference of the survival time was obtained between these latter groups with the corresponding values for the former ones ($P<0.05$). Untreated controls survived for a mean of 36.83±0.75 days, and this result was only statistically different from the one determined for mice given 50 mg/kg and higher doses ($P<0.05$). No evident side effects that could be attributable to E-64 were seen even at doses of 500 mg/kg/day. However, animals treated at 500 mg/kg/day became lethargic. When examining uninfected-treated liver slides, no histological changes that could be linked to the drug were seen.

Assessment of worm fecundity and egg viability

With egg counts, a dose-related trend was also noticed. A 69.5% mean decrease in the number of eggs collected from the gall bladder was observed for infected mice given 50, 100, 200, and 500 mg/kg of E-64, whereas the anti-fecundity efficacy for the 12.5- and 25-mg/kg treatment groups were 16 and 32%, respectively. A statistically significant difference was obtained between these two groups ($P<0.05$). A significant ($P<0.05$) reduction was also observed between each of the latter groups and the corresponding value for groups dosed with 50 mg/kg or more ($P<0.05$). There was also a statistically significant

decrease in ova counts of groups given 50 mg/kg or more when compared to untreated controls ($P<0.05$).

Eggs collected from the 50-, 100-, 200-, and 500-mg/kg treatment groups failed to embryonate in 44, 42, 43, and 43%, respectively. By comparison, 20 and 23% of ova from the 12.5- and 25-mg/kg treatment groups did not develop miracidia, respectively. A significant decrease ($P<0.05$) was observed for groups given 50 mg/kg or more when compared to these two treatment groups. This statistically significant difference was as well determined among the 50 mg/kg or more treatment groups and untreated controls, which developed miracidia at over 85% ($P<0.05$).

Discussion

The intraperitoneal administration of 50 mg/kg/day of E-64 was the lowest possible dose capable of showing the highest anti-proteolytic effects in the liver of *F. hepatica*-infected mice, and was also effective in delaying the progression of this murine model of fasciolosis. Yet, regression analysis suggests a dose of 40 mg/kg as the lowest dose with highest efficacy (87%). Upon in vivo evaluation, higher doses were unable to improve the parameters set by the 50-mg/kg/day dose. Inability of E-64 to reach 99% inhibition of proteolytic activity complies well with findings by Hashida et al. (1982) who reported that the intraperitoneal injection of E-64 to rats inhibited cathepsin B activity to a certain extent only, and when higher doses were applied, further inhibition was not observed. They suggested that E-64 is incorporated into the cytosol in the free form, and then transported to lysosomes, where it binds to cysteine proteases.

E-64, which previously has been shown to be a diagnostic tool in studies of cysteine proteases, has also been proposed as a possible therapeutic agent due to its potent inhibitory activity, stability and permeability into cells and tissues (Powers et al. 2002). For example, the administration of E-64 reduced proteinuria in an experimental model of glomerulonephritis in rats (Baricos et al. 1988) and its effect has also been tested on the resorptive activity of osteoclasts (Delaisse et al. 1987). E-64 analogues have also been employed in biological studies and associated with attenuation of proliferation of parathyroid hormone in osteoblasts (Murray et al. 1997), inhibition coronavirus protein processing and RNA synthesis (Kim et al. 1995), as well as several other biological processes (Powers et al. 2002).

Using FIZ as described in the current study, and considering that E-64 inhibits specifically cysteine proteases (Katunuma and Kominami 1995), it is noted that gelatinolytic activity in a murine model of fasciolosis is due mainly to these enzymes. Nevertheless, incomplete reduction of gelatinolytic activity was observed on liver sections in all E-64-treated mice. It is postulated then that gelatinolysis may be caused by proteases other than E-64-sensitive cysteine proteases. Yet, it may also occur that proteolysis due to cysteine proteases might not be totally inhibited because of the

partial inability of E-64 to fully interact or modify the catalytic domain of these particular *F. hepatica* enzymes.

A dose of 50 mg/kg/day E-64 clearly delayed progression of fasciolosis, as mice lived through the lethal infection induced here, for a mean of almost 11 days more than the infected and untreated mice. These results may therefore be taken to indicate that cysteine protease activity is involved in tissue invasion of *F. hepatica* as suggested by our previous work (Alcalá-Canto et al. 2005). There seems to be a close relationship between the rate of proteolysis and the course of infection, showing that cysteine proteases are an important factor in the in vivo pathological effects of the parasite. The fact that E-64 strongly inhibited proteolysis caused by *F. hepatica* in liver is an encouraging finding, as mortality due to fasciolosis in sheep is a direct consequence of liver pathology caused by migrating flukes (Dalton et al. 1996).

It is interesting to emphasize that E-64 also reduced the egg production and hatching ability of the parasite. A significant ($P<0.05$) decrease in ova counts was observed in the 50-mg/kg or more treatment groups when compared to counts in the untreated groups. Moreover, eggs from these groups had a 43% failure to embryonate. The exact mechanism by which E-64 affects fluke fecundity and egg viability is not known, but it is tempting to speculate that E-64 affects egg production and viability, as a result of the lack of nutrient acquisition by the liver fluke. Dalton et al. (2003) suggested that antibodies produced versus cathepsin L proteases may block cathepsin L activity, limiting parasite feeding, thus preventing amino acid intake and subsequently the synthesis of egg proteins. Vaccination of sheep with cysteine proteases of *F. hepatica* reduced worm fecundity and egg viability (Wijffels et al. 1994).

To the best of our knowledge, this trial describes for the first time the in situ proteolytic activity in relation to worm fecundity and egg viability in fasciolosis, under the effects of E-64. This drug was capable of inhibiting liver proteolysis by 87%, with a 43% mean decrease in embryonation of eggs, and a 69.5% reduction in ova counts, at an experimental dose of 50 mg/kg/day. Analysis of the dose-response relationship yielded a maximum effective dose of 40 mg/kg/day. This efficacy was not linked to hepatic injury. Nevertheless, frequent dosing is required in order to achieve optimal anti-*F. hepatica* proteolysis in liver; hence, further pharmaceutical design is required to obtain a sustained release formulation of E-64 to allow non-daily intraperitoneal administration.

In summary, it is reasonable to suggest that the cysteine proteases of *F. hepatica* remain a promising target for the development of chemotherapeutic agents, and that the administration of E-64 together with other traditional therapies, including immunoprophylaxis, may improve clinical outcomes.

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