**ANTIGIARDIAL ACTIVITY OF THE CYSTEINE PROTEASE INHIBITOR E-64**

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**SUMMARY**

The quest for new antiparasitic alternatives has led researchers to base their studies on insights into biology, host-parasite interactions and pathogenesis. In this context, proteases and their inhibitors are focused, respectively, as druggable targets and new therapy alternatives. Therefore, we proposed to evaluate the *in vitro* effect of the cysteine protease inhibitor E-64 on *Giardia* trophozoites growth, adherence and viability. Trophozoites (10^5) were exposed to E-64 at different final concentrations, for 24, 48 and 72 h at 37 °C. In the growth and adherence assays, the number of trophozoites was estimated microscopically in a haemocytometer, whereas cell viability was evaluated by a dye-reduction assay using MTT. The E-64 inhibitor showed effect on growth, adherence and viability of trophozoites, however, its better performance was detected in the 100 µM-treated cultures. Although metronidazole was more effective, the E-64 was shown to be able to inhibit growth, adherence and viability rates by ≥ 50%. These results reveal that E-64 can interfere in some crucial processes to the parasite survival and they open perspectives for future investigations in order to confirm the real antigiardial potential of the protease inhibitors.

**KEYWORDS:** *Giardia duodenalis,* Protease inhibitor; Trophozoites; Growth; Adherence; Viability.

**INTRODUCTION**

*Giardia duodenalis* (syn. *Giardia lamblia*, *Giardia intestinalis*), a worldwide zoonotic intestinal protozoan, is one of the major causes of non-viral diarrheal disease in humans. Despite giardiasis has a global distribution, higher infection rates (20-60%) have been reported for developing countries, mainly among socially and economically deprived populations. Since in developed areas, infections rates are lower and *G. duodenalis* is often involved in numerous outbreaks that have been attributed to an inappropriate water treatment. Considering the infection impact on health and on socio-economic improvements of developing nation’s population, *Giardia* was recently included in the WHO Neglected Diseases Initiative, a group including pathogens that have a common link with poverty. In these areas, giardiasis is a common cause of diarrhea in nursery and school children, especially in undernourished individuals, which in turn, gives rise to nutritional deficiencies leading to growth failure and cognitive impairment.

So far, drug intervention has been recommended as a preventive strategy to limit and reduce the transmission of giardiasis. Currently, some therapeutic measures have been available in clinical practice for the treatment of giardiasis, highlighting acridine, 5-nitroimidazole, 5-nitrofurans, 5-nitrothiazoles and benzimidazole derivatives. For decades, the most prescribed antigiardial drug is the metronidazole, a 5-nitroimidazole compound that has a recognized antigiardial activity with cure rates ranging from 80 to 95%. Despite its efficiency, treatment shows undesirable side effects, failures are common and evidences suggest the emergence of drug resistance. Therefore, there is an increasing interest for new antigiardial agents that can be effective against the parasite, less harmful for the host and may provide options for refractory cases, especially for children, who may require treatment often because of reinfections.

Nowadays, the quest for new antiparasitic alternatives has led researchers to base their studies on insights into biology, host-parasite interactions and pathogenesis. In light of this, advances in the understanding of the biochemistry and molecular biology of parasites have led to a better understanding of molecules which play a role in biological, metabolic and physiological processes. Among these molecules, the proteolytic enzymes or proteases have excited the researcher’s interest, once they have been identified as important virulent factors as well as potential chemotherapeutic targets in parasites. Considering that proteolytic activity is naturally regulated by specific inhibitors, the selective inhibition of crucial proteases can be a promising strategy to develop new antiparasitic therapies. In this context, it is important to emphasize that proteases have been validated as druggable targets as evidenced by the use of protease inhibitors as effective therapy for hypertension, diabetes, osteoporosis, certain cancers and AIDS. In relation to parasites, several protease targets have been validated by genetic or chemical knockout in protozoan parasites, and many other
enzymes appear promising as targets but they require more investigations for validation, or to identify viable drug leads. So, interesting results have been reported in studies with parasitic infections such as malaria, leishmaniasis and Chagas disease and they have provided important evidences that these substances selectively inhibit parasite proteases without affecting host homologues.11

With respect to *Giardia*, notwithstanding recent studies have shed light on trophozoites proteases and their involvement in metabolism and physiologic processes, relatively little is known about the effect of protease inhibitors on vital processes of this parasite. To date, as far as we know, there are few studies that have evaluated the *in vitro* and *in vivo* performance of protease inhibitors on multiplication and excystation/excystation of the parasite. Thus, considering the aspects above, here we report the *in vitro* effects of E-64, a specific cysteine protease inhibitor, on axenic trophozoites growth and adherence abilities, and viability. The results assembled here may open perspectives to pursue the development of further investigations in order to assess protease inhibitors as potential antigiardial agents.

**MATERIALS AND METHODS**

**Parasite and cultivation conditions**: *G. duodenalis* axenic trophozoites, BTU-11 strain, were axenically cultivated in filter-sterilized TYI-S-33 (Tryptcase, yeast extract, iron serum) medium, modified by KEISTER in 5 mL Vacutainer tubes at 37 °C. The strain isolated in Brazil at the Giardiasis Laboratory (IB/UNESP) in Botucatu, São Paulo was established from cysts of a symptomatic patient presenting diarrhea, flatulence, abdominal cramps and resistance to conventional therapy. Trophozoites harvested in log-phase growth 72 h postinoculation, after chilling in bath ice for 10 min, were collected by centrifugation at 250xg for 15 min at 4 °C. Pooled cells were resuspended in sterile phosphate-buffered saline (PBS; pH 7.2) and the total number of trophozoites was counted microscopically in a haemocytometer (Neubauer cell-counter chamber) and adjusted to an inoculum of 10⁷ parasites.

**Preparation of inhibitor**: E-64 [†-trans-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane], a commercially available cysteine protease inhibitor was purchased from Sigma. The inhibitor was prepared at one mM in aqueous solution and stored at -20 °C, until required. The inhibitor stock solution was diluted in sterile phosphate buffered saline (pH 7.2) at final concentrations of 10, 50 and 100 µM and sterilized in 0.22 µm filter. The assayed concentrations were established according to inhibitor effective concentration.

**Growth inhibition assay**: Firstly, an inoculum of 10⁷ trophozoites were incubated in TYI-S-33 medium (4.5 mL/tube) containing the inhibitor at different concentrations (10, 50 and 100 µM) for 24, 48 and 72 h at 37 °C. After incubation, the tubes were cooled for 10 min, centrifuged at 250xg (10 min, 4°C) and the population density of cultures were estimated by counting in a haemocytometer. As controls, cultures containing only the parasites and cultures treated with metronidazole at 40 µg/mL were included in all the assays and submitted to the same experimental conditions. For each E-64 concentration assessed, three tubes were screened and the protease inhibitor activity on the parasite growth was evaluated comparing the number of organisms in inhibitor-treated cultures with their number in controls. The results were expressed as the trophozoites number and growth inhibition percentage.

**Adherence assay**: The E-64 effect on adherence of *Giardia* trophozoites was analyzed as previously described by EDLIND et al., with slight modifications. An inoculum of 10⁷ organisms was incubated in TYI-S-33 medium for four hours at 37 °C. The medium with unattached cells was replaced with one supplemented with E-64 at 10, 50 and 100 µM. After incubation for 24 and 48 h at 37 °C, adherent cells were dislodged by a 10 min on ice, centrifuged at 250xg and the number of attached cells was determined using a haemocytometer. Control assays were performed under similar experimental conditions with cultures in the absence of E-64 and cultures treated with metronidazole at 40 µg/mL. For each inhibitor concentration, three tubes were assayed and the effect on adhesion capacity was evaluated comparing the number of organisms in E-64-treated cultures with their number in control cultures. The results were expressed as number of attached cells (percentage of control).

**Cell viability assay**: The effect of E-64 on trophozoites viability was evaluated by the quantitative colorimetric MTT-tetrazolium salt technique, as previously described by PONCE-MACOTELA et al. Briefly, trophozoites (10⁷) exposed to each E-64 concentration for 48 and 72 h were transferred to 1.5 Eppendorf tubes, washed with PBS (pH7.4) and incubated for 60 min at 37 °C in a solution containing 40 µL of MTT (5 mg/mL; Sigma) and 20 µL of the catalyst phenazine-methosulfate (PMS 2.5 mg/mL; Sigma). Then, the cells were pelleted by centrifugation and the purple product obtained by the conversion of MTT to the formazan was resuspended in isopropanol/hydrochloric acid. The assay was performed in triplicate and aliquots of 150 µL were collected and the optical densities of each sample were measured at 540 nm. The parasite viability was calculated regarding the untreated cultures (100% viability).

**Statistical analysis**: To evaluate E-64 effect on the growth, adherence and viability of *Giardia* trophozoites, a factorial design layout was employed for counting data following multiple comparisons. Data were submitted to analyze the variance (ANOVA) and the means were compared by Tukey’s test. All analyses were conducted in SAS 9.2 for Windows (SAS Institute Inc., Cary, NC) and p-values less than 0.05 were deemed statistically significant. The inhibitory concentrations at 50% (IC50) were calculated from dose-response curves by linear regression analysis.

**RESULTS AND DISCUSSION**

As many efforts have been made to search new alternatives for *Giardia* infection treatment, the assessment of a drug potential requires suitable models that allow identifying the biological processes on which they can interfere. For this purpose, assays for *in vitro* screening are a preliminary step and advances in trophozoites axenic cultivation have allowed the investigation of antigiardial activity of a range of compounds, especially, in relation to their ability to exert effect on trophozoites growth, adherence and viability. The present study led to some interesting findings concerning the *in vitro* activity of the cysteine protease inhibitor E-64 on axenic trophozoites.

As shown in Figure 1, E-64 exerted inhibitory activity on parasite multiplication in a dose dependent manner. Considering the assayed concentrations of 10, 50 and 100 µM, growth inhibition was observed in
cultures exposed to the highest of them, in which the number of parasites recovered was significantly lower than that obtained in untreated cultures \( (p < 0.05) \). The inhibitory concentrations at 50% (IC50) were 144.29 µM for 24 h, 53.72 µM for 48 h and 27.02 µM for 72 h. At both 48 and 72 h, growth reduction by ≥ 50% and ≥ 70% were observed in cultures treated with 50 and 100 µM, respectively \( (p < 0.05) \). A significant level of inhibition (~80%) was detected in 72 h cultures treated with the 100 µM, but the reduction rate was still lower than that obtained in metronidazole-treated cultures. In adherence assays (Fig. 2), only the concentrations of 50 and 100 µM were able to promote detachment of trophozoites. The IC50 values after 24 and 48 h were 128.65 µM and 33.80 µM respectively. The greater activity was induced by the inhibitor at 100 µM after 48 h \( (p < 0.05) \) when only 10% of trophozoites remained attached. Despite E-64 markedly diminished parasite adherence at 100 µM, the rates of metronidazole inhibition were higher. In both growth and adherence assays, E-64 exhibited a dose-response effect, however by light microscope observations, this activity was not accompanied by marked changes in trophozoites morphology as body swollen and reduction of flagellar beating frequency.

As in the growth and adherence assays, E-64 at 100 µM showed a very significant reduction of viability rate in comparison to non-treated cultures after 48 h (Fig. 3) \( (p < 0.05) \). The highest rates were detected after 48 h, when the inhibitor at 50 and 100 µM was able to kill 80% and 93% of the trophozoites, respectively \( (p < 0.05) \). However, this effect was lower than that obtained by exposure to metronidazole \( (p < 0.05) \).

So far, the most widely used drug against *Giardia* infection is the metronidazole, a 5'-nitroimidazole derivate, although there are some problems related to resistance and toxicity. Once many antiparasitic drugs currently in use show the same disadvantages, recently, investigations have identified promising antiparasitic drug candidates as well as potential targets in parasites. In this context, proteases and their inhibitors are focused, respectively, as druggable targets and new therapy alternatives.

In the last years, several investigations have focused the potential of protease inhibitors in important parasitic infections such as malaria, Chagas’ disease, leishmaniasis and toxoplasmosis\(^{19}\) and they have revealed promising insights on the use of these substances, especially those against thiol or cysteine proteases. Regarding to *Giardia*, advances in the study of its proteases had become clear that this protozoan is actively proteolytic and contain multiple proteases, with the predominance of the cysteine peptidases that are responsible for the main proteolytic activity\(^{4,5,12,26}\).

In the present study, although metronidazole was very effective, the cysteine protease inhibitor E-64 was shown to be also efficient in affecting the parasite growth, adherence and viability. These biological parameters were inhibited by ≥ 50% at concentrations of 50 and 100 µM. With respect to *Giardia*, to date, there are only two studies that evaluate the performance of inhibitors on the life forms of the parasite. In one of these investigations\(^{8}\), antiretroviral protease inhibitors (Kaletra\(^{8}\), ritonavir and saquinavir) were assessed in *vitro* for their activity on trophozoite proliferation and the results revealed that Kaletra\(^{8}\) was the most effective of them. More recently, in *vitro* and in *vivo* assays to evaluate the anti- giardial potential of E-64 showed a significant inhibitory effect on parasite growth and on the efficiency of encystation\(^{11}\).
Interessantemente, E-64 foi também capaz de inibir trofozoite adherência e um marcado detecção de 90% foi observada em culturas expostas à substância a 100 µM. É importante enfatizar que o detaque da *Giardia* trofozoitos para a mucosa intestinal é um fator essencial para sua colonização e sobrevivência no hospedeiro assim como para a giardiase. De acordo com alguns autores, a adherência de *Giardia* é considerada um alvo potencial para terapias alternativas que inibam a entrada e/ou não impedia a sobrevivência do parasita na epitélio intestinal16. Outro ponto a considerar é que substâncias que são capazes de alterar microtúbulo assembly dos trofozoitos têm o potencial de afetar um amplo espectro de processos biológicos como a cilindrícula, locomotiva, aderência e detaque, situações que podem aumentar a viabilidade cellular7.

Apesar do E-64 efeito in vitro, as observações microscópicas não revelaram alterações macroscópicas em trofozoitos. Apesar de E-64 ser uma substância devido ao seu caráter hidrofóbico, é conhecido que sua propriedade de desintegragem e não impede a percolação de camadas celulares. Em contraste a E-64, seu exemplo E-64c e E-64d atuam como proteínas permeantes, que podem percer um membro permeante como a protease trofozoito inhibitors que podem atingir um intento cellular. Recentemente, em um estudo sobre a estrutura e função da *Giardia* endomembrana system e os proteases, DUBOIS10 reportou o crescimento não foi afetado por E-64d até 50 µM. Em adição, segundo este autor, a sobrevivência de trofozoitos na presença de E-64 não diferiu de observado na falta de tratamento. Em vista das observações com E-64d e considerando as observações no estudo do presente em E-64 foi capaz de reduzir a sobrevivência celular por ≥ 50% concentrando 50 e 100 µM, estudos futuros são uma necessidade para elucidar em mais detalhe os efeitos de E-64 e suas derivações in vitro *Giardia* biológicos processos.

As cisteína-proteases estão entre os alvos mais promissores para o desenvolvimento de novos agentes terapêuticos, visto que participam de eventos fundamentais do ciclo de vida de muitos microrganismos, inclusive *Giardia*. Como a atividade das proteases pode ser controlada por inibidores específicos, essas substâncias têm sido avaliadas quanto ao potencial antiparasitário. Diante disso, o presente estudo teve por objetivo avaliar o efeito in vitro do inibidor de cisteína-proteases E-64 sobre o crescimento, a aderência e a viabilidade de trofozoitos de cepa de *Giardia* isolada em Botucatu. Nos ensaios de crescimento e aderência, o número de trofozoitos foi estimado microscópicamente em hemocitômetro, enquanto que a viabilidade celular foi avaliada pelo método de MTT. No presente estudo, embora o metronidazol tenha se apresentado bastante efetivo, o E-64 mostrou ser capaz de inibir o crescimento, a aderência e a viabilidade em taxas superiores a 50%, especialmente nos cultivos expostos à concentração de 100 µM. Devido a preliminares, esses resultados demonstraram que o inibidor E-64 pode interferir em processos primordiais para a sobrevivência do parasita, além do que, abre novas perspectivas para investigações futuras a fim de avaliar o real potencial giardícida dos inibidores de proteases.

RESUMO

**Atividade in vitro do inibidor de cisteína-proteases E-64 sobre trofozoitos de *Giardia***

As cisteína-proteases estão entre os alvos mais promissores para o desenvolvimento de novos agentes terapêuticos, visto que participam de eventos fundamentais do ciclo de vida de muitos microrganismos, inclusive *Giardia*. Como a atividade das proteases pode ser controlada por inibidores específicos, essas substâncias têm sido avaliadas quanto ao potencial antiparasitário. Diante disso, o presente estudo teve por objetivo avaliar o efeito in vitro do inibidor de cisteína-proteases E-64 sobre o crescimento, a aderência e a viabilidade de trofozoitos de cepa de *Giardia* isolada em Botucatu. Nos ensaios de crescimento e aderência, o número de trofozoitos foi estimado microscópicamente em hemocitômetro, enquanto que a viabilidade celular foi avaliada pelo método de MTT.

**ACKNOWLEDGEMENTS**

The authors are grateful to the student’s fellowship granted by the Brazilian agency CAPES.

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