# Parasiticidal effect of synthetic bovine Lactoferrin peptides on the enteric parasite *Giardia intestinalis*

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Parasiticidal effect of synthetic bovine Lactoferrin peptides on the enteric parasite *Giardia intestinalis*

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Abstract

*Giardia intestinalis* is the most common infectious protozoan parasite in children. Despite the effectiveness of some drugs, the disease remains a major worldwide problem. Consequently, the search for new treatments is important for disease eradication. Biological molecules with antimicrobial properties represent a promising alternative to combat pathogens. Bovine lactoferrin (bLF) is a key component of the innate host defense system, and its peptides have exhibited strong antimicrobial activity. Based on these properties, we evaluated the parasiticidal activity of these peptides on *G. intestinalis*. Trophozoites were incubated with different peptide concentrations for different periods of time, and the growth or viability was determined by carboxyfluorescein-succinimidyldiacetate-ester (CFDA) and propidium iodide (PI) staining. Endocytosis of peptides was investigated by confocal microscopy, damage was analyzed by transmission and scanning electron microscopy, and the type of programmed cell death was analyzed by flow cytometry. Our results showed that the LFpeptides had giardicidal activity. The LFpeptides interacted with *G. intestinalis* and exposure to LFpeptides correlated with an increase in the granularity and vacuolization of the cytoplasm. Additionally, the formation of pores, extensive membrane disruption, and programmed cell death was observed in trophozoites treated with LFpeptides. Our results demonstrate that LFpeptides exhibit potent *in vitro* antiangiadial activity.

Keywords: Lactoferrin; LFchimera; parasiticidal; peptides; Giardia; Giardiasis
Introduction

*Giardia intestinalis* (also known as *Giardia lamblia* or *Giardia duodenalis*), is a flagellated unicellular eukaryotic parasite that causes giardiasis, a diarrheal disease, throughout the world (Watkins and Eckmann 2014). Giardiasis is the most common cause of waterborne outbreaks of diarrhea in the United States and is occasionally considered a cause of food-borne diarrhea (Furness et al. 2000). In certain areas of the world, water contaminated with *G. lamblia* commonly causes travel-related giardiasis in tourists (Painter et al. 2015; Watkins and Eckmann 2014). This parasite is particularly problematic in developing countries, where a very high prevalence and incidence of infection has been reported. Data suggest that long-term growth retardation in children can result from chronic giardiasis, in part due to the parasite attaching itself to the lining of the small intestine in humans, where it interferes with the body's absorption of fats and carbohydrates from digested foods (Eckmann 2003). Giardiasis is reported more frequently in young children and immunocompromised or chronically ill individuals, and *G. intestinalis* infection is particularly significant for people with malnutrition, immunodeficiencies, or cystic fibrosis (Painter et al. 2015; Watkins and Eckmann 2014).

Giardia species have two major stages in their lifecycle. Infection with *G. intestinalis* initiates when the cysts are ingested with contaminated water or, less commonly, food or through direct fecal-oral contact. The cyst is relatively inert, allowing prolonged survival in a variety of environmental conditions (Adam 2001; Carranza and Lujan 2010). After exposure to the acidic environment of the stomach, cysts excyst into trophozoites in the proximal small intestine. The trophozoite (the vegetative form) replicates in the small
intestine, causing symptoms of diarrhea and malabsorption. After exposure to biliary fluid, some of the trophozoites form cysts in the jejunum and are passed in the feces, allowing for completion of the transmission cycle by infecting a new host (Adam 2001; Carranza and Lujan 2010).

Standard treatment for giardiasis consists of antibiotic therapy. Metronidazole is the most commonly prescribed drug for this condition. However, metronidazole use has been associated with significant failure rates in clearing parasites from the gut and with poor patient compliance (Watkins and Eckmann 2014). In addition, an increasing incidence of nitroimidazole-refractory giardiasis has been reported in travelers from India (Nabarro et al. 2015). Appropriate fluid and electrolyte management is critical, particularly in patients with large-volume diarrheal losses and in children with acute or chronic diarrhea who manifest a failure to thrive, malabsorption, or other gastrointestinal tract symptoms in whom Giardia organisms have been identified (Dominguez-Lopez et al. 2011; Hill 1993; Vesey and Peterson 1999).

Innate-immunity mechanisms play a role in the control and/or severity of the infection; however, little is known about the mechanisms involved in this immune response (Roxstrom-Lindquist et al. 2006). Breastfeeding protects infants from *G. intestinalis* infection. Breast milk contains detectable titres of secretory IgA, which is protective for infants, especially in developing countries (Eckmann 2003; Morrow et al. 1992). A study from Egypt showed breast-fed infants had a lower incidence of symptomatic and asymptomatic infection. Furthermore, infected infants who were exclusively breast-fed had fewer clinical manifestations than those who were not exclusively breast-fed (Abdel-Hafeez
et al. 2013; Gendrel et al. 1989). Apart from IgA, a milk protein called lactoferrin (LF) has been reported as an immunological factor that kills trophozoites *in vitro* and *in vivo*: both hLf and bLF and peptides derived from their N-terminals (LfcinH and LfcinB) have microbicidal activity against Giardia (Gillin et al. 1983; Ochoa et al. 2008; Turchany et al. 1995; Turchany et al. 1997). However, it is not known if synthetic bLF peptides share this activity and the mechanism of action of Lf-derived peptides is unknown. Thus, the aim of this work was to study the possible microbicidal activity of synthetic bLF-based peptides against *Giardia intestinalis* and to explore the mechanism involved in parasitical effects against Giardia *in vitro*. 
Materials and methods

Bovine lactoferrin, synthetic peptides and chemotherapeutic agents

Bovine LF (bLF, 20% iron saturated) was kindly donated by Morinaga Milk Industries Co (Tokyo, Japan). The purity of bLF (>98%) was checked by SDS-PAGE stained with silver nitrate. Lactoferrin concentration was assessed by UV spectroscopy on the basis of an extinction coefficient of 15.1 (280 nm, 1% solution). The bLF iron saturation was about 20% as detected by optical spectroscopy at 468 nm on the basis of an extinction coefficient of 0.54 (100% iron saturation). LPS contamination of bLF, estimated by Limulus Amebocyte assay (LAL Pyrochrome kit, ThermoFischerScientific, Waltham, MA, USA), was equal to 0.7±0.06 ng/mg of bLF. Synthetic peptides LFcin17-30, LFampin265-284 and LFchimera were obtained by solid-phase peptide synthesis using Fmoc chemistry, as described previously (Bolscher et al. 2012; Bolscher et al. 2009). The chemotherapeutic agents used were metronidazole and albendazole (Sigma Chemical Co., St. Louis, MO, USA). Stock solutions were prepared in phosphate-buffered saline for metronidazole and dimethyl sulfoxide (DMSO) for albendazole. The final DMSO concentration in the culture tubes was <0.5% (v/v).

Giardia intestinalis cyst isolation

Human fecal samples from different patients containing abundant G. intestinalis cysts were obtained from children at Hospital Pediatrico de Sinaloa in Culiacan City. G. intestinalis cysts were purified and concentrated from feces using a combined sucrose flotation and simplified sucrose gradient method (Hautus et al. 1988). The cysts, after being washed twice in distilled water, were resuspended in distilled water and stored at 4°C for a
maximum of 3 days prior to use. The *G. intestinalis*-positive cysts were confirmed by light microscopy and PCR (Elsafi et al. 2013; Stojecki et al. 2014).

**Excystation and axenization**

*G. intestinalis* cysts were purified and concentrated from feces by combining the sucrose flotation method with a simplified sucrose gradient method. The excystation procedure was a modification of the Bingham and Meyer technique performed by Schupp et al. (1988). Briefly, the isolation procedure involved three steps: the concentration and cleaning of cysts by centrifugation in sucrose gradients performed 1 to 3 days after collection, the induction of excystation performed in acid solution from 1 to 5 days after cleaning cyst suspensions, and the culture and axenization in modified TYI-S-33 medium (Schupp et al. 1988).

**Viability and growth inhibition assays**

The effect of bLF, LFpeptides and chemotherapeutic agents on the long-term viability or permabilization of *G. intestinalis* trophozoites was determined by the inclusion of carboxyfluorescein-succinimidyl-diacetate-ester (CFDA) (St. Louis, MO, USA), or the exclusion of the dye propidium iodide (PI).

In one set of experiments, cultures were initiated by the addition of 2.5 x 10⁴ trophozoites in 0.1 ml of medium to vials (15 x 45 mm) containing 3.9 ml of medium containing none (optimal viability) or one of the following agents: 1, 5, 10, 20, or 40 µM of bLF, LFcin17-30, LFampin265-284, or LFchimera. As control of growth inhibition, treatments with metronidazole (1, 5, 10, 20, and 40 µM) were used. The vials were incubated at 37°C for 12 h, chilled on ice to detach trophozoites, and centrifuged at 500 g for 10 min, and the pellet was resuspended in 1 ml of medium. Long-term viability was determined using the fluorescent probe carboxyfluorescein-succinimidyl-diacetate-ester.
(CFDA-SE) (10 µg/ml) and visualized by epi-fluorescence microscopy (dos Santos et al. 2015). Experiments were performed at least three times in triplicate, and the mean and standard deviations are indicated. Comparison of means was done by using a two-tailed t-test for independent samples. A value of P < 0.05 was considered statically significant.

In other experiments, *G. intestinalis* trophozoites (10⁶) were placed in tubes with TYI-S-33 and were then incubated alone (optimal viability) or with 100 µM metronidazole, 5 µM albendazole, or 40 µM LFcin17-30, LFAmpin265-284 or LFchimera for 2 h at 37ºC. Membrane permeabilization by propidium iodide (PI) was used as a measure of trophozoite viability. The total number of organisms per vial was counted and compared to that of parallel untreated cultures. Experiments were performed at least three times in triplicate, and the mean and standard deviations are indicated. Comparison of means was done by using a two-tailed t-test for independent samples. A value of P < 0.05 was considered statically significant.

Tubes with trophozoites were also incubated with combinations of metronidazole ± LFpeptides or albendazole ± LFpeptides or LFpeptides alone. Membrane permeabilization of propidium iodide (PI) measured by flow cytometry was used as a measure of trophozoite viability.

**Confocal microscopy**

Trophozoites (10⁶/ml in TYI-S-33 medium) were incubated with 2 µM FITC-LFcin17-30, FITC-LFAmpin265-284 or FITC-LFchimera for 0, 5, 15, 30, 45 or 60 min at 37ºC. After washing twice in cold PBS, trophozoites were collected by centrifugation, fixed with 4% *p*-formaldehyde (30 min at 37ºC), permeabilized with 0.5% triton X-100, counterstained with PI, washed and processed for analysis by confocal microscopy.
Transmission electron microscopy

*G. intestinalis* trophozoites (approximately $10^6$ cells) were untreated or treated with 40 µM of bLF-peptides for 2 h at 37 ºC. Trophozoites were collected by centrifugation and processed for standard transmission electron microscopy (Vázquez-Nín and Echeverría 2000). Briefly, samples were fixed using 2.5% glutaraldehyde in PBS for 2 h at 4ºC, post-fixed in 1% osmium tetroxide for 1 h, dehydrated in a graded series of ethanol, and embedded in Epon resin. Semi-thin sections (approximately 300-400 nm) were stained with toluidine blue and observed with bright-field microscopy. Sections were mounted on copper grids and contrasted with uranyl acetate and lead citrate. Samples embedded in the resin were cut with an ultramicrotome. Serial thin sections (50-60 nm width) were obtained, and the samples were then stained with uranyl acetate and lead citrate. The sections were observed with a JEOL 1010 transmission electron microscope at 80 kV.

Scanning electron microscopy

*G. intestinalis* trophozoites ($5 \times 10^6$) were incubated with 40 µM of LFpeptides for 2 h at 37 ºC, washed and fixed with 10% formaldehyde for 72 h. Samples were washed and then dehydrated in a graded series of ethanol. Dehydrated samples were placed in acetone and then collocated in desiccator for 30 min. Finally samples were mounted in glass chambers and bombarded with gold particles for 20 min (Perez-Rangel et al. 2013). Processed specimens were observed with a scanning electron microscope JEOL LSM6360LV.

Flow cytometry

To detect early apoptosis or programmed cell death, *G. intestinalis* trophozoites ($5 \times 10^6$) treated with 40 µM LFpeptides for 2 h at 37ºC were stained with Allophycocyanin-Annexin and 7-Amino-Actinomicyn D (BD Pharmingen, San José, CA), following the manufacturer’s instructions. Annexin V (also known as Annexin A5) and 7-AAD (7-amino-
actinomycin D) are indicators of early apoptosis or programmed cell death in Giardia and necrosis or late programmed cell death, respectively. Both fluorescent dyes were measured in *G. intestinalis* using Acoustic Focusing Cytometer Attune™ Blue/Red (Life Technologies).

**Results**

**Effects of bLF, LFpeptides and chemotherapeutics agents on the viability and long-term growth of *Giardia intestinalis***

The effect of LFcin17L30, LFampin265-284 and LFchimera on *G. intestinalis* trophozoites was analyzed using the membrane permeabilization probe PI, as a measure of viability. When the *G. intestinalis* trophozoites were incubated with 40 µM LFpeptides for 2 h, the trophozoites showed a marked PI uptake, indicating a drastic effect on the viability (Figure 1, panel A). LFchimera had the best killing effect (more than 98% of the trophozoites were stained with PI) followed by the other LFpeptides (70-80% of the trophozoites were stained) compared with untreated cells. Furthermore, the parasiticidal effect of LFchimera was higher than the drugs albendazole and metronidazole (controls of viability inhibition) (Figure 1, panel A).

Concentrations of 100 µM of metronidazole or 5 µM of albendazole were needed to permeate more than 95% of *G. intestinalis* cultures. However, only 20 µM and 3 µM of these drugs were needed to reach the same level of membrane permeation when 20, 30 or 5 µM of LFcin17-30, LFampin254-284 or LFchimera, respectively, were added, (Table 1). Thus, the combined effect of lower concentrations of metronidazole or albendazole with LFcin17-30, LFampin265-284 or LFchimera had the best initial parasiticidal activity (Table 1).
Next, the ability of different concentrations of LFcin17-30, LFampin265-284 and LFchimera to inhibit long-term growth was tested using the live-stain CFDA. After 12 h of interaction, the growth of *G. intestinalis* trophozoites in the presence of 5, 10, 20 or 40 µM of LF and LFpeptides was lower than that found in the untreated trophozoites (Figure 1, panel B). Interestingly, LFchimera had better giardicidal activity than the drug metronidazole: 40 µM LFchimera inhibited the growth of cultures with more efficacy than 40 µM metronidazole. Similar results were obtained when *G. intestinalis* cultures were incubated with 40 µM of each treatment for longer periods of time (24, 36 and 48 h, data not shown). Therefore, at 40 µM LFchimera had the best parasiticidal activity, followed by 40 µM of the drug metronidazole and LFcin17-30, LFampin 265-284, and bLF.

**LFcin17-30, LFampin265-284 and LFchimera interact with *Giardia intestinalis***

The majority of live trophozoites incubated with 2 µM FITC-Labeled LFcin17-30, LFampin265-284 or LFchimera showed bright green fluorescence (Figure 2, panels B, C and D, respectively). Trophozoites were counterstained with PI, which exhibits red fluorescence (Figure 2, panels A-D). At 30 min, all FITC-LFpeptides were visible in the trophozoites (green fluorescence, arrowheads), suggesting that the LFpeptides were endocytosed or internalized by *G. intestinalis*. Additionally, it would appear that degraded RNA and DNA are present in the images of trophozoites treated with the peptides (B-D). The controls (untreated trophozoites) were negative for the green fluorescence (A).

**Morphologic effects of LFcin17-30, LFampin265-284 and LFchimera on *Giardia intestinalis* trophozoites**
We examined cells after relatively short times of exposure to observe early and more direct changes, as well as those accompanying or secondary to cell lysis, by transmission electron microscopy (TEM). Untreated cells had a smooth cellular membrane (cm, double-headed arrow), peripheral vacuoles (pv, lines) near the cellular membrane, three pairs of flagella (F, discontinuous arrow), adherent disk (ad), and two nuclei (N) and two nucleoli (no) (Figure 3, panel A). Magnification of the picture shows granules of electron-dense material (asterisk) distributed in the cytoplasm and the arrangement of the microtubules belonging to the adherent disk (ad) (panel B). Exposure to LFcin17-30 led to profound intracellular changes, such as an increase in electron-dense material in the cytoplasm (asterisk), reorganization of the flagella (F, discontinuous arrow) and displacement of the adherent disk (ad, arrow), Figure 3, panel C. In the magnified picture, there are no peripheral vacuoles (pv) near the cellular membrane (cm, double-headed arrow), Panel D. Treatment with LFampin265-284 also caused intracellular damage, including an increase in electron-dense material (asterisk), reorganization of the flagella (F, discontinuous arrow), a large hole in the cytoplasm (arrowhead), and also disruption in the cellular membrane (cm, double-headed arrow) (Figure 3, panel E). The magnified picture shows the large hole induced by treatment with LFampin265-284 (arrowhead, panel F). Trophozoites treated with LFchimera produced the most significant changes and damage in the trophozoites. There were marked changes in the electron-dense material in the cytoplasm (asterisks), the flagella (F, discontinuous arrow) were disrupted and reorganized, and the cytoplasm showed large holes (arrowheads) in which some electron-dense material is visible (Figure 3, panel G). In the magnified picture the larges holes (arrowheads) with aggregates inside them (asterisks) can be seen in more detail (Panel H). Shrunken and distorted peripheral vacuoles (pv, lines) were also observed.
G. intestinalis trophozoites treated with LFpeptides also were analyzed under scanning electron microscopy (SEM). Treated trophozoites exhibited damage on the cell surface (Figure 4). By SEM it was found that G. intestinalis cultures treated with LFcin17-30 (B), LFampin265-284 (C), or LFchimera (D) showed alterations in size, irregular form and perforations (arrows) compared with untreated trophozoites (which had the typical structure of G. intestinalis trophozoites) (A). Additionally, the large hole in the membrane of the trophozoite treated with Lfchimera shown in panel D revealed the presence of unusual aggregates.

**LFcin17-30, LFampin265-284 and LFchimera induced programmed cell death in Giardia intestinalis trophozoites**

LFcin17-30, LFampin265-284 and LFchimera induced early programmed cell death in G. intestinalis trophozoites treated with 40 µM of these peptides for 2 h (Figure 5, panels A, B, and C, respectively). The quadrants represent Q1: Necrotic cells, Q2: Necrosis and apoptotic cells, Q3: Earlier apoptotic cells, Q4: live cells. Cells treated with LFchimera and LFampin265-284 induced early programmed cell death as measured by the liberation of phosphatidylserine (PS) (Q3. 27.9 and 25.5%, respectively), and, to a lesser extent, early programmed cell death was also induced by treatment with LFcin17-30 (Q3. 7.66%). Although the total of the analyzed population were not undergoing programmed cell death, this result and the images observed under electron microscopy reinforce the idea that G. intestinalis is induced to undergo programmed cell death after treatment with LFpeptides.
Discussion

Although giardiasis has been a threat to mankind for thousands of years, this parasitic infection has been, until recently, relatively neglected. *G. intestinalis* is a major cause of parasite-induced diarrhea in humans and animals and is currently an important public health problem, mostly in developing countries but also in developed countries (Escobedo et al. 2010; Painter et al. 2015). Nearly 33% of people in developing countries have had giardiasis, and nearly 2% of adults and 6% to 8% of children have giardiasis worldwide (Escobedo et al. 2010; Furness et al. 2000; Painter et al. 2015). Although giardiasis is considered by most medical practitioners to be an easily treatable infection, prolonged symptoms due to, or following, *G. intestinalis* infection can significantly impact the quality of life (Painter et al. 2015; Vesy and Peterson 1999; Watkins and Eckmann 2014). Symptom recurrence, including abdominal symptoms and fatigue, can result from re-infection, treatment failure, and disturbances in the gut mucosa or post-infection syndromes (Watkins and Eckmann 2014). In developed countries, these sequelae can have an enormous impact on the quality of life; in developing countries, particularly in children, they add yet another burden to populations that are already disadvantaged. Infection with *G. intestinalis* remains latent because only a handful of agents have been used in therapy, and the agents that are available may have adverse effects or be contraindicated in certain clinical situations. Additionally, resistance may play a role in some infections (Vesy and Peterson 1999; Watkins and Eckmann 2014). Thus, research on the development of new compounds to combat giardiasis is needed.

In this work, we demonstrated that synthetic, bLF-derived LFcin17L30, LFampin265-284, and LFchimera have parasiticidal activity against *G. intestinalis* trophozoites in vitro. In...
in vitro giardicidal activity of native human and bovine LF, as well as their derived N-terminal peptides, has been observed previously (Turcany et al. 1995). Treated trophozoites showed ultrastructural damage, with lactoferrin and its N-terminal peptides causing striking and complex morphologic changes in the trophozoite plasmalemma, endomembrane and cytoskeleton and increasing the electron density of the lysosome-like peripheral vacuoles (Turcany et al. 1997). The synthetic peptides used in this work are different from those reported by Turcany et al. (1995), but the giardicidal activity, the binding and endocytosis of the peptides by the trophozoites and the damage induced at the ultrastructural level were similar. Neither Fe$^{3+}$, Fe$^{2+}$, nor other compounds such as MgCl$_2$, or CaCl$_2$, diminished or prevented the parasiticidal activity of LF peptides against *G. intestinalis* trophozoites (unpublished results). While we cannot directly compare our results with Turcany et al. (1995, 1997) because we used cultures from cysts of *G. intestinalis* directly isolated from patients that were forced to excyst in the laboratory, their results support our conclusion that LF peptides exhibit potent *in vitro* antigiardial activity.

LF chimera presented greater giardicidal activity in the viability assays and induced more pronounced damage to *G. lamblia* trophozoites at the ultrastructural level compared to its individual constituent peptides LFcin17-30 and LFampin265-284. All of the peptides at low concentrations had a dramatic parasiticidal effect when they were combined with the pharmacological drugs metronidazole or albendazole (used to treat giardiasis).

Regarding differences found with the two methods to estimate viability (PI and CFDA) in *G. intestinalis* treated with LF peptides, LF and drugs (Figures 1 A and 1 B, respectively), it is known that the fluorogenic dye PI is unable to traverse intact cell membranes; therefore,
only cells with disrupted or broken membranes are counterstained by PI. Consequently, PI is an indirect indicator of cellular viability. On the other hand, it has been established that an intact lipid bilayer slows the leakage of the fluorochrome CFDA from within intact cells, while injured or stressed cells cannot retain or accumulate the fluorochrome CFDA (dos Santos et al. 2015; Schupp et al. 1988). Additionally, the replication time is different in the distinct genotypes of *G. intestinalis*; consequently, the drug sensitivity data from *in vitro* studies in Giardia varies as a function of the replication time and the methodological techniques employed (Hahn et al. 2013). Therefore, both factors (different replication times and methodologies) could explain the differences of results obtained using PI and CFDA (Figures 1 A and 1B). Despite the differences in these methodologies, it is clear that LF and LFpeptides have a parasiticidal effect on *G. intestinalis* isolates, and data obtained by electron microscopy (Figures 3 and 4, respectively) corroborate our conclusion that LF and LF peptides exhibited giardicidal activity.

Regarding the mechanism of action, our confocal microscopy observations demonstrated that LFpeptides were bound and internalized by *G. intestinalis* trophozoites (Figure 2). We speculate that the localization of peptides inside *G. intestinalis* is an event involved in the mechanism of action of LF peptides against this protozoan. It is likely that LFpeptides caused damage to membranes, including internal membranes. In agreement with this idea, LFpeptides appeared to trigger an early programmed cell death or apoptosis-like event in *G. intestinalis* (Figure 5).

In higher eukaryotes, programmed cell-death (PCD) is the death of a cell in any form mediated by an intracellular program. Programmed cell death is a genetically regulated
process that is central to the development, homeostasis and integrity of multicellular organisms (Ameisen 2002). Interestingly, several molecules or pathways that regulate PCD in higher eukaryotes have been implicated in the death of unicellular organisms (Bruchhaus et al. 2007), and apoptosis-like programmed cell death (PCD) has been described in multiple primitive eukaryotes and protists, including unicellular parasites, meaning that unicellular organisms can commit suicide in response to various stimuli (Bruchhaus et al. 2007; Kaczanowski et al. 2011; van Zandbergen et al. 2010). *G. intestinalis* is a divergent amitochondrial eukaryote with a unicellular, bi-nucleated flagellated structure, but even this organism undergoes PCD (Bagchi et al. 2012). However, this is a pathway of autophagy and differs from the classical apoptosis of higher eukaryotes. Annexin-V and 7-AAD staining was used to analyze early programmed cell death in *G. intestinalis* (Bagchi et al. 2012; Ghosh et al. 2009). Annexin V (or Annexin A5) is a member of the annexin family of intracellular proteins and binds to phosphatidylserine (PS). Externalization of PS is an indicator of early apoptosis-like or programmed cell death in Giardia (Ghosh et al. 2009). 7-AAD (7-amino-actinomycin D) has a high DNA binding constant and is efficiently excluded by intact cells and bound by cells in necrosis or late programmed cell death. Both fluorescent dyes were measured in *G. intestinalis* using Acoustic Focusing Cytometer AttuneTM Blue/Red (Life Technologies). Our results demonstrate that all of the peptides induced early programmed cell death in *G. intestinalis* (Figure 5). These data are corroborated by the type of damage observed at the ultrastructural level (Figure 3, Panels C-H, Figure 4, panels B-D).

There is no previous evidence of apoptosis-like or programmed cell death induced by bLF in a parasite, but it has been reported that LF triggered programmed cell death in cells
infected with influenza virus (Pietrantoni et al. 2010), echovirus (Tinari et al. 2005), and
Listeria monocytogenes (Valenti et al. 1999). Additionally, LF triggers apoptosis or
apoptosis-like activity in microorganisms such as Saccharomyces cerevisiae (Acosta-
Zaldivar et al. 2016) and Candida albicans (Andres et al. 2008).

Further studies are needed to determine if LF and LFpeptides have an effect against G.
intestinalis in in vivo models. However, all data to date suggest that LFpeptides are active
compounds with the potential to combat giardiasis, alone or when combined with
chemotherapeutic drugs.

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and Inflammation.
References


Figure 1. LFcin17-30, LFampin265-284 and LFchimera have parasiticidal activity against *Giardia intestinalis* trophozoites. *G. intestinalis* cultures were incubated with 100 µM Metronidazole or 5 µM Albendazole (used as negative controls of viability) or 40 µM LFpeptides for 2 h at 37ºC (Panel A), or with different concentrations of Metronidazole (used as a negative control of culture growth), LF, or LFpeptides for 12 h at 37ºC (Panel B). Then, the samples were washed and viability or culture growth was determined by the exclusion of the dye PI (A) or inclusion of CFDA (B). In both cases (A and B), untreated cultures were used as positive controls of viability or growth. Experiments were performed at least three times in triplicate. The means and standard deviations are indicated in percentages. A value of P<0.05 was considered statistically significant (*).

Figure 2. LFcin17-30, LFampin265-284 and LFquimera are internalized by *Giardia intestinalis*. Trophozoites were untreated (A) or treated with 2 µM FITC-LFpeptides for 30 min at 37 ºC: LFcin17L30 (B), LFampin265L284 (C), and LFchimera (D). Then, samples were fixed, washed and permeabilized with 0.5% triton X-100 and counterstained with PI (red color). Finally, samples were processed for analysis by confocal microscopy. Arrows show the green fluorescence due to the FITC-LFpeptides (B-D). Bars 20 and 50 µm.

Figure 3. LFcin17-30, LFampin265-284 and LFchimera cause damage to *Giardia intestinalis*. Trophozoites were untreated (A, B) or treated with 40 µM LFcin17-30 (C, D), LFampin265-284 (E, F), or LFchimera (G, H), for 2 h at 37 ºC. Then, samples were
processed for analysis by transmission electron microscopy. The sections were analyzed and photographed using a JEOL 1010 transmission electron microscope at 80 kV.

Untreated cells had a smooth cellular membrane (cm, double-headed arrow), peripheral vacuoles (pv, lines) near the cellular membrane, three pairs of flagella (F, discontinuous arrow), adherent disk (ad), and two nuclei (N) and two nucleoli (no) (Figure 3, panel A). Magnification of the picture shows granules of electron-dense material (asterisk) distributed in the cytoplasm and the arrangement of the microtubules belonging to the adherent disk (ad) (panel B). Exposure to LFcin17-30 led to profound intracellular changes, such as an increase in electron-dense material in the cytoplasm (asterisk), reorganization of the flagella (F, discontinuous arrow) and displacement of the adherent disk (ad, arrow), Figure 3, panel C. In the magnified picture, there are no peripheral vacuoles (pv) near the cellular membrane (cm, double-headed arrow), Panel D. Treatment with LFampin265-284 also caused intracellular damage, including an increase in electron-dense material (asterisk), reorganization of the flagella (F, discontinuous arrow), a large hole in the cytoplasm (arrowhead), and also disruption in the cellular membrane (cm, double-headed arrow) (Figure 3, panel E). The magnified picture shows the large hole induced by treatment with LFampin265-284 (arrowhead, panel F). Trophozoites treated with LFchimera produced the most significant changes and damage in the trophozoites. There were marked changes in the electron-dense material in the cytoplasm (asterisks), the flagella (F, discontinuous arrow) were disrupted and reorganized, and the cytoplasm showed large holes (arrowheads) in which some electron-dense material is visible (Figure 3, panel G). In the magnified picture the large holes (arrowheads) with aggregates inside them (asterisks) can be seen in more detail (Panel H). Shrunken and distorted peripheral vacuoles (pv, lines) were also observed.
Figure 4. *LFcin17-30, LFampin265-284 and LFchimera cause damage to Giardia intestinalis.* Trophozoites were untreated (A) or treated with 40 µM of LFcin17-30 (B), LFampin265-284 (C), or LFchimera (D) for 2 h at 37 ºC. Then, samples were processed for analysis. Specimens were observed under a scanning electron microscope JEOL LSM6360LV. Trophozoites treated with LF peptides show alterations in size, irregular form and perforations (arrows, B-C), compared with untreated trophozoites (which had the typical structure of *G. intestinalis* trophozoites, A). Trophozoites treated with LFchimera exhibited aggregates or vesicles and several had a large hole in their membranes (arrow, panel D).

Figure 5. *Programmed cell death in Giardia intestinalis induced by LFpeptides.* Trophozoites of *G. intestinalis* were treated with 40 µM of LFcin17-30 (A), LFampin265-284 (B) or LFchimera (C) for 2 h at 37 ºC. Samples were stained with Allophycocyanin-Annexin (Annexin-APC, to stain Annexin V) and 7-Amino-Actinomycin D (7-AAD), processed and analyzed by flow cytometry. Q1: Necrotic cells, Q2: Necroptotic cells, Q3: Earlier apoptotic cells. Q4: live cells. One of three representative experiments is shown.
Table I. Metronidazole, albendazole and LFpeptides needed alone or in combination to kill >95% of *Giardia intestinalis* trophozoites

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentrations (µM)</th>
<th>LFcin 17-30 (µM)</th>
<th>LFampin 265-284 (µM)</th>
<th>LFchimera (µM)</th>
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<tr>
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<td>-</td>
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<td>5</td>
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<tr>
<td><em>Albendazole</em></td>
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<td><em>Albendazole</em></td>
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<td>LFchimera</td>
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</tr>
</tbody>
</table>
Figure 1

A

B

Viability (%)

Growth (%)

Untreated
Metronidazole
Abnormal
LFcin17-30
LFamp565-284
LFehhiera

1 μM
5 μM
10 μM
20 μM
40 μM

*
Figure 2

(A) 

(B) 

(C) 

(D)
Figure 3
Figure 4
Figure 5