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Natural sesquiterpene lactones induce programmed cell death in *Trypanosoma cruzi*: A new therapeutic target?

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ABSTRACT

Background: Chagas disease or American Trypanosomiasis is caused by the flagellated protozoan parasite *Trypanosoma cruzi* (*T. cruzi*) and is recognized by the WHO as one of the world's 17 neglected tropical diseases. Only two drugs (Benznidazol, Bz and Nifurtimox, Nx) are currently accepted for treatment, however they cause severe adverse effects and their efficacy is still controversial. It is then important to explore for new drugs.
Purpose: Programmed cell death (PCD) in parasites offers interesting new therapeutic targets. The aim of this work was to evaluate the induction of PCD in *T. cruzi* by two natural sesquiterpene lactones (STLs), dehydroleucodine (DhL) and helenalin (Hln) as compared with the two conventional drugs, Bz and Nx.
Material and Methods: Hln and DhL were isolated from aerial parts of *Gaillardia megapotamica* and *Artemisia douglassiana Besser*, respectively. Purity of compounds (greater than 95%) was confirmed by ^{13}C-nuclear magnetic resonance, melting point analysis, and optical rotation. Induction of PCD in *T. cruzi* epimastigotes and trypomastigotes by DhL, Hln, Bz and Nx was assayed by phosphatidylserine exposure at the parasite surface and by detection of DNA fragmentation using the TUNEL assay. Trypanocidal activity of natural and synthetic compounds was assayed by measuring parasite viability using the MTT method.
Results: The two natural STLs, DhL and Hln, induce programmed cell death in both, the replicative epimastigote form and the infective trypomastigote form of *T. cruzi*. Interestingly, the two conventional antichagasic drugs (Bz and Nx) do not induce programmed cell death. A combination of DhL and either Bz or Nx showed an increased effect of natural compounds and synthetic drugs on the decrease of parasite viability.
Conclusion: DhL and Hln induce programmed cell death in *T. cruzi* replicative epimastigote and infective trypomastigote forms, which is a different mechanism of action than the conventional drugs to kill the parasite. Therefore DhL and Hln may offer an interesting option for the treatment of Chagas disease, alone or in combination with conventional drugs.

Introduction

Chagas disease or American Trypanosomiasis is caused by the flagellated protozoan parasite *Trypanosoma cruzi* (*T. cruzi*) and is recognized by the World Health Organization as one of the world's 17 neglected tropical diseases (Guhl and Ramirez 2013; Munoz-Saravia et al. 2012; Schmunis 2007). It has been identified by the Center for Diseases Control in the U.S.A. as one of the five neglected diseases targeted for public health action (http://www.cdc.gov/parasites/npi.html). Currently, around 10 million individuals are infected with *T. cruzi* in endemic areas while it has been increasingly diagnosed in non-endemic areas with an estimation of 325,000 cases in the U.S.A. and about 100,000 in Europe (Le Loup et al. 2011; Lescure et al. 2010; Schmunis and Yadon 2010).

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T. cruzi presents an indirect life cycle, affecting mammals, including man, as its definitive hosts. In these hosts, the parasite develops in two forms: the circulating non-replicative trypomastigotes and the intracellular replicative amastigotes. Chagas disease is mainly transmitted by Reduvidae hematophagous insects that upon feeding on an infected mammal ingest blood trypomastigotes that, in the insect gut, will transform to epimastigotes, the replicative extracellular forms of the parasite (Jimenez et al. 2008; Rassi et al. 2012). These forms differentiate to infective metacyclic trypomastigotes in the hindgut of the bug and are deposited with the feces when the insect feeds on a vertebrate. These parasite forms enter the mammal body when the individual scratches the skin wound inflicted by the insect's bite or through permissive mucosa or conjunctive membranes, being able to invade almost any nucleated cell. Additionally, the parasite can be transmitted congenitally and orally by ingestion of parasite-contaminated food (Schmunis and Yadon 2010; Toso et al. 2011).

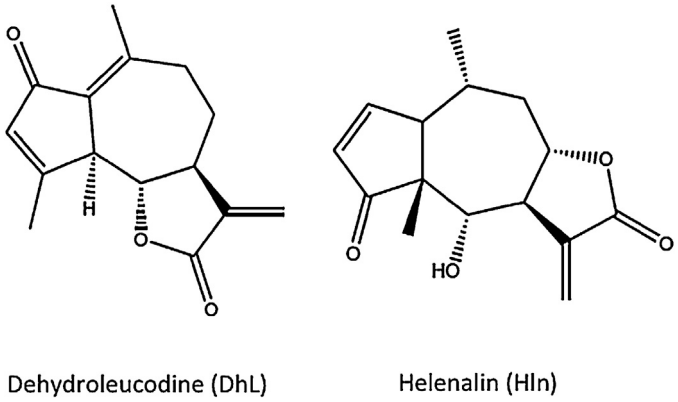
Chagas disease occurs in two phases: acute and chronic. The acute phase can be either asymptomatic or symptomatic with cardiac and neurologic symptoms. Patients who have not cleared the infection enter the chronic phase where around two-thirds of patients will remain in the asymptomatic indeterminate stage, whereas the remaining one-third becomes symptomatic. Of these, on average two-thirds develop a cardiac form of the disease and one-third develops a gastrointestinal form. Progression from the indeterminate phase to a symptomatic form can take years or even decades (Lescure et al. 2010).

Introduced in the 1960s and 1970s nifurtimox (Nx) and benznidazole (Bz), two nitroheterocyclic drugs, are currently used for treatment of Chagas disease although their availability is limited and are not approved by the FDA. Bz and Nx are effective for treating acute infections but both cause undesirable side effects, frequently leading to abandonment of the treatment. Their efficacy during the chronic phase is still controversial with a lack of consensus about the methods for evaluating parasitological cure (Cerecetto and Gonzalez 2002; Mejia et al. 2012; Menna-Barreto et al. 2009). Moreover, treatment failure due to drug inefficacy is known to occur even during acute infection, the stage in which anti-parasitic drug therapy is most effective (Machado et al. 2010).

The limited availability and efficacy of the drugs as well as the absence of an effective and safe vaccine underscore the relevance of developing alternative therapeutic approaches. The current views are based on increasing our understanding about the biology of the host–parasite interaction and on the of discovery new drugs that can selectively target the parasite.

Apoptosis is a form of cell death in which a programmed sequence of events leads to the elimination of cells without inducing or evoking inflammatory responses (Kroemer et al. 2009; Smirlis and Soteriadou 2011). Although it was originally considered a characteristic of multicellular organisms, we among others, have shown, that *T. cruzi* undergoes programmed cell death during the stationary phase of growth in axenic culture or under nutrient deprivation (Jimenez et al. 2008; Smirlis and Soteriadou 2011). Since parasitic protozoa present a complex and multiphase life cycle involving a variety of differentiation events, it has been proposed that regulated cell death operates to limit proliferation, virulence and pathogenicity, thereby promoting transmission of the parasites (Debrabant et al. 2003; Nguewa et al. 2004; Proto et al. 2013).

Cell death in parasitic protozoa shares some morphological features with mammalian apoptosis, although most components involved in the apoptotic pathways of multicellular organisms have no identified homologs in protozoa (Fernandez-Presas et al. 2010). The controversy regarding the proper denomination of this new form of programmed cell death remains, but the presence of a distinct cell death machinery compared with the mammalian hosts represents a great opportunity for exploring new therapeutic targets against trypanosomes (Jimenez et al. 2008).



Dehydroleucodine (DhL) Helenalin (Hln)

Fig. 1. Structure of the sesquiterpene lactones dehydroleucodine and helenalin.

Sesquiterpene lactones (STL) are a group of bioactive substances consisting of a broad spectrum of sesquiterpenes which have been identified in a number of plant families such as Acanthaceae, Apiaceae, Lauraceae, Magnoliaceae, Rutaceae, and Asteraceae (Saeidnia et al. 2013). Several of those compounds present trypanocidal activity (Barrera et al. 2008; Brengio et al. 2000; Jimenez-Ortiz et al. 2005; Saeidnia et al. 2013). There are several mechanisms of action proposed for STL, including DNA alkylation (Amorim et al. 2013) and generation of oxidative stress (Barrera et al. 2013). Here we show that two STLs, dehydroleucodine (DhL) and helenalin (Hln) (Fig. 1) induce cell death with morphological features compatible with programmed cell death in both, the replicative epimastigote form and the infective trypomastigote form of *T. cruzi*. We also show that the anti-parasitic action of the two conventional antichagasic drugs (Bz and Nx) does not show similar characteristics, but the combination of DhL with Bz or Nx increases the trypanocidal activity with promising results.

Materials and methods

Reagents

All chemicals used were of analytical grade. Fetal bovine serum (FBS) (GIBCO®), Culture media (GIBCO®), heminand buffer reagents (SIGMA®); annexin-V FITC and propidium iodide (BD Pharmingen®); TUNEL Kit (Promega®); DAPI (Pierce®); MTT reagents (SIGMA®).

Extraction and purification of sesquiterpene lactones

Hln and DhL were isolated from aerial parts of *Gaillardia megapotamica* and *Artemisia douglassiana Besser*, respectively (Brengio et al. 2000; Jimenez-Ortiz et al. 2005). The purity of the compounds (greater than 95%) was confirmed by ^{13}C-nuclear magnetic resonance, melting point analysis, and optical rotation. Due to limited solubility in water, STL stock solutions were prepared in dimethylsulfoxide (DMSO). For all the experiments, DMSO at a final concentration of 0.25% was added to the parasites as a vehicle control.

Epimastigote culture

T. cruzi epimastigotes (Tulahuen, MF and DM28c strain) were cultured at an initial density of 3×10^6 parasites/ml at 28 °C, in monophasic Diamond's culture medium supplemented with 75 μM

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hemin, 5% fetal bovine serum (FBS), 100 μg/ml sodium penicillin and 100 μ/ml streptomycin.

Cell culture, infection with T. cruzi and trypomastigote harvesting

VERO cells (ATCC® CCL-81) were grown in RPMI medium enriched with 5% FBS and antibiotics (penicillin–streptomycin). Cells were grown at 37 °C in a humid atmosphere at 5% CO_2 for 96 h, replacing the medium every 24 h (Urbina et al. 2003). After confluence, VERO cells were incubated with a culture of epimastigotes in late stationary phase, which increases the percentage of trypomastigotes approximately to 5% (Contreras et al. 1985). Trypomastigotes invade fibroblasts and replicate intracellularly as amastigotes. After 72 h, amastigotes transform back to trypomastigotes that lyse the host cells. Cells and debris were eliminated by low speed centrifugation (500 × *g*), obtaining the trypomastigotes in the supernatant (Villalta and Kierszenbaum 1982).

Cell viability assay

The effect of drug treatments on parasites was evaluated by propidium iodide exclusion or by the MTT viability assay. Briefly, the parasites were cultured in the absence or presence of the STLs, which were dissolved in 1% dimethylsulfoxide (DMSO) (final concentration less than 0.25% v/v), for 24 h.

Propidium iodide exclusion

Parasites were pelleted at 1000 × *g* for 5 min and washed once with phosphate-buffered saline (PBS). They were then re-suspended in 1 ml of 1 μg/ml propidium iodide in PBS pH 7.2 and kept for 2 min at room temperature before observation. The percentage of stained cells (dead parasites) was determined by fluorescence microscopy.

MTT

10 μl of 5 mg/ml tetrazolium dye (MTT; 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) plus 0.22 mg/ml phenazine metosulfate (as electron carrier), was added to aliquots of parasite suspension in 100 μl RPMI 1640 without phenol red. After incubation for 4 h at 37 °C, the water insoluble formazan dye generated was solubilized by addition of 100 μl of 10% w/v SDS in 0.01 M HCl. The plates were further incubated overnight at 37 °C, and optical density (OD) of the wells was determined using a microplate reader (Labsystems Multiskan MS®) at 570 nm. Under these conditions, the OD is directly proportional to the viable cell number in each well (Muelas-Serrano et al. 2000).

Exposed phosphatidylserine detection

Exposed phosphatidylserine (PS) was detected using Annexin-V FITC (BD Pharmingen®). Parasites were collected by centrifugation at 1000 × *g*, washed twice in PBS and re-suspended in 100 μl of binding buffer (140 mM NaCl, 5 mM $CaCl_2$, 10 mM HEPES-Na pH 7.4). Annexin-V FITC and propidium iodide were added at the final concentration indicated by the manufacturer. Fluorescence was measured by FACS analysis in a FACSort apparatus (Becton Dickinson & Co®). Data were processed with WinMDI 2.8 software considering parasites suffering programmed cell death, those stained by annexin-V (propidium iodide positive or negative) and, as necrotic, those only positive to red stain.

DNA fragmentation

We analyzed DNA double-strand ruptures *in situ* by TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling) assay according to manufacturer's protocol (DeadEnd Fluorometric TUNEL system, Promega). Briefly, parasites were collected by centrifugation at 1000 × *g*, washed twice in PBS and resuspended in the same buffer. Once placed on a slide and dried at room temperature, the cells were fixed with 4% p-formaldehyde and washed in PBS. After permeabilization with 0.2% Triton X-100, the cells were incubated with reaction mix containing dUTP-FITC (fluorescein isothiocyanate). Nuclei were counterstained with DAPI (4,6-diamidin-2-phenylindol) (1 μ/ml) or propidium iodide (1 μ/ml). Fluorescence was observed in a Nikon Eclipse E400 microscopy and pictures were captured with a Nikon Coolpix 4500 digital camera. Results were quantified counting about 200 cells in duplicate from three independent experiments. Percentage of TUNEL positive cells was obtained from the ratio between TUNEL positive and total cells.

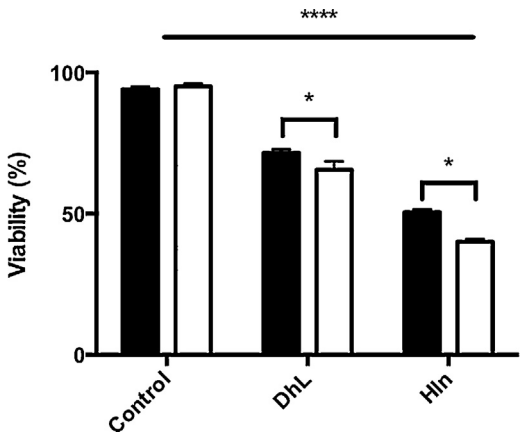

Fig. 2. DhL and Hln are cytotoxic against *T. cruzi* epimastigote and trypomastigote forms: Viability of *T. cruzi* epimastigotes (black bars) and trypomastigotes (white bars) was determined by exclusion of propidium iodide. Parasites were incubated in presence and absence of 10.2 μM DhL or 3.8 μM Hln for 24 h. All values are given as mean ± S.D. and correspond to at least 3 independent experiments carried out in triplicates. Data were analyzed by ANOVA followed by Dunnett's post-test; in addition, the differences between the epimastigote and trypomastigote forms respect to the same treatment, was analyzed by Student *t* test (*), ****$p < 0.0001$; *$p < 0,05$.

Statistics

Results are expressed as mean ± S.D of at least three independent experiments, in triplicate. Statistical significance was evaluated by ANOVA followed by Dunnett's post-test or Student's *t*-test as indicated.

Results

DhL and Hln have cytotoxic activity against T. cruzi epimastigote and trypomastigote forms

We have shown previously that DhL and Hln (Fig. 1) are cytotoxic agents against epimastigotes, the not infective form of the parasite (Brengio et al. 2000; Jimenez-Ortiz et al. 2005). Here we have compared the effect of DhL and Hln on both, epimastigotes (Fig. 2 black bars) and infective trypomastigotes (Fig. 2 white bars). After 24 h of incubation, control epimastigotes presented a viability of 94.5 ± 1.3% while parasites exposed to DhL (10.2 μM) or Hln (3.8 μM) showed a significant decrease in viability to 71.4 ± 1.4% and to 50.5 ± 1.1%, respectively. In trypomastigotes both DhL and Hln have a more pronounced effect, decreasing their viability to 65.4 ± 3.2% and to 40.1 ± 1.2%, respectively (Fig. 2, white bars). These results suggest that infective trypomastigotes are more susceptible to the effect of the STLs than replicative non-infective epimastigotes. STLs are more potent against trypomastigotes than the current antichagasic agents Bx and Nx that only decreased the

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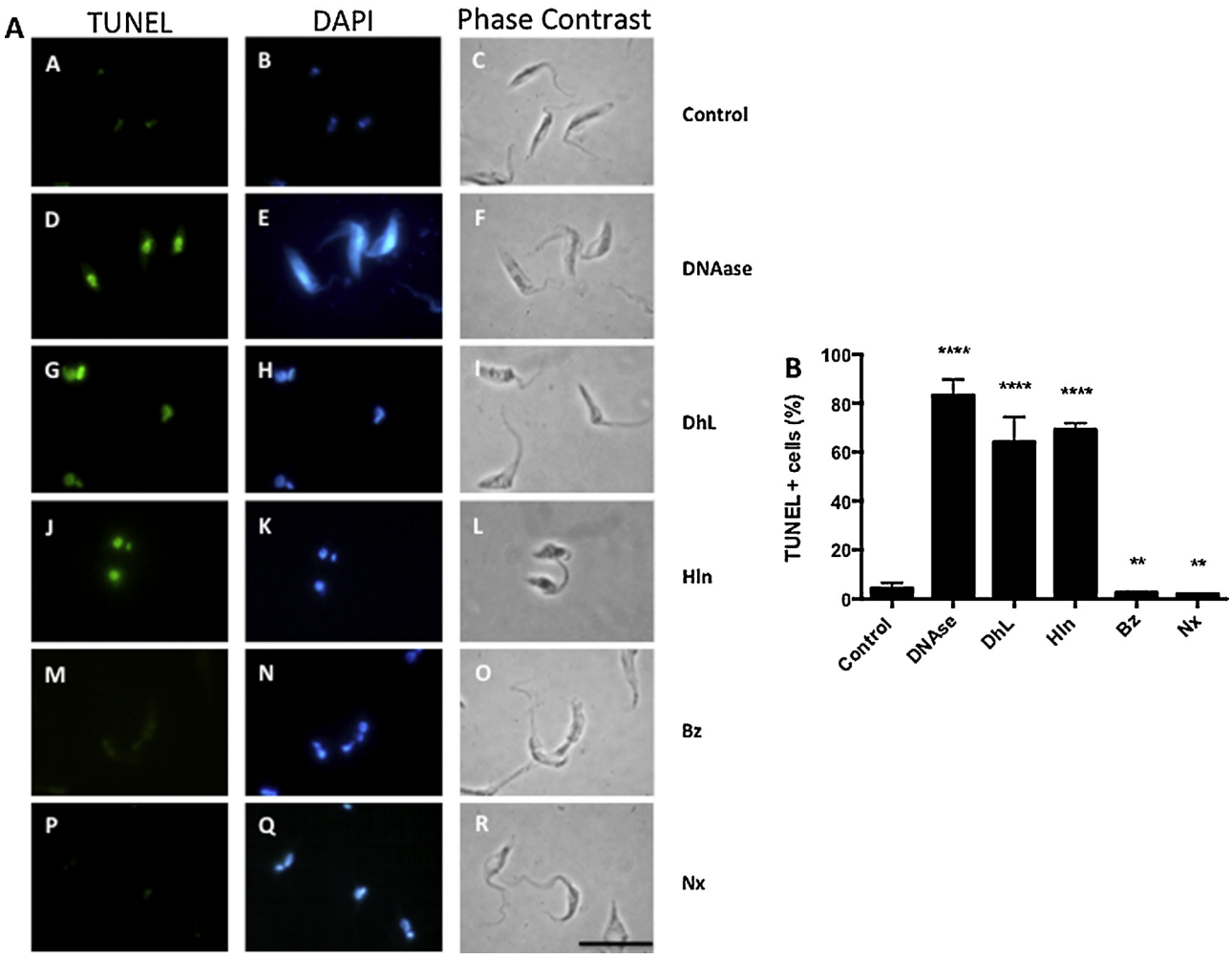

Fig. 3. DhL and Hln induce DNA fragmentation in *T. cruzi* epimastigote forms: A. TUNEL assay: DNA fragmentation was detected by the TUNEL method (Kit Promega) (A, D, G, J, M, P) and nuclei were counterstained with DAPI (B, E, H, K, N, Q). Panels C, F, I, L, O, R corresponds to phase contrast images. *T. cruzi* epimastigotes incubated with 10.2 μM DhL (G–I) or 3.8 μM Hln (J–L) during 24 h showed a significant increase in TUNEL(+) cells as compared to non-treated parasites (Control: A–C). Contrarily, *T. cruzi* epimastigotes incubated with 20 μM Bz (M–O) or 10 μM Nx (P–R), showed no increase in DNA fragmentation. Parasites were treated with DNAse as positive control (DNAse: D–F). B. Percentage of TUNEL (+) cells for each condition. Data are means ± SD and were analyzed by ANOVA followed by Dunnett's post-test (****$p \leq 0.0001$; **$p \leq 0.01$). Scale bar 20 μm.

viability of infective forms to 63.1 ± 6.3 and 67.5 ± 4.0%, respectively (Fig. 6).

Our previous studies had shown that Hln induces some morphological changes compatible with apoptotic cell death (Jimenez-Ortiz et al. 2005). Therefore, we studied whether the cytotoxic effect of DhL and Hln was associated with programmed cell death features in this parasite.

DhL and Hln induce morphological and biochemical changes compatible with programmed cell death

Internucleosomal fragmentation of DNA occurs in the late phase of apoptotic cell death by activation of nucleases (Kroemer et al. 2009) and is one of the distinctive characteristics of apoptotic cell death. We evaluated by TUNEL assay whether this phenomenon is present in parasites treated with STLs. In *T. cruzi* control epimastigotes there is no significant DNA fragmentation (Fig. 3A-Control) compared with parasites treated with DhL (Fig. 3A-DhL), Hln (Fig. 3A-Hln) or DNAse (Fig. 3A-DNAse). Control cells showed 4.4 ± 2.0% positive TUNEL parasites while 64.1 ± 10.3% and 69.2 ± 2.5% of TUNEL positive parasites were observed after 24 h incubation with DhL and Hln, respectively (Fig. 3B). In presence of DNAse the percentage of TUNEL positive increases to 83.3 ± 6.4, $p \leq 0.0001$ (Fig. 3B). Contrarily, parasites treated with the conventional antichagasic drugs, Bz (Fig. 3A and B: Bz) and Nx (Fig. 3A and B: Nx) showed no significant number of TUNEL positive cells (2.5 ± 0.5% and 1.9 ± 0.05%, respectively).

We also evaluated the effect of STLs on infective trypomastigotes. Parasites incubated with both STLs for 24 h showed an increase in DNA fragmentation (Fig. 4: DhL; Hln) compared with the controls (Fig. 4: Control). The morphology of parasites was also deeply affected by the STLs treatment. Cells where smaller, rounded and formed aggregates, similar to what has been established as morphological features of apoptosis (Kroemer et al. 2009).

Phosphatidylserine (PS) exposure in the outer side of the plasma membrane is widely considered as an early marker of apoptotic cell death (Galluzzi et al. 2012). In *T. cruzi* epimastigotes (Tulahuen strain) incubated with DhL (Fig. 5B, D) or Hln (Fig. 5C, D) PS exposure significantly increased (DhL 22.1 ± 1.0% and Hln 28.4 ± 2.3%) compared with control parasites (3.9 ± 0.5%, Fig. 5A, D). Again, these results are suggesting that STLs cytotoxic effect could be mediated by programmed cell death induction.

The trypanocidal activity of DhL is increased when combined with Bz or Nx

Conventional antichagasic drugs, have a trypanocidal effect based on the generation of cytotoxic metabolites in the parasites

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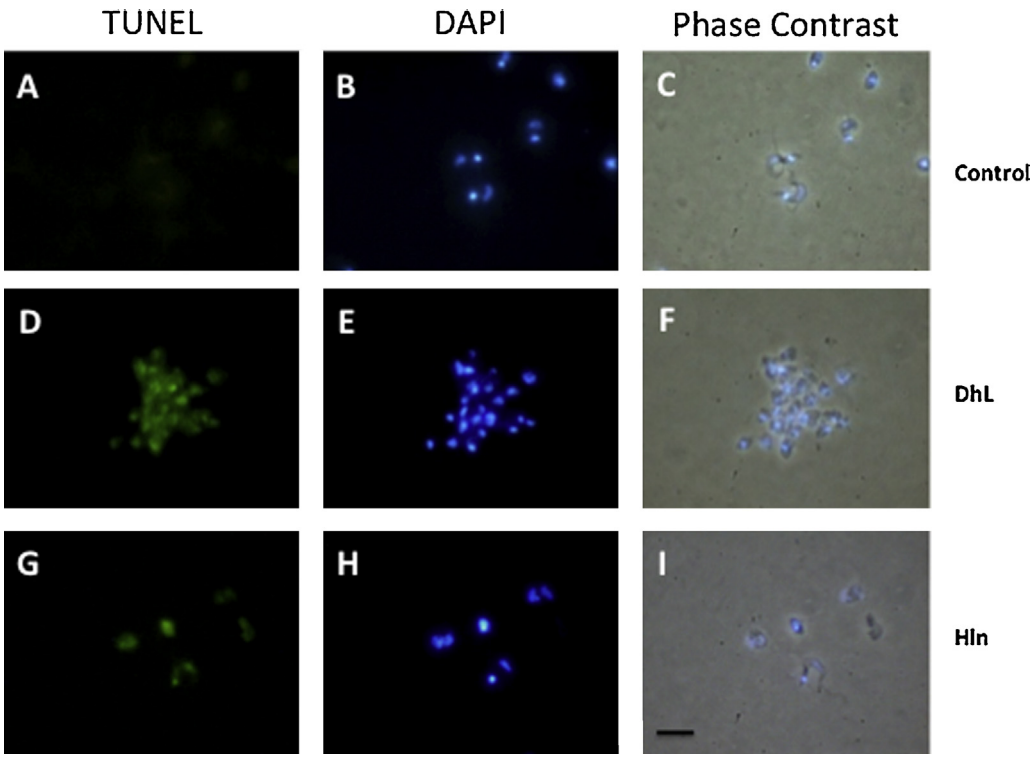

Fig. 4. DhL and Hln induce DNA fragmentation in *T. cruzi* trypomastigote forms: *T. cruzi* trypomastigotes incubated with 10.2 μM DhL (D–F) or 3.8 μM Hln (G–I) during 24 h showed a significant increase in TUNEL(+) cells as compared to control parasites (A–C). DNA fragmentation was detected by TUNEL assay (Kit Promega) (A, D, G) and nuclei were counterstained with DAPI (B, E, H). Panels C, F, I corresponds to phase contrast images. Scale bar 20 μm.

(Hall et al. 2011; Maya et al. 2010) but they do not induce DNA fragmentation (Fig. 3B). Our results show that STLs cytotoxicity seems to be the result of the induction of programmed cell death. Since both types of compounds have a different mechanism of action, we then assayed the combined effect of DhL with Bz or Nx. *T. cruzi* trypomastigotes (DM28c strain) were incubated with one or a combination of drugs using their respective IC_{50} values (Fig. 6). Bz and Nx decreased the viability of trypomastigotes to 63.0 ± 6.2 and 67.5 ± 4.0%, respectively. DhL alone decreases the viability of the parasites to 42.9 ± 7.3% while in combination with Bz and Nx the viability decreased to 20.9 ± 3.2 and 14.7 ± 2.0%, respectively. This decrease in the viability may be the result of the cumulative cytotoxic effect from both drugs on the parasites. Moreover, the magnitude of the combined effect does not indicate a trend toward synergy or potentiation, but suggests a potential additive effect. In any case, a checkerboard experimental design would be required to perform a proper isobolographic analysis of synergy. Assays on the effect of Bz or Nx with 10.2 μM DhL on mammalian fibroblast (VERO® cells) did not show cytotoxic effects (data not shown).

Discussion

Current treatment of Chagas disease relies on two drugs, Bz and Nx, discovered empirically more than three decades ago (Boiani et al. 2010; Cerecetto and Gonzalez 2002; Mejia et al. 2012). The aims for treatment of Chagas disease are (1) to cure the infection in the acute phase; (2) to prevent organ damage in chronic asymptomatic infection, and (3) to limit incapacity and prevent morbidity and mortality once the disease is already clinically manifested (Rassi et al. 2012). Traditionally associated to endemic countries, the epidemiology of Chagas disease is changing with an increase of the number of cases in non-endemic countries, mainly, due to human immigration and relocation of the vectors. This underscores the global impact of the disease as well as the urgent need for the development of new anti-trypanosomal agents with lower toxicity and higher activity, particularly for the chronic phase of the disease (Izumi et al. 2011; Veiga-Santos et al. 2013).

Natural products area growing source of new drugs with activity against different types of pathogens. Ancient customs have led to modern plant research because, for centuries, plant extracts were the only known medicines available. Today, a large number of natural compounds have been investigated (Izumi et al. 2011). The search for better-tolerated and cheaper compounds is particularly relevant in countries in which Chagas disease is endemic.

STLs are a large and structurally diverse group of plant metabolites; many members of which display anti-tumor effects (Costantino et al. 2013) and anti-protozoan activities, among others (Amorim et al. 2013; Barrera et al. 2008; Jimenez-Ortiz et al. 2005; Saeidnia et al. 2013).

The alkylation of nucleophiles (such as thiol-containing cysteine residues in proteins, free intracellular GSH and DNA) by the electrophilic moieties of the STLs is the most common mechanism leading to macromolecular dysfunction (Amorim et al. 2013). In addition to DNA alkylation, disruption of a calcium pump in the endoplasmic reticulum, increased generation in iron-dependent free radicals, control of nuclear factor kB (NFkB), activation of the tumor suppressor p53 and alteration of the epigenetic code have been described as mechanisms of action of STLs (Costantino et al. 2013). The induction of those mechanisms explains the cytotoxicity of the STLs and may also sustain, depending on the concentration or type of compound used, the type of cell death.

DhL reduces cell proliferation and induces apoptotic cell death in cancer cells. HeLa cells treated with DhL show increased focal organization of DNA-damage sensors (c-H2AX and 53BP1) in addition to increased levels and activity of p53. It has been proposed, that the apoptosis induced by DhL might be preceded by a failure to progress normally through the cell cycle in cells with high levels of damaged DNA (Costantino et al. 2013). Similarly, here we

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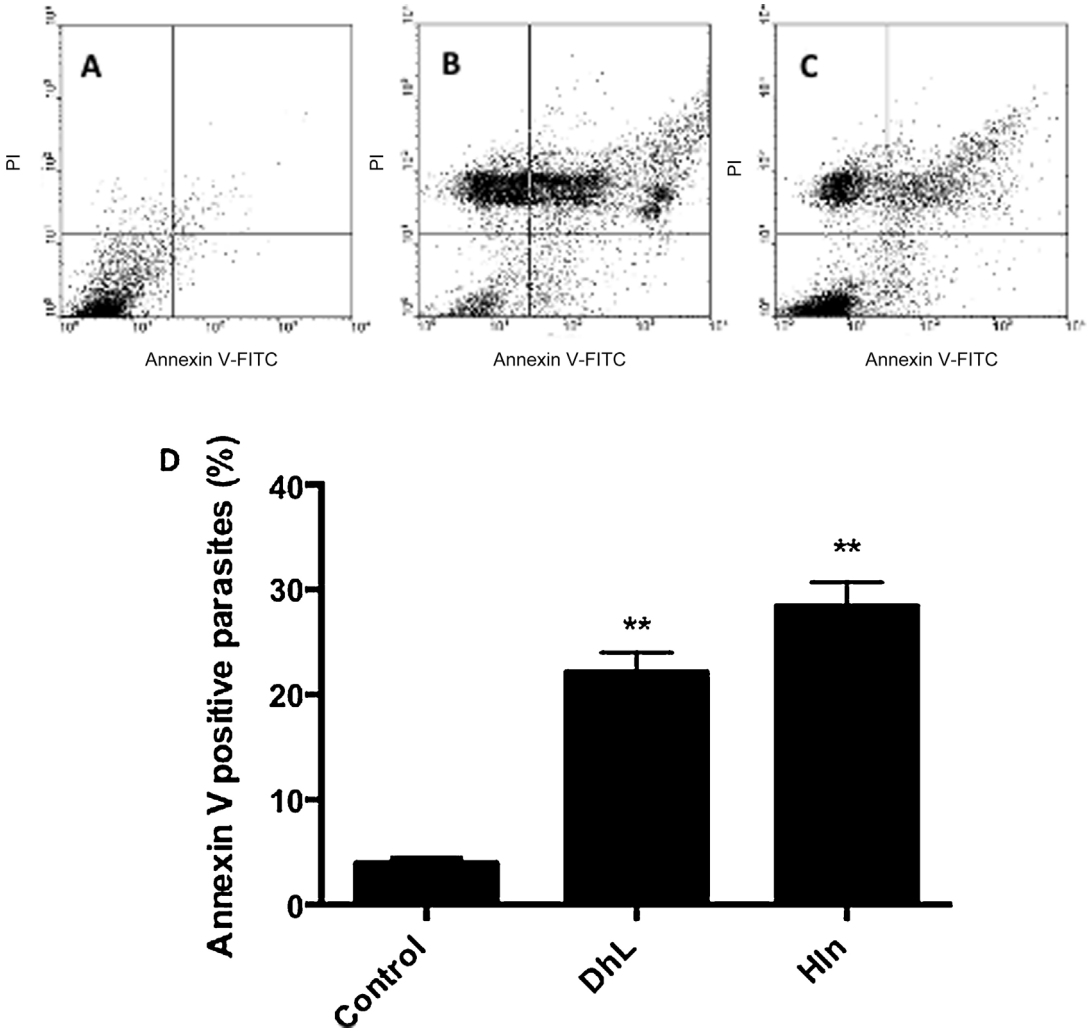

Fig. 5. DhL and Hln induce extracellular phosphatidylserine exposure in *T. cruzi* epimastigotes: *T. cruzi* epimastigotes incubated with 10.2 μM DhL (B, D) or 3.8 μM Hln (C, D) during 24 h were double stained with annexin V-FITC and propidium iodide. Phosphatidylserine exposure was measured by FACS analysis. Parasites incubated with DhL or Hln showed a significant increase in Annexin V(+) cells as compared to control parasites. Panels A–C show representative FACS analysis of three independent experiments. B shows the quantification of phosphatidylserine exposure. Data are means ± SD and were analyzed by ANOVA followed by Dunnett's post-test (**$p \leq 0.01$).

show that DhL induces cell death, DNA damage, PS exposure and morphological features compatible with programmed cell death in both replicative epimastigote and non-replicative trypomastigote forms of the parasite.

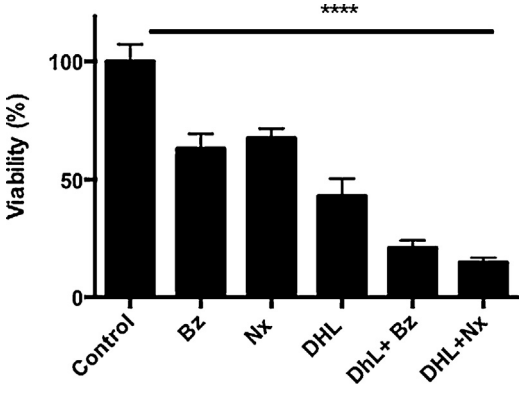

Fig. 6. The trypanocidal activity of DhL is increased when combined with Bz or Nx: *T. cruzi* trypomastigote were incubated in absence or in presence of 20 μM Bz or 10 μM Nx with and without 10.2 μM DhL. Parasite viability was assayed by the MTT method. Data are means ± SD and were analyzed by ANOVA followed by Dunnett's post-test (****$p \leq 0.0001$).

Helenalin induces apoptosis in several cancer cell lines such as Caki (human renal clear cell carcinoma), ACHN (human renal carcinoma), HT29 (human colon carcinoma) and HCT116 (human colon carcinoma) *via* reactive oxygen species (ROS) production (Jang et al. 2013). In activated CD4+ T cells, Hln induces cell death by triggering the mitochondrial apoptotic pathway by rapid stabilization of p53, nuclear localization of p53 and AIF, and an increase in ROS production that results in loss of mitochondrial membrane potential (Berges et al. 2009). In addition, it has been described that Hln mediates autophagic cell death by inhibition of NF-κ B p65 in other cancer cell lines, specifically in A2780 (human ovarian cancer cell line), RKO (colon carcinoma cancer cell line) and MCF-7 (breast adenocarcinoma cancer cell line) (Lim et al. 2012). It has been previously shown that STLs reduce the glutathione content and increase the production of ROS in trypanosomatids (Barrera et al. 2013) and that the cytotoxic effect of the STLs can be prevented by adding reducing agents like DTT or GSH (Brengio et al. 2000; Jimenez-Ortiz et al. 2005). It is reasonable to speculate that the cytotoxic effect that we observed in *T. cruzi* is the result of an increase in the production of ROS that could induce mitochondrial calcium overload, leading to mitochondrial dysfunction and activation of nucleases triggering programmed cell death in the parasites. This could explain the features observed in parasites treated with

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DhL and Hln, but additional experimental evidences are necessary to support this hypothesis.

The activation or prevention of cell death constitutes a critical factor in the outcome of an infection since it can facilitate or difficult the pathogen control and spreading. Apoptosis in the hosts can be managed during the infection with microorganisms, such as bacteria, viruses and protozoa (de Souza et al. 2003). Pathogen induced modulation of the host cell-death pathway serve to eliminate key immune cells or evade host defences that can act to limit the infection (Duaso et al. 2011; Weinrauch and Zychlinsky 1999).

Induction of programmed cell death in *T. cruzi* is a particularly interesting mechanism of action for an antichagasic drug, due to the above mentioned fact, that apoptosis suppresses the inflammatory response. In chronic chagasic myocarditis the maintenance of a pro-inflammatory response is the cause of an excessive or uncontrolled heart inflammation (Rosenberg et al. 2010; Vieira et al. 2012). Therefore inducing apoptosis-like cell death in the parasite not only allows killing the pathogen but also could contribute to the modulation of the inflammatory response, with beneficial effects on the infected host tissues.

T. cruzi presents a variety of invasion and infection mechanisms and is able to parasitize different tissues and organs which have their own particular responses against the parasite. Therefore treatment strategies should consider more than one therapeutic target. Probably, the success of future treatments for parasitic diseases lays on the combination of the conventional anti-parasitic drugs with other synthetic or natural compounds, which, for example, could modulate the immune response (Castillo et al. 2013; Molina-Berrios et al. 2013; Molina-Berríos et al. 2013) or presents a different mechanism of action. Therefore, a multi-drug approach could be more effective and better-tolerated for the treatment of these neglected tropical diseases.

Conclusion

The STLs DhL and Hln have a cytotoxic effect and induce programmed cell death features in *T. cruzi* epimastigote and trypomastigote forms, showing a different mechanism of action than the conventional anti-chagasic drugs. Therefore DhL and Hln may offer an interesting option for treatment of Chagas disease, alone or in combination with conventional drugs and this therapeutic possibility as well as the study of molecular pathways involved in cell death deserves further exploration.

Conflict of interest

The authors have declared no conflict of interest.

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