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CYTOTOXIC SCREENING AND IN VITRO EVALUATION OF PENTADECANE AGAINST *LEISHMANIA INFANTUM* PROMASTIGOTES AND AMASTIGOTES

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ABSTRACT: Pentadecane is an organic compound that is made up of primarily carbon and hydrogen atoms. Pentadecane is a floral volatile found in many different plants and essential oils and also in crude extracts of some plants, and it shows antimicrobial activity. This study investigated in vitro effects of pentadecane in *Leishmania infantum* parasites and found that it decreases growth by 86 ± 2% both in promastigotes (half maximal inhibitory concentration [IC_{50}] = 65.3 μM) and in amastigotes (IC_{50} = 60.5 μM), resulting in a reduction of macrophage infection; growth inhibition was 77% at 300 μM. Analysis of propidium iodide incorporation in *L. infantum*, treated with pentadecane at 48 hr, suggested that cells were arresting in the sub-G0/G1 and G1 phases of the cell cycle, whereas cytotoxicity assay of pentadecane in immortalized cells lines DH82 and U937 and in primary epithelial cells of Cercopiteco showed that it caused negligible cytotoxic effect. This study shows that pentadecane has antimicrobial activity against *L. infantum* parasites in in vitro culture.

Leishmaniasis, caused by the intracellular protozoan *Leishmania infantum* and transmitted by the bite of phlebotomine sand flies, is endemic in 98 countries, with more than 350 million people at risk. Visceral leishmaniasis (VL) caused by parasites of the *Leishmania donovani* complex is a severe human disease that often leads to death if left untreated (World Health Organization, 2010; Maia et al., 2013). Domestic dogs are considered the major host of *Leishmania infantum*, one of the species from the *L. donovani* complex, and this species plays a central role in the transmission cycle to humans by phlebotomine sand flies (Ashford, 2000; Courtenay et al., 2002; Manna et al., 2006). In the absence of effective human and canine vaccines, the only feasible way to treat and control leishmaniasis is through the use of affordable medications. The principal treatment against several forms of leishmaniasis (Fouladvand et al., 2011) is pentavalent antimonials, which are used in much of the world (Croft et al., 2006a; Croft and Olliaro, 2011). In the last decade, significant progress has been made in the development of various formulations of amphotericin B and the oral agent miltefosine for the treatment of VL, although miltefosine is a teratogen and therefore not suitable for women of child-bearing age (World Health Organization, 2010). Liposomal amphotericin B is highly efficient against the disease, with minimum toxicity to the host, and it is currently used as a first-line drug in VL treatment in Europe (Gradoni et al., 2008; Maia et al., 2013). Some of the newer therapies, such as the lipid formulations of amphotericin B, are extremely expensive; this high expense has important implications because, in general, leishmaniasis affects people in the developing world (Meena et al., 2010). These pharmaceutical problems point toward the need to develop new antileishmanial drugs. During the last decade, an increasing interest in the study of organic compounds as chemotherapeutic agents has emerged. Natural products are a potential source of compounds for drug discovery as they contain a large number of molecules, some of which may be bioactive. There have been several reports on the activity of various plant extracts used in herbal medicines against protozoa (Yabu et al., 1998; Hoet et al., 2004; Aderbauer et al., 2008; Shuaibu et al., 2008).

Pentadecane is an organic compound that is made up primarily of carbon and hydrogen atoms. It is a floral volatile of different plants (El-Baroty et al., 2007; Szmigielski et al., 2012). Previous data demonstrated that a pentadecane derivative inhibits progression of disease in *Leishmania*-infected BALB/c mice (Mbati et al., 1994). Many essential oils and crude extracts of some plants containing pentadecane have shown an interesting antimicrobial activity (Genovese et al., 2009).

The aim of this study was to analyze and evaluate the in vitro activity and efficacy of pentadecane against *L. infantum* promastigotes and amastigotes. We describe the effects of pentadecane in the cell cycle of *Leishmania* parasites via flow cytometry assay. Finally, to assess the cytotoxic action of pentadecane, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay was performed on immortalized cells lines DH82 and U937 and on cultures of primary epithelial cells of Cercopithecus (CPE).

MATERIALS AND METHODS

Parasites

Leishmania infantum promastigotes (MHON/TN/80/IPT1 MON1) received from the Higher Institute of Health, Rome, Italy, were treated in phosphate-buffered saline (PBS) and cultured at 25 C and pH 7.18 in RPMI-PY medium that consisted of RPMI 1640 (R0883, Sigma, St. Louis, Missouri) supplemented with an equal volume of Pepton-yeast medium (Limoncu et al., 1997), 10% fetal bovine serum (FBS, Gibco® RPMI Media 1640, Invitrogen, Carlsbad, California), 1% glutamine, (G6392, Sigma) 250 μg/ml gentamicin (G3632, Sigma), and 500 μg/ml of 5-fluorocytosine (F7129, Sigma) (Castelli et al., 2014). Temperature, differentiation time, and acidification of the medium were used as variables for preconditioning of the amastigote cultures. The influence of temperature was evaluated by incubating the promastigotes at 37 C. The conditioning time for the promastigotes varied from 24 to 72 hr and the pH was acidified with 1 N HCl to 5.4 to obtain amastigotes parasites (Da Luz et al., 2009).

Susceptibility assays

Susceptibility of cultures of Leishmania: Flasks containing 5 ml of culture medium were inoculated with 4×10^6/ml *Leishmania* promastigotes or amastigotes and treated with serial concentrations of pentadecane (50, 100, 150, 200, 250, and 300 μM). Pentadecane was dissolved in ethanol as recommended by the manufacturer (Sigma) and diluted out in appropriate culture media. After 48 hr of treatment, the percentage of

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vitality of *Leishmania* was observed by counting in a Bürker hemocytometer (VWR International, Albertslund, Denmark) and compared with that of the control culture (100% viability).

Susceptibility of intracellular amastigote–infected macrophages: Macrophages (5×10^5 cells/ml) derived from human Caucasian histiocytic lymphoma U937 cell line (ECACC 85011440, European Collection of Cell Cultures, Porton Down, U.K.) in the logarithmic phase of growth were incubated in 2-chamber Lab-Tek culture slides (Sigma) in 2.5 ml of RPMI 1640 (Sigma) plus 10% fetal calf serum medium (complete RPMI) containing 25 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) for 18 hr to induce differentiation (Maia et al., 2007, 2013). After 18 hr, the U937 cell line differentiated into non-dividing adherent cells, and medium containing PMA was removed by washing with RPMI 1640. The medium was removed and cells were resuspended with 2.5 ml of complete RPMI containing promastigotes (stationary phase) of *L. infantum* at a 5:1 parasite:macrophage ratio. The cultures were incubated at 37 C in 5% CO_2–95% ambient air for 24 hr and then washed with PBS (pH 7.2) twice. Infected U937 cells were washed three times with fresh medium before addition of the serial dilutions of the different drugs. Cytotoxicity of each studied drug for U937 cells was determined as described previously (Maia et al., 2007, 2013). In brief, maximum tolerated dose was 300 μM pentadecane.

After 48 hr of incubation, cells were washed with PBS twice, fixed with methanol, and stained with acridine orange (100 μg/ml)–ethidium bromide (100 μg/ml). The number of cells infected was determined by examining 3 coverslips for each treatment. At least 100 macrophages were counted by visual examination under ×40 magnification by using a DM 4000B fluorescence microscope (Leica, Heerbrugg, Switzerland). Results are expressed as percentage of reduction in macrophages infection: reduction in macrophages infection (%) = percentage of infected macrophages of treated macrophages/percentage of infected macrophages of control).

Evaluation of live and dead L. infantum: To evaluate the leishmanicidal activity of pentadecane, the percentage of live and dead parasites was determined morphologically after labeling with acridine orange (100 μg/ml) and ethidium bromide (100 μg/ml). After 48 hr of exposure to each compound, parasites (1×10^6) were centrifuged, and the pellet was resuspended in 25 μl of the dye mixture. Ten microliters of the mixture was examined in oil immersion with the ×100 objective of an Eclipse E200 fluorescence microscope (Nikon, Amstelveen, The Netherlands). Live parasites were determined by the uptake of acridine orange (green fluorescence) and exclusion of ethidium bromide (red fluorescence). Dead parasites were determined by the uptake of ethidium bromide (red fluorescence). The percentage of parasite viability was determined after counting at least 300 parasites (Tolomeo et al., 2013).

Flow cytometry analysis of cell cycle: Promastigotes of *L. infantum* were incubated for 24 hr with pentadecane at 26 C. Afterward, the parasites were washed 3 times with PBS containing 0.02 M EDTA to avoid clumps and were then fixed with cold methanol for 24 hr. The parasites were resuspended in 0.5 ml of PBS containing RNase I (50 μg/ml) and propidium iodide (PI) (25 μg/ml) and were then incubated at 25 C for 20 min. The material was kept on ice until analysis. The stained parasites were analyzed in single-parameter frequency histograms by using a FACScan flow cytometer (BD Biosciences, San Jose, California) (Tolomeo et al., 2013).

Cytotoxicity assessment via MTT viability assay: Potential cytotoxic action of the pentadecane was checked via MTT viability assay in DH82 and U937 immortalized cells lines (Carmichael et al., 1987) and in CPE primary epithelial cells. DH82 cells (CRL-10389, American Type Culture Collection, Manassas, Virginia) were propagated in minimum essential medium (MEM) with nonessential amino acids, 2 mM L-glutamine, and 10% FBS (MEM growth media). Cells were grown at 37 C in 5% CO_2 and passaged semiweekly.

U937 cells (ECACC 85011440, European Collection of Cell Cultures) were propagated in RPMI 1640 (R0883, Sigma) plus 10% FBS with 50 ng/ml PMA (Sigma) for 48 hr to induce differentiation (Maia et al., 2007). Cells were grown at 37 C in 5% CO_2 and passaged semiweekly. CPE cells was cultured in RPMI 1640 (R0883, Sigma) supplemented with 10% FBS (Gibco RPMI Media 1640, Invitrogen), penicillin (100 IU/ml, P3032, Sigma), and streptomycin (100 mg/ml, S9137, Sigma). In each experiment, exponentially growing cells were plated in 100-μl aliquots of growth medium into 96-well plates at 10^5 cells per well and then incubated for 24 hr. The cells in the 96-well plates were incubated with pentadecane at concentrations of 50, 150, 200, 250, and 300 μM. After 48 hr of incubation, MTT solution (5 mg/ml) was added to each well, and the formazan precipitate was dissolved in 100 μl of dimethyl sulfoxide after 4 hr of incubation (Kong et al., 2009). The absorbance was measured using a Spectrostar Nano microplate reader (BMG Labtech Inc., Ortenberg, Germany) at 570 nm.

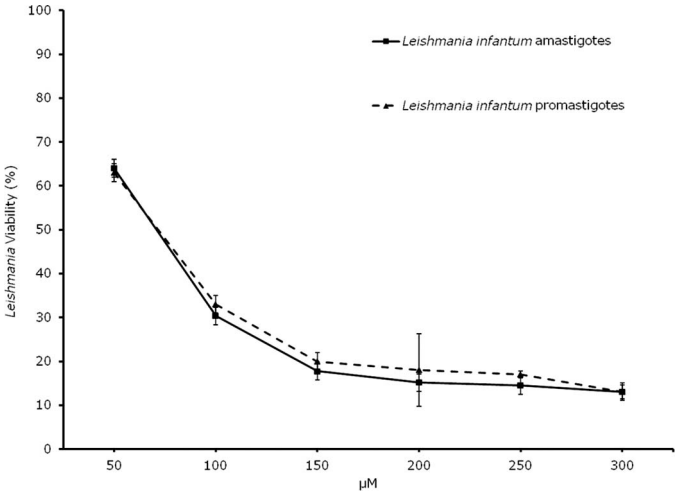

FIGURE 1. Effects of pentadecane on viability of *Leishmania infantum*. Leishmania cultures were incubated with different concentrations of pentadecane for 48 hr. The percentage of viability of *L. infantum* promastigotes and amastigotes was calculated by defining the viability of cells without pentadecane treatment as 100%. Data are the results of 3 independent experiments and are presented as the mean ± SD. Data obtained are statistically significant at $P < 0.001$.

Statistical analysis

All experiments were performed by 2 observers in 3 replicates and repeated with 3 new batches of parasites. The mean and SE of at least 3 experiments were determined. The differences between the mean values obtained for experimental groups were evaluated by Student's *t*-test. *P* values ≤0.05 are considered significant. The half maximal inhibitory concentration (IC_{50}) values were calculated using Prism® 5 (Version 5.01, GraphPad Software, Inc., San Diego, California).

RESULTS

Antileishmanial activity of pentadecane against promastigotes and amastigotes

Pentadecane was evaluated for anti-leishmania activity against promastigotes and amastigotes of *L. infantum* IPT1 strain. Parasites were incubated with different concentrations of pentadecane, and the *L. infantum* viability was compared to that of the control after incubation for 48 hr (see Materials and Methods).

Controls containing the equivalent concentrations of ethanol to those in different pentadecane doses were included and showed no adverse effect on *L. infantum* viability (data not shown). Pentadecane showed anti–*L. infantum* activity in a dose-dependent manner: at 50, 100, 150, 200, 250, and 300 μM, it reduced parasite viability by 63, 33, 20, 18, 17, and 13%, respectively, for promastigotes and by 64, 30, 18, 16, 14, and 13% for amastigotes. The concentration that caused 50% reduction in survival in comparison to the control was estimated by Prism to be 60.5 μM for *L. infantum* amastigotes and 65.3 μM for *L. infantum* promastigotes (Fig. 1).

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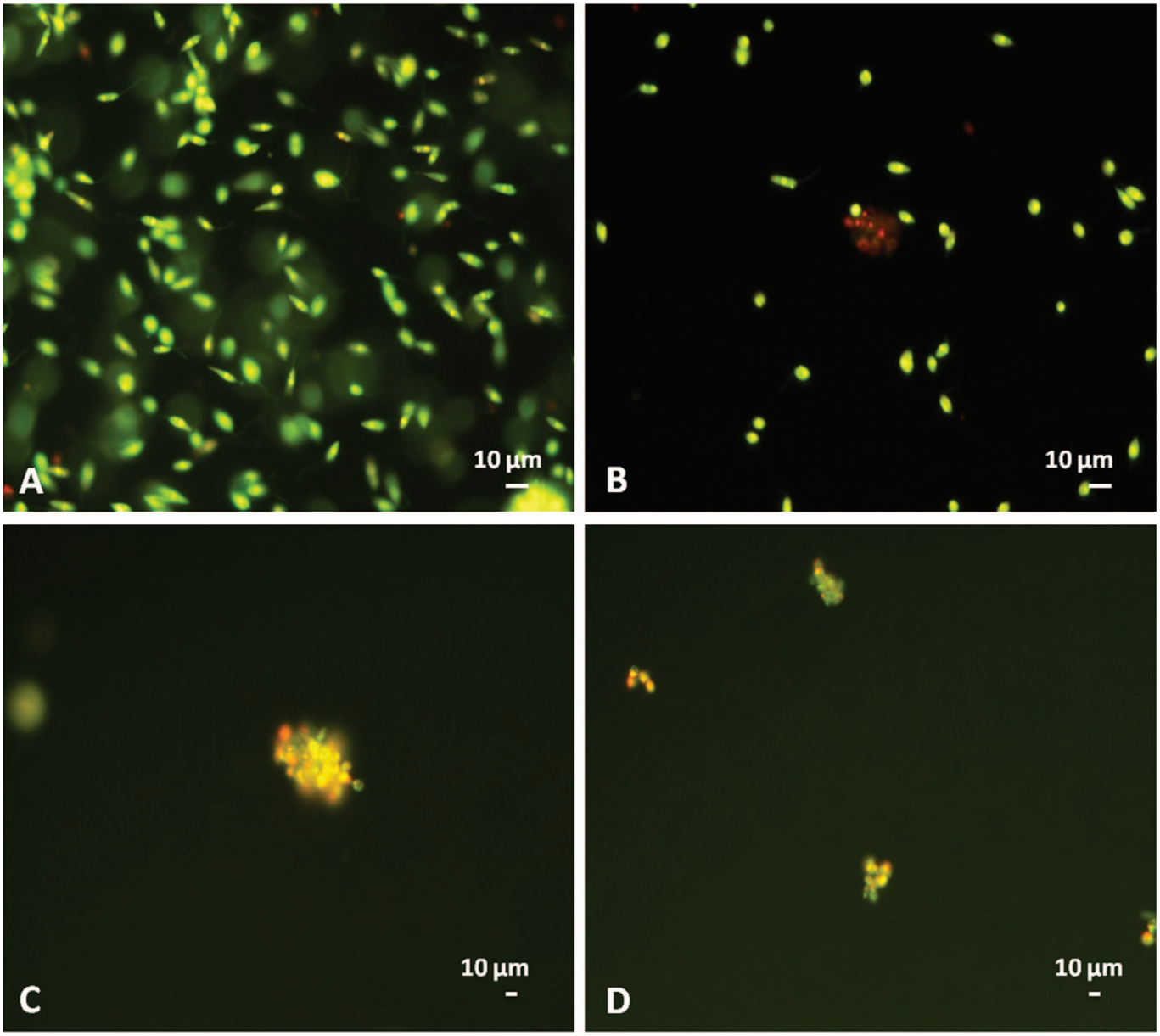

FIGURE 2. Leishmanicidal activity of pentadecane. Morphological changes observed in *Leishmania infantum* after exposure to pentadecane. Cells were exposed to a mixture of acridine orange and ethidium bromide as described in Materials and Methods. Live parasites were determined by the uptake of acridine orange (green fluorescence) and exclusion of ethidium bromide (red fluorescence). Dead parasites were determined by the uptake of ethidium bromide (red fluorescence). (**A**) Control. (**B**) Promastigotes exposed to 65.3 µM pentadecane for 48 hr. (**C**, **D**) Promastigotes exposed to 300 µM pentadecane for 48 hr. Parasites take up ethidium bromide, showing a loss of volume and nuclear condensation. Color version available online.

Leishmanicidal activity of pentadecane

Leishmania infantum exposed to pentadecane 48 hr and stained with an acridine –ethidium bromide mixture showed changes in morphology (e.g., loss of cell volume and nuclear condensation) that share many characteristics with apoptotic cell death in metazoans (Fig. 2).

Cell cycle: The DNA content of *L. infantum* cells was monitored by flow cytometry after PI staining. When *L. infantum* promastigotes were incubated with 65.3 µM pentadecane for 24 hr, the proportion of cells in the sub-G0/G1 phase increased to compare that of the controls (17.41 vs. 7.24%). In contrast, an increase in the number of cells in the sub-G0/G1 phase led to a decrease in the cell number in the G2/M phase compared with untreated cells (0 vs. 3.2%) (Table I). The increased ratio of cells in the sub-G0/G1 phase, after 24 hr, showed that apoptosis via pentadecane in *L. infantum* promastigotes resulted in DNA degradation.

Cytotoxicity of pentadecane: In a first series of experiments, 2 tumorigenic cell lines, dog macrophage DH82 and human lymphoblast U937, and also normal CPE primary epithelial cells were chosen to determine the cytotoxic activity of different concentrations of pentadecane. A vitality higher than 60% was

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TABLE I. Cell cycle of *Leishmania infantum* after treatment with 65.3 μM pentadecane for 24 hr.

	Proportion of *L. infantum* cells (%) in cell cycle phase			
Group	Sub-G0/G1	G1	S	G2/M
Control	7.24	34.24	62.56	3.2
Treated	17.41	39.49	60.51	0

TABLE II. IC_{50} values for growth inhibition assays (promastigote and amastigote cultures and intracellular amastigotes).

Life stage	IC_{50} (μM) of pentadecane in *L. infantum*
Promastigote	63.5 ± 0.5
Amastigote	60.5 ± 0.6
Intracellular amastigote	194.8 ± 0.6

observed (Fig. 3), suggesting a non-toxic action both in immortalized cells (DH82, U937) and in primary cells (CPE).

Antileishmanial activity of pentadecane against intracellular amastigotes: The susceptibility of *Leishmania* parasites (intracellular amastigotes) to pentadecane was determined in 3 individual experiments with 2 replicates. After 48 hr, the percentage of infected macrophages was higher for the control than for the infected macrophages that were treated with pentadecane. The mean number of internalized parasites for macrophages treated with pentadecane was markedly lower than that for the control. Thus, pentadecane showed 50% inhibition of cell survival (IC_{50}) at a concentration of 194.8 μM (Table II), with a growth inhibition of 77% at 300 μM.

DISCUSSION

Although chemotherapy is the only effective treatment for leishmaniasis, the anti-leishmanial drugs available are, in general, toxic, expensive, and require long-term treatment. These side effects and disadvantages demonstrate the necessity to identify new, effective, and safe compounds for the treatment of this disease (Croft et al., 2006b; Rodrigues et al., 2014). The domestic dog is the main reservoir for the transmission of the visceral form of leishmaniasis, thereby accounting for its widespread occurrence in the human population (Courtenay et al., 2002). At present, leishmaniasis is an incurable and complex zoonosis, whose true incidence is not known and, for ethical reasons, drastic measures (e.g., stamping-out) are not to be proposed for animal health. Furthermore, there is a large difference between the immunocompetent patients, who are healed completely after treatment, and patients in which recurrence is a regular occurrence regardless of the drug treatment. The current drugs decrease the parasitic charge and promote the cell-mediated immune response, but they fail to eradicate the disease. Therefore, the development of new therapeutic protocols for the treatment of leishmaniasis and new drugs with leishmanicidal and immunomodulatory activity are needed to achieve the elimination of *L. infantum*. However, it is also necessary to develop economical drugs for developing countries where the impact of leishmaniasis is greatest. In this study, we showed the action of pentadecane as an antileishmanial agent and provided data indicating it did not have a cytotoxic effect in immortalized DH82 and U937 cell lines and in primary cells CPE.

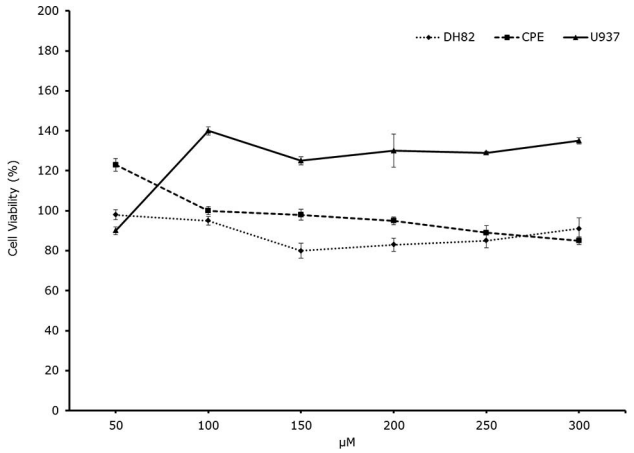

FIGURE 3. Effects of pentadecane on DH82 and U937 cell lines and primary epidermal cells of CPE. The cells were exposed to the indicated concentrations of pentadecane for 48 hr, and then cell viability was monitored by MTT assay.

Pentadecane treatment decreased the viability of *L. infantum* promastigotes and amastigotes up to 14% at 300 μM (IC_{50} promastigotes, 65.3 μM; IC_{50} amastigotes, 60.5 μM). Pentadecane caused a reduction of macrophage infection and its effect on infected macrophages should be of great interest; in fact, pentadecane was even more effective against intracellular amastigotes, with a growth inhibition of 77% for 300 μM after 48 hr of treatment. A cytotoxicity assay of pentadecane in immortalized cells lines DH82 and U937 and in primary epithelial cells of CPE showed that it caused negligible cytotoxic effects.

The DNA content in cells had a direct relationship with the amount of fluorescence intensity and DNA degradation of the cell by apoptosis results into PI intensity lower G1 cells (sub-G1 peak). *Leishmania infantum* promastigotes exposed to 24 hr with 65.3 μM pentadecane increased 17% cells proportion in the sub-G1 peak region, whereas in the control only 7% were found in this region. Furthermore, *L.* promastigotes treated with 65.3 μM pentadecane for 24 hr induced DNA degradation.

This selectivity assay showed that the action of pentadecane is specific for protozoans and is not toxic for mammalian cells. This study shows a potent leishmanicidal activity of pentadecane that may provide promising treatment options against leishmaniasis. The mechanism of action of pentadecane is currently unknown, but is the subject of intense investigation in our laboratory. A better understanding of the mechanisms of pentadecane action may help in finding new targets for the treatment of *Leishmania* parasites. Further studies are required to evaluate whether pentadecane can be used as single anti-leishmanial compound or as a fortifying agent with existing synthetic compounds for the development of anti-leishmanial agents.

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