The HSP90 inhibitor 17-AAG potentiates the antileishmanial activity of the ether lipid edelfosine

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Abstract

HSP90 is an abundant protein in *Leishmania* parasites that plays a major role in the parasite survival under stress conditions. Here we found that the HSP90 inhibitor 17-AAG (≥100 nM 17-AAG) induced cell cycle arrest at G_0/G_1 in *L. infantum* and *L. panamensis* promastigotes, and highly potentiated the induction of cell death by an apoptotic-like process mediated by the ether phospholipid edelfosine (5-20 μM). These data suggest that the combined treatment of 17-AAG and edelfosine might be a novel and effective approach of combination therapy in the treatment of leishmaniasis.

Keywords: HSP90 inhibitor; 17-AAG; edelfosine; cell cycle arrest; apoptosis-like cell death; *Leishmania spp.*

1. Introduction

The current therapy of leishmaniasis, caused by the protozoan parasite *Leishmania*, includes a rather small number of drugs and is not satisfactory. The emergence of drug resistance and toxicity urges the identification of new targets and drugs in the treatment of this disease.

Heat shock proteins (HSPs) comprise several highly conserved families of protein folding facilitators that play important roles in the maintenance of cell functionality and in stress cell response, with HSP90 being of particular impact in the regulation of key steps in a multitude of biological processes (Queitsch et al., 2002; Whitesell and Lindquist, 2005). HSP83, the *Leishmania* ortholog of the human HSP90, is highly and constitutively expressed in *Leishmania spp.*, where represents about 2.8% of the total cell protein (Brandau et al., 1995). Amino acid sequence alignment analysis shows high level of identity of the HSP90 proteins of *L. braziliensis*, *L. major* and *L.*

donovani (~93%), which in turn show ~63% identity with human HSP90 (Silva et al., 2013). *L. infantum* JPCM5 HSP83 protein (NCBI Reference Sequence: XP_003392729.1) (Peacock et al., 2007) shows 62% identity and 78% similarity with human HSP90 (GenBank: AAA36025.1) (Rebbe et al., 1987) by BLAST analysis, thus suggesting that *Leishmania* HSP83 might exert a similar function as human HSP90. In mammalian cells, HSP90 is involved in the stabilization and correct folding of a number of different client proteins, many of them playing a major role in cell survival, cell proliferation, and cancer cell growth (Whitesell and Lindquist, 2005). In this regard, HSP90 has become a valuable target in cancer chemotherapy as its inhibition promotes proteasomal degradation of client proteins required for tumor cell survival (Whitesell and Lindquist, 2005). Despite HSP90 is promiscuous in its action, and therefore caution should be taken into account to avoid undesired effects on chemotherapy (Nieto-Miguel et al., 2008), a wide number of studies have shown that HSP90 inhibitors enhance the cytotoxic action of some anticancer drugs (George et al., 2005; Neckers, 2002). Similarly, inhibition of HSP90 in *Leishmania* parasites using specific inhibitors, such as geldanamycin, radicicol and 17-*N*-allylamino-17-demethoxygeldanamycin (17-AAG, tanespimycin) has been shown to affect transformation of promastigotes into rounded amastigote-like forms and to kill intracellular amastigotes (Li et al., 2009; Petersen et al., 2012; Wiesgigl and Clos, 2001a). These data suggest that HSP90 inhibition might be a new therapeutic approach in the treatment of leishmaniasis.

On the other hand, the ether lipid edelfosine (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine), a rather selective antitumor drug that acts at the cell membrane level (Gajate et al., 2004; Gajate and Mollinedo, 2007; Mollinedo et al., 2010a), exerts *in vitro* antiparasitic activity against different species of *Leishmania* (Azzouz et al., 2006; Azzouz et al., 2005; Croft et al., 2003; Lux et al., 2000).

Furthermore, we have recently found following *in vitro* and *in vivo* studies that edelfosine represents a promising drug against leishmaniasis, killing *Leishmania* parasites through an apoptosis-like process (Varela-M et al., 2012). In our previous study (Varela-M et al., 2012), we found striking differences in the sensitivity of promastigotes from different species of *Leishmania* to edelfosine, including sensitive promastigotes, such as *L. panamensis*, and rather resistant ones, such as *L. infantum*. On these grounds, we analyzed here whether the combination of the HSP90 inhibitor 17-AAG (tanespimycin), currently in clinical trials as an antitumor agent (Katragadda et al., 2013; Neckers and Workman, 2012), and edelfosine could improve the antileishmanial activity of the ether lipid.

2. Materials and methods

2.1. Drugs

Edelfosine was from R. Berchtold (Biochemisches Labor, Bern**,** Switzerland). Stock sterile solution of edelfosine (2 mM) was prepared as previously described (Mollinedo et al., 1997) in complete RPMI-1640 culture medium (Invitrogen, Carlsbad, CA), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS). 17-AAG was purchased from InvivoGen (San Diego, CA). An 8 mM 17-AAG stock solution in dimethyl sulfoxide (DMSO) (Sigma, St Louis, MO) was aliquoted and stored at -20°C until use.

2.2. Leishmania cells and culture conditions

The following *Leishmania* strains were used in this study: *L. infantum* (MCAN/ES/96/BCN150) and *L. panamensis* (MHOM/CO/87/UA140). *Leishmania* promastigotes were grown in RPMI-1640 culture medium, supplemented with 10%

FBS, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin at 26ºC. Promastigotes were treated with the indicated compounds during their logarithmic growth phase (1.5 or 2 x 10^6 parasites/ml) at 26ºC.

2.3. Growth inhibition assay

The inhibition of promastigote growth was determined by using the XTT (sodium 3,3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) cell proliferation kit (Roche Molecular Biochemicals, Mannheim, Germany) as previously described (David-Cordonnier et al., 2005; Varela-M et al., 2012). Cells were resuspended in FBS-containing RPMI-1640 culture medium (1.5 x 10^6 cells/ml) and plated (100 µl/well) in 96-well flat-bottomed microtiter plates at 26ºC, in the absence and in the presence of 10 µM 17-AAG. After 17-h incubation at 26ºC, the percent of growth inhibition was calculated with respect to untreated controls. Measurements were done in triplicate.

2.4. Analysis of apoptosis-like cell death by flow cytometry

Leishmania spp. promastigotes were treated for different times in the absence and in the presence of the indicated concentrations of 17-AAG at 26ºC. Edelfosine was then used at the indicated concentrations for 24 h. In combination therapies, 17-AAG was preincubated for 1 h before adding edelfosine. Thus, promastigotes were untreated or treated with different concentrations of 17-AAG for 1 h, and then incubated in the absence or presence of edelfosine for 24 h in combined treatments. Then, parasites were pelleted by centrifugation (1000 x g) for 6 min, and analyzed for apoptosis-like DNA breakdown by flow cytometry following a protocol previously described (Gajate et al., 2000). Quantitation of apoptotic-like cells was monitored as the percentage of cells in

the sub-G_0/G_1 region (hypodiploidy) in cell cycle analysis, using a fluorescence-activated cell sorting (FACS)Calibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 488 nm argon laser. WinMDI 2.8 software was used for data analysis. In addition, photographs of untreated and drug-treated parasites, before starting their preparation for flow cytometry assays, were taken using a Zeiss Axioplan microscope (Carl Zeiss GmbH, Oberkochen, Germany) at 40X magnification.

3. Results

3.1. 17-AAG inhibits cell cycle in Leishmania spp. promastigotes

We found that 10 μM 17-AAG arrested *L. infantum* and *L. panamensis* promastigotes at the G_0/G_1 phase of cell cycle, as assessed by flow cytometry (Figs. 1A and 1B), which led to a complete growth inhibition (Fig. 1C). Treatment of *Leishmania spp.* promastigotes with 10 μM 17-AAG induced dramatic changes in cell morphology, leading to stubby-shaped bodies, rounding-up, and changes in the flagellum (Fig. 2). Following a concentration-response analysis (10 nM – 10 μM), we found that incubation with ≥100 nM 17-AAG promoted a concentration-dependent cell cycle arrest at the G_0/G_1 phase (Table 1), and morphological changes similar to those described above. Removal of 17-AAG by washing off the drug and subsequent reculture of the cells in drug-free medium for 3 days allowed a partial recovery of the normal cell cycle profile (data not shown).

3.2. 17-AAG pretreatment sensitizes Leishmania spp. parasites to edelfosine-induced cell death

We have recently reported that the ether phospholipid edelfosine induces cell death in *Leishmania* parasites through an apoptosis-like process (Varela-M et al., 2012). Here

we found that pretreatment of *Leishmania* promastigotes with 10 μM 17-AAG highly potentiated the anti-*Leishmania* activity of edelfosine, used at 5 μM in *L. panamensis* and 20 μM in *L. infantum*. Distinct drug concentrations were initially used because of the distinct drug sensitivity of *L. panamensis* and *L. infantum* promastigotes as previously reported (Varela-M et al., 2012). The anti-*Leishmania* activity was assessed by an increase in the percentage of hypodiploid cells (protozoa at the sub-G_0/G_1 region of cell cycle) (Figs. 3A and 3B), suggesting an apoptosis-like cell death. In order to determine the lowest concentration of 17-AAG able to potentiate the anti-*Leishmania* activity of edelfosine, we used the HSP90 inhibitor in the range 10 nM-10 μM. The potentiation of edelfosine-induced apoptosis was detected after incubation with 17-AAG at a concentration ≥100 nM and at 5 μM edelfosine (Table 2). Lower concentrations of 17-AAG or edelfosine were not effective. Incubation with 10 nM - 10 μM 17-AAG at 26ºC did not promote apoptosis in both *Leishmania* species, but the percentage of hypodiploid cells was increased following incubation with 5 μM edelfosine, which per se was unable to elicit cell death in the absence of HSP90 inhibitor in *L. infantum* promastigotes or induced a weak cell death response in *L. panamensis* promastigotes (Table 2). These results suggest that 17-AAG facilitates the induction of apoptosis-like cell death mediated by edelfosine.

4. Discussion

The data reported here indicate that the combined treatment of 17-AAG and edelfosine largely potentiates the killing activity of the ether phospholipid edelfosine on *Leishmania spp.* promastigotes. Interestingly, *L. infantum* promastigotes were previously reported rather resistant to the action of the ether lipid, requiring drug concentrations of ≥20 μM (Varela-M et al., 2012), but here we have found that

pretreatment with 17-AAG turned *L. infantum* promastigotes into edelfosine-sensitive protozoa. Our present data show that *L. infantum* promastigotes become sensitive to the combined use of 17-AAG (≥100 nM) and 5 μM edelfosine, thus lowering the edelfosine concentration required to induce cell death in this *Leishmania* species. Our results indicate that both *L. panamensis* and *L. infantum* promastigotes become sensitive to similar concentrations of edelfosine, thus suggesting that incubation with the HSP90 inhibitor decreases the differences in drug resistance between both *Leishmania* species. The data reported here indicate that 17-AAG preincubation increases the edelfosine-mediated cytotoxic response in *L. panamensis* promastigotes and turns *L. infantum* promastigotes into sensitive cells, thus suggesting that HSP90 inhibition facilitates or leads to a higher upregulation of the cell death response triggered by edelfosine in *Leishmania* promastigotes. On these grounds, 17-AAG sensitizes *Leishmania* parasites to edelfosine, and it might be envisaged that HSP90 inhibition could be an interesting way to potentiate antiparasitic therapy. Because 17-AAG is currently in clinical trials (Katragadda et al., 2013; Neckers and Workman, 2012; Usmani et al., 2009)], and preclinical assays indicate a rather lack of significant toxicity for edelfosine (Gajate et al., 2004; Mollinedo et al., 2010a; Mollinedo et al., 2010b; Mollinedo et al., 2009), our data suggest that the combination of 17-AAG and edelfosine could be a new approach to treat leishmaniasis that warrants further investigation.

Previous studies have shown that HSP90 inhibitors geldanamycin and radicicol induce growth arrest and differentiation from the *L. donovani* promastigote to the amastigote stage, suggesting that disturbances in HSP90 homeostasis are a signal for the onset of stage differentiation in *Leishmania* parasites (Wiesgigl and Clos, 2001a; Wiesgigl and Clos, 2001b). On the other hand, Li *et al.* (Li et al., 2009) have previously shown that incubation of *L. donovani* promastigotes with the HSP90 inhibitor

geldanamycin at 37ºC in pH 5.5 induced cell cycle arrest at G_0/G_1 followed by an apoptosis-like cell death. In relation to the above observations, we also found that the HSP90 inhibitor 17-AAG induced morphological changes that could be similar to amastigote conversion, as well as cell cycle arrest at G_0/G_1. We have previously found that *L. infantum* axenic amastigotes are more sensitive to edelfosine than the promastigote forms (Varela-M et al., 2012), and therefore it is tempting to suggest that HSP90 inhibition could mimic stage conversion and thereby might increase edelfosine sensitivity of *L. infantum* parasites. Similarly to the data reported in *L. donovani* promastigotes by Li *et al.* (Li et al., 2009), we found here that 17-AAG incubation leads to cell cycle arrest, but, unlike what was found in *L.donovani* parasites, we were unable to detect apoptosis-like cell death after 24 h treatment in both *L. panamensis* and *L. infantum* promastigotes. A putative explanation for this apparent discrepancy lies in the different experimental conditions used, that is incubation at 37ºC in pH 5.5 in *L. donovani* promastigotes (Li et al., 2009) and incubation at 26ºC and pH 7.5 in the data herein reported. Furthermore, HSP90 inhibitors have been reported to increase upregulation of HSPs (Wiesgigl and Clos, 2001a) that could prevent cell death in the absence of the ether lipid.

Taken together, our present and previous data (Varela-M et al., 2012) indicate that 17-AAG leads to cell cycle arrest and edelfosine promotes an apoptosis-like response in *Leishmania spp.* The cell cycle arrest, inhibiting cell proliferation, might decrease the probability to render drug resistance, thus potentiating the cell death response triggered by a cytotoxic drug. This could represent a good example of combination therapy, bringing together both cytostatic and proapoptotic agents in the treatment of leishmaniasis. HSP90 protects and stabilizes a wide number of proteins involved in cell survival (Whitesell and Lindquist, 2005). Thus, it might be envisaged

that inhibition of HSP90 leads to the degradation of some critical proteins in *Leishmania* survival that would facilitate the action of a proapoptotic agent, such as edelfosine. Our results reported here indicate that the combined addition of 17-AAG and edelfosine is effective against *Leishmania* parasites causing visceral (*L. infantum*) and cutaneous (*L. panamensis*) leishmaniasis. Interestingly, a recent study has shown that 17-AAG is able to inhibit growth of *L. amazonensis* (IC_{50}, ~65 nM), *L. major* (IC_{50}, ~80 nM) and *L. infantum* (IC_{50}, ~169 nM) axenic amastigotes, and reduced intracellular *L. amazonensis* viability in infected macrophages in a dose- and time-dependent manner (Petersen et al., 2012). Our data indicate that 17-AAG at a similar concentration range (100 nM) arrests cell cycle and facilitates the killing activity of edelfosine on *Leishmania* promastigotes, further supporting a beneficial action of 17-AAG as a putative drug in anti-*Leishmania* therapy by affecting both *Leishmania* promastigotes and amastigotes. However, because the assays reported here were conducted with promastigote cells, further experiments are needed to evaluate the feasibility of the combined therapy in amastigote cells and host conditions. Furthermore, both 17-AAG (Petersen et al., 2012) and edelfosine (Mollinedo et al., 2009) have been reported to lower or inhibit inflammation that, in turn, could be beneficial on leishmaniasis treatment. This inhibitory action would be of particular importance in the treatment of cutaneous and muco-cutaneous leishmaniasis, which are characterized by an intense inflammatory response causing severe tissue destruction (Lopez Kostka et al., 2009; Tasew et al., 2010).

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FIGURE LEGENDS

Figure 1. **17-AAG inhibits proliferation of *Leishmania* promastigotes.** (A) Dot-plot histogram representing the distinct phases of cell cycle in control untreated *L. infantum* (upper) and *L. panamensis* (lower) promastigotes, and after treatment with 10 μM 17-AAG for 17 h by flow cytometry analysis. (B) Profiles of cell cycle analysis from the above dot-plot histograms showing the distinct cell cycle phases of control untreated (black) *L. infantum* and *L. panamensis* promastigotes, and after treatment with 10 μM 17-AAG for 17 h (red). The position of the G_0/G_1 phase is indicated. (C) *L. panamensis* and *L. infantum* promastigotes were incubated in the absence and in the presence of 10 μM 17-AAG for 17 h and then the percentage of cell growth inhibition was determined by the XTT method. Representative experiments of three performed are shown.

Figure 2. 17-AAG induces changes in *Leishmania* promastigote morphology. *L. infantum* and *L. panamensis* promastigotes, untreated (Control) and treated with 10 μM 17-AAG for 17 h were analyzed for changes in cell morphology. Red arrows show different forms of parasite morphology.

Figure 3. 17-AAG pretreatment potentiates the antileishmanial activity of edelfosine. *L. infantum* (A) and *L. panamensis* (B) promastigotes were untreated (Control) or treated with 10 μM 17-AAG for 1 h, and then incubated in the absence or presence of edelfosine (EDLF; 5 μM for *L. panamensis*; 20 μM for *L. infantum*) for 24 h. Apoptosis-like cell death was then quantitated as the percentage of promastigotes in the sub-G_0/G_1 region by flow cytometry, and indicated in each histogram. The positions of the G_0/G_1 and sub-G_0/G_1 regions are indicated. Representative experiments of three performed are shown.

Table 1. Induction of G_0/G_1 arrest in *L. panamensis* and *L. infantum* promastigotes upon 17-AAG treatment.

	% cells in G_0/G_1	
17-AAG	***L. panamensis***	***L. infantum***
No addition	65.7 ± 2.3	66.1 ± 3.7
17-AAG (10 nM)	65.8 ± 3.1	66.3 ± 3.9
17-AAG (100 nM)	78.3 ± 2.9	76.2 ± 3.3
17-AAG (1 μM)	82.4 ± 3.0	78.3 ± 3.2
17-AAG (10 μM)	82.6 ± 4.2	79.5 ± 4.8

The percentages of cells at G_0/G_1 were determined by flow cytometry upon incubation with the indicated concentrations of 17-AAG for 24 h at 26ºC. Data are means ± SD of three independent experiments.

Table 2. Induction of apoptosis-like cell death in *L. panamensis* and *L. infantum* promastigotes by the combined treatment with 17-AAG and edelfosine.

	% cell death (sub-G_0/G_1)	
HSP inhibitor	***L. panamensis***	***L. infantum***
	+ Edelfosine (5 μM)	**+ Edelfosine (5 μM)**
No addition	19.6 ± 5.3	3.2 ± 1.1
17-AAG (10 nM)	19.8 ± 3.5	3.5 ± 1.4
17-AAG (100 nM)	38.3 ± 4.5	27.4 ± 3.9
17-AAG (1 μM)	44.2 ± 6.1	30.4 ± 4.3
17-AAG (10 μM)	46.3 ± 7.2	33.5 ± 5.2

Promastigotes were untreated or treated with the indicated concentrations of 17-AAG for 1 h, and then incubated in the presence of 5 μM edelfosine for 24 h at 26ºC. Apoptosis-like cell death was determined by flow cytometry as the percentage of cells at the sub-G_0/G_1 region. Cells incubated only with 17-AAG for the same period of time did not induce a significant apoptosis-like cell death response. Data are means ± SD of three independent experiments.

Figure 1
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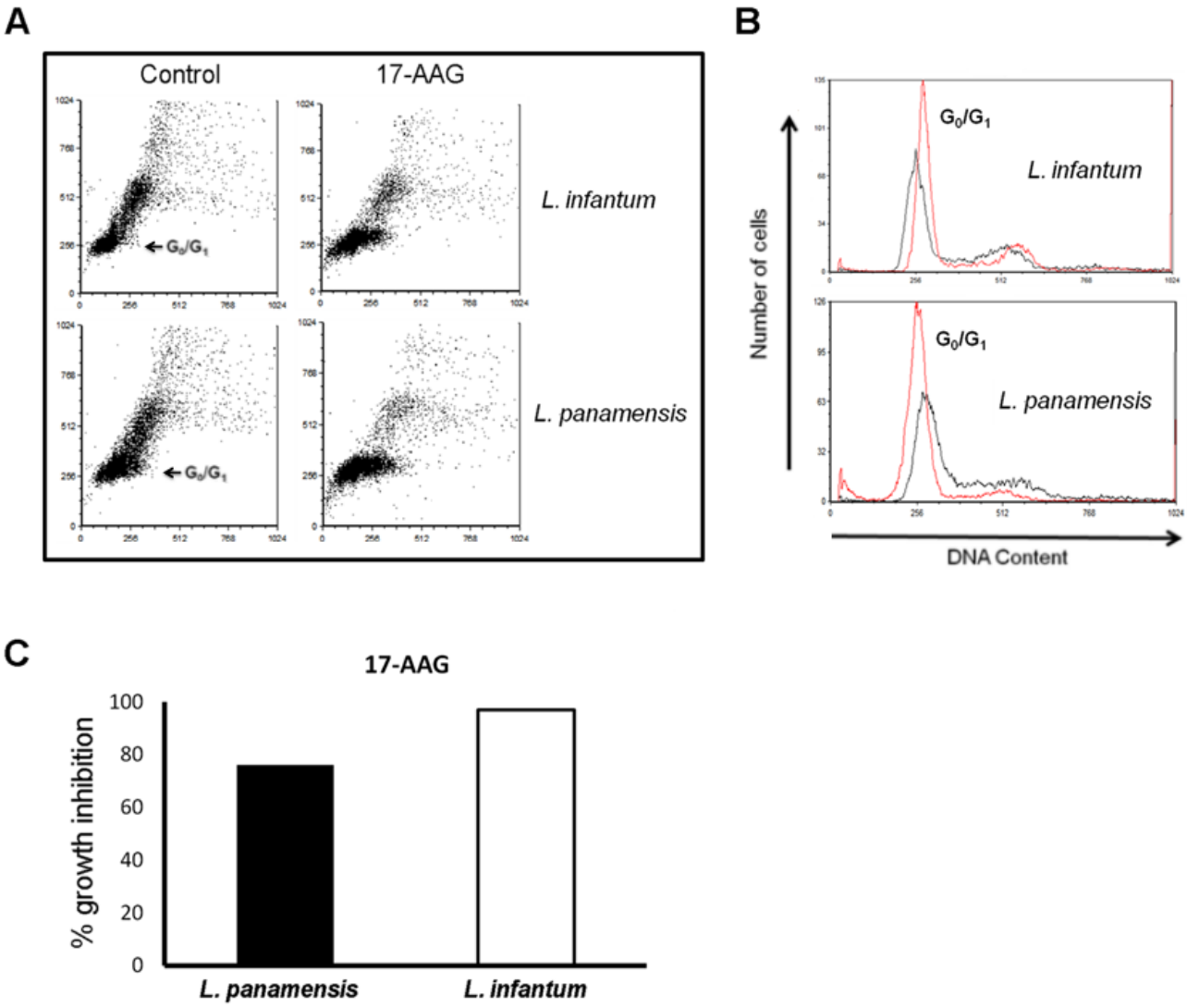

Figure 2
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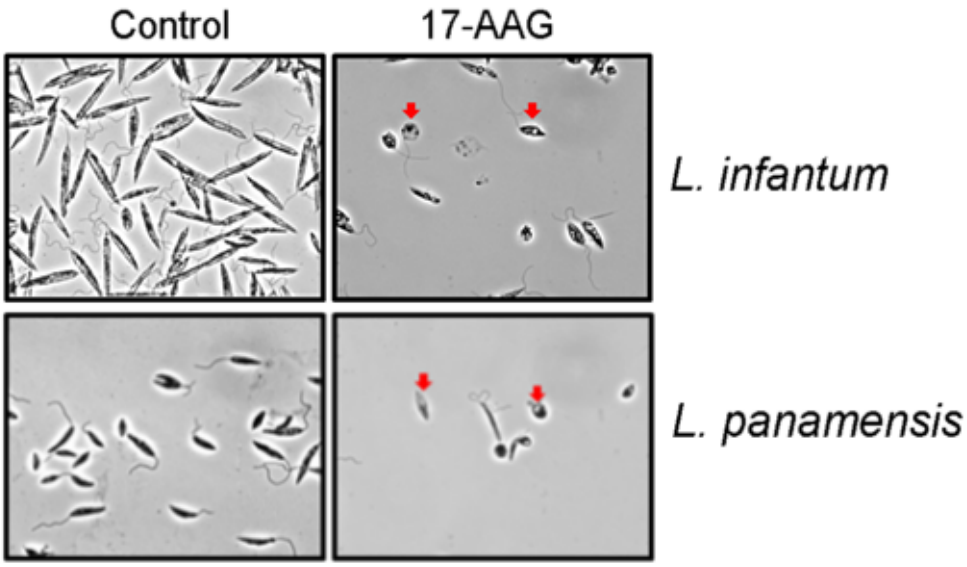

Figure 3
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