SUPPLEMENTARY MATERIAL

Styrylpyrone, isolated from an Amazon plant, induces cell cycle arrest and autophagy in *Leishmania amazonensis*

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Styrylpyrone, isolated from an Amazon plant, induces cell cycle arrest and autophagy in *Leishmania amazonensis*

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Abstract: The search for bioactive compounds against diseases is imperative and the richness of the Amazon provides a large source to be explored. Current therapies for the treatment of parasitic infections have severe side effects and low efficacy, which makes the development of an effective chemotherapy extremely important. In this study, we describe the isolation of styrylpyrone 4-methoxy-6-(11,12-methylenedioxy-trans-styryl)-2-pyrone (SP), from the Amazonian tree species, *Aniba panurensis*, the *in vitro* activity against *Leishmania amazonensis* promastigotes, and its *in silico* pharmacokinetics properties. The results showed morphological and ultrastructural alterations, cell cycle impairment, increased reactive oxygen species production, accumulation of lipid bodies and formation of autophagic vacuoles in SP-treated parasites. *In silico* studies revealed that the compound has a high drug-score, which is encouraging for further investigation. Our results indicate that SP is a promising drug candidate, which induces alterations in *L. amazonensis* leading to parasite death through cell cycle arrest and autophagy.

Keywords: Neglected diseases, cell death, Leishmaniasis, parasite, Amazonia, 4-methoxy-11,12-methylenedioxy-6-*trans*-styryl-pyran-2-one

Caption

Experimental Section

Chemicals

Bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), monodansylcadaverine (MDC), wortmannin (WTM), 3-[4,5-dimethylthiazol-2-yl]-2,5phenyltetrazolium bromide (MTT), H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate) and Nile Red were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), Warren's medium (brain–heart infusion plus hemin and folic acid; pH 7.2) and RPMI 1640 were obtained from Invitrogen (Roswell Park Memorial Institute - Gibco Invitrogen, Grand Island, NY, USA). Propidium iodide (PI) and RNase were also obtained from Invitrogen (Eugene, OR, USA). All the solvents and other reagents were of analytical grade. Sodium Cacodylate buffer (CaCo), 1% osmium tetroxide (OsO4), 0.8% potassium ferrocyanide, 10 mM $CaCl_2$, lead citrate, uranyl acetate, 25% glutaraldehyde and EPON resin were obtained from EMS – Electron Microscopy Sciences.

Plant material and compound isolation

Aniba panurensis collection, preparation and ethanolic extracts were obtained from the stems, and the compound was isolated and identified as described previously (da Silva et al. 2019). SP was solubilised in <1% DMSO.

Parasite and macrophage maintenance

L. amazonensis (strain WHOM/BR/75/JOSEFA) were isolated from a patient with diffuse cutaneous leishmaniasis by Dr. Cesar A. Cuba (Universidade de Brasília, Brazil) (Cuba et al. 1985). Promastigotes were cultured in Warren's medium (brain heart infusion, hemin, and folic acid; pH 7.0) supplemented with 10% inactivated FBS at 25 ºC.

Murine monocytic lineage macrophages J774.A1 (Rio de Janeiro Cell Bank – BCRJ) were maintained in tissue flasks with RPMI-1640 pH 7.6, supplemented with 10% of FCS and 0.4% of penicillin-streptomycin antibiotics, incubated at 37 °C, 5% CO_2 tension and humidified atmosphere.

Antiproliferative and cytotoxicity assays

Promastigote forms in the logarithmic phase of growth (1×10^6 cells/mL) were cultured on a 24-well plate in Warren's media supplemented with FBS in the presence or absence of

increasing concentration (3.6 to 360 µM) of SP, after previous solubilisation with DMSO (<1%). Amphotericin B was used as a positive control. The activity was evaluated for 96 h of incubation where viable cells were counted every 24 h with hemocytometer. The inhibitory concentration was calculated as IC_{50} and IC_{90}, *i.e.* the concentrations that inhibited the growth by 50% or 90% compared to the control (not treated) for each time point. For the follow up experiments, the IC_{50} and IC_{90} of 72 h were used (Scariot et al. 2017).

The cytotoxicity effect of the compound was evaluated against J774G8 macrophage with the colorimetric MTT method (Mosmann 1983). For this, a suspension of 5×10^5 cells/mL was cultured in RPMI-1640 medium supplemented with 10% FBS in a 96-well microplate. The plate was incubated at 37 °C, 5% CO_2 tension and humidified atmosphere. After 24 h, the compound, solubilized in 0.5% DMSO, was added at increasing concentrations (1 to 300 µM) to the macrophages. The plates were incubated for 48 h at 37 °C, 5% CO_2 tension and humidified atmosphere. After the treatment, the medium was removed and the macrophage monolayer was washed with phosphate-buffered saline (PBS), and 50 µL of MTT 2 mg/mL) was added. The plate was incubated for 4 h in a 5% CO_2-air mixture at 37 ºC, and then, 50 µL of DMSO was added. The absorbance was read in a 96-well plate reader (BIO-TEK Power Wave XS spectrophotometer) at 570 nm. A dose-response curve was generated and the percentage of viable cells was calculated compared to controls (not treated cells) and the CC_{50} determined (*i.e.*, 50% cytotoxicity concentration) (Mosmann 1983). The CC_{50} was used to calculate the selective index (SI): $SI = (CC_{50\ macrophage}/IC_{50\ compound})$.

Optical and electron microscopy

Optical and electron microscopy were carried out in order to analyse the morphological and ultrastructural changes after treatment with the compound. Briefly, the promastigote forms of the parasite (1×10^7 cells/mL) were cultured in the presence or absence (control) of 95.7 µM and 187.5 µM (concentration related to IC_{50} and IC_{90} for 72 h of treatment). For optical microscopy, promastigotes were washed in PBS and fixed in methanol for 15 minutes, stained with 5% Giemsa solution and mounted in slides.

For electron microscopy, after treatment, parasites were washed with PBS and fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for 24 h at room temperature.

For scanning electron microscopy, poly-l-lysine was used to fix the parasites on the coverslips, which were dehydrated in different concentrations of ethanol (30, 40, 50, 60, 70,

80, 90, 95 and 100%), critical-point dried with CO_2, sputter coated with gold, and observed in a Shimadzu SS-550 scanning electron microscope.

For ultrastructural evaluation, treated and fixed parasites, as described above, were post-fixed with 1% osmium tetroxide and 0.8% potassium ferrocyanide at room temperature for 60 min. The samples were washed in 0.1 M sodium cacodylate buffer, dehydrated in increased concentration of acetone (50, 70, 80, 90, 95 and 100%) and embedded in Epon resin. Thin sections (60–70 nm) were obtained on ultramicrotome (Power Tome X RMC Products), contrasted with 5% uranyl acetate and 2% lead citrate. Finally, the samples were analysed by transmission electron microscope (TEM) JEOL – JEM 1400.

DNA content analysis of L. amazonensis promastigotes

Promastigotes (1×10^7 cells/mL) were treated with increasing concentrations of SP (1, 10 and 100 μM) for 72 h at 25°C. The promastigotes were washed in PBS plus 5 mM EDTA, resuspended in 70% methanol and kept at 4ºC overnight. Promastigotes were then washed in PBS, resuspended in 1 mL PBS plus 5 mM EDTA with 10 μg/mL PI and 10 μg/mL RNase A, and incubated for 45 min. The samples were analysed on a FACSCalibur flow cytometer (Becton Dickinson, Rutherford, NJ, USA), at least 10,000 events were acquired using CellQuest software (Joseph Trotter, The Scripps Research Institute, La Jolla, CA, USA). Data were analysed with FlowJo software.

Generation of reactive oxygen species (ROS)

Leishmania amazonensis promastigotes (1×10^7 cells/mL) were treated or not treated (control) with the compound SP at concentration of 95.7 μM and 187.5 μM for 24 h, at 25 °C. After treatment, 10 μM of H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate)was added, and the culture incubated in theabsence of light i for one hour. This probe is a non-fluorescence dye that is chemically reduced by intracellular esterases, generating the fluorescence compound 2', 7'-dichlorofluorescein (DFA) and indicating the presence of ROS. Fluorescence intensity was quantified on fluorimeter Perkin-Elmer Victor X3, at λ_{ex} = 488 nm and λ_{em} = 530 (Scariot et al. 2017).

Detection of lipid droplets accumulation in L. amazonensis promastigotes after treatment with SP

Promastigote forms of *L. amazonensis* (1×10^7 cells/mL) were treated for 24 h with concentrations that corresponded to the IC_{50} and IC_{90} of SP. Treated parasites were then harvested, washed twice in PBS, and directly stained with 10 μg/mL Nile Red for 30 min at room temperature. The cytoplasmic lipid bodies in the parasites were detected with a fluorescence microscope Olympus BX51 (Olympus) and images were captured using a UC30 camera (Olympus). In addition, after stained, parasites were analysed in a fluorescence microplate reader (Victor X3, PerkinElmer, Finland).

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Autophagic vacuoles detection

Autophagic vacuoles were evaluated with monodansylcadaverine (MDC) marker, that accumulated in autophagic vacuoles (Munafó & Colombo 2001). Promastigotes (1×10^7 cells/ml) were treated as described above. After 24 h of incubation, the cells were incubated with 0.05 μM of MDC in PBS for 1 h. Next, the cells were washed twice in PBS. MDC stain was analysed using an Olympus BX51 fluorescence microscope, and images were captured using a UC30 camera. As a control of the autophagic process, the cells were also pre-treated with wortmannin (200 nM), a PI3 kinase inhibitor involved in the regulation of early autophagy process. Light field images were acquired in order to confirm the fluorescence inside the parasites.

In silico physicochemical parameters and potential biological activity of SP

Molinspiration (http://www.molinspiration.com/docu/miscreen/druglikeness.html) was used to calculate physicochemical properties of the compound and the software Osiris (https://www.organic-chemistry.org/prog/peo/druglikeness.html) was used to predict toxicity and evaluate the *drug-likeness* and *drug-score* of SP, as well as the antileishmanial drugs, Miltefosine and Amphotericin B.

A drug candidate with good oral bioavailability is considered with the following properties: molecular weight ≤500 μM; cLogP (computed partition coefficient) <5; number

of hydrogen bond acceptors ≤10; number of hydrogen bond donors ≤5; total polar surface area (TPSA) (≤140 Å), and the number of rotatable bonds (≤10).

The toxicity prediction of Osiris considers the tumorigenic, irritant, mutagenic and reproductive risks of compounds. The software also calculates the *drug-likeness* of the compounds (Tian et al. 2015): positive values indicating a good *drug-likeness*. The physicochemical properties, together with the toxicity prediction and *drug-likeness,* results in the *drug-score*. This parameter evaluates how much a compound qualifies as a drug, with values close to 1 indicating a good *drug-score*.

Statistical analysis and figures preparation

The data presented in the graphs are expressed as the mean ± standard error of at least three independent experiments. The data were analysed using analysis of variance (ANOVA). Significant differences among means were identified using Tukey *post hoc* test. Values of $p \leq 0.05$ were considered statistically significant. The statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA). All the figures were created using Inkscape Free and Open Source Software licensed under the GPL. GIMP (GNU Image Manipulation Program) was used for re-scale and format changes, when needed.

Table S1. Molinspiration calculations of physicochemical and *druglike* properties of the compound 4-methoxy-6-(11,12-methylenedioxy -trans-styryl)-2-pyrone (SP).

	Physicochemical properties						*Drug-likeness*					
Compound	TPSA	AH	DH	VIOL	ROTB	VOL	GPC	ICM	KI	NRK	PI	EI
SP	**30.21**	**2**	**0**	**0**	**2**	**183.42**	**-0.73**	**-0.52**	**-0.81**	**-0.71**	**-0.76**	**-0.22**
Amphotericin B	**319.61**	**18**	**13**	**3**	**3**	**865.48**	**-3.06**	**-3.51**	**-3.54**	**-3.45**	**-2.45**	**-2.95**
Miltefosine	**58.60**	**5**	**0**	**0**	**20**	**432.32**	**0.40**	**0.41**	**0.02**	**-0.31**	**.09**	**0.49**

TPSA: total polar surface area; AH: hydrogen bond acceptors; DH: hydrogen bond donors; VIOL: number of violations; ROTB: numbers of rotatable links; VOL: volume; GPC: G protein-coupled receptor; ICM: ion channel modulator; KI: kinase inhibitor; NRL: nuclear receptor ligand; PI: protease inhibitor; EI: enzyme inhibitor.

Table S2. Osiris calculations of physicochemical properties and prediction of toxicity of the compound 4-methoxy-6-(11,12-methylenedioxy -trans-styryl)-2-pyrone (SP).

		Toxic risks				*Drug-score*			
Compound	MW	MUT	TUMO	IRRIT	REP	CP	S	DL	DS
SP	272.26	lefjkf	lefjkf	Lefjkf	lefjkf	1.99	-2.9	-1.09	0.57
Amphotericin B	923.0	lefjkf	lefjkf	Lefjkf	lefjkf	0.32	-5.08	-0.14	0.27
Miltefosine	407.0	lefjkf	lefjkf	Lefjkf	lefjkf	0.12	-2.39	-54.74	0.42

MW: molecular weight; Lefj: no risk; 000: moderate risk dsd: high risk; MUT: mutagenic; TUMO: tumorigenic; IRRIT: irritant; REP: reproductive; CP: cLogP; S: solubility; DL: *Drug-likeness*; DS: *Drug score*.

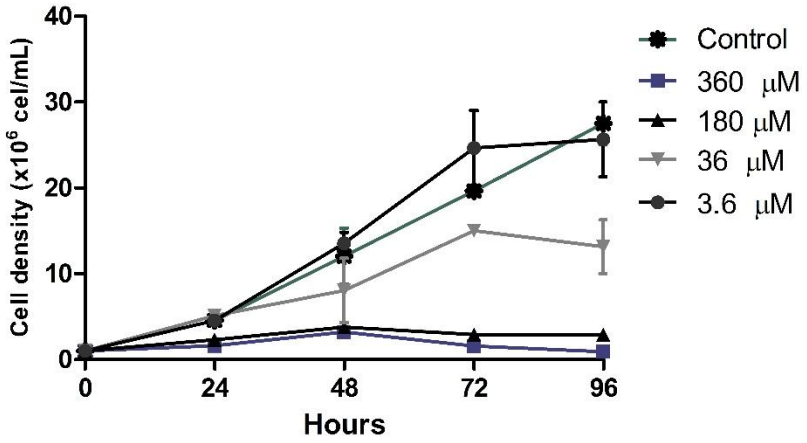

Figure S1. Cell density of *L. amazonensis* promastigote forms treated with different concentrations of SP for 96 h. Errors bars represent SE obtained from three independent experiments performed in triplicate. Treatment with 180 and 360 μM significantly reduced growth at 48, 72 and 96 h ($p < 0.05$) and 36 μM reduced growth only at 96 h ($p < 0.001$).

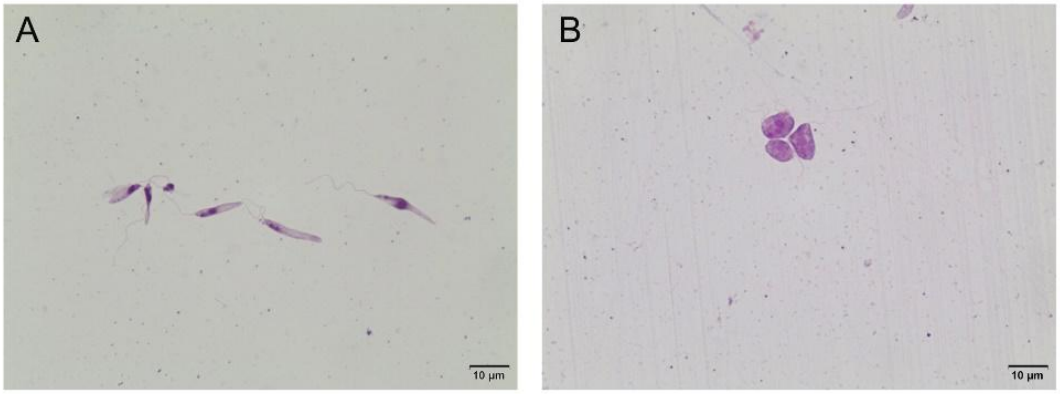

Figure S2. Morphology of untreated (A) and SP-treated (B) promastigote forms of *L. amazonensis*. Alterations in *L. amazonensis* after treatment with 10 μM of SP for 72 h were stained by Giemsa and observed by optical microscopy.

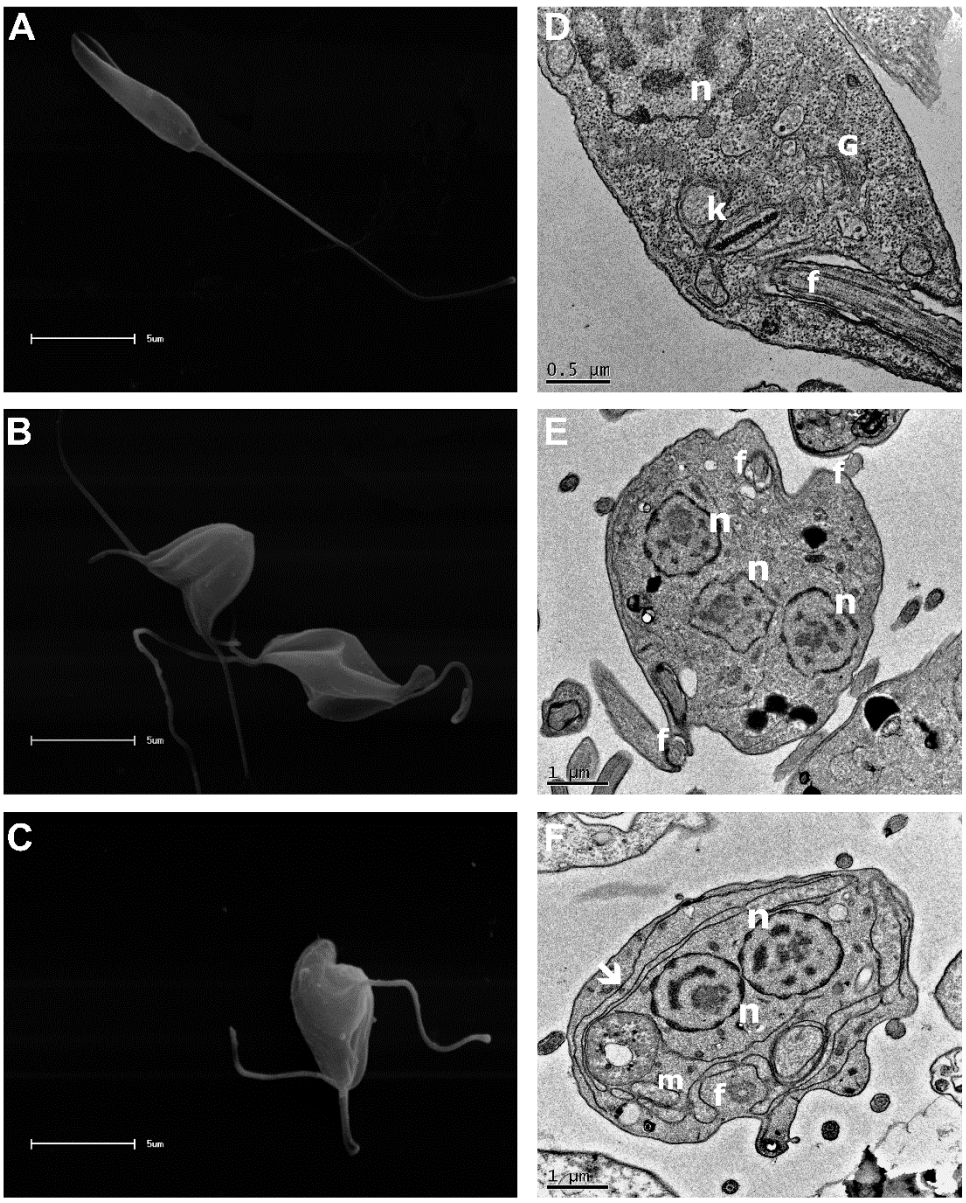

Figure S3. Structural changes in promastigote forms of *L. amazonensis* treated with SP for 72 h. Alternation observed by SEM (A-C) and TEM (D-F). (A, D) control (untreated parasites); (B, E) treated with the IC_{50} concentration, 95.7 µM; (C, F) treated with the IC_{90} concentration, 187.5 µM. N: nucleus; f: flagellum; k: kinetoplast; m: mitochondria; arrows: autophagic vacuoles.

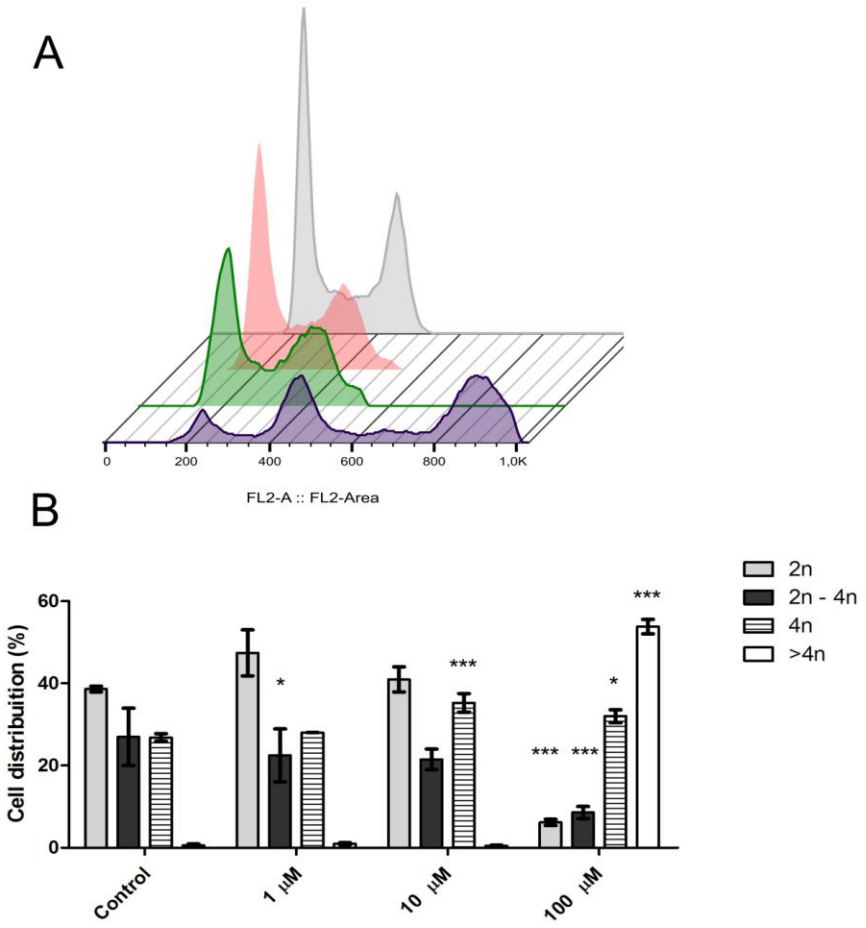

Figure S4. DNA content of *L. amazonensis* promastigotes untreated (grey) and treated with 1 µM (red), 10 µM (green) and 100 µM (blue) of SP for 72 h. (A) DNA content profile of untreated parasites showed normal DNA content with the population presenting the G0/GI (2n), S (2n-4n) and G2/M (4n) phases. Treatment with 1 µM (red) induced a small decrease in S population compared to control. After treatment with 10 µM SP (green) there is an increase in the G2/M population by 8.41% compared to the control. Treatment with 100 µM SP (blue) also increased the G2/M population by 5.1% and induced aneuploidy with 53.8% of population >4n. (B) Bar graphs showing the percentage of the population within each phase of the cell cycle. The level of significance was determined using Two-way ANOVA and Bonferroni post-tests. ***p <0.001, **p <0.01, and *p <0.05.

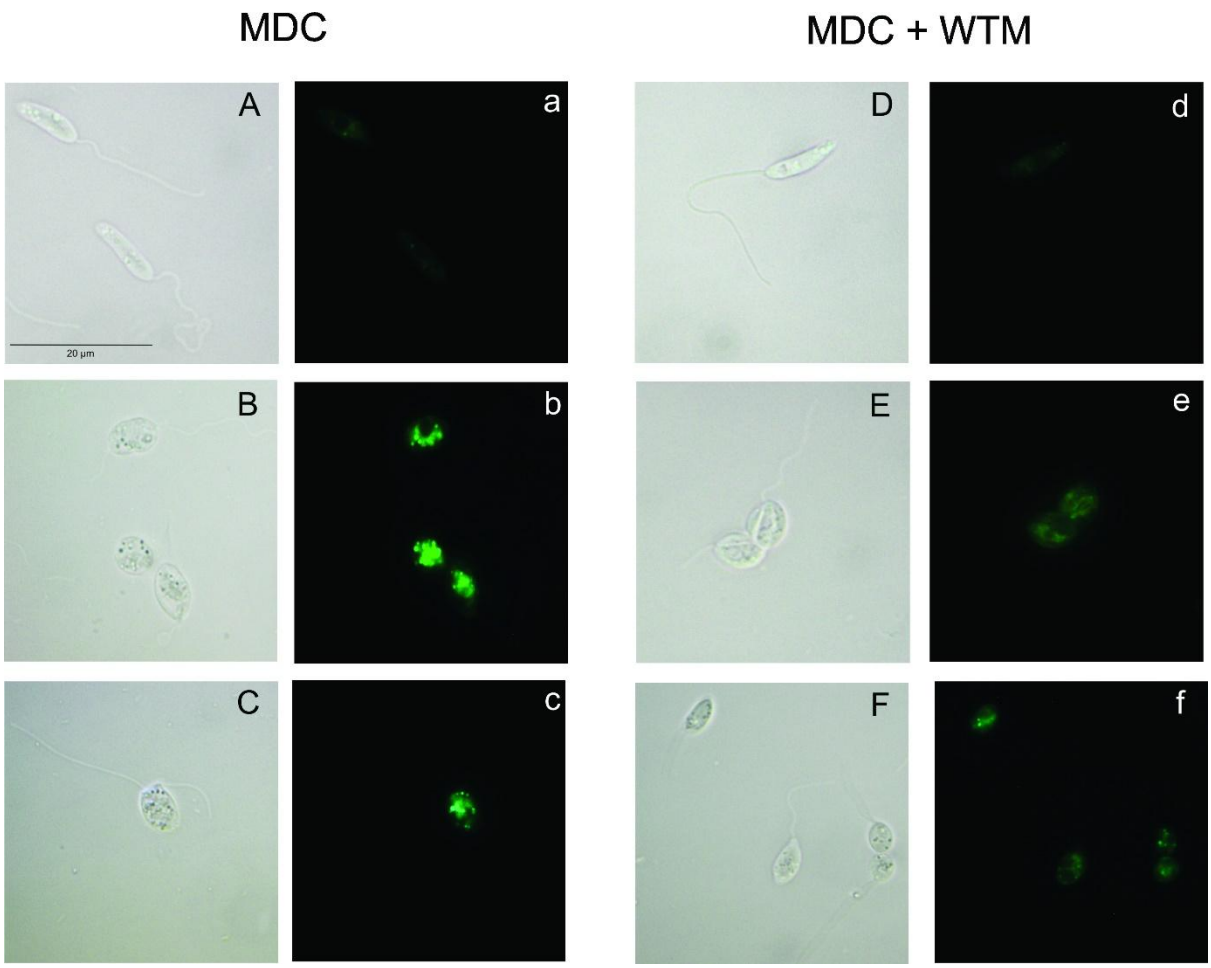

Figure S5. Generation of autophagic vacuoles in promastigote forms of *L. amazonensis* treated with Styrylpyrone (SP) for 24 h. (A-F) Differential interference contrast microscopy (DIC); (a-f) fluorescence microscopy. (A, a) control parasites; (B, b) treated with the IC_{50} concentration; (C, c) treated with the IC_{90} concentration; (D e d) Untreated parasites treated with wortmannin; (E, e, F, f) treated with SP + wortmannin (WTM).

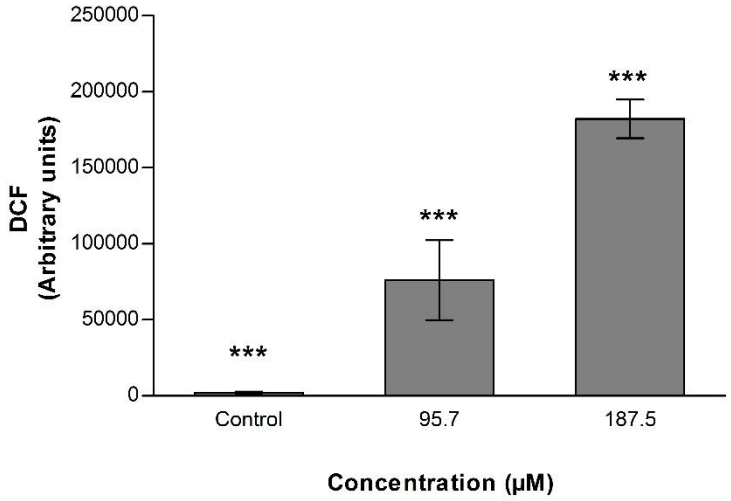

Figure S6. Reactive oxygen species (ROS) production in promastigote forms of *L. amazonensis* treated with SP for 24 h. The protozoa were treated with the IC_{50} concentration of 95.7 µM and the IC_{90} concentration of 187.5 µM for 24 h and were analysed on a microplate reader. Error bars represent standard error from at least three independents experiments. ***p <0.0001.

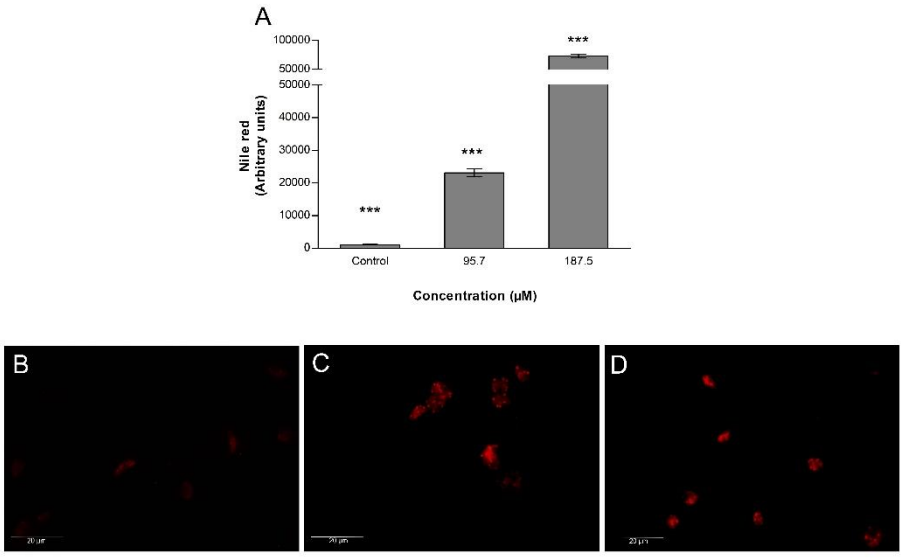

Figure S7. Lipid droplet (LD) accumulation in promastigote forms of *L. amazonensis* treated with SP for 24 h. (A) Quantitative fluorescence evaluation on promastigotes treated with the IC_{50} and IC_{90} concentrations of SP and labelled with Nile red. (B-D) Fluorescence microscopy of untreated promastigotes (B) or treated with the IC_{50} concentration, 95.7 µM

(C) and the IC_{90} concentration, 187.5 μM. (D). Error bars represent standard error from at least three independents experiments *** p <0.0001.

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