Studies *in vitro* on infectivity and sensitivity to antileishmanial drugs in New World *Leishmania* species transfected with the green fluorescent protein [pIR3(-)-eGFP]

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SUMMARY

Current chemotherapeutic agents for leishmaniasis have several disadvantages interfering with the effective treatment and therefore more and better antileishmanial drugs are needed. Discovery of candidates for leishmaniasis treatment requires not only accurate and precise methodologies but also well-known biological system to measure infectivity of parasites and antileishmanial activity of the new compounds. Significant variation in the *in vitro* and *in vivo* infectivity and sensitivity to established and experimental drugs in *Leishmania* strains are reported. This work reports the *in vitro* biological behavior and antileishmanial drugs sensitivity of different green fluorescent protein transfectant *Leishmania*s strains. The *in vitro* growth kinetic and infectivity to U937 cells vary slightly in the *Leishmania* transfectant strains in comparison with their correspondant wild-type. However, the insertion of the pIR3(-)-eGFP may affect the sensitivity of the parasites to meglumine antimoniate (MA) and miltefosine but not to amphotericin B (AMB) and pentamidine isethionate. In consequence, AMB or pentamidine isethionate but not MA or miltefosine should be used as antileishmanial control drugs during *in vitro* assays of antileishmanial activity. Furthermore, is recommended to test compounds against more than one *Leishmania* strain in order to verify that the antileihmanial activity of these compound is similar among species.

Keywords: *Leishmania*, *leishmaniasis*, *L. panamensis*, *L. guyanensis*, *L.braziliensis*, *L.mexicana*, *L.amazonensis*, infectivity, drug sensitivity.

INTRODUCTION

Leishmaniasis is a worldwide-expanded zoonotic disease caused by the infection with protozoan parasites of the genus *Leishmania* (Ross, 1903), which include 30 species classified in two subgenera, *Leishmania* and *Viannia*; approximately 20 are pathogenic for humans (WHO, 2010). These species generally present different epidemiological and clinical characteristics related to different genetic and phenotypic profiles. The clinical manifestation of the disease depends on the specie that is infecting. In the new world seven species of the parasite have been associated with localized, disseminated or diffuse cutaneous but also mucocutaneous leishmaniasis: *Leishmania braziliensis*, *Leishmania panamensis*, *Leishmania guyanensis* and *Leishmania peruviana*, belong to the subgenus *Viannia* and *Leishmania mexicana* and *Leishmania amazonensis* belonging to *Leishmania* subgenus (WHO, 2010).

Current chemotherapy agents for leishmaniasis include pentavalent antimonials, miltefosine, pentamidine and amphotericin B (WHO, 2010). However, these drugs are either toxic, expensive, or both, interfering with the effective treatment of people around the world and therefore more and better antileishmanial drugs are currently needed. The identification of new potential drug compounds and molecular targets throughout *in vitro* and *in vivo* systems are the first steps toward new therapeutic approaches and rational drug design strategies. Development of new therapeutic approaches for leishmaniasis treatment requires not only accurate and precise methodologies, but also well-known biological system to measure cytotoxicity and antileishmanial activity of the new compounds both *in vitro* and *in vivo*.

During the last decade, more accurate and precise methodologies have been developed and implemented through the use of recombinant parasites expressing diverse reporter genes such as β-lactamase, β-galactosidase, firefly luciferase or green fluorescent protein (GFP) genes. This recombinant parasites are used for *in vitro* high-through put methods and *in vivo* assays for antileishmanial drug screening (Buckner *et al*. 1996; Roy *et al*. 2000; Buckner and Wilson, 2005; Lang *et al*. 2005; Bolhassani *et al*. 2011; Pulido *et al*. 2012). Although in spite that it is known that the infectivity and susceptibility of the different *Leishmania* species to the established

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and experimental drugs is variable even in the same *Leishmania* species, reports come from several studies, each of them done by different authors and using different methodologies, parasite biological stages or *Leishmania* species and strains, making difficult the generation of adequate conclusions.

Here, the infectivity and sensitivity to commonly used antileishmanial drugs was tested in transfected *L. braziliensis*, *L. panamensis*, *L. guyanensis*, *L. mexicana* and *L. amazonensis*, the most prevalent *Leishmania* species causing cutaneous leishmaniasis in Colombia and Latin America, expressing the GFP gene (EGFP). Differences and similarities related to the growth characteristics in culture as promastigotes, the infectivity and the sensitivity to current antileishmanial drugs of these *Leishmania* species are discussed.

MATERIALS AND METHODS

Cell line and culture conditions

U-937 promonocytes (CRL1593.2™) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in standar conditions at 37 °C, 5% CO_2, with change of medium every 3 days until use. U-937 cells (suspension) were cultured in RPMI-1640 with 10% fetal bovine serum (FBS) and 1% antibiotics (100 U mL^{-1} penicillin and 0·1 mg mL^{-1} streptomycin).

Leishmania strains and in vitro *culture*

The EGFP-transfected *Leishmania* species were made in a previous work (see Pulido *et al*. 2012). During selection of fluorescent parasites, promastigotes were grown in Schneider's medium supplemented with 10% FBS, 1% antibiotics and nourseothricin (50 or 100 μg mL^{-1} to *Viannia* or *Leishmania* subgenus, respectively). Then, both wild-type and EGFP transfectants *L. panamensis* (MHOM/CO/87/UA140), *L. braziliensis* (MHOM/CO/88/UA301), *L. guyanensis* (MHOM/CO/CL007), *L. mexicana* (MHOM/MX/95/NAN1) and *L. amazonensis* (IFLA/BR/67/PH8) were grown at 26 °C in biphasic medium of Novy–MacNeal–Nicholle (NNN) medium and an overlay of phosphate buffered saline-phosphate-buffered saline (PBS) and glucose, pH 6.9.

Growth curves

Three days old promastigotes were harvested and adjusted at 5×10^4 parasites mL^{-1} liquid phase. One mL were placed into each well of 24 well-plate containing the NNN medium. Parasites were incubated at 26 °C during 12 days. Daily, liquid phase of two wells was harvested and diluted 1:10 in PBS, parasites were counted in an haemocytometer and the parasite average determined for each day to build growth curve.

Evaluation of macrophage infection

To establish the maximal amounts of parasites : cell ratio that gives the 50% infection of cells, commonly named Infectious Dose 50 (ID_{50}), U-937 cells were cultured in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA), pH 7.2 supplemented with 10% FBS and 1% antibiotics at 37 °C, 5% CO_2. After 2–3 days growth the U937 cells were washed with a PBS solution and adjusted at 3×10^5 cells mL^{-1} of RPMI medium containing 0·1 µg mL^{-1} of phorbolmyristateacetate (PMA) (Sigma-Aldrich, St Louis, MO, USA). The cells were dispensed in 24-well cell-culture plate with and without a sterile 12 mm diameter cover slide glass, then plates were incubated 72 h at 37 °C and 5% CO_2. Subsequently the cells were infected with early stationary phase promastigotes at 5 : 1, 10 : 1, 20 : 1, or 40 : 1 parasite : cell ratio following a previously described methodology (Robledo *et al*. 1999). After incubation for 3 h at 34 °C, extracellular promastigotes were removed by four washes with pre-warmed fresh medium and plates were incubated during 24 additional hours. Then, medium was removed and the cells were washed twice with 1 mL cold PBS, cells were fixed with methanol and stained with 10% Giemsa (Merck S. A, Bogotá, Colombia) and analysed under a light microscope (1.000×) to determine the infection percentage. Each dose of parasites was tested in triplicate in at least two independent experiments. Infectivity of each *Leishmania* species was determined according to the infected cells percentages obtained for each dose of parasites. The results were expressed as the ID_{50} calculated by the Probit method (Finney, 1978).

In vitro *cytotoxicity of antileishmanial drugs on U937 cells*

The cytotoxic activity of antileishmanial drugs meglumine antimoniate, miltefosine, pentamidine isethionathe and amphotericin B was assessed based on the cell growth (viability) of U937 cells and evaluated by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method as described previously (Taylor *et al*. 2010). Briefly, into each well of a 96-well cell-culture dishes were dispensed 100 000 cells 100 μL^{-1} in RPMI-1640 supplemented with 10% FBS and 100 μL of the corresponding concentrations of antileishmanial drugs. Six double serial diluted concentrations were evaluated starting at 200 μg mL^{-1}. The cells were incubated at 37 °C with 5% CO_2 for 72 h in the presence of each antileishmanial drug, and then the effect was determined by measuring the activity of the mitochondrial dehydrogenase by adding 10 μL $well^{-1}$ of MTT solution (0·5 mg mL^{-1}) and incubating at 37 °C for 3 h. The reaction was stopped by adding 100 μL $well^{-1}$ of dimethylsulfoxide for 30 min. Cell growth was determined based on the quantity of formazan

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produced, which was measured at 570 nm in a reader plate spectrophotometer (Varioskan Flash, Thermo Scientific, Waltham, MA, USA). Cells cultured in the absence of any antileishmanial drug were used as cell growth controls (negative control), while cells cultured in presence of doxorubicin were used as cytotoxicity control (positive control). Each concentration was tested in triplicate in at least two independent experiments.

Cytotoxicity was determined according to the percentages of cell growth (viability) obtained for each tested compound or medium alone. Percentages of viability were calculated using equation (1):

$$\% \text{ Viability} = [(\text{O.D. of treated cells})/(\text{O.D. of untreated cells})] \times 100, \quad (1)$$

where the O.D. of the untreated cells corresponds to 100% viability. In turn, the percentage of cell growth inhibition is calculated using the equation (2):

$$\% \text{ Cell growth inhibition} = 100 - \% \text{ of Viability}. \quad (2)$$

Percentage of cell growth inhibition was used to calculate the lethal concentration 50 (LC_{50}) that corresponds to the concentration of drug that gives the half-maximal inhibition of the cell growth by the Probit method (Finney, 1978). Cytotoxicity of antileishmanial drugs was graded according to the LC_{50} values, as follows: High cytotoxicity: $LC_{50} < 50\ \mu g\ mL^{-1}$; Moderate cytotoxicity $LC_{50} > 50$ but $<200\ \mu g\ mL^{-1}$ and potential non-cytotoxicity: $LC_{50} > 200\ \mu g\ mL^{-1}$.

In vitro *leishmanicidal activity on intracellular amastigotes*

The effect of antileishmanial drugs against intracellular amastigotes of *Leishmania* species was evaluated by flow cytometry using the methodology described by others (Taylor *et al*. 2010; Pulido *et al*. 2012). Intracellular amastigotes were obtained after infection of U937 cells with promastigotes of each *Leishmania* species; in brief, U937 cells were dispensed in 24-well plates at a concentration of 300 000 cells per well and were treated with 1 μM of PMA for 72 h at 37 °C. Then, cells were infected with promastigotes in stationary growth phase (day 5) at a ratio previously established for each *Leishmania* species and incubated 3 h at 34 °C, 5% CO_2. Cells were washed twice with PBS to eliminate non-internalized parasites and fresh RPMI-1640 was added into each well (1 mL); plates were incubated again at 34 °C and 5% CO_2 to allow intracellular differentiation to amastigotes form. After 24 h of infection of U937 cells, culture medium was replaced by fresh RPMI-1640 medium containing meglumine antimoniate, miltefosine, pentamidine or amphotericin B at the corresponding concentration (4-fold dilutions that is prepared starting at a concentration not exceeding the LC_{50}, as previously determined). Infected and treated cells were maintained at 34 °C and 5% CO_2 for 72 h. After 72 h of incubation at 37 °C, 5% CO_2 cells were removed using trypsin/EDTA solution and washed twice with PBS by centrifuging 10 min at 1100 rpm, 4 °C. Then, cells were analysed in an Argon laser flow cytometer (Cytomics FC 500MPL, Beckman Coulter. Pasadena, CA, USA) by reading at 488 nm excitation and 525 nm emission. Ten thousand events were counted from each well. The percentage of infected cells was determined by dot plot analysis and the mean fluorescence intensity in those infected cells by using histogram analysis (Pulido *et al*. 2012). Infected cells incubated in culture RPMI 1640 medium alone were used as control for infection. Each concentration was assessed in triplicate in at least two independent experiments.

Antileishmanial activity was determined according to the reduction of infected cells percentages obtained for each experimental condition. The infection percentage and infection inhibition for each concentration of each compound were calculated according to equation (3):

$$\% \text{ Infection} = \% \text{ Infected(treated cells)}/\% \text{ Infected (untreated cells)} \times 100. \quad (3)$$

In turn, the percentage of inhibition was calculated using equation (4):

$$\% \text{ Inhibition} = 100 - \% \text{ of infection}. \quad (4)$$

The results were expressed as the effective concentration 50 (EC_{50}) that corresponds to the concentration of drug that gives the half-maximal inhibition of the intracellular parasites calculated by the Probit method from % of inhibition data (Finney, 1978). The degree of antileishmanial activity was established as convenience according to the EC_{50} values, as follows: High activity: $EC_{50} < 20\ \mu g\ mL^{-1}$; moderate activity: $EC_{50} > 20$ but $<70\ \mu g\ mL^{-1}$; and low activity: $EC_{50} > 70\ \mu g\ mL^{-1}$. The index of selectivity (IS) was calculated by dividing the cytotoxicity and the antileishmanial activity using the equation (5):

$$IS = LC_{50}\ (\text{Cytoxicity})/EC_{50}\ (\text{Activity}). \quad (5)$$

Statistical analysis

Parasite growth curves were performed in duplicate, while the *in vitro* experiments for ID_{50}, LC_{50} and EC_{50} were performed in triplicate in at least two independent assays. Data represent the mean value ± S.D. The statistical significance of differences between experimental groups was determined using Mann–Whitney, Wilcoxon matched pair or

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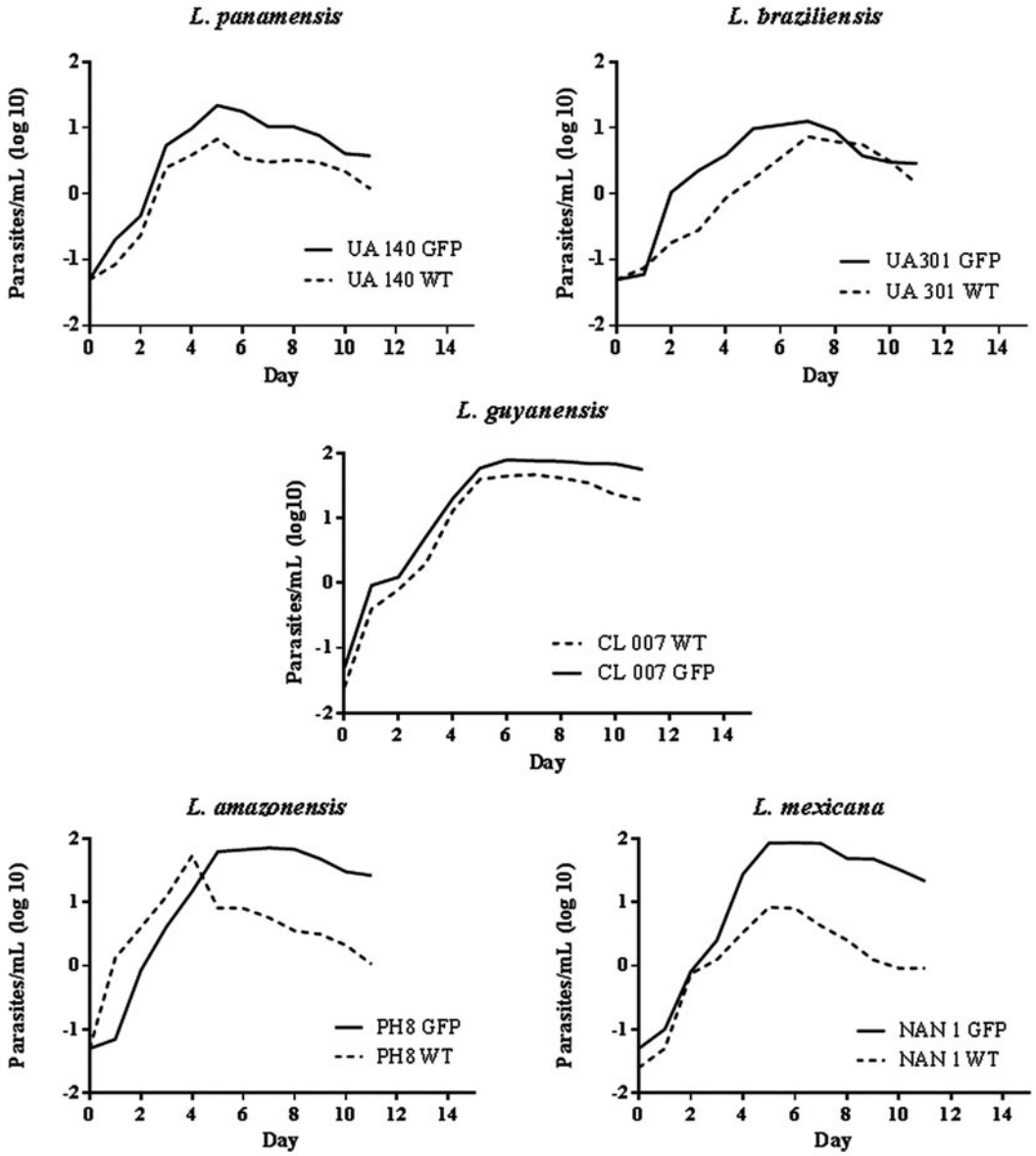

Fig. 1. Growth of *Leishmania* species in NNN biphasic medium. Data represent X + S.D. of parasites amount per day of growth. NNN, Novy–MacNeal–Nicholle

Student's *t*-test were used when suitableusing Graph Pad Prism 6 software (San Diego CA, USA). A *P*-value below 0·05 was considered statistically significant.

RESULTS

Leishmania species growth curve

Results were obtained by counting daily the promastigotes number of each *Leishmania* strain (wild-type and EGFP transfectant) grown in NNN medium. Standar deviations for each test point were below 5% of the corresponding mean values. Growth curves for promastigotes of both wild-type and EGFP-*Leishamnia* strains are showed in Fig. 1. With the initial population densities of $5{\cdot}0 \times 10^4$ parasites mL^{-1} all the evaluated species of *Leishmania* entered into the stationary phase at the same time. Nevertheless, the number of parasites at the stationary phase was always lower with *L. panamensis*-EGFP and *L. braziliensis*-EGFP than *L. mexicana*-EGFP, *L. amazonensis*-EGFP and *L. guyanensis*-EGFP. Growth curves of *L. amazonensis*-EGFP and *L. mexicana*-EGFP were very similar among them.

Differences in the growth rate were observed between *L. panamensis*-EGFP and *L. braziliensis*-EGFP in comparison with *L. guyanensis*-EGFP, *L. amazonensis*-EGFP and *L. mexicana*-EGFP. After 2 or 3 days, *Leishmania* promastigotes growth of all species was exponential and peak of growth was reached after 5 days; on day 6 promastigotes motility and refraction of *L. panamensis* started to decrease until day 9 (Fig. 1). In *L. amazonensis*-EGFP, motility of parasite started to decrease until day 8. In turn, early exponential growth of *L.*

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Table 1. Infectivity of *Leishmania* species to human U-937 macrophages

Leishmania strains	EGFP	WT
L. amazonensis	19·6 ± 5·2	12·0 ± 2·0
L. braziliensis	13·2 ± 1·6	15·0 ± 1·0
L. Mexicana	16·8 ± 6·8	20·0 ± 4·0
L. guyanensis	30·6 ± 1·4	35·0 ± 2·0
L. panamensis	30·0 ± 0·0	25·0 ± 1·0

Data represent X + S.D. of the Infective Dose 50 (ID_{50}) in *Leishmania* expressing the GFP gene (EGFP) and wild-type (WT) of at least two independent experiments.

guyanensis-EGFP and *L. mexicana*-EGFP, achieved in day 3 with stationary phase occurring in days 5–6 or 5–7 of growth, respectively, and decrease of motility and refraction occurred in days 9 and 11, respectively. Lastly, in *L. braziliensis*-EGFP the stationary phase was achieved in days 5–7 and growth decreased occurred in day 11.

Infectivity of Leishmania species

Leishmania braziliensis-EGFP showed higher infective capability evidenced by lower amounts of promastigotes needed to achieve the 50% of infection of the cell population (Table 1). The infective capability of *L. mexicana*-EGFP and *L. amazonensis*-EGFP was slightly lower than *L. braziliensis*-EGFP. In turn, *L. panamensis*-EGFP and *L. guyanensis*-EGFP showed the lowest infective capability because these *Leishmania* species requires higher amount of parasites to infect 50% of cells. The infective capability of *L. panamensis*-EGFP and *L. guyanensis*-EGFP was statistically different than that showed by L. *braziliensis*-EGFP, *L. mexicana*-EGFP and *L. amazonensis*-EGFP.

Sensitivity of human U937 macrophages to antileishmanial drugs

The sensitivity of human U937 cells to meglumine antimoniate, miltefosine, pentamidine isethionate and amphotericin B was assessed to identify the maximum concentration of drug to which cells should be exposed. The U937 macrophages were highly sensitive to pentamidine isethionate (LC_{50} 8·3 ± 1·1 $\mu g\ mL^{-1}$) and moderately sensitivity to miltefosine and amphotericin B (LC_{50} 104·6 ± 11·7 and 137·7 ± 0·6 $\mu g\ mL^{-1}$, respectively). The U937 cells were not susceptible to meglumine antimoniate, showing a LC_{50} value of >200·0 $\mu g\ mL^{-1}$.

Sensitivity of Leishmania *species to current antileishmanial drugs*

The sensitivity of *Leishmania* parasites to the antileishmanial drugs currently used to treat leishmaniasis varied among *Leishmania* species and type of drug. As shown in Table 2, all *Leishmania* strains were highly sensitive to amphotericin B with small differences in the EC_{50} values that were not statistically significant ($P > 0·01$). On the contrary, all EGFP-transfected *Leishmania* species showed a moderate sensitivity to miltefosine with EC_{50} values ranging from 36·3 ± 1·1 to 88·7 ± 3·1 $\mu g\ mL^{-1}$, *L. panamensis*-EGFP was the most sensitive to pentamidine isethionate followed by *L. amazonensis*-EGFP and *L. braziliensis*-EGFP (EC_{50} values <28·0 $\mu g\ mL^{-1}$), while *L. guyanensis*-EGFP and *L. mexicana*-EGFP were less sensitive (EC_{50} > 40·0 $\mu g\ mL^{-1}$). Differences in sensitivity to pentamidine isethionate were statistically significant ($P < 0·01$). Overall, amphotericin B was the most active antileishmanial drug for all EGFP-transfected *Leishmania* strains, while meglumine antimoniate showed the lowest activity. Differences between amphotericin B *vs* other antileishmanial drug was statistically significant ($P <$ 0·01).

None of the EGFP-transfected *Leishmania* were responsive to meglumine antimonite, while the corresponding wild-type strain did respond. As seen by others sensitivity to meglumine antimoniate in wild-type strain varies among species being *L. panamensis* more sentitive to meglumine antomoniate while *L. braziliensis* showed the less sensitivity (Table 3).

DISCUSSION

In the present work, we compared some biological characteristics of *L. braziliensis*-EGFP, *L. panamensis*-EGFP, *L. guyanensis*-EGFP, *L. mexicana*-EGFP and *L. amazonensis*-EGFP that are species that cause cutaneous and mucosal leishmaniasis in the American region. Few studies have been conducted to understand the differences in growth kinetic, infective capability and drug sensitivity among *Leishmania* species and even fewer to determine if these biological criteria are affected by parasite transfection process. These studies are important to establish parameters to compare methodologies and variables included in biological assays used for *in vitro* studies of infectivity and antileishmanial activity.

This work was compared, simultaneously, with the behavior of five *Leishmania*-EGFP-transfected strains respect to the growth kinetic, infection capability and sensitivity to antileishmanial drugs. The *in vitro* growth of all the five *Leishmania*-EGFP strains was quite similar in terms of days to reach exponential or stationary growth. However, multiplication of *L. braziliensis*-EGFP and *L. panamensis*-EGFP was lower than that of *L. mexicana*-EGFP, *L. amazonensis*-EGFP and *L. guyanensis*-EGFP. Indeed, with comparable initial densities, there is a difference of about one log in growth between the time at which *L. braziliensis*-EGFP and *L. panamensis*-EGFP entered to stationary phase in comparison

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Table 2. Sensitivity of *Leishmania* EGFP-transfected strains to antileishmanial drugs

	EC_{50} $\mu g\ mL^{-1}$ ($\mu\mu$)				
Drug[a]	*L. panamensis*	*L. braziliensis*	*L. amazonensis*	*L. mexicana*	*L. guyanensis*
AMB	0·22 ± 0·06 (0·238 ± 0·064)	0·032 ± 0·07 (0·035 ± 0·075)	0·34 ± 0·08 (0·368 ± 0·087)	0·20 ± 0·01 (0·216 ± 0·011)	0·23 ± 0·04 (0·249 ± 0·043)
MA	>200·0 (>446)	>50·0 (>111·5)	>50·0 (>111·5)	308·0 ± 3·6 (686·9 ± 8·03)	243·5 ± 9·3 (543 ± 20·74)
MIL	88·7 ± 3·1 (217·6 ± 7·6)	36·3 ± 1·1 (89·8 ± 2·7)	46·2 ± 5·4 (113 ± 13·25)	39·1 ± 6·0 (95·93 ± 14·72)	44·0 ± 7·1 (107·96 ± 17·42)
PEN	2·95 ± 0·28** (8·6 ± 0·82)**	27·2 ± 0·6 (79·9 ± 1·76)	12·2 ± 0·6** (35 ± 1·76)**	44·5 ± 5·3 (130 ± 18·5)	40·6 ± 6·3 (0·12 ± 0·02)

Data represent the median effective concentration (EC_{50}) in $\mu g\ mL^{-1}$ for each compound ± S.D.

[a] AMB: amphotericin B; MA: meglumine antimoniate; MIL: Miltefosine; PEN: pentamidine in *Leishmania*-EGFP strains.

*$P < 0{\cdot}01$ AMB *vs* other drugs; **$P < 0{\cdot}01$ *L. panamensis vs* other *Leishmania*-EGFP strains.

with *L. mexicana*-EGFP, *L. amazonensis*-EGFP and *L. guyanensis*-EGFP.

Interestingly, *L. panamensis*-EGFP behaves in the same way as does *L. braziliensis*-EGFP (both species belonging to *Viannia* subgenus) whereas *L. guyanesis*-EGFP (*Viannia* subgenus) display grown patterns similar to that *L. amazonensis*-EGFP and *L. mexicana*-EGFP, which belongs to *Leishmania* subgenus. Knowing the growth curve of parasites optimizes *in vitro* infection of macrophages. Here we found that the growth kinetics of the analysed species are similar with respect to time to late log phase occurs; moreover, the fifth day was detected as the ideal to do *in vitro* infection assays with any of these *Leishmania* strains. Recently, clinical isolates of *L. braziliensis* and *L. amazonensis* showed that metacyclogenesis occurs in 6 days old parasite cultures (Ovalle-Bracho *et al.* 2015).

Differences in infectivity between the EGFP strains were observed. One explanation could be that the number of infective parasites is different for each strain at the time of infection. Although early stationary phase parasites for all strains were used, the fraction of infective parasites may not be the same. Interestingly in the growth kinetics experiment *L. braziliensis* seem to reach stationary phase at a lower density and slightly earlier than the other strains and this strain has the lowest infective dose. This could easily be the result of a higher fraction of fully differentiated metacyclic parasites.

There are very few published work in which the infective capacity of *Leishmania* species using the *in vitro* infection of U937 cells was studied. However, in these papers a *Leishmania*:parasite cell ratio similar to that observed here was detected even that parasite used were from different strains. In those identified studies the infective parasite amount per U937 cell used was the following: 10:1 with *L. amazonensis* (LV78 strain) (Misra *et al.* 2005; Da Silva *et al.* 2015), 15:1 (Ovalle-Bracho *et al.* 2015) or 10:1 (Da Silva *et al.* 2015) with *L. braziliensis* (M2903 strain), and 20:1 with *L. panamensis* (LS94 strain) (Puentes *et al.* 2000), *L. guyanensis* (M4147 strain) (Puentes *et al.* 2000) and *L. mexicana* (M379 strain) (Bertholet and Mauël, 2000).

Overall, the sensitivity of EGFP-transfected *Leishmania* strains to antileishmanial drugs did not show big variations among strains. All *Leishmania*-EGFP strains had high sensitivity to amphotericin B and pentamidine and moderate sensitivity to miltefosine. Only pentamidine and miltefosine did show a small difference in sensitivity between *L. panamensis*-EGFP in comparison with the other EGFP transfectant *Leishmania* strains. However, further studies are required to determine the basis of these differences.

Surprisingly, all EGFP-transfected *Leishmania* strains were not sensitive to meglumine antimoniate while the corresponding wild-type strains were sensitives. Because these experiments on sensitivity to

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Table 3. Sensitivity of *Leishmania* wild-type strains to meglumine antimoniate

EC_{50} $\mu g\ mL^{-1}$ (μM)				
L. panamensis	*L. braziliensis*	*L. amazonensis*	*L. mexicana*	*L. guyanensis*
25·80 ± 3·3 (0·07 ± 0·0)	35·47 ± 5·6 (0·1 ± 0·01)	32·40 ± 3·5 (0·09 ± 0·0)	32·16 ± 4·5 (0·09 ± 0·01)	32·19 ± 4·1 (0·09 ± 0·01)

Data represent the median effective concentration (EC_{50}) in $\mu g\ mL^{-1}$ for meglumine antimoniate (MA) ± S.D. in *Leishmania* wild-type strains. *$P < 0·01$ *L. panamensis vs* other *Leishmania* strains.

meglumine antimoniate in EGFP transfectant and wild-type *Leishmania* strains were carried out using the same stock of meglumine antimoniate, this finding suggest that the insertion of the pIR3 (-)-eGFP construct may affect the sensitivity of *Leishmania* to meglumine antimoniate.

Although the cellular mechanism of the antileishmanial activity of antimonials has not been fully understood, one posible explanation for the reduced sensitivity of GFP-expressing parasites to antimonials may be associated with the competition of the overexpressed eGFP with the SbIII, the active form of the drug, for the trypanothione reductase (TR), which play important roles in the intracellular redox system of the parasite. In this scenario, we propose that the overexpressed GFP could compete with SbIII for the main intracellular reduction system, the TR, rendering the TR inaccessible to SbIII and therefore reducing the toxic effects of this compound inside the cell.

It has been shown that SbIII inhibits the TR activity compromising the thiol-redox potential in promastigotes and amastigotes. On the other hand, it has been suggested that fluorescent proteins overexpression in bacteria and mammalian cells induce a myriad of physiological effects that impair the response to different antibiotics and compounds, respectively (Liu *et al*. 1999; Allison and Sattenstall, 2007; Tao *et al*. 2007). One of the proposed toxic mechanisms of GFP and other fluorescent proteins has been associated with the aggregation propensity of GFP inside the cell through intermolecular disulphide bond formation, where the protein aggregates may induce toxicity in the cell associated with oxidative stress. In order to preserve the auto-fluorescence and prevent cell toxicity the fluorescent protein should be reduced by non-described reduction systems inside the cell. As shown in our previous works and in the current report, GFP-transfected parasites are highly and homogeneously fluorescent implying an effective reduction process of the overexpressed protein inside the cell regardless the high transcription rate of the used promoter.

Concluding remarks

The *in vitro* growth kinetic, the number of promastigotes needed to infect the U937 cells and the level of sensitivity to a specific antileishmanial drug may varyin the *Leishmania*-EGFP strains. Moreover, the insertion of the pIR3(-)-eGFP may affect the sensitivity of the transfectant leishmanias to meglumine antimoniate and miltefosine but not to amphotericin B and pentamidine isethionate. In consequence, amphotericin B or pentamidine isethionate are the most adequated drugs to use as control use *in vitro* assays for antileishmanial activity, while neither meglumine antimoniate nor miltefosine should be used in *in vitro* assays as antileishmanial control drugs. Furthermore, during screening of drug candidates is necessary to test each compound against more than one *Leishmania* strain in order to verify that the antileihmanial activity of the tested compound is similar or not in those parasites. Interestingly the finding that GFP expressing parasites are refractory to antimonials activity may indicate a physiological alteration in the genetically modified parasites. It has been suggested that fluorescent proteins overexpression in bacteria and mammalian cells induce a myriad of physiological effects associated with the aggregation prone properties of fluorescent proteins through intermolecular disulfide bond formation. We hypothesize that TR may be involved in the reduction of the monomeric forms of eGFP in order to prevent its aggregation as can be inferred by the high and homogeneous fluorescence inside the parasites. However, simultaneous studies are needed to verify changes in the antileishmanial drug sensitivities of these transfected strains with respect to the correspondent wild-type strains.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interests regarding the publication of this paper.

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