Anti-parasitic effect of the diuretic and Na^+-ATPAse inhibitor furosemide in cutaneous leishmaniasis

N. ARRUDA-COSTA[1], D. ESCRIVANI[1], E. E. ALMEIDA-AMARAL[2], J. R. MEYER-FERNANDES[3] *and* B. ROSSI-BERGMANN[1]*

[1] *Instituto de Biofísica Carlos Chagas Filho, Av Carlos Chagas Filho 373, 21941-901 Rio de Janeiro, Brazil*
[2] *Fundação Oswaldo Cruz, Av Brazil 4365, 21040-900, Manguinhos, Rio de Janeiro, Brazil*
[3] *Instituto de Bioquímica Médica Leopoldo de Meis, Av Carlos Chagas Filho 373, 21941-901 Rio de Janeiro, Brazil*

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SUMMARY

Leishmania amazonensis promastigotes are known to express furosemide (Lasix®)-sensitive *P*-type membrane Na^+-ATPase. In the present study, furosemide activity was studied in intracellular amastigotes and infected BALB/c mice to investigate its efficacy in cutaneous leishmaniasis (CL). Intracellular parasites, but not macrophages, were found to be sensitive to killing by furosemide ($IC_{50} = 87\,\mu M$ *vs* $CC_{50} \gg 1000\,\mu M$, respectively). Although furosemide did not induce nitric oxide production or intracellular pH changes in infected macrophages, it led to a significant reactive oxygen species (ROS) burst. Freshly isolated tissue parasites expressed a high degree of Na^+-ATPase activity that decreased with culture, indicative of a higher enzyme expression in amastigotes than in promastigotes. Both intraperitoneal and oral treatment of *L. amazonensis*-infected mice with furosemide dosages equivalent to that prescribed as a diuretic significantly reduced the parasite's growth compared with the situation in untreated mice. Combination with oral furosemide increased the efficacy and safety of intraperitoneal treatment with sodium stibogluconate (SSG). To summarize, furosemide control of intracellular leishmanial growth by means of parasite Na^+-ATPase inhibition, and macrophage ROS activation may help explain its sole and SSG-combined therapeutic effect against murine CL.

Key words: leishmaniasis, Na^+-ATPase, furosemide.

INTRODUCTION

Leishmaniasis is a complex of diseases caused by *Leishmania* protozoans with a spectrum of clinical manifestations ranging from morbid cutaneous leishmaniasis (CL) to lethal visceral leishmaniasis (VL) (WHO, 2012). To date, no vaccines have been approved for human use, and chemotherapy remains the most effective control measure for both forms of the disease. However, in comparison with VL, there are limited treatment options for CL. The response to multiple injections with the pentavalent antimonials, sodium stibogluconate (SSG) and meglumine antimoniate, liposomal amphotericin B formulation and pentamidine is unpredictable. Likewise, the efficacy of the more easily administered topical paromomycin and oral miltefosine is variable and dependent on the parasite species (Obonaga *et al*. 2014; Sundar and Chakravarty, 2015). New drug discovery has not been a simple task, but drug repositioning appears as a promising alternative, owing to the reduced time and costs in comparison with new drug research and development (Kaiser *et al*. 2015).

Leishmanial parasites exist as flagellated extracellular promastigotes in the sandfly, and intracellular amastigotes in the vertebrate host. Survival is correlated with the ability of parasites to adapt to hostile environments within both hosts, where they are exposed to extreme pH changes. Under these harsh conditions, transport systems regulating intracellular ion concentrations such as Na^+ are fundamental for cell homoeostasis (Rodríguez-Navarro and Benito, 2010). *P*-type Na^+-ATPases (ENA-ATPases) that induce Na^+ efflux from lower eukaryote cells have been identified in *Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania donovani* and *Leishmania amazonensis* (Stiles *et al*. 2003; De Almeida-Amaral *et al*. 2008). In this respect, *L. amazonensis* promastigotes have been shown to be killed by the Na^+-ATPase inhibitor furosemide but not K^+Na^+-ATPase inhibitor ouabain (De Almeida-Amaral *et al*. 2008). ENA-ATPase is relatively conserved among all trypanosomatid parasites, and its encoding gene identified in *T. cruzi* (*TcENA*) is absent in mammals (Iizumi *et al*. 2006).

The correlation of ENA-ATPase as a putative anti-leishmanial drug target led to the assumption that furosemide had therapeutic potential in the treatment of CL.

Furosemide (Lasix®, Fig. 1), the first of the loop diuretics, was introduced in the USA in 1966 for the treatment of congestive heart failure, oedema and hypertension. After half a century of use, furosemide has become one of the most frequently prescribed medications in the world, including neonates

* Corresponding author: Instituto de Biofísica Carlos Chagas Filho, Av Carlos Chagas Filho 373, 21·9410901 Rio de Janeiro, Brazil. Email: bartira@biof.ufrj.br

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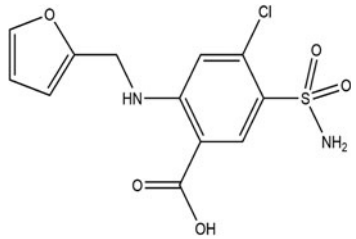

Fig. 1. Structure of furosemide.

(Pacifici, 2012). In vertebrates, the diuretic action is related to blockage of the Na–K–2Cl symporter in the kidneys' loop of Henle (Giménez, 2006), a target that is different from the leishmanial Na^+-ATPase (De Almeida-Amaral *et al*. 2008).

Therefore, in view of the reported *in vitro* leishmanicidal activity, differential mammal and parasite drug targets, and the fact that furosemide is long and safely used in humans for diabetes treatment, we proposed to study the mode of action of furosemide in the host cell and in CL.

METHODS

Drugs

Furosemide was purchased from Sigma-Aldrich, St Louis, USA for *in vitro* experiments and from Sanofi Aventis, Suzano, Brazil (Lasix®, 20 mg/2 mL solution for injection) for *in vivo* experiments. SSG (Pentostam®) was a donation from GlaxoSmithKline, Brentford, UK. Drug powders were prepared as a 20 mg mL^{-1} stock solution in culture medium and kept at 4 °C for no more than 30 days. On the day of use, the stock solutions were vortexed and immediately diluted in culture medium to the desired concentration.

Animals

BALB/c mice originally obtained from Jackson Laboratory (Bar Harbor, Maine, USA) were bred and maintained at the animal facilities at Universidade Federal do Rio de Janeiro under controlled temperature, sterilized bedding, filtered water and pelleted rodent food. Female mice at 8 weeks of age (approximately 20 g of body weight) were used in all experiments. All experiments were performed in conformity with the Brazilian Council for the Control of Animal Experiments based on the Guide for the Care and Use of Laboratory Animals [National Institutes of Health (NIH)] and were approved by the Committee for Ethics in Animal Use of the Federal University of Rio de Janeiro under the code CAUAP180.

Parasites

Promastigotes of *L. amazonensis* (WHOM/BR/75/Josefa) originally isolated from a human case of diffuse CL in Brazil were used throughout this work. They were periodically isolated from mouse lesions and maintained in culture at 26 °C in medium 199 (Cultilab, Brazil) supplemented with 10% heat-inactivated fetal calf serum (HIFCS, Invitrogen, USA), 50 μg mL^{-1} streptomycin, 50 U mL^{-1} penicillin and 2% human urine as a source of hypoxanthine, herein complete *Leishmania* medium (CLM). Parasites were always used when the stationary phase of growth was reached.

Parasite Na^+-ATPase activity

This was carried out as described previously (De Almeida-Amaral *et al*. 2008). Promastigotes (2×10^8 cells) were washed by centrifugation (1500 ***g***) three times in 12 mL of ice-cold phosphate-buffered saline (PBS). The protein concentration was determined by the Lowry method. The standard assay medium (0·1 mL final volume) contained: 20 mM HEPES–Tris, pH 7.0, 10 mM $MgCl_2$ and 5 mM ATP. The final osmolality was adjusted with choline chloride to 370 mOsm kg^{-1}. The ATPase activity was assayed by measuring the hydrolysis of [$^{\gamma 32}$P] ATP (10^4 Bq nM^{-1} ATP). The reaction was started by the addition of 0·5 mg mL^{-1} of parasite homogenate and stopped after 1 h by the addition of 0·5 mL charcoal activated by HCl (0·1 N). The [^{32}P]Pi released was measured in an aliquot of the supernatant (0·2 mL) obtained after centrifugation of the charcoal suspension for 15 min at 1500 ***g*** at 4 °C and addition of 10 mL of scintillation fluid (2 g of PPO in 1 L of toluene). The ATPase activity was calculated after subtracting the non-specific ATP hydrolysis measured in the absence of homogenate. All experiments were carried out in the presence of 1 mM ouabain and in the absence of added K^+ for complete inhibition of all (Na^+/K^+)-ATPase isoforms (Felibertt *et al*. 1995).

Anti-amastigote activity

Resident mouse peritoneal macrophages (10^6 cells) were allowed to adhere to round glass coverslips in 24-well culture plates for 2 h at 37 °C. Then, 5×10^6 promastigotes in 500 μL of CLM were added to each well for 4 h at 34 °C. After extensive washing to remove non-internalized parasites, the infected cells were incubated for a further 48 h at 37 °C with varying concentrations of the drugs (0, 62·5, 125, 250, 500 and 1000 uM) in medium 199 plus 10% HIFCS. At the end of the treatment, the cell monolayers were stained with Giemsa. The intracellular parasite loads were expressed as the total numbers of amastigotes in 200 total macrophages per sample. The 50% inhibitory concentration (IC_{50}) was determined by logarithmic regression analysis (sigmoidal dose–response) using GraphPad Prism software.

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Cytotoxicity to macrophages

Mouse peritoneal macrophages (10^6 cells) were plated and treated with varying concentrations of furosemide as above. Control cultures were treated with 1% Triton X-100 (maximum release) or medium alone (minimum release). At the end of the 48 h treatment, cell supernatants were gently centrifuged (500 ***g*** for 5 min) and assayed for lactate dehydrogenase (LDH) activity adapting the manufacturer's instructions (Doles, Brazil) for the microplate scale. The results were expressed as percentage-specific release = (maximum release − test release)/(test release − spontaneous release) × 100. The 50% cytotoxic concentration (CC_{50}) was obtained by logarithmic regression analysis (sigmoidal dose–response) assuming the Triton value as CC_{100}.

Nitric oxide assay

Bone marrow-derived macrophages (BMDM) were obtained from mouse femurs and differentiated *in vitro* using L929-cell-conditioned medium (LCCM) as a source of granulocyte/macrophage colony-stimulating factor as described previously (Marim *et al*. 2012). Cells were plated in 96-well culture plates at 2×105 well^{-1} and allowed to rest in the absence of LCCM for 24 h, when they were infected or not with10^6 *L. amazonensis* promastigotes (1:5) for 4 h at 34 °C. Infected macrophages were washed twice with pre-warmed PBS and incubated for a further 48 h at 37 °C in the presence of varying concentrations of furosemide or LPS (Sigma-Aldrich, 1 mg mL^{-1}) in 200 μL. The supernatants were collected for the colorimetric measurement of $NaNO_3$ end product using a modified Griess method (Wu and Yotnda, 2011).

Reactive oxygen species assay

BMDM were obtained and infected or not with *L. amazonensis* as above for nitric oxide (NO). After two washes, to remove non-internalized parasites, the cells were incubated at 37 °C in the presence of varying concentrations of furosemide or 250 μg mL^{-1} of zymosan (Sigma-Aldrich) for 24 h, when 10 μM of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Invitrogen) was added in the last 20 min in the dark. Oxidation of non-fluorescent H_2DCFDA to the highly fluorescent 2′,7′-dichlorofluorescein was measured at 485 nm (excitation) and 528 nm (emission) using a fluorescence microplate reader (BioTek). For all measurements, basal fluorescence relative to reagents alone was subtracted (Wu and Yotnda, 2011).

Macrophage intracellular acidity

Mouse peritoneal macrophages were plated and infected or not with *L. amazonensis* promastigotes (1:5) in a 24-well plate as for anti-amastigote activity. After removal of non-internalized parasites, the cells were cultured for 24 h at 37 °C to allow for full parasitophorous vacuole formation. Cells were treated with varying concentrations of furosemide for 24 h. Lysotracker Red dye (100 nm, Invitrogen) was added in the last 30 min of culture. Then, cells were washed and gently scraped off in PBS for fluorescence intensity (acidity) analysis by flow cytometry using Flowing software.

Mouse infection and treatment

Mice were infected subcutaneously (s.c.) in the footpads with 2×10^6 *L. amazonensis* promastigotes in 20 μL of PBS. When indicated, they were infected s.c. in the ear pinna with 10^6 parasites in 10 μL of PBS. Treatments started on day 7 of infection. Animals were given furosemide or SSG by the intraperitoneal (i.p.) route (0·4 mg/100 μL = 20 mg kg^{-1}), a total of nine injections in timely spaced intervals between days 7 and 28. Alternatively, mice were given furosemide by the oral route using intragastric gavage (1 mg/100 μL = 50 mg kg^{-1}), five times a week, a total of 30 doses between days 7 and 49. Controls received PBS alone.

Infection follow-up

Lesion sizes were periodically measured using a dial caliper and expressed as the difference between infected and contralateral uninfected footpad/ear thicknesses. For the determination of parasite loads, animals were sacrificed by deep isoflurane anaesthesia followed by cervical dislocation, and the parasites were quantified in the infected tissue by limiting-dilution assay (Lima *et al*. 1997). In brief, infected feet or ears were cleaned with 70% ethanol and aseptically removed at the joint. They were then individually minced and homogenized with 1 mL of CLM using a glass grinder. Quadruplicate serial dilutions made in 96-well plates were incubated at 26 °C for 14 days. The original numbers of parasites in each foot/ear were calculated from the highest dilution at which motile promastigotes were seen, indicative of a single amastigote at the start of incubation.

Toxicity

Mice were infected with *L. amazonensis* in the footpads and treated with oral furosemide and/or i.p. SSG as above. Controls were left untreated or received a single i.p. injection with 100 μL of 1% CCl_4 (Otsuka *et al*. 2002). One day after the termination of treatment, animals were bled under deep isoflurane anaesthesia, and their sera were assayed for creatinine and the enzymes aspartate transaminase (AST) and alanine transaminase (ALT) using

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commercial laboratory kits (Doles) according to the manufacturer's instructions.

Statistical analysis

Data were analysed using Student's *t*-test and one-way analysis of variance, with the software GraphPad Prism 5. Values of $P < 0{\cdot}05$ were considered statistically different.

RESULTS

Furosemide displays selective anti-amastigote activity

Furosemide IC_{50} activity against *L. amazonensis* promastigotes was previously determined as 840 μM (De Almeida-Amaral *et al*. 2008). Interestingly, activity against the intracellular amastigotes was found to be than that of promastigotes ($IC_{50} = 87 \pm 4$ μM) and only slightly lower than the reference drug SSG (62 ± 5 μM, $P < 0{\cdot}05$) (Fig. 2A). The 10-fold higher amastigote/promastigote activity was not due to cytotoxicity to macrophages, since these looked healthy and firmly attached. Indeed, subtle LDH release revealed CC_{50} well above 1000 μM (Fig. 2B), equal to an extrapolated value of 2200 μM. The selectivity index (SI = IC_{50} amastigote/EC_{50} macrophage) was estimated at 25, a high SI. These results show that the intracellular amastigotes are much more sensitive to furosemide than macrophages. Despite the relatively high anti-amastigote IC_{50}, the high but well-tolerated therapeutic doses justify the continuation of furosemide studies in *Leishmania*.

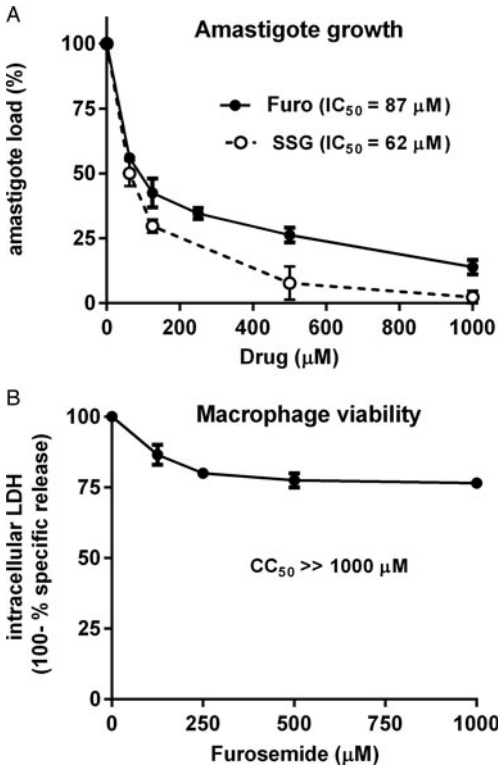

Fig. 2. Inhibition of intracellular amastigote growth. (A) Macrophages were infected with *Leishmania amazonensis* (1:5 cell ratio) for 4 h, washed and then incubated with the indicated concentrations of furosemide (Furo) or sodium stibogluconate (SSG) for 48 h. At the end of treatment, cells were stained with Giemsa, and the numbers of amastigotes in 200 total macrophages were counted. Untreated controls (100%) were 395 ± 50 amastigotes. Percentage of infected macrophages = 91%. (B) Uninfected macrophages were treated with furosemide or 1% Triton X-100. At the end of the 48 h culture time, the collected supernatants (500 µL) were colourimetrically assayed for lactate dehydrogenase activity. Maximum (Triton) and minimum (medium alone) release optical densities were $0{\cdot}951 \pm 0{\cdot}04$ and $0{\cdot}237 \pm 0{\cdot}02$. CC_{50} extrapolated value was 2200 μM. Means ± S.D. ($n = 3$). Data are representative of three independent experiments.

Furosemide activates reactive oxygen species (ROS) but not NO oxidative functions in infected macrophages

NO and ROS are important mechanisms by which macrophages kill intracellular *Leishmania* parasite (Mukbel *et al*. 2007). To investigate whether or not macrophage activation caused the anti-amastigote activity to be unexpectedly 10 times higher than the anti-promastigote activity, the production of NO and ROS was measured in furosemide-treated macrophages. Figure 3A (white bars) shows that, as expected, leishmanial infection rendered macrophages less responsive to LPS for NO production (Balestieri *et al*. 2002). Likewise, although uninfected macrophages can respond to 50 μM furosemide with detectable NO production, infected cells are non-responsive. On the other hand, infected and uninfected macrophages equally responded to furosemide with a significant increase in ROS production (Fig. 3B). ROS activation was maximal with 100 μM furosemide, a concentration sufficient to kill more than 50% of the intracellular amastigotes (Fig. 2). These results suggest that activation of ROS but not NO, may contribute to the observed *in vitro* anti-amastigote activity of furosemide.

Intracellular acidity of infected macrophages is not affected by furosemide

Unlike other intracellular pathogens such as *Legionella pneumophila* and *Toxoplasma gondii* that block phagosome acidification (Sibley *et al*. 1985; Sturgill-Koszycki and Swanson, 2000), *Leishmania* maintains an acidic pH around 5.0, which seems vital for their survival and replication inside the parasitophorous vacuole (Antoine *et al*. 1990). To investigate whether furosemide affects macrophage intracellular acidity in infected cells, infected and non-infected macrophages were treated with furosemide together with Lysotracker Red dye used as

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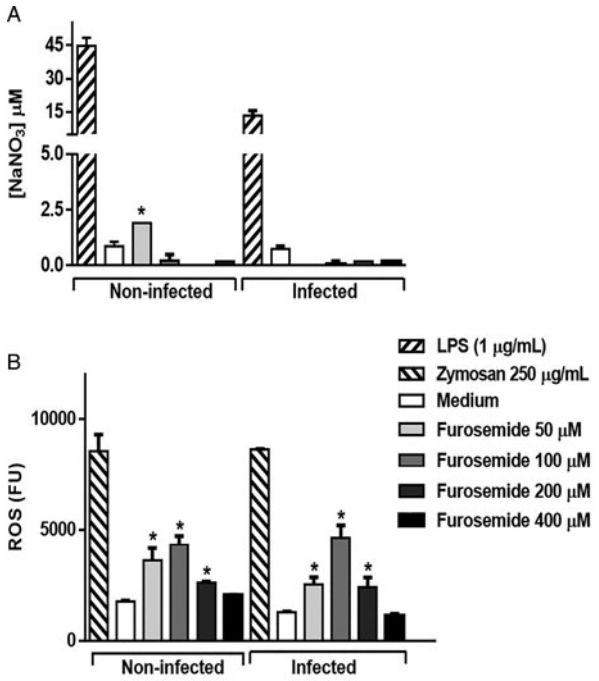

Fig. 3. Macrophage production of reactive nitrogen and oxygen products. *Leishmania amazonensis*-infected and non-infected macrophages (2×10^5) were treated with the indicated concentrations of furosemide, LPS or zymosan. (A) After 48 h of culture, the supernatants (200 μL) were collected and assayed for sodium nitrite ($NaNO_3$). (B) After 24 h of culture, H_2DCFDA (10 μM) was added, cells were cultured for 20 min in the dark, and ROS formation was expressed as fluorescence units (FU). Means ± S.D. ($n = 3$). *$P < 0{\cdot}05$ in relation to medium alone.

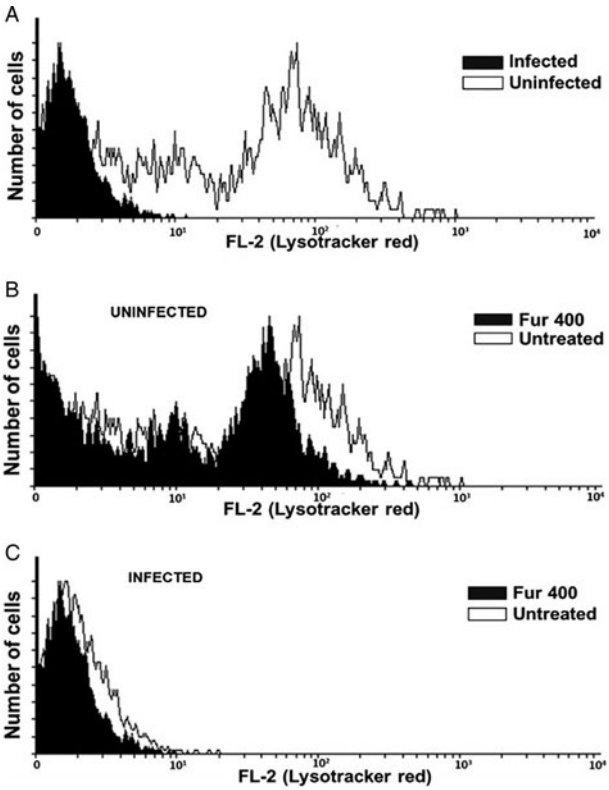

Fig. 4. Macrophage intracellular acidity. Macrophages were infected or not with *Leishmania amazonensis* promastigotes (1:5) for 28 h, when they were treated or not with 400 μM of furosemide for 24 h. After addition of Lysotracker Red dye (100 nm) in the last 20 min of culture, cells were individually analysed by flow cytofluorometry. The intracellular acidity was expressed as fluorescence intensity in FL-2. (A) Untreated: infected *vs* uninfected; (B) uninfected: treated *vs* untreated; (C) infected: treated *vs* untreated. Histograms are representative of triplicate cultures.

an H^+-sensitive probe. Figure 4A shows that *L. amazonensis* infection alone decreased the intracellular acidity. Furosemide concentrations ranging from 50 to 400 μM were tested, but an intracellular pH change was seen only with the highest 400 μM concentration. In that sense, 400 μM furosemide decreased the acidity in uninfected (Fig. 4B) but not infected cells (Fig. 4C). Therefore, it appears that intracellular pH change does not contribute to the observed *in vitro* anti-amastigote activity of furosemide.

Furosemide is effective in mice by both i.p. and oral routes

To optimize drug bioavailability, the efficacy of furosemide was first assessed by the i.p. route in *L. amazonensis*-infected mice (20 mg kg^{-1}, nine doses, ending day 28). As compared with the PBS controls, i.p. furosemide significantly ($P < 0{\cdot}05$) delayed the onset of lesion growth in infected footpads (Fig. 5A). Parasite burdens measured 2 months after termination of treatment were 80% smaller in relation to untreated footpads ($P < 0{\cdot}01$, Fig. 5B). Parasites isolated from i.p.-treated mouse lesions exhibited a 62% higher Na^+-ATPase activity than those from untreated mice (PBS). Whether or not the enhanced Na^+-ATPase activity hampered or favoured furosemide efficacy remains to be determined.

The effect of oral treatment was also investigated. A dose regimen (50 mg kg^{-1}, 30 doses, ending on day 49) more intensive than that used via the i.p. route was applied to compensate for intestinal excretion. Oral furosemide also significantly retarded the onset of lesion growth (Fig. 6A) and reduced the parasite burdens by 86% in relation to PBS-treated mice ($P < 0{\cdot}01$, Fig. 6B). As seen via the i.p. route, parasites from orally treated mice also showed a higher Na^+-ATPase activity than those from untreated mice. Freshly isolated parasites (P3) showed a 23% higher Na^+-ATPase activity than PBS parasites. However, upregulated Na^+-ATPase was gradually lost during culture, reaching control levels after five passages (P5, Fig. 6C). Together, these findings show that the *in vitro* furosemide anti-parasitic activity is extensively *in vivo*.

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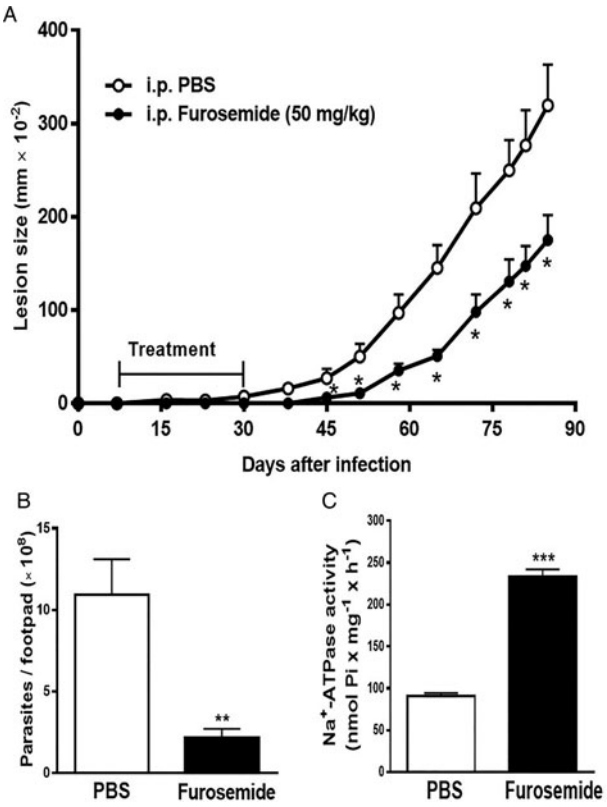

Fig. 5. Intraperitoneal treatment of cutaneous leishmaniasis with furosemide. Mice (5/group) were infected in the footpad with 2×10^6 *Leishmania amazonensis* promastigotes. During days 7–28 of infection, animals were given a total of nine i.p. injections with furosemide (50 mg kg^{-1}, 3 × week/3 weeks). (A) Course of infection in untreated and furosemide-treated mice. The lesion sizes were measured on the indicated days. (B) On day 85 of infection, the parasite loads were determined in the tissue homogenates of individual footpads by a limiting-dilution assay. Means ± S.D. ($n = 5$). (C) Parasites isolated in B were cultured as promastigotes at 26 °C for three passages, when they were assayed for Na^+-ATPase activity. Means ± S.D. ($n = 5$). $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

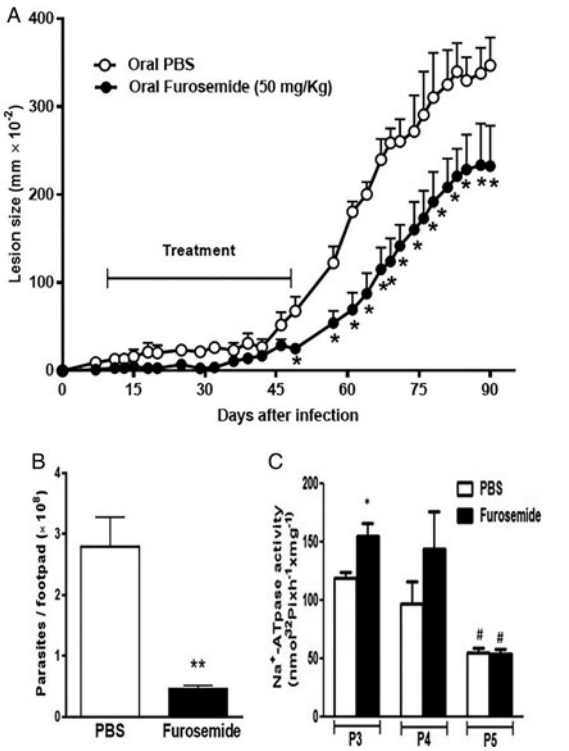

Fig. 6. Oral treatment of cutaneous leishmaniasis with furosemide. Mice were infected in the footpads with 2×10^6 with *Leishmania amazonensis*. During days 7–49 of infection, they were given 30 doses of furosemide (50 mg kg^{-1}, 5 × week/6 weeks) by oral route. (A) Course of infection in untreated and furosemide-treated mice. The lesion sizes were measured in the indicated days. Means ± S.D. ($n = 5$). (B) On day 90 of infection, the parasite loads in the footpad homogenates were individually determined by limiting-dilution assay. Means ± S.D. ($n = 5$). (C) Amastigotes isolated from tissue homogenates in B were expanded at 26 °C as promastigotes for 3 (P3), 4 (P4) or 5 (P5) passages, when they were assayed for Na^+-ATPase activity. Means ± S.D. ($n = 5$). $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$ in relation to PBS; $\#P < 0{\cdot}01$ in relation to control P3.

Efficacy of antimony therapy is improved with oral furosemide

Combination therapy has appeared as a strategy to reduce pentavalent antimonial toxicity (Shanehsaz and Ishkhanian, 2015). To evaluate the adjunct effect of oral furosemide, infected mice were given a suboptimal dose of SSG (20 mg kg^{-1}, nine doses) alone or in combination with oral furosemide (50 mg kg^{-1}, 30 doses). All the treated groups developed lesions significantly smaller than untreated controls, but not easily differentiated among themselves (not shown). Parasite burdens, by far the most critical and sensitive infection parameters, are shown in Fig. 7. Although infection of the ears was milder (Fig. 7A) than infection of the footpads (Fig. 7B), the efficacy of both drugs was less pronounced in the former. However, when combined with furosemide, SSG treatment further reduced the parasitic burden irrespective of the infection site. To explore combination therapy toxicity, on termination of treatment the serum levels of ALT, AST and creatinine were measured as markers of liver and kidney toxicity. Although no elevation in AST and creatinine levels was detected during sole SSG treatment, the ALT was increased, indicative of SSG liver toxicity. However, the ALT level was normal when SSG was given with furosemide (Fig. 8). Together, these results show that the combination with oral furosemide improved antimony therapy and reduced its toxicity.

DISCUSSION

In this work, we investigated the repositioning potential of the loop diuretic furosemide for the treatment of CL. To date, the only oral drug approved for leishmaniasis treatment is miltefosine, but high rates of clinical failure in VL, inconsistent

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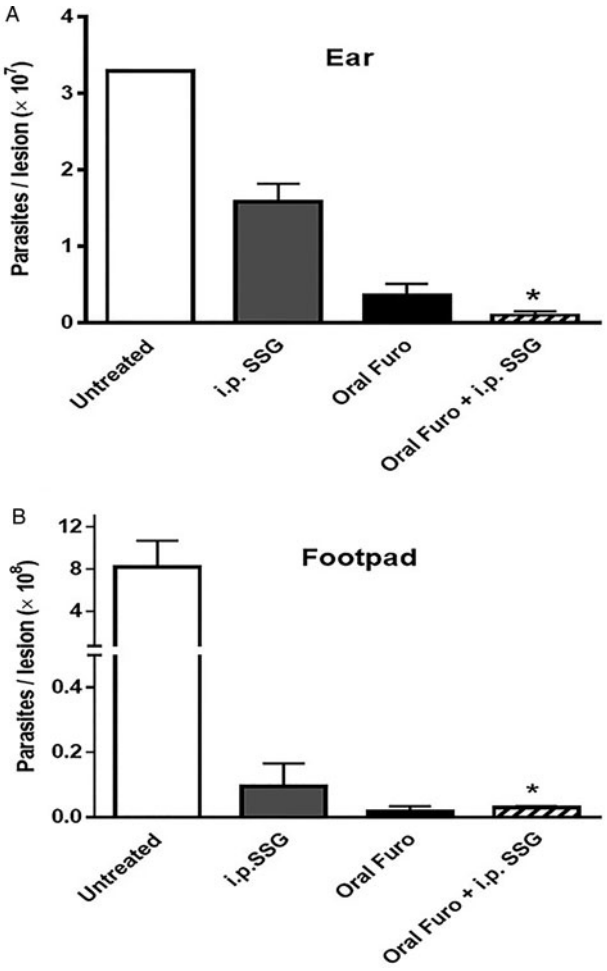

Fig. 7. Combination therapy of oral furosemide with intraperitoneal sodium stibogluconate (SSG). Mice were infected in the ear (A) or footpad (B) with 10^6 or 2×10^6 parasites, respectively. Starting on day 7 of infection, the animals were given nine i.p. injections with SSG (20 mg kg^{-1}, 3 × week), 30 doses of oral furosemide (50 mg kg^{-1}, 5 × week) or a combination of both drugs using the respective dose regimens. On day 86 of infection, the parasite loads in the ears and footpads were individually determined by limiting-dilution assay. Means ± S.D. ($n = 5$). *$P < 0{\cdot}05$ in relation to i.p. SSG.

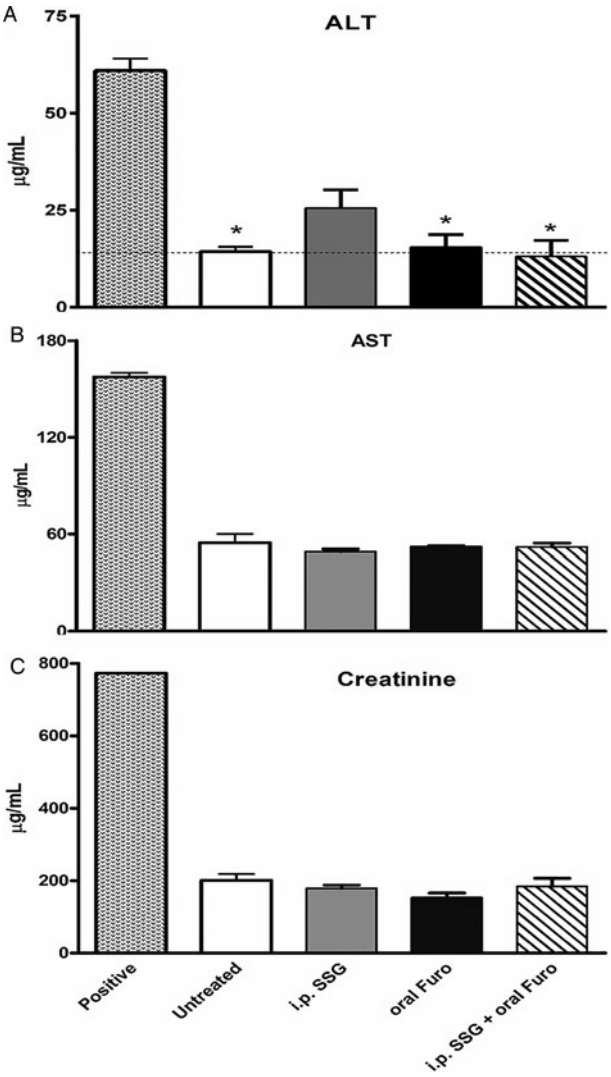

Fig. 8. Toxicity after single and combination therapy. Mice were infected in the footpads and treated i.p. with sodium stibogluconate (SSG, 20 mg kg^{-1}), oral furosemide (50 mg kg^{-1}) or a combination of both, as in Fig. 7. One day after termination of treatment (day 50 of infection), their sera were individually assayed for (A) alanine transaminase (ALT), (B) aspartate transaminase (AST) and (C) creatinine. Positive controls received a single i.p. injection with 1% CCl_4 on day 84. The dotted line indicates the reference level. Means ± S.D., $n = 5$. *$P <$ 0·05 in relation to i.p. SSG.

efficacy in CL, and adverse gastrointestinal events and teratogenesis have been the main drawbacks (Obonaga *et al*. 2014; Monge-Maillo and Lopez-Velez, 2015). Unlike miltefosine, furosemide is well tolerated by patients given the recommended intravenous and oral diuretic dosage. For chronic use, as required for leishmaniasis, oral treatment is preferred for better patient compliance. In mice, the oral treatment with 50 mg kg^{-1} day^{-1} of furosemide for 4 weeks was slightly better in reducing the leishmanial burden than the i.p. treatment with 20 mg kg^{-1} per every 2–3 days during the same period of time. The chosen murine oral dosage is clinically acceptable, considering that it is equivalent to 4 mg kg^{-1} day^{-1} in man using the body surface area for dose conversion between species, that is less than half the maximum recommended human daily dose (10 mg kg^{-1} day^{-1}) (Reagan-Shaw *et al*. 2008). Formulation in appropriate delivery systems will possibly allow a reduction in the number of oral doses (Nielsen *et al*. 2016). Furosemide therapeutic doses did not affect serum toxicity parameters, contrary to SSG that led to elevated ALT levels compatible with liver toxicity commonly found in humans under antimonial therapies (Oliveira *et al*. 2011). When SSG was used in combination with furosemide, better efficacy and safety were achieved. It is not known whether or not the furosemide diuretic effect contributed to the

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decreased SSG toxicity, but in addition to improving anti-leishmanial therapy, combination with furosemide may also favourably control antimony-associated hypertension (Lawn *et al*. 2006).

This study made use of a lesion suppression model in which the drugs were compared for their capacity to prevent the development of the cutaneous lesions. Although infection of BALB/c mice with 2×10^6 *L. amazonensis* used here represents an extremely susceptible animal model of CL (Mears *et al*. 2015), evaluation of furosemide effect in already-established lesions will likely provide a better dimension of its therapeutic range. Validation against other parasite species will also add to the spectrum of anti-leishmanial action. In this respect, our preliminary findings demonstrate that both dermotropic (*L. amazonensis* and *Leishmania braziliensis*) and viscerotropic (*Leishmania infantum* and *L. donovani*) species are sensitive to furosemide (unpublished).

Amastigotes were found to be much more sensitive to killing by furosemide ($IC_{50} = 87\ \mu M$) than extracellular promastigotes ($IC_{50} = 840\ \mu M$). This unusual effect, considering the intracellular amastigote location, was not due to killing of the host cell, since furosemide concentrations as high as 1000 μM did not significantly promote macrophage cytotoxicity, in agreement with its safety with human leucocytes at 5 mM (Yuengsrigul *et al*. 1999). Thus, it seems logical that contrary to the situation with the parasite, the furosemide-inhibitable Na^+-ATPase pump is not vital for the mammalian cell. Despite this, uninfected macrophages were able to respond to lower furosemide concentrations with NO and ROS production (Fig. 3). A maximal oxidative response coincided with an effective anti-amastigote concentration of 100 μM. The decreased response with higher concentrations is possibly due to a more dominant anti-oxidative effect (Lahet *et al*. 2003), rather than to a cytotoxic effect since at 400 μM of furosemide a 25% decrease in cell viability during 48 h (Fig. 2) would not explain the 75% decrease in ROS production during 24 h of incubation (Fig. 3). However, macrophage capacity to produce ROS was preserved, suggesting that ROS, unlike NO, contributed to intracellular amastigote killing. In any case, the use of anti-oxidants would help validate the involvement of macrophage oxidative mechanisms in furosemide activity. Although the level of Na^+-ATPase expression was not directly compared between promastigote and amastigote forms, their differential sensitivity to furosemide is likely due to a greater Na^+-ATPase dependence by the later. This assumption is supported by the finding that Na^+-ATPase expression was higher in freshly isolated parasites than in cultured parasites (Fig. 6). Indeed, a higher Na^+-ATPase expression would help amastigote survival in the highly protonated intravacuolar environment (Fig. 4) and, together with the indirect effect of ROS, would explain the higher sensitivity of amastigotes to furosemide in relation to promastigotes.

In conclusion, we show in this study the potential therapeutic effect of furosemide in CL. Since some leishmaniasis patients may be under diuretic therapy, the possibility that furosemide interferes in a positive way in the anti-leishmanial therapy should be always considered.

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