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Leishmanicidal activity of primary *S*-nitrosothiols against *Leishmania major* and *Leishmania amazonensis*: Implications for the treatment of cutaneous leishmaniasis

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Abstract

Nitric oxide (NO) is considered a key molecule in the defense against intracellular pathogens, particularly *Leishmania*. The expression of inducible nitric oxide synthase and consequent production of NO by infected macrophages has been shown to correlate with leishmaniasis resistance in the murine model as well as in human patients. Nitric oxide donors have been used successfully in the treatment of cutaneous leishmaniasis in humans, although their mechanisms of action are not fully understood. In the present work, the dose-dependent cytotoxic effects of the NO-donors *S*-nitroso-*N*-acetyl-L-cysteine (SNAC) and *S*-nitrosoglutathione (GSNO) against *Leishmania* were evaluated. GSNO inhibited the growth of *Leishmania major* and *Leishmania amazonensis* with in vitro 50% inhibitory concentrations (IC_{50}) of 68.8 ± 22.86 and 68.9 ± 7.9 μmol L^{-1}, respectively. The IC_{50} for SNAC against *L. major* and *L. amazonensis* were, respectively, 54.6 ± 8.3 and 181.6 ± 12.5 μmol L^{-1}. The leishmanicidal activity of GSNO, but not of SNAC, was reversed by ascorbic acid (AA) and dithiothreitol (DTT), suggesting that the mechanism of action of GSNO is related to the transnitrosation of parasite proteins. These results demonstrate that SNAC and GSNO have leishmanicidal activity, and are thus potential therapeutic agents against cutaneous leishmaniasis.

Keywords: Nitric oxide; *S*-Nitrosoglutathione; *S*-Nitroso-*N*-acetyl-L-cysteine; Cutaneous leishmaniasis; S-Nitrosation

Parasites of the genus *Leishmania* inhabit mammalian macrophages where they survive and grow intracellularly as non-flagellated amastigotes. This specific ability is the result of molecular mechanisms evolved by the parasites to inhibit macrophage activation by preventing the production of antimicrobial molecules and pro-inflammatory cytokines [1]. Nitric oxide (NO), which is known to mediate numerous physiological and physiopathological processes in mammals [2,3], is recognized as a key molecule in the antimicrobial activity of macrophages. It has been demonstrated that NO produced by the cytokine-inducible isoform of NO synthase (iNOS or NOS2) controls the effects of macrophages in inflammatory and immune responses, including the defense against intracellular *Leishmania* [4,5]. The mechanisms of *Leishmania* survival in infected host macrophages are related to the parasite's ability to inhibit iNOS expression or activity both directly and by an inhibitory effect on cytokines regulating iNOS production [6,7].

Several NO donors like *S*-nitroso-*N*-acetylpenicillamine (SNAP), *S*-nitrosoalbumin, *S*-nitrosoglutathione (GSNO), and *S*-nitroso-*N*-acetyl-L-cysteine (SNAC) have already been shown to kill *Trypanosoma cruzi* [8,9] and *Plasmodium*

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falciparum [10] in vitro, while glyceryl trinitrate (GTN), SNAP or acidified nitrite cream were used in the treatment of a few cutaneous leishmaniasis patients [11–13]. It has been assumed that the anti-parasite activity of these NO-donors depends upon the release of free NO, but their precise mechanism of action has not been clarified.

Except for GTN, all the other NO-donors mentioned above are *S*-nitrosothiols (RSNOs) and some of them, like *S*-nitrosoalbumin, GSNO, and *S*-nitrosohemoglobin, were already identified as endogenous NO carriers and donors in mammals [14–16]. In such species, NO is covalently bound to a sulfur atom in a C–S–NO moiety and can be released through the homolytic or heterolytic S–N bond cleavage. The homolytic cleavage is able to release free NO that can be transferred to specific receptors like iron-containing enzymes, to which it can coordinate as a ligand (nitrosylation reactions). Treatment of *Leishmania* promastigotes or amastigotes with authentic NO gas or with NO donors has been shown to lead to inhibition of mitochondrial respiration with decreased aconitase activity, probably triggered by iron loss [5,17].

The heterolytic reaction, on the other hand, allows the transfer of NO directly to thiol-containing proteins, to which it can bound as a nitrosonium ion (NO^+) in transnitrosation reactions [18,19]. RSNOs are involved in the storage and transport of NO and are responsible for its preservation against reactive species, such as dioxygen, superoxide, and oxyhemoglobin [20,21]. This property is probably a key factor in the already demonstrated role that RSNOs play in cellular defense [22].

An increasing amount of evidence indicates that the RSNOs exert their main biological actions by this direct transfer of NO to NO receptors and not by generating free NO. In fact, the ability of RSNOs to S-nitrosate cysteine residues of proteins has emerged as an important mechanism of protein activation and inactivation via posttranslational protein modification [23,24]. For example, S-nitrosation has been shown to inhibit the activity of caspases [23] and denitrosation is required for enzymatic activity of these enzymes. GSNO is able to S-transnitrosate the insulin receptor in hepatocyte cultures, leading to insulin resistance [25]. It has also been demonstrated that NO-donors inhibit *Leishmania* cysteine proteinase (CP) activity [26–28]. The reversal of proteinase inactivation by dithiothreitol (DTT) and L-ascorbic acid (AA) [27] suggests that S-transnitrosation is the main process for parasite CP inactivation by NO donors.

While SNAP is an exogenous RSNO, whose stability is linked to the fact that the sulfur atom is bound to a tertiary carbon, GSNO and SNAC are primary RSNOs (their sulfur atoms are bound to primary carbons), whose intrinsic stabilities are linked to structural factors and the existence of intramolecular hydrogen bonds with the S–NO moiety [29]. Their relative stability, ability to undergo transnitrosation and similarity to the endogenously found RSNOs, makes them good candidates for testing as leishmanicidal agents.

In the present study, the dose-dependent cytotoxic effects of the *S*-nitrosothiols SNAC and GSNO against two *Leishmania* species were evaluated: *L. major*, which causes cutaneous leishmaniasis in Asia and *L. amazonensis*, one of the most important causes of cutaneous leishmaniasis in South America and the most important agent of diffuse cutaneous leishmaniasis, which is usually unresponsive to treatment. The results obtained demonstrate that SNAC and GSNO have leishmanicidal activity and are thus potential agents for the treatment of cutaneous leishmaniasis.

Experimental procedures

Materials

Glutathione (γ-Glu-Cys-Glu, GSH), *N*-acetyl-L-cysteine (NAC), sodium nitrite ($NaNO_2$), amphotericin B, phosphate-buffered saline (PBS), pH 7.4, dithiothreitol (DTT), and L-ascorbic acid (AA) were purchased from Sigma, St. Louis, MO, USA and used as received. All the experiments were carried out using analytical grade water from a Millipore Milli-Q Gradient filtration system.

Synthesis of GSNO and SNAC

GSNO was synthesized as described previously [30]. In brief, reduced glutathione was reacted with an equimolar quantity of sodium nitrite in aqueous HCl solution, under stirring, in an ice bath for 40 min. The final solution was precipitated with acetone, filtered and washed with cold water, acetone and the final precipitate was further freeze-dried for 24 h. GSNO was stored at freezer temperature (−20 °C) protected from light. Aqueous SNAC stock solution was prepared by reacting equimolar (1.0×10^{-2} mol L^{-1}) NAC solution with sodium nitrite. The final solution was stirred at room temperature for 15 min protected from light with aluminum foil. SNAC solutions were used immediately after synthesis.

Thermal decomposition of GSNO and SNAC solutions

Spectral changes of GSNO and SNAC solutions were monitored in the range 220–1100 nm in the dark, referenced against air, using a diode array spectrophotometer (Hewlett–Packard, Model 8453, Palo Alto, CA, USA). Kinetic curves of GSNO and SNAC decomposition were obtained from the absorption changes at 336 nm in time intervals of 30 min, at 25 °C for 24 h, for solutions (500 μmol L^{-1}) in PBS. Initial rates (I_R) of GSNO and SNAC decomposition were obtained by linear regression of the slopes of the initial sections (less than 10% of the reaction) of the kinetic curves.

Parasites

Leishmania major (MHOM/IL/1981/Friedlin) and *L. amazonensis* (MHOM/BR/1973/M2269) promastigotes

were grown in liquid culture at 25 °C in Medium 199 (Gibco-BRL) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco-BRL). Promastigotes were collected at the late exponential phase of growth.

Assay of in vitro antiproliferative activity

Inhibition of cell growth was tested in vitro by cultivating promastigotes (5×10^6) in the presence of increasing concentrations of GSNO or SNAC in 24-well culture dishes (Corning Life Sciences, NY, USA) for 24 h. Freshly prepared SNAC and GSNO solutions were diluted in PBS to reach 1.0×10^{-2} mol L^{-1}. Aliquots ranging from 1 to 100 μL were added onto the wells containing the promastigote cultures in M-199. The final concentrations of SNAC and GSNO in the wells were in the range 50 to 1500 μmol L^{-1}. Cell viability was assessed by measuring the cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma–Aldrich, St. Louis, MO, USA) by metabolically active cells as described elsewhere [31]. Briefly, cells were incubated in 3-(*N*-morpholino) propanesulfonic acid (MOPS)-buffered saline (30 mmol L^{-1} MOPS, pH 7.2, 116 mmol L^{-1} NaCl, 5.4 mmol L^{-1} KCl, 0.8 mmol L^{-1} $MgSO_4$, and 5.5 mmol L^{-1} D-glucose) containing 5 mg ml^{-1} MTT for 2 h at 25 °C. MTT cleavage was measured by using a multiwell scanning spectrophotometer (Labsystems, Multiskan EX) with a reference wavelength of 690 nm and a test wavelength of 595 nm. Assays were performed in triplicate and results are expressed as the mean percent reduction of parasite numbers compared to untreated control wells calculated for at least three independent experiments. The 50% inhibitory concentration (IC_{50}) was determined from sigmoidal regression of the concentration–response curves.

Effects of AA and DTT on the kinetics of decomposition of SNAC and GSNO solutions

The effects of the reducing agents DTT and AA on the decomposition of SNAC and GSNO solutions were characterized by following the absorbance decays of the RSNOs at 336 nm using the same RSNO/AA and RSNO/DTT molar ratios used in the recovery of *L. amazonensis* assay, i.e., with SNAC and GSNO solutions 500 μmol L^{-1} in the presence of DTT 100 and 200 μmol L^{-1} and AA 2 and 20 mmol L^{-1}. The percentages of SNAC and GSNO remaining in the solutions after 40 min of incubation with DTT or AA were calculated from the kinetic curves. The reaction time of 40 min was employed since the reaction between RSNOs and the reducing agents occurs within this period (data not shown). After this period of time, the concentration of remaining RSNOs in solution decreases by the thermal S–NO bond cleavage.

The kinetics of RSNOs decomposition in the presence of DTT and AA were compared to the kinetics obtained in aqueous PBS solution.

Preservation and recovery of L. amazonensis viability by DTT and L-ascorbic acid

The effects of DTT and AA in the preservation of the viability of *L. amazonensis* promastigotes exposed to RSNOs were evaluated in the co-incubation of the parasites with RSNOs (100 and 250 μmol L^{-1}) and DTT (50 and 100 μmol L^{-1}) or AA (1 and 10 mmol L^{-1}) for 24 h at 25 °C. Viability was assessed by the MTT assay as described above. Recovery from GSNO induced damage was analyzed by incubating parasites pretreated for 4 h and then incubated in the presence of DTT and AA for 16 h at 25 °C. Viability was evaluated as described above. Control experiments with amphotericin B were performed incubating parasites with the drug (50 and 100 nmol L^{-1}) in the presence or absence of DTT (50 and 100 μmol L^{-1}) or AA (1 and 10 mmol L^{-1}) for 24 h at 25 °C. Viability was evaluated by counting aliquots of cultures in a hemocytometer, since amphotericin B interferes with the spectrophotometric reading at 595 nm.

Results and discussion

Synthesis and stability of GSNO and SNAC solutions

GSNO and SNAC were synthesized through the S-nitrosation reaction of aqueous GSH and NAC solutions, respectively. S-nitrosation in this case was performed by nitrous acid (HONO), which is formed in the acidified $NaNO_2$ solution as described previously [32]. The formation of GSNO and SNAC was confirmed by their two characteristic absorption bands at 336 and 545 nm, assigned to the $\pi \rightarrow \pi^*$ and $n_N \rightarrow \pi^*$ electronic transitions, respectively [29,33].

The stability of RSNO solutions, regarding the spontaneous thermal decomposition of aqueous buffered SNAC and GSNO solutions, was characterized by monitoring the spectral changes at 336 nm associated with the homolytic cleavage of S–N bond. Fig. 1 shows the kinetic curves

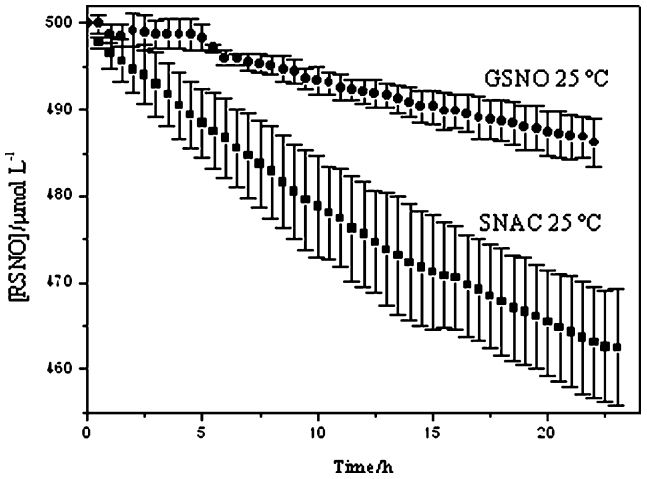


Fig. 1. Kinetic curves of thermal decomposition of GSNO and SNAC (500 μmol L^{-1}) in PBS (pH 7.4) at 25 °C.

corresponding to thermal decomposition of GSNO and SNAC solutions (500 μmol L^{-1}) at 25 °C during 22–23 h. The kinetic curves show that GSNO is more stable than SNAC, confirming previously reported results [29]. The rate of decomposition of the SNAC solution was found to be 2.4-fold higher compared to the rate obtained for GSNO solutions at 25 °C ($1.56 \pm 0.04 \times 0.65 \pm 0.01$ mol L^{-1} h^{-1}, respectively).

Activity of SNAC and GSNO against L. major and L. amazonensis in vitro

Incubation of *L. amazonensis* and *L. major* promastigotes in the presence of increasing concentrations of GSNO and SNAC led to a progressive decrease in the viability of the parasites (Fig. 2). Morphological indications of the activity of GSNO and SNAC were soon apparent after the addition of the drugs as decreased flagellar movements; eventually, treated parasites remained deposited at the bottom of the wells (data not shown).

The sensitivity of both *Leishmania* species to GSNO was similar (Figs. 2A and B) with IC_{50}s of 68.8 ± 22.86 and 68.9 ± 7.9 μmol L^{-1} for *L. major* and *L. amazonensis*, respectively. Interestingly, even at doses approximately 10-fold higher than the IC_{50}, about 10% of the parasites incubated with GSNO remained viable.

A similar profile was observed for *L. major* promastigotes incubated with SNAC (Fig. 2C) while a less potent activity was observed against *L. amazonensis* promastigotes, particularly at low concentrations (Fig. 2D). On the other hand, at doses equal or higher than 500 μmol L^{-1} SNAC exerted a more toxic action than GSNO against *L. amazonensis*. The dose–response curves for SNAC allowed the estimation of IC_{50}s of 54.6 ± 8.3 and 181.6 ± 12.5 μmol L^{-1} for *L. major* and *L. amazonensis*, respectively.

Similarly to what was observed in the case of GSNO, incubation with SNAC at high concentrations also allowed the survival of a relatively constant percentage of parasites although less pronounced for *L. amazonensis* than *L. major*, suggesting that the susceptibility of this asynchronous parasite culture is not homogeneous. These surviving parasites may represent a subpopulation in the culture in a different developmental stage, possibly metacyclic promastigotes.

Recovery of L. amazonensis viability by DTT and L-ascorbic acid

The leishmanicidal action of NO donors (especially RSNOs) may be due to the release of free NO or to the direct transfer of the NO group to parasite proteins. The latter was studied by evaluating whether the reversal of S-nitrosation leads to the recovery of promastigotes viability.

Two nitrosative modifications of proteins leading to their inactivation must be considered: the primary nitrosation of the cysteine residue (PCys-SH), generating an S-nitrosated protein (PCysSNO) (Eq. (1)), and the subsequent decomposition of the S-nitrosated protein, yielding their corresponding dimers (PCysS-SCysP) (Eq. (2)).

$$\text{PCys-SH} + \text{RSNO} \rightarrow \text{PCysSNO} + \text{RSH} \quad (1)$$

$$2\text{PCysSNO} \rightarrow \text{PCysS-SCysP} + 2\text{NO} \quad (2)$$

where P represents the protein and CysSH represents the cyteine residue.

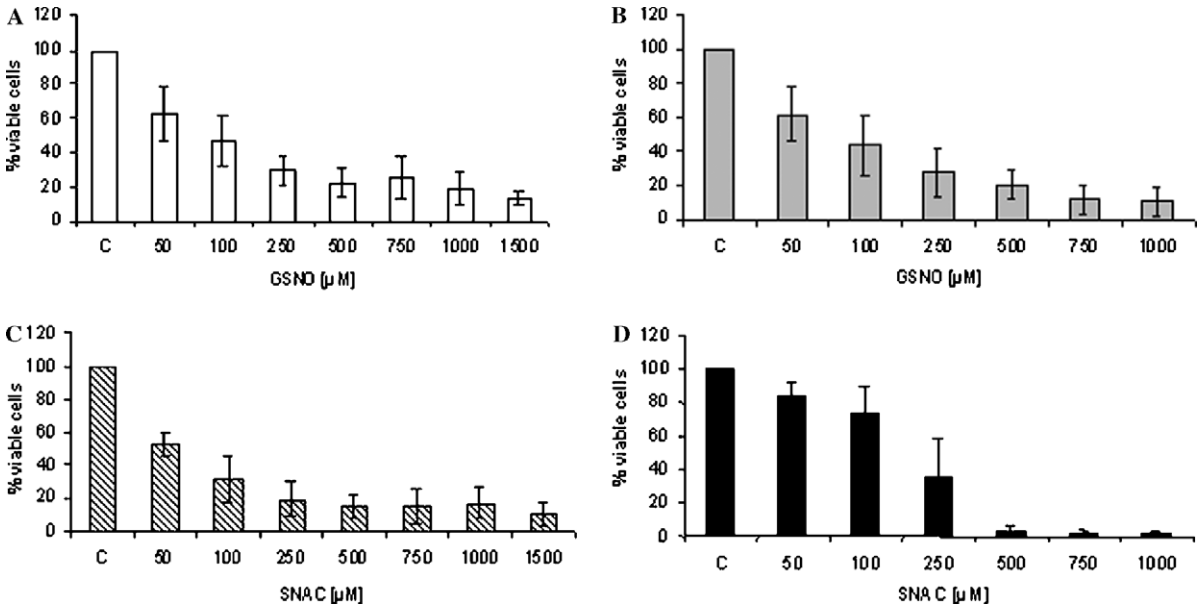


Fig. 2. Effect of RSNOs on the survival of *Leishmania*. Cell viability of *L. major* (A and C) or *L. amazonensis* (B and D) promastigotes was evaluated after 24 h incubation with different concentrations of GSNO (A and B) or SNAC (C and D). Cell viability was calculated as the percentage of values obtained for control parasites incubated in medium alone. Cultures were tested in triplicates and results shown are the average of at least three independent experiments.

In the first case, the protein S-nitrosation can be reversed by an agent capable of reducing the sulfur atom of the SNO group to its former thiol. Two reducing agents have been used for this purpose in other works: AA and DTT [34]. AA is a well-known reducing agent, which is able to transfer two hydrogen atoms per molecule in a redox reaction, yielding dehydroascorbic acid (DAA). Its reduction potential is expected to allow the reduction of S-nitrosated proteins according to Eq. (3).

$$2\text{PCysSNO} + \text{AA} \rightarrow 2\text{PCysSH} + \text{DAA} + 2\text{NO} \quad (3)$$

SNO groups can also be reduced to free thiols by the more powerful reducing agent DTT, which has two free thiols per molecule. These thiols establish a sulfur bond between themselves, transferring their hydrogen atoms to the sulfur atoms of SNO moieties; a reaction favored by the negative enthalpy variation associated with the formation of a stable five-member ring in the oxidized DTT (oxiDTT) (Eq. (4)).

$$2\text{PCysSNO} + \text{DTT} \rightarrow 2\text{PCysSH} + \text{oxiDTT} + 2\text{NO} \quad (4)$$

Although AA is not capable of reducing sulfur bonds of PCysS-SCysP to their corresponding free thiols, such reaction can be performed by DTT [35] (Eq. (5)).

$$\text{DTT} + \text{PCysS-SCysP} \rightarrow \text{oxiDTT} + 2\text{PCysSH} \quad (5)$$

To investigate if AA and DTT could reverse the toxicity of the RSNOs, the effect of these agents on the viability of *Leishmania* had to be established. *L. amazonensis* promastigotes were incubated with increasing concentrations of AA and DTT for 24 h and cell viability tested by the MTT method (data not shown). The maximal concentration of DTT allowing growth was 100 μmol L^{-1} while AA up to 10 mmol L^{-1} did not result in measurable toxicity. Fig. 3 shows the effects of AA and DTT on the viability of *L. amazonensis* exposed to GSNO and SNAC. AA significantly recovered the viability of parasites exposed to both 100 and 250 μmol L^{-1} of GSNO. This reversal of effect was also observed with DTT, although at higher DTT concentrations the recovery was incomplete, due to the intrinsic toxicity of this reducing agent (Fig. 3A). In contrast, the simultaneous incubation of SNAC with either AA or DTT was not effective in blocking parasite killing (Fig. 3B).

As a further control regarding the ability of AA and DTT of reversing the toxicity of GSNO as an indication of its mode of action, we tested the effects of these reducing agents on promastigotes incubated with amphotericin B, a polyene antibiotic which binds preferentially to ergosterol, the major sterol of *Leishmania* [36]. The mode of action of this leishmanicidal agent is therefore unrelated to the generation of NO or nitrosation of proteins. The survival rates of *L. amazonensis* promastigotes incubated with 50 and 100 nmol L^{-1} amphotericin B were 52.3 and 3.84% of the control, respectively. No significant increase in survival was observed when parasites treated with either 50 or 100 nmol L^{-1} amphotericin B were incubated with AA (1 or 10 mmol L^{-1}) or DTT (50 or 100 μmol L^{-1}), as expected (data not shown).

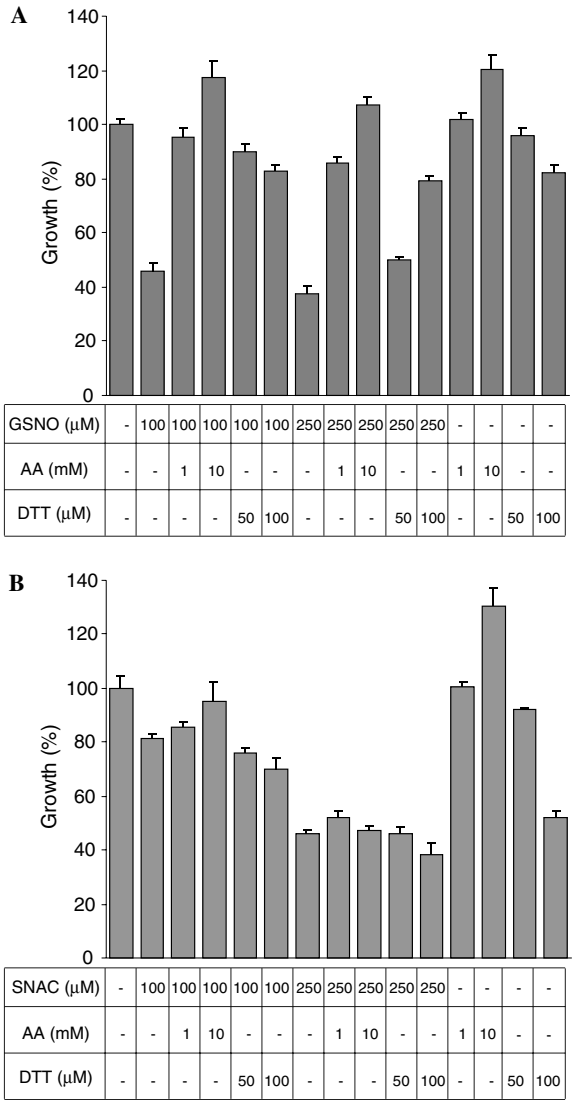


Fig. 3. Effect of AA and DTT on the viability of promastigotes treated with GSNO or SNAC. Cell viability of *L. amazonensis* promastigotes was evaluated after 24 h incubation with the indicated concentrations of GSNO (A) or SNAC (B) in the presence of AA or DTT and is expressed as the percentage of values obtained for control parasites incubated in medium alone. Results shown are the average and standard deviations of triplicates in one experiment, representative of three independent experiments.

As both RSNOs and reducing agents were added to the cultures simultaneously, we had to investigate whether AA and DTT were reducing the RSNOs prior to their interaction with parasites.

Fig. 4 shows the percentage of remaining GSNO and SNAC (500 μmol L^{-1}), after incubation with AA (2 and 20 mmol L^{-1}) and with DTT (100 and 200 μmol L^{-1}) for 40 min. The incubation with 2 and 20 mmol L^{-1} of AA led to percentages of remaining GSNO of 65 and 25%, respectively, and of remaining SNAC of 50 and 25%, respectively. The greater reducing potential of DTT is reflected in the percentages of remaining RSNOs (65 and 55% for GSNO and 80 and 65% for SNAC) obtained in

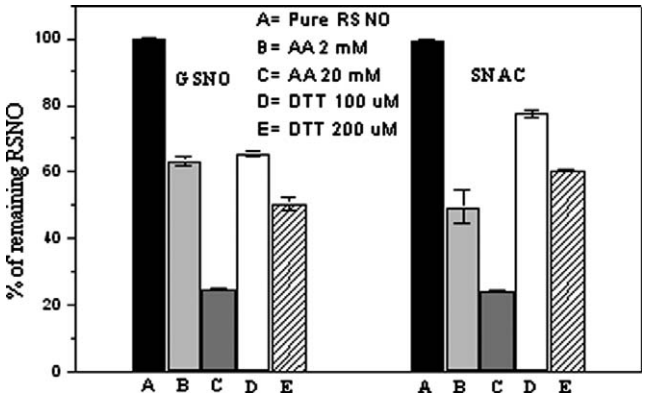
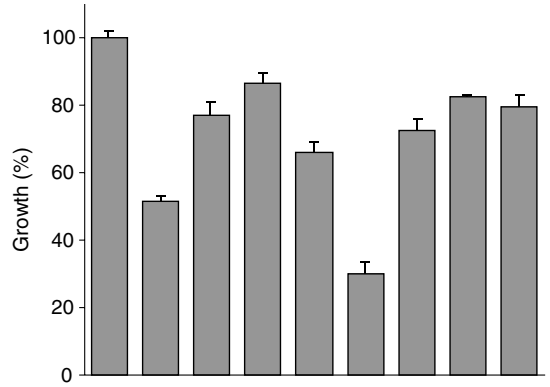


Fig. 4. Percentage of remaining GSNO and SNAC (initial concentration of 500 μmol L^{-1}) after 40 min incubated in PBS (A) and in PBS/AA (2 (B) and 20 (C) mmol L^{-1}) and in PBS/DTT (100 (D) and 200 (E) μmol L^{-1}).



Pretreatment									
GSNO (μM)	-	100	100	100	100	250	250	250	250
GSNO (μM)	-	100	-	100	100	250	-	250	250
AA (mM)	-	-	-	1	-	-	-	1	-
DTT (μM)	-	-	-	-	100	-	-	-	100

Fig. 5. Effect of AA and DTT on the leishmanicidal activity of GSNO. *L. amazonensis* promastigotes were preincubated with 100 or 250 μmol L^{-1} GSNO for 4 h. Cells were then washed, resuspended in fresh media containing the concentrations of GSNO, AA, and DTT indicated on the table below the graph and incubated for 20 h. Cell viability was calculated as the percentage of values obtained for control parasites incubated in medium alone. Results shown are the average and standard deviations of triplicates in one experiment, representative of three independent experiments.

incubations with DTT 100 and 200 μmol L^{-1}, respectively, which are 20- to 100-fold less concentrated than AA.

These results indicated that in addition to reverting the S-nitrosation of proteins, AA and DTT may also directly decompose SNAC and GSNO, even if partially. Thus, although an appreciable concentration of the drug remained intact, the recovery of GSNO-treated promastigotes on Fig. 3A could be associated not only with the reversal of S-nitrosation but also with the partial reduction of GSNO before S-nitrosation of the proteins.

To test whether the effects of GSNO on promastigotes could in fact be reversed by the incubation with AA or DTT, these compounds were added to cultures preincubated for 4 h with 100 or 250 μmol L^{-1} GSNO (Fig. 5). At the end of the preincubation period, the effect of GSNO on parasite motility was already evident with decreased flagellar movement in about 20% of the parasites incubated with 100 μmol L^{-1} and 80% of the cells treated with 250 μmol L^{-1} GSNO. To ascertain that the effect of adding the reducing agents was not due to drug inactivation, we included control cultures pretreated with the drug for 4 h and then kept on drug-free media for additional 16 h.

The treatment of *L. amazonensis* promastigotes with 100 μmol L^{-1} GSNO for 24 h resulted in 48.5% mortality. Cultures pretreated with 100 μmol L^{-1} GSNO for 4 h and then incubated for 16 h with GSNO plus 1 mmol L^{-1} AA or 100 μmol L^{-1} DTT showed 13.3 and 34.4% mortality, respectively. The reversal in the toxic effect was clearly observed with the addition of AA even in comparison with cultures pretreated with 100 μmol L^{-1} GSNO and then kept on drug-free medium for 16 h (mortality 23.0%) (Fig. 5). The same was observed when parasites were pretreated with 250 μmol L^{-1} GSNO for 4 h and then incubated with the reducing agents: mortality was reduced from 69.9% in the control cultures to 17.4% on the AA-treated cells and to 20.9% after DTT incubation. Taken together, results shown in Figs. 3–5 indicate that the *S*-nitrosothiols GSNO and SNAC are active against *Leishmania* promastigotes through distinct mechanisms: the reversibility of GSNO's toxic effects by reducing agents suggests that transnitrosation of parasite proteins is the main target for this nitrosothiol. The results obtained for GSNO are in accordance with data reported by Salvati et al. [26] in which simultaneous incubation of DTT and NO-donors with active CPs prevented the enzyme inhibition showing that *Leishmania* CPs are at least one of the targets of transnitrosation.

Conversely, the lack of effect of AA and DTT on preventing the killing of parasites by SNAC indicates that S-nitrosation is probably not operating as the primary mechanism of action in this case. A faster rate of thermal homolytic decomposition for SNAC and consequent generation of higher concentrations of free NO may shift the mechanism of action to a NO-related toxicity. NO-mediated metabolic inhibition of promastigotes and axenic amastigotes has been related to iron loss from enzymes with iron–sulfur prosthetic groups, in particular aconitase [5,17] and to the activation of a pathway similar to apoptosis [37]. However, the small difference in stability between SNAC and GSNO may not justify distinct mechanisms of action. It has been recently shown that the nitrosothiol *S*-nitrosocysteine (CSNO), but not GSNO, can be taken up by cells through a stereoselective transporter [38]. This implies specific transmembrane movement of intact L-CSNO but not of

GSNO. It is possible that SNAC and GSNO also differ in their properties regarding diffusion and permeation through membranes and that may explain distinct mechanisms of action. Future studies will be aimed at identifying specific molecular targets of both SNAC and GSNO against *Leishmania*.

Conclusions

The primary *S*-nitrosothiols SNAC and GSNO exhibit leishmanicidal activity against *L. major* and *L. amazonensis*. SNAC solutions decompose faster than GSNO solutions through NO releasing reactions. The growth arrest of promastigotes by GSNO proceeds via S-nitrosation reactions involving vital substrates, the best candidates being the cysteine proteases. This idea is reinforced by the observation that both ascorbic acid and DTT revert the toxicity of GSNO through their ability to reduce SNO groups back to their parent active free thiols. These results indicate that primary *S*-nitrosothiols have potential to be tested as therapeutic agents against cutaneous leishmaniasis.

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