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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