Leishmanicidal Activity of Two Naphthoquinones against *Leishmania donovani*

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Received May 7, 2012; accepted June 30, 2012

Here we studied ability of two naphthoquinones to inhibit *Leishmania* growth (2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone (TR 001) and 2,3-dibromo-1,4-naphthoquinone (TR 002)). TR 001 was more efficient than TR 002 in inducing killing of promastigotes and intracellular amastigotes. These values compare well to those obtained with the standard first-line antileishmanial agent sodium stibogluconate (SSG). TR 001 also induced significantly more nitric oxide (NO) production than TR 002 or SSG. Taken together, these data show that TR 001 and TR 002 could be promising new drugs for treatment of visceral leishmaniasis.

Key words *Leishmania*; naphthoquinone; macrophage

Naphthoquinones are a class of chemical compounds exhibiting a variety of anti-carcinogenic, immunomodulatory and antimicrobial activities and several have shown activity against the protozoan parasite *Leishmania*.1–5 Infections caused by different species represent a wide range of clinical manifestations, including cutaneous (CL), mucocutaneous and visceral leishmaniasis (VL). This disease is transmitted by the bite of sandflies.6–7 The disease is mostly zoonotic in nature, but an anthropotic mode of transmission also exists in some parts of Europe, Asia and other parts of the world.8,9 According to WHO, the worldwide prevalence of this disease is estimated at 12 million cases, with an annual mortality of about 60000 and the population at risk is estimated to be about 350 million.9 The systemic pentavalent antimonials still remain the recommended drugs for treatment in most endemic countries, but these are toxic and have poor patient compliance because they require daily injections periods for 3 or more weeks. Treatment of CL or VL with antimonials is even more difficult in human immunodeficiency virus (HIV)-infected individuals and is associated with frequent relapses because these drugs require healthy immune system and CD4+ T cells for optimal anti-parasitic activity.10 In addition, parasite resistance to antimonial and other first line drugs is rapidly emerging in VL endemic regions of the world, particularly India.6,9–13 Therefore, there is an ongoing need for developing new drugs for CL and VL.6,8,14–16 Currently, there is not any commercial preparation to treat any form of human leishmaniasis based on the chemical structure of naphthoquinones or their chemical derivatives. However, several studies suggest that this class of compounds have potential for development as anti-leishmanial agents. A number of monomeric and dimeric naphthoquinones present antileishmanial activity in vitro.17 For example, 8,8′-diplumbagines isolated from the plant *Pera benensis* showed activity comparable to that of the reference drug Glucantime™ towards *L. amazonensis*-infected mice,18 and 2-phenoxyl-1,4-naphthoquinones showed activity against *L. donovani* in vitro.19 Our rationale for testing 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone (TR 001) and 2,3-dibromo-1,4-naphthoquinone (TR 002) arose from the fact that these compounds are affordable and could be cheaply obtained in large quantities, which is critical for any drug developed against a neglected tropical disease.

MATERIALS AND METHODS

**Parasites** We generated a line of *L. donovani* strain LV82 expressing firefly luciferase and a red fluorescent protein. Briefly, clonal transfectants were obtained by homologous integration of a LUC-DSRed2 cassette (Swal fragment from plasmid pIRISAT-LUC-DSRed2 (strain B5947) into the ribosomal RNA (rRNA) locus as described previously for *L. major*17. Expression of DSRed2 was confirmed in both promastigotes and intracellular amastigotes by fluorescent microscopy. A number of clonal lines showing similar DSRed2 and LUC expression were obtained and used in these studies. This line will be referred to as DSRed2-*L. donovani*. Prior to each screening assay, fluorescence of parasites was verified by immunofluorescence microscopy. Direct counting of promastigotes co-cultured for 48h with TR 001 or TR 002 was performed using a Neubauer chamber.

**Leishmanicidal Assay** For screening leishmanicidal activity of drugs, we incubated 10⁶ DSRed2-*L. donovani* promastigotes with different concentrations of sodium stibogluconate, TR 001 or TR 002 at 28°C for 48h. Parasites were centrifuged at 3000 RPM for 5 min, re-suspended in 1mL of phosphate buffered saline (PBS) and parasite killing was measured by flow cytometry quantifying proportion of dead parasites assessed by loss of the fluorescence. Untreated or sham treated (2% dimethylsulfoxide (DMSO)) parasites were used as live cell control and parasites treated with 100 µg/mL of saponin from *Q. saponaria* (Sigma-Aldrich) for 30 min were used as the dead/permeabilized cell control.

**Preparation of Bone Marrow Derived Macrophages (BMDM)** We isolated BMDM from long bones (femurs and tibias) of C57BL/6 mice, as described previously.20 More than 90% of differentiated cells were CD11b+ macrophages.

The authors declare no conflict of interest.

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as determined by flow cytometry. 5×10^5 BMDM in 1mL of complete RPMI 1640 were seeded into the wells of a 24 wells plate, each well containing a glass coverslip at the bottom. Macrophages were infected overnight with promastigotes of DsRed2-L. donovani at a 1:5 ratio (1 macrophage/5 promastigotes), and extracellular parasites were washed off with fresh warm media. Infected macrophages were treated with different concentrations (0 to 100 µM) of TR 001 or TR 002 at 37°C in a CO₂ incubator for 48h. Supernatants were collected and adherent macrophages on the coverslips were fixed and stained by Giemsa to quantify infection rates as described previously. Sham-treated macrophages infected with DsRed2-L. donovani were used as controls.

**Macrophage Cytotoxicity** We cultured 5×10^5 BMDM in the presence of different concentrations (0–100 µM) of sodium stibogluconate (SSG, Pentostam™), TR 001, TR 002 or 2% DMSO (Control) for 48h and then we tested viability by trypan blue exclusion test. Macrophage cytotoxicity (CC_{50}) was

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**Fig. 1.** Chemical Structure of Two Naphthoquinones Tested for Leishmanicidal Activity: 2,3-Dichloro-5,8-dihydroxy-1,4-naphthoquinone (TR 001, A) and 2,3-Dibromo-1,4-naphthoquinone (TR 002, B)

**Fig. 2.** Axenic DsRed2-L. donovani Promastigotes (A) and DsRed2-L. donovani Amastigotes Inside of Bone Marrow Derived Macrophages (BMDM) from C57BL/6 Mice and Counterstained with DAPI (B)

**Fig. 3.** Flow Cytometry Analysis of Promastigotes of DsRed2-L. donovani Promastigotes Treated with 100 µM of Sodium Stibogluconate (Reference Drug, C), 2,3-Dichloro-5,8-dihydroxy-1,4-naphthoquinone (TR 001, D) or 2,3-Dibromo-1,4-naphthoquinone (TR 002, E) Negative control (treated with DMSO 2%, A) and positive control (treated with 100 µg/mL of saponin, B) were included. Numbers of promastigotes co-cultured with TR 001 or TR 002 were included (F). Data shown are representative of two different experiments performed in triplicate.
calculated as drug concentration needed to kill 50% of macrophages. Therapeutic index (TI) for each drug was calculated by the formula: TI = CC50/IC50 for intracellular parasites.29,30

Nitric Oxide (NO) Determination NO quantitation was performed by mixing the Griess reagent with samples and reading at 570 nm. Results so obtained were extrapolated from a standard curve prepared with Na NO2 at different concentrations (0 to 400 μM).18

Statistical Analysis Student t-test was performed to determine level of significance. IC50 values for promastigotes and amastigotes inside of BMDM were determined with the use of a computer program LdP Line® and Prism 5®.

RESULTS

The chemical structure of TR 001 and TR 002 are presented in (Fig. 1).

In Figs. 2A and B we show fluorescent promastigotes and amastigotes inside of BMDM, respectively. Promastigote death correlated with loss of fluorescence measured by flow cytometry. Figures 3A and B correspond to negative and positive controls. Figs. 3C–E correspond to promastigotes treated with SSG, TR 001 or TR 002 respectively and Fig. 3F corresponds to parasite numbers after treatment with TR 001 or TR 002 for 48h. We found that TR 001 or TR 002 presented IC50 = 5.4±2.4 and 7.04±0.15 μM respectively and SSG displayed antileishmanial activity of IC50 = 306.85±5.06 μM against L. donovani promastigotes (Table 1). Direct counting of promastigotes after 48 h co-culture with TR 001 or TR 002 showed a significant reduction in their number (Fig. 3F). Furthermore, leishmanicidal activity of SSG was much lower for extracellular parasites than TR 001 or TR 002 (Table 1).

Next, we examined the efficacy of TR 001 and TR 002 against intracellular amastigotes grown in BMDM as described previously. These compounds presented IC50 for amastigotes inside of BMDM of 0.069±0.02 μM for TR 001 and IC50 = 0.26±0.18 μM for TR 002. The reference drug (SSG) presented IC50 = 13.32±5.14 μM (Table 1). Both TR 001 and TR 002 displayed significantly higher antileishmanial activity against intracellular amastigotes than the reference drug SSG and TR 001 was more efficient than TR 002 to kill intracellularized parasites. We also recorded the TI of SSG, TR 001 and TR 002. As can be observed in Table 1 both naphthoquinones presented higher TI than SSG. As killing intracellular Leishmania is often mediated by NO, NO quantification in supernatants from macrophages cultures (48 h) was performed. Both TR 001 and TR 002 induced NO production in BMDMs, 32-fold and 7-fold respectively (16.7 vs. 0.5 μM; p<0.0001 for TR 001 and 3.3 vs. 0.5 μM; p=0.2 for TR 002), compared to control BMDMs treated with 2% DMSO (Fig. 4).

Discussion

In summary, we have demonstrated that two naphthoquinones, TR 001 and TR 002 display better antileishmanial activity against extracellular as well as intracellular L. donovani in macrophages as compared to SSG. Furthermore, we show that TR 001 displays significantly better leishmanicidal activity against intracellular parasites as compared to TR 002. This antiparasitic activity correlates with an increase in NO production. Our findings also suggest that antileishmanial activity of TR 002 against intracellular parasites is likely to be mediated via NO-dependent mechanism. Cytotoxicity of the two compounds was similar to SSG, but their therapeutic indexes were much higher. Additional in vivo work is necessary using animal models of experimental VL exploring different routes of administration (oral, intravenous or intramuscular injections) in order to perform lead optimization. Selection of TR 001 and TR 002 was considered because they have similar structure to plumbagin which is a well known antileishmanial compound. Naphthoquinones have been tested for their anti-parasite potential,25) and in most cases tested compounds correspond to dimeric and monomeric naphthoquinones such as plumbagin originally isolated from Pera benensis.5) Interestingly, TR 001 and TR 002 (two related monomeric naphthoquinones) displayed significantly better anti-Leishmania

<table>
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<th>Compound</th>
<th>Promastigote killing (IC50)</th>
<th>Amastigote killing** (IC50)</th>
<th>Macrophage cytotoxicity§ (CC50)</th>
<th>In vitro therapeutic index¶</th>
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<td>49.2±2.7</td>
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IC50 is the concentration of drug (μM) to achieve 50% killing of parasites per 10^6 axenic promastigotes or 2.5×10^6 amastigotes inside of 0.5×10^6 BMDM after 48h of culture.1) Drug concentration (μM) per 0.5×10^6 BMDM to achieve 50% killing of macrophages. 11) Therapeutic index=CC50/IC50*. 13) Significantly different (p<0.05) as compared to SSG.
activity against intracellular amastigotes of *L. donovani* as compared to extracellular promastigotes. Similar compounds have been used for *in vivo* treatment of certain types of cancer in mice with no signs of unacceptable toxicity. Taken together, our findings suggest that these compounds could be further explored as therapeutic agents for VL.

Acknowledgements This work was developed with funding from NIH Grants A1 076309, AT 004160, A1 090–803 and RC4 A1 09264 to ARS, A1 21903 and A1 29646 to SMB and CONACYT (Fondo Sectorial Salud 140091) Grants to APIM and CMLD. TR 001 and TR 002 were kindly provided by GJK and purchased at Sigma-Aldrich.

REFERENCES


