Asiaticoside induces tumour-necrosis-factor-\(\alpha\)-mediated nitric oxide production to cure experimental visceral leishmaniasis caused by antimony-susceptible and -resistant Leishmania donovani strains

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Objectives: The aim of this study was to investigate and characterize the efficacy of asiaticoside in an experimental model of visceral leishmaniasis caused by antimony-susceptible (AG83) and -resistant (GE1F8R and K39) Leishmania donovani.

Methods: The effect of asiaticoside was evaluated by microscopic counting of intracellular amastigotes in cultured macrophages stained with Giemsa. The antileishmanial effect of the compounds was assessed in infected BALB/c mice by estimation of splenic and liver parasite burdens in Leishman Donovan units. Cytokines were measured by real-time PCR and ELISA. Intracellular tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) was measured by fluorescence-activated cell sorting. Nitric oxide was measured by the Griess reaction.

Results: Besides effectively inhibiting in vitro replication of the parasite within macrophages, asiaticoside treatment resulted in almost complete clearance of the liver and splenic parasite burden when administered at a dose of 5 mg/kg \times 10 starting on day +30 of challenge with antimony-susceptible (AG83) and -resistant (GE1F8R and K39) L. donovani. Asiaticoside treatment was associated with a switch in the host from a Th2- to a Th1-type immune response accompanied by the induction of TNF-\(\alpha\)-mediated nitric oxide production, all of which are important elements for macrophage function in antileishmanial defence mechanisms.

Conclusions: These results suggest that oral therapy with asiaticoside shows promising antileishmanial efficacy in animals infected by antimony-susceptible (AG83) and -resistant (GE1F8R and K39) L. donovani.

Keywords: antileishmanial drugs, macrophage function, T cell response, cluster of differentiation 14

Introduction

Protozoa of the genus Leishmania are obligate intracellular parasites of mononuclear phagocytes. Promastigotes injected intra-dermally during insect feeding are rapidly taken up by the mononuclear phagocytes, where they transform into amastigotes. While resting macrophages support parasite growth, once they are activated, specific cellular events result in destruction of the intracellular parasites. First-line therapy includes drugs containing pentavalent antimony, such as sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime). Other drugs that have been used include amphotericin B and miltefosine. Therapy of leishmaniasis poses problems because of the parasite’s drug resistance, the most widely used of these drugs—pentavalent antimony compounds—are now of little use in northern India, which alone accounts for 50% of the world’s burden of visceral leishmaniasis.1 Thus, the identification of new and safer drugs against both antimony-susceptible and -resistant strains of Leishmania is of economic and medical importance.

Centella asiatica Linnaeus is commonly known as Indian pennywort. The plant is widely distributed in Asia, Africa and North and South America. C. asiatica has been reported to have both immunomodulating and macrophage-activating properties.2,3 C. asiatica induces the production of interleukin (IL)-2, tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and nitric oxide (NO) in macrophages.2,4 The primary active constituents of C. asiatica include triterpenoid saponins, asiaticoside and sapogenins, aspartic acid and madecassic acid. The many biological activities of C. asiatica include wound healing activity,5 anti-ulcer activity,5 anti-herpes simplex virus activity6 and anti-hepatoma activity.7

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Immunomodulatory and antitumoral effects of triterpenoid saponins have been documented. Antileishmanial activity has been reported for Hedera, Dracaena and Yucca saponins, but their value as drug candidates cannot be fully assessed since in vivo data from animal models have not been generated. The triterpenoid saponin asiaticoside has been identified as the most active compound in the C. asiatica plant associated with the healing of wounds and duodenal ulcers, and immunomodulation. It has been used in traditional medicine in India for the treatment of leprosy; asiaticoside dissolves the waxy coating of the leprosy bacteria, allowing the immune system to destroy the bacteria. The presence of rhamnose as its end sugar makes it a potential macrophage activator by virtue of rhamnose-CD14 binding, leading to TNF-α induction. Asiaticoside induces apoptosis of tumour cells. Control of the protozoan parasite *Leishmania* depends upon the development of a Th1 response, which is the major source of macrophage-activating interferon-γ (IFN-γ). IFN-γ-activated macrophages kill intercellular parasites in an L-arginine- and TNF-α-dependent mechanism involving NO production. The cluster of differentiation 14 antigen (CD14) expressed on monocytes and macrophages acts as a high-affinity receptor for lipopolysaccharide (LPS) and LPS binding protein (LBP). CD14 has been shown to bind polysaccharides with rhamnose as an end sugar and to induce TNF-α and NO production. Drug-induced NO production as a means of therapeutic treatment of visceral leishmaniasis has been well documented. Meglumine antimoniate indirectly increases NO production through increased TNF-α production. The leishmanicidal action of sodium antimony gluconate, sitafuside and patomycin involves NO production. The antileishmanial effects of Kalanchee and fucodian are also due to the activation of reactive nitrogen intermediates.

Control of *L. donovani* infection depends on the expansion of Th1 cells, macrophage activation through the production of IFN-γ and the subsequent generation of TNF-α-dependent NO and reactive oxygen species (ROS). This prompted us to test the therapeutic efficacy of asiaticoside in both in vitro and in vivo models of visceral leishmaniasis. Our data demonstrated that asiaticoside therapy was associated with a switch in the profile of parasite-derived cytokines in BALB/c mice infected with anti-monocyto-susceptible and -resistant strains of *L. donovani*.

**Materials and methods**

**Parasites, cell culture and infection**

*L. donovani* anti-monocyto-susceptible (Sb7) strain AG83 (MHOM/IN/1983/AG83) and anti-monocyto-resistant (Sb6) strains GE1F8R (MHOM/IN/89/GE1F8R) and K39 (isolated from a sodium antimony gluconate (SAG)-unresponsive patient) were grown in medium M199 (Invitrogen Life Technologies) and maintained in golden hamsters as described previously. Complete soluble antigen (CSA) was prepared from NP-40-solubilized promastigote extracts as described previously. Promastigotes obtained after transforming amastigotes from infected spleen were maintained in M199 supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal calf serum (FCS) at 22°C. Macrophages were collected by peritoneal lavage from female BALB/c mice (20–25 g) given an intra-peritoneal injection of 0.5 mL of 4% thiglycolate broth 5 days before harvest. The cells were washed, re-suspended in complete RPMI medium (RPMI 1640 supplemented with 10% FCS, 100 μg/mL streptomycin and 100 IU/mL penicillin) and were grown on glass coverslips (2 × 10^6 cells/2 mL) in six-well culture plates. After 2 h of incubation, non-adherent cells were removed by extensive washing. Adherent cells were then infected with stationary-phase second-passage *L. donovani* promastigotes at a parasite/macrophage ratio of 20:1. Forty-eight-hour parasitized cells were used throughout the study.

Cell viability was assessed using an MTT-based colorimetric assay kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer’s instructions. Asiaticoside was purchased from Sigma Chemicals (St Louis, MO, USA). Asiaticoside, solubilized beforehand in DMSO, was added to the culture medium at the selected concentrations for in vitro work; the final concentration of DMSO was 1%. All reagents, including asiaticoside (100 mg/L), were tested for endotoxin contamination by the Limulus amoebocyte lysate (LAL) endpoint assay (QCL-1000; Bio-Whitaker, MD, USA) following the manufacturer’s manual and were less than 0.1 EU/mL. For animal experiments, female BALB/c mice (20–25 g) were injected via the intra-cardiac route with 10^7 second-passage promastigotes as described. Experiments using animals were done with the permission of the Animal Ethics Committee of the Indian Institute of Chemical Biology (accreditation no. of the institute 147/1999/CEPSEA).

**Cytokine analysis by real-time PCR**

Total RNA was isolated from peritoneal macrophages or splenocytes of BALB/c mice according to the RNeasy Mini Kit isolation procedure (Qiagen) and was individually analysed (five animals/group) by real-time RT–PCR. Samples of 2 μg RNA from different experimental groups of mice were first used for cDNA synthesis by random hexamers (Invitrogen) using Superscript II (Invitrogen). The synthesized cDNA was subjected to real-time PCR with SYBR Green JumpStart Taq ReadyMix (Sigma) and a gene-specific primer set in an iCycler PCR detector (Bio-Rad) according to the manufacturer’s instructions. The relative quantification of products was determined by the comparative threshold cycle method using the equation 2^(-ΔΔCT), where ΔΔCT = [CT(target) - CT(18S)]treated - [CT(target) - CT(18S)]control, to determine the fold increase in product. Each gene of interest was normalized to the β-actin gene and the fold change was compared relative to the normal control. The data are represented as the mean ± SD of all five animals of the group under consideration.

**Cytokine production by ELISA**

In a final volume of 0.2 mL, 1 × 10^6 spleen cells from individual mice (five animals/group) were seeded in 96-well plates and incubated for 24 h at 37°C in the presence of 50 μg/mL CSA. The release of cytokines was measured in the supernatants using commercial ELISA kits (Quantikine M; R&D Systems, Minneapolis, MN, USA; IL-18, e-Bioscience). The detection limit of these assays was <2.0, <2.5, <5.1, <1.5 and <4.6 pg/mL for IFN-γ, IL-12p70, TNF-α, IL-10 and transforming growth factor-β (TGF-β), respectively. The data are represented as the mean ± SD of all five animals of the group under consideration.

**IL-2 assay**

Splenocytes (5 × 10^5 cells/well) derived from individual mice (n = 5) of different experimental groups were stimulated for 24 h with or without CSA (50 μg/mL) in complete RPMI 1640 medium in a 5% CO2 incubator at 37°C. The culture supernatants were analysed for
the presence of IL-2 by proliferation of the IL-2-dependent murine cell line HT-2 and the extent of proliferation was measured as [³H]thymidine uptake. A total of 10⁵ HT-2 cells/well were incubated with 100 µCi of culture supernatant for 48 h. The cells were then pulsed with 1 µCi of [³H]thymidine (6.7 Ci/mM) for 18 h. Incorporation of radioactive thymidine was assessed with a liquid scintillation counter (Tri-Carb 2100TR; Packard Instrument). The data are represented as the mean±SD of all five individual animals of the group under consideration.

Measurement of ROS and NO

The level of ROS was monitored using the cell-permeant probe H2DCFDA as described previously.²⁷ Splenocytes (10⁵/mL) from individual mice (n=5) of different groups were suspended in phenol red-free RPMI medium and incubated with CSA (50 µg/mL) or without CSA for 72 h in a 5% CO₂ incubator at 37°C. The supernatant was collected for NO assay and the cells were taken up in RPMI medium and incubated with H2DCFDA (2 µg/mL) at room temperature for 20 min in the dark. Relative fluorescence was measured in a PerkinElmer LS50B Spectrofluorometer at an excitation wavelength of 510 nm and emission wavelength of 525 nm. For each experiment, fluorometric measurements were performed in triplicate and expressed in fluorescence intensity units. The culture supernatant was analysed for its nitrite (NO₂⁻) content using the Griess reagent as described previously.²⁷ Measurement of nitrite was an indication of NO production by these cells. The data are represented as the mean±SD of all five animals of the group under consideration.

Lymphocyte proliferation assay

The T cell proliferation assay was performed as described.²⁷ Single-cell suspensions of splenocytes (10⁵ cells/well in 96-well plates) from asiaticoside-treated or infected individual animals (n=5/group) were allowed to proliferate for 3 days at 37°C in a 5% CO₂ incubator in the presence or absence of CSA (50 µg/mL). Control cells were incubated with 3 µg/mL concanavalin A. Eighteen hours before they were harvested, cells were pulsed with 1 µCi (6.7 Ci/mM) [³H]thymidine/well. [³H]Thymidine uptake, as an index of proliferation, was measured with a liquid scintillation counter (Tri-Carb 2100TR; Packard Instrument). The data are represented as the mean±SD of all five animals of the group under consideration.

Immunofluorescent staining

For asiaticoside-mediated TNF-α responses, L. donovani-infected splenocytes cultured with either asiaticoside at 25 mg/L or no antigen (as a negative control) were stained for intra-cytoplasmic TNF-α cytoplasts treated with either asiaticoside at 25 mg/L or no antigen (as a negative control) were stained for intra-cytoplasmic TNF-α. The data were represented as the mean±SD of all five animals of the group under consideration.

Phosphatidylinositol-specific phospholipase C (PI-PLC) treatment

Cells (1×10⁶/mL) were incubated for 60 min at 37°C in RPMI 1640 containing (i) bovine serum albumin (2 mg/mL), 2-mercaptopethanol (5×10⁻⁵ M) and PI-PLC (10 U/mL) or (ii) bovine serum albumin (2 mg/mL), 2-mercaptopethanol (5×10⁻⁵ M) and Tris-acetate (50 mM, pH 7.4) only. Cells were washed twice in RPMI and viability was evaluated. The functionality of macrophages was monitored by their phagocytic activity.

Phagocytosis

Zymosan (35 mg) in 100 mL PBS (Sigma Chemicals) was boiled for 30 min and washed twice with PBS prior to use. Zymosan particles were resuspended in PBS containing Ca²⁺ and Mg²⁺ (14 mg/mL). For opsonization, 0.5 mL of zymosan particles (14 mg/mL PBS) were mixed in 0.5 mL normal mouse serum and incubated for 30 min at 37°C. The opsonized zymosan particles were then washed, resuspended in PBS at a concentration of 1 mg/mL and stored for up to 7 days at 4°C. Peritoneal macrophages were incubated with 10 mL PBS containing opsonized zymosan in the presence of glucose (5 mM) and glutamine (2 mM) for 30 min at 37°C. Phagocytosis was quantified by counting (in a counting chamber) the percentage of cells that had phagocytosed more than four particles of zymosan.

RNA interference

CD14 expression was down-regulated using 100 nM specific small interfering RNA (siRNA) sequence (Santa Cruz Biotechnology) and the electroporation system from Amaxa Biosystems (Gaithersburg, MD, USA), which uses cell type-specific buffers and reagents to allow high transfection efficiency in primary immune cells. Non-targeting sequences (control siRNA; Santa Cruz Biotechnology) were used as a negative control. At 48 h post-transfection, the effects of siRNA transfection on CD14 expression level were confirmed by flow cytometry.

Statistical analysis

A paired two-tailed Student’s t-test was used for statistical analysis of the data. Differences between means were assessed for statistical significance and P values of <0.05 were considered statistically significant.

Results

Asiaticoside-induced NO production and a protective Th1 response in L. donovani-infected peritoneal macrophages

We investigated whether asiaticoside could induce NO production and a protective Th1 response in murine peritoneal macrophages. Asiaticoside induced NO production in peritoneal macrophages in a concentration- and time-dependent manner (Figure S1a and b; Figure S1 is available as Supplementary data at JAC Online). Maximal NO generation was observed with 25 mg/L asiaticoside at 36 h (22.83±1.36 µM/10⁶ cells). LPS was used as a positive control. Meglumine antimonicate indirectly increases NO production through increased TNF-α production.²⁵ Similarly to its effect on NO production, asiaticoside treatment increased TNF-α production in infected peritoneal macrophages in a concentration- and time-dependent manner, with a maximum increase produced by 25 mg/L asiaticoside at 36 h (523±25.86 pg/mL) (Figure S1c). We next investigated asiaticoside-mediated induction of the pro-inflammatory cytokines IL-12 and IL-2 in uninfected peritoneal macrophages. Maximal production of IL-12 (437±15.52 pg/mL, Figure S1d) and IL-2 (5.76±0.21 pg/mL, Figure S1e) was induced by 25 mg/L asiaticoside in peritoneal macrophages at 36 h. Supernatants of peritoneal macrophages infected with the Sb⁵ L. donovani strain GE1F8R or K39 or the Sb⁵ strain AG83 were assayed for NO generation and cytokine production. L. donovani-infected peritoneal macrophages showed no increase in NO production compared with uninfected cells. In contrast, there was 7.1-, 6.29- and 6.24-fold up-regulation of NO generation in asiaticoside-treated AG83-, GE1F8R- and K39-infected peritoneal macrophages, respectively (Figure 1a). The vehicle DMSO had no effect on nitrite production. The presence of increased NO in supernatants of asiaticoside-treated
L. donovani-infected peritoneal macrophages appeared to be related to the increase in inducible NO synthase (iNOS) transcript in these cells. At 25 mg/L, asiaticoside induced a 9-fold increase in the expression of iNOS in uninfected peritoneal macrophages, and a 7.73-, 6.73- and 7-fold increase in iNOS expression in AG83-, GE1F8R- and K39-infected peritoneal macrophages, respectively. Besides NO, intracellular parasites can be destroyed by reactive oxygen intermediates. Hence, we next checked the generation of ROS following asiaticoside treatment. There was no ROS generation on asiaticoside treatment (data not shown).

In experimental visceral leishmaniasis, besides the induction of macrophage microbicidal activity, the efficacy of conventional antileishmanial chemotherapy depends on T cells and endogenous Th1 cytokines. Compared with the uninfected peritoneal macrophages, the level of the cytokines did not change on L. donovani infection. Asiaticoside treatment following infection augmented IL-2, IL-12 and TNF-α production. Asiaticoside treatment enhanced the production of TNF-α (12.75-, 11.88- and 13.13-fold increase in AG83-, GE1F8R- and K39-infected macrophages, respectively, P < 0.0001, Figure 1b). To assess the influence of TNF-α on NO production, NO production was measured in the presence of anti-TNF-α antibody (20 ng/mL; clone MP6XT22, R&D Systems). NO production was largely blocked in the presence of anti-TNF-α antibody (Figure 1a). This result indicated that NO production on asiaticoside treatment was increased via increased TNF-α production. Asiaticoside treatment also enhanced the production of IL-12 (9.21-, 11.97- and 13.81-fold increase in AG83-, GE1F8R- and K39-infected macrophages, respectively, 25(a)).
respectively, P<0.0001, Figure 1b) and IL-2 (19.53-, 14.1- and 16.78-fold increase in AG83-, GE1F8R- and K39-infected macrophages, respectively, P<0.0001, Figure 1c). Success of chemotherapy depends on the up-regulation of IL-12 and IFN-γ synchronized with down-regulation of IL-10 and TGF-β. Asiaticoside treatment resulted in a 92%–94% decline in IL-10 and TGF-β production in L. donovani-infected peritoneal macrophages (Figure 1d). Asiaticoside did not have any direct effect on the viability of AG83, GE1F8R or K39 promastigotes, as judged by the MTT assay, at concentrations of 25 and 50 mg/L (data not shown).

**Asiaticoside induces TNF-α production through CD14**

We next investigated the role of CD14 in asiaticoside-mediated TNF-α production. Experiments were done to study the release of TNF-α by asiaticoside in peritoneal macrophages in the presence of anti-CD14 blocking antibody (10 μg/mL, rat anti-mouse, clone 4C1, BD Biosciences). The CD14 dependence of asiaticoside-mediated TNF-α production was confirmed by the inhibition by anti-CD14 antibody (Figure 1e), but not by the isotype-matched control antibody. To further confirm the role of CD14 in asiaticoside-mediated TNF-α production, peritoneal macrophages were pretreated with PL-PLC, which is known to remove cell surface CD14. Figure S2(b) (Figure S2 is available as Supplementary data at JAC Online) shows that 10 μM PL-PLC removed known glycosylphosphatidylinositol (GPI)-anchored CD14 on peritoneal macrophages. A transmembrane (non-GPI anchored) protein, CD54 (intracellular adhesion molecule 1, ICAM-1), was not removed by PL-PLC (data not shown). The specificity of the PI-PLC used was tested by inactivating the enzyme by boiling for 30 min. Once inactivated, PI-PLC failed to remove CD14 expression (data not shown). In a typical experiment, the results showed that enzymatic removal of CD14 reduced TNF-α-positive asiaticoside-stimulated peritoneal macrophages by 76.34% (Figures 1e and S2c). A critical function of macrophages is their ability to phagocytose. The viability and phagocytic ability of the peritoneal macrophages treated with PI-PLC remained unaltered (data not shown). To further confirm the involvement of CD14 in asiaticoside-mediated TNF-α production, we down-regulated CD14 and investigated asiaticoside-mediated TNF-α production. Like cells treated with PI-PLC, CD14-silenced cells showed a significant reduction in TNF-α production (Figure 1e). Transfection efficiency was monitored by immunofluorescent staining of cell surface CD14 for in vitro flow cytometric analysis (Figure S2d). C. asiatica grows along ditches and in low wet areas and hence frequently suffers from high levels of bacterial contamination. Endotoxin (LPS) contamination of asiaticoside purified from C. asiatica was excluded as the mediator of TNF-α production because: (i) all reagents (including asiaticoside) were negative in the Limulus assay; and (ii) induction of TNF-α by asiaticoside was not blocked by polymyxin B, in contrast to LPS-induced TNF-α production. Polymyxin B (10 μg/mL), which binds and inactivates endotoxin (LPS), did not significantly alter asiaticoside (25 mg/L)-induced TNF-α production (520.33 ± 14.36, 549.33 ± 25.42 and 556.66 ± 21.01 pg/mL in AG83-, GE1F8R- and K39-infected macrophages, respectively, P<0.0001). Asiaticoside (25 mg/L)-induced TNF-α production in AG83-, GE1F8R- and K39-infected macrophages was 513.33 ± 10.50, 511.00 ± 2.00 and 525 ± 9.64 pg/mL, respectively, P<0.0001. In contrast, as expected, LPS-induced (100 ng/mL) TNF-α secretion (469.33 ± 25.03 pg/mL) by peritoneal macrophages decreased to <15% of initial values (P<0.0001) in the presence of polymyxin B (69.67 ± 9.87 pg/mL).

**Antileishmanial activity of asiaticoside in vitro depends on TNF-α-mediated NO production**

We checked the effect of asiaticoside on L. donovani amastigotes of all three strains in peritoneal macrophages. Asiaticoside (6.25–50 mg/L) was added after 48 h of infection and cultures were examined after 36 h. A significant, concentration-dependent decrease in infectivity index was observed, with inhibition as high as 94%, 93% and 96% of AG83, GE1F8R and K39 amastigote multiplication, respectively, for 25 mg/L asiaticoside after 36 h (Table 1). No obvious cytotoxicity was noted against peritoneal macrophages at concentrations ranging from 5 to 250 mg asiaticoside/L/10^6 cells (data not shown). The 50% inhibitory concentration (IC50) of asiaticoside (calculated by sigmoidal regression analysis using Microsoft Excel, 2007) against intramacrophage amastigotes of L. donovani was 0.109 ± 0.003, 0.109 ± 0.011 and 0.104 ± 0.002 nm for AG83, GE1F8R and K39, respectively (Table 1). We hypothesized that the antileishmanial activity of asiaticoside was associated with the production of TNF-α-induced NO in infected macrophages. Hence, the infectivity index was measured in the presence of the iNOS inhibitor L-NAME (L-NAME) and anti-TNF-α neutralizing antibody (20 ng/mL). The in vitro inhibitory effect of asiaticoside was markedly reduced in the presence of 500 μM L-NAME (96.24%, 93.95% and 95.08% for AG83, GE1F8R and K39, respectively) at 25 mg/L asiaticoside. The antileishmanial activity of asiaticoside was also significantly blocked by neutralizing antibody against TNF-α (82.65%, 88.91% and 85.95% for AG83, GE1F8R and K39, respectively) at 25 mg/L asiaticoside. To confirm the protective efficacy of asiaticoside, we observed CD14-dependent TNF-α production in response to asiaticoside treatment. We used an anti-CD14 blocking antibody to examine the CD14-dependent antileishmanial activity of asiaticoside. The protective efficacy of asiaticoside was considerably abrogated in the presence of anti-CD14 blocking antibody (Table 1).

**In vivo antileishmanial efficacy of asiaticoside in animal models of visceral leishmaniasis**

Inhibition of numerous macrophage functions, particularly those involved in immune surveillance and macrophage activation and the repression of pro-inflammatory cytokine production allows Leishmania to escape many of the immune responses that would otherwise be directed against it. The immunostimulatory and macrophage-activating properties of asiaticoside make it a potent candidate to mount a protective immune response against leishmaniasis. Hence, we wanted to confirm our in vitro data in an animal model of L. donovani infection. We investigated the antileishmanial activity of asiaticoside (1–10 mg/kg/day) on infected BALB/c mice at week 4 post-infection. Asiaticoside was administered orally for 10 consecutive days and animals were sacrificed 20 days after the last treatment. At 1 mg/kg/day, there was 56.37%, 50% and 57.93% reduction in liver parasite load and 53.59%, 41.76% and 46.96% reduction in spleen parasite load for AG83, GE1F8R and K39 parasites, respectively (Figure 2a and b). At 5 mg/kg/day there was almost complete clearance of the parasite burden from liver and spleen for both...
Table 1. Comparison of the antileishmanial activity of asiaticoside against the intracellular amastigote form of *L. donovani* \(^{Sb^5}\) strain AG83 and \(Sb^R\) strains GE1F8R and K39 *in vitro*

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Time of exposure to asiaticoside (h)</th>
<th>Amastigotes/100 peritoneal macrophages</th>
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<tr>
<td></td>
<td>24</td>
<td>36</td>
</tr>
<tr>
<td>AG83</td>
<td></td>
<td></td>
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<tr>
<td>GE1F8R</td>
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<tr>
<td>K39</td>
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<tr>
<td>0 nM AS</td>
<td>95.66 ± 8.51</td>
<td>119.18 ± 2.52</td>
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<tr>
<td>1.30 nM AS</td>
<td>49.33 ± 4.51**</td>
<td>40.00 ± 1.00***</td>
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<td>2.61 nM AS</td>
<td>24.33 ± 2.52**</td>
<td>23.33 ± 2.08**</td>
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<tr>
<td>5.21 nM AS</td>
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<tr>
<td>(IC_{50}) (nM)</td>
<td>0.164 ± 0.062</td>
<td>0.109 ± 0.003³</td>
</tr>
<tr>
<td>(CC_{50}) (nM)</td>
<td>&gt;52.13³</td>
<td>&gt;52.13³</td>
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<tr>
<td>(SI)</td>
<td>&gt;317.87</td>
<td>&gt;478.26</td>
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<tr>
<td>5.2 nM AS + 500 μM (L) -NMMA</td>
<td>85.0 ± 2.0³</td>
<td>111.67 ± 4.16³</td>
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<td>5.2 nM AS + 20 ng/mL (anti-TNF-\alpha)</td>
<td>83.78 ± 4.65³</td>
<td>97.74 ± 8.56³</td>
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<td>10.4 nM AS + 10 μg/mL (anti-CD14)</td>
<td>84.34 ± 6.22³</td>
<td>97.88 ± 5.03³</td>
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</table>

AS, asiaticoside; \(CC_{50}\), concentration required to reduce cell growth by 50%.

³There was <2% inhibition at 52.13 nM asiaticoside at 48 h.

³Highest concentration tested.

³The selectivity index (SI) is defined as the ratio of \(CC_{50}\) on murine peritoneal macrophage cells to \(IC_{50}\) on *L. donovani* intramacrophage amastigotes.

*P* < 0.0001, **P* < 0.001 and ***P* < 0.01 versus infected control group.

*P* < 0.0001 versus the 5.21 nM AS-treated group.
Sb\textsuperscript{5} and Sb\textsuperscript{5} strains of *L. donovani* (Figure 2a and b). During the experiment, all the animals remained healthy and no marked effect on body weight was noted in any of the experimental groups. One of the difficulties with the mouse as a model for visceral leishmaniasis is that the infection is chronic but not fatal. Golden hamsters are considered to be the best models to study experimental visceral leishmaniasis, because it produces clinicopathological features of human visceral leishmaniasis. Infection with *L. donovani* leads to visceral disease and death, making it an ideal tool for most experimental studies, including vaccine and drug testing. To ascertain the long-lasting efficacy of asiaticoside, golden hamsters were infected with the three strains of *L. donovani* and 4 weeks post-infection the animals were treated orally with asiaticoside (5 mg/kg/day, 10 consecutive days). All the treated hamsters of the three protected groups survived and remained healthy until the termination of the experiment at 12 months post-therapy (the end of our study period), whereas all the non-treated hamsters succumbed to virulent *L. donovani* challenge within 6 months (Figure S3a; Figure S3 is available as Supplementary data at JAC Online). In the absence of an adequate host immune response, treatment is frequently unsuccessful and relapses are common.\textsuperscript{32} Hence, we set out to examine whether asiaticoside therapy could prevent disease relapse. We had seen that at 4 weeks post-treatment asiaticoside reduced the parasite burden in hamsters by \textasciitilde99\% . Up to 12 months later, the asiaticoside-treated hamsters were still protected, as illustrated by their very low splenic and hepatic parasite loads (Figure S3b and c). It is well known that successful chemotherapy in leishmaniasis is accompanied by the maintenance of specific long-term immune responses associated with resistance against *Leishmania* re-infection.\textsuperscript{33,34} To ascertain that asiaticoside therapy confers long-lasting immunity, groups of 30 day infected (*n*=5) mice were treated with asiaticoside (5 mg/kg/day) for 10 days or used as control. Eight weeks after the primary infection, animals were re-infected (intra-cardiac) with 1×10\textsuperscript{7} *L. donovani* promastigotes. Untreated infected mice were kept as control-1 (white bars). Animals were sacrificed 8 weeks after re-infection and the (c) liver and (d) spleen parasite burden was determined. Controls were infected but not treated. Data represent the means \pm SD of five animals per group and are representative of three independent experiments. \textsuperscript{*}P<0.0001 compared with respective infected control groups at all timepoints; paired two-tailed Student’s t-test.

**Figure 2.** *In vivo* efficacy of asiaticoside in BALB/c mice infected with Sb\textsuperscript{5} (AG83) and Sb\textsuperscript{5} (GE1F8R and K39) strains of *L. donovani*. Asiaticoside was given at a dosage of 1–10 mg/kg/day orally for 10 consecutive days starting on day +30 after infection. Animals were sacrificed 20 days after treatment and (a) liver and (b) spleen parasite load was determined for all groups. (c and d) Course of re-infection with *L. donovani* (Sb\textsuperscript{5} and Sb\textsuperscript{5}) in mice treated with curative doses of asiaticoside. Naive age-matched control BALB/c mice (black bars) and asiaticoside-treated animals (hatched bars) were administered 1×10\textsuperscript{7} *L. donovani* promastigotes (intra-cardiac). Untreated infected mice were kept as control-1 (white bars). Animals were sacrificed 8 weeks after re-infection and the (c) liver and (d) spleen parasite burden was determined. Controls were infected but not treated. Data represent the means \pm SD of five animals per group and are representative of three independent experiments. \textsuperscript{*}P<0.0001 compared with respective infected control groups at all timepoints; paired two-tailed Student’s t-test.
Antileishmanial therapy with asiaticoside

(a) IL-2 (kcpm)

(b) Fold change expression of IL-2

(c) Fold change expression of IFN-γ

(d) Fold change expression of IL-12

(e) Fold change expression of TNF-α

(f) Fold change expression of IL-10

(g) Fold change expression of TGF-β

(h) IFN-γ, pg/mL

(i) IL-12, pg/mL

(j) TNF-α, pg/mL

(k) IL-10, pg/mL

(l) TGF-β, pg/mL

(m) NO2 generation (μM/10^6 cells)
**Leishmania antigens induces T cell proliferation and a T cell-mediated immune response in asiaticoside-treated mice**

Visceral leishmaniasis is associated with impaired cell-mediated immunity and marked T cell anergy specific to *Leishmania* antigens. To determine whether asiaticoside treatment could reverse the T cell effect, we determined the effect of asiaticoside therapy in an *L. donovani* antigen-specific proliferative response. Splenocytes from AG83-, GE1F8R- and K39-infected asiaticoside-treated mice (n=5/group) showed 25.5-, 13.5- and 14.1-fold enhanced T cell proliferation, respectively, compared with infected saline-treated animals at 50 µg/mL CSA (Figure 3a). It has been shown that impairment of IL-2 generation and a depressed splenic T cell response are associated in experimental as well as clinical visceral leishmaniasis.5,36 Hence, we analysed the functional activity of IL-2 in terms of [³H]thymidine uptake in HT-2 cells in asiaticoside-treated infected mice. When spleen cells from all groups of mice 20 days post-therapy were stimulated ex vivo with or without CSA (50 µg/mL) for 24 h and the supernatants were tested for IL-2 activity, it was seen that the IL-2 level was significantly higher in culture supernatants from treated animals compared with infected mice (Figure 3b). In the case of AG83 infection, asiaticoside treatment showed 11.66-fold greater IL-2 production than the corresponding infected group at 20 days post-therapy. IL-2 production was sustained up to 8 weeks post-therapy in asiaticoside-treated animals. At 8 weeks post-therapy, asiaticoside-treated animals also showed 14.33-fold greater IL-2 production than the corresponding infected group. Asiaticoside-treated GE1F8R- and K39-infected mice showed ~13.77- to 15.27- and ~15.1- to 17-fold greater IL-2 production, respectively, than the corresponding infected BALB/c mice for the different experimental groups.

The cytokine profile of asiaticoside-treated and control infected mice was compared. Expression of IFN-γ, IL-12 and TNF-α was up-regulated 3.5- to 4-, 5- to 5.5- and 10.5- to 11.5-fold for AG83, GE1F8R and K39, respectively, in the asiaticoside-treated mice 20 days post-therapy (Figure 3c–e). Expression of IFN-γ, IL-12 and TNF-α was sustained at this level 8 weeks post-therapy (Figure 3c–e). The extremely high expression of IL-10 in the infected animals (Figure 3f) declined 9-, 9.9- and 10.1-fold for AG83, GE1F8R and K39, respectively, in the asiaticoside-treated mice 20 days post-therapy and remained at this level up to 8 weeks post-therapy. The expression of TGF-β in the infected animals (Figure 3g) declined 16.1-, 14.3- and 13.8-fold for AG83, GE1F8R and K39, respectively, in the asiaticoside-treated mice 20 days post-therapy and also remained at this level up to 8 weeks post-therapy. Cytokine production at the protein level in the spleen cells of infected and asiaticoside-treated animals 20 days and 8 weeks post-therapy reflected a similar pattern, with a 5.5- to 6-, 7- to 8.5- and 11- to 12.5-fold increase in IFN-γ, IL-12 and TNF-α production, respectively, for AG83, GE1F8R and K39 (Figure 3h–j), and a 13- to 15- and 12- to 13-fold reduction in IL-10 and TGF-β (Figure 3k and l) levels, respectively, for AG83, GE1F8R and K39. Interestingly, splenocyte culture supernatants of the re-infected asiaticoside-treated animals showed a similar cytokine pattern. This demonstrated that the IFN-γ and IL-12 produced in an IL-10 down-regulated environment inhibited parasite replication in these animals.

**Induction of iNOS transcript and reactive nitrogen intermediates in asiaticoside-treated mice**

The importance of NO produced by iNOS in controlling leishmaniasis is well established.37 In the case of *L. donovani*-infected asiaticoside-treated mice, we found a 6- to 6.5-fold increase for AG83, GE1F8R and K39 in the expression of iNOS transcript compared with the respective saline-treated infected control groups (Figure 3m). Since the asiaticoside-treated mice showed elevated expressions of IFN-γ and TNF-α, the induction of NO/nitrite by these cytokines in a down-regulated IL-10 environment was also examined. NO was estimated in the culture supernatants of splenocytes isolated from *L. donovani*-infected and asiaticoside-treated mice. Asiaticoside-treated Sb² and Sb⁸ *L. donovani*-challenged asiaticoside-treated mice showed induction of 22-fold and 20-fold higher levels of NO, respectively (Figure 3n).

**Discussion**

Leishmaniasis is a major tropical disease with a wide clinical spectrum of cutaneous, mucocutaneous and visceral involvement. The outcome of infection depends on the parasite species and the host’s immunological response. IL-10 plays a major role in suppressing T cell responses in active disease and cure is associated with the induction of IL-12-driven expansion of Th1 cells, production of IFN-γ, macrophage activation and subsequent generation of reactive nitrogen and oxygen species, and a fall in IL-10 production. The organic pentavalent antimonials, which are the clinical drugs most frequently employed against leishmaniasis, have been associated with considerable toxicity to humans. The spread of drug-resistant Leishmania strains in several endemic areas stresses the urgent need to identify new innovative and alternative therapies against leishmaniasis.

In the present study, the antileishmanial activity of asiaticoside against intracellular amastigotes of both antimony-susceptible and -resistant strains of *L. donovani* was confirmed. The results show that asiaticoside treatment results in a significant elevation in the expression of NO and iNOS in *L. donovani*-infected mice. The enhanced expression of iNOS in asiaticoside-treated mice may be due to the up-regulation of IFN-γ and IL-12, which are known to induce iNOS expression. The increased iNOS expression in asiaticoside-treated mice may be responsible for the observed decrease in parasite burden and the induction of a Th1 response.

**Figure 3.** Effect of asiaticoside on T cell response of *L. donovani* (LD)-infected mice. (a) BALB/c mice were infected with *L. donovani* promastigotes and treated with asiaticoside as described in the legend to Figure 2. Splenocytes (1×10⁵ cells) from different groups of experimental mice were isolated at 20 days post-therapy and incubated with 50 µg/mL CSA in a 5% CO₂ incubator at 37°C for 72 h. T cell proliferation was measured by [³H]thymidine incorporation. (b–n) Splenocytes (2×10⁵ cells) from different groups of experimental mice were isolated at the indicated timepoints and incubated with 50 µg/mL CSA in a 5% CO₂ incubator at 37°C for 72 h. (b) Expression of IL-2 was determined in terms of proliferation of the IL-2-dependent murine cell line HT-2. Expression of (c) IFN-γ, (d) IL-12, (e) TNF-α, (f) IL-10 and (g) TGF-β mRNA was evaluated by real-time PCR. mRNA levels were normalized to β-actin and expressed as fold changes compared with control. Levels of (h) IFN-γ, (i) IL-12, (j) TNF-α, (k) IL-10 and (l) TGF-β in culture supernatants were determined by ELISA. In vivo expression of iNOS (m) and generation of NO (n) from infected asiaticoside-treated mice. Results are representative of one of three individual experiments. Data represent means ± SD. *P<0.0001 versus the infected control; paired two-tailed Student’s t-test.
and -resistant L. donovani in vitro was demonstrated. This inhibition of intracellular amastigote replication was concentration and time dependent, reaching >98% with 25 mg/L asiaticoside at 36 h of treatment. Asiaticoside showed leishmanicidal activity against intracellular amastigotes through TNF-α-mediated NO production, but no activity against promastigotes was observed. The potent in vitro activity of asiaticoside against intracellular L. donovani amastigotes in the absence of obvious cytotoxicity in host macrophage cells formed the basis for extension of these in vitro data to therapy-based studies in vivo.

The antileishmanial activity of asiaticoside was validated in L. donovani-infected BALB/c mice and golden hamsters. At 5 mg/kg/day there was almost complete clearance of parasites from the liver and spleen in both SbS and SbR strains of L. donovani. There was no evidence of toxic effects on the hamsters studied. All treated hamsters in the three protected groups survived and remained healthy until the termination of the experiment at 12 months post-therapy (the end of our study period), without any appreciable enhancement in parasite number, while the control animals succumbed within 6 months. To our knowledge, only one report has described the in vivo effect of asiaticoside in hamsters infected with L. donovani (AG83 strain). The study showed that a dose of 4 mg/kg of asiaticoside suppressed the splenic parasite load by only 47%. The use of liposome-encapsulated asiaticoside led to a 62% reduction of splenic parasite load, and the increased efficacy of the liposome-encapsulated asiaticoside could be attributed to the effect of the liposome, since the reduction in spleen parasite load with the empty liposome was ~12%–14%. However, the study reports neither the treatment modalities nor the mechanisms by which the treatment regimens work.

Therapy with asiaticoside induced polarized Th1 responses with enhanced IFN-γ, IL-12 and NO and reduced Th2-associated cytokine IL-10 and TGF-β responses for both SbS and SbR strains of L. donovani. An effective leishmanicidal response against L. donovani is dependent on an IL-12-driven Th1 response leading to induction of IFN-γ production and, in turn, macrophage activation and parasite killing. IFN-γ plays a pivotal role in the activation of macrophages to kill pathogens and protect the host cell from infection. Leishmania parasites inhibit the initial IL-12 production by their host macrophage, thereby impairing the signalling cascade. We too observed that IL-12 was down-regulated in all three groups of infected mice (AG83, GE1F8R and K39) but was significantly elevated at both the RNA and protein levels in asiaticoside-treated groups. In parallel with the observed increase in IL-12 expression/production, the reduction in the parasite load after asiaticoside treatment was associated with increased expression/production of IFN-γ. Independently of IFN-γ, IL-12 also induces the production of TNF-α in macrophages. TNF-α and IFN-γ are important cytokines for protection against leishmaniasis. Asiaticoside treatment increased the level of TNF-α in all three groups of mice compared with the infected control groups. Asiaticoside-induced TNF-α production appeared to be mediated in a CD14-dependent manner. Healing in visceral leishmaniasis is also associated with the development of strong cell-mediated immunological responses, such as T cell responses and NO production, which helps in activating macrophages to kill the intracellular parasites. Asiaticoside therapy restored lymphocyte proliferation in splenocytes of both SbS- and SbR-infected animals. Asiaticoside-mediated leishmanicidal activity directly correlated with TNF-α-induced NO production.

In conclusion, our results indicate that asiaticoside treatment successfully reduced organ parasite burden in animal models of visceral leishmaniasis. Reduction in parasite load following asiaticoside treatment was associated with the induction of TNF-α-mediated NO production. The Th2-to-Th1 switch as a result of therapy probably accounts for the resistance to disease relapse and long-lasting efficacy of asiaticoside. Most of the currently available antileishmanials have serious side effects and are challenged by the emergence of drug resistance. An attractive alternative strategy is the use of immunomodulators that can augment host immune responses during infectious disease. These immunomodulators offer the potential for a broad spectrum of activity against other infectious diseases. Thus, asiaticoside offers an important immunotherapeutic option for immunocompromised patients, in whom traditional antimicrobials often work poorly.

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Transparency declarations
None to declare.

Supplementary data
Figures S1, S2 and S3 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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