Novel immunomodulatory function of 1,3,4-thiadiazole derivatives with leishmanicidal activity

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Objectives: Previously, some nitroheteroaryl-1,3,4-thiadiazole derivatives were identified to have potent activity against Leishmania sp. The present aim was to complete the in vitro analysis, thereby investigating the in vivo efficiency of the analogues 15a, 21a and 21b against infected BALB/c mice.

Methods: Following parasite inoculation and intraperitoneal drug administration (5 and 20 mg/kg/day) for 5 days, the course and size of cutaneous lesions, histopathology of the liver, parasite loads in the spleen through limiting dilution assay as well as spleen cell activation assays through cytokine secretion profiles were studied in BALB/c mice, over a period of 23 and 30 days post-drug injections.

Results: The analogues significantly decreased lesion size and progression of infection in the liver and spleen, and were associated with granuloma formation, which correlates with disease regression in the liver of murine hosts. Moreover, the analogues had immunomodulatory effects, stimulating interferon-γ expression and suppressing interleukin-10 and interleukin-5 production, favouring type-1 immune responses and resolution of the parasitic infection.

Conclusions: Our results highlight marked differences between the responses of key anatomical organs to the thiadiazole derivatives in comparison with the current antileishmanial drug, meglumine antimoniate. The in vivo observations provide further evidence on the efficiency of the compounds for Leishmania treatment. The immunomodulatory function plays an essential role in enhancing cell-mediated immunity for complete clearance of the pathogen.

Keywords: Leishmania, immunomodulatory effects, type-1 response, type-2 response

Introduction

Parasitic diseases have an overwhelming impact on public health in developing regions. Many of these infections are caused by protozoan parasites. Malaria, leishmaniasis and trypanosomiasis are the protozoal parasitic diseases that are targeted for control or eradication by the WHO’s Division of Control of Tropical Diseases.1 Leishmaniasis is a complex of diseases caused by ≥17 species of the protozoan parasite Leishmania. Human leishmaniasis is distributed worldwide, but mainly in the tropics and subtropics, with a prevalence of 12 million cases and an approximated incidence of 0.5 million cases of visceral leishmaniasis (VL) and 1.5 million cases of cutaneous leishmaniasis (CL).2,3 The chemotherapy currently available for leishmaniasis is far from satisfactory. Resistance to the pentavalent antimonial, which have been the recommended drugs for the treatment of both VL and CL for >50 years, is now widespread throughout the world.3,4 Although new drugs have become available in recent years for the treatment of VL, including a highly expensive liposomal formulation of amphotericin B and miltefosine, which has now been registered in India, treatment problems and toxic side effects remain.3,5

Cure of leishmaniasis, probably even during chemotherapy, appears to be dependent upon the development of an effective immune response that activates macrophages to produce reactive nitrogen and oxygen metabolites to kill the intracellular amastigotes. The immunomodulator imiquimod has proved to be an adjunct for CL therapy.5-7 It has been shown that mammalian host protection against leishmanial infection is dependent on the development of T helper 1 (Th1) responses, which trigger enhanced leishmanicidal activity by infected macrophages.8 Th1 responses are associated with healing and parasite killing, whereas T helper 2 (Th2) responses are associated with non-healing diseases and uncontrolled parasite growth.9,10

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Depending on their activation state, macrophages can either host or kill Leishmania. Inducible nitric oxide synthesis (iNOS) and the presence of an oxidative burst are of crucial importance to parasite killing.\(^{11,12}\) These pathways are competitively regulated by the cytokines secreted by Th1 and Th2 cells. Experiments have revealed that nitric oxide (NO)-stimulated production by interferon (IFN)-\(\gamma\)-activated macrophages is the key effector for killing pathogens, particularly the intracellular parasite Leishmania. Th1 cytokines induce iNOS, which leads to oxidative stress, whereas Th2 cytokines suppress oxidative-inducing pathways.\(^{8,11,13,14}\)

The resolution of infection is mediated by Th1 responses and IFN-\(\gamma\) production. IFN-\(\gamma\) induces NO production in phagocytic cells that harbour Leishmania major (principally macrophages), which leads to the destruction of the parasite, while BALB/c mice develop Th2 responses, causing severe and uncontrolled lesions to become lethal. All these observations have been attributed to the distinct genetic background of BALB/c mice inducing the Th2 response characterized by elevated levels of interleukin (IL)-4, IL-5 and IL-10.\(^{15-17}\)

Research in the past two decades has shown that some chemical drugs possess diverse biological and pharmacological properties, such as antioedema, anti-inflammatory, anticarcinogenic, immunomodulatory and antitumour activities. The medicinal values of numerous antimicrobial and anticancer agents have led to them being referred to as immunomodulators, which are biological response modifiers that enhance Th1- and Th2-associated cytokine secretion by spleen cells or macrophages, i.e. they are able to modulate multiple intercellular and intracellular molecular signals involved in the functioning of the immune system. Studies have shown that biological immunomodulators can provide a potent strategy to enhance drug activity in the treatment of VL and CL. Recently, a new generation of immunopotentiating drugs has shown potential for leishmaniasis treatment. One of the imidazoquinolines has been found to induce NO production in macrophages. The compound was shown to have antileishmanial activity via macrophage activation in experimental models and in clinical studies on CL in combination with antimonials.\(^{18-21}\) Recent studies have reported the selective cytotoxicity of nitroheteroaryl-1,3,4-thiadiazole-based compounds, including 1-[5-(5-nitrofuran-2-yl)-1,3,4-thiadiazol-2-yl]-4-arylpiperazines and 1-[5-(5-nitroimidazole-2-yl)-1,3,4-thiadiazol-2-yl]-4-arylpiperazines, against Leishmania and a possible molecular mechanism of action for initiating parasite cell death.\(^{22-24}\) The aim of the present work was to assess more precisely the in vivo effectiveness and cytotoxicity of the most potent nitroheteroaryl-1,3,4-thiadiazoles.

**Ethics**

We complied with the relevant institutional and national standards for animal care and experimentation. The assay procedures, administration route and doses of the test compounds were selected according to standard operating procedures to cause no pain and distress due to corrosive or severely irritating actions, were approved by Yazd Cardiovascular Research Center, Shahid Sadoughi University of Medical Sciences and have been described previously.\(^{25-28}\)

**Parasites**

Promastigotes of *L. major* (strain MRHO/IR/75/ER; Pasteur Institute, Tehran, Iran) were used in the experiments. The infectivity of the parasites was maintained by regular passaging in susceptible BALB/c mice. The parasites were grown in blood agar cultures at 25°C, and recultured at 2 × 10\(^6\) cells/mL density in RPMI 1640 medium (Sigma–Aldrich Co. LLC, USA) supplemented with 1% heat-inactivated fetal bovine serum (FBS) (Sigma–Aldrich Co. LLC, USA), 2 mM glutamine (Sigma–Aldrich Co. LLC, USA), 100 U/mL penicillin and 100 \(\mu\)g/mL streptomycin (Sigma–Aldrich Co. LLC, USA), pH \(\sim 7.2\). For the experiments described here, stationary parasites at the virulence stage were washed three times with PBS and the parasite concentration adjusted to 2 × 10\(^7\) cells/mL; then, 100 \(\mu\)L was inoculated into 6-8-week-old BALB/c mice.

**Experimental animals**

The healthy male BALB/c mice were obtained from the Pasteur Institute, Tehran (Iran). The animals were weighed (\(\sim 20\) g), housed in groups of eight and screened every day throughout the study.

**In vivo activity assessments**

The assay procedures, administration route and doses of the test compounds were selected according to the standard operating procedures that have been described previously.\(^{25-28}\) All compounds were injected by the intraperitoneal (ip) route, based on previous experience that demonstrated that abdominally administered test compounds have the greatest potential to show activity. The selected standard dosage should be high enough to be effective, but only moderately toxic doses are to be used; the administration of doses that are expected to be lethal or to cause marked pain and distress, due to corrosive or severely irritating actions, should be avoided.

The compounds tested may have a direct effect on the cells of the immune system, as has been demonstrated by in vivo experiments with several drugs; therefore, the lymphocyte-proliferative and cytokine responses of the spleen were examined.\(^{25-31}\)

**BALB/c mice infection with *L. major*, drug administration, lesion monitoring and tissue processing**

BALB/c mice were infected subcutaneously, via the lateral tail with 2 × 10\(^6\) stationary-phase promastigotes of *L. major* in a final volume of 100 \(\mu\)L (in sterile PBS). At 14 days post-infection, after immune granuloma ulcers and skin lesions were observed at the site of injection, the mice were randomly sorted into groups of five and were injected ip with 5 or 20 mg/kg/day doses of the thiadiazole analogues (dissolved in DMSO) for 5 consecutive days. The following treatments were used as controls: treatment with the standard drug meglumine antimoniate (Glucantime), administered ip at 56 mg/kg/day (dissolved in water) for 5 days; injection with 100 \(\mu\)L of DMSO; and no injection. The lesion sizes of the treated and untreated mice were measured regularly with a vernier caliper. The mice were also examined regularly to detect cutaneous ulcers and secondary lesions. At selected timepoints, on days 23 and 30 post-injection, all
examined mice were killed by ether inhalation. The spleens and livers were collected for determination of their parasite loads, histological studies, or both (see below). The livers and spleens were weighed; livers were then immediately fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin by routine methods for histopathology and parasite infectivity studies. Glass slides with affixed 4–5 μm thick tissue sections were prepared and stained with haematoxylin and eosin. Spleens were aseptically removed and teased into single-cell suspensions in RPMI medium for limiting dilution analysis, and lymphocyte-proliferative and cytokine-response assays, as described above.

**Limiting dilution analysis**

To evaluate the frequency of *L. major*-infected cells in the main lymphatic organ, the spleen, groups of mice were killed at intervals after infection and treatment for determination of the parasite burdens. The spleens were excised, weighed and then homogenized with a sterile tissue grinder in 4 mL of RPMI medium prepared as described above. Under sterile conditions, serial 4-fold dilutions ranging from 1 to 12 were prepared in wells of 96-well microtiter plates containing 150 μL of Navy- MacNeal- Nicolle (NNN) culture medium. After 7 and 15 days of incubation at 25°C, the plates were examined with an inverted microscope at a magnification of ×100 or ×200. The presence or absence of mobile promastigotes was recorded in each well. The final titre was the last dilution for which the well contained at least one parasite. The number of parasites per gram (parasite burden) in the corresponding organ was calculated as follows: parasite burden = (geometric mean of reciprocal titres from each duplicate/weight of homogenized cross-section) × 400, where 400 is the reciprocal fraction of the homogenized organ inoculated into the first well.32

**Splenocyte culture and mitogen-induced lymphoproliferative responses**

Spleens were aseptically removed and teased into single-cell suspensions in RPMI 1640 supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, 50 mM 2-mercaptoethanol, 2 mM L-glutamine and 10% heat-inactivated FBS. Red blood cells were removed by lysis with 0.83% (w/v) NH₄Cl. The remaining cells were washed twice with culture medium and the viable mononuclear cell number was determined by counting trypan blue unstained cells in a haemocytometer. Splenocyte suspensions (1×10⁷ cells/mL) were dispensed into 96-well culture plates and incubated for 24 h with *Salmonella Typhi* lipopolysaccharide (LPS; Sigma–Aldrich Co. LLC, USA) at 5 μg/mL as the B cell mitogen or with concanavalin A (ConA; Sigma–Aldrich Co. LLC, USA) as the T cell mitogen; these mitogen concentrations have been shown to induce optimum splenocyte proliferation. After incubation at 37°C in 5% CO₂, the proliferation of spleen cells was measured by colorimetric reading of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reduction, as described previously.31

**Cytokine production by splenocytes**

The method used for cytokine production was that described previously.35 Briefly, as described above, spleen lymphocytes were prepared in RPMI medium. The spleen cell number was adjusted to 1×10⁶ cells/mL and cells were cultured in 24-well plates, containing either 10 μg/mL ConA for IFN-γ or 10 μg/mL LPS for IL-12/IL-10/IL-5 production, for 72 or 24 h, respectively. After incubation, the lymphocyte culture supernatants were harvested and stored at −70°C for further analysis of cytokine secretion.

**Assay for cytokine production by stimulated lymphocytes**

Thereafter, culture supernatants were analysed for IFN-γ, IL-10 and IL-5 by ELISA (BD Biosciences and R&D Systems, sensitivity 8–2500 pg/mL), according to the manufacturer’s instructions.

**Statistical analysis**

Student’s t-test, with significance at P<0.05, was used to compare the in vivo compound susceptibilities of the parasite.

**Results**

**BALB/c mice inoculated subcutaneously with *L. major***

The infected mice, inoculated via the base of their tails, developed a detectable lesion (papule or ulcer) during weeks 1–2. The lesion size progressed steadily to become severe swellings and necrotizing skin lesions at the site of injection. The progression of the lesion was monitored by measuring its length and width at the tail base, until 3–4 weeks after the last drug administration. The control animals that were injected daily with DMSO or 56 mg/kg/day meglumine antimoniate for 5 consecutive days developed larger and necrotizing lesions, which showed progression during the course of infection. In contrast, the daily administration of analogues 15a, 21a and 21b at 5 or 20 mg/kg/day for 5 days resulted in regression and inhibited the development of the lesion. In the first set of experiments (on day 23 post-injection), all control groups (untreated group, meglumine antimoniate treatment and DMSO injection) produced rapidly developing lesions (with lethality in BALB/c mice), which continuously increased in size (lesion size ~1.06–1.44 cm², P<0.005) and showed no signs of recovery (Figure 1a). The data presented here indicate that 5 day treatment with 5 mg/kg/day 21b gave greater suppression of lesion development (lesion sizes were ~0.14 cm² for 21b to ~0.657 cm² for 21a, P<0.005) in comparison with 20 mg/kg/day doses (lesion sizes were ~0.3 cm² for 21b to ~0.785 cm² for 15a, P<0.005); however, with both doses the growth of lesions was greatly controlled and regressed (maximum lesion size ~0.785 cm²) (Figure 1a). Further, in the second set of experiments (on day 30 post-injection), no sign of parasite regression was observed in the controls, even in the meglumine antimoniate treatments (lesion size ~1.7 cm², P<0.005–0.01) (Figure 1b), whereas thiadiazole treatments demonstrated greater resolution of the lesions, especially with the 5 mg/kg/day doses (lesion sizes were ~0.12 cm² for 21b and ~0.37 cm² for 21a, P<0.005) (Figure 1b). Interestingly, a long time after the last administration of 21a, the cutaneous infection started to regress and the lesion growth was controlled (lesion size ~0.37 cm² at 30 days post-treatment, which was significantly different from the un.injected and meglumine antimoniate controls, P<0.005, or the DMSO injection controls, P<0.01) (Figure 1b).

**Course pattern of lesion development in drug-treated mice**

For better interpretation of the differences in lesion development between the treatment groups, data analysis was conducted, as illustrated in Figure 1(a and b). Long after exposure of the mice to the analogues 15a, 21a and 21b, following infection with the
parasite, there were similar observations in the cutaneous lesions, correlating with cutaneous Leishmania healing. Therefore, treatment of BALB/c mice with the test compounds was found to greatly abrogate ulcerated lesions, while in the control groups we observed progression to large lesions. In general, the nitroimidazole analogues 15a and 21a showed a long period of activity against the parasitic infection (Figure1a and b). Interestingly, untreated, DMSO-injected and meglumine antimoniate-treated mice were not able to control parasite numbers in the lesions, and maintained a large lesion size throughout the period of observation (Figure1a and b).

**Drug effects and parasite load in spleens of infected BALB/c mice**

To investigate if there was a correlation between lesion development and parasite replication in the spleen, the parasite load was estimated using a limiting dilution assay. The animals were killed, spleens weighed and homogenized in 4 mL of RPMI medium, and then plated in 96-well plates containing NNN medium. To assess parasite loads, parasite expansion was observed at weeks 1 and 2 post-treatment. Active VL is characterized by enlargement of the spleen and compromised immunology with increased susceptibility to bacterial superinfections. Five weeks after intradermal injection with the parasite, considerable decreases in spleen weights were observed in the thiadiazole-treated groups, e.g. the smallest spleen weight (~0.18 g) was related to 21a treatment, which reached a 2-fold decrease in spleen weight (P<0.01) in comparison with the controls; however, enlarged spleens were observed in the meglumine antimoniate- or DMSO-administered mice (spleen weights ~0.33 and ~0.39 g, respectively; Figure 2). In agreement with cutaneous prevention of lesion development in the thiadiazole analogue-treated mice, small spleens also indicated a prevention of visceral progression of the parasite (Figure 2). Likewise, a significant positive correlation (R² ~1) was observed between lesion size and parasite burden in the spleens. In the infection model, typical parasite burdens at days 7 and 14 in the untreated controls were 6857 and 1143, respectively, whereas the 5 and 20 mg/kg/day analogue treatments exhibited parasite burdens of 38–78 and 14–145, respectively, at day 7. At day 14, parasite expansions increased, reaching 133–314 for the 5 mg/kg/day analogue doses and 100–368 for the 20 mg/kg/day doses, which demonstrated that the parasite’s ability to regenerate was very weak. However, outcomes of

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**Figure 1.** Effect of thiadiazole analogues on the cutaneous lesion size of L. major-infected BALB/c mice was monitored at weeks 3 (a) and 4 (b), after drug treatment. The bars represent the mean lesion size of five infected mice per group, treated with the indicated doses for 5 consecutive days in a representative experiment. Control corresponds to un.injected mice. Glucantime corresponds to mice treated daily with 56 mg/kg meglumine antimoniate dissolved in water. DMSO corresponds to mice that received 100 µL of the solvent. 15a, 21a and 21b correspond to mice treated with the tested compounds dissolved in DMSO. Data are the means ± SEM of five experiments. *P≤0.05 and **P<0.005 in comparison with the uninjected, meglumine antimoniate- and DMSO-treated controls.

**Figure 2.** Weights of spleens during the 30 days after L. major infection in BALB/c mice and drug treatments. Results are expressed as means ± SD of five BALB/c mice, with a significant difference of P<0.01 (Student’s t-test). Glucantime corresponds to mice treated daily with 56 mg/kg meglumine antimoniate. DMSO corresponds to mice that received 100 µL of the solvent. Control corresponds to uninjected mice. 15a, 21a and 21b correspond to mice treated with the test compounds.
Histological suppression of visceral infection induced by L. major in BALB/c mice

Histological studies were performed at 4 and 5 weeks post-infection on livers collected from analogue-treated and untreated (as control) mice. At 30 days post-treatment, the parasite distributions in the livers of control and drug-treated mice were determined (Figure 3 and Figures S2 to S5, available as Supplementary data at JAC Online). For each thiadiazole analogue (15a, 21a and 21b), the treatment induced suppression of the parasites in comparison with the control. Parasites were also abundantly found in the secondary lesions in the liver (Figures 3e and f, S3c, S4 and S5), attesting to the extensive parasite spreading and visceralization of L. major infection in BALB/c mice; however, this was not observed in analogue-treated mice (Figures 3a–c, S2 and S3a and b). Granuloma-like structures were visible deep in the 5 week post-infection lesions of analogue-treated mice (Figures 3a–c, S2 and S3a and b), but rarely in DMSO- or meglumine antimoniate-treated BALB/c mice (Figures 3d, e, S4 and S5). Plasma cells and red pulp were increased in the sinusoids and liver area of both DMSO- and meglumine antimoniate-treated mice (Figures 3f, S4 and S5). In untreated controls and DMSO-injected mice, follicular hyperplasia of the lymphatic pulp with increased number and size of hepatic nodules appeared in 5 week post-infection lesions of BALB/c mice; also, there was a marked increase in the red pulp area in the sinusoids of meglumine antimoniate-treated mice (Figures 3f and S5). Vacuolated histiocytes containing parasites were seen in the red pulp in untreated controls, and DMSO- and meglumine antimoniate-treated mice, sometimes forming granuloma-like patterns. In the liver, a portal inflammation with blood cell infiltration and sinusoid dilatation was also seen in untreated BALB/c mice. The high density of parasites observed in the red pulp of the liver indicates a bloodstream dissemination of the parasites to other internal organs (Figures 3d–f, S3d, S4d and S5). In contrast, the thiadiazole-treated mice succeeded in eliminating the parasitic infection from the primary cutaneous lesions by the formation of granulomas that healed. Cutaneous fibrosis and granulomatous inflammation were observed, which are associated with the healing process, and the total elimination of parasites from the liver was noted (Figures 3a–c, S2 and S3b).

Thiadiazole analogue-modulated production of Th1-associated cytokines from activated spleen cells

Because L. major-infected BALB/c mice present clinical and histopathological features similar to VL in humans, these mice are considered as a putative model for clinical spectrum and immunological studies. The immune response to both forms of leishmaniasis (VL and CL) has been extensively studied in rodent models, and it has been shown that infected macrophages are activated to kill intracellular parasites through elevated levels of reactive oxygen metabolites and NO.7–9,11,12,15–18 This macrophage activation occurs through Th1 responses and the production of cytokines, most critically IFN-γ.9,10 Infected leads to the down-regulation of these signals and the up-regulation of negative feedback signals, such as IL-10 and

<table>
<thead>
<tr>
<th>Drug treatmentsa</th>
<th>Parasite growth (5 mg/kg/day)</th>
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<tr>
<td></td>
<td>7 day expansionb</td>
<td>14 day expansion</td>
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<tr>
<td>15a</td>
<td>57c</td>
<td>256</td>
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<td>21a</td>
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<td>21b</td>
<td>78</td>
<td>314</td>
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a Groups of male BALB/c mice (n=4) infected with L. major on day 0 were treated on days 7–11, when papule or ulcers appeared at the site of parasite injection.
b Parasite burden was measured after 7 and 14 days of expansion by microtitration assay.

The number of parasites per gram was calculated after 7 and 14 days of expansion by microtitration assay as follows: parasite burden (geometric mean of reciprocal titres from each duplicate/weight of homogenized cross-section)×400, where 400 is the reciprocal fraction of the homogenized organ inoculated into the first well.11

c Mice injected with 100 μL of DMSO solvent.
d Mice injected with 56 mg/kg/day meglumine antimoniate for 5 days as a control.
e This group received no treatments.

DMSO and meglumine antimoniate administration showed a marked parasite burden (~19000 and ~8500) due to the high visceral loading of L. major (Table 1). In general, all treatments inhibited parasite progression (>90% suppression); in contrast, meglumine antimoniate and DMSO induced positive effects against infection (>24%–180% parasite progression) (Table 2).

%Parasite growth: [(mean number of parasites in treated mice culture×100)/(mean number of parasites in untreated mice culture)]−100.

Data represent the means±SD of four animals.
P<0.005 versus untreated control.

Table 2. Comparison of thiadiazole effects on the parasite’s ability to regenerate in optimum conditions, after induction of VL in mice, 5 weeks post-infection and 30 days after drug treatment

<table>
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<tr>
<td>15a</td>
<td>−99.2±3</td>
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</tr>
<tr>
<td>21a</td>
<td>−98.9±0.9</td>
<td>−72.5±4</td>
</tr>
<tr>
<td>21b</td>
<td>−98.4±2</td>
<td>−77.6±3</td>
</tr>
<tr>
<td>DMSO</td>
<td>180±4</td>
<td>−25±2</td>
</tr>
<tr>
<td>Glucantime</td>
<td>24.4±6</td>
<td>129.7±4</td>
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IL-5. One rational approach to therapy is to modulate the immune response to overcome the negative control systems and to boost the positive killing responses. The immunomodulatory effects of some chemical structures have been shown to induce stimulatory signals in T cells and macrophages.

Enhancement of IFN-γ expression in splenocytes of L. major-infected BALB/c mice

Herein, we aimed to identify and validate the stimulatory effects of the compounds on IFN-γ, measured 23 days post-injection using ELISA kits specific for the cytokine. We examined the effect of in vivo treatment with thiadiazoles on IFN-γ production as an indicator of Th1 activation and macrophage killing of the pathogen (Figure 4). The 5 day treatment with 5 mg/kg/day thiadiazole analogue doses significantly (P<0.05) increased the ConA-driven response in comparison with the untreated control (300–450 versus ≏150 pg/mL, respectively), while the responses induced by the 20 mg/kg/day doses, meglumine antimoniate and DMSO were similar to those of the control (<150 pg/mL; Figure 4). Likely, the enhancement of IFN-γ cytokine production in 21b-treated mice may be related to considerable activity against the parasite burden in cutaneous lesions in BALB/c mice.

Figure 3. Parasite loading in livers from L. major-infected BALB/c mice treated with 5 mg/kg/day 15a (a), 21a (b) or 21b (c), untreated (d), or treated with 100 µL of DMSO (e), or treated with 56 mg/kg/day meglumine antimoniate (f) for 5 consecutive days. Staining with haematoxylin and eosin (magnification: ×400). The arrows point to granulomas formation by immune cells for parasite regression, but the circles refer to parasite invasion. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
Decrease in IL-10 production in splenocytes of L. major-infected BALB/c mice

We determined the stimulatory effects of the test compounds on IL-10, measured 23 days post-injection. In comparison with 21b, IL-10 Th2-activating cytokine production was significantly suppressed by the analogue 21a, which resulted in a greater reduction of the parasite burden in the liver. Whereas, compound 21b enhanced IFN-\(\gamma\) production and thereby regressed lesion size significantly. However, compounds 15a and 21b at 5 mg/kg/day did not considerably affect IL-10 expression (cytokine concentration \(\approx\) 180–300 pg/mL). Following meglumine antimoniate and DMSO treatment, IL-10 production was measured to be \(\approx\) 100–300 pg/mL (Figure 5). It seems that limiting the IL-10 production was the main mechanism for regressing the parasite-visceral progression in 21a treatments. In general, 20 mg/kg/day treatments increased IL-10 cytokine activity (concentration \(\approx\) 350 pg/mL), while reducing IFN-\(\gamma\) expression (Figure 5).

Figure 4. Th1-associated cytokine production by ConA-activated splenocytes from infected BALB/c mice treated as indicated. Mice were inoculated with L. major promastigotes. At 4–5 weeks post-infection, spleens were pooled and lymphocyte cells were pre-incubated with ConA for 72 h before media supernatants were analysed. Data represent the concentration of IFN-\(\gamma\) (pg/mL) in comparison with the untreated control. 15a, 21a and 21b correspond to thiazole treatments. Glucantime, DMSO and control correspond to mice treated with 56 mg/kg/day meglumine antimoniate for 5 days, mice treated with 100 \(\mu\)L of DMSO and untreated mice, respectively. The data represent the means \(\pm\) SD of three independent experiments, performed with four mice per group, \(P\leq 0.05\).

Figure 5. Th2-associated cytokine production by LPS-activated splenocytes from infected BALB/c mice treated as indicated. Mice were inoculated with L. major promastigotes. At 4–5 weeks post-infection, spleens were pooled and lymphocyte cells were pre-incubated with LPS for 24 h before media supernatants were analysed. Data represent the concentration of IL-10 (pg/mL) in comparison with the untreated control. 15a, 21a and 21b correspond to thiazole treatments. Glucantime, DMSO and control correspond to mice treated with 56 mg/kg/day meglumine antimoniate for 5 days, mice treated with 100 \(\mu\)L of DMSO and untreated mice, respectively. The data represent the means \(\pm\) SD of three independent experiments, performed with four mice per group, \(P\leq 0.05\).

Increase in IL-10 production in splenocytes of L. major-infected BALB/c mice

We determined the stimulatory effects of the test compounds on IL-10, measured 23 days post-injection. In comparison with 21b, IL-10 Th2-activating cytokine production was significantly suppressed by the analogue 21a, which resulted in a greater reduction of the parasite burden in the liver. Whereas, compound 21b enhanced IFN-\(\gamma\) production and thereby regressed lesion size significantly. However, compounds 15a and 21b at 5 mg/kg/day did not considerably affect IL-10 expression (cytokine concentration \(\approx\) 180–300 pg/mL). Following meglumine antimoniate and DMSO treatment, IL-10 production was measured to be \(\approx\) 450–350 pg/mL (Figure 5). It seems that limiting the IL-10 production was the main mechanism for regressing the parasite-visceral progression in 21a treatments. In general, 20 mg/kg/day treatments increased IL-10 cytokine activity (concentration \(\approx\) 350 pg/mL), while reducing IFN-\(\gamma\) expression (Figure 5).
Responses showed an equivalence with analogues 15a and 21b (Figure 7b). An analogue 21a (observed in mice treated with 20 and 5 mg/kg/day doses of analogues) showed a high level of Th1-related activity was compared with controls, a high level of Th1-related cytokine secretion (Figure 7b). In comparison with controls, a high level of Th1-related cytokine secretion (Figure 7b). The degree of drug-induced Th1 immune response was expressed as the stimulation index or index of Th1 activity, which represented the fold increase in Th1-associated cytokine production relative to Th2-associated cytokine production (Figure 7a). The results represented a 3–4-fold increase in type-1 immune responses in genetically type-2 competent mice (Figure 7a). The results represented a 3–4-fold increase in type-1 immune responses in genetically type-2 competent mice (Figure 7a). The results represented a 3–4-fold increase in type-1 immune responses in genetically type-2 competent mice (Figure 7a).

Enhancement of thiadiazole-induced Th1 response in infected BALB/c mice

The degree of drug-induced Th1 immune response was expressed as the stimulation index or index of Th1 activity, which represented the fold increase in Th1-associated cytokine production relative to Th2-associated cytokine production (Figure 7a). The results represented a 3–4-fold increase in type-1 immune responses in genetically type-2 competent mice (Figure 7a). Another stimulation index corresponding to thiadiazole treatments. Glucantime, DMSO and control correspond to mice treated with 56 mg/kg/day meglumine antimoniate for 5 days, mice treated with 100 μL of DMSO and untreated mice, respectively. The data represent the means±SD of three independent experiments, performed with four mice per group, P≤0.05.

Discussion

Leishmaniasis is a complex of diseases and different clinical forms, which has traditionally been classified in three different clinical degrees: VL, CL and mucocutaneous leishmaniasis, each with different immunopathologies and degrees of morbidity and mortality. The parasite exists in two forms: the flagellated promastigote in the female phlebotomine sand fly vector; and the amastigote in the mammalian host. Amastigotes are obligate intracellular parasites of macrophages (and rarely other cell types), where they survive and multiply within a phagolysosome compartment. The target for chemotherapy is the intracellular amastigote that survives and divides in immune cells, particularly in macrophages, thereby causing coinfection with HIV virus.

One of the major aspects in the drug development process for novel antipathogenic agents is the investigation of the chemical–biological interaction in animal models, to obtain lead compounds that can enter the development process. Few of the investigations have been followed by work on the compound in an animal model. Research aimed at identifying and validating new drug targets is important, since the cell biology of Leishmania and mammalian cells differ considerably, and this distinctness extends to the biochemical level. This provides the promise that many of the parasite’s proteins should be sufficiently different from anything in the host to be successfully exploited as drug targets. Many potential drug targets have been identified in biochemical and molecular studies, and some have been validated. The search for new drugs continues, with bisphosphonates, e.g. risedronate and pamidronate, and plant derivatives, such as licochalcone A and quinoline alkaldoids, being reported to have activity against experimental animal infections.

In addition, cure of leishmaniasis during chemotherapy has been shown to depend upon the development of an effective immune response that activates macrophages to produce toxic metabolites, reactive nitrogen species (RNS) and reactive oxygen species (ROS) to kill the intracellular amastigotes. Treatment efficacy is compromised when there is immunosuppression induced by the parasite. This can lead to exacerbation of the disease or emergence from latent infection; the depleted immune capability means that standard chemotherapy is frequently unsuccessful.

The immune response to both VL and CL has been extensively studied in rodent models, which has offered paradigms of how cell-mediated immunity activates the infected macrophages to kill the intracellular parasites through elevated levels of ROS and RNS. This is carried out through activation of the correct population of T lymphocytes (Th1) and production of cytokines (IL-12 and IFN-γ) to, in turn, activate the macrophages. These factors clearly have implications for chemotherapy. Another competent immune response that is important for effective chemotherapy, of both CL and VL, is extensive granuloma formation around the infected macrophages, which influences the healing induced by the drug. Liver granulomas form by antigen-specific lymphocytes and parasite-specific T cells producing IFN-γ that develop type-1 response and compromise the ability of macrophages to kill the parasites. However, these processes are suppressed by the infection itself, which down-regulates the requisite signalling between macrophages and T cells, e.g. the production of IFN-γ, up-regulates NO release from IFN-γ-activated macrophages, which result in the complete suppression of spleen parasite burden, or the presentation of major histocompatibility complex and costimulatory molecules at the macrophage surface. Studies conducted from
the 1980s until now have shown that biological immunomodulators can provide a missing signal and enhance the treatment of VL and CL. The immunomodulatory effects of some drugs with antileishmanial activity have been reported, including their activity as a costimulatory signal for T cell and macrophage activation in vitro, and as an enhancer of IFN-γ production and NO in peritoneal macrophages after they are triggered with mitogens.

Previously, we reported that nitroheteroaryl-1,3,4-thiadiazole-based compounds, including 1-[5-(5-nitrofuran-2-yl)-1,3,4-thiadiazol-2-yl]-4-arylpiperazines and 1-[5-(5-nitroimidazole-2-yl)-1,3,4-thiadiazol-2-yl]-4-arylpiperazines of thiadiazole derivatives, possess potent leishmanicidal activity with selective inhibitory effect against parasite topoisomerase. In this paper, attempts were made to study the in vivo efficacy of the thiadiazole analogues with particular attention to cutaneous lesion size development, visceral progression of the parasite and modulatory effects on murine immune effector cells. We have previously shown that the analogues exerted greater efficiency against the macrophage-intracellular parasite in vitro, possibly through inducing NO and ROS formation (F. Pourrajab, S. K. Forouzannia and S. A. Tabatabaei, unpublished data). Similarly, we also showed that thiadiazoles were non-toxic for mouse macrophage cells at concentrations up to 100 μM. Interestingly, some of the potent thiadiazoles were found to enhance the NO and ROS production pathways inside macrophages and spleen lymphocytes.

The present results for granuloma formation and parasite clearance from the liver are in agreement with an earlier study showing that the compounds could significantly increase macrophage and lymphocyte activity for killing the intracellular parasite. Granulomatose phagocytic cells produce high concentrations of NO and ROS as well as IL-12 and they prime antigen-specific T cells to produce effector cytokines [IFN-γ and tumour necrosis factor (TNF)-α]. The resolution of disease in the livers of mice infected with L. major correlates with the local formation of granulomas, which normally form during disease regression in the liver, and are poorly formed in immunodeficient murine and human hosts. Competent immune responses are needed for the successful control of parasite replication in infection sites, as well as to contribute to the induction of leishmanicidal activity within macrophages infected with L. major, as was shown previously in vitro. Parasite-specific CD4+ and CD8+ T cells induce liver granuloma formation and, subsequently, develop Th1-type immune responses. The previous attempts have been followed by the work to establish whether the compound has useful activity against leishmaniasis in an animal model. The results obtained here, may reflect simply that the compounds in the doses injected, show no cytotoxicity for the host, whereas ensuring potent activity to resolving Leishmania infection in an animal model.

Moreover, compound-induced type-1 cytokine expression, IFN-γ, IL-12 and reduced IL-10 expression in spleen cells in vivo, by stimulating pathways in immune cells, prevents visceral development of the pathogen as well as resolving cutaneous infection and lesion regression by parasite healing. Another report also demonstrated that the production of IL-10 by spleen cells was not induced in the spleens of drug-administrated mice, possibly to balance the effector responses (e.g. oxidative burst) that control the parasite burden in visceral organs and the regulatory cytokines, in order to limit collateral tissue damage due to the increased release of certain cytokines, which result in macrophage and lymphocyte activation. However, the compounds had negative effects on type-2 response by reducing expression of Th2-associated cytokine IL-5, significantly. In our present study, the test compounds could enhance immune response-inducing cytokine production.

Figure 7. Relative IFN-γ to IL-5 (a) and IL-10 (b) expression in activated spleen cells from mice treated with thiadiazole. Inability of mitogen-stimulated spleen cells Th2-associated cytokine production. 15a, 21a and 21b correspond to thiadiazole treatments. Glucantime, DMSO and control correspond to mice treated with 56 mg/kg/day meglumine antimoniate for 5 days, 100 μL of DMSO and untreated mice, respectively.
One speculation is that the analogues might be able to enhance the binding of the mitogen to the receptors so as to achieve the synergistic effect on Th1-associated cytokine production. Another speculation is that the analogues might be able to increase the number of receptors for mitogen binding, thus enhancing immune responses. The finding that the thiadiazole analogues can enhance the effect of mitogens for the activation of spleen cells is not surprising, since an earlier report also demonstrated that some compounds are able to exert immunomodulatory effects in humans to resolve drug-resistant CL. However, the analogues were indicated to induce little IL-10 production and would also down-regulate Th1 response. The apparently contradictory effects might also reflect a differential immunomodulatory efficacy of the drugs to reduce a potentially harmful effect (respiratory burst), thereby reducing the toxic side effects. Actually, the results obtained from the present studies show that the in vivo administration of the new synthetic thiadiazole analogues alone into mice would have no significant cytotoxic effects in vivo and also no potent in vivo efficiency to regress parasite infection; however, they would have an enhancing effect on effector immune cell activation (F. Pourrajab, S. K. Forouzannia and S. A. Tabatabaee, submitted for publication).

Additionally, we observed granuloma formation, which correlates with the local regression of Leishmania infection in mammalian organs. The parasite is controlled within functional granulomas (based on experimental models and the examination of asymptomatic animals). The results demonstrate that the tested compounds can exert in vivo immunomodulatory activity on immune cells to fight against pathogens, whilst inducing no toxicity or visceral damage. In general, we investigated innate and adaptive immune responses to L. major infection in genetically susceptible BALB/c mice, and compared these with responses in drug-administered mice. The results of our study provide compelling evidence that the selected compounds provide efficient parasite control by innate and adaptive immune responses. Collectively, our findings demonstrate that the analogues can exert immunomodulatory effects on macrophages and lymphocytes, both in vitro and in vivo. Finally, analysis of all the results shows that compound 21a has more effective leishmanicidal activity than the other analogues tested and meglumine antimoniate. The results indicate a need for the clinical applicability of these compounds in the treatment of leishmaniasis to be explored in the future.

A new paradigm in immunology is that conserved microbial structures or pathogen-associated molecular patterns can be recognized by a broad array of host cellular pattern-recognition receptors (TLRs). Such recognition is believed to serve both for the immediate activation of innate immune defences and as a bridge for establishing acquired immunity. The finding that the thiazole analogues can enhance the effect of mitogens for the activation of spleen cells is not surprising, since an earlier report also demonstrated that some compounds are able to exert immunomodulatory effects in humans to resolve drug-resistant CL. However, the analogues were indicated to induce little IL-10 production and would also down-regulate Th1 response. The apparently contradictory effects might also reflect a differential immunomodulatory efficacy of the drugs to reduce a potentially harmful effect (respiratory burst), thereby reducing the toxic side effects. Actually, the results obtained from the present studies show that the in vivo administration of the new synthetic thiazole analogues alone into mice would have no significant cytotoxic effects in vivo and also no potent in vivo efficiency to regress parasite infection; however, they would have an enhancing effect on effector immune cell activation (F. Pourrajab, S. K. Forouzannia and S. A. Tabatabaee, submitted for publication).

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A new paradigm in immunology is that conserved microbial structures or pathogen-associated molecular patterns can be recognized by a broad array of host cellular pattern-recognition receptors (TLRs). Such recognition is believed to serve both for the immediate activation of innate immune defences and as a bridge for establishing acquired immunity. As a consequence the ability to promote Th1-activated cytokine (IFN-γ) excretion, which potentiates the immune response severalfold. According to our data, it appears that certain chemical compounds may induce TLR-mediated immunomodulatory effects, in contrast to the parasite exploiting part of this to escape the host’s antimicrobial mechanisms. However, there are many questions that remain to be answered regarding TLR-mediated immunomodulatory effects. Also, further studies on the clinical applicability of the compounds are needed, which will allow us to explore the relationship between TLRs and drugs.

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Transparency declarations
None to declare.

Supplementary data
Figures S1 to S5 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References


45 Kima PE. The amastigote forms of Leishmania are experts at exploiting host cell processes to establish infection and persist. Int J Parasitol 2007; 37: 1087–96.

