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In vitro Immunobiological Studies of Novel 5-(5-nitrofuran-2-yl)-1, 3, 4-Thiadiazoles with Piperazinyl-Linked Benzamidine Substituents against *Leishmania Major*

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ABSTRACT

It was recently demonstrated that 5-(5-nitrofuran-2-yl)-1, 3, 4-thiadiazoles with piperazinyl-linked benzamidine substituents are effective *in vitro* against *Leishmania major*.

Following on this evidence, we used colorimetric assay of acid phosphatase activity in the promastigotes as an indicator for cell viability. Also we studied the effect of these compounds on induction of nitric oxide (NO) in macrophage and production of reactive oxygen species (ROS) in lymphocyte that have important role in activation of immune response against *Leishmania* and elimination of parasite.

Results showed that these compounds decrease the viability of the parasite and increase ROS and NO production in lymphocyte and macrophage respectively.

These compounds can induce parasite killing, directly by decreasing the parasite viability and indirectly by exhibiting a significant increase on immune system.

Keywords: 5-(5-nitrofuran-2-yl)-1,3,4- thiadiazoles piperazinyl-linked benzamidine substituents; Acid phosphatase; Leishmaniasis; Nitric Oxide; Reactive oxygen species

INTRODUCTION

Leishmania is a trypanosomatid protozoan parasite which causes a variety of diseases in mammals known as leishmaniasis in tropical and subtropical regions of the world. The parasite alternates between sandfly and mammalian hosts in two developmental forms; motile promastigote that multiplies in the sandfly gut and nonmotile amastigote that lives and replicates in the phagolysosomal compartment of mammalian macrophages.[1]

The current treatment of leishmaniasis is primarily based on chemotherapeutic agents including pentavalent antimonials, amphotericin B, pentamidine isothionate, and miltefosine. The utilization of these compounds have some disadvantages such as high cost and duration of treatment, lack of patient adherence to

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treatment, and the development of resistant parasite strains to some of these medicines.[2] During the last 10 years, the global burden of leishmaniasis and pattern of infections in the world has been extensively increased due to its co-infection with HIV and development of resistant strains. To date, no vaccine exists yet against leishmaniasis. As a result, academic researchers try to develop new anti-leishmania agents that are inexpensive, safe and orally available.[3-5]

In the previous study, we demonstrated that 5-(5-nitrofuran-2-yl)-1, 3, 4-thiadiazoles with piperazinyl-linked benzamidine substituents have antiproliferative activities against promastigote and amastigote form of *Leishmania major*. Also these compounds showed low level of toxicity against macrophages.[6]

It has been suggested that secretory products of *Leishmania* play a major role in the survival of these parasites.[7] It is interesting to know that promastigotes of all pathogenic species of *Leishmania* constitutively secrete acid phosphatase into the culture medium during *in vitro* growth.[8-10] Amastigotes were also shown to produce acid phosphatase, which might make the survival of the parasite longer within macrophage by dephosphorylating critical elements involved in lysosomal function and oxidative killing mechanism.[11]

Studies on the murine model of leishmaniasis have indicated that induction of NO is one of the main effector mechanisms of macrophages for elimination of *Leishmania* parasites.[12,13] On the other hand, activated macrophages produce IL-12 which induces Th1 differentiation and proliferation. Th1 cells have activatory effect on macrophages by IFN-γ production, which is essential for the elimination of intracellular pathogens.[14-16] Also studies showed that low level of ROS can stimulate lymphocytes for IFN-γ secretion.[17] Elevated level of IFN-γ induces production of ROS and NO in phagocyte cells that harbor *Lieshmania* and IL-12 leading to potentiating of type-1 response and destruction of the parasite.

For these reasons within the current study we investigated the effect of these compounds on the viability of the parasites by measuring the acid phoshatase activity on different growth phases and ROS and NO production in mice lymphocytes and macrophages respectively to evaluate the effect of 5-(5-nitrofuran-2-yl)-1, 3, 4-thiadiazoles with piperazinyl-linked benzamidine substituents on immune cells.

MATERIALS AND METHODS

Test Compounds

All 5-(5-nitrofuran-2-yl)-1, 3, 4- thiadiazoles with piperazinyl-linked benzamidine substituents derivatives (Figure 1) tested in the series of experiments were synthesized in the laboratory, and the synthesis details, characterization, IC_{50} and their inhibitory effect on amastigote and promastigote form of parasite have been described previously.[6]

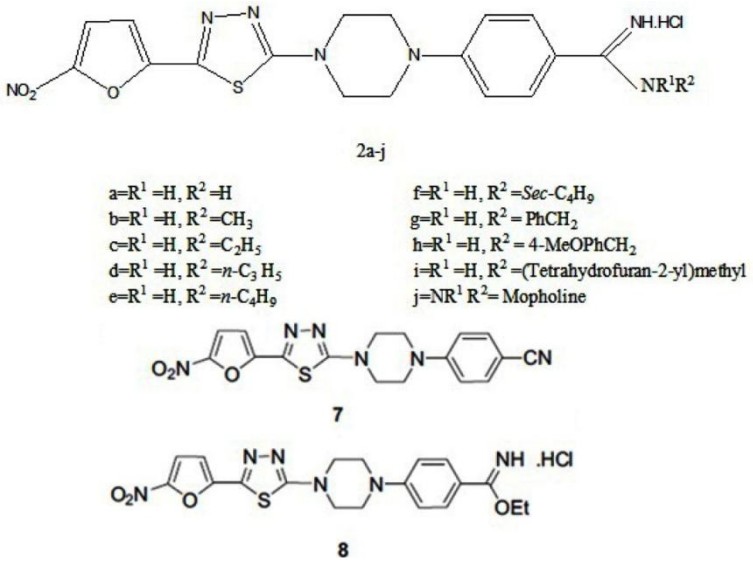

Figure 1. Structure of novel 5-(5-nitrofuran-2-yl)-1, 3, 4- thiadiazoles with piperazinyl-linked benzamidine substituent ' s derivatives.

Table 1. Acid phosphatase activity assay of *L. major* Promastigotes at two different growth phases, treated with the selected compounds and drug solvent DMSO as a negative control for 24 h.

Compounds[a]	IC_{50}(µM)	Logarithmic phase*	Stationary phase*	Logarithmic phase±	Stationary phase±
7	104 ± 0.7	0.191 ± 0.033	0.332 ± 0.04	3.5 ± 0.91	2.4 ± 0.18
8	48 ± 0.42	0.421 ± 0.014	0.275 ± 0.015	2.1 ± 0.19	2.9 ± 0.36
2b	23 ± 0.25	0.313 ± 0.013	0.371 ± 0.014	2.7 ± 0.125	2.6 ± 0.23
2c	33 ± 0.62	0.429 ± 0.036	0.425 ± 0.065	2.3 ± 0.23	2.95 ± 0.48
2d	10 ± 0.65	0.235 ± 0.045	0.401 ± 0.04	3.6 ± 0.77	2.3 ± 0.42
2e	11 ± 1.4	0.228 ± 0.02	0.519 ± 0.01	4.1 ± 0.43	2.2 ± 0.46
2f	33 ± 0.21	0.337 ± 0.01	0.446±0.02	2.6 ±0.102	2.1 ±0.065
2h	80 ± 1.6	0.62 ± 0.032	0.641±0.126	2.4 ±0.15	1.4±0.031
2i	95 ± 0.8	0.317 ± 0.002	0.382±0.015	2.8 ±0.207	2.5 ±0.23
2j	93 ± 0.9	0.562 ± 0.05	0.571±0.076	2.4 ±0.27	1.5 ±0.21
DMSO		0.935 ± 0.009	0.941±0.052		

AcP: Acid phosphatase

a: Promastigotes were treated with IC_{50} doses of thiadiazols

*The results in these two vertical columns are OD (optical density)

±The results in these two vertical columns are; AcP activity of control sample/AcP activity of drug-treated sample

Promastigote Culture and Treatment

The strain of *L. major* used in this study was the vaccine strain (MRHO/IR/75/ER), obtained from Pasteur Institute, Tehran (Iran). The infectivity of the parasites was maintained by regular passage in susceptible BALB/c mice. The parasite was grown in blood agar culture medium at 25 ºC. Promastigotes of *L. major* strain (MRHO/IR/75/ER) were cultured as described previously.[18] Briefly, 2×10^6 cells/ml were routinely inoculated and cultured in complete RPMI 1640 medium pH 7.2, containing 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma), enriched with 10% heat-inactivated fetal calf serum (FCS), at 25°C. Based on growth curve, logarithmic phase appeared at 48h and stationary phase appeared at 96h.

For treatment purpose, IC_{50} doses of compounds were added to the logarithmic or stationary phases of parasites (Table 1). Drug solutions were made in 2% DMSO as stock and diluted to make appropriate concentrations in the medium immediately before adding to the cell culture. Glucantime is used to treat cutaneous and visceral leishmaniasis in humans. In order to show the specific effect of our compounds, we used 60 mg/ml glucantime which is its IC_{40} on the strainof *L.major* used in this experiment and 2% DMSO as vehicle control.

Acid Phosphatase Activity Assay to Determine 5-(5-Nitrofuran-2-yl)-1, 3, 4- Thiadiazole with Piperazinyl-Linked Benzamidine Substituents Toxicity

Acid phosphatase activity is a reliable indicator for the parasite growth rate as well as for its virulent potency.[19,20]

The acid phosphatase activity of promastigotes in either logarithmic or stationary phases in the presence or absence of IC_{50} concentration of compounds for 24h was measured; the cells reached the logarithmic and stationary phases of growth at day 2 and 4, respectively. Promastigote (2×10^6) dispensed in microplate wells with 200 µl of medium containing IC_{50} dilutions of test drugs for 24h at 25ºC. Total acid phosphatase activity (secretory, membranous and cytoplasmic) was determined as follows:[21,22] After 24 hours incubation, 20 µl of lysis buffer (1M sodium acetate, pH 5.5 and 1% Triton X-100) containing 10 mg/ml *p*-nitrophenyl phosphate was directly added to each well. Incubation was continued for a further 6h at 37°C, and the production of *p*-nitrophenol was determined by optical density measurements at 405nm using microplate reader (Bio Tek Power Wave XS2).

Evaluation of NO Production by Treated Peritoneal Macrophages

Macrophages were obtained from BALB/c mice by lavage of peritoneal cavity. The cells were cultured in 24 well plates (10^6 cells/ml, 1ml /well) in complete RPMI 1640 medium and incubated at 37ºC, with 5% CO_2 for 2h and non-adherent cells were removed. Then the compounds at a concentration of equal to its IC_{50} value were added to wells. The supernatants were collected after 48h and NO production was determined by Griess reagent.[23] Since NO is unstable and is rapidly converted to nitrate and nitrite, it was necessary to determine both nitrate and total nitrite concentrations in samples. In order to convert nitrates to nitrites 100 µl vanadium chloride (400mg were prepared in 50 ml HCl 1M) was used. Briefly, to 100 µl of culture medium, 100 µl of vanadium chloride (III) and 50 µl of Griess reagents [1:1 (v/v) of 0.1% naphthylethylenediaminedihydrochloride (NEDD) in H_2O+2% sulphanilamide in 5% H3PO4] were added and incubated at 37°C for 40 min and the absorbance was read at 540 nm using microplate reader (Bio Tek Power Wave XS2).

Evaluation of ROS Production by Treated Mice Lymphocytes

Intracellular ROS level was measured in treated and untreated lymphocytes using fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (DCFH2DA).[24] Briefly, lymphocytes were obtained from spleen of BALB/c mice and cultured in 24-well plates (10^6 cells/ ml, 1ml/ well) in RPMI 1640 and 10% FCS at 37ºC with 5% CO_2 in the presence of IC_{50} concentrations of the compounds. The plates were incubated for additional 24h. At the end of incubation, a final concentration of 25 µM of DCFH2DA was added and the cells were incubated at 37ºC, with 5% CO_2 in humidified incubator for 30 min to allow loading of the DCFH2DA. After entering the cell membrane, DCFH2DA was converted to DCFH2 by cellular esterase. Peroxidases, cytochrome c and Fe^{2+} oxidize DCFH2 to 2, 7-dichlorofluorescein (DCF), a highly fluorescent compound, in the presence of hydrogen peroxide. After 30 min, the cells were washed three times with phosphate-buffered saline (PBS) and suspended in PBS. Accumulation of DCF in the cells was measured (Ex=485, Em=530) using Cary eclips, Varian Fluorospectrometer.

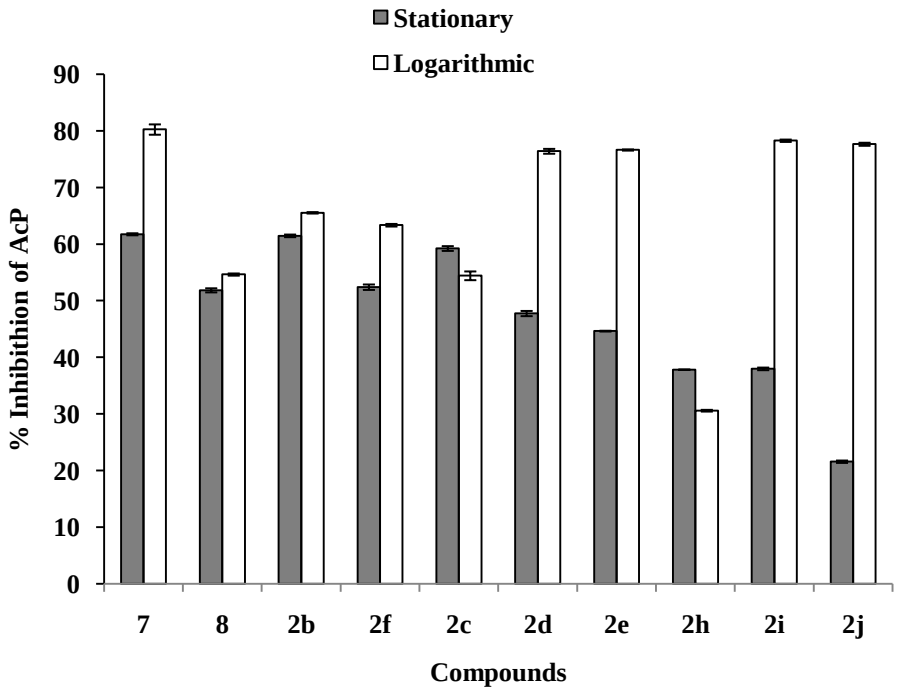

Figure 2. Acid phosphatase activity assay of *L.major* promastigotes at two different growth phases, stationary phase and logarithmic phase, treated with the IC_{50} concentration of selected compounds and 2% DMSO as a vehicle control for 24h.

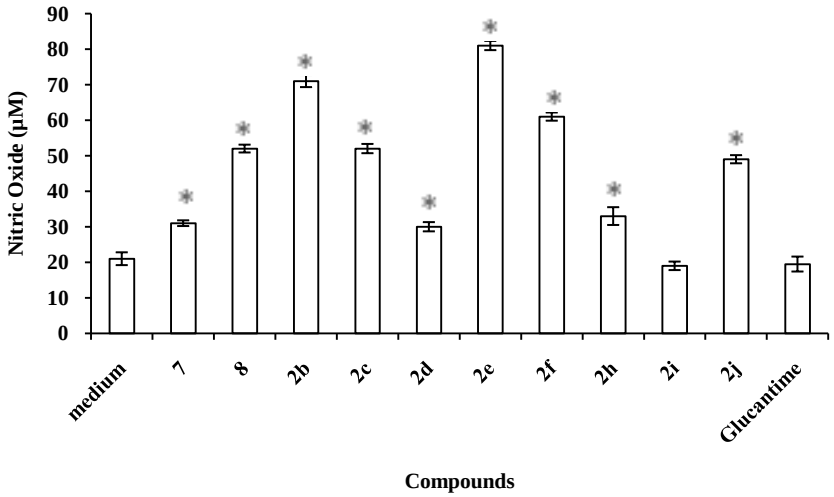

Figure 3. The effect of thiadiazole derivatives on NO production in mice macrophages. Macrophages were cultured in RPMI medium in the present of IC_{50} concentration of selected compounds and compared with untreated cells (medium). The bars are mean +/- SD for three times study which are significantly different from control (*P≤0.02*).

Statistical Analysis

Statistical Analysis was performed by student t-test (Microsoft Excel).

RESULTS

Significant Decrease of Acid Phosphatase

L.major contained considerable acid phosphatase activity related to its viability, which is a reliable method for assessing the parasite growth rate, also as an indicator of virulent potency that contributes to block the phagocyte antimicrobial response; NO and ROS production.[19,20] The acid phosphatase of *L.major* has a broad substrate specificity hydrolyzing glycerol phosphatase, mono- and di-phosphorylated sugars,[25,26] inositol phosphate and phosphorylated proteins.[27] Thus inhibition of acid phosphatase activity has important role in diminishing of intracellular parasite growth. However, to facilitate the characterization of compound impact on the parasite viability and their growth inhibitory effects, we measured acid phosphatase activity during logarithmic and stationary growth phases. Treatment with 5-(5-nitrofuran-2-yl)-1,3,4-thiadiazoles with piperazinyl-linked benzamidine substituents compounds significantly decreased acid phosphatase activity on both logarithmic (~ 2.7 folds) and stationary (~2.2 folds) promastigotes with higher effect against the logarithmic phase although parasites at the stationary phase had relatively higher level of acid phosphatase activity (Table 1 and Figure 2). However, as previously we reported, glucantime had smaller effect on logarithmic phase (2.1±0.41) and stationary phase (1.1±1.64) comparing with 5-(5-nitrofuran-2-yl)-1, 3, 4-thiadiazoles with piperazinyl-linked benzamidine substituents.[28]

Compounds Induce NO Production in Macrophages

The macrophages were cultured in complete RPMI medium and stimulated with IC_{50} concentrations of the compounds. After 48h incubation, the supernatants were collected and analyzed for NO production. Data showed that all of compounds except 2i and glucantime significantly ($p\leq0.02$) stimulate macrophages to induce NO production (Figure 3). It is noteworthy that compounds 2e, 2b and 2f showed more stimulatory effect on NO production.

Compounds Induce Production of ROS in the Mice Lymphocytes

ROS production was analyzed using spectrofluorometer. As shown in Figure 4, all tested compounds in IC_{50} concentration significantly ($p<0.05$) induced ROS production in lymphocytes. Maximum ROS production was observed with 2c, 2e.

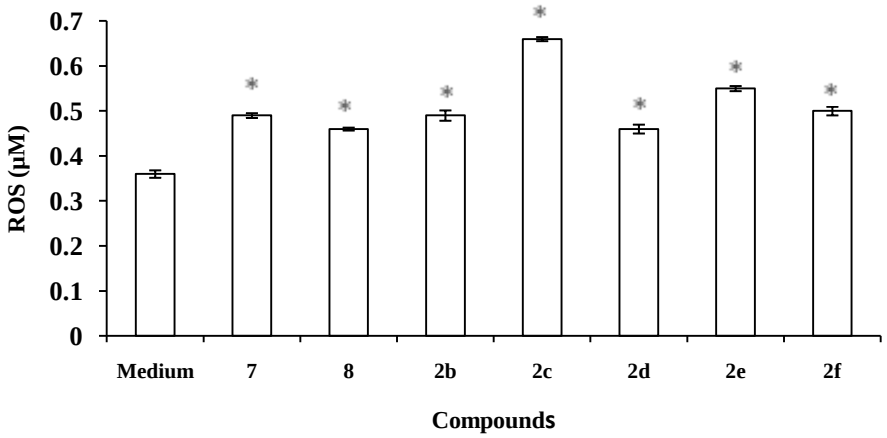

Figure 4. The effect of thiadiazole derivatives on ROS production in lymphocytes treated with IC_{50} concentration of compounds. Lymphocytes (10^6 cells/ml) were cultured in RPMI 1640 in the presence of compounds and compared with untreated cells (medium). After 24 h, the cells were analyzed for ROS production by spectrofluorometer. All compounds can increase ROS production in lymphocytes (*p<0.05*) in comparison to vehicle control (2%DMSO). The bars are mean +/- SD for three experiments.

DISCUSSION

In the previous study, we reported significant effect of thiadiazole derivatives against promastigote and amastigote form of *L.major*.[6] In this study, we evaluated the effect of these compounds on acid phosphatase activity and induction of NO in macrophage as a major mechanism in elimination of intracellular parasite.[12,13] Also we evaluated the effect of these compounds on lymphocytes for ROS production because ROS can stimulate lymphocytes for IFN-γ secretion.[17] *Leishmania* promastigotes have two growth stages including stationary and logarithmic growth phases. In the logarithmic growth phase, parasites have a low virulence but high growth rate; in the later stationary growth phase, parasites exert a high disease-developing potential but low growth rate.[29-31]

Promastigotes from logarithmic and stationary phase cultures of *Leishmania* produce and secrete acid phosphatase outside the cell but the expression and activity of the enzyme is much higher in the stationary phase which is important for the establishment of the disease in this phase. The part of the enzyme located on the cell surface of *Leishmania* involves in parasite adhesion to host cells and promotes the entry of parasite into the cells. The portion of acid phosphatase secreted into the phagosome produces inorganic phosphate as a source of nutrition and disables hydrolyses and is essential for survival of the parasite.[20] It was found that 5-(5-nitrofuran-2-yl)-1,3,4-thiadiazoles with piperazinyl-linked benzamidine substituents exhibited potent anti-leishmanial activity on both parasite growth stages and decreased cell viability of parasites. In the previous study, cytotoxicity of these compounds were investigated using MTT test but in this study the parasite viability was evaluated using acid phosphatase activity assay.[1] Results showed that all compounds decreased acid phosphatase activity on both logarithmic (~ 2.7 folds) and stationary (~2.2 folds) promastigotes of *leishmania*.

There is now a good clinical and experimental body of evidence that control of cutaneous leishmaniasis is obtained through the following circuit: activated macrophages produce IL-12 which drives Th1 cell differentiation and proliferation. Th1 cells produce IFN-γ which activates macrophages to kill *Leishmania* parasites through NO production. It is well documented that NO production is the major mechanism in leishmaniacidal activity of murine macrophages.[32-34] We evaluated the effect of the compounds inducing NO production in macrophages which were infected by amastigotes. All compounds except 2i could induce macrophage NO production after 48h. Compounds 2e and 2b which had the highest power of induction of NO

production (about 70 µM), had also significant effect on amastigote killing within macrophages (Infectivity Index less than 30).[6]

Therefore, it appears that there is a direct relationship between increased NO production in macrophages and decreased intracellular parasites. It seems that induction of NO in macrophages is one of the main mechanisms through which these compounds cause intracellular parasites to be killed. Glucantime, as a positive control was not capable of inducing NO production in macrophages. If the new synthetic derivatives considerably increase the amounts of NO in macrophages, they will have great advantage over drug Glucantime which is commonly used.

T cell response to *Leishmania* infection by the production of cytokines is responsible for the activation of macrophages to promote killing of intracellular *Leishmania* parasites.[17] ROS production in lymphocytes after treatment with these compounds was also evaluated. There exists a huge body of data concerning the cell-damaging role of ROS.[35] The generation of ROS has been connected to stress responses, apoptosis, aging and death.[36,37] In recent years, however, the "bad reputation" of H_2O_2 and other ROS molecules has changed. These molecules are now being recognized as molecules of life that are essential to the proper development and proliferation of cells. It has been known for some time that low doses of H_2O_2 have mitogenic effects and can mimic the function of growth factors.[38,39] In the immune system, the activation of lymphocytes often requires a close cellular contact between two cells forming a synapse. Such a synapse is formed between an antigen-presenting cell and a T cell as well as between B cells.[40,41]

It seems that H_2O_2 also functions as a secondary messenger between cells.[42] Thus, increased ROS production in lymphocytes under the influence of these compounds is considered as an advantage for these derivatives which can cause lymphocytes to be activated and finally they activate the immune system against *Leishmania*. The compounds can increase ROS production in lymphocyte and among them, compounds 2e and 2c have more stimulatory effect.

The mechanism of action described in this study needs more attention and should be considered in future experiments in animal and clinical studies. One of our next goals will be to investigate *in vivo* efficacy and cytotoxicity of these compounds.

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