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Research Article

The Potential Use of Nitazoxanide in the Treatment of Cutaneous Leishmaniasis: In-Vitro Assays Against Leishmania Tropica

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ABSTRACT

Here we designed to assess the effect of nitazoxanide (NTZ) alone and along with meglumine antimoniate (MA) against amastigote and promastigote forms of *Leishmania tropica in vitro.* The antileishmanial activity of effects of NTZ alone and along with MA on *L. tropica* promastigote and amastigote forms have been determined via a colorimetric MTT method and on the J774-A1 macrophage cells, respectively. Also, the effect of NTZ on the infection rate of *Leishmania* to J774-A1 cells was determined. The results of the 50% inhibitory concentrations (IC50) showed that NTZ + MA (25.8 ± 1.57 µg/ml) possess a more anti-leishmanial effect than MA (65.3 ± 3.12 µg/ml) or MTX alone (80.2 µg/ml) on promastigotes of *L. tropica*. The MTX combined with MA triggered a significant reduction ($P<0.05$) in the mean number of amastigotes forms in J774-A1 cells in comparison with control group. The results also demonstrated that rate of infectivity of promastigotes was considerably ($P<0.05$) decreased after it pre-incubation with NTZ. The obtained findings revealed that the great effectiveness and a synergistic effect of NTZ alone and along with MA on the growth of *L. tropica* promastigote and amastigote forms. However, further clinical studies are mandatory to examine this synergistic effect on a therapeutic scale.

Keywords: cutaneous leishmaniasis, combination therapy, promastigote, amastigote, infectivity rate.

INTRODUCTION

Leishmaniasis is a common disease in the tropical and subtropical regions which is caused by a protozoan parasite of genus *Leishmania*. This protozoan is transmitted by the bite of female sandflies to humans and other reservoir hosts, including the dogs and desert rodents (1). Considering the lesions caused by this disease, leishmaniasis can take four forms: cutaneous, muco-cutaneous, disseminated cutaneous, and visceral or kala-azar, each of which is created by different species of *Leishmania*. The genus *Leishmania* is divided into different subgenera, one of which is *Leishmania tropica* complex that includes *Leishmania tropica* and *Leishmania major*. These two parasites cause anthroponotic cutaneous leishmaniasis (ACL) and zoonotic cutaneous leishmaniasis (ZCL), respectively (2).

There are various methods for the treatment and control of the disease, such as the use of various medications and preventive measures, but no effective treatment has yet been discovered for the cutaneous leishmaniasis (3). One of the drugs used to treat the disease is made by the compounds such as meglumine antimoniate (MA) or glucantime and sodium stibogluconate (SSG), which are used by topical injection. However, these drugs have problems such as high toxicity, long-term treatment, and low efficacy. In addition to these compounds, there are other drugs that are not used owing to the high cost and side effects (4-6). Other treatments include ice therapy for small lesions and local removal of the lesion or curettage (7).

As mentioned, new compounds should be used due to the lack of effective treatment for this

disease. Different methods are used to produce new drugs, and one of these methods is the combination therapy. In this method, two or more drugs are used to treat a disease, which aims to achieve greater efficacy with the least amount of medication and less toxicity. This method has been used to treat many diseases such as HIV, malaria and various types of cancer (8).

Nitazoxanide (N-(5-nitrothiazol-2-gammal) salicylamide, NTZ) is a new compound based on nitrothiazole benzamide. This compound is a non-competitive inhibitor of pyruvate ferredoxin oxidoreductase (PFOR), and the inhibition of the disulfide isomerase protein is another mechanism of action of this drug. This compound is used in viruses to inhibit their replication, which affects hepatitis B and C viruses. Also, in the viruses such as influenza, it degrades the hemagglutinin protein (9). Previous studies have shown that this compound has a substantial impact on a wide range of worms and protozoa, such as *Cryptosporidium spp.*, *Giardia, Ascaris lumbricoides*, and *Hymenolepis nana* (9). Nitazoxanide can also be effective in treating the diarrhea on account of the rotaviruses. This compound can also be used to treat hepatitis B and C and as an antibacterial agent against *Helicobacter pylori* (9). Due to the antimicrobial, especially antiparasitic, properties of NTZ and the need to produce a new agent to treat leishmaniasis, it was chosen to study the effects of NTZ and MA in combination with each other and alone in the *in-vitro* conditions.

MATERIALS AND METHODS

Parasite culture

L. tropica promastigotes (MHOM/IR/2002/Mash2) were cultured in RPMI1640 improved with 2 mM L-glutamine, penicillin (100 IU/ml), streptomycin (100 µg/ml), and 15% heat inactivated fetal calf serum (FCS, Gibco, Eching, Germany) at 37 °C and 5% CO_2.

Cell culture

The J774-A1 macrophage cells were provided from Pasteur Institute of Iran and were cultured at Dulbecco's modified eagle's medium (DMEM) improved with 10% FBS at 37°C in 5% CO_2.

Anti–proliferation effect against promastigotes forms

The ability of NTZ (Sigma-Aldrich, St Louis, MO, USA) to inhibit the proliferation of promastigotes alone and in combination with MA (Rhône, Poulenc, France) against *L. tropica* was measured through colorimetric cell viability MTT test (10). On hundred µl of different concentrations of NTZ alone (12.5-200 µg/ml), MA alone (12.5-100 µg/ml) and along with NTZ (25 µg/ml) was added to 96-well culture plate containing the promastigotes in the logarithmic growth phase (10^6 cells/ml) and incubated at 24°C for 3 days. After adding 10 µl of MTT solution (5 mg/ml) to the wells were and incubating for 3 h, the absorbance of each well was determined by means of an ELISA reader (BioTek-ELX800) at 490 nm. Non-treated promastigotes and complete medium without any promastigote were considered as the control and blank, respectively.

Effect on intramacrophage amastigote forms

At the first step, 0.2 ml of the J774-A1 cells (10^5cells/ml) was added to the each well of 6-chamber slides. The promastigotes (stationary phase) of *L. tropica* with a ratio of 10parasites / cell and incubated at 37°C and 5% CO_2 for 24 h. The infected J774-A1 cells were then treated with some concentrations of MA or NTZ alone (12.5-200 µg/ml) and various concentrations of MA along with 25 µg/ml of NTZ at 37°C for 72 h. After this time, dried slides were fixed with methanol and stained with Giemsa and tested under light microscopy. Non-treated J774-A1 cells and non-infected cells were considered as the positive and negative controls, respectively. To investigate the effect of anti-*Leishmania* 100 macrophages were considered and the number of amastigotes in macrophages were counted with light microscope (12).

Inhibition of infection in murine macrophages

The inhibitory effect of NTZ on invasion of *L. tropica* was determined according to the method described elsewhere (12). NTZ at the concentration of 25 µg/ml and in combination with MA was used to pre-incubation of promastigotes (10^6 cells/ml) for 2 h at the 21°C. Promastigotes were then exposed with J774-A1 cells for 4 h at the 21°C. Finally, after staining the J774-A1 cells by Giemsa, the mean infection rate of cells was measured via counting 100 cells in comparison with control (12).

Statistical analysis

In the present study, data were indicated as the means ± standard deviations (SD) of three independent tests. The 50% inhibitory concentrations (IC50) in each stage were measured based on the Probit test through the software SPSS ver. 22 (Chicago, IL, USA). The obtained data were considered by one-way ANOVA tests and Scheffe Post Hoc tests, in software SPSS ver. 22 (SPSS Inc., Chicago, USA). Additionally, t-test was applied to compare the IC^{50} values of tested groups. $P<0.05$ was also considered significant.

RESULTS

Anti - promastigote effects

As shown in Fig. 1, NTZ, particulary in combination with MA significantly ($P<0.05$)

inhibited the rate of growth of promastigotes and indicated a dose-dependent inhibition as compared with MA. The IC_{50} values of MA and NTZ were 65.3 and 80.2 µg/ml, respectively. These IC_{50} are meaningfully ($P<0.05$) upper than the determined IC_{50} values for different concentrations of MA+NTZ (25 µg/ml) against (25.8 µg/mL), demonstrating lower effects of MA or NTZ alone in comparison with NTZ+ MA on *L. tropica* promastigotes.

Effects on intramacrophage amastigotes

Here, to assess the *in vitro* anti-amastigote effects of NTZ, we calculated the number of amastigotes in each macrophage infected by promastigote of *L. tropica*. Findings revealed that combination of MA+NTZ (25 µg/ml) powerfully decreased ($P<0.05$) the number of amastigotes in each macrophage in comparison with MA or NTZ alone (Fig. 2). Table 1 showed he IC_{50} values of MA or NTZ alone and MA+NTZ (25 µg/ml) which exhibited higher effectivity of MA+NTZ combination against amastigote stage of *L. tropica* in comparison with MA or NTZ alone.

Inhibition of infection in macrophages

The results showed that generally more than 70% of the macrophages were infected by promastigotes of *L. tropica*. While, promastigotes of *L. tropica* treated with MA or NTZ alone and MA+NTZ were able to infect 29%, 52% and 18.5% of macrophages, respectively; indicating that promastigote infectivity meaningfully *($P<0.05$)* decreased with NTZ pre-incubation.

Table 1: IC_{50} values between different concentrations of meglumine antimoniate (MA), nitazoxanide (NTZ*)* and NTZ (25 µg/ml) + MA against the growth rate of promastigote and amastigote forms of *L. tropica*. Data are expressed as the mean ± SD (n = 3)

Chemicals	IC_{50} (µg/ml)	
	Amastigote	Promastigote
MA	18.6± 1.62	65.3 ± 3.12
NTZ	38.6±2.51	80.2 ± 2.52
NTZ (25 µg/ml)+MA	6.5±0.15	25.8 ± 1.57

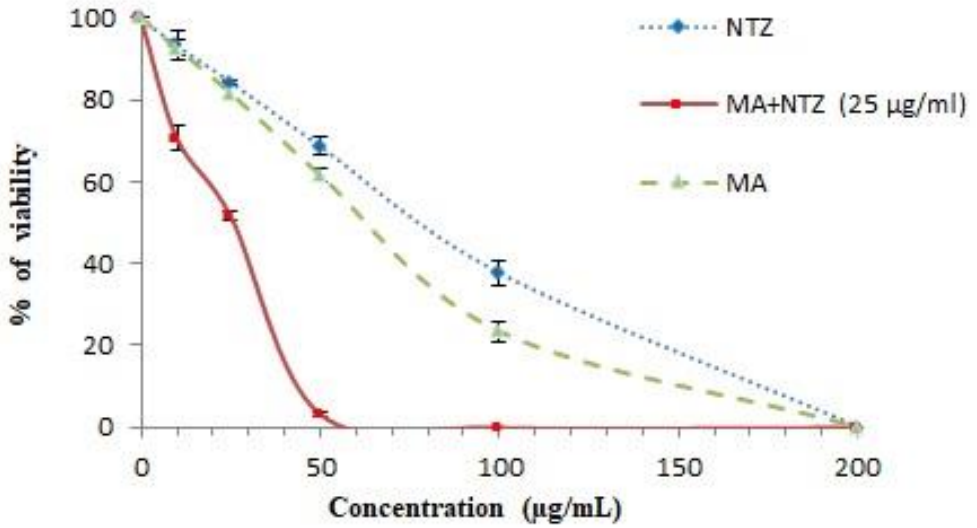

Fig.1: The viability of promastigotes of *L. tropica* in the presence of various concentrations of meglumine antimoniate (MA), nitazoxanide (NTZ) and NTZ (25 µg/ml)+MA after 72 h incubation. Data are expressed as the mean ± SD (n = 3)

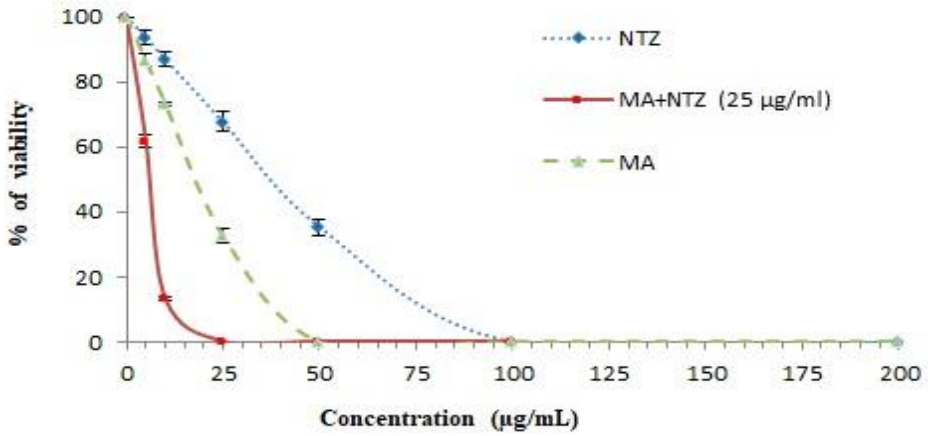

Fig.2: The effect of various concentrations of meglumine antimoniate (MA), nitazoxanide (NTZ) and NTZ (25 µg/ml)+MA on the mean number of amastigotes of *L. tropica* in each macrophage (amastigotes viability) in comparison with infected macrophages with no treatment as positive control. Data are expressed as the mean ± SD (n = 3)

DISCUSSION

Leishmaniasis is considered as one of the seven most important tropical diseases and many concerns regarding the economics and public health are made; whereas the disease is endemic in nearly 100 countries and affects millions of people worldwide (14). Currently, several methods are used for the treatment of leishmaniasis, one of which is the medication. The drugs such as meglumine antimoniat (MA) and sodium stibogluconate (SSG) as an illustration have been used for a long period of time to enhance the lesions owed to the disease. However, these drugs have problems such as high toxicity, long-term treatment and low efficacy. In virtue of this reason, researchers seek to corroborate an ideally effective treatment for this disease only then would the side effects of existing drugs be vanished (6,15,16).

In recent years, one of the methods that have been considered for the treatment of many diseases is combination therapy. This method can also be used to treat leishmaniasis, as former studies have substantiated that various drugs in combination with MA have been used for this disease (8,17). In this study, we used a combination of nitazoxanide and MA. Nitazoxanide is a non-competitive inhibitor of PFOR. In previous studies, it has been demonstrated to have antibacterial as well, especially antiparasitic. The results of the experiments reveal that the inhibitory potential of these two drugs in combination with each other is much higher than their use alone, as by combining these two drugs, the half maximal inhibitory concentration (IC50) is equal to 25.8 µg/ml against the promastigotes of *L. tropica*, which is much higher in the use of these two drugs alone. Moreover, the concentration in the inhibition of amastigotes in the macrophages is 6.5 µg/ml, which is much lower than the IC_{50} of the two drugs alone. It suggests that the more drugs combined, the more inhibition can be achieved with less concentration.

Many studies have reported the numerous properties of nitazoxanide, such as the potential use of the drug against the anaerobic gram-negative bacteria and aerobic gram-positive bacteria and also its effect on a wide range of viruses. Previous studies have also validated that the drug can be used to treat the worm infections and even the protozoan infections.

Me´Graud et al. (1998) suggested the widespread use of this drug on Helicobacter pylori and anaerobic bacteria such as Clostridium species (18). Additionally, Keeffe et al. (2009) confirmed that nitazoxanide has a significant effect on hepatitis B and C and is able to inactivate these viruses through various mechanisms (19). Rossignol (2014) supplementary noted that according to the conducted research, nitazoxanide is capable of eliminating a wide range of viruses such as the influenza A and B viruses, including pH1N1 and H7N9 (20). It can also inhibit the replication of viruses such as RSV, parainfluenza, rotavirus, coronavirus, and Japanese encephalitis (20).

According to the studies conducted by Fox et al. (2005), nitazoxanide has the ability to counteract the effects of worm infections such as *Ascaris lumbricoides*, *Hymenolepis nana*, *Tenia saginata*, *Enterobius vermicularis*, *Fasciola hepatica* and coupled with protozoan infections such as *Cryptosporidium parvum*, *Giardia lamblia*, *Entamoeba histolytica*, *Entamoeba dispar*, and *Isospora belli* (21). Abaza et al. (1998) found that

in addition to the aforementioned protozoa, the drug can eliminate the amoebas and infections caused by *Balantidium* (22).

Due to the significant effect of nitazoxanide on a variety of microorganisms, different mechanisms are expressed for its efficacy. One study found that the antibacterial mechanism of nitazoxanide, especially on Helicobacter pylori, inhibits the DNA synthesis (18). Another study suggested that nitazoxanide can affect the hepatitis B and C viruses by activating positive PKR regulation and elf2-alpha phosphorylation (19). It can also eliminate the influenza virus by inhibiting the maturation of hemagglutinin protein, stimulating the production of interferon type 1 by host fibroblasts (20) and degrading the hemagglutinin protein in the influenza virus (9). As mentioned before, there is also a similar mechanism in rotavirus where the inhibition of VP7 protein maturation occurs (20). For the noticeable effect of nitazoxanide on parasites, many researchers have found that the drug can heal the people against the parasitic infections and the infections caused by anaerobic bacteria, such as *Clostridium* species, by inhibiting the PFOR and also the enzyme that is essential for the anaerobic energy metabolism (21). The mechanism of nitazoxanide action on *Leishmania* seems to be similar.

Since infectivity is one of the most important pathogenic and biological criteria of *Leishmania* parasites, the effects of NTZ alone and in combination with MA on the infectivity of promastigotes of *L. tropica* to murine macrophages were evaluated (12). The results showed that generally more than 70% of the macrophages were infected by promastigotes of *L. tropica*. While, promastigotes of *L. tropica* treated with MA or NTZ alone and MA+NTZ were able to infect 29%, 52% and 18.5% of macrophages, respectively; indicating that the infectivity of promastigotes of *L. tropica* significantly *($P<0.05$)* reduced with NTZ pre-incubation.

Considering the toxicity of NTZ, previous studies showed that nitazoxanide does not cause significant side effects. However, the gastrointestinal symptoms such as abdominal pain, diarrhea, nausea, or headache may occur if it is used in excess of the required dose. Of course, these symptoms are usually not severe and are transient. It has been reported that using up to 4 g of this drug has no side effects, and by taking more than this amount, the side effects will increase as well (21,23).

CONCLUSION

The obtained findings revealed that the high potency and a synergistic effect of NTZ alone and combined with MA in inhibiting growth of promastigote and amastigote stages of *L. tropica*. However, further clinical studies are mandatory to examine this synergistic effect on a therapeutic scale.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not Applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not Applicable.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest to disclose.

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