The in vitro Effects of Azithromycin and Clarithromycin on Promastigotes and Amastigotes of Leishmania tropica

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Abstract

Leishmania (L.) tropica is one of the most common species responsible for cutaneous leishmaniasis (CL) in the Old World including Turkey. The pentavalent antimonials are widely used as intralesional and/or intramuscular in the treatment of CL, but increase in resistance to these agents led to investigations on alternative drugs. In vitro antileishmanial activities of two macrolides, azithromycin and clarithromycin were evaluated on promastigotes in RPMI 1640 medium and amastigotes in macrophage series of L. tropica. ED_{50} values of azithromycin and clarithromycin were found to be 5 µg/ml and <5 µg/ml on promastigotes, and 50-75 µg/ml and <3 µg/ml on amastigotes, respectively, while ED_{90} values of the same drugs were 75 µg/ml and 25 µg/ml on promastigotes and 100 µg/ml and 10 µg/ml on amastigotes, respectively. Our data suggested that clarithromycin and azithromycin were effective on both L. tropica promastigotes and amastigotes in vitro. Clarithromycin was found to be more effective than azithromycin at lower concentrations on promastigotes and amastigotes. In vivo studies should be planned to detect intracellular concentrations of these drugs for the effective route and dosage.

Keywords: Azithromycin, Clarithromycin, Leishmania tropica, treatment, in vitro

INTRODUCTION

Leishmaniases are vector-borne diseases caused by the genus Leishmania. Several clinical syndromes are represented under the term leishmaniasis. Visceral, cutaneous, and mucosal leishmaniases result from replication of the Leishmania amastigotes in macrophages, mononuclear phagocyte systems of dermis, and naso
in Afghanistan 3.

Cutaneous leishmaniasis (CL) was reported to be epidemic
within different phagocytic cells, especially macrophages.

We aimed to evaluate the in vitro effects of azithromycin and
clarithromycin on the promastigotes in RPMI 1640 medium
containing 106 promastigotes in 100 ml of RPMI was added
to remove free parasites, and were put into new Petri
plates. Fresh RPMI, with or without antibiotics, at different
concentrations (azithromycin 40-200 μg/ml; clarithromycin
7,8 and Cryptosporidium parvum 5,6, 7,8, and Plasmodium
species 9; azithromycin was also reported to have an anti-leishmanial
activity 10 because it may reach high concentration levels
within different phagocytic cells, especially macrophages.

We aimed to evaluate the in vitro effects of azithromycin and
clarithromycin on the growth and viability of L. tropica
promastigotes in a cell-free medium and amastigotes in
murine macrophages.

The two macrolides, azithromycin and clarithromycin,
were found to be effective on intracellular parasites,
such as Pneumocystis carinii and Toxoplasma gondii 6,5.
Cryptosporidium parvum 7,8 and Plasmodium species 6,7.
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MATERIAL and METHODS

Promastigotes

The strain of L. tropica (MON-53), used in the study,
was isolated from a patient with cutaneous leishmaniasis
in Şanlıurfa, a city located in the southeast of Turkey, and
identified by isoenzyme analysis in Montpellier, France.
Promastigotes were grown in RPMI 1640 medium
(Biological Industries Cat No: 01-106-1A), after adding
20% fetal calf serum (Biological Industries Cat No: 01-121-1B) and 2% antibiotic solution (Sigma P-3539).
Promastigotes were then washed twice in phosphate buffered
saline (PBS; pH: 7.0) solution, centrifuged 10 min at 2,000
rpm and adjusted as 109/ml promastigotes.

Antibiotics and Controls

Azithromycin base was obtained from Pfizer® and
first dissolved in 1 ml of acetonitrile solution, then 9 ml
of sterilized PBS solution was added to get 1 mg/ml of
azithromycin stock solution (AZTSS). Totally, 14 different
concentrations of azithromycin, between 5 and 250 μg/
ml, were prepared with AZTSS. Two different controls
containing only acetonitrile solution and only culture
medium were also prepared.

Lyophilized clarithromycin was received as 500 mg/
vial for intravenous administration (Klacid®, Abbott®) and
reconstructed in distilled water and diluted with PBS
solution to obtain 1 mg/ml of clarithromycin stock solution
(CSS). Totally, 10 different concentrations of clarithromycin
between 5 and 750 μg/ml, were prepared with CSS. Only
culture medium was used as control.

A total of 24 different antibiotic concentrations were
put in the wells of a plate and 50 μl of RPMI 1640, contain-
ing 106 promastigotes, were added to each well. The plate
was kept in an incubator at 25ºC and number of pro-
 mastigotes in each well was counted every day until the
end of 7th day. The procedures were performed twice at
different times and their results were compared.

Culture solutions (RPMI 1640) alone and with aceto-
nitril (100 μl acetonitril + 850 μl RPMI 1640) were used as
culture medium.

Infection of Macrophages with Promastigotes
and Antibiotic Treatment of Amastigotes

Mouse macrophages, J774G8, were kindly provided
from Prof. K.-P. Chang, Department of Microbiology, Finch
University, Chicago, and cultivated in RPMI 1640 medium
in flasks. Macrophages were washed and concentrated
by centrifugation at 1,300 rpm, for 15 min. The number of
macrophages was 107 and were put on a glass
coverslip, placed in a 35 mm sterile plastic Petri dish
and incubated at 37ºC/5% CO 2 overnight. The medium
was aspirated, and the coverslips were washed inside the
Petri dish with 2 ml of RPMI 1640, containing
10% inactivated fetal calf serum (FCS) and antibiotic
solution, and taken into new Petri dishes. The suspension
containing 106 promastigotes in 100 ml of RPMI was added
to the coverslips in each Petri dish. The promastigote/
macrophage ratio was adjusted as 10:1. The Petri dishes
were incubated again for 24 h. Then, the medium was
aspirated; the coverslips were washed in 2 ml of PBS
to remove free parasites, and were put into new Petri
dishes. Fresh RPMI, with or without antibiotics, at different
concentrations (azithromycin 40-200 μg/ml; clarithromycin
3-50 μg/ml) were added onto the coverslips. Petri dishes
were reincubated for 24 h. The coverslips were stained
with Giemsa and examined under x1000 magnification.
The ratio of infected macrophages was calculated as
previously reported 11.

In vitro anti-leishmanial activities of azithromycin and
clarithromycin on the promastigotes in RPMI 1640 medium
and amastigotes in macrophages were assessed in different
concentrations and their ED50 and ED90 values were
determined.

Statistical Analysis

The data were evaluated by SPSS v15.0 for Windows
6.1®. Differences between the averages of quantitative
variables were evaluated by Student’s t test. \( p<0.05 \) was accepted as statistically significant.

**RESULTS**

*Effects of Antibiotics on L. tropica Promastigotes*

Azithromycin: On the 7th day, all promastigotes were lysed in the plate, containing between 100 and 250 μg/ml, and lysis ratio varied between 55% and 96% in lower concentrations (Fig 1).

Clarithromycin: Lysis was observed at all concentrations over 75 μg/ml, 50 μg/ml and 25 μg/ml on the first, second and fourth days, respectively (Fig. 2).

*Effects of Antibiotics on L. tropica Amastigotes*

The amastigote/100 macrophages ratios, \( E_{50} \) and \( E_{90} \) values and viability ratios of both azithromycin and clarithromycin were demonstrated in Table 1. As demonstrated in Fig. 3 and Fig. 4, clarithromycin was found to be more effective on amastigotes than azithromycin at lower concentrations.

**DISCUSSION**

Pentavalent antimonials still represents the first-line of treatment of leishmaniases and other effective agents are amphotericine B, pentamidine and paromomycin. Pentavalent antimonials have some disadvantages such as serious side effects, high cost, non-oral formulation and long-term hospitalization. They were found to be ineffective in some immunocompromised patients with VL, either with AIDS or receiving immunosuppressive therapy and resistance against these drugs causes significant clinical problems and increases the cost of the treatment.

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Fig 1. *In vitro* activity of azithromycin on promastigotes. (C: Control, AN-C: Acetonitril control)

Şekil 1. Azitromisinin promastigotlar üzerindeki *in vitro* aktivitesi (C: Kontrol, AN-C: Asetonitril kontrol)

Fig 2. *In vitro* activity of clarithromycin on promastigotes. (C: Control)

Şekil 2. Klaritromisinin promastigotlar üzerindeki *in vitro* aktivitesi (C: Kontrol, AN-C: Asetonitril kontrol)
The in vitro Effects of ... Clarithromycin and azithromycin act through the inhibition of protein synthesis and could be concentrated and carried in tissue macrophages. They have important advantages, including long half-life, oral and injectable administration, relatively safe usage in children and pregnant women and benign toxicity profile. Both drugs were approved by FDA for respiratory tract and skin infections, but they may also be used in mycobacterial infections and toxoplasmosis with HIV/AIDS. Clarithromycin was shown to be effective on Cryptosporidium spp., Pneumocystis carinii and Toxoplasma gondii. Azithromycin is effective for intra-cellular parasites, such as T. gondii, C. parvum and Plasmodium spp.
In vivo and in vitro trials have been performed not only by commonly used anti-leishmanial agents but also by other intracellular active compounds and antibiotics for their activities on Leishmania spp. Some of these trials revealed promising results as in miltefosine studies 17-25.

It was reported that ciprofloxacin inhibited the reproduction of L. major promastigotes, while amphotericin B inhibited the L. donovani promastigotes in vitro 18,26. Amphotericin B was found to be more effective than pentamidine in inhibiting promastigote reproduction 26.

There is a raising demand for studies using intra-macrophage Leishmania amastigotes, for their easy usage in the interpretations of in vivo effects of new agents on Leishmania spp. The ED₅₀ value of megalomycin, a new macrolide, against L. donovani and L. major promastigotes were reported as 3 and 8 μg/ml, respectively 27.

Azithromycin concentrates in tissues, especially in macrophages that are infected by Leishmania parasites, and can reach concentrations 100 to 200 times higher than in serum 10. Azithromycin was reported to have no inhibitory effect on the phagocytic capacity of mouse peritoneal macrophages and a significant (P<0.05) increase in leishmaniacidal activity was detected at the concentrations of 0.1, 0.3 and 0.6 mg/ml. Azithromycin did not provide any contribution to the phagocytosis of L. major promastigotes in macrophages in vitro, but it increased the intracellular killing rates of amastigotes. It was reported that azithromycin had a potential anti-leishmanial effect, and could provide a significant advantage in the treatment of the disease 29. Therefore, our findings were compared with the results of other studies, concerning agents acting similarly. It was reported that the ED₅₀ value of azithromycin on T. gondii was 140 μM and azithromycin had toxic effects on macrophages 29. It was also reported that, azithromycin could inhibit the protein synthesis in both intracellular and free trophozoites of T. gondii 30.

In the present study, the onset of lysis of promastigotes was observed 24 h after the addition of azithromycin into medium, and became more intense with increasing drug concentrations. ED₅₀ and ED₉₀ values were found as 5 μg/ml and 75 μg/ml for azithromycin, and <5 μg/ml and 25 μg/ml for clarithromycin respectively on promastigotes. Lysis of promastigotes was started 24 h after the addition of azithromycin and clarithromycin, and intensified with the increase in drug concentrations. ED₅₀ and ED₉₀ values were found as 50-75 μg/ml and 100 μg/ml with azithromycin, and <3 μg/ml and 10 μg/ml with clarithromycin, respectively on amastigotes. Similar to our results of azithromycin trial, the potential antileishmanial activity of azithromycin against three New World Leishmania species (L. amazonensis, L. braziliensis, L. chagasi) was previously assessed using in vitro models and it is reported that azithromycin decreased viability of promastigote cultures as well as in amastigote intracellular cultures, a significant decrease in infected macrophages counts was observed for all three species with IC₅₀ of 20.83, 2.18 and 6.12 mg/mL, respectively 31.

Besides in vitro studies, the clinical studies were also carried out using azithromycin. Two small series 32,33 of L. braziliensis infected patients have been described: azithromycin (500-1,000 mg/d, for 2 to 10 d/mo, and a maximum of 4 months of treatment) cured 85% of the patients.

Two other clinical studies using azithromycin in the treatment of patients with old world CL (L. major and L. tropica) have also been published 34,35 and both of them reported that azithromycin is not effective for the treatment of old world CL. In an Iranian study, 17 out of 21 CL patients were reported to be treated using azithromycin. The drug was administrated orally 500 mg/day for 3 weeks. At the end of therapy only 2 (11.8%) patients showed complete cure, while 4 (23.6%) patients showed partial cure and 11 (64.7%) patients did not respond to the treatment 34. On the other hand, in a case report from France, successfull treatment of a 10-year-old boy with CL (L. major) was reported with oral azithromycin 38. We notified that in the clinical studies using systemic and topical treatments for CL, more efforts are needed for understanding the efficacy of macrolid antibiotics.

Here, we report that, azithromycin and clarithromycin are effective agents on both amastigotes and promastigotes of L. tropica in vitro. Clarithromycin was more effective than azithromycin on both forms of Leishmania parasites at lower concentrations. According to our data, we suggest that further studies are required to reveal the efficacy of these agents for the chemotherapy of patients with leishmaniasis and/or chemoprophylaxis among people traveling endemic areas.

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