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RESEARCH ARTICLE

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

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Summary

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*

Leishmania tropica Promastigotları ve Amastigotları Üzerine Azitromisin ve Klaritromisinin *in vitro* Etkisi

Özet

Leishmania (L.) tropica, Türkiye'de dahil olmak üzere Eski Dünya'da kutanöz leishmaniasisden (KL) sorumlu en önemli türdür. KL tedavisinde intralezyoner ve intramuskuler yoldan beş değerlikli antimon bileşikleri yaygın olarak kullanılmaktadır, fakat bu ajanlara karşı artan direnç alternatif ilaçların geliştirilmesini gerekli kılmaktadır. Bu çalışmada, makrolid grubundan iki antibiyotik olan azitromisin ve klaritromisinin *L. topica*'nın RPMI 1640 besiyerindeki promastigotlar ve makrofaj serisindeki amastigotlar üzerine olan antileishmanial aktiviteleri değerlendirilmiştir. Azitromisin ve klaritromisinin promastigotlar üzerindeki ED_{50} değerleri sırasıyla 5 µg/ml ve <5 µg/ml olarak, amastigotlar üzerindeki ED_{50} değerleri sırasıyla 50-75 µg/ml ve <3 µg/ml olarak bulunurken ED_{90} değerleri promastigotlar üzerinde 75 µg/ml ve 25 µg/ml olarak, amastigotlar üzerinde ise 100 µg/ml ve 10 µg/ml olarak bulunmuştur. Bu çalışmada, azitromisin ve klaritromisinin *in vitro* olarak *L. tropica* promastigot ve amastigotları üzerine etkili olduğu gösterilmiştir. Klaritromisin her iki parazit formunda da daha düşük dozlarda azitromisinden daha etkili olduğu belirlenmiştir. Bu ilaçların kullanım şekli ve dozajlarının belirlenmesi için hücre içi konsantrasyonlarının saptanması amacıyla *in vivo* çalışmaların planlanması gerektiği kanısına varılmıştır.

Anahtar sözcükler: *Azithromycin, Clarithromycin, Leishmania tropica, tedavi, 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-

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oropharyngeal mucosa, respectively [1].

Recently, there has been an increase in the number of cases with visceral leishmaniasis correlated with the increases in number of immunocompromised patients, traveling to and immigration from endemic regions, and resistance against pentavalent antimonial compounds. About 100.000 deaths due to visceral leishmaniasis (VL) were estimated among 280.000 people in the epidemic area of southern Sudan between 1984 and 1994 [2], and cutaneous leishmaniasis (CL) was reported to be epidemic in Afghanistan [3].

Pentavalent antimony compounds are still the first drugs of choice in the treatment of leishmaniasis; but the recent increase in the number of resistant cases, especially in visceral leishmaniasis, and failures of treatment in immunocompromised cases, directed the researchers to alternative drug investigations [4].

The two macrolides, azithromycin and clarithromycin, were found to be effective on intracellular parasites, such as *Pneumocystis carinii* and *Toxoplasma gondii* [5,6], *Cryptosporidium parvum* [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.

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 cultivated 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 10^6/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, containing 10^6 promastigotes, were added to each well. The plate was kept in an incubator at 25°C and number of promastigotes 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 acetonitril (100 µl acetonitril + 850 µl RPMI 1640) were used as controls.

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 10^5 and were put on a glass coverslip, placed in a 35 mm sterile plastic Petri dish and incubated at 37°C/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 10^6 promastigotes in 100 ml of RPMI was added on 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 ED_{50} and ED_{90} 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, ED_{50} and ED_{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 paromomycine. Pentavalent antimonials have some disadvantages such as serious side effects, high cost, non-oral formulation and long-term hospitalization [12,13]. 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 [14].

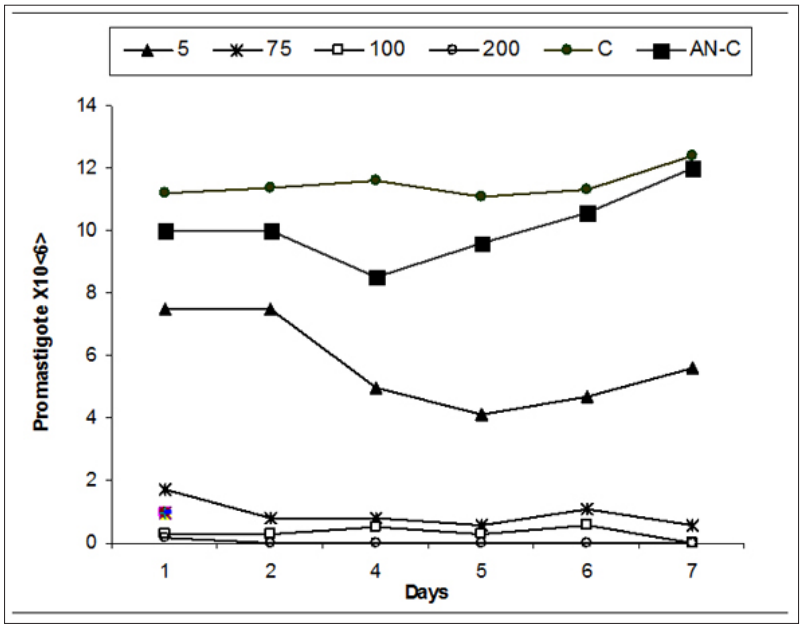

Fig 1. *In vitro* activity of azithromycin on promastigotes. (C: Control, AN-C: Acetonitril control)

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

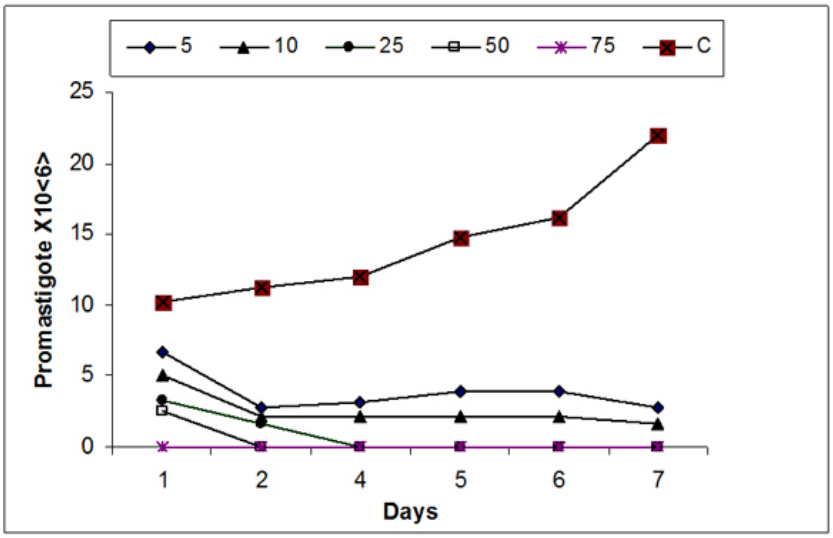

Fig 2. *In vitro* activity of clarithromycin on promastigotes. (C: Control)

Şekil 2. Klaritromisinin promastigotlar üzerindeki *in vitro* aktivitesi (C: Kontrol, AN-C: Asetonitril kontrol)

Table 1. *Comparison of the in vitro activities of azithromycin and clarithromycin*
Tablo 1. *Azitromisin ve Klaritromisinin in vitro aktivitelerinin karşılaştırılması*

Parasite Stage	Effective Dose	Azithromycin	Clarithromycin
Promastigotes	ED_{50}	5 mg	<5 mg
	ED_{90}	75 mg	25 mg
Amastigotes	ED_{50}	50-75 mg	3 mg
	ED_{90}	100 mg	10 mg
Amastigote/100 macrophage (min-max)		5-117	2-100
Viability ratio (%) (min-max)		3-79	1-33
Drug Conc. (mg/ml)		200-40	50-3

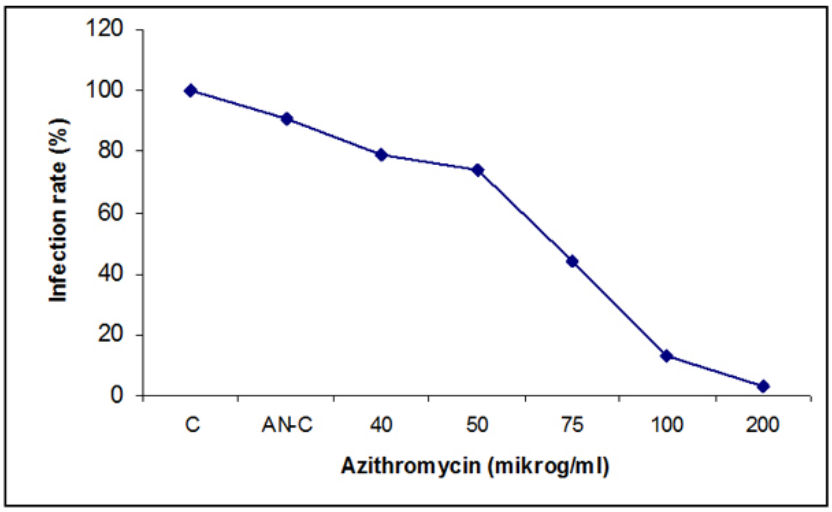

Fig 3. *In vitro* activity of azithromycin on amastigotes. (C: Control; AN-C: Acetonitril control)

Şekil 3. Azitromisinin amastigotlar üzerindeki *in vitro* aktivitesi (C: Kontrol, AN-C: Asetonitril kontrol)

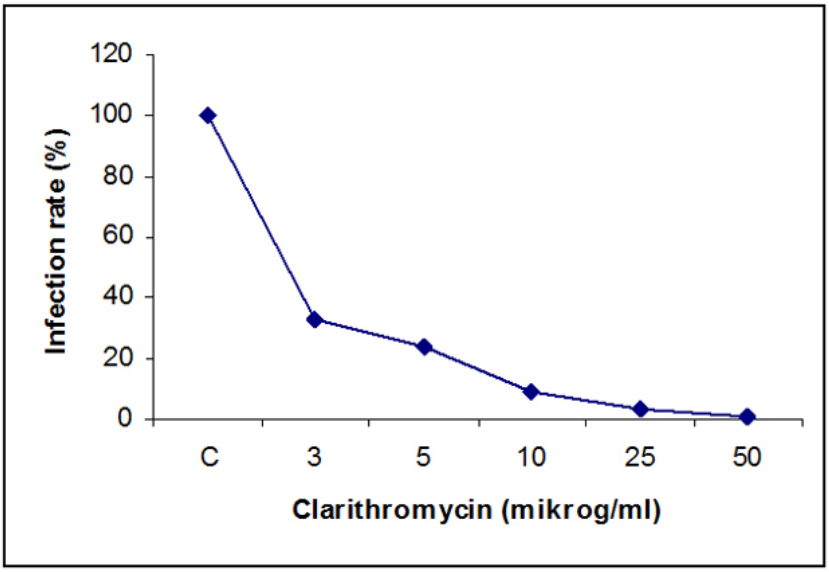

Fig 4. *In vitro* activity of clarithromycin on amastigotes. (C: Control)

Şekil 4. Klaritmosinin amastigotlar üzerindeki *in vitro* aktivitesi (C: Kontrol, AN-C: Asetonitril kontrol)

Clarithromycin and azithromycin act through the inhibition of protein synthesis and could be concentrated and carried in tissue macrophages [15]. They have important advantages, including long half-life, oral and injectable administration, relatively safe usage in children and pregnant women and benign toxicity profile [10]. 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 [16]. Clarithromycin was shown to be effective on *Cryptosporidium* spp.[7], *Pneumocystis carinii* and *Toxoplasma gondii* [6]. Azithromycin is effective for intracellular parasites, such as *T. gondii* [5], *C. parvum* [8] and *Plasmodium* spp.[9].

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 ED_{50} 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 [28]. Therefore, our findings were compared with the results of other studies, concerning agents acting similarly. It was reported that the ED_{50} 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. ED_{50} and ED_{90} 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. ED_{50} and ED_{90} 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 azithromycine. 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 administered 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 [36]. 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 leishmaniases and/or chemoprophylaxis among people traveling endemic areas.

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