In vitro susceptibility of *Leishmania donovani* promastigotes to some antimicrobials compared with meglumine antimoniate

-May A. Omran (M. Sc.) Dept. of Microbiology, college of medicine, Kufa University, Iraq.

-Ali M. Jasim (M. Sc.) Dept. of Microbiology, college of medicine, Kufa University, Iraq.

-Sundus N. Jasim (M. Sc.) Dept. of Microbiology, college of medicine, Kufa University, Iraq.

-Saif J. Yasir (M. Sc.) Dept. of Microbiology, college of medicine, Kufa University, Iraq.

الخلاصة

أجريت هذه الدراسة التجريبية لتقييم فاعلية بعض المضادات الجرثومية ضد الطور الأمامي السوط لطفيلي اللشمانيا الحشوية خارج الجسم الحي . وقد تم اجراؤها في كلية الطب بجامعة الكوفة خلال شهري تشرين الاول وتشرين الثاني عام ٢٠٠٨. تضمنت الدراسة تنمية الطفيلي المذكور في الوسط الزرعي الخاص به والمسمى (RPMI) مدعما بمصل جنين البقر بنسبة (١٠) بالمئة ولمدة (١٤) يوما مع الازثرومايسين بتراكيز (٦و١٢ و١٨) مايكروغرام/مل و مع مزيج الترايميثوبريم-سلفاميثوكسازول بتركيز (٠,٥-٩,٥) مايكروغرام/مل ومع نورفلوكساسين بتركيز (١) مايكروغرام/مل ومع مجلومين انتيمونيات بتركيز (٢٠) مايكروغرام/مل. بينما لم تعامل مجموعة السيطرة بأي مضاد جرثومي.

قسمت النماذج الى مجموعات وكل مجموعة تتمثل بعشرين أنموذجا بحيث عوملت كل منها وتحت ظروف قياسية بعقار واحد (عدا مجموعة السيطرة) بتركيز معين بحيث يكون ضمن التركيز التثبيطي الأدنى لذلك العقار. وقد تمت عملية حساب عدد سوطيات اللشمانيا الحشوية أثناء الطور اللوغاريتمي من منحنى النمو وتحديدا في اليوم الثامن من بداية الزرع،وقد قسمت الطفيليات حسب حيويتها الى متحركة و خاملة و ساكنة اثناء عملية الحساب، وقورنت نتائج المجاميع المعاملة بالمضادات الجرثومية مع مجموعة السيطرة.

بينت النتائج ان معدل عدد سوطيات اللشمانيا الحشوية المتحركة في كل مليلتر في مجموعة السيطرة قد بلغ (129.2×10^{4}) وفي مجموعات الازثرومايسين قد بلغ (20.4×10^{4}، 9.2×10^{4}, 5.6×10^{4}) بينما كان في مجموعة الترايميثوبريم-سلفاميثوكسازول (10.64×10^{4}) اما في مجموعة النورفلوكساسين فكان (1.6×10^{4}) وفي مجموعة مجلومين انتيمونيات بلغ(4×10^{4}).لقد كان هناك انخفاضا معنويا في معدل عدد سوطيات اللشمانيا الحشوية المتحركة في مجموعات الأدوية مقارنة بمجموعة السيطرة، الا انه غير معنوي اذا ما قورن مع مجموعة مجلومين انتيمونيات. وطبقا لهذه النتائج، يبدو من الواضح ان هذه المضادات الجرثومية فعالة بما فيه الكفاية لتجربة أستعمالها لعلاج الفئران المصابة لضمان استعمالها في المستقبل كمضادات للشمانيا داخل جسم الحي.

ABSTRACT :

This experimental study was designed to evaluate the effectivness of some antimicrobial agents against *Leishmania donovani* promastigotes (LDP) in vitro. It was performed in College of Medicine in Kufa University during october and november 2008. It included treatment of LDP, cultured in Roswell Park Memorial Institute (RPMI) medium enriched with 10% fetal calf serum (FCS) for 14 days, with azithromycin (6, 12, and 18 µg /ml), trimethoprim-sulfamethoxasol (0.5-9.5 µg/ml), norfloxacin (1 µg /ml) and meglomin antimoniate (20 µg/ml).The control group contained no antimicrobial with the LDP.

Samples were grouped so that each group (20 samples) treated under standardized conditions with one drug (except the control group) in certain concentration that is within its minimal inhibitory concentration (MIC). Promastigotes were counted during the log phase of the growth curve (at day 8). They were subdivided into motile, sluggish and immotile during the counting process and data from the drug-treated groups were compared with that of the control group.

The results showed that mean count of motile LDP at day 8 of cultivation in the control group was (129.2×10^4)LDP/ml, while mean counts of motile LDP in the treated groups were as follows: (20.4×10^4, 9.2×10^4, 5.6×10^4) LDP/ml for azithromycin group, (10.64×10^4) LDP/ml for trimethoprim-sulfamethoxasol group, (1.6×10^4) LDP/ml for norfloxacin group and (4×10^4) LDP/ml for meglomin antimoniate group. There was a significant reduction in motile LDP count in all treated groups compared to the control group ($p < 0.05$), though it is insignificant in comparison to meglumine group ($p > 0.05$). According to these data, it is clear that these antimicrobials may be effective enough as an experimental trial on infected mice then to be used in vivo as antileishmanial agents in the future.

INTRODUCTION

Parasites of the genus *Leishmania* are transmitted by sand flies that ingest the parasite in the amastigote stage resident within macrophages of human, then inoculate the promastigote stage into other hosts .[1]

Therapies are needed to supplement or replace currently available therapies. Proven therapies against human leishmaniasis include pentavalent antimonials and meglumine antimoniate. [2]. It is necessary to seak for other drugs because many studies revealed increasing resistance to meglumine antimoniate among *Leishmania* strains. Furthermore, repeated antimonial treatments of animal reservoirs with signs of leishmaniasis was dangerous, as these may produce a permanent reservoir of parasites unsusceptible to the drugs that used for clinical uses. [3]

It was showed that there was a highly variable *in vitro* response of *Leishmania* parasites to pentavalent antimony, from patients with treatment failure, higher than that of strains from patients in whom treatment is successful. This confirms previous observations that the susceptibility of *Leishmania* strains to pentavalent antimony decreases in patients undergoing treatment with meglumine antimoniat[4]. The range of MIC for pentavalent antimony against *Leishmania* parasites is from 15-30µg/ml.[5]

Azithromycin, an azalide antibiotic from a family of macrolides substances, has the potential for effective anti-leishmanial activity. The range of MIC of azithromycin was from 2 µg /ml to more than 30 µg /ml [5]. One of its key characteristics is that the drug concentrates in tissues, especially in macrophages, and may reach levels 100 to 200 times higher than those in serum [6]. Other advantages include (1)the possibility of oral and injectable administration,(2)good oral bioavailability and long half-life, [7–9] (3)relative safety for use in children and pregnant women , and (4) a benign toxicity profile. [10]

Several different protozoan infections of humans have been shown *in vitro* and *in vivo* to be susceptible to azithromycin in varying degrees, The site of azithromycin concentration in macrophages coincides with the intracellular location of *Leishmania*, suggesting a potential efficacy against these infections. [11,12]

Nofrloxacin, a synthetic quinolone derivative, was shown to be a potent antibacterial fluoroquinolone with marked activity against *Leishmania donovani* in vivo. [13]

The range of MIC of norfloxacin was from 0.064 µg /ml to more than 32 µg /ml [14]. Trimethoprim/sulfamethoxazole is another synthetic agent with reliable efficacy against aerobic bacteria and *Pneumocystis carinii* . The known MIC of trimethoprim-sulfamethoxasol against *E. coli* was 0.5-9.5 µg /ml. [5]

MATERIALS AND METHODS

Antimicrobials:

Azithromycin (Zithromax; Pfizer, New York, NY) was obtained in lyophilized form as 500 mg/vial for intravenous administration. The vial content was reconstituted with sterile water and diluted to the appropriate concentration in medium (Roswell Park Memorial Institute with supplements) to be used for *in vitro* treatments. The target final concentrations of azithromycin were 6, 12 and 18 µg / ml in three separated groups of samples.

Norfloxacin (Neofloxin, Julphar, UAE) was obtained as 500-mg powder dissolved in distilled water, sterilized with millipore filteration and diluted to appropriate concentration (1 µg/ml).

Trimethoprim/sulfamethoxazole (Furaprim, Furat, Iraq) was obtained as 480-mg powder containing 80 mg trimethoprim and 400 mg sulfamethoxazole powder was dissolved in distilled water, sterilized with millipore filteration and diluted to appropriate concentration (0.5-9.5 µg/ml).

Meglomin antimoniate (Glucantim, Leo, France) was obtained as 2-ml vials containing 20 mg/ml antimony as active ingredient diluted down to 20 µg/ml as a final concentration in the culture medium.

Parasites:

Leishmania donovani promastigotes were isolated in biphasic medium (Roswell Park Memorial Institute with supplements) from infected mice and then cultivated with or without an antimicrobial for 14 days at 6°C in (RPMI) medium supplemented with 10% fetal calf serum (FCS), 2 mM of glutamine, 100 U/ml of penicillin, and 100 µg/ml of streptomycin.[1]

Assays on promastigotes were performed briefly, as promastigotes were cultured in RPMI/10% FCS medium . Serial dilutions of the drug in promastigote culture medium were performed in sterile tubes each contained 5 ml of the medium. Promastigote count in the original culture (biphalic medium) was 3200 $\times 10^4$ LDP/ml .From which 0.1 ml was taken and put in each tube with 5 ml of RPMI / 10% FCS medium and incubated at 26°C for 14 days with or without the antimicrobial agent to perform the test in each group.

Growth was measured through serial promastigotes counting 24 hourly and counting on day 8 was estimated to represent main study data and subjected for further statistical analysis. All assays were performed in duplicate at least twice.

Counting of Leishmania donovani promastigotes:

On day 8 of LDP cultivation, a wet film preparation was done after putting the growth tube in magnetic stirrer for 15 seconds for even cell distribution, and counting procedure was followed using ordinary slide chamber method .[1,2]

Results :

Data were constituted depending on LDP counts in the control and drug-treated groups of samples at day 8 of incubation. Mean counts ± standard errors were calculated for the number of motile, sluggish and immotile LDP per a milliliter for each group. Chi square test of more than two means was employed to predict the significant difference (if any) among the mean motile LDP counts of the control and the drug-treated groups, and among the meglumin- and other drug-treated groups (Table: 1).

Results showed a significant difference among mean LDP counts of the drug treated groups and that of the control ($p < 0.05$). While it was insignificant when comparing mean LDP counts of the meglumin-treated group with that of other drug-treated groups ($p > 0.05$), however, azythromycine at 18 µg / ml recorded the best LDP inhibitory effect and was nearest to that of meglumine antimoniate .

Table (1): Mean LDP counts in control and drug –treated culture groups of *L. donovani* in RPMI / 10% FCS (each group contains 20 samples) at day 8 of incubation.

Antimicrobial	Conc. in culture medium(µg/ml)	Mean LDP count ($\times 10^4$ /ml)± SE		
		motile	sluggish	immotile
Non (control)	-------	129.2±5.94	98±2.95	29.2±3.97
Azithromycin	6	20.4±2.88	28.8±4.26	27.6±3.38
Azithromycin	12	9.2±1.94	56.8±4.23	38.8±4.32
Azithromycin	18	5.6±1.07	27.6±5.19	49.6±5.58
Norfloxacin	1	1.6±0.51	30.4±3.43	31.2±4.41
Trimethoprim/ sulphamethoxazol	0.5/9.5	10.64±1.34	24±2.5	52±3.77
meglomin antimoniate	20	4±0.81	42±3.1	78.4±4.74

Key:

LDP: *Leishmania donovani* promastigotes.; **RPMI/ 10% FCS**: Rosewell park memorial institute medium enriched with 10% fetal calf serum.; **SE:** Standard error.

DISCUSSION

Orally administrable drugs have sometimes been used to treat human leishmaniases without prior demonstration of efficacy in experimental models Because needing for active anti-leishmanial agents. The anti-leishmanial activity of such agents was tested against promastigotes of *Leishmania donovani* (a cause of visceral leishmaniasis) in vitro.

To test the susceptibility of *Leishmania* to different drugs, promastigotes were cultured in cell-free medium in the presence of variable concentrations of antimicrobials. Parasite levels were measured by microscopic observation of the living promastigotes in cell-free culture. [15,19]

The current study has shown that azithromycin, trimethoprim-sulfamethoxazole and norfloxacin have a significant *in vitro* activity against the promastigotes of *L. donovani* as far as meglumine antimoniate. There was a statistically significant reduction in the survival of promastigotes in cell-free culture, RPMI. Many in vivo and in vitro experimental trials suggested similar antileishmanial activity for such antimicrobials.Although unresponsiveness to antimonial drugs in human leishmaniasis appears to be increasing and it is mandatory to search for another agent, resistance to antimony in *Leishmania spp.* is not well documented. [3]

Trimethoprim plus sulfamethoxazole and isoniazid plus rifampin have been reported as efficacious orally in certain human studies of cutaneous leishmaniasis , however, these drugs were ineffective in vitro (<40% parasite elimination) at peak achievable serum levels. Both the negative and the positive data of this report may aid in selection of effective orally active agents for in vivo trials. [20]

On the other hand, it was found that the antileishmanial activity of norfloxacine was studied in a relatively limited extent, but it its marked in vitro activity brings it as a reliable agent to be tried instead of other therapies in case of drug resistance or as an oral drug dosage form.[13]

The effect of azithromycin against promastigotes was dose dependent, with an ED50 of 12 µg/ml. Because much larger amounts of drug were needed to control the promastigotes in cell-free culture, the more pronounced effect in macrophage culture may be due to the ability of the macrophage to concentrate the drug actively inside the cells. Treatment of susceptible BALB/cByJ mice reduced the lesion size and parasite numbers in the lesions; however, no effect was seen in the normally resistant C57/BL6J mice. [15,16]

Azithromycin has been shown to have immunomodulatory activity by preventing the production of pro-inflammatory mediators and cytokines.[21] In addition, effects on macrophage functions include stimulation of phagocytosis, chemotaxis, and cytocidal activity.[22]

On this basis, it has been suggested that the ability of azithromycin to accumulate in the tissues and especially in phagocytes can act synergistically with the host immune system. The diversity of human responses to infection with *Leishmania* is one of the integral characteristics of this disease; it is possible that the portion of the population that requires drug intervention because of an inadequate immune response may benefit from treatment with azithromycin. [23]

In conclusion, it has been shown that azithromycin, T-S and norfloxacin have a reliable activity against LDP *in vitro*. Given the severity of the disease and the limitations of the available therapeutic agents, these agents may have a significant role in the treatment of visceral leishmaniasis.

Further prospective studies with larger cohorts of patients are required to establish the clinical significance and the impact of treatment and prophylaxis on the development of *Leishmania* strains resistant to antimonials and possible use of orally effective antimicrobials for treatment of leishmaniasis. The results of such studies may reveal the need to re-examine current therapy policies in *Leishmania*–infected patients and/or the use of alternative drugs during relapses or development of drug resistance in such patients.

REFERENCES

1.Berman, J.D.,1996: Treatment of New World cutaneous and mucosal leishmaniases. *Clin Dermatol* **14***:*519 –522.

2.Berman, J.D.,1997: Human leishmaniasis: Clinical, diagnostic, and chemotherapeutic developments in the last 10 years. *Clin Infect Dis* **24***:* 684 – 703.

3.Gramiccia, M. ; Gradoni, L. and Orsini, S. 1992: Decreased sensitivity to meglumine antimoniate (Glucantime) of Leishmania infantum isolated from dogs after several courses of drug treatment. Ann Trop Med Parasitol. **86**(**6**): 20-613.

4.Faraut-Gambarelli, F.; Piarroux, R.; Deniau, M.; Giusiano, B.; Marty, P.; Michel, G. *et al*. 1997. *In vitro* and *in vivo* resistance of *Leishmania infantum* to meglumine antimoniate: a study of 37 strains collected from patients with visceral leishmaniasis. *Antimicrob Agents Chemother* **41**: 30-827.

5.Darrell, R.; Gary, J.; Robert, L.; Dawn, M.; Robert, A.; Keith, R.; *et.al*., 2002: Mosby drug consult, section III: Drug information. Copyright 2002 Mosby, Inc. 20-2607.

6.Gladue, R.P.; Bright, G.M.; Isaacson, R.E.; Newborg, M.F.;1989: In vitro and in vivo uptake of azithromycin by phagocytic cells: possible mechanism of delivery and release at sites of infection. *Antimicrob Agents Chemother* **33**: 277 –820.

7.Luke, D.R.; Foulds, G.; Cohen, S.F.; Levy, B.;1996. Safety,toleration, and pharmacokinetics of intravenous azithromycin. *Antimicrob Agents Chemother* **40:** –815-2577.

8.Lalak, N.J.; and Morris, D.L. 1993. Azithromycin clinical pharmacokinetics. *Clin. Pharmacokinet* **25:** 370 –374.

9.Foulds, G.; Shepard, R.M.; Johnson, R.B.;1990: The pharmacokinetics of azithromycin in human serum and tissues. *J. Antimicrob Chemother* **25:**73 –82.

10. Steigbigel, N.H.;1995. Macrolides and clindamycin. In Mandell G.L., Bennett J.E., Dolin R., eds. *Mandell, Douglas and Bennett's Principles and Practice of Infectious Diseases.* Churchill Livingstone, NY : 334 –336.

11. Jha, T.K.; Sundar, S.; Thakur, C.P.; Bachmann, P.; Karbwang, J. ; Fischer, C.; Voss, A.; Berman, J. 1999: Miltefosine,an oral agent, for the treatment of Indian visceral leishmaniasis. *N Engl J Med* **341***:* 1795 –1800.

12.Alrajhi, A.A.; Ibrahim, E.A.; De Vol, E.B.; Khairat, M.; Faris, R.M.; Maguire, J.H. 2002: Fluconazole for the treatment of cutaneous leishmaniasis caused by *Leishmania major .N Engl J Med* **346** : 891 –895.

13. Raether, W.; Seidenath, H.; and J. Hofmann , 1989: Potent antibacterial fluoroquinolones with marked activity against *Leishmania donovani* in vivo. J. Parasit. Research, **75(5):** 412-13.

14.Sugita, K.;2002: Comparative in vitro activities of several antimicrobial agents against Helicobacter pylori. Jpn J Antibiot **55(6)**: 771-7.

15.Berman, J.D.; Lee, L.S.;1984: Activity of antileishmanial agents against amastigotes in human monocyte –derived macrophages and in mouse peritoneal macrophages. *J. Parasitol* **70:**220 –225.

16.Berman, J.D.; Wyler, D.J.;1980: An in vitro model for investigation of chemotherapeutic agents in leishmaniasis. *Infect Dis* 142*:*83 –86.

17. Berman , J.D.;Dwye,r D.M.; Wyler, D.J. 1979: Multiplicationof *Leishmania* in human macrophages in vitro. *Infect Immun* **26***:*375 –379.

18. Carrió, J.; Riera, C.; Gállego, M.;. Ribera, E and M. Portús**.** 2001: *In vitro* susceptibility of *Leishmania infantum* to meglumine antimoniate in isolates from repeated leishmaniasis episodes in HIV-coinfected patients .J. of Antimicrob. Chemother. **47**: 120-121.

19. Carrió, J.; de Colmenares, M.; Riera, C.; Gállego, M.;Arboix, M. & Portús, M. 2000: *Leishmania infantum*: stage-specific activity of pentavalent antimony related with the assay conditions. *Experimental Parasitology* **95**, 209–14.

20. Berman, J. D. and L. S. Lee,1983 :Activity of Oral Drugs against *Leishmania Tropica* in Human Macrophages in Vitro.Am. J. Trop. Med. Hyg., **32(5)**, : 947-951.

21.Ianaro, A.; Ialenti, A.; Maffia,P.,; Sautebin, L.; Rombola, L.; Carnuccio, R.;Iuvone T.; D'Acquisto, F.; Di Rosa, M.; 2000: Anti-inflammatory activity of macrolide antibiotics. *J. Pharmacol Exp Ther* **292:**156 –163.

22. Xu, G.; Fujita, J.; Negayama, K.; Yuube, K.; Hojo, S.; Yamaji ,Y.; Kawanishi, K.; Takahara, J.,1996: Effect of macrolide antibiotics on macrophage functions. *Microbiol Immunol* **40:** 473 –9.

23. McDonald, P.J.; Pruul, H.; 1992: Macrolides and the immune system; Activity of azythromycin against *Leishmania major. Scand J Infect Dis.***83(l)***:* 34–40.