An In-vitro Study to Predict the Activity of Clotrimazole against *Lishmania Donovani* Promastigotes

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Abstract

Study design and objective: this in vitro experiment was done in Kufa Medical College during March through July 2008 to predict the effect of clotrimazole on *Leishmania donovani* promastigotes (LDP) in term of growth inhibition and this effect was compared with that of meglumine antimoniate and placebo (control).
Method: *Leishmania donovani* promastigotes were isolated and cultured in Rosewell Park Memorial Institute (RPMI) medium supplemented with 10% fetal calf serum (FCS). Promastigotes were then transferred in two 96-well plates with equal cell count (1 milion LDP/ml). Clotrimazole in final concentrations 5 and 10 µg/ml was added to the two test groups (30 wells each), and meglomine antimoniate (20 µg/ml) was added in other 30-well group, and a forth group was kept as a control.
Results: After 3 days of incubation, LDP in the tests and control groups were counted to predict the growth inhibition effect of the clotrimazole (5 and 10 µg/ml) which were about 79% and 95% respectively, compared to that of meglumine antimoniate which eliminated about 89% of LDP at 20 µg/ml.
Conclusion: clotrimazole was shown to have a reliable antileishmanial activity in vitro and it is recommended to be used as systemic (oral) treatment for visceral leishmaniasis in future.

الخلاصة

نوع الدراسة و الهدف منها: أجريت هذه الدراسة التجريبية خارج جسم الحي في كلية الطب بجامعة الكوفة خلال المدة من شهر آذار الى تموز عام ٢٠٠٨. لتقييم فاعلية عقار الكلوتريمازول ضد الطور الأمامي السوط لطفيلي اللشمانيا الحشوية اعتمادا على نسبة تثبيط النمو ومقارنة هذه الفاعلية مع تأثير عقار المجلومين أنتيمونيات ومع مجموعة السيطرة.
طريقة اجراء الدراسة: تضمنت الدراسة عزل و تنمية الطفيلي المذكور في الوسط ألزرعي الخاص به والمسمى (RPMI) مدعما بمصل جنين البقر بنسبة (١٠) بالمئة. ثم تم نقل الطفيلي الى صفائح الحضن المكونة من ٩٦ اخدودا وبتراكيز متساوية وبواقع ١ مليون خلية لكل مل. تمت اضافة الكلوتريمازول بتراكيز ٥ و ١٠ مايكروغرام/مل لمجموعتين تتألف كل منها من ٣٠ اخدودا. وقد اضيف مجلومين انتيمونيات بتركيز (٢٠) مايكروغرام/مل للمجموعة الثالثة بينما لم تعامل المجموعة الرابعة (مجموعة السيطرة) بأي مضاد جرثومي.
النتائج: بعد ثلاثة ايام من الحضن تم حساب تراكيز الطور الأمامي السوط لطفيلي اللشمانيا الحشوية في كل من مجموعات الفحص ومجموعة السيطرة لتقييم نسبة تثبيط النمو للكلوتريمازول بتراكيز ٥ و ١٠ مايكروغرام/مل والتي بلغت 79% و 95% على التوالي مقارنة مع مجلومين انتيمونيات والذي ثبط حوالي ٨٩% من نمو الطفيليات المذكورة.
الاستنتاج: بينت النتائج ان الكلوتريمازول يمتلك فاعلية مضادة للشمانيا خارج الحي بقدر يمكن الاعتماد عليه ويوصى باستعماله بالفم كعلاج لمرض اللشمانيا الحشوية في المستقبل.

Introduction

Leishmania is a protozoan parasite that is responsible for several pathologies collectively known as leishmaniasis. According to the latest WHO, 12 million people are affected

by leishmaniasis worldwide and 2 million new cases occur each year [1].

The parenteral treatment currently available for visceral leishmaniasis (VL) shows non promising results and potentially exposes patients to serious side effects in addition to the development of resistant strains to the current therapy[2,3]. Thus, effective, alternative oral therapy would be valuable.

Clotrimazole is an imidazoline-derived antifungal which is well absorbed after oral administration (as lozenges). It acts against pathogen by causing leakage of intracellular phosphorus and subsequent breakdown of cellular nucleic acids [4].

Clotrimazole was found to be effective in a dose range of 15 to 60 μM (IC_{50}: 11 and 23·5 μM)[5].

In the current study, the efficacy of clotrimazole was assessed in term of promastigotes' growth inhibition *in vitro*.

Material and method

Parasite

Leishmania donovani promastigotes were isolated in biphasic medium from infected rabbit obtained from Research Unit in Al-Nahrain University and then cultivated for 10 days at 26°C in (RPMI) medium to a final parasite count of about 1 milion LDP/ml[6].

Culture medium

Rosewell Park Memorial Institute (RPMI) medium (BME, England) supplemented with 10% fetal calf serum (FCS), 2 mM of glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin was prepared to be used for LDP cultivation and sensitivity testing[6].

Antimicrobials

Clotrimazole as solution 10 mg/ml was diluted to final concentrations of 5 and 10 μg/ml in the culture medium to be used for further sensitivity test.

Meglumine 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[4].

Samples preparation

Assays on LDP were performed as follows: promastigotes were cultured in RPMI /10% FCS medium. Test of the drugs' effect against promastigotes in culture medium was performed in 96-well microtitre plates (Costar 3595; Corning Costar, Cambridge, MA, USA). Promastigotes (10^5) in their logarithmic growth phase were then added to each well (100μL) and incubated at 26°C for 3 days. Wells were subdivided into four 30-well groups; two contained clotrimazole (5 and 10 μg/ml), a third group included meglumine antimoniate (20 μg/ml) and the forth group was kept as a control with no drug. Growth was measured in each well through counting of LDP after 3 days by the conventional slide chamber method[7].

Results

Mean LDP counts in all test and control groups were determined by slide chamber method of counting (Table 1), and results were as follows; mean LDP count for the control group was 2,400,000 LDP/ml, while in clotrimazole groups 280,000 and 113,000 LDP/ml, (i.e.) clotrimazole inhibited about and 95% of LDP growth in concentrations 5 and 10 μg/ml, respectively. While mean LDP count in meglomine antimoniate group was 260,000 LDP/ml, that's to say meglomine antimoniate has eliminated

about 89% of LDP in concentration 20 µg/ml (Figure 1).

Table 1. Mean LDP counts and growth inhibition ratio in all test and control groups (each comprises 30 samples) determined by slide chamber method of counting after 3 days of incubation with the drug

Group	Drug conc. (µg/ml)	Mean LDP count (x1000 LDP/ml)	Growth inhibition ratio
Clotrimazole groups	5	280	79%
	10	113	95%
Meglumine antimoniate group	20	260	89%
Control group	---------	2,400	0%

Key:- LDP: Leishmania donovani promastigotes.

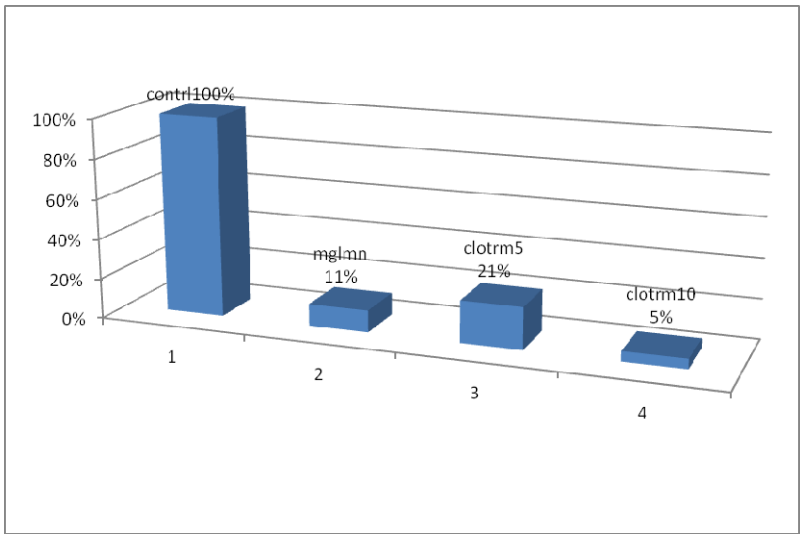

Figure 1. Representative LDP growth levels (as percent of the control) in all test and control groups (each comprises 30 samples) determined by slide chamber method of counting after 3 days of incubation with the drug:

Key:- LDP: Leishmania donovani promastigotes; mglmn: meglumine antimoniate group; clotrm 5and 10: clotrimazole(5 and 10 µg/ml) groups.

Discussion

In view of the public health importance of VL and the inherent difficulties of conventional therapeutic techniques, the effectiveness of clotrimazole was evaluated for this purpose in the present study.

The anti-leishmanial activity of clotrimazole has been determined here in LDP cultures. Clotrimazole eliminated about 79% and 95% of the parasites at drug concentrations 5 and 10 µg/ml, respectively. Those drug concentrations are serum concentrations that are achievable in vivo. While meglumine antimoniate eliminated about 89% at its peak achievable concentration, 20 µg/ml[4].

Studies made on conventional antileishmanial agents reported a percentage of cure from leishmaniasis of 85%, using meglumine antimoniate[8,9]. Despite the high efficacy of these drugs, they present many disadvantages such as parenteral administration, and, reversible secondary effects such as nausea,

vomiting, muscular and abdominal pain, cardiac problems, a rise in the concentration of hepatic amino-transferases, and chemical pancreatitis[2,3].

Additionally, the adherence to the treatment is affected by its duration (several weeks) and its availability by the restriction in its distribution. Therapeutic alternatives of second line have been proposed; amphotericin B and pentamidine have been used with excellent results, nevertheless their high cost, little availability, the necessity to hospitalize the patients for their administration and the severity of their secondary effects have limited their uses[3,10].

In the last decade new treatments for leishmaniasis have been developed, using oral agents such as mefloquine, itraconazole, miltefosine, paromomycin, ketoconazole, allopurinol and dapsone, however, they have not shown enough evidence of their effectiveness[9,11-13].

Because of the need for orally active antileishmanial agents, orally administrable drugs have sometimes been used to treat human leishmaniases without prior demonstration of efficacy in experimental models[14].

In a clinical trial for cutaneous leishmaniasis treatment, activity of clotrimazole was tested in vivo against meconazole, another member of imidazoline derivatives, and it was found that no side effects to be observed and was concluded that clotrimazole was the most effective among the imidazoline compounds and is recommended as initial treatment for simple lesions[15].

Conclusions and Recommendations

The results of this study demonstrated that clotrimazole has anti-leishmanial activity in a model system, and suggested that it could be considered for in vivo trials in animal models of the disease and even for clinical trials in human. Further prospective studies with larger cohorts of patients are required to establish the clinical significance of, and the impact of clotrimazole on treatment and prophylaxis of *Leishmania* strains resistant to antimony. 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 in such patients

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