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## Efficacy of Amphotericin B drug Against Promastigote and Axenic Amastigote of *Leishmania tropica* in Vitro

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### Abstract

*Leishmania* are protozoan parasites belonging to the family *Trypanosomatidae* that cause high morbidity and mortality levels with a wide spectrum of clinical syndrome. This study aimed to investigate the effect of liposomal amphotericin B (AmBisome) drug on promastigote and axenic amastigote stages of *Leishmania tropica*. From the 20 isolates of cutaneous leishmaniasis collected from patients attended to the AL-Karama Teaching Hospital in Baghdad during the period from October 2013 until February 2014, only three isolates successfully transformed to motile promastigote stage in the culture media. The most active one is included in this study. Different concentrations of liposomal amphotericin B (AmBisome) and pentostam Sb (V) drugs were investigated against *Leishmania tropica* promastigote and axenic amastigote. The IC<sub>50</sub> values of SbV and AmBisome drugs on promastigote were 5.42 mg/ml and 2.14 µg/ml, respectively, while they were 0.88µg/ml and 0.75 µg/ml respectively, for axenic amastigote. The present study concluded that axenic amastigote was more sensitive than promastigote against both drugs, and AmBisome drug showed high effectiveness against both stages with low concentrations in comparison with pentostam.

**Keywords:** *Leishmania* , AmBisome , pentostam , cutaneous leishmaniasis

## تأثير عقار الامفوترسين بي ضد الطور الامامي السوط وعديم السوط لليشمانيا الجلديه خارج جسم الكائن الحي

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### الخلاصه

الليشمانيا طفيليات ابتدائية تعود الى عائلة المثقبيات التي تسبب معدلات عاليه من الامراضيه والوفيات مع مدى واسع من الاعراض السريره. هدفت الدراسه الى اختبار فاعلية عقار الامفوترسين الشحمي على الطور امامي السوط وعديم السوط خارج الخلو لليشمانيا الجلديه. تم عزل 20 عينه ليشمانيا جلديه من المرضى المراجعين لمستشفى الكرامه التعليمي في بغداد للفترة من تشرين الثاني 2013 الى شباط 2014 وقد نجحت ثلاث عزلات فقط في التحول الى الطور امامي السوط المتحرك في الوسط الزرعى واختيرت العزله الاكثر نشاطاً لاستخدامها في هذه الدراسه. اختيرت تراكيز مختلفه من عقار البنتوستام والامفوترسين الشحمي ضد الطورين الامامي السوط وعديم السوط خارج الخلو من اللشمانيا الجلديه و كانت قيم IC<sub>50</sub> لهذين العقارين

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هي 5.42 ملغم /مل و 2.14 مايكروغرام /مل للطور الامامي السوط على التوالي بينما كانت قيمها 0.88 مايكرو غرام /مل و 0.75 مايكرو غرام /مل للطور عديم السوط على التوالي. اظهرت النتائج ان الطور عديم السوط خارج الخلوي اكثر حساسيه تجاه العقارين من الطور امامي السوط. وأن الامفوتيريسين الشحمي فعال ضد الطورين وبتراكيز اقل منها للبتنوستام.

## Introduction

*Leishmania* species is eukaryote parasites, transmitted by the bite of female insect belongs to the family *Phlebotominae*; *Leishmania* generates two forms during its life cycle which required an invertebrate and vertebrate hosts to be completed; flagellated insect stage form called promastigotes and vertebrate non-flagellated form called amastigote [1].

The different clinical manifestations vary from cutaneous self-healing lesions to visceral disease, fatal if left untreated. Leishmaniasis is considered to be the second most important protozoal disease and one of the neglected diseases that have become a special focus WHO [2]. The transmission of *Leishmania* occurs through hematophagus vectors from *Phlebotomus* genus in the Old World and *Lutzomyia* in the New World [3]. In cutaneous leishmaniasis (CL), the patient generally presents with one or several ulcer(s) or nodule (s) in the skin. Different species of *Leishmania* can infect the macrophages in the dermis, with variable clinical presentations and prognoses.[4,5].The ulcers heal spontaneously- although slowly - in immunocompetent individuals, but cause disfiguring scars, Primary prevention relies on managed control of the maintenance host and sand fly bite prevention measures. Secondary and tertiary prevention are dependent on the medical assistance using the clinical guidelines and adequate treatments. Pentavalent antimonials sodium stibogluconate (Sb) or (Pentostam) and meglumine antimoniate (Glucantime), the most standard drugs recommended 60 years ago, despite the low efficacy and adverse reactions, they remain the first-line treatment in most parts of the world. The Sb resistance now is likely to be due to the widespread misuses of Sb drugs with low dosing, interrupted treatment courses, short duration of treatment and possibly also the quality of drugs[6]. In search of less toxic, possibly more effective formulations, lipid associated formulations of amphotericin B were developed. Amphotericin B deoxycholate (Fungizone) is a systemic antifungal and a highly active antileishmanial. Due to the increasing resistance to antimonials, it is used as an alternative drug for leishmaniasis [7].This study aimed to demonstrate the effectiveness of different concentrations of liposomal formulation of amphotericin B drug (AmBisome) against *L. tropica* promastigote and axenic amastigote stages comparing with sodium stibogluconate (Pentostam or Sb) *in vitro* conditions.

## Materials and methods

Samples of cutaneous leishmaniasis used in this study include 20 samples collected from patient diagnosed as cutaneous leishmaniasis depending on clinical and laboratory test (skin smear). They attended AL-Karama Teaching Hospital in Baghdad during the period from October 2013 until February 2014. Some patient information was recorded such as (age, sex, number of lesion, location of lesion, history of family, and duration of infection).

The aspirated material collected from cutaneous lesions was inoculated in two culture tubes containing five ml of semi solid or M199 media. All inoculated tubes were incubated at 26°C. Cultures were examined for 15-30 days before being considered negatives. Patients were positively diagnosed for cutaneous leishmaniasis when actively motile promastigotes were seen in culture [8].

## Promastigote cultivation

To obtain a large amount of parasites in promastigote stage *in vitro*, inoculums of 1ml was transferred from culture contain growth to screw tube vials contain 5ml of media (m199) with 10% Fetal Calf Serum (FCS), and then incubated at 26°C. After 3 days the culture was examined under light microscope to ensure the growth of parasites and the absence of any other contamination, added amount of media to the culture if need, by this way gain the active parasites in log phase ( 3-4 days after cultivation) [9].

## Axenic amastigote cultivation

Axenic amastigote growths were induced by cultivation log phase promastigote in M199 medium under certain circumstances (ph 5.2, 5% of CO<sub>2</sub>, 35-36°C for cutaneous leishmaniasis) [9].

### Drug concentrations

A stock solution (100mg/ml) of sodium stibogluconate was used in this study. The following concentrations (2, 4, 8, 16, 32 and 64 mg/ml) were prepared for promastigote and (0.2, 0.4, 0.6, 0.8, 1 and 1.2  $\mu\text{g} / \text{ml}$ ) for axenic amastigote, while for AmBisome drug the following concentrations (0.6, 1.2, 2.4, 4.8, 8.16, 16.32 and 32.64  $\mu\text{g} / \text{ml}$ ) were prepared for promastigote and (0.2, 0.4, 0.6, 0.8, 1 and 1.2  $\mu\text{g} / \text{ml}$ ) for axenic amastigote.

### Statistical analysis:

The results of each experiment were analyzed by the method described by both Hills *et al.*, [10] and Huber and Koella [11]. Briefly, Hills proposed finding two concentrations,  $x_1$  and  $x_2$ , such that the parasite density,  $y_1$ , at concentration  $x_1$  (and all lower concentrations) was more than half of the density found in the control,  $y_0$ , and that the parasite density,  $y_2$ , at concentration  $x_2$  (and all higher concentrations) was less than half of  $y_0$ . The IC<sub>50</sub> value for each drug was then found by linear extrapolation between  $x_1$  and  $x_2$ :

$$\text{Log (IC}_{50}) = \log(x_1) + [(y_1 - y_0/2) / (y_1 - y_2)] [\log(x_2) - \log(x_1)].$$

### Results and discussion

Drug susceptibility of *Leishmania tropica* to liposomal amphotericin B (AmBisome) and pentostam Sb (V) drugs was determined on both stages: the promastigote and the axenic amastigote.

Pentostam SbV (2, 4, 8, 16, 32 and 64 mg/ml) concentrations against *L. tropica* promastigotes showed rapid decreased in the number of the parasite from the first day in all concentrations of the drugs compared with control as shown in figure (1). The effect of lower concentrations (0.2, 0.4, 0.6, 0.8, 1 and 1.2  $\mu\text{g}/\text{ml}$ ) of Sb (V) drug showed gradual decline in the number of *L. tropica* parasites as shown in figure 2.

Experimental chemotherapy of Leishmaniasis must involve both forms of *Leishmania*; the promastigote and the amastigote. The use of promastigote form is of limited value; because the parasite exists naturally in the vertebrate host as amastigote intracellular form [12, 13]. Indeed, most studies on drug susceptibility used the promastigote, rather than the amastigote model, mainly because promastigotes are easier to culture than amastigotes, which require macrophages as host cells in a highly acidic intracellular environment [14].

Pentavalent antimonial drugs were used worldwide for the treatment of visceral leishmaniasis VL and CL for over six decades with little evidence of resistance. The reason for the emergence of resistance is widespread misuse of the drug. On many occasions the daily dose of drug is split into two injections, to be given twice daily. These practices presumably expose the parasites to drug pressure, leading to progressive tolerance of the parasite to Sb (V). Pentavalent antimony Sb (V) undergoes biological reduction to much more active/toxic trivalent form of antimony Sb (III) that exhibits antileishmanial activity.

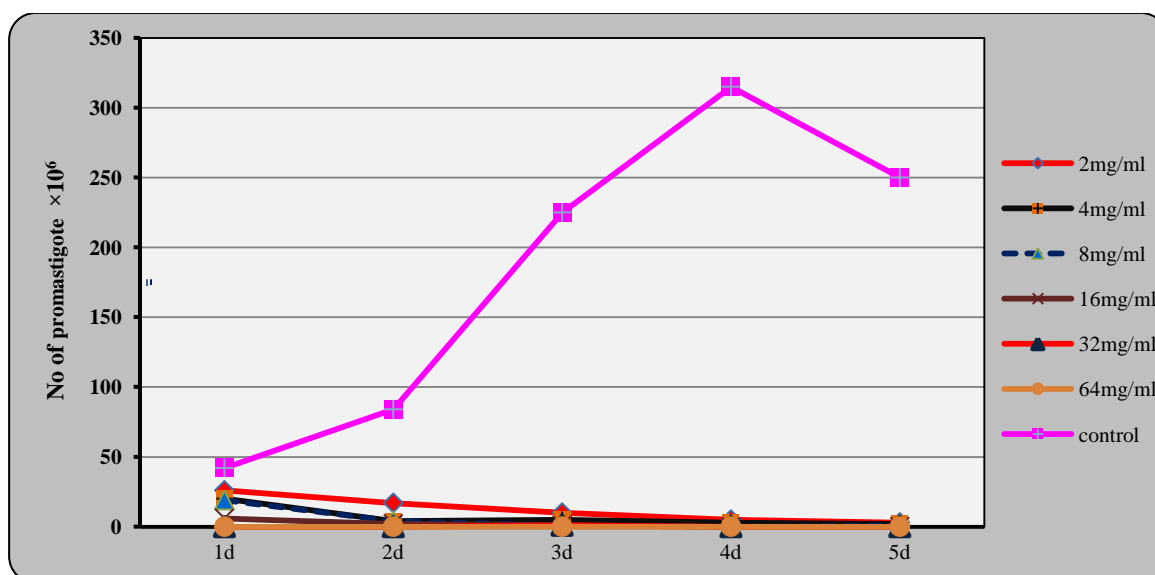


Figure 1-The effect of different concentrations of Sb (V) drug on promastigote of *L. tropica*.

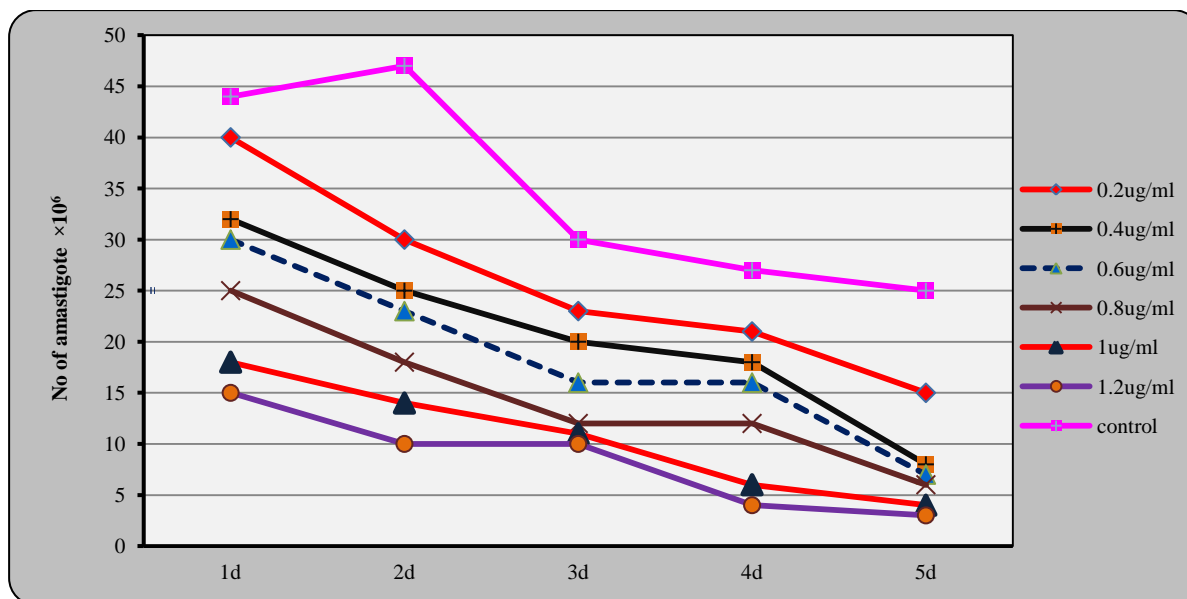


Figure 2-The effect of different concentrations of Sb (V) drug on axenic amastigote of *L. tropica*

Pentavalent antimony Sb (V) undergoes biological reduction to much more active/toxic trivalent form of antimony Sb (III) that exhibits antileishmanial activity. However, the site of (amastigote or macrophage) and the mechanism of reduction (enzymatic or nonenzymatic) remain controversial. For instance, amastigotes but not promastigotes can reduce Sb (V) to Sb (III). This explains why amastigotes are more susceptible to Sb (V) but promastigotes are not [15, 16]. In the present study both forms of the parasite susceptible to Sb(V) drug, but the drug effectiveness on axenic amastigote was more than those for promastigote ,because the former was more sensitive to low drug concentrations in comparison to high drug concentrations used against promastigote form.

Different concentrations (0.6, 1.2, 2.4, 4.8, 8.16, 16.32 and 32.64  $\mu\text{g/ml}$ ) of liposomal amphotericin B (AmBisome) drug were investigated against the promastigote of *L. tropica* parasites.

All AmBisome used concentrations revealed high activity on decreasing *L. tropica* promastigote, as shown in figure 3. On the other hand, lower concentrations (0.2, 0.4, 0.6, 0.8, 1 and 1.2  $\mu\text{g/ml}$ ) of AmBisome drug were investigated against axenic amastigote of *L. tropica*. This stage also susceptible to all AmBisome used concentration as figure 4.

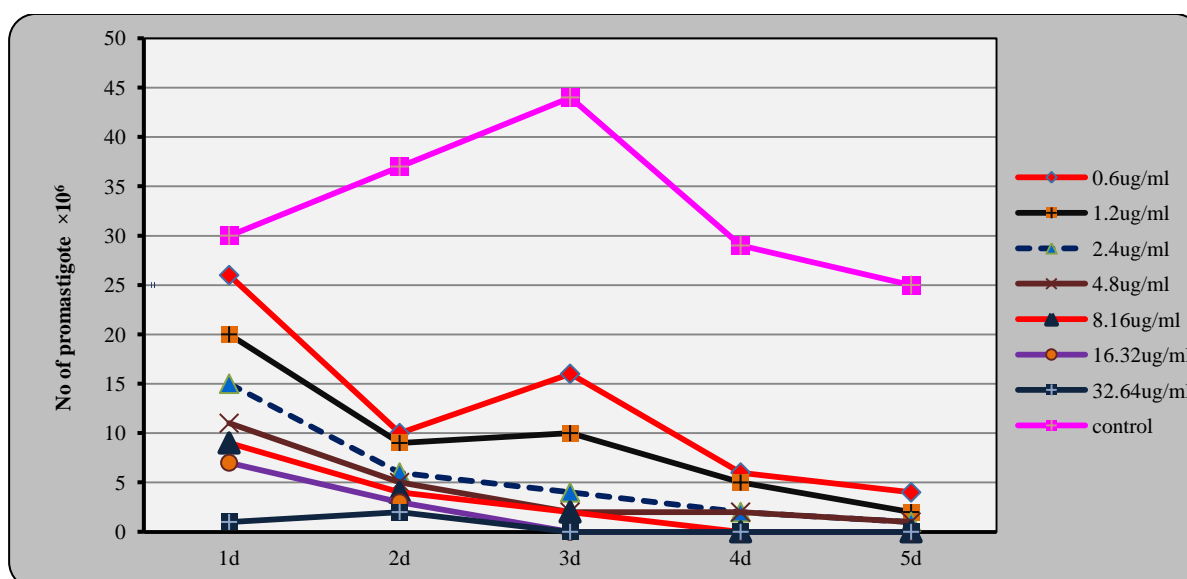
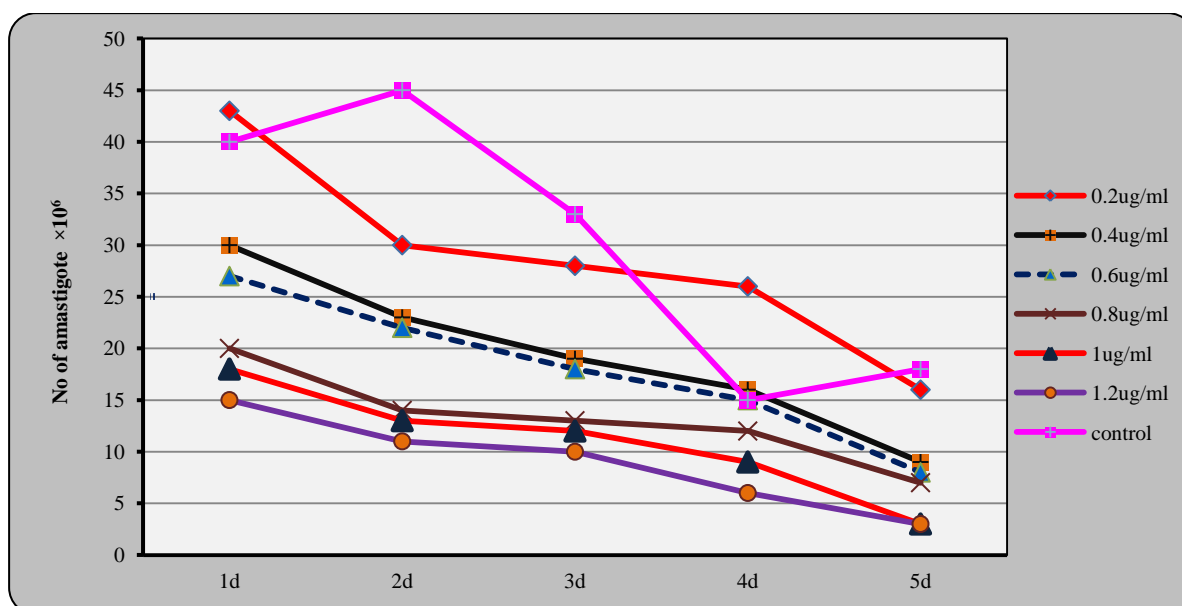


Figure 3-The effect of different concentrations of AmBisome drug on promastigote of *L. tropica*.



**Figure 4-**The effect of different concentrations of AmBisome drug on axenic amastigote of *L. tropica*.

Amphotericin B has excellent leishmanicidal activity and constitutes an option in patients that showed resistance to treatment with antimonials. The major limiting factor about the use of this drug is due to their toxicity. Currently, toxic effects of amphotericin B have been largely ameliorated with the advent of lipid formulations. In these formulations, deoxycholate has been replaced by other lipids that mask amphotericin B from susceptible tissues, thus reducing toxicity, and facilitating its preferential uptake by reticuloendothelial cells. Thus, this drug delivery result in increasing efficacy and reduced toxicity [17]. The mechanism of action of amphotericin B might be based on the peculiar metabolism of sterols of *Leishmania* species. In contrast to mammalian host, 24-ergosterol is the main sterol synthesized and existing in fungal and *Leishmania* membranes. Polyene macrolides bind to these molecules, creating pores that leak ions [18]

Previous studies done by Pucadyil *et al.*, [19], Tewary *et al.*, [20] and Rodriguez *et al.*, [21] have shown the requirement of host membrane cholesterol in the binding and internalization of *Leishmania* promastigotes into macrophage cells. So the physical depletion of cholesterol from macrophages leads to inhibition in binding of *Leishmania* promastigotes into macrophage cells [19,21]. Other study on the sterol-binding agent AmBisome suggests that mere sequestration of host plasma membrane cholesterol can inhibit leishmanial infection. Sequestration of membrane cholesterol with AmBisome could lead to a reduction in the availability of free cholesterol in the host plasma membrane that is essential for parasite entry. Taken together, these results reinforce the crucial requirement of membrane cholesterol in host cells for leishmanial infection. The molecular mechanism of how cholesterol supports binding of the parasite and its subsequent entry into host macrophages continues to be a key issue. The involvement of multiple membrane-bound receptors in the entry of the parasite into host cells has been mentioned earlier [22, 23].

In the present study, the leishmanias at both parasite stages were found to be highly susceptible to AmBisome, with the amastigote forms being in general more sensitive. These results are in substantial agreement with those obtained with a macrophage model [24], and with the results of Lariviere *et al.*, [25] how found that amphotericin B inhibits the growth of *L. tropica* promastigotes and decreases the number of viable parasites *in vitro*.

The IC<sub>50</sub> is the concentration of drug which decreases cell numbers by 50% compared to numbers of control cells grown in the absence of drug. The current study showed clear differences between the IC<sub>50</sub> values for the drugs on promastigote and axenic amastigote form of *L. tropica*. The axenic amastigote was more susceptible for both drugs than promastigote. As clarified in (table 1). The number of the parasites was inhibited at concentrations less than the inhibited concentrations for promastigote for Sb (V) and AmBisome, this due to the susceptibility of this stage in comparison to promastigote.

**Table 1**-The total values SbV and AmBisome drugs IC<sub>50</sub> against *L.tropica* promastigote and axenic amastigote

Drugs \ Stages	Promastigote	Axenic amastigote
SbV	5.42 mg/ml	0.88 µg/ml
AmBisome	2.14 µg/ml	0.75 µg/ml

A study done by Kinuthia *et al.*, [26] showed that Sb (V) and Ambisome had IC<sub>50</sub> values of (0.26 µg/ml) and (0.82 µg/ml) respectively against promastigotes, and therefore very little quantities of these drugs were inhibited *L. major* promastigote. Kamau-Ngure *et al.*, [27] recorded the IC<sub>50</sub> of pentostam against *L. donovani* and *L. major* promastigote which was (28.41 and 139.31 µg/ml) and it was for AmBisome (49.90 and 2.38 µg/ml) respectively. The variations in the previous results of these studies and the present study are due to many effected factors, such as the used strain, used drug (type, concentrations and the origin of the drug), and other condition of the test used. The IC<sub>50</sub> for AmBisome drug is approximately the same IC<sub>50</sub> value for Sb (V) drug against the same stage. So due to the less toxicity and more safety of AmBisome, it could be the best alternative drug for Sb (V) leishmanial resistant cases.

The overcome of present study indicated that AmBisome drug is an effective against both promastigote and axenic amastigote of *L. tropica*, and it was highly effective upon axenic amastigote from promastigote when it's used concentrations against axenic amastigote were too low.

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