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The *in Vitro* effect of clarithromycin on amastigote of *Leishmania donovani*

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Abstract: ***Backgrounds and objectives:*** *Leishmania donovani* is one of the most common species responsible for visceral leishmaniasis (VL) in India, Bangladesh and Sudan. The pentavalent antimonials are widely used as intramuscular route in the treatment of VL, but increase in resistance to this agent led to investigation of new drugs. We undertook this study to identify an alternative to current leishmaniasis treatment.

Methods: The *in vitro* activities of clarithromycin, amphotericin B, sodium stibogluconate and paromomycin were evaluated against drug- sensitive visceral *Leishmania* (amastigote) strain. A standard two fold serial dilution method in a 24-well plate was used to determine the 50% inhibitory concentration (IC_{50}) against intracellular amastigotes in mouse peritoneal macrophages.

Results: We determined the susceptibilities of both extracellular and intracellular drug sensitive amastigotes to clarithromycin and compared with amphotericin B, sodium stibogluconate and paromomycin. IC_{50} values of clarithromycin were found to be 87μM and 51μM for extracellular and intracellular amastigotes, respectively. 50% cytotoxic concentration (CC_{50}) of clarithromycin in mouse peritoneal macrophage was found to be 1596μM. Selectivity indexes in cellular model for both clarithromycin and paromomycin were found to be 31, whereas selectivity index for sodium stibogluconate was found to be 17.

Interpretation and conclusion: Our data suggested that clarithromycin was effective on *L. donovani* amastigotes in extracellular and cellular models. Clarithromycin was found to be equipotent to paromomycin when selectivity index was considered. Moreover, clarithromycin was more potent than sodium stibogluconate. Activity of clarithromycin against *L. donovani* may offer an alternative to current leishmaniasis treatment.

Keywords Amastigote- clarithromycin- cytotoxicity- *Leishmania donovani*- selectivity index-visceral leishmaniasis

Introduction

Visceral leishmaniasis (VL), a vector borne disease is caused by the genus *Leishmania*. Several clinical symptoms are represented under the term leishmaniasis. Visceral leishmaniasis results from the replication of the *Leishmania donovani* amastigotes within macrophages. Recently, there has been an increase in the number of cases with visceral leishmaniasis correlating with the increase in number of immune-compromised patients, travelling to and migration from the endemic regions, and resistance against pentavalent antimonial compounds. The disease is currently endemic in 88 countries of the developing world and showing increasing prevalence in immunosuppressive conditions such as HIV/AIDS[1]. The World Health Organization has put out an ardent appeal for the development of drugs and delivery devices against leishmaniasis[2]. Pentavalent antimonial compounds are still the first drug of choice in the treatment of leishmaniasis; but the recent increase in the number of resistance cases, especially in visceral leishmaniasis, and failures of treatment in immune-compromised cases, directed the researchers to

alternative drug investigations[3]. The two macrolides, azithromycin and clarithromycin, were found to be effective on intracellular parasites, such as *Pneumocystis carnii*, and *Toxoplasma gondii*[4,5], *Cryptosporidium parvum*[6,7] and *Plasmodium* species[8]. We aimed to evaluate the *in vitro* effects of clarithromycin in *L. donovani* amastigotes as axenic culture and in murine macrophages.

Materials and Methods

***Parasites and culture conditions*:** The strain of *Leishmania donovani* (MHOM/IN/83/AG83) was Indian VL isolates obtained from Indian Institute of Chemical Biology, Council of Scientific and Industrial Research, Kolkata, India. Promastigotes were cultured in M 199 medium supplemented with 20% heat inactivated fetal calf serum (FCS) and 2% antibiotic solution (Sigma P-3539) at 24°C.

***Antibiotics and control*:** Clarithromycin base was obtained from Sigma–Aldrich and dissolved in PBS (140mM) solution to obtain 1mg/ml of clarithromycin stock solution. Different concentrations of clarithromycin between 10 to 500 µM were prepared with stock solution. Culture medium was used as control. amphotericin B, sodium stibogluconate (SSG) and paramomycin (PMM) were used as standard antileishmanial drugs and for comparision with clarithromycin.

***Generation of axenic amastigotes*:** *Leishamnia donovani* amastigote forms were grown and maintained as described by Debrabant *et al.*[9]. Axenically grown amastigotes of *L. donovani* were maintained at 37°C in 5% CO_2 /air by weekly sub-passages in MAA/20 (medium for axenically grown amastigote) at pH 5.5 in petridishes[10]. Under these conditions, promastigotes differentiated to amastigotes within 120 hours. Cultures were maintained by 1:3 dilutions once in a week.

Animals: BALB/c mice of either sex, weighing 20–25 g and of approximately the same age were used for the study. The experimental protocols were approved by the Jadavpur University Animal Ethics Committee, and procedures followed were in accordance with the Committee's guidelines, with necessary humane care. Mice were housed in polypropylene cages and fed with a standard diet and water ad libitum. Mice were exposed to a normal day and night cycle.

***Axenic amastigote drug susceptibility assay*:** Axenic amastigote drug susceptibility determinations were performed using direct counting growth inhibition assay[11]. Amastigotes were seeded at an initial concentration equivalent to early log phage ($3x10^5$ amastigotes/ml) and allowed to multiply for 72 hours either in medium alone or in presence of serial dilutions of drugs until late logarithmic phage ($1x10^6$ cells/ml). Axenic amastigote numbers increased about three to four times during the assay which indicated that cells were viable. Drug susceptibility experiments were performed in the maintenance media. Amastigotes were counted using a hemocytometer after being passed through a 27-gause niddle three times in order to separate clumps, as needed, for accurate cell determinations. All experiments were repeated three times, unless otherwise indicated.

***Amastigote in macrophage assay*:** BALB/c mice were injected intraperitoneally with 1.5 ml of 3% thioglycolate medium. After 96 hours, the peritoneal macrophages were harvested by peritoneal lavage using cold RPMI-1640 medium. Cells were counted, centrifuged and re-suspended at a concentration of $4x10^5$/ml in RPMI-

1640 medium without supplements. Sterile round glass cover slips (12 mm) were placed in each well of 24-well culture plates. Macrophages were pipetted at a volume of 500 µl/well and allowed to attach for 2 hours at 37°C in 5% CO_2/air. After 2 hours, the medium was removed from the wells and replaced with 500 µl of warm (37°C) RPMI medium supplemented with 10% FCS and penicillin (50 U/ml) and streptomycin (50 µg/ ml). At the following day, a suspension of $4x10^6$/ml amastigotes in RPMI was added in a 500 µl volume to each well (macrophage/parasite ratio of 1:10). The plates were incubated for 4 hours at 37°C in 5% CO_2/air and the medium was aspirated to remove free parasites. Fresh RPMI (1 ml) without or with drugs at the appropriate concentration was added in triplicate wells. Plates were incubated for 72 hours at 37°C in 5% CO_2/air. The medium was aspirated and the cover slips were removed, methanol fixed and air dried. After staining with Giemsa, 100 cells on the glass disks were counted along the borderline. Three independent experiments in triplicate for each concentration were performed to evaluate the efficacies of drugs. Results are presented as the ratio between the infection proportions of treated and untreated macrophage culture.

Cytotoxicity against mammalian macrophages: Macrophage cells were cultured in minimum essential medium (MEM, Gibco), supplemented with 20 mM L-glutamate, 16 mM NaHCO3, 5% FCS and penicillin–streptomycin. The assay was performed in 96-well tissue culture plates in the presence of standard counts of macrophages. The wells were seeded with test solutions and the viable macrophages were counted microscopically.

Statistical analysis: Experiments were repeated at least three times. After calculating parasites survival percentage, SPSS software, and version 11.5 (SPSS Inc., Chicago, IL, USA) was used to compare the results in all statistical procedures. Results were expressed as the mean ± SD and $p<0.05$ was considered significant. IC_{50} was calculated by linear regression analysis or linear interpolation.

Results

As an initial test of biological activities of clarithromycin, we tried to evaluate antileishmanial activity in axenic and intracellular amastigote models and the toxicity of clarithromycin in mouse peritoneal macrophage. We also included amphotericin B, sodium stibogluconate (SSG) and paromomycin (PMM) in our study as standard antileishmanial drugs to compare their antileishmanial activities and selectivities with those of clarithromycin (Table1). It was shown previously that axenically grown amastigotes could be useful tool for the investigation of antileishmanial agent[10] and thus represent a powerful model that can be used to investigate the activities of drugs against active and dividing populations of amastigote forms. It offers numerous advantages, and in particular, coupled with *in vitro* models with macrophages, the influence of the latter on drug activity may be analyzed. It was shown that the extracellular amastigote forms of different *Leishmania* species clearly resembled the intracellular amastigotes according to their ultra structural, biochemical, and immunological properties[12]. Moreover, the characterized amastigotes, like intracellular ones, differed from promastigotes in a variety of biochemical characteristics, including proteinase and dehydrogenase activities and nitric oxide susceptibility[13,14]. The development of *in vitro*

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models of drug susceptibility has greatly facilitated studies on the molecular basis of drug susceptibility. Unfortunately, most of the mechanisms responsible for antileishmanial drug action were characterized with the promastigote form, which are mainly encountered in the insect vector[15-17] 50% inhibitory concentrations (IC_{50}) of SSG, PMM and clarithromycin in cellular model were much less than those in axenic model. The 50% cytotoxic concentration (CC_{50}) of each drug was measured using uninfected mice peritoneal macrophages culture as the test system. In cellular evaluation model selectivity indexes (SIs) of all the drugs increased compared to those in axenic evaluation model.

SI is the ratio of CC_{50} and IC_{50}. Higher value of SI represents the greater or higher selectivity. In that respect amphotericin B showed the SI of 70 and 93 in axenic and cellular amastigote models repectively (Table 1). SSG showed SI of 7.5 and 17 in axenic and cellular models repectively, lowest among amphotericin B, PMM and clarithromycin. Selectivity of clarithromycin appeared to be equal to PMM in cellular model. Here it is noteworthy that CC_{50} value of clarithromycin was much higher than those of amphotericin B, SSG and PMM.

It appeared from Figure 1 that SIs of amphotericin B, SSG, PMM and clarithromycin in cellular evaluation model were much higher than those in axenic evaluation model.

Discussion

There is an urgent need for alternative drugs for leishmaniasis treatment that provide effectiveness, safety and easy administration. Pentavalent antimonials still represent the first- line drug of choice for the treatment of leishmaniasis and other drugs are amphotericin B and paramomycin. Pentavalent antimonials have some disadvantages such as serious side effects, non–oral formulation and long-term hospitalization[18,19]. They are found to be ineffective in some immune-compromised patients with VL, either with AIDS or receiving immunosuppressive therapy and resistances against these drugs cause significant clinical problems and increase the cost of treatment[20].

Clarithromycin acts through the inhibition of protein synthesis and is concentrated and carried to tissue macrophages where amastigote resides and multiply[21]. In visceral leishmaniasis miltefosine has been administered by oral route and also has undergone clinical trial. Clarithromycin has important advantages such as long half-life, oral and injectable administration, relatively safe use in children and benign toxicity profile[22]. Clarithromycin was approved by FDA for respiratory tract and skin infections, but may also be used in mycobacterial infections and toxoplasmosis with HIV/ AIDS[23]. Clarithromycin was shown to be effective on *Criptosporiun spp*[24], *Pneumocystis carnii* and *Toxoplasma gondii*[5].

Clarithromycin and azithromycin concentration in tissues, especially in macrophages that were infected by *Leishmania* parasites, could reach concentrations 100 to 200 times higher than in serum[22]. Here, we report that, clarithromycin is effective agent on both axenic and intracellular amastigotes of *L. donovani in vitro*. Clarithromycin was equipotent to PMM with respect to SIs (Table1, Figure 1) and was more effective than azithromycin on *L. donovani* in cellular amastigote model[25]. Regarding the leishmanicidal activity, a study by Tanyuksel and others suggested an independent phagocytic increasing capacity effect[26].

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Clarithromycin is present within the main parameters of an effective medication, comprising simple oral administration and good tolerance. The drug has been commercialized worldwide. The possibility of an oral treatment is a highly desirable factor, mainly because of difficulties involved in attending health services by most people infected by *Leishmania spp*. Clarithromycin may be a valuable alternative treatment of VL. In conclusion, this study provides data that clarithromycin exhibits antileishmanial properties with minimal toxicity for mammalian cells and its unique and potent activity profiles represent an important new asset in search for novel antileishmanial drugs. According to our data, we suggest that further studies are required to reveal the efficacy of clarithromycin for chemotherapy of patients with VL.

Table 1: Drug sensitivity profile against axenic and intracellular amastigotes

Drugs tested	IC_{50} (mean ± SD) µM				
	Axenic evaluation model		Cellular evaluation model		Cytotoxicity CC_{50} (µM) (Macrophage cells)
	WT[a]	SI[c]	WT[a]	SI[c]	
Amphotericin B	0.2±0.05* (n=3)	70±6.12	0.15±0.05* (n=3)	93±4.43	14
SSG(SbV)[b]	3.6±0.40 (n=4)	7.5±0.05	1.6±0.20 (n=4)	17±0.9	27
PMM	10±2.03* (n=3)	25±2.45	8±2.11* (n=4)	31±2.5	248
Clarithrimycin	87±5.17* (n=4)	18.34±1.6	51±4.12* (n=4)	31.29±3.1	1596

[a]WT, Wild Type.
[b]Values for antimonial agents are in µg Sb /ml,
[c]Selectivity Index (SI) was calculated by dividing the CC_{50} by that of IC_{50}.
*p<0.05 significant difference compared with SSG.

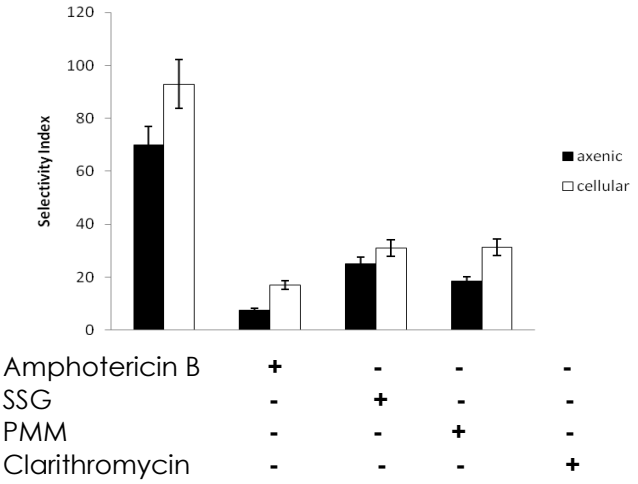

Figure 1: Mean selectivity index of amphotericin B, sodium stibogluconate,paromomycin and clarithromycin against axenic and intracellular amastigote. The data represent mean ± SD of three performed experiment.

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Conflict of interest

The authors have declared that no competing interests exist.

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