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Effect of Miltefosine on Growth and Metabolism of *Leishmania donovani* and *Leishmania tropica* Promastigotes

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Abstract

Miltefosine was found to be potent inhibitor of *Leishmania donovani* and *Leishmania tropica* promastigotes growth in vitro. Miltefosine was found to be equally effective against both parasites with ED50> 0.0001 mg/ml. The results demonstrated that the effect of miltefosine may be attributed to the inhibition of the enzyme diethylacetonephosphate acetyltransferase, that lead to the damaging membrane phospholipid biosynthesis, thus causing cellular death. The results suggest that miltefosine might be a promising potential as antileishmanial drug.

Introduction

Leishmaniasis is a vector borne disease caused by various members of the genus Leishmania, a protozoan parasite, belong to the order Kinetoplastida and the family of Trypanosomatidae (Sharma and Singh, 2008; Hassan and Mahmood, 2017). The clinical presentation ranges from simple cutaneous lesions to life threatening visceral forms (Gonzalez *et al*., 2008). The disease is endemic in 88 tropical and subtropical countries around the world (El-beshbishy *et al*., 2013), and according to WHO estimates 350 million people are at the risk with 12 million people are affected (Chppius *et al*., 2007). For the treatment of cutaneous and visceral leishmaniasis, pentavalent antimonial drugs have been the therapy of choice (Haldar *et al*., 2011; Junior *et al*., 2012).

In cases where pentavalent antimonials treatment fails, second line drugs such as pentamidine, amphotericin B and ketoconazole can be used. Frequently, however, these drugs fail to eradicate the parasite due to problems

relating toxicity and lower efficacy (Pujol and Riera, 2014; Frézard *et al*., 2009). It is generally accepted that there is a great need to develop more effective and less toxic drugs for the treatment of leishmaniasis (Croft and Yardley, 2002). New hope for an oral treatment came with the discovery of miltefosine, which appears to be highly efficient, less toxic, and can be administered orally (Oullette *et al.*, 2004). If clinical trials are successful, this drug will be very beneficial, especially in many countries.

Materials and methods

Parasite growth

The Promastigote forms of *Leishmania tropica* and *Leishmania donovani* were grown at 26 C in PY medium as described in Table 1. Gentamicin sulphate was added at 25 mg\ml to inhibit bacterial growth.

Drug susceptibility experiments

Cultures were initiated at 5×10^5 parasite/ ml and drugs were added to appropriate concentrations, 24 hr. later. Drug solutions were freshly prepared and sterilized using a Millipore filter (0.22 μm). The number of motile parasite present in the cultures were counted daily, using an improved Neubauer hemocytometer, until parasite growth entered stationary phase. The efficacy of the drugs is given in terms of the ED50 and ED90 the minimum concentration of drug used that reduce the number of promastigotes present after 3 days by 50% and 90% respectively.

Tabl.1 Components of culture media (PYF) in (gram /100 milliliter)

Components	Concentrations
NaCl	0.9
Na_2HPO_4	0.75
Peptone	1
Yeast extract	0.25
Folic acid	0.004
Human urine	5 % (v/v)

Estimation of nucleic acids

To determine RNA and DNA contents, promastigotes grown in the presence (absence) of drug concentration causing 50% inhibition of growth were harvested by centrifugation at 3000 Xg at 4℃ for 10 min and washed twice with 0.85% (w\v) saline. It was then precipitated by resuspension in 5 ml of 0.2 N perchloric acid (PCA) at 0℃, and extracted twice at 0℃ for 30 min with 0.2 N PCA. Lipid was then removed by two extraction at 45℃ first with 75% (v/v) ethanol, and then with 10 ml of ethanol /ether (1:1), Finally, nucleic acids were extracted at 70℃ for 40 min with 10 ml of 0.5 N PCA. The extract was then stored at 4℃ for 48 hr., after which centrifuged at 3000 Xg for 15 min with the resultant supernatant (RNA and DNA) and pellet (phospholipid) being containing deoxyribonuclease (1 mg/ml) to the standard volume (15 ml). The amount of RNA was determined by the method of Plumer(1988) using an Orcinol reagent with yeast RNA as standard whereas the amount of DNA was determined by the method of Giles and Mayer (1965) using diphenyl amine reagent.

Determination of protein concentration

To determine of protein content, promastigotes grown in the presence (absence) of drug concentration causing 50% inhibition of growth were harvested and washed as described above. It was then homogenized with 5 ml of cold 5% Trichloroacetic acid (TCA) to precipitate the protein. The homogenate was centrifuged at 3000 Xg for 10 min with resultant supernatant and pellet being separated. The supernatant was decanted and the pellet was washed with 5 ml of 5% TCA three times. The precipitated protein was then solubilized by 1N NaOH for 5 hr. with continuous shaking. The supernatant which contains the protein was taken and diluted with 1N NaOH to the standard volume (15 ml). The protein content was estimated by the method of Lowery *et al.*, (1951) with bovine serum albumin as standard.

Preparation of cell extract

Mid log phase promastigotes (10^8 cells /ml) were centrifuged at 4500 Xg for 10 min at 4℃. Pellets were washed twice in phosphate buffered saline and lysed by sonication involving two 15 sec periods separated by a 30 sec cooling period using MSE soniprep 150 sec fitted with an exponential microprobe at 4 amplitude microns, Crude homogenates were centrifuged at 105000 Xg at 4℃ for 1 hr. and the resultant supernatant was used as source of the enzyme.

Extraction of fatty acids

Pellet of promastigotes ($8-9 \times 10^7$ cells/ml) were dried using cooling lyophilizer and then 200 mg of dried promastigotes were dissolved in solvent (4% NaOH in methanol-benzene 6:4 v/v) and heated at 105℃ for 90 minutes. The mixture is cooled then 12 ml distilled water is added and pH adjusted to pH 2 with sulfuric acid (20%). Fatty acids were extracted by (30-50) ml Diethyl ether using separating funnel. The ether layer containing fatty acids was separated and dried using rotary vacuum evaporator at 50℃.

Thin –layer chromatography (TLC) of fatty acids

Fatty acids were separated by TLC using silica gel as the separating medium. Samples (10μl) were spotted on to silica gel sheets in the presence of standard fatty acids as chromatograms. The plates were developed by ascending chromatography in one of two solvent system. Solvent I, Benzene / diethyl ether (75/25 by volume); solvent II, acetic acid /distilled water (90 /10 by volume). The chromatogram spots were sprayed lightly with sulfuric acid (50%), then heated at 110-120℃ for 10 minute and rate of flows (Rf) values were determined. All spots form dark brown to black on a white background.

Results and Discussion

Effect of Miltefosine on the growth of Leishmania species

Pentostam, ketoconazole and miltefosine proved to be potent growth inhibitors even at low concentration (Table 2). For instance, miltefosine was the most potent compound and caused at 10 mg/ml 94% and 98% growth inhibition of *Leishmania tropica* and *Leishmania donovani* promastigotes, respectively (fig 1 and fig2). Pentostam exhibited antileishmanial activity and at 100 mg/ml reduced the number of *Leishmania tropica* and *Leishmania donovani* promastigotes by 3 days by 57% and 78% respectivel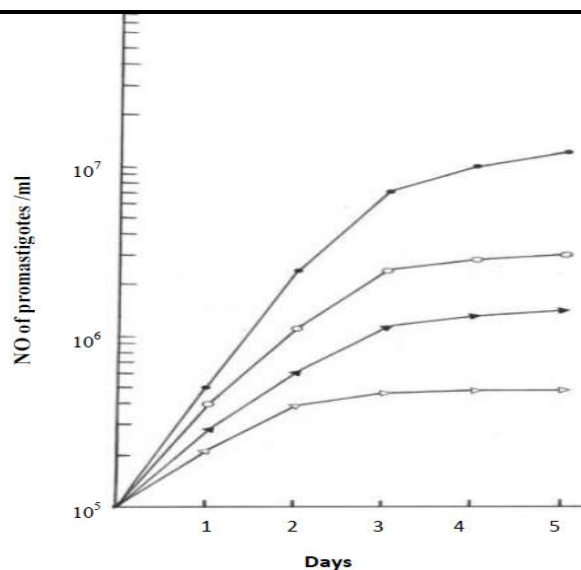y. Ketoconazole was almost equally effective against leishmanial promastigotes and at 10 mg/ml reduced the number *Leishmania tropica* and *Leishmania donovani* promastigotes by 96% and 97% respectively.

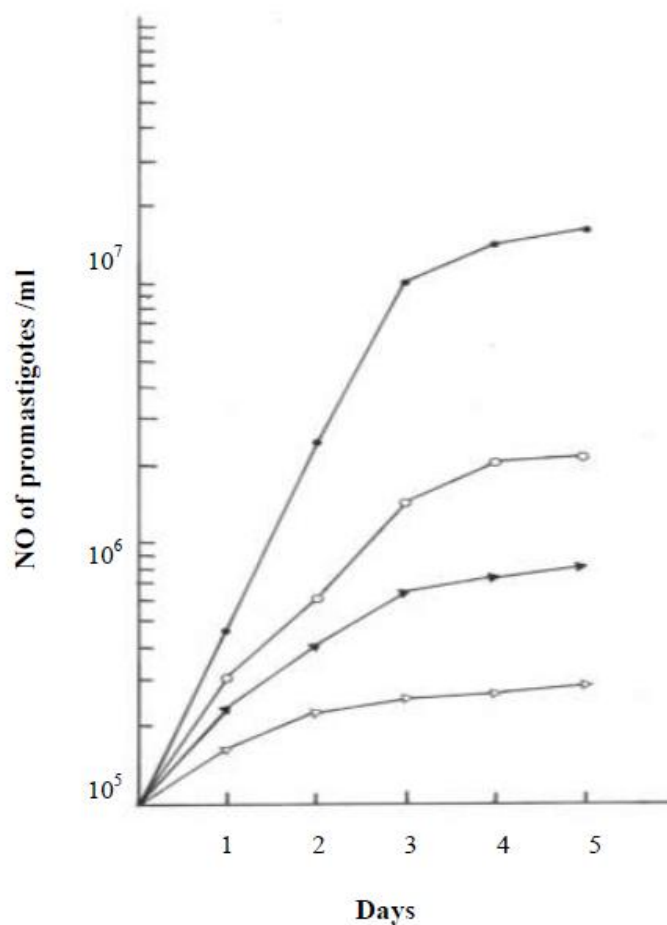### Effect of miltefosine upon nucleic acid, protein synthesis and fatty acids

To determine the effect of miltefosine, we have measured the nucleic acid content and protein synthesis of drug treated promastigotes (Table 3,4,5). Protein content did not reduced by miltefosine, pentostam and ketoconazole, whereas nucleic acid contents (RNA and DNA) were decreased by more than 20-30% by the all three drugs.

Table.2 ED50 and ED90 for miltefosine and other antileishmanial agents against the promastigotes of Leishmania tropica and Leishmania donovani

Compound	ED50(mg/ml		ED90 (mg/ml)	
	Leishmania tropica	*Leishmania donovani*	*Leishmania tropica*	*Leishmania donovani*
Pentostam	<0.1	<0.1	10.1	0.98
Ketoconazole	<0.001	<0.001	<0.001	<0.001
Miltefosine	<0.0001	<0.0001	0.0096	0.00096

Effectiveness of miltefosine on *Leishmania tropica* promastigote (•Control)
(∘, 0.0001 mg\ml) (▲, 0.001 mg\ml) (Δ, 0.01 mg\ml)

Effectiveness of miltefosine concentration on *Leishmania donovani* promastigote (•Control) (∘, 0.0001 mg\ml) (▲, 0.001 mg\ml) (Δ, 0.01 mg\ml)

Table.3 Effect of miltefosine and antileishmanial agents upon leishmanial protein synthesis

Treatment	*Leishmania tropica*		*Leishmania donovani*	
	mg protein /10^8	% decrease	mg protein /10^8	% decrease
Control	382 ±12		426 ± 16	
Pentostam	361 ± 9	5.5	415 ± 11	2.6
Ketoconazole	372 ± 12	2.7	418 ± 14	1.9
Miltefosine	368 ± 13	3.7	420 ± 18	1.5

Table.4 Effect of miltefosine and antileishmanial agents upon *Leishmania tropica* nucleic acid contents

Treatment	mg DNA /10^8	Inhibition %	mg RNA /10^8	Inhibition %
Control	24 ± 3		75 ± 4	
Pentostam	18 ± 2	25	49 ± 6	35
Ketoconazole	22 ± 2	8	62 ± 5	7
Miltefosine	16 ± 1	33	53 ± 4	29

Table.5 Effect of miltefosine and antileishmanial agents upon *Leishmania donovani* nucleic acid contents

Treatment	mg DNA /10^8	Inhibition %	mg RNA /10^8	Inhibition %
Control	31 ± 2		96 ± 3	
Pentostam	22 ± 4	29	66 ± 4	31
Ketoconazole	24 ± 1	23	73 ±5	24
Miltefosine	19 ± 3	39	69 ± 3	28

Table.6 Effect of miltefosine upon fatty acid contents of *Leishmania tropica* and *Leishmania donovani*

Treated	Fatty acids	Oleic acid C18:1Δ9	Palmitic acid C16:0	Myristic acid C14:0	Stearic acid C18:0	Caproic acid C6:0
Leishmania tropica	Control	+	+	+	-	+
	Miltefosine	+	+	-	-	-
Leishmania donovani	Control	+	+	+	+	-
	Miltefosine	+	-	-	-	+

Table.7 Effect of miltefosine and antileishmanial agents upon leishmanial acetyltransferase activity

Drugs	*Leishmania tropica*		*Leishmania donovani*	
	Enzyme activity	% Inhibition	Enzyme activity	% Inhibition
Control	24 ± 2		36 ± 2	
Pentostam	23.6 ± 3	2	35 ± 2	3
Ketoconazole	23.5 ± 4	3	34 ± 1	6
Miltefosine	2 ± 0.2	91.7	2 ± 0.9	94.5

As presented in Table (6) the fatty acids contents of miltefosine treated promastigotes of Leishmania tropica and Leishmania donovani have shown the presence of palmitic and caproic acid respectively in addition to oleic acid.

To determine whether the leishmanistatic effect of miltefosine was due to inhibition of enzymes involved in the metabolism of lipid treated cells were extracted and assayed for acetyltransferase activity. A significant inhibition of diethylacetonephosphate acetyltransferase was seen in promastigotes of *Leishmania tropica* and *Leishmania donovani* exposed to miltefosine (Table 7).

The pentavalent antimonial, namely sodium stibogluconate (pentostam) has been the front line drug for treatment of all forms of human leishmaniasis for many years (Haldar *et al*., 2011; Mudawi *et al*., 2009). In cases where pentostam treatment fails, second line drugs such as ketoconazole, amphotericin B and pentamidine can be used (Ouellette *et al*., 2004; Ashutosh *et al*., 2007). Frequently, however,these drugs fail to eradicate the parasite because of problems relating to toxicity and/or lower efficacy (AL-Makki, 2013; Croft and Coombs, 2003). It is generally accepted that there is a great need to develop more effective and less toxic drugs for the treatment of leishmaniasis (Croft and Yardley, 2002; Berman, 2005). Recent efforts in the search for new drugs has been the demonstration that miltefosine (hexadecylphosphocholine) is active in several models (Paris *et al*., 2004). It has also been demonstrated that the miltefosine is the only oral drug that was safe and effective in cutaneous and visceral leishmaniasis (Sunder *et al*., 2003).

The data presented here suggest that the miltefosine inhibit the growth of leishmanial promastigotes by inhibition of the enzyme diethylacetonephosphate acetyltransferase which involved in the phospholipid biosynthesis. Similarly, it has been demonstrated that the effective of miltefosine for leishmanial promastigotes may in part due to the damage to the intracellular membranes and defects in phospholipid synthesis (Bari, 2006). In this respect, the present finding may provide a novel approach to increasing the effectiveness of miltefosine as antileishmanial.

References

AL-Makki, A.Q. 2013. In vitro study of the effect of Some drugs on *leishmania donavani* replication rate. *J. Thi-qar University,* 8(2): PP: 62-75.

Ashutosh, Sundar, S. and Goyal, N. 2007. Molecular mechanisms of antimony resistance in *Leishmania*. *J. Med. Microb*., 56: PP: 143-153.

Bari, A.U. 2006. Miltefosine: a breakthrough in treatment of Leishmaniasis. *J. Pak. Ass. Dermat.,* 16: 225-230.

Berman, J. 2005. Recent Developments in *Leishmaniasis*: Epidemiology, Diagnosis, and Treatment, *Curr. Infect. Dis. Reports*, 7: 33-38.

Chappuis, F., Sundar, S., Hailu, A., Ghalib, H., Rijal, S., Peeling, R. W., Alvar, J. and Boelaert, M. 2007. Visceral Leishmaniasis: what are the needs for diagnosis, treatment and control?. *Nature Rev. no,* 11: 5, pp. 57-66.

Croft, S.L. and Coombs G.H. 2003. Leishmaniasis – current chemotherapy and recent advances in the search for novel drugs. *Rev. Trends in Parasit. Elsever,* 19(11): 502-508.

Croft, S.L., and V. Yardley. 2002. Chemotherapy of leishmaniasis. *Curr. Pharm. Des.*, 8: 319–342.

El-Beshbishy, H.A., Al-Ali, K.H. and El-Badry, A.A. 2013. Molecular characterization of cutaneous Leishmaniasis in Al-Madinah Al-Munawarah province, western Saudi Arabia. *Int. J. Infect. Dis.,* 17(5): pp. 334-338.

Frézard, F., Demicheli, C. and Ribeiro, R.R. 2009. Pentavalent Antimonials: New perspectives for old drugs. *J. Mol.,* 14. PP: 2317-2336.

Giles, K. and Mayer, A. 1965. An improved diphenylamine reagent for estimation of DNA concentration, *Nature,* 20: PP: 93.

Gonzalez, U., Pinart, M., Reveiz, L., and Alvar, J. 2008. Interventions for Old World cutaneous leishmaniasis. *Cochrane Database Syst. Rev.*, CD005067.

Haldar, A.K., Sen, P. and Roy, S. 2011. Use of antimony in the treatment of leishmaniasis current status and future directions. *Res. Mol. Biol. Inter.,* PP: 1-23.

Hassan, H.F. and Mahmood, I.N. 2017. Leishmania. Baghdad Boil and Black Fever. Fuzuli Pub., Kirkuk, Iraq.

Junior, L.H.F., Chatelain, E., Kim, H.A. and Neto, J.L.S. 2012).Visceral leishmaniasis treatment: What do we have, what do we need and how to deliver it?. *Inter. J. Parasit*., *Drugs and Drug Resistance,* 2: PP. 11-19.

Lowrey, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.,* Vol. 193, PP: 265-275.

Mudawi, M.M.E., Shaddad, S.A.I., Eltayeb, I.B., Khalil, E.A.G., Musa, A.M. and Muddathir, A.K. 2009. The

Pharmacodynamic interactions of sodium stibogluconate (SSG) and paromomycin (PM) on various isolated tissues preparations. *Res. J. pharmacol.,* 3(2): PP. 26-30.

Ouellette, M., Drummelsmith, J., and Papadopoulou, B. 2004. Leishmaniasis: drugs in the clinic, resistance and new developments. *Drug Resist. Updat.,* 7: 257-266.

Paris, C., Loiseau, P.M., Bories, C., and Breard, J. 2004. Miltefosine induces apoptosis – like death in *Leishmania donovani* promastigotes. *Antimicrob. Agents Chemother.,* 48: 852 – 860.

Plummer, D.T. 1988. An introduction practical Biochemistry, 3rd ed, Tata Mc Graw-Hill Pub.Co., New Delhi.

Pujol, A. and Riera, C. 2014. Review and new therapeutic alternatives for the treatment of cutaneous Leishmaniasis, Research signpost, Barcelona, Spin, PP. 188.

Reissig, J.L, Strominger, J.L., and Leloir, L.F. 1955. A modified colorimetric method for the estimation of N-Acetylamino sugers. *J. Biol. Chem.,* 217: 959-966.

Sharma, U. and Singh, S. 2008. Insect vectors of Leishmania: distribution, physiology and their control. *J. Vector Borne Dis.,* 45: p. 255–272.