RESEARCH ARTICLE

Antileishmanial Activity of Formycin Analogs on Promastigotes of *Leishmania Donovani* and *Leishmania Tropica*

Husain F. Haassan, Rana A. Ahmed*

Department of Biology, College of Science, University of Kirkuk, Kirkuk, Iraq

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ABSTRACT

The analogs of formycin have growth inhibitory effect on the *Leishmania tropica* and *Leishmania donovani* promastigotes. The formycin B was found to be more active than formycin A, allopurinol riboside, thioformycin B and tubercidin. Treatment of promastigotes of leishmanial species with formycin analogs (except tubercidin) decreased protein amounts by more than 50% and nucleic acid synthesis by 25–50%. The formycine analogs also resulted in inhibiting the enzymes adenylosuccinate lyase, adenylosuccinate synthetase and nucleoside kinase.

The postulated possibility of the inhibitory effect of formycin analogs may be due to the ability of promastigotes to convert these analogs to their nucleotide triphosphates which are incorporated into RNA resulting in blocking of the synthesis of protein and in causing death of the parasite.

Keywords: Antileishmanial activity, Formycin, *Leishmania*, Promastigotes.

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Conflict of interest: None

INTRODUCTION

Leishmania is the hemoflagellate trypanosomatid of the order Kinetoplastida, causing various disease manifestations, collectively known as leishmaniasis, that are currently threatening 350 million men, women, and children in 88 countries throughout temperate, subtropical and tropical regions of the world.[1]

Leishmaniasis is a disease with different symptoms, including cutaneous lesions, mucocutaneous ulceration, or visceral infection, potentially fatal if left untreated.[2] The drugs of choice for treating leishmaniasis are pentavalent antimonals sodium stibogluconate (Pentostam) and methylglucamine antimoniate (Glucantime), which are being used for over fifty years.[3] But frequently, they are failed to eradicate the parasite due to resistance, toxicities, adverse side effects, and lower efficacy.[4] Consequently, there is an urgent requirement for new antileishmanial agents to treat drug-resistance cases and avoid the toxic side effects of those in current clinical use.

Extensive studies have shown that formycin compounds appear to be highly efficient and non-toxic to mammalian cells and, however, can be potential of a rational approach to leishmanial chemotherapy.

MATERIALS AND METHODS

Cultivation of *Leishmania* in PY Medium

As described previously, Promastigotes of *L. donovani* and *L. tropica* were also cultivated *in-vitro* at 26°C in PY medium.[5]

The media consists of NaCl (0.9 gm), Na_2HPO_4 0.75 gm, yeast extract (0.25 gm), peptone (1-gm), and folic acid 0.004 gm in 100 mL distilled water. The pH of the media is adjusted to 7.2, sterilized by autoclaving at 121 for 15 minutes, and when cool, 100U/mL of penicillin and 0.1 mg/mL streptomycin were added. Fresh human urine was collected from a single adult male volunteer, cleared by centrifugation at 2000 g for 5 minutes, and sterilized using an 0.22 µm membrane filter. The sterilized urine was added to the media at a concentration of 5% (v/v).

Preparation of Cell Extract

Promastigotes of *L. donovani* and *L. tropica* were harvested at the mid-logarithmic phase by centrifugation at 3000x g at 4 for 10 minutes and washed twice with ice-cold 50 mM Tris-HCl buffer, pH 7.2, containing 0.85% (w/v) NaCl (Buffer A). For most studies, the pelleted cells were suspended at a density of approximately $3x10^9$ cells/mL in Buffer A containing 0.1 mM dithiothreitol and disrupted by sonicator for two periods of

*Author for Correspondence: hamody2009210@gmail.com

15 seconds, separated by 10 seconds cooling period, using an MSE Soniprep 150 fitted with an exponential probe at 4 µm amplitude. The crude homogenates produced were either used directly or fractionated by centrifugation at 105,000 xg at 4°C for 1 hour, with the resulted supernatants and pellets being separated and the latter resuspended in the above buffer to the volume of the supernatant fraction.

Exposure of Leishmanial Promastigotes to Drugs

The test protocol used in these experiments was as follows: Cultures were initiated at 2–5 x 10^5 promastigotes/mL, and drugs were added to appropriate concentrations 24 hours later. Drug solutions were freshly prepared and sterilized using a millipore filter (0.2 µm). The numbers of promastigotes present in the cultures were counted daily, using an improved Neubauer hemocytometer, until parasite growth entered the stationary phase. The efficacy of some compounds is given as LD50, the minimum concentration of drugs used in reducing promastigotes numbers present after 96 hours by 50%.

Isolation of Nucleic Acids from *L. donovani* and *L. tropica*

Nucleic acids were isolated from *L. donovani* and *L. tropica* promastigotes using the procedure mentioned in previous researches.[6] Promastigoes are grown in the presence (or absence) of drug concentration causing 50% inhibition of growth were harvested by centrifugation at 3000xg at 4°C for 10 minutes 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°C, and extracted twice at 0 for 30 minutes with 0.2 N PCA. Two extractions then removed lipid at 45°C, the 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 minutes with 10 mL of 0.5 N PCA. The extract was then stored at 4°C for 48 hours, after which centrifuged at 3000 xg for 15 minutes with the resultant supernatant (RNA and DNA) and pellet (phospholipids) being separated and the supernatant diluted with 0.5 N PCA to the standard volume (15 mL).

Estimation of Deoxyribonucleic Acid (DNA)

The amount of DNA was estimated using the diphenylamine reaction described by some researchers[6] with calf thymus DNA as standard. To one mL test solution was added two mL of diphenylamine reagent. The mixture was incubated at 30°C overnight, and the resulting blue color read at 595 nm on spectrophotometer against blank (water).

Estimation of Ribonucleic Acid (RNA)

The amount of RNA was determined by the method of[7] using an orcinol reagent with yeast RNA as standard. This method depends on the reaction of ribose sugar with strong acid to produce furfural that will condense with orcinol to form a characteristic green color compound with an absorption peak at 660 nm. Therefore, the procedure is to add 3 mL of orcinol reagent to 2 mL of test solution. After boiling for 20 minutes, the mixture is cooled, and the optical density is read at 660 nm against a blank (water).

Determination of Protein Concentration

To determine of protein content, promastigotes grown in the presence (or absence) of drug concentration causing 50% inhibition of growth were harvested by centrifugation at 3000 xg at 4°C for 10 minutes, washed twice with 0.85%(w/v) saline and homogenized with5ml of cold 5% trichloroacetic acid (TCA) to precipitate protein. The homogenate was centrifuged at3000 xg for 10 minutes, with the resultant supernatant and pellet being separated. The supernatant was decanted, and the pellet was washed with 5 mL of TCA three times. 1 N NaOH then solubilized the precipitated protein for 5 hours with continuous shaking. The supernatant which contains the protein, was taken and diluted with 1 N NaOH to the standard volume (15 mL). The method[8] estimated the protein content with bovine serum albumin as standard.

Enzyme Analyses

Spectrophotometric Assays

Adenosine kinase (AK), adenylosuccinate lyase, and adenylosuccinate synthetase were assayed spectrophotometrically.[9] The rate of change in absorbance resulting from the conversion of substrates to products was monitored at the appropriate wavelength using PyeUnicam SP 800 Ultraviolet spectrophotometer. Final assay mixtures having a total volume of 1 mL and 1 cm pathlength quartz cuvettes, were used. Reaction mixtures were preincubated to 26°C and the reaction usually was started by the addition of enzyme.

RESULTS AND DISCUSSION

Effect of Formycin A on Growth of Cultured Promastigotes of L. tropica and L. donovani

The results of exposing the promastigotes of *L. tropica* and *L. donovani* to Formycin A for 6 days are seen in Figures 1 and 2. Formycin A inhibited *L. tropica* by 86%, whereas growth of *L. donovani* was inhibited by 80%.

Effect of Formycin B on Growth of Cultured Promastigotes of *L. tropica* and *L. donovani*

The results of treating promastigotes of *L. tropica* and *L. donovani* by formycin B for six days are seen in Figures 3 and 4 respectively. Formycin B was highly active than Formycin A against promastigotes of both leishmanial species. However, formycin B inhabited 91% of growth *L. tropica*. The growth of promastigote of *L. donovani* was also inhibited by formycin B, although to a lesser extent than that of L. tropica and it inhibited 84%.

Effect of Drugs on Total Protein Content of Leishmanial Promastigotes

The total protein content of *L. tropica* and *L. donovani* promastigotes exposed to a drug concentration that reduces 50% of growth over six days are represented in Tables (1 and 2). Amongst the antileishmanial agents, only pentostam caused about 30 and 27% decrease in protein content of *L. tropica* and *L. donovani* , respectively. Amongst the formycin analogs tested, all except tubercidin showed significant inhibitory activity.

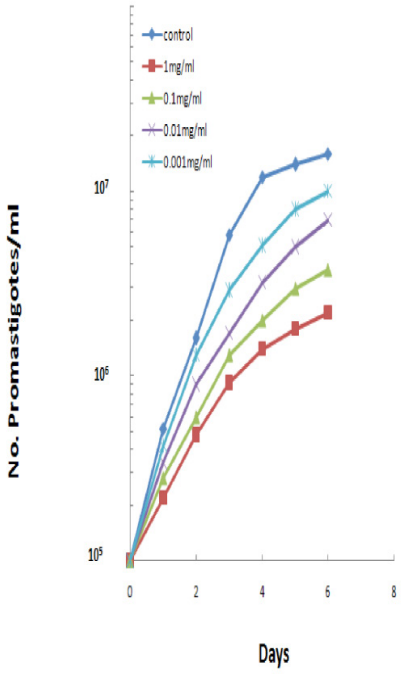

Figure 1: The effect of formycin A on the growth of L.tropica promastigotes

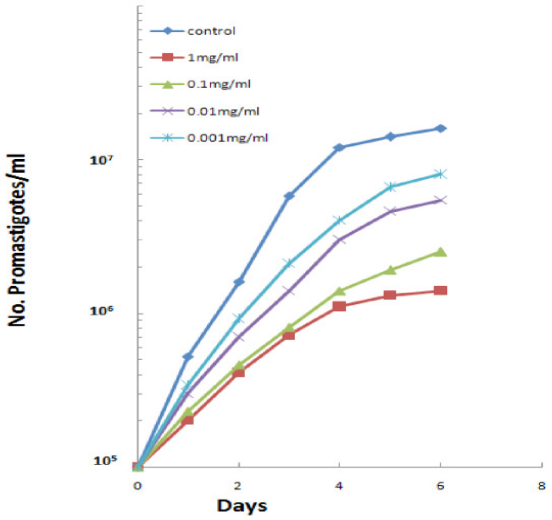

Figure 2: The effect of formycin A on the growth of L.donovani promastigotes

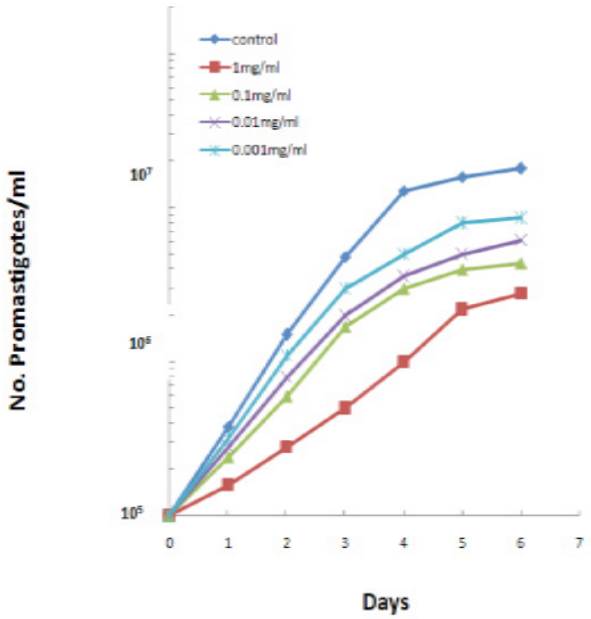

Figure 3: The effect of formycin B on the growth of L.tropica promastigotes

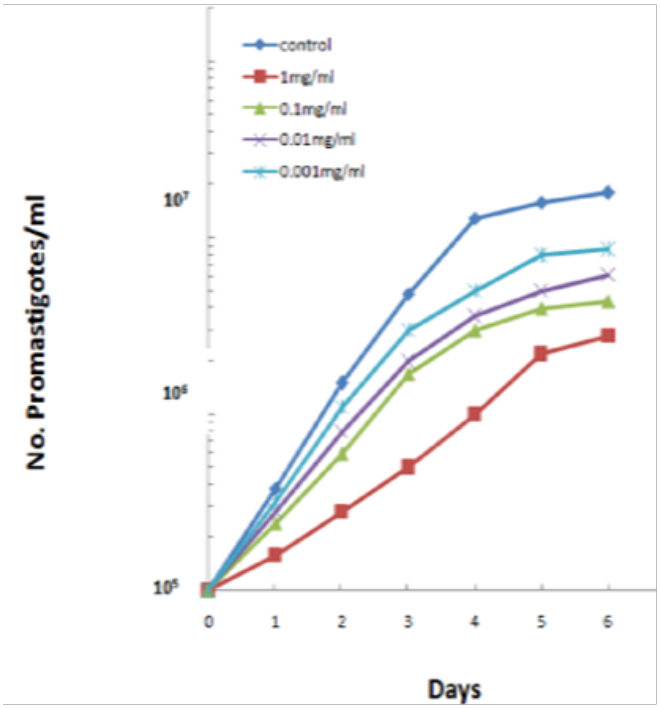

Figure 4: The effect of formycin B on the growth of L.donovani promastigotes

Effect of Antileishmanial Agents and Formycin Analogs on Leishmanial Promastigotes Enzymes

To determine whether the reduced RNA content of formycin analogs –treated cells were due to inhibition of purine salvage enzymes, cells were extractedand assayed for enzymatic activities. Significant inhibition of nucleoside kinase, adenylosuccinate synthetase and adenylosuccinate lyase were seen in promastigotes of both species of Leishmania, exposed to inosine analogs (Tables 3 and 4), respectively.

For leishmaniasis, the pentavalent antimonials are still in use and while they have saved countless lives and untold misery over the years, they are inconvenient and dangerous to use and are not always effective.[10] Pentamidine and ketoconazole also play important roles in treating various types of leishmanial

Table 1: Effects of drugs upon protein synthesis in *L. tropica* promastigotes

Treatment	*Mg protein /10^8 cells*	*%Decrease*
Control	352 ± 8	—
Pentostam	248 ± 11	30
Ketoconazole	345 ± 9	2
Formycin B	64 ± 2	82
Formycin A	96 ± 4	73
Allopurinol riboside	126 ± 6	64
Thioformycin B	104 ± 7	70
Tubercidin	332 ± 9	6

Table 2: Effects of drugs upon protein synthesis in *L. donovani* promastigotes

Treatment	*Mg protein /10^8 cells*	*% Decrease*
Control	368 ± 7	—
Pentostam	268 ± 8	27
Ketoconazole	359 ± 9	2
Formycin B	59 ± 4	84
Formycin A	92± 6	75
Allopurinol riboside	88 ± 7	76
Thioformycin B	72± 5	80
Tubercidin	336 ± 11	9

Table 3: Effect of antileishmanial agents and formycin analogs on *L. tropica* purine salvage enzymes

	%Inhibition		
Drugs	*Kinase*	*Lyase*	*Synthetase*
Control	36 ± 2	9 ± 1	6 ± 2
Pentostam	36 ± 4(NI)	9 ± 2(NI)	6 ± 1(NI)
Ketoconazole	36 ± 3(NI)	9 ± 1(NI)	6 ± 1(NI)
Formycin B	8 ± 1(78)	2 ± 0.1(78)	1 ± 0.1(83)
Formycin A	14 ± 2(61)	5 ± 1(44)	3 ± 0.1(50)
Allopurinol riboside	10 ± 2(72)	3 ± 0.4(67)	2 ± 0.2(67)
Thioformycin B	11 ± 2(69)	3 ± 0.3(67)	3 ± 0.3(50)
Tubercidin	16 ± 3(56)	6 ± 1(45)	4 ± 0.5(33)

Table 4: Effect of antileishmanial agents and formycin analogs on *L. donovani* purine salvage enzymes

Drugs			
	Kinase	Lyase	
Control	42 ± 3	12 ± 1	11 ± 2
Pentostam	42 ± 4(NI)	12 ± 2(NI)	11 ± 2(NI)
Ketoconazole	42 ± 3(NI)	12 ± 1(NI)	11 ± 1(NI)
Formycin B	7 ± 1(83)	2 ± 0.5(83)	3 ± 0.2(73)
Formycin A	16 ± 3(62)	5 ± 0.4(58)	6 ± 1(45)
Allopurinol riboside	9 ±1(79)	3 ± 0.1(75)	4 ± 1(64)
Thioformycin B	12 ± 3(71)	4 ± 0.1(67)	3 ± 1(73)
Tubercidin	20 ± 4(52)	6 ± 1(50)	5 ± 1(55)

infections.[11] To say that these medications are far from satisfactory is by no means to deny their status as valuable contributions to medicine. Thus, the need for safer remedies is acute.[12]

The metabolic differences between the leishmanial parasites and its mammalian host offer opportunities to design new drugs; the new approaches to chemotherapy may soon bear fruit.[13]

Recent efforts to find alternative antileishmanial agents have produced several interesting leads, including the demonstration that some purine analogs such as formycin B, formycin A, and allopurinol riboside, have significant antileishmanial activity.[14] Their being useful chemotherapeutic agents results from Leishmania's inability to synthesize purine and from being remarkably nontoxic to the host. Formycin B, Formycin A and allopurinol riboside have shown significant antileishmanial activity.Similar findings have also been reported for other Leishmania species promastigotes and trypanosomes.[15] In this respect, the selective toxicity of these agents for Leishmania depends upon the phosphorylation of formycin B, formycin A and allopurinol riboside by nucleoside kinases to it's ribotide. Which in turn gets aminated by adenylosuccinate synthetase and adenylosuccinate lyase and then phosphorylated to their diphosphate and triphosphate ribotides. It is finally incorporated into the parasites RNA.[16] Neither, formycin B nor allopurinol riboside is phosphorylated by nucleoside kinases found in mammalian cells and are nontoxic to mammals.[17]

It is relevant to note that the inhibition of nucleoside kinase,adenylosuccinate synthetase and adenylosuccinate lyase by formycin Band its analogues could be due to their competing as substrate for the parasite enzymes.[18] These findings provide a possible explanation of how purine analogs such as hypoxanthine and inosine might antagonize the antileishmanial activity of formycin.

The results presented here suggest that formycin B and its analogs provide good models for designing agents with antileishmanial activity. The formycin B molecule is of considerable interest because it has a carbon-carbon bond instead of carbon-nitrogen bond to bound the riboside moiety to the five-membered ring. This bond at 7–8 position is not broken in mammalian cells and thus, formycin B should resist metabolic degradation in humans.[19] The present study confirmed that formycin analogs possess an in vitro antileishmanial activity against *L. tropica* and *L. donovani* promastigotes.

CONCLUSIONS

1. Pentostam and ketoconazole have significant *in-vitro* antileishmanial activity against both *L. tropica* and *L. donovani* promastigotes.
2. Formycin B, formycin A, and allopurinol riboside proved to be potent chemotherapeutic agents for Leishmania.
3. Purine salvage enzymes appear to be targeted for chemotherapeutic attacks.

REFERENCES

1. Kumar A. Leishmania and leishmaniasis. Springer science, Business media, New York;2013.

2. Tiuman TS, Santos AO, Ueda Nakamura T, Dias Filho BP Celso Recent advances in leshmaniasis treatment. Intrernational Journal of Infection Diseases. 2011;15(8):525-532.
3. Hadighi R, Boucher P, Khamesipour A, Meamar AR, Roy G, Ouellette M, and Mohebali M Glucantimeresistant lieshmania tropica isolated from Iranian patients with cutaneous leishmaniasis are sensitive to alternative antilieshmania drugs. Parasitol.Res., 2007;101(5):1319-1322.
4. Sundar S, and Chatterjee M Visceral leishmaniasis – current therapeutic modalities. Indian.J. Med.Res. 2006;123:345352.
5. Hassan H, and Abdulla M Effect of miltefosine on growth and metabolism of Leishmania donovani and Leishmania tropica. Int. J. Curr. Res.Acad. Rev. 2017;5:95-101.
6. Burton K. Determination of DNA concentration with diphenylamine. Methods Enz., XII, 1967;163-166.
7. Plumer D An Introduction to practical Biochemestry. 2nd. ed., Mc Grawhill Book Com., UK,L td.Maiden Head,England, 1978;357.
8. Lowery OH, Rosebrough N, Farr A, and Randall R Protein measurement with the folin –phenol reagent. J. Biol.Chem. 1951;193:265-275.
9. Hassan H, and Coombs G Comparative study of the purine and pyrimidine metabolizing enzymes of rang of Trypanosomatids. Comp. Biochem. Physiol., 1986;84B:217-230.
10. Kirigi GM, Buchi MW, Mbui JK, Rashid JR, Kinoti DM, Nioroge SN, Basive F, Magiri C A successful treatment of a Kenyan case of unresponsive cutaneous leishmaniasis with a combination of pentostam and oral allopurinol :case report. East African Medical Journal, 2010;87(12):521-524.
11. Castinetti F, Guignat L, Giraud P, Muller M, Kamenicky P Ketaconazolein Cushing's disease: is it worth a try?. The journal of Clinical Endocrinology &Metabolism 2014;99(5): 1623-1630.
12. Patel RP, Patel H, Baria AH Formation and evalution of liposomes of ketoconazole. Int. J. Drug DelivTechnol. 2009;1(1):16–23.
13. McGwire BS, Satoskar AR. Leishmaniasis: clinical syndromes and treatment. QjM : An International journal of medicine, 2014;107)1):714.
14. Mondal S, Roy JJ, and Bera T Generation of adenosine triphosphate in Leishmania donovaniamastigotes forms. Acta parasitological 2014;59(1):1116.
15. Carson DA, Cottam HB, Jin Wu GCN, Takabayashi K Purine analogs.US Patent APP. 2013;13:175-791.
16. Vincent I M, and Barrett M P Metabolomic – based strategies for anti parasite drug discovery. Journal of biomolecular screening 2015;20(1):44-55.
17. Carter NS, Yates P, Arendt CS, Boitz JM, and Ullman B Purine and Pyrimidine Metabolism in Leishmania. Drug targets in kinetoplastid parasites, 2008;141-154.
18. Azzouz S, Lawton P. In vitro effect of purine and pyrimidine analogoues on Leishmania donovani and Leishmania infantum promastigotes and intracellular amastigotes. Acta parasitological, 2017;62(3):582-588.
19. Dorla TPC, Balasegaram M, Beijnen JH, Vries PJ Miltefosine: A review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis. Journal of Antimicrobial Chemotherapy, 2012;67(11):2576-2597.