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Antileishmanial activity of inosine analogs on promastigote forms of Iraqi *Leishmanial* species

Husain F. Hassan* and Iman N. Mahmood

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

Corresponding author

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A B S T R A C T

The growth inhibitory effects of inosine analogs, allopurinol, thiopurinol, allopurinol riboside, formycin B, thioformycin B, 7-deazainosine, 9-deazainosine, 8-azainosine and thio-7-deazainosine were tested against promastigotes of *Leishmania tropica*, *L.major* and *L.donovani*. All ten analogs were active against all the three species. The efficacy and metabolism of the formycin B, allopurinol riboside, 7-deazainosine and 9-deazainosine in leishmanial species were evaluated. These analogs affect several metabolic processes. First, is the increased catabolism of RNA and consequent reduction of protein synthesis. Second, inhibition of adenosine kinase, adenylosuccinate synthetase and adenylosuccinate lyase which reduces the organisms ability to synthesize ATP. Third, inhibition of IMP dehydrogenase and GMP reductase ought to be sufficient to half the interconversion of adenine nucleotides to guanine nucleotides and vice versa. The results suggests that such inosine analogs may have promise as antileishmanial agents.

Introduction

Pentavalent antimonials, the current drugs of choice, are widely used for treatment of leishmaniasis but frequently are not effective, often show adverse side effect and have associated toxicities at high dosages (1-3). Therefore, new classes of more effective and less toxic chemotherapeutic agents that are active against *Leishmania* are needed.

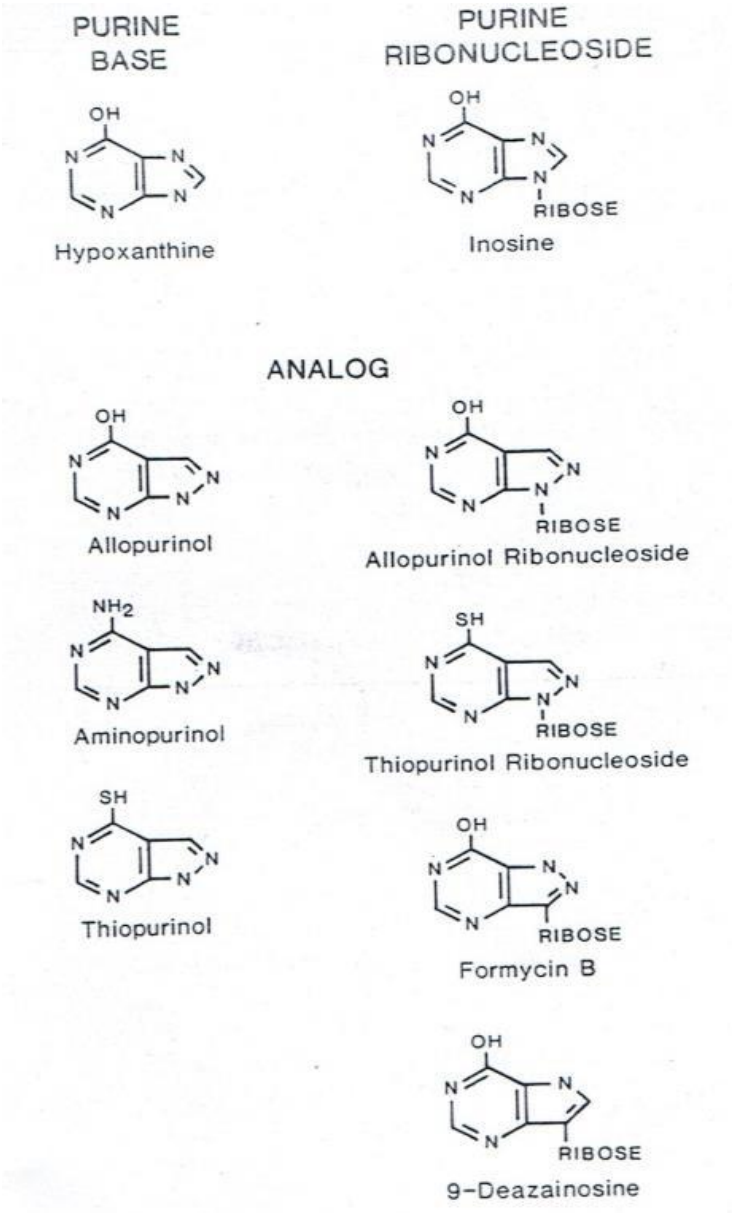
Purine metabolism in pathogenic hemoflagellate appears to offer chemotheraputically exploitable oppurtunities, not only it is different from their counter parts in human beings but also it is inhibited by pyrazolopyrimidine analogs (4-5). In addition, the enzymes of the salvage pathways are capable of accepting

the pyrazolopyrimidine ring as a purine and metabolic inhibitor (6). This property of the hemoflagellate enzymes does not occur in humans (7) and, for this reason, pyrazolopyrimidines offer promise as potential chemotherapeutic agents for management of leishmaniasis.

The prototype of this class of compounds, allopurinol [4-hydroxypyrazolo (3,4-d) pyrimidine] is non toxic to human beings and is active against *Leishmania* and *Trypanosoma* (8-9). Further investigation have demonstrated the therapeutic efficacy of allopurinol in cutaneous leishmaniasis and visceral leishmaniasis caused by *L.tropica* and *L. donovani* respectively.

Since allopurinol is an inosine analog (Fig. 1), we investigated several modifications of the inosine structure to determine which features of the molecule are important for activity against *Leishmania*.

Fig.1 Inosine analogs



Materials and Methods

Growth of organisms

Promatigotes of *L.tropica, L.major,L. donovani* were grown at 26CO in HOMEM medium supplemented with 10% (v/v) heat inactivated foetal calf serum as described previously (10). Streptomycin at 5 µg/ml and penicillin at 5 U/ml were added to culture media to inhibit bacterial growth.

Antileishmanial effect of inosine analogs

Cultures were initiated at 1x106 promastigotes /ml. The drugs were dissolved in IN NaOH at 50-100 times the concentrations used for experimentation and were sterilized by filtration (0.22µM Millipore filter). One day after seeding, the promastigotes were counted using an improved Neubauer hemocytometer, and the compound to be tested was aseptically added at the desired concentration taking care to adjust the pH with few drops of IN HCl to about 7.2. Each test was made in duplicate. The number of motile parasites present in the cultures were counted daily using hemocytometer, and the counts were compared with those of controls grown in the absence of the drugs. The 50% inhibitory dose (ID50), the drug concentration that caused a 50% reduction in growth compared with that in untreated control cultures, was determined from plots of cell growth versus drug concentration.

Estimation of ribonucleic acid (RNA)

To determine RNA content, promastigotes grown in the presence (alosence) of drug concentration causing 50% inhibition of growth were harvested by centrifugation at 3000 Xg at 4Co 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 0Co, and extracted twice at 0 Co for 30 min with 0.2 N PCA. Lipid was then removed by two extraction at 45 Co first with 75% (v/v) ethanol, and then with 10 ml of ethanol/ether (1:1), Finally, nucleic acids were extracted at 70 Co for 40 min with 10 ml of 0.5 N PCA. The extract was then stored at 4 Co 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 (11) using an orcinol reagent with yeast RNA as standard.

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 5ml 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 IN NaOH for 5 hr with continuous shaking. The supernatant which contains the protein was taken and diluted with IN NaOH to the standard volume (15ml). The protein content was estimated by the method of lowery *et al* (12) with bovine serum albumin as standard.

Preparation of cell extract

Mid log phase promastigotes(108cells/ml) were centrifuged at 4500 Xg for 10 min at 4 CO. 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 a MSE Soniprep 150 sec fitted with an exponential microprobe at 4 amplitude microns. Crude homogenates were centrifuged at 105000 Xg at 4 Co for 1 hr and the resultant supernatant was used as source of the enzyme.

Enzyme assays

The composition of the assay mixtures and the extinction coefficients used to calculate the enzyme activities are summarised in Table 1.All enzyme activities were assayed spectrophotometrically at 26Co (13) and expressed as nmol/ min / mg protein.

Results and Discussion

Effects of inosine analogs on growth of *Leishmania* species

As indicated in Table 2, all of these inosine analogs were inhibitory in vitro for the promastigotes of all three species of *Leishmania*, but their effects varied greatly from one species to another. *Leishmania tropica* was the most sensitive, *L.major* was the intermediate and *L.donovani* was the most resistant of the three species.The growth reduction rate took one generation (about 18 to 21 h) to become fully established. When observed under the light microscope, the inosine analogs- treated promastigotes were initially elongated and they became immobile and spherical.

The inhibitory effect of the inosine analogs was leishmanistatic for all three species, because promastigotes eventually resumed their normal growth rates, reaching control cellular growth in a 4-day period, when subcultured into medium free of the drug. In general, formycin B had the lowest 50% effective doses of the ten compounds tested in vitro. Thioformycin B was less active than formycin B. The estimated ID50 for thiopurinol was similar to that for allopurinol and the value for thiopurinol ribonucleoside was similar to that for allopurinol ribonucloside. The ID50 for 7-deazainosine and 9-deazainosine were low and they compares favorably with formycin B in this regard. The thio dervative of 7-deazainosine was relatively inactive, as was 8-azainosine against three species of *Leishmania*.

A variety of purine bases (adenine, guanine, xanthine and hypoxanthine) and purine nucleoside (adenosine, guanosine, xanthosine and inosine) were used in an attempt to reverse the action of the most effective inosine analogs (formycin B, allopurinol riboside, 7-deazainosine and 9-deazainosine) on three leishmanial species, at concentrations of 10 times that of the inosine analogs (Table 3 and Table 4).

Inosine was markedly effective at reversing the growth inhibition induced by inosine analogs in all three species. None of the purine bases or other purine nucleosides reversed the growth inhibition by inosine analogs in leishmanial species.

Effects of inosine analogs upon RNA and protein synthesis

To determine whether inosine analogs affect RNA metabolism, we have measured the RNA content of drug treated cells (Table 5). Formycin B, allopurinol riboside, 7-deazainosine and 9-deazainosine reduced the RNA content.These effects upon RNA content are reflected in protein synthesis. Formycin B, allopurinol riboside, 7-deazainosine and 9-deazainosine caused more than 80% decrease in protein content of *L.tropica*, *L.major* and *L.donovani* promastigotes (Table 6).

Table.1 Spectrophotometric assay conditions

Enzyme	EC	Substrate	Final concentration (mM)	Other constituent of reaction mixture[a]	product	nm(mM^{-1} cm)[b]
Kinase	2.7.1.20	Nucleoside	0.5	5 mM KCl, 1mM MgCl2, 0.1 mM PEP, 0.1 mM NADH, 1mM ATP, 0.1 UPK, 0.1 ULDH	Nucleotide	340 (-18.66)
Adenylosuccinate lyase	4.3.2.2	Succino AMP	0.1	5mM EDTA	AMP	280 (-10.7)
IMP dehydrogenase	1.2.1.14	IMP	1	100mM KCL, 0.5 mM NAD	XMP	340 (6.22)
GMP reductase	1.6.6.8	GMP	0.1	1mm EDTA, 10 MM DTT, 0.1 MM NADPH	IMP	340 (-6.22)

Abbreviations used are: IMP , inosine-5-monophosphate; GMP, guanosine-5-monophosphate; AMP, adenosine-5-monophosphate; 5-AMP, succinyladenosine-5-monophosphate; XMP, Xanthosine-5-monophosphate, PK, pyruvate kinase; LDH, lactate dehydrogenase. .
a- The buffer used was 50mM Tris-HCl, pH 7.2
b- Wavelength monitored (extinction coefficient).

Table.2 ID50 for various inosine analogs tested against the promastigote forms of *L.tropica*, *L.major* and *L.donovani*

Compound	ID_{50} (µM)		
	L. tropica	*L. major*	*L. donovani*
Allopurinol	75	80	100
Thiopurinol	70	88	96
Allopurinol riboside	5	10	17
Thiopurinol riboside	8	12	19
Formycin B	0.06	0.11	0.8
Thioformycin B	13	16	22
7-Deazainosine	0.5	0.9	1.5
9-Deazainosine	0.3	0.7	1.2
Thio-7-deazainosine	35	36	41
8-Azainosine	50	61	75

Note. The concentration of 9- deazainosine was 10 µg /ml/ and that of the antagonists was 100 µg/ml.

Table.3 Reversal of the action of formycin B (FoB) on three species of *Leishmania* by purines

Addition	Growth of organisms compared with control (%)		
	L.tropica	*L.major*	*L.donovani*
No FoB	100	100	100
FoB	9	18	34
FoB+adenine	8	19	37
FoB+ guanine	11	20	35
FoB+hypoxanthine	10	18	38
FoB+xanthine	11	16	31
FoB+adenosine	12	17	36
FoB+ guanosine	13	19	34
FoB+inosine	68	62	73
FoB+xanthosine	7	17	35

Note. For the experiments with *L.tropica,* FoB was present at a concentration of 5 µg/ml/. and the antagonists at concentrations of 50 µg/m/.For the experiments with *L.major* and *L.donovani*,FoB was present at a concentration of 10 µg /ml and the antagonists at concentrations of 100 µg/ml. The presence of the antagonists alone had no significance influence on the growth of the organisms.

Table.4 Reversal of the action of 9-deazainosine on three species of Leishmania by purines

Addition	Growth of organisms compared with control (%)		
	L.tropica	*L.major*	*L.donovani*
No 9-deazainosine	100	100	100
9- deazainosine	24	32	48
9- deazainosine+adenine	20	34	46
9- deazainosine+ guanine	22	30	47
9- deazainosine+hypoxanthine	26	34	49
9- deazainosine+xanthine	24	29	44
9- deazainosine+adenosine	25	33	48
9- deazainosine+ guanosine	26	30	50
9- deazainosine+inosine	72	66	61
9- deazainosine+xanthosine	22	32	40

Note: The concentration of 9- deazainosine was 10 µg /ml/ and that of the antagonists was 100 µg/ml.

Table.5 Effect of inosine analogs upon RNA synthesis

Treatment	*L. tropica*		*L. major*		*L. donovani*	
	µg RNA/10^8	% Decrease	µg RNA/10^8	% Decrease	µg RNA/10^8	% Decrease
Formycin B	121(120-122)*	46	128(124-132)	40	131(125-131)	44
Allopurinol riboside	124(119-129)	45	135(133-137)	36	154(150-158)	34
7-Deazainosine	113(110-116)	50	130(126-134)	39	160(158-162)	31
9-Deazainosine	142(139-145)	37	146(145-147)	31	157(153-161)	32
Control**	224±11	-	212±9	-	232±14	-

*Data expressed as a mean of two experiments.
**The mean value of control (µg/108 cells) is also shown.

Table.6 Effect of inosine analogs upon protein synthesis

Treatment	*L. tropica*		*L. major*		*L. donovani*	
	µg Prot/10^8	% Decrease	µg Prot/10^8	% Decrease	µg Prot/10^8	% Decrease
Formycin B	55(53-57)*	84	48(46-50)	85	51(50-52)	86
Allopurinol riboside	62(60-64)	82	58(55-61)	82	60(56-64)	83
7-Deazainosine	59(55-63)	83	64(58-70)	80	56(52-60)	84
9-Deazainosine	64(60-68)	81	60(57-63)	81	62(59-65)	82
Control**	340±13	-	322±11	-	352±15	-

*Data expressed as a mean of two experiments.
**The mean value of control (µg/108 cells) is also shown.

Table.7 Effect of inosine analogs on *Leishmania tropica* purine salvage enzymes

Purine analogs	% Inhibition*				
	Kinase	Lyase	Synthetase	Dehydrogenase	Reductase
Formycin B	72	65	79	85	57
Allopurinol riboside	76	59	81	80	50
7-Deazainosine	69	61	73	89	55
9- Deazainosine	70	63	74	76	51
Control**	25±1	3±0.5	5±1	51±6	11±2

***** Inhibitors were preincubated with the enzyme for 10 min at 26 Co and the reaction was initiated by the addition of substrate.
******The mean value of control represent the specific activity expressed as nmol/min/mg protein

Table.8 Effect of inosine analogs on *Leishmania major* purine salvage enzymes

Purine analogs	% Inhibition*				
	Kinase	Lyase	Synthetase	Dehydrogenase	Reductase
Formycin B	69	57	82	76	52
Allopurinol riboside	67	59	78	75	53
7-Deazainosine	70	62	70	69	54
9- Deazainosine	72	65	73	72	50
Control**	19±1	2±0.1	6±0.5	63±3	16±3

***** Inhibitors were preincubated with the enzyme for 10 min at 26 Co and the reaction was initiated by the addition of substrate.
******The mean value of control represent the specific activity expressed as nmol/min/mg protein.

Table.9 Effect of inosine analogs on *Leishmania donovani* purine salvage enzymes

Purine analogs	% Inhibition*				
	Kinase	Lyase	Synthetase	Dehydrogenase	Reductase
Formycin B	73	61	74	80	58
Allopurinol riboside	61	66	70	83	56
7-Deazainosine	68	59	71	77	57
9- Deazainosine	67	60	76	74	53
Control**	31±5	6±1	9±2	59±4	20±2

***** Inhibitors were preincubated with the enzyme for 10 min at 26Co and the reaction was initiated by the addition of substrate.

******The mean value of control represent the specific activity expressed as nmol/min/mg protein.

To determine wether the reduced RNA content of inosine analogs – treated cells was due to inhibition of purine salvage enzymes, cells were extracted and assayed for enzymatic activites. A significant inhibition of nucleoside kinase, adenylosuccinate synthestase, adenylosuccinate lyase and IMP dehydrogenase was seen in promastigotes of all three species of *Leishmania* exposed to inosine analogs (Table 7-9). These results indicate that the increased catabolism of RNA caused by the inosine analogs modulates the utilisation of exogenous purine.

The observation reported here suggest that inosine analogs provide good models for the design of agents with antileishmanial activity. Of the ten different analogs tested, four compounds (formycin B, 9-deazainosine, 7-deazainosine and allopurinol riboside) were the most promising, as they strongly inhibited multiplication of culture forms of three species of the *Leishmania*. In addition, it was found that *L.donovani* was less sensitive to inosine analogs than *L.tropica* and *L.major*. The reason for this differences in unclear, but it may reflect quantitative differences in either transport or enzymatic activites between the three organisms. Similar difference among various Trypanosoma species with respect to inosine analogs sensitivity have also been found (7).

The effects of allopurinol, thiopurinol and their ribonucleosides on the growth of these leishmaniae were somewhat different. Allopurinol riboside and thiopurinol riboside were more active than the bases allopurinol and thiopurinol, against *L.tropica*, *L.major* and *L.donovani*. The inhibition by allopurinol riboside and thiopurinol riboside were reversed by inosine, whereas inosine had no effect on that caused by allopurinol and thiopurinol. The similar antileishmanial activity of allopurinol to thiopurinol and of allopurinol riboside to thiopurinol riboside in this model confirms the data from infected tumer macrophages(14-15). However, the apparent mechanism of allopurinol riboside and thiopurinol riboside is that they are metabolized to ribotide by the parasites nucleoside kinase, which then incorporate in to RNA (16). It has been reported here, that allopurinol riboside and thiopurinol riboside also inhibit nucleoside kinase activity, and this could be due to their competing as substrates for the parasite enzymes(6). These observations provide one possible explanation as to how inosine antagonise the antileishmanial activities of allopurinol riboside and thiopurinol riboside.

The vastly increased antileishmanial activity of formycin B compared with allopurinol riboside indicates that leishmanial promastigotes clearly distinguish these two close analogs of inosine. Promastigotes in

this model do not distinguish between allopurinol riboside and thiopurinol riboside, but the organisms do distinguish formycin B from thioformycin B and 7-deazainosine from thio-7-deazainosine. However, the differential activity of these inosine analogs compared with their thio derivatives might not be clinically expoitable.

7-deazainosine and 9-deazainosine are of considerable interest not only because they supports the general theme of inosine analogs as potential chemotherapeutic agents for several protozoan diseases but also because they have a carbon-carbon bond between the heterocyclic ring and the ribose. This bond is not broken in mammalian cells and these compounds should resist metabolic degradation in humans (7).

The data presented here suggest that inosine analogs (Formycin B, 9-deazainosine, 7-deazainosine and allopurinol riboside) inhibit the growth of leishmanial promastigotes by inhibition of enzymes which carry out interconversion of purine nucleotide and by the mechanisms related to decrease in the net amount of RNA per cell and its loss of function, as measured by protein synthesis. Promastigotes are surrounded by high concentrations of host cell ATP and GTP. Presumably, these nucleotides are utilized by the parasite following cleavage to their respective ribonucleosides or bases and under these conditions purine interconversion enzymes would be necessary. The decreased in amount of RNA and the inhibition of protein synthesis which are related to the inosine analogs concentration suggest that there are one or more other mechanisms of inhibition of cell growth active at the level of RNA function. An inosine analog which can be phosphorylated and aminated by a protozoan system, but not by a mammalian system would be an excellent candidate agent for antiprotozoan chemotherapy.

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