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Inosine Analogs as Anti-Leishmanial Agents

Petrie Rainey¹, Patricia A. Nolan², Leroy B. Townsend³, Roland K. Robins⁴, Jack J. Fox⁵, John A. Secrist III⁶ and Daniel V. Santi^{2,7}

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Abstract: Several criteria were used to select a number of inosine analogs as potential growth inhibitors of the protozoan parasite *Leishmania tropica*. Of nine compounds tested, seven showed a high degree of selective toxicity towards *L. tropica* promastigotes as compared to mouse L1210 cells; these include analogs of formycin B, 7-substituted analogs of 7-deazainosine and analogs of inosine in which the sugar moiety has been modified to confer metabolic stability. The metabolism of 7-deazainosine in *L. tropica* promastigotes was shown to involve conversion to cytotoxic adenosine nucleotide analogs (tubercidin derivatives) that become incorporated into RNA. The results suggest several new classes of compounds which have potential as anti-leishmanial agents.

Inosine analogs such as allopurinol riboside (1–3), thiopurinol riboside (2, 4), formycin B (2, 3, 5–8) and 3'-deoxyinosine (9) inhibit the growth of the pathogenic protozoan *Leishmania* at concentrations that have little effect on mammalian cells. Accordingly, these and related compounds offer promise as chemotherapeutic agents for the treatment of leishmaniasis. Metabolic studies of the aforementioned analogs have revealed features important for their apparent selective toxicity. First, these analogs possess modified sugar or base

moieties that confer resistance towards catabolism. Second, the selectivity appears to result from differences in purine salvage pathways in the host and parasite. *Leishmania* has a nucleoside phosphotransferase that effectively converts these inosine analogs to their corresponding nucleoside 5'-monophosphates (1, 4, 6–9), whereas mammalian cells are either ineffective or less effective in phosphorylation of these compounds (8, 10). Third, the 5'-monophosphates of these analogs are either directly cytotoxic to *Leishmania* or are further converted to other cytotoxic metabolites in these organisms. Thiopurinol riboside-5'-monophosphate is believed to be the cytotoxic metabolite of thiopurinol in *Leishmania* (4), whereas the effects of allopurinol riboside and formycin B appear to result from conversion of their 5'-monophosphates to corresponding cytotoxic adenosine nucleotide metabolites and their subsequent incorporation into RNA (1, 6–8). 3'-Deoxyinosine is converted to adenosine nucleotide analogs, but is not incorporated into RNA (9).

The aforementioned findings provide a rationale for the design of additional potentially useful anti-leishmanial agents. In the present work, we describe the growth inhibitory effects of a number of inosine analogs towards *L. tropica* promastigotes and L1210 mouse leukemia cells. In addition, we have examined the metabolism of one of these analogs, 7-deazainosine, and found it to be similar to that reported for allopurinol riboside and formycin B.

Materials and Methods

L. tropica promastigotes (Clone POJ2 of Iran strain 252, obtained from B. Ullman) were grown at 26°C in room air supplemented to 9% CO₂ using a defined medium consisting of Dulbecco's modified Eagle's medium containing 1% glucose, 0.3% bovine serum albumin, 5 mg/l hemin and 10 μM hypoxanthine. Stock cultures were maintained by reseed-

¹Department of Laboratory Medicine, School of Medicine, University of California, San Francisco, CA 94143, USA.

²Department of Biochemistry and Biophysics and Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94143, USA.

³Department of Medicinal Chemistry, College of Pharmacy, University of Michigan, Ann Arbor, MI 48109, USA.

⁴Cancer Research Center, Department of Chemistry, Brigham Young University, Provo, UT 84602, USA.

⁵Laboratory of Organic Chemistry, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA.

⁶Southern Research Institute, P.O. Box 55305, Birmingham, Alabama 35255, USA.

⁷To whom correspondence should be addressed.

into fresh medium at 3 to 4 day intervals. L-1210 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat treated horse serum (11).

Growth inhibition studies were carried out in standard 12-well plates (area 2.0 cm²/well, Flow Laboratories) using 1.0 ml of growth medium containing varying amounts of inhibitor. Cells were seeded at 5 x 10⁵ cells/ml for *L. tropica* promastigotes and 5 x 10⁴ cells/ml for L-1210 cells. Cells were counted daily for three to four days during logarithmic growth using a Coulter Counter ZBI. The percentage of control growth is 100 times the slope of the plot of the logarithm of the number of cells versus time, divided by the slope of the plot for the untreated control culture. EC₅₀ refers to that concentration of drug resulting in a growth rate equal to 50% of the rate in a drug-free medium.

Formycin B, tubercidin, tubercidin 5'-monophosphate, *C. atrox* venom and *C. adamanteus* venom were obtained from Sigma. [³H]Tubercidin (11 Ci/mmol) was obtained from Moravék Biochemicals. 1-Methylformycin B (2) (12), 2-methylformycin B (3) (13), 7-thioformycin B (4) (14, 15), 7-selenoformycin B (5) (16), 7-deazainosine (6) (17), 7-bromo-7-deazainosine (7) (18), 7-carboxamido-7-deazainosine (8) (19), 7-cyano-7-deazainosine (9) (19), and carbocyclic inosine (10) (20) were compounds whose syntheses were previously described. 2'-Fluoro-ara-hypoxanthine (11) (21) was provided by J. J. Fox.

[³H]7-Deazainosine was prepared by treating 440 nmol [³H]tubercidin (1.0 Ci/mmol) in 0.3 ml water with 80 mg sodium nitrite and 130 μl glacial acetic acid at 75°C for 1 h. The resulting mixture was concentrated under an argon stream to 0.25 ml and purified by HPLC on a Lichrosorb RP-18 column (4.5 x 250 mm; Altex) using a 100 ml linear gradient of 0–50% aqueous methanol as previously described (7). The radioactive peak corresponding to 7-deazainosine (retention volume 19 ml) was collected. Rechromatography of an aliquot of the purified material with authentic 7-deazainosine confirmed comigration and demonstrated that less than 0.01% of [³H]tubercidin could have been present. 7-Deazainosine 5'-monophosphate was prepared for use as a chromatographic marker from tubercidin 5'-monophosphate by nitrous acid deamination as previously described for the preparation of formycin B 5'-nucleotides (7).

The separation, quantitation and identification of the metabolites formed upon treatment of *L. tropica* promastigotes with [³H]7-deazainosine was carried out by procedures analogous to those described for the determination of the metabolites of [³H]formycin B (7). Briefly, 40 x 10⁶ cells were treated with 8.0 μM [³H]7-deazainosine (1.0 Ci/mmol) for 4 h. The cells were then extracted with 0.6 M trichloroacetic acid. After neutralization, the extract was subjected to HPLC on a Partisil SAX column using a phosphate gradient. Metabolites were identified by comparison of their retention volumes with authentic compounds or in the case of tubercidin 5'-di- and triphosphates, by their appearance in regions of the chromatogram characteristic of nucleoside 5'-di- and triphosphates and by their hydrolysis to tubercidin 5'-monophosphate. All metabolites were further characterized by UV spectrum and by degradation to the parent nucleoside using *C. atrox* venom. RNA incorporation was determined by hydrolyzing the RNA in the trichloroacetic acid insoluble pellet with the use of 0.3 M potassium hydroxide, followed by reprecipitation of protein and DNA with 0.6 M trichloroacetic acid. The resulting RNA extract was neutralized and digested with alkaline phosphatase and *C. adamanteus* venom and the resulting nucleosides

analyzed by HPLC. Incorporation into DNA was determined from radioactivity remaining in the acid insoluble pellet after removal of RNA.

Results

Table I lists the EC₅₀ values of a number of inosine analogs for growth inhibition of *L. tropica* promastigotes. Also provided are inhibitory properties of these analogs on the growth of L-1210 mouse cells which were used as a model to assess the effects of these compounds on mammalian cells.

Formycin B (1) has previously been shown to be an excellent inhibitor of *L. tropica* growth (7) and serves here as a standard for evaluation of the other analogs. With exception of the 2-methyl analog, the formycin B derivatives are all quite potent

Table I. Growth Inhibition of *L. tropica* Promastigotes and L-1210 Cells by Inosine Analogs.

Compound	EC ₅₀ (μM)	
	<i>L. tropica</i>	L-1210
1 formycin B	0.025	80
2 1-methylformycin B	0.60	> 100 ^a
3 2-methylformycin B	100	— ^b
4 7-thioformycin B	0.40	70
5 7-selenoformycin B	0.20	10
6 7-deazainosine	2.7	10
7 7-bromo-7-deazainosine	2.4	100
8 7-carboxamido-7-deazainosine	1.1	>100 ^c
9 7-cyano-7-deazainosine	40	>100 ^a
10 carbocyclic inosine	0.15	>100 ^d
11 2'-fluoro-ara-hypoxanthine	0.6	>100 ^d

^a10% inhibition at 100 μM. ^bNot determined. ^c20% inhibition at 100 μM. ^dNo inhibition at 100 μM.

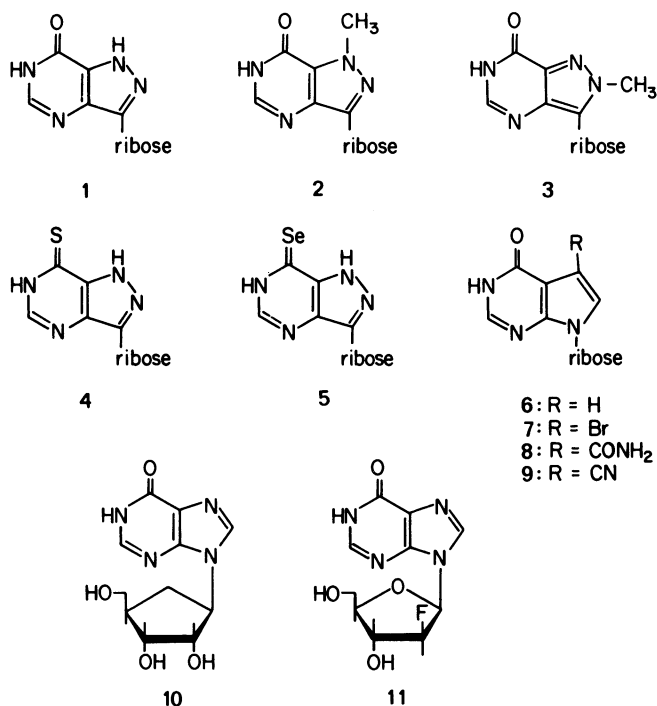


Fig. 1 Structures of inosine analogs.

inhibitors of *L. tropica* with EC_{50} values in the sub-micromolar range; 2-methylformycin B (**3**) showed an EC_{50} of 100 μ M. Towards L-1210 cells, selenoformycin B (**5**) was somewhat toxic, having an EC_{50} value only 50-fold higher than that seen with *L. tropica*. Thioformycin B (**4**) had only a moderate effect on the growth of L-1210 cells and 1-methylformycin B (**3**) showed only 10% inhibition of growth at 100 μ M.

In accord with previous reports (2, 3), 7-deazainosine (**6**) was found to be a good inhibitor of *L. tropica* growth, but it was also relatively toxic towards the L-1210 cell line. 7-Deazaadenosine (tubercidin), an alternate precursor of the adenosine nucleotide metabolites of 7-deazainosine (see below), had EC_{50} values of 0.5 and 0.05 μ M, respectively, for *L. tropica* and L-1210 cells. Placement of bromo- or carbox-amido-groups at the 7-position of 7-deazainosine had little effect on the growth inhibition of *L. tropica*, but greatly reduced the toxicity towards L-1210 cells. In contrast, the 7-cyano substituent significantly reduced the inhibition of growth in *L. tropica* as well as L-1210 cells.

Carbocyclic inosine (**10**) and 2'-fluoro-ara-hypoxanthine (**11**), both of which have modifications in the ribose moiety, inhibited the growth of *L. tropica* with EC_{50} values under 1 μ M and showed no inhibition of L-1210 cell growth at a concentration of 100 μ M.

Figure 2 shows the HPLC profile of the extract obtained from *L. tropica* promastigotes after a 4 h exposure to 8.0 μ M 7-[3 H]deazainosine. The major metabolites include 7-deazainosine monophosphate (0.45 pmol/ 10^6 cells), tubercidin diphosphate (0.42 pmol/ 10^6 cells) and tubercidin triphosphate (1.56 pmol/ 10^6 cells). The 7-deazainosine and tubercidin monophosphate peaks overlapped and together comprised 0.89 pmol/ 10^6 cells. Three minor radioactive peaks with retention volumes of 51, 69 and 75 ml were not identified. In addition, radioactive tubercidin was found to be incorporated into RNA to an extent of 0.50 pmol/ 10^6 cells. No radioactivity was detected in DNA.

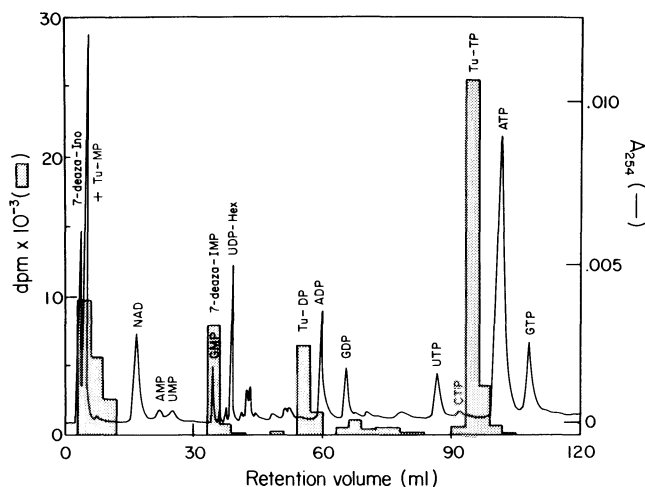


Fig. 2 HPLC of the acid-soluble extract from 1.4×10^7 *L. tropica* promastigotes treated with 8.0 μ M [3 H]7-deazainosine for 4 h. The extract was separated on a Partisil SAX column using a phosphate gradient as previously described (7). The absorbance of the column effluent was continuously monitored at 254 nm. The histogram bars indicate the radioactivity in each 3.0 ml fraction. 7-deza IMP, 7-deza inosine 5'-monophosphate; Tu-MP, Tu-DP, TU-TP, tubercidin 5'-mono-, di-, and triphosphate, respectively; UDP-Hex, UDP-glucose, or UDP-galactose, or both.

Discussion

A number of inosine analogs have been reported which are potent and specific inhibitors of *Leishmania* (1–9). Studies of the metabolism of such inhibitors have suggested criteria which aid in the selection and design of other inosine analogs as potential anti-leishmanial agents. First, such analogs should have the capability of being converted to their corresponding 5'-monophosphates by the nucleoside phosphotransferase that is unique to the parasite. Second, the resulting nucleotide analog must either itself be cytotoxic or have the ability to be further converted to a cytotoxic agent. Third, the analog should be modified so that the bond between the sugar and base moieties is resistant towards cleavage. The compounds selected for the present study are inosine analogs that possess stable linkages between the ribose and purine moieties and also have the potential of fulfilling the former two criteria. They include analogs of formycin B, 7-substituted analogs of 7-deazainosine, and analogs of inosine in which the sugar moiety has been modified to confer metabolic stability.

Formycin B (**1**) is a potent inhibitor of *L. tropica* and has little effect on the growth of mammalian cells in tissue culture (2, 3, 5–10). 1-Methylformycin B (**2**), thioformycin (**4**) and selenoformycin (**5**) were all potent inhibitors of *L. tropica* growth with EC_{50} values in the submicromolar range; in contrast, methylation at the 2-position of formycin B resulted in a significant decrease in cytotoxicity. All of the formycin analogs that were active against *Leishmania* were significantly less toxic towards the mammalian cells examined and warrant further examination.

7-Deazainosine (**6**) has previously been shown to be a good inhibitor of growth of both *L. donovani* promastigotes and *L. tropica* amastigotes, but produced cytotoxic effects in mammalian cells at only slightly higher concentrations (2, 3). Likewise, we found that it strongly inhibited both *L. tropica* promastigotes and L-1210 cells. However, introduction of a substituent at the 7-position markedly reduced toxicity for L-1210 cells in all three compounds tested. With the 7-bromo- (**7**) and 7-carboxamido (**8**) derivatives this was achieved without any decrease in potency against the *Leishmania*. These results suggest that substitution at the 7-position of 7-deazainosine could provide promising compounds with selective anti-leishmanial activity.

The formycin B and 7-deazainosine analogs have been modified in the purine moiety to confer stability to the sugar-base linkage. This stability can also be accomplished by modification of the sugar moiety, as in carbocyclic inosine (**10**) and 2'-fluoro-ara-hypoxanthine (**11**) (22). We have shown here that these two compounds inhibit the growth of *L. tropica* at sub-micromolar concentrations but show no inhibition of L-1210 cells at concentrations as high as 100 μ M. Also, it has been recently reported that a similar analog, 3'-deoxyinosine, strongly inhibits *L. tropica* but is only mildly cytotoxic towards mammalian cells (9). Clearly, analogs of inosine that are modified in the sugar moiety are good candidates for selective anti-leishmanial agents.

The metabolism and mechanism of action of the compounds studied here remain largely undetermined because of the unavailability of radiolabeled compounds. However, we were able to prepare [3 H]7-deazainosine from the available [3 H]tubercidin and have demonstrated that 7-deazainosine (**6**) undergoes the same metabolic conversions seen with formycin B and allopurinol riboside, namely, phosphorylation to an IMP analog followed by conversion to AMP, ADP and ATP

analogs and incorporation into RNA. The adenosine nucleotide analogs are derivatives of tubercidin, an extremely cytotoxic compound (23) for both *L. tropica* ($EC_{50} = 0.5 \mu\text{M}$) and L-1210 cells ($EC_{50} = 0.05 \mu\text{M}$).

It seems likely that 1-methylformycin B (2) and the 7-substituted 7-deazainosine analogs (7-9) are metabolized by the parasite to cytotoxic nucleotides in a manner similar to the parent compounds. 7-Thioformycin B (4) and 7-selenoformycin B (5) are chemically more similar to thiopurinol riboside and, like the latter, are probably not metabolized beyond their 5'-monophosphate derivatives. Carbocyclic inosine (10) and 2'-fluoro-ara-hypoxanthine (11) both possess an intact hypoxanthine moiety and are likely metabolized to adenosine nucleotide analogs in the same manner as inosine, formycin B (1) and 7-deazainosine (6), and may also be incorporated into RNA, or in the case of 2'-fluoro-ara-hypoxanthine (11), perhaps DNA. These mechanisms are speculative and must be confirmed with radiolabeled compounds.

In summary, we have used criteria that have emerged from studies of the metabolism and toxicity of several inosine analogs in *Leishmania* to select nine new compounds for screening as selectively toxic agents. Of these, seven have demonstrated potent anti-leishmanial activity at concentrations where their effects on L-1210 cells were minimal. These results suggest several classes of compounds that warrant further examination as anti-leishmanial agents. These include 1-substituted analogs of formycin B and thioformycin B, 7-substituted analogs of 7-deazainosine, and inosine analogs with modifications in the ribose moiety that confer stability on the glycosidic bond.

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