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Biological Action of Inosine Analogs in *Leishmania* and *Trypanosoma* spp.

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Previous investigations have suggested that inosine analogs would be good models for the development of chemotherapeutic agents active against pathogenic hemoflagellates. We have systematically modified the five-membered heterocyclic ring of six inosine analogs and tested them for their antiprotozoal activities and toxicity to a mammalian cell line. All six analogs were very active against the three protozoan pathogens *Leishmania donovani*, *Trypanosoma cruzi*, and *Trypanosoma gambiense*. Two of the six, 9-deazainosine and allopurinol ribonucleoside, had very little toxicity for mouse L cells and offer promise as potential chemotherapeutic agents.

Purine metabolism in the pathogenic hemoflagellates is unique in that it can be inhibited by pyrazolopyrimidine analogs. This is due, in part, to the fact that there is no de novo synthesis (2, 6, 10, 18, 31) and, more importantly, to the fact that the enzymes of the salvage pathways will accept the pyrazolopyrimidine ring as a purine. This property of the hemoflagellate enzymes does not occur in humans (7, 12, 22) and, for this reason, pyrazolopyrimidines offer promise as potential chemotherapeutic agents for the management of leishmaniasis and some forms of trypanosomiasis.

The prototype of this class of compounds is allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine), which was the first such compound shown to be active against leishmania (14, 21, 25) and subsequently against *Trypanosoma cruzi* (1, 16) and the African trypanosomes (15). Further investigation demonstrated that the ribonucleosides of pyrazolopyrimidines were as active as, and in some cases more active than, the corresponding bases. Allopurinol ribonucleoside (4-hydroxypyrazolo[3,4-*d*]pyrimidine ribonucleoside) (24) and 4-thiopyrazolo pyrimidine ribonucleoside (17) were the first pyrazolopyrimidine ribonucleosides demonstrated to have antileishmanial action in vitro. Subsequently, formycin B (7-hydroxypyrazolo[4,3-*d*]pyrimidine ribonucleoside) was shown to have antileishmanial activity (5). Its metabolism is identical to that of allopurinol ribonucleoside (23, 26).

Since these compounds are inosine analogs (Fig. 1), we investigated several modifications of the inosine structure to determine which features of the molecule are important for activity against leishmania and trypanosomes yet prevent toxicity to mammalian hosts. The five-membered heterocyclic ring was chosen for modification since both the 3,4-*d* (allopurinol and thiopurinol ribonucleoside) and the 4,3-*d* (formycin B) configuration of the pyrazolopyrimidine ring were active against the hemoflagellates (Fig. 1).

Organisms were grown as described previously (8, 16, 18) in the presence or absence of the compounds in question (10 μg/ml), and the results were expressed as a percentage of the control. Doses effective against 50% of the organisms were determined by triplicate counting of organisms in a Coulter ZBI counter and determining a mean value. Agents which inhibited growth to less than 50% of the control were tested further by titration from 10 to 0.1 μg/ml. Concentration curves were done with mouse L cells as the mammalian model and with three major pathogenic hemoflagellates: *Leishmania donovani*, *Trypanosoma cruzi*, and *Trypanosoma gambiense*. *L. donovani* S1 and *T. cruzi* Costa Rica were obtained from Stuart Krassner, Department of Developmental and Cell Biology, University of California, Irvine. *T. gambiense* TH114 from the Institut fur Schiffsund Tropenkrankjeiten, Hamburg, West Germany, was obtained from R. Brun, Schweizer Tropeninstitut, Basel, Switzerland. The allopurinol ribonucleoside and 8-azainosine were obtained from Burroughs-Wellcome Co., Research Triangle Park, N.C.; formycin B was purchased from Sigma Chemical Co., St. Louis, Mo.; and the other compounds were synthesized at the Sloan-Kettering Institute, New York, N.Y. 9-Deazainosine was synthesized by the procedure of Lim et al. (13), 7-thia-7,9-dideazainosine was synthesized by the method of Wren et al. (34), and 7-deazainosine was synthesized by the method of Mizuno et al. (19).

All of these inosine analogs were active against the three pathogens in vitro (Table 1). In general, the compounds had 50% effective doses in the range of 1 to 10 μM and were comparable to allopurinol ribonucleoside, which already has been shown to be active (15). Formycin B had the lowest 50% effective doses, but the only major difference was with respect to *T. gambiense*, against which it was very effective. Allopurinol ribonucleoside was relatively inactive against *T. gambiense*, as was 8-azainosine against *L. donovani*. Three of these analogs, 7 deaza-, 8-aza-, and 7-thia-7,9-dideazainosine, were relatively toxic to mouse L cells. There is no common theme among these that is evident from the structures. Of the three remaining agents, two have already been shown to have considerable antiprotozoal activity (allopurinol ribonucleoside and formycin B (5, 15, 23, 24, 26). The former is not metabolized by mammalian cells as a nucleotide and is virtually without toxicity. The latter, although substantially more active against *T. cruzi* and *T. gambiense*, is relatively more toxic to L cells. It is neither phosphorylated nor cleaved by some mammalian systems (32, 38), and the 50% lethal dose in mice after the intrave-

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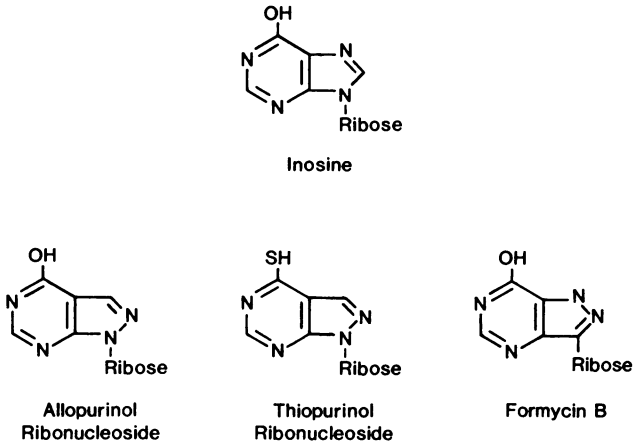
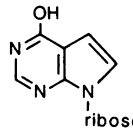
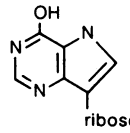
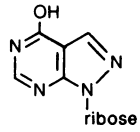
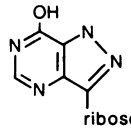
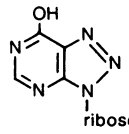
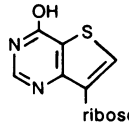


FIG. 1. Pyrazolopyrimidine analogs of inosine.

nous injection of formycin B is about 1,000 mg/kg (30). In that system, formycin B was two to four times less toxic than formycin A, the adenosine analog. It is known, however, to inhibit several enzymes of nucleic acid metabolism (20, 28) and, in mouse L cells, it is incorporated into RNA (T. Spector, T. E. Jones, S. W. LaFon, D. J. Nelson, R. L. Berens, and J. J. Marr, Biochem. Pharmacol., in press). This latter study demonstrated that mouse L cells are capable of phosphorylating formycin B and converting this nucleotide to all of the corresponding adenine nucleotide analogs. The conversion of formycin B to the nucleotides of formycin A raises the possibility that formycin A could be generated from formycin B in humans. Indeed, Berman et al. (4) have demonstrated that formycin A nucleotides can be formed from formycin B in human macrophages, and the toxicity of a series of formycin analogs to *L. tropica* was paralleled by their toxicities to these macrophages (3). Glazer and Lloyd (9) have shown that formycin A can be

TABLE 1. Cytotoxicity of inosine analogs[a]

Compound	Mouse L Cells	Leishmania donovani	Trypanosoma cruzi	Trypanosoma gambiense
7-Deazainosine 	20	<2	3	6
9-Deazainosine 	>1000	1	1	1
Allopurinol riboside 	>2000	7	2-10	20
Formycin B 	200	1	0.5	0.02
8-Azainosine 	2	35	4.5	9
7-Thia 7,9-Dideazainosine 	15	<2	7	7

[a]Numbers represent effective doses for 50% of cells (micromolar concentrations).

incorporated into the DNA of human colon carcinoma cells. Formycin A is a cytotoxic analog of adenosine (11, 32) and is one of the most effective analogs to replace adenosine in cellular reactions (33). The triphosphate of this compound can substitute for ATP as a substrate for aminoacyl-tRNA synthetase (33). Robbins et al. (27) have shown that formycin A has a close steric resemblance to adenosine and predicted that it would resemble the purine nucleoside in its enzymatic reactions. Formycin B also has shown some toxicity to mouse macrophage tissue culture (J774) (5). The evidence of toxicity for mammalian tissue culture systems was supported in this study since the toxic concentration for mouse L cells, although much higher than the 50% effective dose for the protozoans, was also much lower than that found for allopurinol ribonucleoside and 9-deazainosine.

This latter nucleoside is of considerable interest not only because it supports the general theme of inosine analogs as potential chemotherapeutic agents for several protozoan diseases but also because it has a carbon-carbon bond between the heterocyclic ring and the ribose. This bond is not broken in mammalian cells (11, 33), and 9-deazainosine should resist metabolic degradation in humans. The toxicity of this compound is low in mouse L cells, in excess of 1 mM, and it compares favorably with allopurinol ribonucleoside in this regard. The latter has been shown to be without toxicity to animals and humans in relatively high doses (unpublished data).

These data and published investigations indicate that inosine analogs are very reasonable models for the design of agents with antiprotozoal activity. The metabolism by and toxicity to animal cells will vary from one compound to another and, at this time, cannot be predicted from the structural formulas. If the inosine analogs are converted to the corresponding adenosine analog by mammalian cells, these agents probably will be very toxic (29). It has been shown, for example, that 9-deazaadenosine is quite toxic to human pancreatic carcinoma and human colon carcinoma cells (M. Y. Chu, L. B. Landry, and G. W. Crabtree, Abstr. Proc. Am. Assoc. Cancer Res., p. 306, 1983; R. I. Glazer and K. Hartman, Abstr. Proc. Am. Assoc. Cancer Res., p. 294, 1983), owing presumably to the fact that it does not react well with mammalian adenosine deaminases. In this study with human pancreatic carcinoma cells, it was shown that the toxicity was due to the incorporation of this compound into nucleoside mono-, di-, and triphosphates and RNA. It also inhibited the biosynthesis of nucleoside phosphoderivatives and DNA (Chu et al., Abstr. Proc. Am. Assoc. Cancer Res.). The investigation with human colon carcinoma cells also demonstrated that 9-deazaadenosine was toxic and that this toxicity was associated with the incorporation of the compound into RNA and DNA (Glazer and Hartman, Abstr. Proc. Am. Assoc. Cancer Res.). 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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LITERATURE CITED

1. **Berens, R. L., J. J. Marr, F. Steele Da Cruz, and D. J. Nelson.** 1982. Effect of allopurinol on *Trypanosoma cruzi*: metabolism and biological activity in intracellular and bloodstream forms. Antimicrob. Agents Chemother. **22**:657–661.
2. **Berens, R. L., J. J. Marr, S. W. LaFon, and D. J. Nelson.** 1981. Purine metabolism in *Trypanosoma cruzi*. Mol. Biochem. Parasitol. **3**:187–196.
3. **Berman, J. D., L. S. Lee, R. K. Robins, and G. R. Revankar.** 1983. Activity of purine analogues against *Leishmania tropica* within human macrophages in vitro. Antimicrob. Agents Chemother. **24**:233–236.
4. **Berman, J. D., P. Rainey, and D. V. Santi.** 1983. Metabolism of formycin B by *Leishmania* amastigotes in vitro. Comparative metabolism in infected and uninfected human macrophages. J. Exp. Med. **158**:252–258.
5. **Carson, B. A., and K.-P. Chang.** 1981. Phosphorylation and antileishmanial activity of formycin B. Biochem. Biophys. Res. Commun. **100**:1377–1383.
6. **Ceron, C. R., R. A. Caldas, C. F. Feliz, M. H. Mundim, and I. Roitman.** 1979. Purine metabolism in trypanosomatids. J. Protozool. **26**:479–483.
7. **Elion, G. B., A. Kovensky, and G. H. Hitchings.** 1963. Metabolic studies of allopurinol, an inhibitor of xanthine oxidase. Biochem. Pharmacol. **15**:863–867.
8. **Fish, W. R., J. J. Marr, and R. L. Berens.** Purine metabolism in *Trypanasoma cruzi*. Antimicrob. Agents Chemother. **22**:288–292.
9. **Glazer, R. I., and L. S. Lloyd.** 1982. Effects of 8-azaadenosine and formycin on cell lethality and the synthesis and methylation of nucleic acids in human colon carcinoma cells in culture. Biochem. Pharmacol. **31**:3207–3214.
10. **Gutteridge, W. E., and N. Gaborek.** 1979. A re-examination of purine and pyrimidine synthesis in the three main forms of *Trypanosoma cruzi*. Int. J. Biochem. **10**:415–422.
11. **Hori, M., E. Ito, T. Takeda, G. Koyama, T. Takeuchi, and H. Umezawa.** 1964. A new antibiotic, formycin. J. Antibiot. (Tokyo) **17A**:96–99.
12. **Krenitsky, T. A., R. A. Strelitz, and G. H. Hitchings.** 1967. Ribonucleosides of allopurinol and oxoallopurinol. J. Biol. Chem. **242**:2675–2682.
13. **Lim, M.-I., R. S. Klein, and J. J. Fox.** 1980. Synthesis of the pyrrolo[3,2-d] pyrimidine C-nucleoside isostere of inosine. Tetrahedron Lett. **21**:1013–1016.
14. **Marr, J. J., and R. L. Berens.** 1977. Antileishmanial effect of allopurinol. II. Relationship of purine biosynthesis in *L. donovani* and *L. braziliensis* to action of allopurinol. J. Infect. Dis. **136**:724–732.
15. **Marr, J. J., and R. L. Berens.** 1983. Pyrazolopyrimidine metabolism in the pathogenic Trypanosomatidae. Mol. Biochem. Parasitol. **7**:339–356.
16. **Marr, J. J., R. L. Berens, and D. J. Nelson.** 1978. *Trypanosoma cruzi*: antitrypanosomal effect of allopurinol through *in vivo* conversion to amino pyrazolopyrimidine nucleotides. Science **202**:1018–1020.
17. **Marr, J. J., R. L. Berens, D. J. Nelson, and T. A. Krenitsky.** 1982. Antileishmanial action of thiopurinol and its ribonucleoside. Biochem. Pharmacol. **31**:143–148.
18. **Marr, J. J., D. J. Nelson, and R. L. Berens.** 1978. Purine metabolism in *Leishmania donovani* and *Leishmania braziliensis*. Biochim. Biophys. Acta **544**:360–371.
19. **Mizuno, Y., M. Ikehara, K. A. Watanabe, and S. Sazaki.** 1963. Synthetic studies of potential antimetabolites. X. Synthesis of 4-hydroxy-7-β-D-ribofuranosyl-7H-pyrrolo[2,3-d]pyrimidine, a tubercidin analog. J. Org. Chem. **28**:3331–3336.
20. **Muller, W. E. G., H. J. Rohde, R. Steffen, A. Maidhof, M. Lachman, R. K. Zahn, and H. Umezawa.** 1975. Influence of formycin B on polyadenosine diphosphoribose synthesis in vitro and in vivo. Cancer Res. **35**:3673–3681.
21. **Nelson, D. J., C. J. L. Bugge, G. B. Elion, R. L. Berens, and J. J. Marr.** 1979. Metabolism of pyrazolo(3,4d)pyrimidines and 4-aminopyrazolopyrimidine in *Leishmania braziliensis* and *Leishmania donovani*. J. Biol. Chem. **254**:3959–3964.
22. **Nelson, D. J., C. J. L. Bugge, H. C. Krasney, and G. B. Elion.** 1973. Formation of nucleotides of [6-^{14}C]allopurinol and oxypurinol in rat tissue and effects on uridine nucleotide pools. Biochem. Pharmacol. **22**:2003–2022.

23. **Nelson, D. J., S. W. LaFon, T. E. Jones, T. Spector, R. L. Berens, and J. J. Marr.** 1982. The metabolism of formycin B in *Leishmania donovani* Biochem. Biophys. Res. Commun. **108**:349–354.
24. **Nelson, D. J., S. W. LaFon, J. V. Tuttle, W. H. Miller, R. L. Miller, T. A. Krenitsky, G. B. Elion, R. L. Berens, and J. J. Marr.** 1979. Allopurinol ribonucleoside as an antileishmanial agent: biological effects, metabolism and enzymatic phosphorylation. J. Biol. Chem. **254**:11544–11549.
25. **Pfaller, M. A., and J. J. Marr.** 1974. Antileishmanial effect of allopurinol. Antimicrob. Agents Chemother. **5**:469–472.
26. **Rainey, P., and D. V. Santi.** 1983. Metabolism and mechanism of action of formycin B in leishmania. Proc. Natl. Acad. Sci. U.S.A. **80**:288–292.
27. **Robbins, R. K., L. B. Townsend, F. Cassidy, J. F. Gerster, A. F. Lewis, and R. L. Miller.** 1966. The structure of the nucleoside antibiotic formycin, formycin B and laurusin. J. Heterocycl. Chem. **3**:110–114.
28. **Sheen, M. R., B. K. Kim, and R. E. Parks.** 1968. Purine nucleoside phosphorylase from human erythrocytes. III. Inhibition by the inosine analogue formycin B of the isolated enzyme and of nucleoside metabolism in intact erythrocytes and sarcoma. Mol. Pharmacol. **4**:293–299.
29. **Suhadolnik, R. J.** 1970. Nucleoside antibiotics, p. 354–389. John Wiley & Sons, Inc., New York.
30. **Takeuchi, T., J. Iwanaga, T. Aoyagi, and H. Umezawa.** 1966. Antiviral effect of formycin and formycin B. J. Antiobiot. (Tokyo) **19A**:286–287.
31. **Trager, W.** 1974. Nutrition and biosynthetic capabilities of flagellates: problems of in vitro cultivation and differentiation. Ciba Symp. **20**:225–245.
32. **Umezawa, H., T. Sawa, Y. Fukagawa, I. Homma, M. Ishizuka, and T. Takeuchi.** 1967. Studies on formycin and formycin B in cells of Ehrlich carcinoma and *E. coli*. J. Antibiot. (Tokyo) **20**:308–316.
33. **Ward, D. C., W. Fuller, and E. Reich.** 1969. Stereochemical analysis of the specificity of pancreatic RNAse with polyformycin as substrate: differentiation of the transphosphorylation and hydrolysis reactions. Proc. Natl. Acad. Sci. U.S.A. **62**:581–588.
34. **Wren, W.-Y., M.-I. Lim, B. A. Otter, and R. S. Klein.** 1982. Synthetic studies of the thieno[3,2-d] pyrimidine C-nucleoside isostere of inosine. J. Org. Chem. **47**:4633–4637.