Activity of Inosine Analogs against *Pneumocystis carinii* in Culture

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Three analogs of inosine, formycin B, allopurinol ribonucleoside, and 9-deazainosine, were tested for their ability to suppress proliferation of *Pneumocystis carinii* in culture with WI-38 cells. The organism was inhibited by 9-deazainosine at 10 µg/ml, and there was some inhibition at 1 µg/ml. Formycin B was effective only at 40 µg/ml. Allopurinol ribonucleoside had little effect.

Current antimicrobial agents used for prophylaxis and therapy of pneumocystis pneumonia, although efficacious in the majority of clinical situations, often are accompanied by serious side effects which require that use of the agent be halted. Trimethoprim-sulfamethoxazole often causes reactions, especially in acquired immune deficiency syndrome patients, and some infections do not respond to therapy (6). Pentamidine isethionate is toxic and thus cannot be used for prophylaxis. Not all active infections respond to pentamidine isethionate, and in some cases therapy must be stopped because of toxicity (1). Rapid systematic evaluation of new agents for use in *Pneumocystis* pneumonia has been difficult with patients or with animal models. We developed a screening system to evaluate ability of antimicrobial agents to suppress *Pneumocystis carinii* proliferation in culture with WI-38 cells (2); this system compares growth curves of drug-treated and control cultures. Using this system, we found that formycin B and 9-deazainosine were active against *P. carinii*.

The susceptibilities of *P. carinii* to allopurinol ribonucleoside, formycin B, and 9-deazainosine were evaluated in culture by using WI-38 human embryonic lung fibroblast cell monolayers (3) adapted for measuring antimicrobial susceptibility (2). Cultures incorporating trimethoprim plus sulfamethoxazole were included because that drug combination is known to be effective against *P. carinii*. Briefly, the WI-38 cells were grown in 12-well tissue culture plates. After monolayers formed, the cells were inoculated with a homogenate of infected rat lungs. A portion (150 to 200 mg) was ground in culture medium and added to the culture medium for five plates. The inoculum contained approximately 7×10^5 trophozoites per well. After 7 to 8 days of growth, supernatants were removed and new media were added. For control cultures, new medium was free of drugs; experimental cultures received media containing the desired concentrations of test drugs. Concentrations of drugs are given below (see Fig. 1 and 2). Wells were sampled on days 1, 3, 5, 7, and 10 by removing 10 µl of supernatant fluid which was then spread in a 1-cm square on a glass slide, air dried, fixed in methanol, stained with Giemsa, and counted. Each slide was counted as an unknown by two individuals, and the counts were averaged. The values shown are average counts of multiple fields at $\times 1,000$ oil immersion magnification. Data points are expressed as raw counts but may be con-

verted to number of organisms per milliliter by using a constant as previously described (2, 3). A total of four wells was used for each drug concentration and for each control at every time point.

Data were expressed as means $\pm$ standard errors of the mean for the numbers of organisms in the culture supernatants at the various times and conditions. Data are plotted with least-squares regression lines. Actual counts of treated and control cultures may show overlap; however, comparisons of slopes as determined by linear regression analysis show significant and reproducible differences for effective drugs as compared with controls.

Allopurinol ribonucleoside was a gift of the Burroughs Wellcome Co., Research Triangle Park, N.C.; formycin B was purchased from Sigma Chemical Co., St. Louis, Mo.; and 9-deazainosine was synthesized at Sloan-Kettering Institute, New York, N.Y., by the procedure of Lim et al. (11).

Numbers of *P. carinii* in supernatants of control cultures increased linearly over 10 days (Fig. 1 and 2). The value for the slopes of control cultures in seven experiments was 0.51 ± 0.10 (mean $\pm$ standard error of the mean; data not shown).

In the presence of 20 µg of 9-deazainosine per ml growth was inhibited, and the slope of the curve dropped to -0.067 in one experiment and 0.002 in another (data not shown). Concentrations of 10 µg/ml gave comparable results (Fig. 1A), and 1-µg/ml concentrations produced a growth curve with 75% of the slope of the control (data not shown).

In the presence of 40 µg of formycin B per ml growth of *P. carinii* was inhibited. The slope of the curve fell to 0.113 in one experiment (Fig. 1B) and to -0.009 in another. At 1 µg/ml the drug had no effect on growth, whereas at 10 µg/ml the effect was variable and less inhibitory than with 40 µg/ml. Allopurinol ribonucleoside had no effect at concentrations from 25 to 200 µg/ml (data not shown).

Sulfamethoxazole plus trimethoprim, at concentrations of 200 and 50 µg/ml, respectively, inhibited growth of *P. carinii*. The slope of the curve was -0.18 as compared with the control slope of 1.45 (Fig. 2).

Little is known about the metabolism of *P. carinii*, a probable protozoan. Most protozoans studied cannot synthesize purines de novo and must salvage these compounds from various sources (7, 8, 16). For these reasons, we studied inosine analogs, 9-deazainosine, allopurinol ribonucleoside, and formycin B, which are known to inhibit purine salvage pathways in the pathogenic hemoflagellates (10, 12, 13).

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The most active of the three, 9-deazainosine, completely inhibited *P. carinii* at concentrations of 20 and 10 $\mu\text{g/ml}$, and even at 1 $\mu\text{g/ml}$ there was some inhibition. This compound also had the greatest antiprotozoan effect against the pathogenic hemoflagellates and little toxicity to mouse L cells used as a model for mammalian cell toxicity studies (10). The carbon-carbon bond between the five-membered heterocyclic ring and the ribose in 9-deazainosine is not broken in mammalian cells (9, 17).

Formycin B, although active at 40 $\mu\text{g/ml}$ (Fig. 1B), had no effect at lower concentrations and cannot be considered a therapeutic agent because of its toxicity (4, 5, 9, 14, 15). Allopurinol ribonucleoside, although active against a variety of pathogenic hemoflagellates (12), had little effect.

Drugs that are known to be effective against *P. carinii* in vivo, e.g., trimethoprim-sulfamethoxazole and pentamidine isethionate (2), also were inhibitory in this system. Thus, the culture screening procedure may correlate with efficacy in vivo and may suggest which drugs should be further studied in animal models.

The study demonstrated that some inosine analogs act against *P. carinii* in a tissue culture system, suggesting that there is some purine salvage carried out by these organisms

FIG. 1. Trophozoites in supernatants of cultures treated with 9-deazainosine (A) and formycin B (B). Counts are means $\pm$ standard error. The data are plotted with least-squares regression lines.

FIG. 2. Trophozoites in supernatants of cultures treated with trimethoprim-sulfamethoxazole. Counts are means $\pm$ standard error. The data are plotted with least-squares regression lines.

and that they may use inosine or hypoxanthine as a purine source. The reason for the differential effects among the three inosine analogs is unclear but probably is caused by differences in transport or metabolism of the three compounds. Further metabolic studies are in progress to clarify this point, to study other purine and purine nucleoside analogs, and to investigate the relative importance of the various purine salvage pathways which may be present in this organism. Animal studies with 9-deazainosine, currently being evaluated, indicate inhibition of *P. carinii* in vivo as well.

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LITERATURE CITED

1. Anonymous. 1985. Pentamidine for *Pneumocystis carinii* pneumonia. Med. Lett. 27:6-7.
2. Bartlett, M. S., R. Eichholtz, and J. W. Smith. 1985. Antimicrobial susceptibility of *Pneumocystis carinii* in culture. Diagn. Microbiol. Infect. Dis. 3:381-387.
3. Bartlett, M. S., P. A. Verbanac, and J. W. Smith. 1979.

- Cultivation of *Pneumocystis carinii* with WI-38 cells. J. Clin. Microbiol. 10:796-799.
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. Glazer, R. L., 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.
 6. Gordin, F. M., G. L. Gwynn, C. B. Wofsy, and J. Mills. 1984. Adverse reactions to trimethoprim-sulfamethoxazole in patients with the acquired immunodeficiency syndrome. Ann. Intern. Med. 100:495-499.
 7. Hammond, D. J., and W. E. Gutteridge. 1984. Purine and pyrimidine metabolism in the Trypanosomatidae. Mol. Biochem. Parasitol. 13:243-263.
 8. Hitchings, G. H. 1982. The purine metabolism of protozoa. Adv. Enzyme Regul. 20:375-386.
 9. Hori, M., E. Ito, T. Takeda, G. Koyana, T. Takeuchi, and H. Umezawa. 1964. A new antibiotic, formycin. J. Antibiot. Ser. A 17:96-99.
 10. LaFon, S. W., D. J. Nelson, R. L. Berens, and J. J. Marr. 1985. Inosine analogs: Their metabolism in mouse L cells and in *Leishmania donovani*. J. Biol. Chem. 260:9660-9665.
 11. Lim, M.-I., R. S. Klein, and J. J. Fox. 1980. Synthesis of the pyrolo (3,2-d) pyrimidine C-nucleoside isostere of inosine. Tetrahedron Lett. 21:1013-1016.
 12. Marr, J. J., and R. L. Berens. 1983. Pyrazolopyrimidine metabolism in the pathogenic Trypanosomatidae. Mol. Biochem. Parasitol. 7:339-356.
 13. Marr, J. J., R. L. Berens, N. K. Cohn, D. J. Nelson, and R. S. Klein. 1984. Biological action of inosine analogs in *Leishmania* and *Trypanosoma* spp. Antimicrob. Agents Chemother. 25:292-295.
 14. 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.
 15. Umezawa, H., T. Sawa, V. Fukagawa, I. Homma, M. Ishizuka, and T. Takeuchi. 1967. Studies on formycin and formycin B in cells of Erlich carcinoma and *E. coli*. J. Antibiot. Ser. A 20:309-316.
 16. Wang, C. C. 1981. Recent advances in parasite biochemistry. Annu. Rep. Med. Chem. 16:269-280.
 17. Ward, D. C., W. Fuller, and E. Reich. 1969. Stereochemical analysis of the specificity of pancreatic RNase with polyformycin as a substrate: differentiation of the trans-phosphorylation and hydrolysis reactions. Proc. Natl. Acad. Sci. USA 62:581-588.