ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1993, p. 1227–1231
0066-4804/93/061227-05$02.00/0
Copyright © 1993, American Society for Microbiology
Vol. 37, No. 6

Inhibition of *Pneumocystis carinii* Dihydropteroate Synthetase by *para*-Acetamidobenzoic Acid: Possible Mechanism of Action of Isoprinosine in Human Immunodeficiency Virus Infection

JOSEPH A. KOVACS,[1]* FRANCOISE POWELL,[1] DONNA VOELLER,[2] AND CARMEN J. ALLEGRA[2]

Critical Care Medicine Department, Clinical Center,[1] and Navy Medical Oncology Branch, National Cancer Institute,[2] National Institutes of Health, Bethesda, Maryland 20892

Received 12 October 1992/Accepted 9 March 1993

Isoprinosine has been reported to decrease progression to AIDS, primarily by preventing *Pneumocystis carinii* pneumonia (PCP), in human immunodeficiency virus-infected patients, but the mechanism of action is unknown. *para*-Acetamidobenzoic acid (PAcBA), one component of isoprinosine, is structurally related to *para*-aminobenzoic acid (PABA), a precursor of de novo folate synthesis. This pathway is known to be important for *P. carinii* because sulfonamides, which are effective anti-*P. carinii* agents, inhibit incorporation of PABA into folate precursors by the enzyme dihydropteroate synthetase (DHPS). Inhibition of *P. carinii* DHPS by PAcBA was investigated by using two assays. In short-term cultures of *P. carinii* from rats, [^{3}H]PABA incorporation into reduced folates was inhibited by both isoprinosine (mean ± standard error 50% inhibitory concentration [IC_{50}], 20 ± 8.4 μM) and PAcBA free acid (IC_{50}, 240 ± 100 μM); a soluble PAcBA salt was more potent than PAcBA free acid alone (IC_{50}, 29 ± 48 μM). The activity of PAcBA free acid was confirmed in a cell-free DHPS inhibition assay (IC_{50}, 120 ± 120 μM). Inosine and dimethylaminopropanol, two other components of isoprinosine, were poor inhibitors of PABA incorporation (IC_{50}, >1,000 μM). PAcBA free acid also showed activity in inhibiting the DHPS of *Toxoplasma gondii*, but was a poor inhibitor of the DHPSs of *Escherichia coli* and *Saccharomyces cerevisiae*. In a rat model of PCP, the PAcBA salt administered intraperitoneally demonstrated no activity against established PCP either alone or when used in combination with trimethoprim; the lack of efficacy in this model may be due to the rapid metabolism of the drug. Prevention of PCP by PaCBA through inhibition of *P. carinii* DHPS may explain the activity of isoprinosine in decreasing the progression to AIDS in human immunodeficiency virus-infected patients.

Isoprinosine has recently been shown in a randomized, placebo-controlled trial to decrease the progression to AIDS in human immunodeficiency virus (HIV)-infected patients (14). Although isoprinosine has immunomodulatory properties in vitro, no immunological changes that correlated with clinical benefit were seen in that randomized trial (12). Because the primary benefit seen was a decrease in the incidence of *Pneumocystis carinii* pneumonia (14), we investigated the possibility that the benefit of isoprinosine was based on a specific anti-*P. carinii* effect.

Isoprinosine is composed of inosine and the *para*-acetamidobenzoic acid (PAcBA) salt of *N,N*-dimethyl-2-aminopropanol, in a 1:3 ratio (2). PAcBA is structurally related to *para*-aminobenzoic acid (PABA), a precursor of de novo folate synthesis (Fig. 1). Sulfonamides and sulfones, which are used to treat *P. carinii* pneumonia, exert their effects by inhibiting the incorporation of PABA into folate precursors by inhibiting the catalytic activity of the enzyme dihydropteroate synthetase (DHPS). Because PAcBA is structurally related to PABA, we hypothesized that PAcBA could also inhibit *P. carinii* DHPS. We have previously used in vitro screening assays to examine the relative abilities of sulfonamides and sulfones to inhibit the DHPSs of both *P. carinii* and *Toxoplasma gondii* (1, 7). In the study described here we have demonstrated that isoprinosine inhibits *P. carinii* DHPS and *T. gondii* DHPS and that this inhibition is attributable exclusively to PAcBA.

(This study was presented in part at the Annual Meeting of the American Federation for Clinical Research, Seattle, Wash., 3 May 1992, abstr. 143A.)

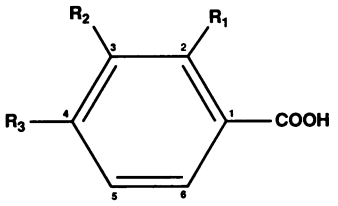
MATERIALS AND METHODS

Organisms. *P. carinii* organisms were obtained from immunosuppressed Sprague-Dawley rats and were partially purified by Ficoll-Hypaque density gradient centrifugation as described previously (11). The RH strain of *T. gondii* was maintained by intraperitoneal passage in mice (9).

PABA uptake studies. PABA incorporation studies were performed as described previously (8). Briefly, *P. carinii* organisms were resuspended in RPMI–10% fetal calf serum, incubated with or without drugs for 4 h, pulsed with 0.6 μM [^{3}H]PABA (specific activity, 50 Ci/mmol), harvested after 18 h, washed, and pelleted. Folates were extracted and [^{3}H]folates were resolved by reverse-phase high-pressure liquid chromatography and quantitated as described previously (8). In each experiment a single flask was used for each concentration.

DHPS assay. Enzyme extracts were prepared by sonication of partially purified organisms and were assayed for DHPS activity as described previously (1). The assay mixture contained 5 mM $MgCl_2$, 5 mM dithiothreitol, 10 μM 6-hydroxymethyl-7,8-dihydropterin pyrophosphate, 1 μM [^{3}H]PABA (final specific activity, 2 Ci/mmol), and 40 mM

* Corresponding author.



	R_1	R_2	R_3
PABA	H	H	NH_2
PAcBA	H	H	$CH_3C(=O)NH-$
Benzoic acid	H	H	H
3-Aminobenzoic acid	H	NH_2	H
3,4-Diaminobenzoic acid	H	NH_2	NH_2
4-Chlorobenzoic acid	H	H	Cl
4-Dimethylaminobenzoic acid	H	H	$(CH_3)_2NH-$
2,4-Dichlorobenzoic acid	Cl	H	Cl

FIG. 1. Structures of PABA and its analogs.

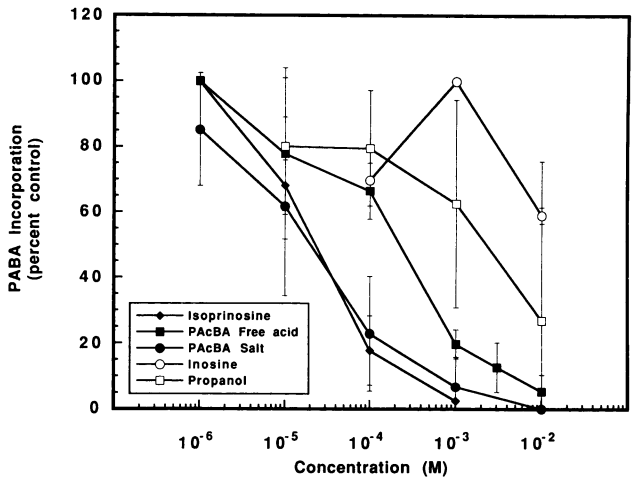


FIG. 2. Inhibition of de novo folate synthesis of *P. carinii* by isoprinosine and its components. Cultures of *P. carinii* without (control) or with the indicated inhibitor were pulsed for 18 h with [^{3}H]PABA, and then folates were extracted and identified as described in Materials and Methods. Incorporation of [^{3}H]PABA, expressed as a percentage of the control, is shown along the left axis, and the inhibitor concentration is shown along the bottom. Results represent the means ± standard deviations of two experiments for isoprinosine, six experiments for PAcBA free acid, six experiments for PAcBA salt, three experiments for inosine, and four experiments for *N,N*-dimethyl-2-aminopropanol. Not all concentrations were examined in each experiment.

Tris-HCl (pH 8.3), plus enzyme extract, in a final volume of 100 μl. Drugs were added at various concentrations (one sample per concentration) as indicated in the Results. After incubation for 30 min at 37°C, the sample was placed on ice, spotted onto Whatman 3MM chromatography paper, and resolved by descending chromatography. The origin, which contained the labeled product, was cut out and the radioactivity was counted in a scintillation counter.

Studies in animals. For pharmacokinetic studies, rats received PAcBa intraperitoneally as the *N,N*-dimethyl-2-aminopropanol salt (PAcBA salt), and blood samples were collected from three rats sacrificed at 1, 2, and 8 h. Serum was diluted in an equal volume of phosphate-buffered saline, and total PAcBA was extracted, processed, and quantitated by the methods described above for folate extraction (8). The concentration of PAcBA was determined on the basis of a comparison with a standard curve by using PAcBA free acid and was corrected for the efficiency of extraction; PABA was used as an internal standard.

Therapy studies examining the efficacy of PAcBA salt in the treatment of *P. carinii* pneumonia were performed as described previously (10). Rats were immunosuppressed for 6 weeks, and the presence of *P. carinii* pneumonia was confirmed by sacrificing two animals and examining impression smears of the lung that had been stained with Diff-Quik for *P. carinii* organisms. Groups of rats (12 to 15 animals per group) were then placed on one of the following treatment regimens for 3 weeks: no therapy; oral trimethoprim-sulfamethoxazole (10 mg of trimethoprim plus 50 mg of sulfamethoxazole per animal per day given in the drinking water); intraperitoneal PAcBA salt (500 mg/kg of body weight [1.8 mM/kg] twice a day); PAcBA salt (500 mg/kg twice a day) given intraperitoneally plus trimethoprim (10 mg/day) given orally. This dose of PAcBA was determined in preliminary experiments to be the maximum dose that could be tolerated without causing morbidity or mortality. All surviving animals were sacrificed at day 21; approximately 0.5-cm^2 pieces of lung were removed from the right and left lower lobes, fixed in formaldehyde, and stained with methenamine silver.

Drugs. PAcBA free acid, *N,N*-dimethyl-2-aminopropanol, and other PABA analogs were obtained from Sigma (St. Louis, Mo.). Isoprinosine was obtained from the Division of AIDS, National Institute of Allergy and Infectious Diseases.

Data analysis. Mean ± standard error 50% inhibitory concentrations (IC_{50}s) for both PABA incorporation inhibition and DHPS inhibition were determined by using the ALLFIT program (3) on a personal computer (International Business Machines).

RESULTS

Inhibition of folate metabolism in *P. carinii* was investigated by two assays. In a short-term tissue culture assay evaluating the ability of isoprinosine to inhibit incorporation of [^{3}H]PABA by intact *P. carinii* into reduced folates, isoprinosine was able to inhibit incorporation by *P. carinii* in a dose-dependent manner; nearly complete inhibition was seen at a concentration of 10^{-3} M, and partial inhibition was seen at 10^{-4} and 10^{-5} M (Fig. 2). No inhibition was seen at 10^{-6} M. The mean ± standard error IC_{50} of isoprinosine in this assay was 20 ± 8.4 μM (Table 1). The free acid of PAcBA could also inhibit [^{3}H]PABA incorporation by *P. carinii*, although at concentrations greater than those of isoprinosine (Fig. 2), with an IC_{50} of 240 ± 100 μM. PAcBA free acid is poorly soluble in water, while PAcBA salt has a greater solubility in water. This salt was as effective as isoprinosine in inhibiting [^{3}H]PABA incorporation (IC_{50}, 29 ± 48 μM). Inosine and *N,N*-dimethyl-2-aminopropanol, the

TABLE 1. Inhibition of DHPSs from *P. carinii* and *T. gondii* by PABA analogs

Organism	Drug	IC_{50} (μM)	
		Intact organisms	Cell-free DHPS
P. carinii	Isoprinosine	20 ± 8.4 (2)[a]	
	PAcBA free acid	240 ± 100 (6)	120 ± 120 (4)
	PAcBA salt	29 ± 48 (6)	
	Inosine	>1,000 (3)	
	N,N-Dimethyl-2-aminopropanol	>1,000 (4)	
	Sulfamethoxazole		0.71 ± 0.17[b]
	Benzoic acid		170 ± 26 (2)
	3-Aminobenzoic acid		1,700 ± 2000 (2)
	3,4-Diaminobenzoic acid		220 ± 200 (2)
	4-Chlorobenzoic acid		>1,000 (2)
	4-Dimethylaminobenzoic acid		>1,000 (2)
	2,4-Dichlorobenzoic acid		>1,000 (2)
T. gondii	Isoprinosine		23 ± 7 (3)
	PAcBA free acid		54 ± 18 (5)
	Sulfadiazine		19 ± 5[b]

[a] Numbers in parentheses are the number of experiments used to determine the value.
[b] Values are from previous studies (1, 7).

other two components of isoprinosine, demonstrated no consistent inhibition of [^{3}H]PABA incorporation by *P. carinii* at these concentrations (IC_{50}, >1,000 μM; Fig. 2).

In an assay evaluating the inhibition of *P. carinii* DHPS in crude cell enzyme extracts, the free acid of PAcBA was found to be an effective inhibitor at concentrations similar to those seen in the intact cell assays (IC_{50}, 120 ± 120 μM; Table 1). In a single experiment, *N,N*-dimethyl-2-aminopropanol and inosine had no effect at concentrations up to 1,000 μM. The IC_{50} of sulfamethoxazole, previously determined by the identical assay (7), was 0.71 ± 0.17 μM.

To determine whether other non-sulfa analogs of PABA were able to inhibit *P. carinii* DHPS, a number of other compounds (Fig. 1) were evaluated in the enzyme assay. Benzoic acid and 3,4-diaminobenzoic acid were inhibitory at concentrations similar to that of isoprinosine (Table 1); the remaining compounds showed substantially less activity.

To examine the effect of PAcBA on the DHPSs of other organisms, enzyme extracts of *T. gondii*, *Escherichia coli*, and *Saccharomyces cerevisiae* were used in DHPS inhibition assays. *T. gondii* DHPS was inhibited in a manner similar to that of *P. carinii* (Fig. 3) by isoprinosine, the free acid of PAcBA, and PAcBA salt. The IC_{50}s of isoprinosine and PAcBA free acid in the *T. gondii* DHPS assay were 23 ± 7 and 54 ± 18 μM, respectively. The IC_{50} of sulfadiazine, the standard sulfonamide used in the treatment of toxoplasmosis, was previously determined to be 19 ± 5 μM by the same assay (1). PAcBA free acid was a poor inhibitor of *E. coli* and *S. cerevisiae* DHPSs (IC_{50}s, approximately 300 and 400 μM; Fig. 4). In comparing the inhibition of DHPSs from the four organisms, only *P. carinii* and *T. gondii* DHPSs were inhibited at 10^{-4} M PAcBA free acid (Fig. 4), although the DHPSs of all four organisms were at least partially inhibited by 10^{-3} M PAcBA free acid.

The pharmacokinetics of PAcBA were determined following single-dose administration of 500 to 2,700 mg of PAcBA salt per kg (1.8 to 9.6 mM/kg) to nonimmunosuppressed rats. There was an approximately linear relationship between the PAcBA dose and the peak concentration of PAcBA in plasma, described by the equation $y = 1.1x - 278$. The relationship between PAcBA levels in serum to the dose of PAcBA salt administered can be described as follows. For PAcBA doses of 500 (1.8), 1,350 (4.8), and 2,700 (9.6) mg/kg (mM/kg), peak concentrations in plasma were 0.18 ± 0.03, 1.29 ± 0.43, and 2.70 ± 1.70 mM (mean ± standard error of the mean; each value represents the mean for three animals). The half-life of PAcBA in plasma was approximately 75 min.

To examine the potential therapeutic benefit of PAcBA, immunosuppressed rats were treated parenterally for 21 days with PAcBA salt, 500 mg/kg (1.8 mM/kg) twice a day, alone or in combination with trimethoprim. No decrease in the severity of *P. carinii* pneumonia was seen in either PAcBA treatment group compared with that seen in controls with no drug treatment, as evaluated by methenamine silver staining of lung specimens. In the group treated with trimethoprim-sulfamethoxazole, the infection cleared in all animals.

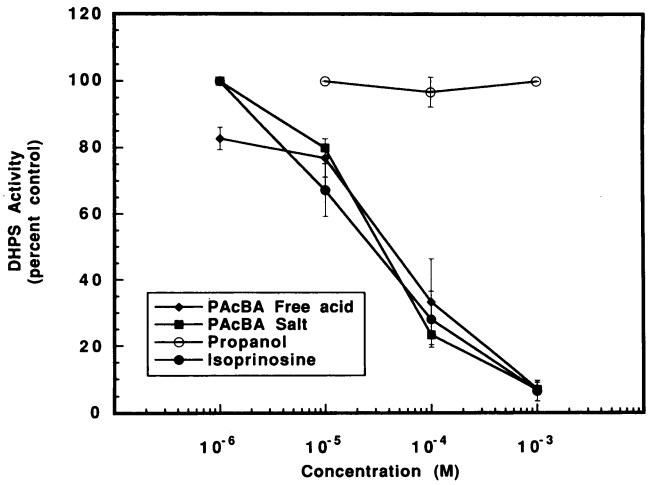


FIG. 3. Inhibition of *T. gondii* DHPS by isoprinosine, PAcBA free acid, the salt of PAcBA and *N,N*-dimethyl-2-aminopropanol, and *N,N*-dimethyl-2-aminopropanol alone. Results represent the means ± standard deviations of five experiments for PAcBA free acid, three experiments for isoprinosine, two experiments for *N,N*-dimethyl-2-aminopropanol, and a single experiment for the PAcBA salt. Not all concentrations were examined in each experiment.

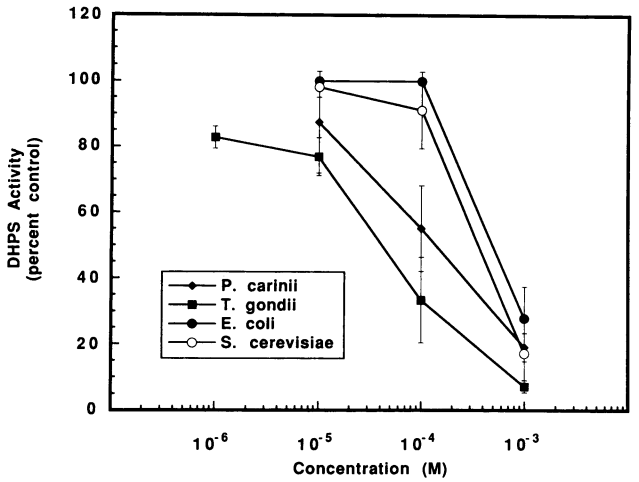


FIG. 4. Comparison of inhibition of the DHPSs of *P. carinii*, *T. gondii*, *E. coli*, and *S. cerevisiae* by PAcBA free acid. Results represent the means ± standard deviations of four experiments for *P. carinii*, five experiments for *T. gondii*, and three experiments each for *E. coli* and *S. cerevisiae*. The curve for *T. gondii* is the same as that in Fig. 3.

DISCUSSION

The present study demonstrated that isoprinosine can inhibit the DHPS of *P. carinii* and that this inhibition can be attributed to PAcBA, one of the components of isoprinosine. Furthermore, in an intact short-term culture system, incorporation of PABA into reduced folates was inhibited by isoprinosine and PAcBA salt at similar concentrations of each drug, demonstrating that the drugs are taken up and active in intact organisms. The present study thus suggests that the beneficial effects of isoprinosine seen in a randomized clinical trial of human immunodeficiency virus-infected patients may have been due to the prevention of *P. carinii* pneumonia (14). The inhibitory activity of PAcBA salt or free acid was substantially less than that which we found for sulfamethoxazole, the standard sulfonamide used for treating *P. carinii* pneumonia, in a previous study (7). Nonetheless, the lack of toxicity seen with isoprinosine in clinical trials suggests that high, potentially effective doses of PAcBA can be safely administered. It is of interest that the free acid of PAcBA was apparently 10-fold less effective than the PAcBA salt; it is conceivable that entry into the organism is facilitated by formation of the neutrally charged salt.

Although no beneficial effects of the PAcBA salt were seen in an animal model of *P. carinii* pneumonia when it was administered alone or in combination with trimethoprim, metabolism and elimination of drugs in humans and rodents differ, and this may account for the observed lack of efficacy of the PAcBA salt. The PAcBA salt has a short half-life in rats (approximately 75 min); sufficient concentrations of drug in serum may not have been maintained for a long enough period of time to achieve inhibition of *P. carinii* growth in vivo, despite the twice-daily administration of drug. Because the levels of PAcBA achieved in humans following administration of isoprinosine have been poorly defined, we are unable to determine how the levels achieved in rats compare with those found in humans. Additionally, the drug may be more active as a prophylactic agent than as a therapeutic agent. Other drugs, such as trimethoprim-sulfamethoxazole, have been shown to be effective prophylactically in humans at much lower doses than are used for the treatment of acute pneumonia (5). In one series of experiments, however, no prophylactic benefit of the PAcBA salt was seen in the rat model (1a).

Even if PAcBA is ineffective in treating or preventing *P. carinii* infections in vivo, the identification of an inhibitor of *P. carinii* DHPS that is not related to sulfonamides is a critical finding of the present study, since such a compound could greatly improve the treatment and prevention of *P. carinii* pneumonia. The currently used inhibitors of DHPS, which include the sulfonamides and sulfones, are associated with a high incidence of adverse reactions, especially in patients with human immunodeficiency virus infection. It is possible that the sulfur contributes to these adverse reactions. In previous studies non-sulfur-containing analogs of PABA were reported to be poor inhibitors of DHPSs from bacteria, and alteration of the *para*-amino group has been reported to result in the loss of inhibitory activity (13). PAcBA was found to have no inhibitory effects on the DHPS of *E. coli* in one report (16), although the concentrations assayed were not stated. Other studies have found that compounds with a modification in the amino group of PABA were active against intact *E. coli* or streptococcal species (6). Phosphanilic acid (*p*-aminobenzene phosphonate), which is a non-sulfur-containing PABA analog in which the amino group is intact but a phosphonate group has replaced the carboxy group, has been reported to inhibit the DHPS activities and replication of *E. coli* and *Pseudomonas aeruginosa*, as well as to inhibit the growth of *Mycobacterium tuberculosis* (4, 15). As for other organisms, the data presented in this report demonstrate that substitutions at the 4 position of PABA (other than an acetamido group) result in a substantial decrease in inhibition of the *P. carinii* DHPS.

The observation in the present study that DHPSs from different organisms differ in their susceptibilities to PAcBA free acid supports the concept that these enzymes are structurally different and raises the possibility that agents with limited activity against bacterial DHPS may be clinically active against *P. carinii* or *T. gondii*. These studies suggest that the screening of PABA analogs, which have been dismissed as clinically ineffective on the basis of studies with bacteria, is warranted. The recent cloning of *P. carinii* DHPS (17) should facilitate such studies and may ultimately lead to the identification of effective, nontoxic agents for the treatment of these devastating infections.

REFERENCES

1. **Allegra, C. J., D. Boarman, J. A. Kovacs, P. Morrison, J. Beaver, B. A. Chabner, and H. Masur.** 1990. Interaction of sulfonamide and sulfone compounds with *Toxoplasma gondii* dihydropteroate synthase. J. Clin. Invest. **85**:371–379.
1a. **Bartlett, M.** Personal communication.
2. **Campoli-Richards, D. M., E. M. Sorkin, and R. C. Heel.** 1986. Inosine pranobex: a preliminary review of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy. Drugs **32**:383–424.
3. **DeLean, A., P. J. Munson, and D. Rodbard.** 1978. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiologic dose-response curves. Am. J. Physiol. **235**:E97–E102.
4. **Eagon, R. G., and A. T. McManus.** 1989. Phosphanilic acid inhibits dihydropteroate synthase. Antimicrob. Agents Chemother. **33**:1936–1938.
5. **Hughes, W. T., G. K. Rivera, M. J. Schell, D. Thornton, and L. Lott.** 1987. Successful intermittent chemoprophylaxis for *Pneumocystis carinii* pneumonitis. N. Engl. J. Med. **316**:1627–1632.
6. **Johnson, O. H., D. E. Green, and R. Pauli.** 1944. The antibac-

terial action of derivatives and analogues of *p*-aminobenzoic acid. J. Biol. Chem. **153**:37–47.

7. **Kovacs, J. A., C. J. Allegra, J. Beaver, D. Boarman, M. Lewis, B. Chabner, and H. Masur.** 1988. Evaluation of sulfonamides and sulfones as inhibitors of *Pneumocystis carinii* and *Toxoplasma gondii* dihydropteroate synthetase. Program Abstract 28th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1235.
8. **Kovacs, J. A., C. J. Allegra, J. Beaver, D. Boarman, M. Lewis, J. E. Parrillo, B. Chabner, and H. Masur.** 1989. Characterization of de novo folate synthesis in *Pneumocystis carinii* and *Toxoplasma gondii*: potential for screening therapeutic agents. J. Infect. Dis. **160**:312–320.
9. **Kovacs, J. A., C. J. Allegra, B. A. Chabner, J. C. Swan, J. Drake, M. Lunde, J. E. Parrillo, and H. Masur.** 1987. Potent effect of trimetrexate, a lipid-soluble antifolate, on *Toxoplasma gondii*. J. Infect. Dis. **155**:1027–1032.
10. **Kovacs, J. A., C. J. Allegra, S. Kennedy, J. C. Swan, J. C. Drake, J. E. Parrillo, B. Chabner, and H. Masur.** 1988. Efficacy of trimetrexate, a potent lipid-soluble antifolate in the treatment of rodent *Pneumocystis carinii* pneumonia. Am. J. Trop. Med. Hyg. **39**:491–496.
11. **Kovacs, J. A., J. L. Halpern, J. C. Swan, J. Moss, J. E. Parrillo, and H. Masur.** 1988. Identification of antigens and antibodies specific for *Pneumocystis carinii*. J. Immunol. **140**:2023–2031.
12. **Kweder, S. L., R. A. Schnur, and E. C. Cooper.** 1990. Inosine pranobex—is a single positive trial enough? N. Engl. J. Med. **322**:1807–1809.
13. **Northey, E. H.** 1948. The sulfonamides and allied compounds. Reinhold Publishing Corporation, New York.
14. **Pedersen, C., E. Sandstrom, C. S. Petersen, G. Norkrans, J. Gerstoft, A. Karlsson, K. C. Christensen, C. Hakansson, P. O. Pehrson, J. O. Nielsen, H. J. Jurgensen, and The Scandinavian Isoprinosine Study Group.** 1990. The efficacy of inosine pranobex in preventing the acquired immunodeficiency syndrome in patients with human immunodeficiency virus infection. N. Engl. J. Med. **322**:1757–1763.
15. **Smith, M. I., E. W. Emmart, and B. B. Westfall.** 1942. The action of certain sulfonamides, sulfones, and related phosphorous compounds in experimental tuberculosis. J. Pharmacol. Exp. Ther. **74**:163–174.
16. **Thijssen, H. H. W.** 1977. *p*-Aminobenzoic acid derivatives as inhibitors of the cell-free H_2-pteroate synthesizing system of *Escherichia coli*. J. Med. Chem. **20**:233–236.
17. **Volpe, F., M. Dyer, J. G. Scaife, G. Darby, D. K. Stammers, and C. J. Delves.** 1992. The multifunctional folic acid synthesis *fas* gene of *Pneumocystis carinii* appears to encode dihydropteroate synthase and hydroxymethyldihydropterin pyrophosphokinase. Gene **112**:213–218.