

ACTIVITY OF ANTIFOLATES AGAINST
PNEUMOCYSTIS CARINII DIHYDROFOLATE REDUCTASE
AND IDENTIFICATION OF A POTENT NEW AGENT

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Pneumocystis carinii (PC) is a frequent cause of pneumonia in immunosuppressed patients, particularly those with the acquired immunodeficiency syndrome (AIDS) (1–3). Very little is known about this organism because attempts to cultivate it have been unsuccessful. With few exceptions, it has been difficult to obtain purified and viable organisms from infected tissues (4, 5). As a result, little is known about the biology of this organism. The development of chemotherapeutic agents has been guided by the presumption that the organism is a protozoan because of its morphologic characteristics. Pentamidine isethionate, pyrimethamine plus sulfa, trimethoprim plus dapsone, and trimethoprim plus sulfamethoxazole have been used to treat pneumocystis pneumonia, but any of these regimens is successful in only 50–75% of cases, and adverse reactions occur in up to 60% of patients, particularly when trimethoprim-sulfamethoxazole is used in AIDS patients (2, 3, 6, 7). Thus, there is a clear need for development of more effective, less toxic antipneumocystis agents.

Although little is known regarding the metabolism of PC, both trimethoprim-sulfamethoxazole and pyrimethamine-sulfa presumably act on pneumocystis by the same mechanisms as they act on classic protozoa and bacteria. The diaminopyrimidines (trimethoprim and pyrimethamine) inhibit dihydrofolate reductase (DHFR; EC 1.5.1.3), while sulfa blocks the conversion of paraaminobenzoic acid to dihydrofolate through inhibition of the enzyme dihydropteroate synthetase. Because of the classification of pneumocystis as a protozoan, it has been assumed that the DHFR in the PC organism is similar to other protozoal and bacterial DHFR in its sensitivity to the diaminopyrimidine inhibitors.

We have entertained the possibility that other antifolates that possess a more classical pteridine-like structure, such as those used in cancer chemotherapy, might be superior to diaminopyrimidines for the treatment of PC. Highly charged folate analogues such as methotrexate require a folate-specific membrane carrier for transport (8). Such a mechanism is present in mammalian cells but is generally not found in bacteria or protozoa. Thus, antifolates that preserve the basic folate structure have not been effective antibacterial or antiprotozoan agents because they cannot enter these microorganisms. A newly developed reductase inhibitor,

trimetrexate, is a quinazoline analogue of methotrexate that has the pharmacologic advantage of readily penetrating cellular membranes because of its lipid solubility (9). This report describes the kinetics of trimetrexate uptake and DHFR binding by *P. carinii*, and strongly suggests that trimetrexate has substantial potential as an antipneumocystis drug.

Materials and Methods

Isolation of PC DHFR. PC organisms were isolated from the homogenized lungs of infected Sprague-Dawley rats by a two-step procedure: an initial slow-speed (260 g) centrifugation for 5 min, which permitted removal of most of the mammalian cells, followed by further purification by layering the supernatant onto a Ficoll-Hypaque gradient and sedimenting at 1,000 g for 12 min. The intact PC organisms were then harvested from the buffer/Ficoll-Hypaque interface. Organisms prepared in this manner were found to contain only rare mammalian cells by light microscopy. PC DHFR was prepared as a 100,000 g cytosolic preparation of sonicated (0.65 mA/s for 60 s at 4°C) PC organisms in the presence of 50 µg/ml each of the protease inhibitors leupeptin and chymostatin (Sigma Chemical Co., St. Louis, MO) (10). The specific activity of the PC DHFR was found to be 3.9 ± 1.6 nmoles/min/mg protein at 37°C.

DHFR Catalytic Activity Assay. A spectrophotometric assay (11) was used to measure the reaction catalyzed by either PC or rat liver DHFR (sp. act. 3.7 U/mg). Each 1-ml assay contained 0.15 µmoles of NADPH (Sigma Chemical Co.) in 160 mM Tris-HCl, pH 7.2, and 160 mM KCl with 2.3 U of DHFR and various concentrations of inhibitor. After temperature equilibration (37°C), the reaction was initiated by the addition of 0.075 µmoles of dihydrofolic acid (Sigma Chemical Co.), and the reaction velocity measured by the disappearance of NADPH at 340 nm.

Relative DHFR Binding Affinity Assay. The ability of the antifolates to compete with radiolabeled methotrexate for binding to either rat liver or PC DHFR was used as an additional measure of the potency of interaction with the enzyme for each of the inhibitors. The relative potency of binding was calculated from the ratio of the labeled methotrexate concentration (2×10^{-9} M) to the concentration of inhibitor required to displace one-half of the enzyme-bound methotrexate. Each 450 µl assay contained 0.15 µmole of NADPH, 1 pmole of [³H]methotrexate (sp. act. 18 Ci/mmol; Moravек Radiochemicals, Brea, CA), various concentrations of competitors (inhibitors), and 50 mM KH₂PO₄, pH 7.4. The reaction was begun with the addition of enzyme, and after 10 min of equilibration at 21°C the unbound ligand was adsorbed using acid-washed activated charcoal, and separated by filtration (12, 13). The protein-bound [³H]methotrexate was then counted in a liquid scintillation counter.

Data Analysis. All values from both the catalytic and binding assays were calculated using ALLFIT, a least-squares, curve-fitting program capable of simultaneous curve-fitting (14).

Drug Uptake Assay. The uptake of radiolabeled methotrexate (sp. act. 18 Ci/mmol), trimetrexate (Drug Synthesis and Chemistry Branch, NCI, Bethesda, MD; sp. act. 13.1 mCi/mmol), and leucovorin (sp. act. 1.5 Ci/mmol) was measured in both a human promyelocytic cell line (HL-60) and intact PC organisms using standard techniques (9, 15). *l*-5-[³H]Formyltetrahydrofolic acid (leucovorin) was prepared from [3', 5', 7, 9-³H]-folic acid (Moravек Biochemicals) by enzymatic reduction to tetrahydrofolic acid, followed by formylation and purification (16). Intact HL-60 cells or PC organisms were suspended in 300 µl of 160 mM HEPES/2 mM MgCl₂ at 21°C and exposed to 1 µM concentrations of labeled compounds for up to 30 min. After the exposures, the cells/organisms were sedimented through 1 ml of F50 silicon fluid (General Electric, Waterford, NY) at 15,000 g for 1 min. The cell pellets were dissolved in 0.5 ml of 1 N NaOH, and radioactivity was counted in a liquid scintillation counter. Nonspecific background counts were established for each radiolabeled compound by adding the radiolabeled compound to the cells and then immediately quenching the transport of the radiolabeled compound by the addition of a 1000-fold excess of unlabeled compound.

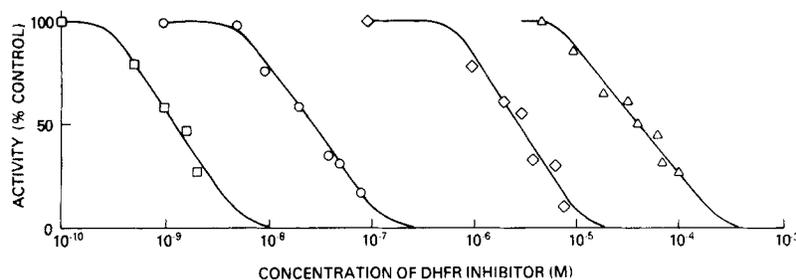


FIGURE 1. Inhibition curves of the reaction catalyzed by 2-3 U of PC DHFR (1 U = 1 nmole/min at 37°C) by methotrexate (□), trimetrexate (○) pyrimethamine (◇), and trimethoprim (△) are illustrated as a percent of control. Assays were performed using the methods outlined under Table I. Each point represents the mean of at least four separate experiments with a standard error of <15%, as shown in Table I.

TABLE I
Comparative Inhibition of DHFR from *P. Carinii* and Rat Liver by Antifolates

Inhibitor	Rat liver		<i>P. carinii</i>	
	Inhibition of enzyme activity (ID ₅₀)	Relative potency of binding	Inhibition of enzyme activity (ID ₅₀)	Relative potency of binding
	<i>nM</i>		<i>nM</i>	
Trimethoprim	390,000 ± 150,000	0.0003	39,600 ± 3,800	0.000011
Pyrimethamine	2,400 ± 900	0.0069	2,800 ± 300	0.000098
Trimetrexate	5.7 ± 1.1	0.35	26.1 ± 2.2	0.17
Methotrexate	1.4 ± 0.5	1	1.4 ± 0.2	1

The tabulated ID₅₀ values represent the concentration of each inhibitor required to inhibit the DHFR-catalyzed reaction by 50% ID₅₀. The binding values represent the relative ability of each inhibitor to compete with labeled MTX for enzyme binding, and are expressed as values relative to MTX where MTX = 1.0. All catalytic reactions were performed with 7.5×10^{-5} M dihydrofolate as substrate, and each inhibitor was preincubated with enzyme before initiation of the reaction to obviate potential differences in the rate of interaction of the inhibitors with the enzyme. Each experimental point represents the mean of at least four separate experiments.

Results and Discussion

PC organisms were harvested from the lungs of infected rats after 6 wk of oral steroid therapy. The PC DHFR was isolated as a crude cytosolic preparation free of contaminating mammalian reductase. Proof of the purity of the preparation is dependent on several lines of evidence, including (a) the uniphasic nature of inhibition of the reductase (Fig. 1); (b) inhibition constants that were significantly different from those of rat DHFR (Sigma Chemical Co.) (Table I); (c) lack of detectable DHFR activity in preparations of steroid-treated rat lung exudate lacking pneumocystis organisms but containing the same contaminating elements, i.e., rat lung cells, yeast, rat red cells, and rat white cells; and (d) inability to inhibit the presumptive PC DHFR by a polyclonal antibody (a generous gift of Dr. J. Bertino) (17) capable of fully inhibiting the catalytic activity of rat DHFR. The antifolates trimethoprim, pyrimethamine, methotrexate, and trimetrexate were tested as inhibitors of the reaction catalyzed by the PC DHFR and by rat liver DHFR (Fig. 1 and Table I). Trimethoprim, the antifolate currently used to treat PC pneumonia, was the weakest inhibitor, inhibiting the reaction by half at a concentration of 39,600 nM. Pyrimethamine was 10-fold more potent, but the antineoplastic antifolates trimetrexate and methotrexate, were 1,500-fold and

TABLE II
Steady-state Levels of Leucovorin and Antifolates in Intact P. Carinii and HL-60 Cells

Cells	Leucovorin	Trimetrexate	Methotrexate
		<i>pmoles/mg</i>	
<i>P. carinii</i>	0	76.0 ± 20.8	0
HL-60	16.0 ± 3.5	366.0 ± 68.5	10.3 ± 0.5

All experiments were performed using single-cell suspensions in transport media and 1 μ M concentrations of drugs. Steady-state levels of trimetrexate were reached within 2 min of exposure, while MTX and leucovorin required 30 min to reach steady-state conditions. Each experimental point represents the mean \pm SEM of at least three separate experiments.

28,000-fold more potent than trimethoprim, respectively. Methotrexate and trimetrexate were also more potent inhibitors of mammalian DHFR than were the diaminopyrimidines. In contrast, bacterial DHFR was exquisitely sensitive to the diaminopyrimidines (18).

These results were confirmed by studies of the ability of the various antifolates to form a tight-binding complex with DHFR and NADPH, as determined by direct measurement of the complex (Table I). Trimetrexate was found to be almost 10,000- and 2,000-fold more potent than trimethoprim and pyrimethamine, respectively, in its affinity for binding to the PC DHFR.

Classic folate structures such as the reduced folate leucovorin (5-formyltetrahydrofolic acid) and methotrexate require a specific active transport system to cross cellular membranes (8). This system is present in mammalian cells but is lacking in most bacteria and protozoa, and thus precludes the use of methotrexate for the treatment of microbial infections. To determine whether the more lipid-soluble trimetrexate would be taken up by pneumocystis, we compared the uptake of this compound, the physiologic folate leucovorin, and methotrexate in the intact PC organisms and in a human promyelocytic cell line, HL-60. Table II illustrates that, at 1- μ M concentrations of the compounds, leucovorin and methotrexate penetrated only the mammalian cell line, while trimetrexate entered both HL-60 cells and PC organisms, with steady-state amounts of 366 and 76 pmoles/mg, respectively.

These experiments demonstrate that PC DHFR is only weakly inhibited by trimethoprim, the antifolate commonly used to treat clinical pneumocystis infection. The classic antifolate methotrexate and its lipid-soluble analogue trimetrexate are 1500- to 28,000-fold more potent inhibitors, with ID_{50} s of 1.4 and 26.1 nM, respectively. Transport studies indicate that the PC organisms do not possess the membrane mechanism required for the transmembrane transport of folates (leucovorin) and methotrexate, but the hydrophobic nature of trimetrexate allows this drug ready intracellular access. These findings suggest that a more effective, nontoxic therapeutic approach may be to use the potent PC DHFR inhibitor trimetrexate, with or without sulfa, simultaneously with specific rescue of the host by leucovorin. Reduced folates such as leucovorin have well-documented ability to reverse the toxic effects of antifolate drugs on mammalian tissues (8). The efficacy of trimetrexate with sulfa has been compared to trimethoprim with sulfa in rats that had been maintained on corticosteroids for 8 wk.

Groups of 30 leucovorin-treated animals were randomized to receive a 21-d course of nothing additional, oral trimethoprim-sulfa, or oral trimetrexate-sulfa while corticosteroids were continued. Pneumocystis scores at the end of treatment (based on the percent of alveoli involved with *P. carinii*) (19) demonstrated that either treatment greatly diminished the quantity of organisms. Trimetrexate-sulfa-treated rats had fewer organisms, with 23 of 30 animals having <1% infected alveoli, while trimethoprim-sulfa-treated rats had 16 of 30 animals, and controls only 3 of 30 animals with <1% infected alveoli (our unpublished observations). A clinical trial is in progress to evaluate the efficacy of trimetrexate-leucovorin with or without sulfa as an alternative to conventional therapy for the treatment of PC pneumonia in man (20).

Summary

The therapy of *Pneumocystis carinii* (PC) pneumonia is often unsuccessful, particularly in patients with acquired immune deficiency syndrome (AIDS). Because of difficulties in growing the organism in vitro or obtaining purified organisms, current treatment choices have been made with little information on the metabolic effects of therapeutic agents on PC. This report quantitates the effects of the commonly used antifolates as well as the classic antineoplastic antifolate methotrexate and a lipid-soluble analogue, trimetrexate, on the target enzyme, dihydrofolate reductase (DHFR), in the PC organisms. Trimethoprim and pyrimethamine were found to be weak inhibitors ($ID_{50} = 39,600$ and $2,800$ nM, respectively), while methotrexate and trimetrexate were potent reductase inhibitors ($ID_{50} = 1.4$ and 26.1 nM, respectively). Transport studies with radiolabeled compounds showed that compounds with the classic folate structure (methotrexate and leucovorin) were not taken up by the intact PC organisms. In contrast, trimetrexate exhibited rapid uptake. These results suggest a major therapeutic advantage may be gained by combining a potent, readily transported PC DHFR inhibitor such as trimetrexate with the reduced folate leucovorin to achieve a highly potent antiprotozoan effect while preventing toxicity to mammalian cells.

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