ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1988, p. 430–433
0066-4804/88/040430-04$02.00/0

Vol. 32, No. 4

Potent Antipneumocystis and Antitoxoplasma Activities of Piritrexim, a Lipid-Soluble Antifolate

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Received 3 August 1987/Accepted 14 January 1988

Piritrexim, a lipid-soluble antifolate, was evaluated for its activity against *Pneumocystis carinii* and *Toxoplasma gondii*. The concentration of piritrexim needed to inhibit 50% of the catalytic activity of *P. carinii* dihydrofolate reductase (DHFR) was 19.3 nM, and that for *T. gondii* DHFR was 17.0 nM, concentrations that were 40- to over 1,000-fold less than those needed for the inhibition of activity by trimethoprim and pyrimethamine, the antifolates conventionally used in treating these organisms. Piritrexim was able to inhibit replication of *T. gondii* in a mouse peritoneal macrophage model at concentrations of 0.1 to 1.0 μM. Leucovorin, a reduced folate that can bypass the inhibition of DHFR by antifols in mammalian cells but not in protozoa, did not affect the ability of piritrexim to inhibit *T. gondii* replication. The addition of sulfadiazine, which alone was ineffective, to piritrexim allowed inhibition of *T. gondii* replication at lower concentrations of piritrexim than when piritrexim was used alone. These results suggest that piritrexim, alone or combined with a sulfonamide, may be a highly potent antitoxoplasma and antipneumocystis agent that could provide major pharmacologic and clinical advantages over available agents.

Pneumocystis carinii and *Toxoplasma gondii* are major causes of morbidity and mortality in patients with the acquired immunodeficiency syndrome (AIDS), as well as in patients with other immunosuppressive disorders (7, 9, 13, 16). The only regimens with documented efficacy for the treatment of these infections are pentamidine, trimethoprim-dapsone, and trimethoprim-sulfamethoxazole for *P. carinii* and pyrimethamine plus a sulfonamide for *T. gondii* (10, 12, 15, 17). Adverse reactions associated with these regimens occur frequently, especially in patients with AIDS, and often necessitate termination of therapy. New regimens are needed since there are no regimens documented to be effective for patients with toxoplasma infection who cannot tolerate sulfonamides and for patients with pneumocystis infection who cannot tolerate sulfonamides or pentamidine.

Rational development of therapeutic alternatives requires an understanding of metabolic pathways that may be a target for directed therapy. Dihydrofolate reductase (DHFR) is an enzyme that is essential in purine and thymidylate metabolism; and as such, it provides an ideal target for therapeutic intervention, especially since mammalian and protozoan DHFRs have markedly different affinities for individual DHFR inhibitors. Moreover, mammalian cells can utilize leucovorin, a reduced folate that bypasses the inhibition of DHFR by antifols, while certain protozoa cannot. DHFR inhibitors such as trimethoprim and pyrimethamine are known to be effective in combination with sulfonamides for treating pneumocystis and toxoplasma. It is logical to consider structural analogs of these drugs to find more potent and less toxic compounds. Trimetrexate, a lipid-soluble analog of methotrexate, has recently been shown to be 100 to 10,000 times more potent than trimethoprim and pyrimethamine in inhibiting the DHFRs of *P. carinii* and *T. gondii* (2, 3). Subsequently, results of studies in both rats and humans have documented that trimetrexate is an effective antipneumocystis agent when used either alone or in combination with a sulfonamide (1). Trimetrexate has also been shown to have potent antitoxoplasma activity in tissue culture and mouse studies, as well as in limited human trials (8).

Piritrexim (BW301) is a lipid-soluble analog of methotrexate that differs from trimetrexate primarily because it is a dimethoxy rather than a trimethoxy analog and has a shorter bridging link between the two ring structures (Fig. 1). The structures of trimetrexate and piritrexim more closely resemble folates than do the diaminopyrimidines trimethoprim and pyrimethamine (Fig. 1). Piritrexim has undergone phase 1 studies as an antineoplastic agent in humans and has been found to have a shorter half-life than trimetrexate (H. Iland, J. Laszlo, W. Brenckman, V. Currie, C. Young, T. Williams, C. Sigel, A. Guaspari, R. Blum, and S. Liao, Proc. Am. Soc. Clin. Oncol, 3:29 [abstr.], 1984; J. Laszlo, W. Brenckman, V. Currie, M. O'Hehir, T. Williams, A. Guaspari, C. Sigel, and R. Blum, Proc. Am. Assoc. Cancer Res. 26:158 [abstr.], 1985). Because these properties may offer therapeutic advantages over trimetrexate, such as a lower incidence of toxicity, as well as over the currently used DHFR inhibitors, a series of studies was undertaken to evaluate the ability of piritrexim to inhibit the DHFRs of *P. carinii* and *T. gondii*, as well as to inhibit the in vitro replication of *T. gondii*.

MATERIALS AND METHODS

Source of organisms. *P. carinii* was obtained from the homogenized lungs of Sprague-Dawley rats that were treated with dexamethasone for 6 to 10 weeks, as described previously (2). Organisms were partially purified from host cells by Ficoll-Hypaque density gradient centrifugation and sonicated in the presence of protease inhibitors (leupeptin, 50 μg/ml; chymostatin, 50 μg/ml; benzamidine, 2 mg/ml; apro-

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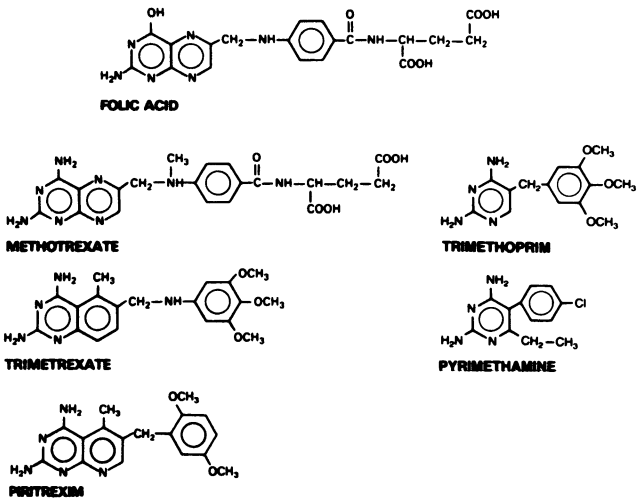

FIG. 1. Chemical structures of folic acid and inhibitors of DHFR.

tinin, 100 μg/ml; phenylmethylsulfonyl fluoride, 5 μg/ml). Phenylmethylsulfonyl fluoride was obtained from Boehringer-Mannheim Biochemicals (Indianapolis, Ind.). The other inhibitors were obtained from Sigma Chemical Co. (St. Louis, Mo.). The supernatant obtained following centrifugation at 27,000 × *g* was used in enzyme assays.

The RH strain of *T. gondii* was passaged every 3 to 4 days by intraperitoneal inoculation of BALB/c mice. For enzyme inhibition or in vitro replication inhibition studies, *T. gondii* was harvested by peritoneal lavage 4 days after inoculation. Organisms were partially purified by differential centrifugation as described previously (3). For enzyme studies, organisms were sonicated in the presence of protease inhibitors and processed as described above for *P. carinii*.

DHFR catalytic assay. DHFR catalytic activity was measured by using a standard spectrophotometric assay (5). The standard reaction mixture contained 0.1 mM NADPH, 160 mM Tris hydrochloride (pH 7.2), and 160 mM KCl and various concentrations of inhibitors in a final volume of 0.5 ml. The reaction was initiated after equilibration at 37°C by the addition of 50 nmol of dihydrofolate. The reaction velocity was measured by the disappearance of NADPH at 340 nM. Results from the catalytic assay were calculated by using ALLFIT, a least-squares curve-fitting program capable of simultaneous curve-fitting (6). The standard error of the mean was calculated by determining the average concentration that inhibited 50% of the catalytic activity in at least four individual experiments.

***T. gondii* replication inhibition.** For replication inhibition studies, fresh peritoneal macrophages were harvested from BALB/c mice by peritoneal lavage, suspended at 10^6 cells per ml, and cultured (1 ml per well) in two chambered sterile slides (Lab-tek; Miles Laboratories, Inc., Naperville, Ill.) for 24 h in RPMI 1640 medium supplemented with 10% fetal bovine serum–100 U of penicillin per ml–100 μg of streptomycin per ml (8). Tissue culture media and additives were obtained from MA Bioproducts (Walkersville, Md.). Cells were then infected with 1 ml of *T. gondii* (2×10^6 tachyzoites per ml) for 1 h, washed 5 times, and incubated for 18 h in RPMI 1640 medium supplemented as described above or supplemented with inhibitors. For synergy studies, RPMI 1640 medium was replaced with RPMI 1640 medium without folic acid or *para*-amino benzoic acid, as described previously (8). Slides were fixed in 3% gluteraldehyde, stained with Diff-quik, and counted in a blinded fashion. A total of 200 to 400 cells were evaluated for the following: number of cells infected with *T. gondii*, number of vacuoles per cell, and number of *T. gondii* per vacuole. Results are presented as the mean number of *T. gondii* per vacuole.

TABLE 1. Ability of piritrexim to inhibit *P. carinii* and *T. gondii* DHFRs

Drug	Concn (nM) of drug needed to inhibit 50% of DHFR catalytic activity in:	
	P. carinii	*T. gondii*
Piritrexim	19.3 ± 4.8	17.0 ± 6.7
Trimetrexate[a]	26.1 ± 2.2	1.4 ± 0.2
Trimethoprim[a]	39,600 ± 3,800	14,500 ± 1,900
Pyrimethamine[a]	2,800 ± 300	760 ± 130

[a] The values for trimetrexate, trimethoprim, and pyrimethamine were determined in previously published experiments (2, 3) by using a method similar to that described here. Values shown represent the mean ± standard error of the mean of at least four separate experiments.

Drugs. Piritrexim and pyrimethamine were obtained from Burroughs Wellcome Co. (Research Triangle Park, N.C.). Trimetrexate was obtained from Warner-Lambert (Ann Arbor, Mich.).

RESULTS

Piritrexim was very effective in inhibiting the DHFRs of both *P. carinii* and *T. gondii*. The concentration at which 50% of catalytic activity was inhibited for the *P. carinii* DHFR was 19.3 ± 4.8 nM (mean ± standard error of the mean) and that for the *T. gondii* DHFR was 17.0 ± 6.7 nM (Table 1 and Fig. 2) For comparison, data for trimetrexate, trimethoprim, and pyrimethamine from previous studies (2, 3) are included in Table 1. These results demonstrate that piritrexim and trimetrexate are each more potent than tri-

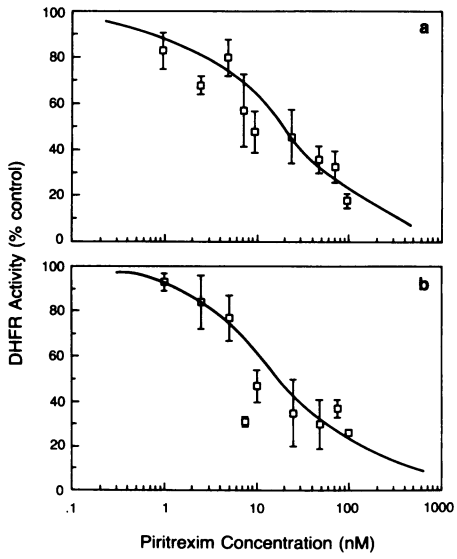

FIG. 2. Concentration of piritrexim versus ability to inhibit catalytic activity of *P. carinii* (a) and *T. gondii* (b) DHFRs. The percentage of control (no drug) activity is shown along the ordinate, and the concentration of piritrexim is shown along the abcissa. Each point represents the mean ± standard error of the mean of up to five separate experiments.

TABLE 2. Inhibition of *T. gondii* replication in vitro by piritrexim, pyrimethamine, and trimetrexate

Drug	Inhibition at the following concn (M)[a]:			
	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Piritrexim	1.5 ± 0.6[b]	2.3 ± 0.3[b]	5.7 ± 0.4	6.1 ± 0.4
Pyrimethamine	3.3 ± 0.8[b]	6.1 ± 0.5		
Trimetrexate		1.6 ± 0.09[b]	3.6 ± 0.2[b]	6.2 ± 0.4

[a] Values represent the mean ± standard error of the mean number of *T. gondii* per macrophage vacuole, as evaluated in 5 to 10 separate experiments for each drug concentration. The value for the control (no drug) was 5.9 ± 0.2.
[b] $P < 0.01$ compared with control.

methoprim and pyrimethamine in inhibiting the DHFRs from these organisms.

Piritrexim was also effective in inhibiting the in vitro replication of *T. gondii* in mouse peritoneal macrophages. Almost total inhibition of relication was seen at 1 μM, and partial inhibition was seen at 100 nM (Table 2). These concentrations were somewhat greater than those needed with trimetrexate, but were approximately 10-fold less than the concentration of pyrimethamine needed to inhibit replication.

Leucovorin at equimolar to 100-fold higher concentrations than piritrexim had no effect on the ability of piritrexim to inhibit the replication of *T. gondii* (data not shown). Sulfadiazine alone at concentrations of 200 μg/ml was not able to inhibit the replication of *T. gondii*. However, when this concentration of sulfadiazine was combined with subtherapeutic concentrations of piritrexim, the combination was more effective in inhibiting *T. gondii* replication than was either drug alone (Table 3).

DISCUSSION

The increased incidence of *P. carinii* and *T. gondii* disease in immunocompromised patients, especially patients with AIDS, combined with the limited number of well-tolerated therapeutic regimens, has prompted a search for more effective, safer, alternative regimens. Recently, trimetrexate, a lipid-soluble analog of methotrexate, has been found (2, 3, 8) to be a very potent inhibitor of *P. carinii* and *T. gondii* DHFRs in vitro, as well as a potent inhibitor of *T. gondii* replication in cultured peritoneal macrophages. Results of preliminary clinical trials with trimetrexate have suggested that it is a potent agent alone or combined with a sulfonamide in the treatment of AIDS patients with *P. carinii* pneumonia (1).

Piritrexim (BW301) is a lipid-soluble analog of methotrexate that is structurally similar to trimetrexate, but that has different pharmacokinetic properties in humans, including a shorter half-life (4.5 h for piritrexim versus 9 to 16 h for trimetrexate) (1, 11; Iland et al., Proc. Am. Soc. Clin. Oncol. 3:29 [abstr.], 1984). Since such differences may be important in determining the efficacy and toxicity of pharmacologic agents, investigation of the therapeutic potential of piritrexim in treating *P. carinii*- and *T. gondii*-related infections was undertaken.

Results of this study have shown that piritrexim can inhibit the DHFRs of *P. carinii* and *T. gondii* at concentrations of 19.3 and 17.0 nM, respectively, values that are similar to those observed for trimetrexate. For comparison, peak levels of 1.7 to 9.6 μM in serum were achieved in nine patients who received 2.5 to 12.5 mg of piritrexim per kg orally once daily for 5 days in phase 1 clinical studies (M. Rogers, Burroughs Wellcome, personal communication, 1987). Both drugs are 40- to over 1,000-fold more effective than trimethoprim or pyrimethamine, the DHFR inhibitors currently used in the treatment of *P. carinii*- and *T. gondii*-related diseases (Table 1) (2, 3).

Both piritrexim and trimetrexate are also more effective than pyrimethamine in inhibiting replication of *T. gondii* in cultured peritoneal macrophages (Table 2). Piritrexim has already undergone phase 1 testing as an antineoplastic agent in patients with a variety of malignant neoplasms (Iland et al., Proc. Am. Soc. Clin. Oncol. 3:29 [abstr.], 1984; Laszlo et al., Proc. Am. Assoc. Cancer Res. 26:158 [abstr.], 1985). As with trimetrexate, piritrexim can be given simultaneously with leucovorin, a reduced folate that can bypass the blockade induced by DHFR inhibitors. Since mammalian cells have the active transport mechanism necessary for uptake of leucovorin, but *P. carinii* and *T. gondii* do not, the concurrent administration of leucovorin with piritrexim would allow prevention of toxicity due to piritrexim in mammalian cells, without interfering with therapeutic efficacy (2, 3).

Results presented in two recent reports (4, 14) support the findings of the current study. In an in vitro culture system used to evaluate antipneumocystis activity and in an animal model of toxoplasmosis, piritrexim exhibited antiprotozoan activity. It is not clear at present whether piritrexim offers any advantage over trimetrexate as an antiprotozoan agent. Only clinical studies will be able to determine whether the structural differences or altered metabolism of piritrexim is associated with increased efficacy or decreased toxicity when used in this manner.

The addition of a sulfonamide to a DHFR inhibitor appears to result in improved therapeutic efficacy. In this study, sulfadiazine, which alone was not effective in inhibiting the replication of *T. gondii*, acted synergistically with piritrexim in inhibiting *T. gondii* replication (Table 3). Similar results have been found with trimetrexate (8). Although patients with AIDS have a high incidence of adverse reactions to sulfonamides, it is possible that a more effective DHFR inhibitor than trimethoprim or pyrimethamine, such as piritrexim, could be combined with lower doses of sulfonamides than are currently being used clinically to minimize toxicity without losing efficacy. Further studies are needed to determine whether structural analogs of methotrexate, such as piritrexim, alone or combined with a sulfonamide,

TABLE 3. Inhibition of *T. gondii* replication in vitro by piritrexim in combination with sulfadiazine

Sulfadiazine concn (μg/ml)	Inhibition at the following piritrexim concn (M)[a]:							
	1×10^{-7}	5×10^{-8}	2.5×10^{-8}	1×10^{-8}	5×10^{-9}	2.5×10^{-9}	1×10^{-9}	None
None	1.9 ± 0.2	2.9 ± 0.4	4.7 ± 0.4	5.5 ± 0.6	5.5 ± 0.8	5.3 ± 0.8	5.9 ± 0.7	
200	1.6 ± 0.1	1.6 ± 0.2	2.2 ± 0.3[b]	2.5 ± 0.2[b]	2.6 ± 0.4[b]	5.0 ± 0.5	5.5 ± 0.4	5.2 ± 1.0

[a] Values represent the mean ± standard error of the mean number of *T. gondii* per vacuole in three separate experiments for each drug concentration.
[b] $P < 0.05$ compared with no sulfadiazine.

will be more effective or less toxic than the current regimens used to treat *P. carinii* and *T. gondii* disease.

ACKNOWLEDGMENTS

We thank Rene Costello and Howard Mostowski for valuable assistance in this project.

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