Activity of Lipid-Soluble Inhibitors of Dihydrofolate Reductase
against *Pneumocystis carinii* in Culture and in a Rat Model of Infection

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Trimetrexate and BW301U (pirimexim isethionate), lipid-soluble inhibitors of dihydrofolate reductase, are potent inhibitors of the growth of *Pneumocystis carinii* in culture with WI-38 cells. Inhibition was observed with 0.1 μg of trimetrexate or BW301U per ml. Trimethoprim is ineffective at 100 μg/ml in this culture system. Both trimetrexate and BW301U were effective as prophylactic agents against *P. carinii* pneumonia in rats; trimetrexate at 7.5 mg/kg protected 9 of 10 rats, and BW301U at 5 mg/kg protected 4 of 10.

Drugs approved for treatment or prophylaxis of pneumonias caused by *Pneumocystis carinii* are unsatisfactory for many patients. Trimethoprim in combination with sulfamethoxazole or other sulfonamides often causes adverse reactions in acquired immunodeficiency syndrome patients (10, 13). The other commonly prescribed drug, pentamidine, causes a variety of mild to severe side effects (16). Other drugs and drug combinations have been used for treatment and prophylaxis of *P. carinii* pneumonia, but the numbers of patients treated have been small and the response to drugs has been difficult to evaluate in many instances. Pyrimethamine with sulfadoxine (Fansidar) is being evaluated (19), and difluoromethylornithine has had limited success in a small number of patients (17). Dapsone plus trimethoprim caused clinical improvement in 15 patients but still caused significant adverse reactions (14).

*P. carinii* does not grow in continuous culture but will undergo several replicative cycles when grown in contact with certain mammalian cell lines (4, 7). The organisms grow extracellularly, some remaining attached to the host cells and some being released into the medium. Growth is less predictable than for many other cultured organisms, but with appropriate controls the system is useful for studying drug effects on *P. carinii*. Previously we have been able to identify drugs with activity against *P. carinii* by incorporating drugs into medium on cultures of WI-38 cells infected with *P. carinii* from rats (2, 3). This culture system has now been used to identify two folate inhibitors that strongly inhibit proliferation of *P. carinii*: trimetrexate and BW301U (pirimexim isethionate). Both drugs were originally developed as anticancer agents (5, 11, 15, 18) and are believed to inhibit dihydrofolate reductase in a manner similar to that of methotrexate but are proposed to enter cells more readily because these new agents are more lipid soluble than methotrexate. These and the other drugs reported in this study were selected for testing because drugs with similar mechanisms of action (trimethoprim and pyrimethamine) are among the most useful agents for treating infections caused by *P. carinii*.

MATERIALS AND METHODS

Cultures of *Pneumocystis carinii* were prepared and evaluated as previously described (2-4). Briefly, human embryonic lung fibroblastic cells (WI-38) were cultured in 12-well tissue culture plates with minimum essential medium containing 10% fetal calf serum. Confluent monolayers were inoculated with homogenates of rat lungs infected with *P. carinii*. The infected rat lungs were obtained from rats immunosuppressed with cortisone acetate by the procedure of Frenkel et al. (9). Lungs from these animals were ground in minimum essential medium, trophozoite numbers were determined by fluorescein diacetate-ethidium bromide viability staining (12), and the inoculum was adjusted to give a final concentration of 3 × 107 to 7 × 107 trophozoites per ml. Each culture plate was divided so that control and drug-treated cultures were on the same plate. For example, in the experiment shown in Figure 2B, cultures in four wells were untreated, four wells received trimetrexate, and four wells received BW301U. A separate plate was harvested each day of the experiment.

The procedure for drug evaluation differed from the published method (2, 3) in that in the current studies the drug was added at the same time the cells were inoculated with pneumocystis-infected lung. The growth of *P. carinii* that occurs during the first 10 days after addition of inoculum is the primary culture. If the medium from the primary culture is replaced with fresh medium, replication will continue for 10 days or longer. This growth period was termed outgrowth culture. We have now established that the primary culture system gives results comparable to those of the outgrowth cultures previously employed. In both systems the number of trophozoites released into the supernatant indicates growth of *P. carinii*.

Plates were incubated at 35°C in 5% oxygen and 5 to 10% carbon dioxide, with the balance nitrogen. Separate plates were harvested for analysis at 1, 3, 5, 7, and 10 days after inoculation. At these times, 10 microliters of culture supernatant were removed from each well, air dried onto 1 cm2 of a slide, fixed with 100% methanol, and stained with Giemsa. These slides were examined at 1,000× magnification for
quantitation of numbers of organisms as described previously (2, 3). Slides prepared from each well were read as unknowns by two persons, each person counting 10 fields to determine the average value; therefore, for each culture condition the data reported were means of eight values for each time point. The data plotted in Fig. 1 and 2 are counts of trophozoites per field; multiplying these counts by a factor of $4 \times 10^5$ yields organisms per milliliter of culture supernatant.

The growth pattern of the primary cultures used in this study differs from the patterns we have previously reported for outgrowth cultures. Whereas the outgrowth cultures show linear growth over 10 days, the primary cultures show a characteristic lag of about 3 days and often plateau by day 10. The shape of the growth curve is dependent upon the inoculum employed. For example the experiments shown in Fig. 1A and 2B were inoculated from the same rat lung and are similar to each other but different from the other experiments shown, which used lungs from different rats as inocula. In all studies, drugs known to be effective against *P. carinii* were included as positive controls.

Control monolayers are routinely evaluated by microscopy for morphological changes caused by drug treatment. The ability of drugs to change the uptake of radiolabeled uridine by the monolayer cells is also determined for each drug. These precautions lessen the possibility of false-positive results caused by drugs that might inhibit growth of *P. carinii* by damaging the WI-38 cell monolayer.

For uridine incorporation studies, monolayers of WI-38 cells were grown on cover slips in the 1-ml wells. After 5 days control wells received fresh medium with 1 μCi of tritium-labeled uridine per well; experimental wells received labeled uridine and homogenate of infected rat lung. The appropriate drugs were also added at this time to both infected and uninfected cultures. At harvest 5 days later the cell monolayers on the cover slips were removed, washed, and counted with 10 ml of Aquasol II scintillation fluid in a Beckman LS 7500 scintillation counter. Uridine is incorporated into both WI-38 cells and *P. carinii* under these conditions, as illustrated by autoradiography, but incorporation into *P. carinii* is less than 1% that of host cells. Therefore, the values for incorporation in the infected culture reported in Table 1 largely represent incorporation into the WI-38 cells.

Trimetrexate, supplied by Warner-Lambert/Parke, Davis & Co., Detroit, Mich., was tested at 0.1, 1, 5, 10, and 20 μg/ml; BW301U, supplied by Burroughs Wellcome Co., Research Triangle Park, N.C., was tested at 0.1, 0.5, and 1 μg/ml. Phase 1 studies on trimetrexate suggested that the maximally tolerated human dose of 120 mg/m² produced plasma concentrations in the same range we tested in vitro (15).

Female Sprague-Dawley rats (Harlan Laboratories, Indianapolis, Ind.) used in these studies were ca. 150 g at the start of immune suppression. All rats received 25 mg of cortisone acetate subcutaneously twice weekly for 8 weeks. Tetracycline was added to drinking water to achieve a dose of 15 mg per rat per day. This model is similar to that described by Frenkel et al. (9). Daily doses of trimetrexate (7.5 mg/kg) or BW301U (5 mg/kg) were divided into two equal doses given 8 to 12 h apart and administered intraperitoneally throughout the period of immune suppression; all animals received 2.5 mg of leucovorin daily. Control animals received cortisone acetate, leucovorin, and oral tetracycline.

To evaluate infection at the end of the study, rats were anesthetized with ketamine hydrochloride and sacrificed by exsanguination. Blood was collected for hematologic evaluation. The lungs were removed aseptically, and a portion of the left lower lobe was fixed in Formalin for histology. A small wedge of lung was used to make impression smears, and the remaining tissue was frozen at $-70^°$C for subsequent culture. Impression smears and sections were stained with a rapid methenamine silver stain by the procedure of Brinn (6) modified as described earlier (4). Duplicate sets of impres-
sion smears were also stained with Giemsa stain, and duplicate sections were stained with hematoxylin and eosin.

Slides were examined as unknowns by two examiners whose readings were averaged to produce a score for each individual animal in each group. The severity of infection was determined on a scale of 0 to 4, with 4 representing more than 10 organisms per 1,000 cells, 3 representing 1 to 10 organisms per field, 2 representing 2 to 9 organisms in 10 fields, 1 representing 1 organism in 10 or more fields, and 0 representing no organisms seen in a search of more than 50 fields. As described, the evaluation scale is roughly logarithmic. With this scale, the numbers of trophozoites were evaluated from the Giemsa stains, and the numbers of cysts were evaluated from the silver stains.

Hematology data were analyzed by one-way analysis of variance and by the unpaired t test.

FIG. 2. Inhibition of growth of cultured P. carinii by trimetrexate and related dihydrofolate reductase inhibitors. (A) Cultures grown in standard medium and supplemented with 10 μM leucovorin, with (●) or without (○) 20 μg of trimetrexate per ml. (B) Cultures grown in standard medium with the indicated concentrations of drugs.

RESULTS

Both trimetrexate and BW301U significantly inhibited growth of P. carinii in vitro (Fig. 1 and 2).

BW301U was tested in the presence of leucovorin to protect the WI-38 monolayer cells from folate depletion. At 0.5 μg/ml the compound was strongly inhibitory to growth of P. carinii (Fig. 1A). Leucovorin alone had no effect on growth of untreated cultures. BW301U at 1 μg/ml was tested in two experiments and was strongly inhibitory (data not shown). Even at 0.1 μg/ml BW301U caused transient inhibition of growth of P. carinii in primary cultures (Fig. 1B).

Trimetrexate strongly inhibited growth of P. carinii in each of the four experiments in which it was tested. This drug was also tested in the presence of 10 μM leucovorin. At concentrations of 10 or 20 μg/ml trimetrexate produced declining numbers of P. carinii within 20 days (Fig. 2A). Lower concentrations of trimetrexate were tested with 10 μM leucovorin to estimate the lowest effective concentration (Fig. 3). At the lowest concentration tested (0.1 μg/ml), trimetrexate was still slightly inhibitory.

Trimetrexate and BW301U were directly compared with each other and with drugs with similar mechanisms of action in one experiment (Fig. 2B). As we had previously observed, trimethoprim alone at 100 μg/ml (345 μM) was no different from the control. BW301U at 0.5 μg/ml (1.6 μM) and trimetrexate at 5 μg/ml (14 μM) showed marked inhibition of similar degree. Pyrimethamine at 5 μg/ml (20 μM) and methotrexate at 10 μg/ml (20 μM) were also tested in this experiment. Growth with pyrimethamine was intermediate between control and trimetrexate curves after day 3. Methotrexate at 10 μg/ml was similar in effect to trimetrexate at 5 μg/ml.

Neither trimetrexate nor BW301U appeared to be grossly toxic to the monolayer cells at the concentrations used in drug studies. No morphologic changes were detected by microscopic evaluation.

Uridine incorporation was measured as an index of the influence of drug on uninfected and infected cultures (Table 1). Uridine incorporation into infected WI-38 cells was
markedly reduced by *P. carinii* infection, an observation that has been confirmed in eight separate experiments. Both infected and uninfected WI-38 cells were affected by the concentrations of trimetrexate and BW301U that inhibit growth of *P. carinii* in culture (Table 1). Relative to the leucovorin controls, both drugs inhibited uridine incorporation by about 30%. In separate experiments under similar conditions, we evaluated the effects of trimethoprim (50 μg/ml) with sulfamethoxazole (200 μg/ml); at these concentrations known to be effective against *P. carinii* in culture (2) the combination reduced incorporation by 10% in control (n = 3) or infected cultures (n = 11).

Both trimetrexate and BW301U were evaluated for the ability to prevent development of *P. carinii* infection in rats during immunosuppression. The doses selected were judged to be well tolerated, based on preliminary studies. Trimetrexate at 7.5 mg/kg protected 9 of 10 rats (Table 2). The one rat showing infection had only an occasional cyst in impression smears. BW301U at 5 mg/kg completely protected 4 of 10 rats; the remaining animals had average scores of less than 1.5 for cysts (silver stain) versus 3.1 in controls. Only two animals in the group had trophozoites in Giemsa-stained impression smears.

Both drugs showed a tendency to reduce leukocyte counts (Table 2), but the effect was not statistically significant (P > 0.05) in this experiment. Hemoglobin values were significantly lowered (P < 0.05) by each drug, but the mean cell hemoglobin content was not changed significantly (Table 2).

**DISCUSSION**

Trimetrexate was originally designed and tested as an anticancer agent (5, 18), as was BW301U (11). Both these agents were developed in an attempt to produce lipid-soluble substitutes for methotrexate that would not be dependent upon uptake systems in the cancer cells (11). Trimetrexate has subsequently been shown to inhibit histamine N-methyltransferase, but BW301U has not; consequently, trimetrexate elevates histamine levels in brain and kidney, but BW301U has no effect (8). Recent phase 1 studies with trimetrexate excluded patients in whom elevated histamine might be harmful (peptic ulcer disease, asthma, or chronic obstructive pulmonary disease); within the selected patient population, there was no evidence of toxicity related to histamine (15).

Trimetrexate and BW301U, both potent inhibitors of dihydrofolate reductase, are inhibitory to *P. carinii* in culture. Although the mechanism of action is presumed to be the same as for trimethoprim, both agents are considerably more potent. Pyrimethamine and methotrexate are also less potent than either trimetrexate or BW301U. When pyrimethamine or trimethoprim is used to treat pneumonia caused by *P. carinii*, sulfonamides are added to the regimen. With the marked increase in potency of trimetrexate and BW301U, therapy might be possible with these agents alone. Prophylaxis with one of these agents alone would also seem possible and appealing. Without the sulfonamide component in the drug regimen, side effects might be greatly reduced.

A recent report on dihydrofolate reductase purified from *P. carinii* confirms the action of trimetrexate directly upon that enzymatic target (1). In that study, methotrexate is extremely effective against the isolated enzyme but fails to be taken up by intact *P. carinii*. Our studies in which the organisms are exposed to the drugs for up to 10 days in culture show less difference in potency. Methotrexate at 22 μM is about as inhibitory as 14 μM trimetrexate.

Uridine incorporation is diminished by both trimetrexate and BW301U in the human cell lines WI-38 and MRC-5. These studies suggest that at doses effective against *P. carinii* some direct effects on host cells may also be evident. Other effective drugs also slightly inhibit uridine incorporation into host cells.

Trimetrexate has been used in a limited trial in acquired immunodeficiency syndrome patients for treatment of pneumonia caused by *P. carinii* (C. J. Allegra, J. Drake, J. Swan, H. C. Lane, B. Chabner, and H. Masur, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 691, 1986). Although therapy appeared effective, the number of patients was small. To our knowledge, use of BW301U for this condition in humans has not been reported.

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**LITERATURE CITED**


