ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1995, p. 2436–2441 Vol. 39, No. 11
0066-4804/95/$04.00+0

Evaluation of Potent Inhibitors of Dihydrofolate Reductase in a Culture Model for Growth of *Pneumocystis carinii*

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Received 16 May 1995/Returned for modification 17 July 1995/Accepted 15 August 1995

Many antifolates are known to inhibit dihydrofolate reductase from murine *Pneumocystis carinii*, with 50% inhibitory concentrations (IC_{50}s) ranging from 10^{-4} to 10^{-11} M. The relationship of the potency against isolated enzyme to the potency against intact murine *P. carinii* cells was explored with 17 compounds that had proven selectivity for or potency against *P. carinii* dihydrofolate reductase. Pyrimethamine and one analog were inhibitory to *P. carinii* in culture at concentrations two to seven times the IC_{50}s for the enzyme, suggesting that the compounds may enter *P. carinii* cells in culture. Methotrexate was a potent inhibitor of *P. carinii* dihydrofolate reductase, but the concentrations effective in culture were more than 1,000-fold higher than IC_{50}s for the enzyme, since *P. carinii* lacks an uptake system for methotrexate. Analogs of methotrexate in which chlorine, bromine, or iodine was added to the phenyl ring had improved potency against the isolated enzyme but were markedly less effective in culture; polyglutamation also lowered the activity in culture but improved activity against the enzyme. Substitution of a naphthyl group for the phenyl group of methotrexate produced a compound with improved activity against the enzyme (IC_{50}, 0.00019 μM) and excellent activity in culture (IC_{50}, 0.1 μM). One trimetrexate analog in which an aspartate or a chlorine replaced two of the methoxy groups of trimetrexate was much more potent and was much more selective toward *P. carinii* dihydrofolate reductase than trimetrexate; this analog was also as active as trimetrexate in culture. These studies suggest that modifications of antifolate structures can be made that facilitate activity against intact organisms while maintaining the high degrees of potency and the selectivities of the agents can be made.

Many antifolates have been tested as inhibitors of dihydrofolate reductase from murine *Pneumocystis carinii* (1, 5, 10–12, 14–16, 19, 24–26, 28). The potencies of these compounds vary widely, with 50% inhibitory concentrations (IC_{50}s) ranging from 10^{-4} to 10^{-11} M. The relationship of the potency against the isolated enzyme to the potency of the compound against intact murine *P. carinii* cells has not been systematically explored.

The potencies of antifolates against the isolated enzyme may not be a good reflection of activity against whole cells because some classes of these compounds penetrate cell membranes poorly (1, 13). For example, trimetrexate and piritrexim, both potent inhibitors of isolated *P. carinii* dihydrofolate reductase, require concentrations more than 1,000-fold higher than the IC_{50} for the enzyme for inhibition of *p*-aminobenzoic acid (PABA) incorporation, an assay that measures the ability of intact *P. carinii* to form folates in the presence of the inhibitor (5, 6, 13). Those studies suggest that understanding the structural features that promote entry into a particular organism is as important as understanding the interaction of the drug with its target: both must be maximized to develop an agent with ideal effects against intact organisms.

In order to address this question, we selected 17 compounds that had been fully characterized with regard to their activities against dihydrofolate reductase from murine *P. carinii*. Pyrimethamine, methotrexate, and trimetrexate were included as reference compounds. The experimental compounds were all chosen on the basis of their significant selectivities for the *P. carinii* enzyme. In addition, the compounds were chosen to represent a wide range of potencies and to represent several chemical classes. The studies presented herein correlate the in vitro inhibition of *P. carinii* dihydrofolate reductase with the inhibition of murine *P. carinii* growth in culture by these compounds and demonstrate that, for some classes of antifolates, activity against whole cells approaches the activity observed against isolated enzyme.

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MATERIALS AND METHODS

Isolation of *P. carinii* dihydrofolate reductase. Cytosolic enzymes of *P. carinii* were isolated as described previously for dihydrofolate reductase (5). The isolated organisms were resuspended in 50 mM phosphate buffer (pH 7.0) containing leupeptin (20 μg/ml), phenylmethylsulfonyl fluoride (9 μg/ml), soybean trypsin inhibitor (50 μg/ml), aprotinin (50 μg/ml), and 20 mM 2-mercaptoethanol, sonicated, and centrifuged at 100,000 × *g*. The supernatant, which contained both dihydrofolate reductase and dihydropteroate synthase activities, was frozen in liquid nitrogen for use in enzyme assays.

Recombinant *P. carinii* dihydrofolate reductase was also produced for enzyme assays. The gene sequence was identical to that reported previously (8). The expression system used pET8C with T7 RNA polymerase (27). Host *Escherichia coli* cells containing the appropriate plasmid construction were grown in Luria broth culture with 75 μg of kanamycin per ml at 37°C on a rotary shaker. The culture was transferred to fresh medium, and the optical density at 590 nm was monitored. When the optical density at 590 nm reached 0.4, the culture was shifted to 42°C for 30 min to induce the gene for T7 RNA polymerase. Rifampin (200 μg/ml) was added to suppress *E. coli* RNA polymerase. After 30 min, the culture was shifted back to 37°C for 90 min. Cells were harvested by centrifugation, washed, and suspended in appropriate buffer containing protease inhibitors and 2-mercaptoethanol as described above. Bacterial cells were ruptured by sonication. The supernatant obtained after centrifugation at 100,000 × *g* was used directly in enzyme assays.

Dihydrofolate reductase assay. The spectrophotometric assay was modified to optimize for temperature and substrate and cofactor concentration. The standard assay contained sodium phosphate buffer (40.7 mM; pH 7.4), 2-mercaptoethanol (8.9 mM), NADPH (0.117 mM), 1 to 3.7 IU of enzyme activity (1 IU = 0.005 optical density units per min), and dihydrofolic acid (0.092 mM). The first four reagents were combined in a disposable cuvet and were brought to 37°C. Dilutions of drug were added at this stage. The enzyme was added 30 s before the

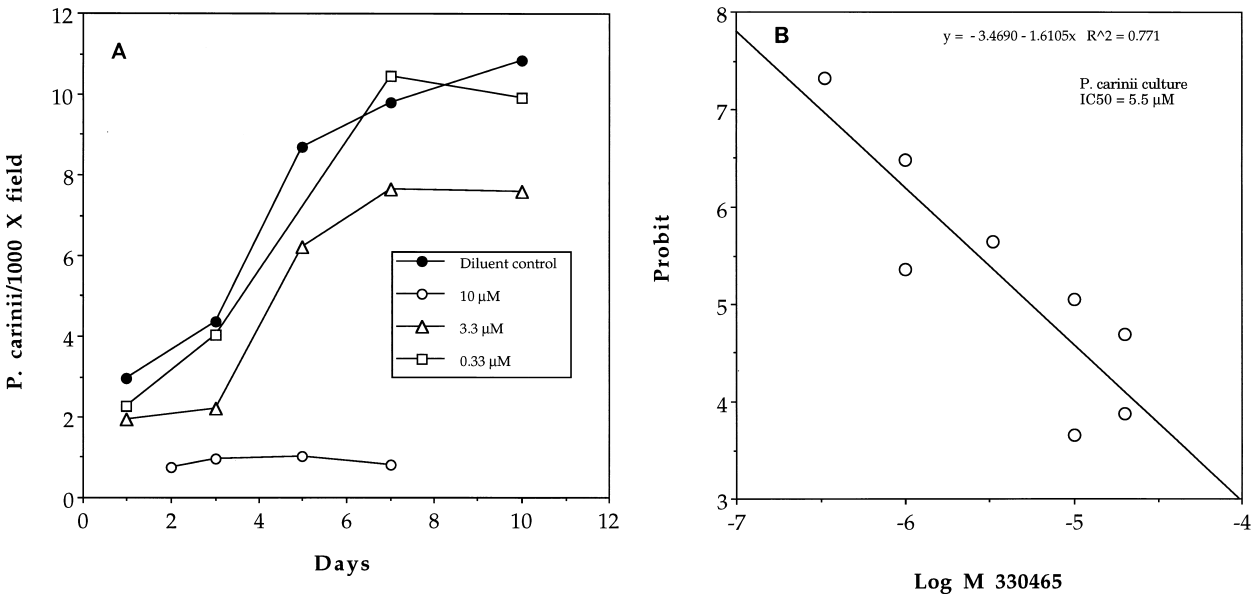


FIG. 1. Inhibition of *P. carinii* growth in short-term culture. (A) *P. carinii* was cultured in the presence of compound 330465 (compound 2) at concentrations of 10, 3.3, or 0.33 μM. The diluent control contained a dilution of DMSO equal to the highest concentration carried in diluents of any of the test drugs. *P. carinii* organisms were counted after Giemsa staining. The number of *P. carinii* organisms per ×1,000 field may be converted to the number of *P. carinii* organisms per milliliter of culture supernatant by multiplying by a factor of 400,000. (B) Data from individual experiments testing growth in the presence of compound 330465 (compound 2) were expressed as a percentage of control growth. The percentages from all experiments were pooled to generate the curve shown. Linear regression was performed to give the line of best fit. The equation of that line was used to calculate the IC_{50}, the concentration at which the probit value was equal to 5.0.

reaction was initiated with dihydrofolic acid. The reaction was followed for 5 min with continuous recording of optical density at 340 nm. The activity under these conditions of the assay is linear with the enzyme concentration over at least a fourfold range.

Determination of IC_{50}s. Determination of the IC_{50}s was performed as described previously (5). Briefly, dihydrofolate reductase activity was measured in the absence of inhibitor and in the presence of various concentrations of inhibitor to yield an inhibition curve ranging from 10 to 90% of the values without inhibitor. The values for percent inhibition were converted to probits, and the equation of the resulting straight line was used to calculate IC_{50}s (probit = 5.0).

Culture of *P. carinii*. Short-term culture of *P. carinii* has been used extensively to screen compounds with activity against the organism (2, 3, 9, 18, 21–23). The culture method used for these studies was described previously (4, 20), with inoculation of approximately 7×10^5 organisms per 1 ml of medium in 24- or 96-well plates containing confluent monolayers of HEL cells. Organisms were evaluated both by direct counting and by enzyme-linked immunosorbent assay (7); proliferation in culture was monitored over 5 to 7 days. Each study contained untreated control cultures as well as cultures containing a range of concentrations of inhibitor. In order to quantitate the degree of inhibition produced by the compounds in culture, the growth in each culture at 5 or 7 days was expressed as a percentage of the control growth; these percentages were then converted to probits and were analyzed as described above.

Assessment of stabilities of compounds in culture. For most of the experimental compounds to be tested, no direct assay exists. In order to estimate the amount of active material in the culture medium, the ability of these drugs to inhibit mammalian dihydrofolate reductase was exploited. Samples from the culture medium were taken at the time of inoculation and at days 1 through 7. These samples were diluted in water to yield a series of concentrations which, when added to the dihydrofolate reductase assay, inhibited rat liver dihydrofolate reductase at levels ranging from 10 to 90%. In order to assess the concentrations of inhibitors in these diluted samples, a standard curve generated with fresh authentic material was created for each inhibitor; the equation of the straight line was used with the probit for the unknowns in order to solve for the concentration present in the diluted sample. This method detects any bioactive material and does not distinguish active metabolites from the original material.

Statistical analysis. Curve fitting was done with Cricket Graph (Computer Associates). Linear regressions and correlation coefficients were obtained with InStat (GraphPad Software).

Chemicals. NADPH, 2-mercaptoethanol, and dihydrofolic acid were purchased from Sigma Chemical Company (St. Louis, Mo.). The compounds from the National Cancer Institute repository were originally synthesized and tested for their anticancer or antimicrobial effects in many different laboratories.

Inhibitors were shipped from the National Cancer Institute repository and were stored refrigerated in a desiccated state in the dark until use. Stock solutions were prepared in dimethyl sulfoxide (DMSO) and were then diluted in water to provide a range of concentrations suitable for testing in the enzyme assay or in culture. The carryover of DMSO into the dihydrofolate reductase assay or into culture was kept to below 1% because higher concentrations caused inhibition. Solutions of the drugs were stored at −70°C in the dark for short periods. Some stock solutions of compounds had to be made fresh on the day of assay.

RESULTS

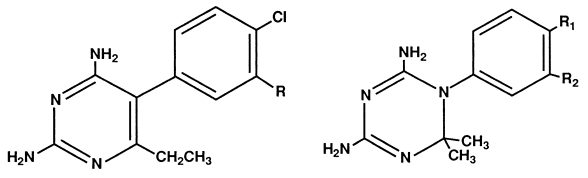
In order to assess the inhibition of growth of cultured *P. carinii*, parallel cultures were established, with a range of concentrations of inhibitor incorporated into the medium at the time of inoculation. Initial experiments established the general range of concentrations appropriate for testing. Figure 1A illustrates the results of such an experiment. Subsequent experiments were performed for each compound to confirm the range and add concentrations in the crucial region around the estimated IC_{50}. Figure 1B shows the IC_{50}s calculated for the culture; the calculation required at least three concentrations producing inhibition levels of between 10 and 90%.

Several groups of structurally related antifolates were evaluated for their effects on cultured *P. carinii*. The compounds were selected to cover a range of potencies against *P. carinii* dihydrofolate reductase as well as a range of lipophilicities. Pyrimethamine and one analog (compounds 1 and 2, respectively; Table 1) have modest potencies against the isolated enzyme, with IC_{50}s in the micromolar range. The concentrations of these compounds required to inhibit growth in culture were similar to the IC_{50}s for the enzyme.

Triazines such as compounds 3 and 4 (Table 1) are more potent inhibitors of *P. carinii* dihydrofolate reductase than pyrimethamine and its analogs. Inhibition in culture could not be demonstrated with compound 4, but compound 3 inhibited *P. carinii* growth in culture with an IC_{50} of 14.1 μM, or 83 times the IC_{50} for the enzyme.

A class of highly potent inhibitors of dihydrofolate reductase is related to methotrexate (compound 5; Table 2). Methotrex-

TABLE 1. Effects of pyrimethamine and related antifolates on *P. carinii* growth in culture



1: R = H (Pyrimethamine) **3:** R_1 = H, R_2 = $O(CH_2)_3OC_6H_4$-pNH_2

2: R = -N=N-$N(CH_3)_2$ **4:** R_1 = OCH_2-C_6H_4 -m$CON(CH_3)_2$, R_2 = Cl

Compound	IC_{50} (μM)		IC_{50} ratio
	DHFR[a]	*P. carinii* culture	
1 (pyrimethamine)	3.65	26.1	7
2 (330465)	2.8	5.5	2
3 (109832)	0.17	14.1	83
4 (139105)	0.021	>60	>2,900

[a] Data are from reference 5.

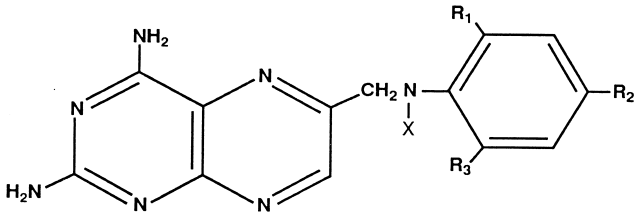
ate is known to require specific uptake mechanisms in mammalian cells and is not well taken up by *P. carinii* (13). The IC_{50} of methotrexate for *P. carinii* dihydrofolate reductase is 1.3 nM, but micromolar concentrations are required to inhibit growth in culture (Table 2). The result is a ratio of IC_{50}s exceeding 3,000. The configuration of the asymmetric carbon of the glutamate at position R-2 of the terminal phenyl ring is L for methotrexate. Introducing the racemic mixture in place of L-glutamate (compound 6) has little effect on the IC_{50}s. Further modification by substituting fluorines at positions R-1 and R-3 (compound 7) had no dramatic effect on the IC_{50} for the enzyme or the activity in culture. Substitution of chlorines instead of fluorines at positions R-1 and R-3 (compound 8) greatly enhanced the activity against the enzyme, but it had little effect on the activity in culture. As a result, for compound 8 the discrepancy between IC_{50}s for the enzyme and culture was even larger than that for methotrexate itself. A mixed halogen substitution at positions R-1 and R-3 with bromine and chlorine, respectively (compound 9), produced a compound with potency against dihydrofolate reductase intermediate between those of compound 8 and methotrexate, but it had a markedly reduced effect in culture compared with that of compound 8. The polyglutamate derivative (compound 11) was highly potent against the enzyme but required 40,000-fold higher concentrations than the enzyme Ic_{50} to inhibit growth in culture. Compound 10 differed from methotrexate only by the substituent at position X; substituting the alcohol for the methyl group at that position had more of an effect on the activity in culture than on enzyme inhibition. A wide discrepancy between IC_{50}s for culture and enzyme activities was also seen with compound 12, which differed from compound 6 only by the iodine at position R-1.

Compounds to which complex substituents were added to introduce lipophilicity to the basic methotrexate ring system were also explored. Compound 13 (Table 3) resembles methotrexate, but the phenyl ring is replaced by a naphthyl ring; this compound was a potent inhibitor of *P. carinii* dihydrofolate reductase and the most potent compound in culture in this series of experiments. Compounds with an unsubstituted naphthyl ring (compound 14) and an *S*-phenyl system (compound 15) both showed much reduced potencies in the enzyme assay and culture, but the correlation of the IC_{50}s for the enzyme and culture was close.

Trimetrexate (compound 16) is a potent, lipophilic antifolate with known antipneumocystis activity (1). The IC_{50} of trimetrexate for *P. carinii* dihydrofolate reductase was greater than that of methotrexate, and the IC_{50} for inhibition in culture was fourfold lower than that of methotrexate; as a result, the ratio of IC_{50}s was 262 (Table 4). A derivative (compound 17) in which the methoxy substituents were replaced by hydrogen (*meta*), chlorine (*para*), and aspartate (*meta*) substituents had similar properties in culture but was more active against the enzyme.

One concern with an evaluation of the effects of inhibitors on the growth of cultured *P. carinii* organisms was that the compounds might not be stable in the growth medium over the course of the experiment. Direct assay of these experimental

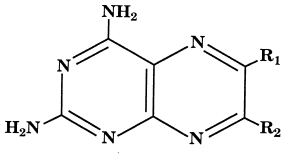
TABLE 2. Activities of methotrexate analogs in *P. carinii* culture



Compound	R-1	R-2[a]	R-3	X	IC_{50} (μM)		IC_{50} ratio
					DHFR[b]	*P. carinii* culture	
5 (methotrexate)	H	CONH**C**(COOH)HCH_2CH_2COOH	H	CH_3	0.0013	4.3	3,308
6 (117356)	H	CONH**C**(COOH)HCH_2CH_2COOH	H	CH_3	0.00088	1.5	1,705
7 (136736)	F	CONH**C**(COOH)HCH_2CH_2COOH	F	CH_3	0.00051	1.5	2,941
8 (29630)	Cl	CONH**C**(COOH)HCH_2CH_2COOH	Cl	CH_3	0.000035	0.72	20,571
9 (98579)	Br	CONH**C**(COOH)HCH_2CH_2COOH	Cl	CH_3	0.00021	8.1	38,571
10 (169531)	H	CONH**C**(COOH)HCH_2CH_2COOH	H	CH_2CH_2OH	0.00035	67.0	191,429
11 (341077)	H	CO[NH**C**(COOH)$HCH_2CH_2CO]_4OH$	H	CH_3	0.000035	1.4	40,000
12 (136735)	I	CONH**C**(COOH)HCH_2CH_2COOH	H	CH_3	0.000104	15.7	150,962

[a] Carbons shown in boldface type are in the L configuration.
[b] Data are from reference 5.

TABLE 3. Effects of complex substituents on methotrexate ring system



Compound	R-1	R-2	IC_{50} (μM)		IC_{50} ratio
			DHFR[a]	*P. carinii* culture	
13 (174121)	$CH_2N(CH_3)$-1-napthyl-4-L-glutamate	H	0.00019	0.10	526
14 (235777)	CH_2NH-1-naphthyl	H	0.13	3.7	28
15 (232965)	CH_2-S-phenyl	H	9.5	75.0	8

[a] Data are from reference 5.

compounds was not possible, but the high degrees of potency of the compounds against mammalian dihydrofolate reductase made it possible to use that enzyme as a detection system for active inhibitor (Table 5). Five compounds were assessed after incubation in medium only. The data in Table 5 show the concentration of drug dissolved in medium for the study and the concentrations calculated on the basis of assays performed before incubation (day 0) or after 1 to 7 days of incubation. Fresh medium alone or spent medium from untreated cultures of *P. carinii* caused no inhibition of mammalian dihydrofolate reductase when it was added directly to the assay mixture. For all five drugs in experiment 1, the agreement between the concentration of drug prepared and the concentration actually assayed was good, suggesting that medium also had no effect in the evaluation of the inhibitors. Significant changes over 4 days of incubation at 37°C were seen with only two drugs. Pyrimethamine concentrations increased approximately 5% daily, which is likely to be caused by evaporation from the wells during incubation. The concentrations of compounds 330465 (compound 2) and 232965 (compound 15) and trimetrexate also tended to increase by comparable amounts, but those changes were not statistically significant. Only compound 235777 (compound 14) showed a significant decrease in concentration over time in this experiment, as assessed by linear regression analysis.

In the second experiment (Table 5), drugs were put into *P. carinii* cultures, and the medium was evaluated as described above. The concentrations of pyrimethamine and compound 330465 (compound 2) were chosen to be near the IC_{50} for *P. carinii* dihydrofolate reductase; for the other three compounds the concentrations were well in excess of the IC_{50}s for the enzyme but were below the IC_{50}s for culture. In the present study the concentration of pyrimethamine again tended to increase, but the change was not statistically significant. The concentrations of both methotrexate and compound 117356 (compound 6) tended to decrease by about 10 to 12% daily, but the change was statistically significant only for compound 117356 (compound 6). There was no significant change in the concentrations of compound 235777 (compound 14). The concentrations of compound 330465 (compound 2) increased significantly from day 0 through day 7, but there was a major discrepancy between the actual concentration (1.63 μM) and the concentration assayed on day 0 (0.27 μM). This decrease may reflect a rapid action of the *P. carinii* inoculum on the drug. The apparent increase in concentration with subsequent incubation is unexplained, but because the assay detects all bioactive materials, the accumulation of active metabolites cannot be ruled out.

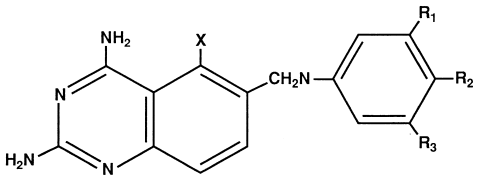
The assays of compounds from culture medium showed that bioactive material remained in culture for the duration of the growth experiments for these seven representative compounds. Therefore, failure of these compounds to act on *P. carinii* in culture cannot be ascribed to rapid destruction of the compound. For example, concentrations of compound 117356 (compound 6) fell to about 40% of the initial concentrations, but the concentration remaining at day 7 was still nearly 40 times the IC_{50} for the enzyme. Likewise, for methotrexate, the final concentration in culture was 17% of the initial concentration, but that concentration equaled the IC_{50} for the enzyme.

DISCUSSION

Among the clinically used antifolates, great variability exists in their abilities to tranverse membranes and reach the site of action within cells. *P. carinii* cells lack the folate-specific membrane transport system and therefore cannot take in charged folates or related antifolates (1). For this reason, effectiveness in culture or in PABA incorporation assays, both of which use whole cells, does not correlate strictly with the IC_{50} for the target enzyme. The present study was designed to explore the properties that confer potency toward intact *P. carinii*.

The compounds selected for the present study were related

TABLE 4. Inhibition of *P. carinii* growth in culture by trimetrexate and its analogs[a]



16: X = CH_3, R_1 = R_2 = R_3 = OCH_3 (Trimetrexate)

17: X = CH_3, R_1 = H, R_2 = Cl, R_3 = $CONHC(COOH)HCH_2COOH$

Compound	IC_{50} (μM)		IC_{50} ratio
	DHFR[b]	*P. carinii* culture	
16 (trimetrexate)	0.042	1.1	26
17 (132483)	0.0054	1.4	259

[a] The carbon in boldface type is in the L configuration.
[b] Data from reference 5.

TABLE 5. Stability of antifolates in *P. carinii* culture[a]

Expt and drug	Concn (μM) in medium[b]	Concn (μM) assayed after incubation[c]						*P* value of regression
		Day 0	Day 1	Day 2	Day 3	Day 4	Day 7	
Expt 1, incubation in medium only								
1 (pyrimethamine)	89	113	118	135	135	135		0.042[d]
2 (330465)	109	108	100	83	172	129		0.362
14 (235777)	19	20	22	13	13	11		0.024[e]
15 (232965)	49	37	50	106	36	90		0.329
17 (trimetrexate)	4.4	3.4	4.4	14	10	7.6		0.217
Expt 2, incubation in *P. carinii* culture								
1 (pyrimethamine)	3.3	4.0	5.6	6.2	6.1	5.6	6.3	0.070
2 (330465)	1.63	0.27	0.20	0.32	0.38	0.46	1.0	0.006[d]
5 (methotrexate)	0.0081	0.0094	0.0029	0.0023	0.0023	0.0017	0.0014	0.051
6 (117356)	0.081	0.058	0.049	0.047	0.039	0.033	0.033	0.032[e]
14 (235777)	2.4		2.4	2.6	2.8	3.6	2.4	0.671

[a] On day 0 dilutions of drug were made in medium to give the desired concentrations and 1-ml aliquots were distributed into the plates for incubation at 37°C. In experiment 1, medium only was used; in experiment 2, the medium was from monolayer cultures of HEL cells inoculated with *P. carinii* organisms from rat lung. To assay the amount of drug in the medium, dilutions were assayed as described in Materials and Methods. No inhibition was detected with supernatant from untreated cultures or with fresh medium.

[b] Concentration of drug in medium incubated at 37°C.

[c] Concentration of drug detected in medium initially (day 0) or after incubation for 1 to 7 days.

[d] Statistically significant increase by linear regression.

[e] Statistically significant decrease by linear regression.

to clinically used inhibitors of dihydrofolate reductase. In addition, these particular compounds were also chosen on the basis of their selectivities toward *P. carinii* dihydrofolate reductase. Selectivity is defined by the ratio of the IC_{50} for mammalian dihydrofolate reductase divided by the IC_{50} for *P. carinii* dihydrofolate reductase. For pyrimethamine, this ratio is 0.6, illustrating that the drug has little selectivity for either form of the enzyme (5). In contrast, compound 2 has a selectivity of nearly 7, showing a preferential inhibition of dihydrofolate reductase from *P. carinii* (5). Both pyrimethamine and compound 2 inhibit *P. carinii* growth in culture at concentrations less than 10-fold greater than the IC_{50} for the enzyme. This close agreement of the IC_{50}s for culture and the enzyme suggests that these compounds penetrate to their site of action efficiently.

Two triazines (compounds 3 and 4) were selected for the present study. These compounds are related to cycloguanil, which has been shown to be inactive against *P. carinii* (29). Of the 23 relatively hydrophilic triazines tested against *P. carinii* dihydrofolate reductase in previous studies, none was selective for the *P. carinii* enzyme (5); compound 3 was chosen for study in culture because the selectivity ratio was near unity, which was the best ratio for the compounds in this class. Compound 3 was active in culture at 83 times the IC_{50} for the enzyme. Compound 4, which has not been described previously, was a more potent inhibitor of *P. carinii* dihydrofolate reductase than the other triazines (5), but the selectivity was only 0.25, which favors the mammalian enzyme. No activity in culture could be demonstrated with this compound. The lack of enzyme selectivity of these hydrophilic triazines and the lack of good activity in culture suggest that these compounds are of limited appeal for development as treatment against this organism. A different series of s-triazine analogs, including several highly lipophilic compounds with selectivity for *P. carinii* dihydrofolate reductase, has recently been reported (17). These highly lipophilic compounds might enter *P. carinii* cells more easily than the hydrophilic s-triazines, but tests with intact *P. carinii* have not been reported.

Methotrexate is a highly potent inhibitor of various forms of dihydrofolate reductase, including that from *P. carinii* (5). Methotrexate is slightly selective for *P. carinii* dihydrofolate reductase, with a selectivity ratio of 1.9. The other seven methotrexate analogs chosen for the present study are more selective, with ratios ranging from 4.0 to 15.9 (5). As has been shown by others (1), methotrexate is not readily taken up by intact *P. carinii* isolates, and this fact is reflected by the high ratio between the IC_{50} for the culture and the IC_{50} for the enzyme. Polyglutamation, which is a mechanism used by cells to lower lipophilicity of folates and thereby retain them within the cell, predictably increased the ratio of the IC_{50} for culture to that for the enzyme (compound 11); the more water-soluble derivative is unlikely to enter the cell in significant amounts. Likewise, bromine, chlorine, or iodine substitutions were associated with ratios of the IC_{50} for the culture to the IC_{50} for the enzyme in excess of 20,000 (compounds 8, 9, and 12). Compound 10, in which the glutamate substitution of methotrexate at position R-2 was retained but in which the *N*-methyl group was replaced with an alcohol, was also poorly active in culture.

Truncated analogs of methotrexate have been reported to be selective for *P. carinii* dihydrofolate reductase (5). Three of these compounds were studied in the culture system (Table 3). Compound 13 contained an L-glutamate, but the amino acid was substituted on a naphthyl ring rather than a phenyl ring, as in methotrexate. This compound had potency toward *P. carinii* dihydrofolate reductase exceeding that of methotrexate and was highly active in the culture model. The ratio of the IC_{50} for the culture to the IC_{50} for the enzyme was 526. A naphthyl derivative that lacked the glutamate substitution (compound 14) was much less potent both against the enzyme and in culture, but the ratio of the two IC_{50}s was only 28. Compound 15 was much less potent than other methotrexate analogs against the isolated enzyme and in culture, but the ratio of the IC_{50}s remained low. With only three compounds, no strict conclusions as to the molecular features that promote activity in the whole cells can be drawn. Nevertheless, the potential of this type of compound is worthy of further exploration with additional analogs.

Trimetrexate is more lipophilic than methotrexate and therefore is more likely to be able to penetrate cell membranes. As this additional lipophilicity would predict, the ratio

of the IC_{50} for the culture to the IC_{50} for the enzyme is 2 orders of magnitude lower for trimetrexate than for methotrexate in our studies (26 versus 3,308). Although trimetrexate has been used in humans, the selectivity of the compound highly favors inhibition of mammalian dihydrofolate reductase (5). This unfavorable selectivity has necessitated combining trimetrexate with folinic acid (leucovorin), which protects the mammalian cells to a degree. The trimetrexate analog chosen for the present study has a much more favorable selectivity than trimetrexate; the ratio of IC_{50}s for mammalian and *P. carinii* dihydrofolate reductases is 0.072 for trimetrexate, showing strong selectivity for the mammalian enzyme, but the ratio is 2.8 for compound 17, showing modest selectivity for *P. carinii* dihydrofolate reductase. Compound 17, in which the amino acid substituent was an L-aspartate in the *meta* position of the phenyl ring, maintained or improved both enzyme and culture activities relative to those of trimetrexate. The results obtained with this compound suggest that other positional analogs of this type might be worthy of exploration, in that potency has been improved in a molecule with much better selectivity for *P. carinii* dihydrofolate reductase and the ability to enter *P. carinii* cells.

Structure-activity studies in which compounds are tested against an isolated target enzyme and its mammalian analog are crucial for determining those molecular features that produce both potency and selectivity. As illustrated in the present study, those compounds with desirable features can be further selected by making use of a culture assay in which the effect of the compound depends on its being taken up by the intact organism. The present studies also point out the need for further studies on the mechanisms of uptake by *P. carinii* cells.

ACKNOWLEDGMENTS

This work was supported in part by the following contracts: NO1-AI-87240 (to S.F.Q.), NO1-AI-35171 (to S.F.Q.), and NO1-AI-72647 (to J.W.S.).

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