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Pneumocystis carinii Dihydrofolate Reductase Used To Screen Potential Antipneumocystis Drugs

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***Pneumocystis carinii* was obtained in high yield from the lungs of immunosuppressed rats by rupturing mammalian host cells, washing away the soluble mammalian dihydrofolate reductase, and harvesting intact organisms in association with the mammalian plasma membranes. *P. carinii* dihydrofolate reductase, measured in the 100,000 × *g* supernatant from sonicated organisms, was obtained in yields ranging up to 62 IU per rat. The enzyme prepared in the presence of protease inhibitors was stable when frozen in liquid nitrogen. *P. carinii* dihydrofolate reductase differed from the mammalian enzyme in that the former was slightly inhibited by 150 mM KCl, whereas the latter was stimulated over twofold by 150 mM KCl. The standard assay for *P. carinii* dihydrofolate reductase contained 0.12 mM NADPH and 92 μM dihydrofolic acid. Under these conditions, the 50% inhibitory concentrations of the known inhibitors trimethoprim, trimetrexate, and pyrimethamine were 12 μM, 42 nM, and 3.8 μM, respectively. These standard compounds were also tested against dihydrofolate reductase from rat liver to allow an assessment of the selectivity of the drugs. Although it was the least potent, trimethoprim was the most selective. Pyrimethamine was more potent but was nonselective. Trimetrexate was extremely potent but was selective for mammalian dihydrofolate reductase. A series of experimental compounds was obtained from the National Cancer Institute and other sources through the Developmental Therapeutics Branch of the Division of AIDS at the National Institute of Allergy and Infectious Diseases. Among the first 87 compounds tested, 11 had 50% inhibitory concentrations below that of trimetrexate and 3 were more selective than trimethoprim. The most promising compounds in this original group were chemically related to methotrexate.**

Therapy for *Pneumocystis carinii* pneumonia has involved representatives of several classes of compounds: pentamidine (13), trimetrexate (1), trimethoprim-sulfamethoxazole (9), and clindamycin-primaquine (10, 15, 19). The mechanisms of action of pentamidine and clindamycin-primaquine remain obscure, but for trimethoprim and trimetrexate, the molecular target is known to be the enzyme dihydrofolate reductase (2).

Trimethoprim given with sulfamethoxazole is effective and causes few side effects in patients without AIDS, but the combination is associated with a high percentage of adverse reactions in AIDS patients (7, 17, 18); moreover, many patients do not respond to therapy with this combination. More potent dihydrofolate reductase inhibitors, such as trimetrexate or piritrexim, suffer from the disadvantage of poor selectivity (2, 8). Patients treated with these agents require concomitant leucovorin administration to avoid unacceptable toxicity. Trials with trimetrexate have demonstrated that toxicity remains a potential problem, even with the coadministration of leucovorin (16); the combination is also very expensive, and relapse is not uncommon.

The ideal dihydrofolate reductase inhibitor for use against *P. carinii* pneumonia should combine features of existing drugs: it should have the selectivity of trimethoprim and a potency approaching that of trimetrexate or piritrexim. We have prepared the dihydrofolate reductase from *P. carinii* and have begun to screen the library of compounds available through the National Cancer Institute and the Division of AIDS, National Institute of Allergy and Infectious Diseases, to attempt to identify compounds with these desired characteristics.

MATERIALS AND METHODS

Inoculation of rats with *P. carinii*. Female Sprague-Dawley rats (115 to 130 g) from Harlan Laboratories colony 202 were immunosuppressed by the administration of dexamethasone (1 mg/liter) in drinking water. The average daily dose in this colony was 30 μg. After 4 to 7 days of immunosuppression, the animals were anesthetized with a ketamine cocktail (ketamine, 12 μg; atropine, 2 μg; and chlorpromazine, 260 μg [per 130-g rat]) and transtracheally inoculated with *P. carinii* in accordance with our published procedure (5, 6). Within 6 to 8 weeks, the animals showed signs of severe pneumocystis pneumonia. When impression smears made from lungs of sentinel animals showed more than 100 organisms per ×1,000 field, the colony was judged ready for harvest.

Isolation of *P. carinii* from rat lungs. Heavily infected rats were anesthetized with a ketamine cocktail before sacrifice. The chest cavity was opened to expose the heart and lungs. A 20-gauge needle was inserted into the right atrium, and the animal was gently exsanguinated until no further blood could be removed. The lungs blanched noticeably during this procedure. In some experiments, the abdominal aorta and vena cava were interrupted and 5 ml of cold phosphate-buffered saline with 10 mM citrate (PBSC) was pushed through. The lungs were removed, dissected free from extraneous tissue, and washed thoroughly in cold PBSC. When all lungs had been harvested, they were washed once more and then minced in approximately 5 volumes of cold PBSC. The minced tissue was homogenized by 10 to 15

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strokes in a Dounce glass-on-glass, hand-held homogenizer with a loose-fitting spherically tipped pestle (Blaessig Glass, Rochester, N.Y.). This procedure ruptured the larger, more fragile mammalian cells but did not disrupt the much smaller *P. carinii* cells, as confirmed by microscopic examination. This homogenization step was similar to the procedure used to prepare the inoculum for cultures of *P. carinii*, illustrating that organisms subjected to homogenization in this way remain not only intact but also viable (14).

The crude homogenate was poured through the following stainless steel sieves in the order shown, and the filtrate was collected: 60 gauge (250-μm openings), 100 gauge (150-μm openings), and 280 gauge (63-μm openings). These sieves are designed to fit together in a stack with tight seals. A stainless steel pan forms the bottom of the stack and catches the filtrate. The whole assembly is kept on ice. Cold PBSC (250 ml) was added to the homogenate to facilitate passage through the sieves. After the upper sieve had drained, the residue in that sieve was agitated and gently pressed against the screen; a total volume of 1.5 liters of cold PBSC was used to wash the sieves. Bronchi and fibrous material retained on the upper sieve were discarded. Rare trophozoites or cysts were detected in this debris when samples were examined microscopically with Giemsa stain. Rare trophozoites were also observed in the residues from the last two sieves. When bacteria were detected microscopically at this stage, the preparation was discarded.

The filtrate was rich in trophozoites, cysts, erythrocytes, nuclei, and plasma membranes. The filtrate was centrifuged at 3,000 × *g* for 25 min. The supernatant, containing mammalian cytoplasmic proteins, was discarded. The pellet had three layers: lower (erythrocyte fraction), middle (pink mixed fraction), and upper (creamy white plasma membrane fraction). The middle and upper layers were pooled and washed twice in 5 to 10 volumes of cold PBSC. In the first four experiments, the upper and middle layers were handled separately. In later experiments, they were pooled. The layers were washed once in 3 volumes of 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, which lysed the remaining erythrocytes. The bright red supernatant was discarded, and the pellet was resuspended in the same buffer with proteinase inhibitors and 2-mercaptoethanol. In pilot experiments, Percoll, Ficoll-Hypaque, or sucrose gradients at this step significantly decreased yields without compensatory gains in purity; consequently, these methods were abandoned. The resuspended, washed pellet contained identifiable cell nuclei, large quantities of amorphous materials indistinguishable from plasma membranes, and clearly identifiable cysts and trophozoite forms of *P. carinii*. As determined by microscopic examination, the concentration of organisms present before sonication was in excess of 6×10^8/ml, for a calculated yield of approximately 4×10^9 per rat. This pellet was sonicated for 1 to 2 min in 10- to 20-s bursts. The material was kept on ice during sonication. No trophozoites were detected intact in the sonicate, but rare cysts were visible microscopically. The sonicate was centrifuged at 100,000 × *g* for 30 min. The supernatant was collected and stored in aliquots in liquid nitrogen or at −70°C. The enzyme retained over 80% of the initial activity over 14 days of storage at −70°C.

Dihydrofolate reductase could be further purified by affinity chromatography with dihydrofolate-Sepharose, but yields of activity from such columns were low; in the absence of any data to suggest the superiority of the purified enzyme for our purposes, the procedure was therefore abandoned in favor of the higher yields in the crude preparation (12).

Dihydrofolate reductase assay. The spectrophotometric assay for dihydrofolate reductase is well characterized and suitable for screening. The published assay was modified to optimize for temperature, substrate concentration, and cofactor concentration (2). K_m and V_{max} values estimated for our preparations were similar to those reported previously (2, 12). Although KCl was not included in the assay used by Allegra et al. (2, 12) for *P. carinii* dihydrofolate reductase, we tested the effect of KCl because some types of dihydrofolate reductase are stimulated by KCl but others are unaffected or are inhibited.

The standard assay contained sodium phosphate buffer (pH 7.4) (40.7 mM), 2-mercaptoethanol (8.9 mM), NADPH (0.117 mM), 1 to 3.7 IU of enzyme (1 IU = 0.005 optical density units per min), and dihydrofolic acid (0.092 mM). The first three reagents were combined in a disposable cuvette and brought to 37°C. Diluted drugs were added at this stage. The enzyme was added 30 s before the reaction was initiated with dihydrofolic acid. The reaction was continuously recorded for 5 min. Activity under these assay conditions was linear with enzyme concentration over at least a fourfold range.

Chemicals. NADPH, 2-mercaptoethanol, and dihydrofolic acid were purchased from Sigma Chemical Co. The compounds from the National Cancer Institute repository were originally synthesized and tested for anticancer effects. Many were tested against a variety of tumor cell lines, including L1210; inhibition data for mammalian dihydrofolate reductase were available for a few of these compounds, but we elected to retest all compounds against dihydrofolate reductase from rat liver in parallel with tests of the compounds against dihydrofolate reductase from *P. carinii*. These compounds represented many of the best-exploited classes of dihydrofolate reductase inhibitors. No data were available on whether any other enzymes were inhibited by these compounds. Inhibitors were shipped from the National Cancer Institute repository and stored refrigerated under desiccation in the dark until use. Stock solutions of each compound were prepared in dimethyl sulfoxide and then diluted in water to provide a range of concentrations appropriate for testing in the enzyme assay. Carryover of dimethyl sulfoxide into the assay was less than 1%; at higher concentrations, dimethyl sulfoxide itself caused inhibition. Solutions were stored at −70°C in the dark; a few compounds had to be made fresh on the day of assay.

Determination of IC_{50}s. Dihydrofolate reductase was assayed with no inhibitor and with a series of concentrations of inhibitors to allow for a range of inhibition from 10 to 90%. At least three concentrations were required for calculation. Semilogarithmic plots of the data showed a normal sigmoidal plot for most inhibitors. These plots were converted to straight lines by converting percent inhibition to probit values which were plotted versus the log of the drug concentration (11). The data were fit by least-squares linear regression. The 50% inhibitory concentration (IC_{50}) is the concentration at which the probit value is 5.0.

For each inhibitor, the IC_{50} was determined against dihydrofolate reductase from rat liver as well as from *P. carinii*. The selectivity ratio for a compound was determined by dividing the IC_{50} for the rat liver enzyme by the IC_{50} for the *P. carinii* enzyme.

TABLE 1. Yields of dihydrofolate reductase activity from *P. carinii*-infected rat lungs

Harvest	No. of rats	Assessment of infection (Giemsa stain score)[a]	Yield of dihydrofolate reductase[b]	
			Total IU	IU/rat
1	27	5.0	483	18
2	54	4.7	1,058	20
3	36	4.5	2,100	58
4	26	4.6	947	36
5	28	4.0	97	4
6	20	4.9	506	25
7	56	4.8	1,193	21
8	41	4.5	517	13

[a] Randomly selected rats were evaluated for degree of infection at times ranging from 6 days before harvest to the day of harvest. Seven to 21 rats in each group were evaluated. Giemsa-stained impression smears of lungs were evaluated with a previously published scale (4–6). Scores were assigned as follows: 5 = >100 organisms per ×1,000 oil immersion field; 4 = 11 to 100 organisms per field. Ten fields selected at random on each slide were evaluated, and the 10 scores were averaged to yield the value shown.

[b] One IU = 1 nmol of NADPH oxidized per min (ca. 0.005 optical density unit/min).

TABLE 2. Effect of KCl on dihydrofolate reductase activity in *P. carinii*, rat lungs, and rat liver

Source of enzyme	NADPH (μM)	IU[a] with:	
		No KCl	150 mM KCl
P. carinii	117	2.85	2.81 (0.99)
	58.5	2.26	1.56 (0.69)
Rat liver	117	2.83	7.45 (2.63)
	58.5	2.65	5.60 (2.11)
Rat lungs			
Plasma membrane fraction of uninfected lungs	117	0.30	0.64 (2.14)
Plasma membrane fraction of uninfected lungs contaminated with erythrocytes	117	0.23	0.74 (3.26)

[a] One IU = 1 nmol of NADPH oxidized per min (ca. 0.005 optical density unit/min). For each source of enzyme and condition of assay, the activity with KCl is divided by the activity measured in the absence of KCl; this ratio appears in parentheses in the last column.

RESULTS

Dihydrofolate reductase activity was obtained in good yield from *P. carinii* harvested from infected rat lungs (Table 1). The eight preparations shown in Table 1 supplied the enzyme used for evaluating the compounds reported in this study. The yield of dihydrofolate reductase was calculated not only as total units recovered but also as total units per rat. In the first four preparations, pellets were separated and handled differently, as the purification method was being standardized, but the last four preparations were all handled the same way; therefore, we may compare yields within this last set of four preparations. The yield of dihydrofolate reductase in fact correlated with the degree of infection with *P. carinii* in the harvested animals, as indicated by infectivity scores of Giemsa-stained impression smears (Table 1). Cysts were also counted in impression smears from these last four preparations; for harvest 5, with a Giemsa stain score of 4.0, the average number of cysts per field was 12, whereas for harvests 6 to 8, the average numbers of cysts ranged from 55 to 68 cysts per field.

The utility of the screening with dihydrofolate reductase from *P. carinii* depends upon the enzyme preparation being substantially free of mammalian dihydrofolate reductase. The initial isolation of *P. carinii* was designed to minimize the carryover of cytosolic mammalian enzymes into the 100,000 × *g* supernatant containing *P. carinii* dihydrofolate reductase. Several experiments were carried out to assess how successfully this goal was achieved.

In the first set of controls, dihydrofolate reductase was isolated from uninfected normal rat lungs by the same procedure as that used for infected lungs. In two separate experiments, the recovery of activity from the uninfected lungs was less than 10% of that from infected lungs (Table 2), suggesting that carryover from the host tissue would be minimal. The data in Table 2 for uninfected lungs are based on assays of a freshly prepared enzyme. One preparation made from uninfected mammalian lungs was frozen at −70°C and reassayed 3 days later; the activity was only 10% of the original activity, suggesting that, unlike the *P. carinii* dihydrofolate reductase, this rat lung enzyme was not stable in storage. In another preparation made from infected rat lungs, dihydrofolate reductase activity was assayed in the supernatant from the last wash before sonication of the pellet containing *P. carinii*. This supernatant contained proteinase inhibitors and 2-mercaptoethanol in the same concentrations as the sonicate. The activity of dihydrofolate reductase in this wash supernatant before sonication was 0.094 IU/min; sonication of the resuspended pellet, which disrupted *P. carinii*, increased the activity to 2.1 IU/min in the same volume.

The properties of the enzymes from rat liver, rat lung, and *P. carinii* were also compared. No striking differences in substrate or cofactor requirements were observed, but the response to KCl was quite different (Table 2). KCl at a concentration of 150 mM was a potent stimulator of mammalian dihydrofolate reductase, whether it was isolated from rat lungs or from rat liver (Table 2). In contrast, KCl had little effect on the *P. carinii* enzyme. This evidence was strengthened by the relationship between the yield of enzyme in our preparations (Table 1) and the severity of infection, as assessed by microscopic examination of lung smears. This relationship was confirmed in a third control experiment in which seven rats free of *P. carinii* (4) were housed in microisolator cages to prevent accidental acquisition of *P. carinii*. The rats received the same dose of dexamethasone as rats in the standard transtracheal inoculation model. At the end of 6 weeks, the rats were sacrificed and confirmed to be free of *P. carinii* by microscopic examination, and the lungs were used to make a dihydrofolate reductase preparation by standard techniques. When the preparation was assayed fresh without KCl, no dihydrofolate reductase activity could be detected, but with KCl added, 0.84 IU/min/100 μl of extract was detected.

An additional test that we applied to the putative *P. carinii* dihydrofolate reductase was to assess its response to known inhibitors of the enzyme. Trimethoprim, trimetrexate, and pyrimethamine were chosen as standard inhibitors because activities of these compounds against mammalian dihydrofolate reductase had been reported by several workers and because all three had been assessed by Allegra et al. in their study of *P. carinii* dihydrofolate reductase prepared by a different method (1). The comparison was valid, because the enzyme assay used in our study was very similar to that used by Allegra et al. For the comparison, the *P. carinii* enzyme from at least three different preparations was used. Data

TABLE 3. Comparison of IC_{50}s

Inhibitor	IC_{50} for dihydrofolate reductase from:			
	Rat liver		*P. carinii*	
	Earlier study (2)	This study	Earlier study (2)	This study
Trimethoprim	3.9×10^{-4} M	1.3×10^{-4} M	4.0×10^{-5} M	1.2×10^{-5} M
Trimetrexate	5.7×10^{-9} M	3.0×10^{-9} M	2.6×10^{-8} M	4.2×10^{-8} M
Pyrimethamine	2.4×10^{-6} M	2.3×10^{-6} M	2.8×10^{-6} M	3.8×10^{-6} M

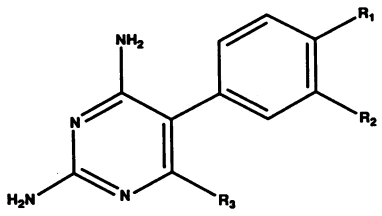
from these three experiments were not significantly different and were pooled for final analysis. The IC_{50}s in Table 3 for both rat liver and *P. carinii* dihydrofolate reductases were very close to published values.

The patterns of inhibition revealed with these compounds were different for the three enzyme sources. Trimethoprim was a poor inhibitor of mammalian dihydrofolate reductase but was 10-fold more potent against *P. carinii* dihydrofolate reductase. This pattern of inhibition was also unlike that observed with bacterial or fungal dihydrofolate reductase, with IC_{50}s for trimethoprim being on the order of 7 nM and 50 μM, respectively (3).

On the basis of the results discussed above, the enzyme that we isolated from *P. carinii*-infected rat lungs was judged to be from *P. carinii* and, when assayed in the absence of KCl, was judged suitable for screening new compounds for selective activity against this organism. The first series of compounds assessed were analogs of pyrimethamine, which itself had potency against *P. carinii* dihydrofolate reductase in the micromolar range and showed no significant selectivity (Table 4). The analogs tested varied from the parent compound in position 5 or position 6. Analogs that differed from pyrimethamine by having bulkier substituents at position 6 (R3) were uniformly less potent (analogs 118203, 211797, 211804, and 212329). Other substitutions or combinations of substitutions usually did not significantly affect potency against *P. carinii* dihydrofolate reductase.

Selectivity was also considered in analyzing these compounds. A few compounds that retained the chlorine in the *para* position on the phenyl ring (e.g., compounds 3062,

TABLE 4. Structure-activity relationships for pyrimethamine analogs[a]

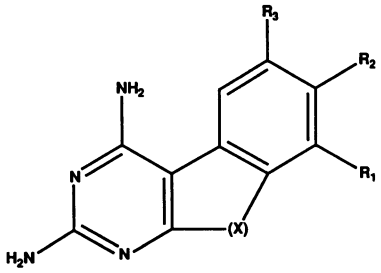


Compound	R_1	R_2	R_3	IC_{50} (μM) for:		Selectivity ratio (RL/PC)
				PC	RL	
Pyrimethamine	—Cl	—H	—Et	3.65	2.3	0.63
3062	—Cl	—Cl	—Et	1.08	0.19	0.18
7364	—Cl	—Cl	—Me	1.68	0.32	0.19
115194	—Cl	—Cl	—CH_2OEt	12.7	0.093	0.007
118203	—Cl	—H	—CH_2-O-(pNO_2-phenyl)	85.1	42.5	0.50
125357	—Cl	—Cl	—CH=$CHCH_2$-phenyl	0.5	0.088	0.18
125358	—Cl	—Cl	—CH_2O-phenyl	13.5	1.28	0.09
125359	—Cl	—Cl	—CH_2O-1-naphthyl	3.9	0.113	0.03
125850	—Cl	—Cl	—CH=CH-2-naphthyl	12.8	2.06	0.16
211797	—Cl	—H	—CH_2CN	490	188	0.38
211804	—Cl	—H	—CF_3	>42	>42	
212329	—Cl	—H	—CH_2NH-(pNO_2-phenyl)	17	1.06	0.06
302325	—Cl	—NO_2	—Et	0.85	0.015	0.02
319947	—Cl	—N_3	—Et	1.33	0.33	0.25
319949	—piperidine	—NO_2	—Et	5.05	0.9	0.18
330463	—Cl	—$N^+\equiv N$	—Et	0.19	0.032	0.17
330465	—Cl	—N=N-$N(Me)_2$	—Et	2.8	18.9	6.75
372950	—Cl	—N=CH-$N(Me)_2$	—Et	10.6	3.5	0.33
372955	—piperidine	—H	—Et	12.0	0.94	0.08
382035	—NO_2	—N(Me)CH_2-phenyl	—Et	1.6	0.0032	0.002
382036	—NH_2	—N(Me)CH_2-phenyl	—Et	3.6	0.022	0.006
382042	—NO_2	—N(Et)CH_2-phenyl	—Et	5.7	0.079	0.014
382046	—NO_2	—CH_2-phenyl	—Et	1.03	0.025	0.024
400654[b]	—Cl	—H	—Et	1.4	0.28	0.20

[a] The IC_{50} for each compound was determined for dihydrofolate reductase from *P. carinii* (PC) in the absence of KCl and for dihydrofolate reductase from rat liver in the presence of KCl (RL). The selectivity of each compound is the ratio of the RL IC_{50} to the PC IC_{50}. Compounds with selectivity for *P. carinii* dihydrofolate reductase have selectivity ratios of >1. Et, ethyl (—CH_2CH_3); Me, methyl (—CH_3).

[b] 2,4-Diamino groups are replaced by NH-SO_2-methyl.

TABLE 5. Structure-activity relationships of tricyclic ring systems[a]



Compound	R_1	R_2	R_3	X	IC_{50} (μM) for:		Selectivity ratio (RL/PC)
					PC	RL	
137187	—H	—OMe	—H	—CH_2CH_2—	6.2	1.25	0.20
137188	—H	—Me	—Me	—CH_2CH_2—	0.59	0.036	0.061
137189	—Cl	—H	—Cl	—CH_2CH_2—	1.07	0.022	0.021
137191	—H	—H	—Cl	—CH=CH—	0.12	0.016	0.133
137192	—H	—Me	—H	—CH=CH—	0.46	0.06	0.13

[a] See Table 4, footnote *a*.

319947, and 372950) showed selectivities near that of the parent compound. Compounds in which the chlorine was replaced by other substituents tended to be less selective. In particular, compounds with bulky substituents at R2 that contained aromatic groups (e.g., compounds 382035, 382036, 382042, and 382046) had greatly enhanced effectiveness against the mammalian enzyme. In this series, only one compound (330465) showed selectivity for *P. carinii*; it retained the chlorine in the *para* position of the phenyl ring and had a *meta*-dimethyl triazene substituent. A compound such as 372950, in which one nitrogen was replaced by CH, lost both potency against *P. carinii* and selectivity. Removal of the dimethyl substituents on the terminal nitrogen at R2 (e.g., compound 319947) also decreased selectivity, primarily by enhancing activity against the mammalian enzyme. A simple diazo substituent at R2 (e.g., compound 330463) significantly increased potency but not selectivity.

With pyrimethamine, the phenyl ring can rotate, but the second series of compounds can be thought of as analogs of pyrimethamine in which the two rings are held in rigid relationship to each other by fusion through carbon 6 of the pyrimidine ring (Table 5). Although some of these compounds were much more potent than pyrimethamine, they were also less selective against *P. carinii* dihydrofolate reductase.

The results with the pyrimethamine analogs suggested that potency against and selectivity for *P. carinii* dihydrofolate reductase were not easily enhanced by manipulations at position 5 or 6 of the pyrimidine ring. Attention was then shifted to a different ring system known to bind avidly to many forms of dihydrofolate reductase. Accordingly, the third series of compounds assessed included methotrexate analogs. Methotrexate was several orders of magnitude more potent than pyrimethamine and showed 1.9-fold selectivity (Table 6). Several of the analogs retained high potency and showed significantly improved selectivity. Halogen substitutions at R1 and R3 improved potency, relative to that of methotrexate, and enhanced selectivity in the following compounds: 29630, 98579, 136735, and 136736. Of these four compounds, only compound 136735 was monohalogenated, with an iodine substitution. Compounds containing a single bromine (compound 98580) or fluorine (compound 107146) at this site had improved potency against mammalian dihydrofolate reductase and thus lost selectivity. Other substitutions at R1, such as —CF_3 and —OCH_3, may result in slightly improved potency while not changing or slightly improving selectivity (compounds 144698 and 152737).

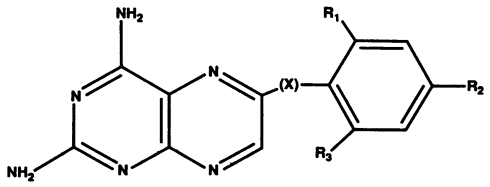
The substitution at R2 in methotrexate is derived from L-glutamate. Compounds such as 29630, 98579, and 98580 retained the L configuration at the asymmetrical carbon in the substituent at R2, but compounds such as 117356, 136735, and 136736, which contained the same substituent at R2 without the L configuration at the asymmetrical carbon, illustrated that the L configuration is not required for potency against *P. carinii* dihydrofolate reductase. Derivatives with a polyglutamyl group at R2 retained activity and were in fact more potent and selective than methotrexate itself (compounds 269401, 341076, and 341077).

All methotrexate derivatives with small non-amino-acid substituents at R2 had greatly impaired potency and showed a loss of selectivity (compounds 131463, 137545, 233903, 233904, 235791, 236642, and 241522). Dropping the methyl group from the linker between the two aromatic systems (designated X in Table 6) had little effect on potency against *P. carinii* dihydrofolate reductase (compound 257456 versus compound 117356). Replacing the methyl group in X with an ethyl group (compound 169531 versus methotrexate) may have slightly improved potency.

A fourth set of compounds had the same fused ring system as methotrexate but differed in substituents at position 7 of the ring (Table 7). Three compounds in this short series showed interesting selectivity. Compound 232965 was highly selective but had an IC_{50} of 9.5 μM against *P. carinii* dihydrofolate reductase. Compound 235777 was more potent and was still selective. Neither of these compounds was as potent as methotrexate, although both were more selective. In contrast, compound 174121 was more potent and more selective than methotrexate. This compound was analogous to methotrexate, with a naphthyl group replacing the phenyl group of methotrexate.

A small number of trimetrexate analogs were also tested (Table 8). All five test compounds had greater potency against *P. carinii* than trimetrexate. Two of the compounds (129516 and 132483) were selective for *P. carinii* dihydrofolate reductase, in contrast to trimetrexate, which was strongly selective for mammalian dihydrofolate reductase.

TABLE 6. Structure-activity relationships for methotrexate analogs[a]



Compound	R_1	R_2[b]	R_3	X	IC_{50} (nM) for: PC	IC_{50} (nM) for: RL	Selectivity ratio (RL/PC)
Methotrexate	—H	—CONHCH(COOH)CH_2CH_2COOH	—H	—CH_2NMe—	1.3	2.5	1.9
29630	—Cl	—CONHCH(COOH)CH_2CH_2COOH	—Cl	—CH_2NMe—	0.035	0.33	9.4
98535	—H	—CONHCH(COOH)CH_2COOH	—H	—CH_2NH—	28	17	0.61
98579	—Cl	—CONHCH(COOH)CH_2CH_2COOH	—Br	—CH_2NMe—	0.21	1.45	6.9
98580	—H	—CONHCH(COOH)CH_2CH_2COOH	—Br	—CH_2NMe—	1.09	0.55	0.50
107146	—H	—CONH(COOH)CHCH_2CH_2COOH	—F	—CH_2NMe—	0.72	0.36	0.50
117356	—H	—CONH(COOH)CHCH_2CH_2COOH	—H	—CH_2NMe—	0.88	6.20	7.0
127977	—H	—CONH(COOH)CH$(CH_2)_4NH_2$	—H	—CH_2NMe—	>8,000	>8,000	
131463	—H	—COOH	—H	—CH_2NMe—	940	110	0.12
136735	—H	—CONH(COOH)CHCH_2CH_2COOH	—I	—CH_2NMe—	0.104	1.65	15.9
136736	—F	—CONH(COOH)CHCH_2CH_2COOH	—F	—CH_2NMe—	0.51	4.80	9.4
137545	—Me	—COOH	—Me	—CH_2NMe—	190	38	0.20
144698	—CF_3	—CONH(COOH)CHCH_2CH_2COOH	—H	—CH_2NMe—	0.31	1.55	5.0
152737	—OMe	—CONH(COOH)CHCH_2CH_2COOH	—H	—CH_2NMe—	0.49	1.1	0.20
169531	—H	—CONHCH(COOH)CH_2CH_2COOH	—H	—CH_2NEt—	0.35	1.4	4.0
233903	—H	—$CONH_2$	—H	—CH_2NH—	153[c]	220	<0.0001
233904	—H	—H	—H	—CH_2NMe—	10,000	830	0.083
233910	—H	—$NHCOCH_3$	—H	—CH_2NH—	7,100	170	0.024
235791	—H	—$COCH_3$	—H	—CH_2NH—	720	75	0.104
236642	—H	—$CON(Me)_2$	—H	—CH_2NH—	>5,000	>10,000	
241522	—H	—CONH-Pr	—H	—CH_2NH—	3,100	480	0.15
257456	—H	—CONH(COOH)CHCH_2CH_2COOH	—H	—CH_2NMe—	0.82	2.54	3.1
269401	—H	—CO[NHCH(COOH)$CH_2CH_2CO]_2$OH	—H	—CH_2NMe—	0.2	1.0	5.0
341076	—H	—CO[NH(COOH)CH$CH_2CH_2CO]_3$OH	—H	—CH_2NMe—	0.2	1.59	7.9
341077	—H	—CO[NH(COOH)CH$CH_2CH_2CO]_4$OH	—H	—CH_2NMe—	0.035	0.35	10.0

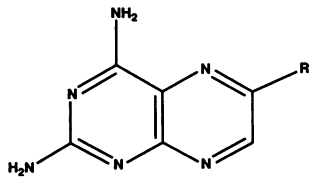
[a] See Table 4, footnote *a*. Pr, propyl.
[b] Asymmetrical carbons in boldface type have the L configuration.
[c] Millimolar.

Finally, a series of compounds containing the S-triazine ring system rather than the pyrimidine ring system was assessed. The potency of these compounds against *P. carinii* dihydrofolate reductase varied widely, but most of them were selective for the mammalian enzyme (Table 9).

DISCUSSION

The studies reported here establish that *P. carinii* dihydrofolate reductase can be isolated in sufficient amounts to allow screening of the hundreds of dihydrofolate inhibitors

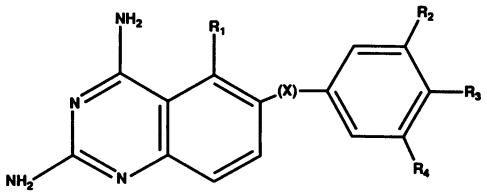
TABLE 7. Structure-activity relationships of truncated or ring-modified methotrexate analogs[a]



Compound	R^b	IC_{50} (μM) for: PC	IC_{50} (μM) for: RL	Selectivity ratio (RL/PC)
79658	—Me	>20	>20	
174121	—CH_2N(Me)-1-naphthyl-4-CONHCH(COOH)CH_2CH_2COOH	0.00019	0.0025	13.2
232965	—CH_2-S-phenyl	9.5	246	25.9
233904	—CH_2N(Me)-phenyl	10	0.83	0.083
233912	—CH_2-O-phenyl	136	13	0.096
235776	—CH_2-NH-phenyl	>1.9	>1.9	
235777	—CH_2NH-1-naphthyl	0.13	1.26	9.7

[a] See Table 4, footnote *a*.
[b] The asymmetrical carbon in boldface type has the L configuration.

TABLE 8. Structure-activity relationships of trimetrexate analogs[a]



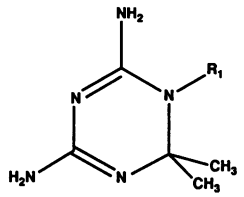
Compound	R_1	X	R_2	R_3[b]	R_4	IC_{50} (nM) for: PC	IC_{50} (nM) for: RL	Selectivity ratio (RL/PC)
Trimetrexate	—Me	—CH_2NH—	—OMe	—OMe	—OMe	42	3	0.072
122870	—Me	—CH_2NH—	—H	—CONHCH(COOH)CH_2COOH	—H	0.42	0.156	0.37
129516	—Me	—CH_2NH—	—H	—$CONHCH(CH_2COOH)_2$	—H	0.22	0.266	1.2
132483	—Me	—CH_2NH—	—H	—Cl	—CONHCH(COOH)CH_2COOH	5.4	15	2.8
184692	—Et	—CH_2NH—	—H	—CONHCH(COOH)CH_2COOH	—H	9.7	3.6	0.37
351521	—Me	—CH_2—	—OMe	—H	—OMe	31	1.5	0.047

[a] See Table 4, footnote *a*.
[b] The asymmetrical carbon in boldface type has the L configuration.

that have been made as anticancer or antimicrobial agents. The enzyme preparation, while by no means pure, is free of significant mammalian dihydrofolate reductase, so that reliable comparisons can be made.

Two techniques make this large-scale enzyme production practical. The first is the rat transtracheal inoculation model, which enables us to produce heavily infected rats within 6 weeks (5, 6). The second technique is the simple, high-yield scheme for the purification of *P. carinii* from lungs. The purification is based on the observation that *P. carinii* cysts and trophozoites are not susceptible to rupturing by mechanical shear forces that break mammalian lung cells. *P. carinii* organisms therefore survive homogenization and, because they are strongly adherent to membranes, are copurified with the membrane fraction. Extensive washing at this step easily removes the cytosolic mammalian enzymes, including

TABLE 9. Structure-activity relationships of S-triazine derivatives[a]



Compound	R_1	IC_{50} (μM) for: PC	IC_{50} (μM) for: RL	Selectivity ratio (RL/PC)
3075	—C_6H_4-pCOOH	>52	>52	
3077	—C_6H_3-m,pCl	0.154	0.029	0.19
5205	—C_6H_3-*ortho*-dimethyl	>20	>20	
96642	—Naphthyl	0.72	0.16	0.22
104124	—C_6H_4-m$(CH_2)_4$-C_6H_3-o,pCl	0.36	0.018	0.05
104129	—C_6H_4-p$(CH_2)_4$-C_6H_3-o,pCl	2.23	0.043	0.02
109832	—C_6H_4-mO$(CH_2)_3OC_6H_4pNH_2$	0.17	0.16	0.94
109834	—C_6H_4-mOCH_2-C_6H_4-mNH_2	1.4	0.25	0.18
112529	—C_6H_4-mO$(CH_2)_2OC_6H_4pNH_2$	0.47	0.041	0.087
113220	—C_6H_4-p$(CH_2)_2CONHC_6H_4pSO_2F$	0.59	0.0031	0.005
115928	—C_6H_4(mCN)	1.6	0.22	0.14
118213	—C_6H_4-p$OCH_2CONHC_6H_4mSO_2F$	1.0	0.074	0.074
122059	—C_6H_3(mCl)p$OCH_2CH_2NHSO_2C_6H_4mSO_2F$	0.34	0.027	0.08
127153	—C_6H_4-p$(CH_2)_4$-phenyl	0.31	0.02	0.06
127752	—C_6H_4-m$(CH_2)_2$-phenyl	0.43	0.041	0.10
127753	—C_6H_4-p$(CH_2)_2$-phenyl	0.57	0.029	0.05
127754	—C_6H_4-m$(CH_2)_4$-O-phenyl	0.27	0.0054	0.02
128189	—$C_6H_4pNH_2$	70.9	26	0.37
132275	—C_6H_4(mCl)-pOCH_2CONH-phenyl	0.3	0.0038	0.013
137765	—C_6H_4-mCH_2CONH-phenyl	0.44	0.28	0.64
158695	—C_6H_4-3,4,5-OMe	>26	>26	
211327	—$C_6H_4pCH(CN)(CH_2)_3$-phenyl	6.1	0.067	0.01
368890	—C_6H_4-3,5-diCF_3	66.9	0.57	0.009

[a] See Table 4, footnote *a*. p, *para*; m, *meta*; o, *ortho*.

small amounts of mammalian dihydrofolate reductase that may initially be present. The final sonication releases soluble enzymes from *P. carinii*. The dihydrofolate reductase released by sonication differs from the host dihydrofolate reductase in its response to KCl and to standard inhibitors (Tables 2 and 3). In addition to dihydrofolate reductase, we have also assayed dihydropteroate synthetase (data not shown), an enzyme not present in the mammalian host.

Using this enzyme preparation, we were able to confirm published IC_{50}s for trimethoprim, pyrimethamine, and trimetrexate (Table 3). Using the criteria of potency and selectivity, we identified 14 compounds of potential interest. All are more selective than methotrexate, pyrimethamine, or trimetrexate, and some are more selective than trimethoprim. Two of the 14 have IC_{50}s for *P. carinii* that are similar to those of trimethoprim (compound 232965) and pyrimethamine (compound 330465). Compound 235777 is more potent, with an IC_{50} of 0.13 μM. The single trimetrexate analog (132483) has a potency in the nanomolar range and a selectivity ratio of 2.8, a value nearly 40 times better than that of trimetrexate itself. The 10 methotrexate analogs (29630, 98579, 117356, 136735, 136736, 144698, 174121, 269401, 341076, and 341077) are highly potent, with IC_{50}s ranging between 0.88 and 0.035 nM, and highly selective, with selectivity ratios ranging from 5.0 to 25.9. On the basis of these results, these compounds are scheduled for further testing in culture systems and animal models, as appropriate, to determine whether any of them possess chemical and pharmacokinetic properties that would make them worthy of further development. Kinetic analysis of inhibition with selected inhibitors is also under way with both the crude preparation used for the initial screening and a more highly purified preparation obtained by affinity chromatography.

The goal of these initial studies was to identify classes of compounds with desirable potency against and selectivity for *P. carinii* dihydrofolate reductase. We identified methotrexate analogs as having the most desirable combination of high potency and high selectivity. A full structure-activity analysis was not possible with only the available compounds, but the data do serve to guide the efforts of organic chemists in synthesizing structures to allow a more complete characterization of *P. carinii* dihydrofolate reductase. These collaborative studies are now under way.

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