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6,7-Disubstituted 2,4-Diaminopteridines: Novel Inhibitors of *Pneumocystis carinii* and *Toxoplasma gondii* Dihydrofolate Reductase

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Four novel, disubstituted diaminopteridines have been identified which antagonize the uptake of a folate precursor (*para*-aminobenzoic acid) by rat-derived *Pneumocystis carinii* maintained in short-term axenic culture at concentrations ranging from 4.5 to 26 μM. The compounds were at least 10 to 100 times more active than trimethoprim in this assay. None of these entities exhibited toxicity to mammalian cell lines at <100 μM. The same structures also caused significant inhibition of *Toxoplasma gondii* tachyzoite replication within Madin-Darby bovine kidney cells at concentrations ranging from 0.1 to 10 μM. Three of the structures (GR92754, AH10639, and AH2504) were at least an order of magnitude more potent than the standard anti-*T. gondii* agent, pyrimethamine. All three entities were also significantly more potent and selective than pyrimethamine as inhibitors of *T. gondii* dihydrofolate reductase (DHFR), with 50% inhibitory concentrations within the range of 0.018 to 0.033 μM. One of these compounds, 6,7-dibutyl-2,4-diaminopteridine (GR92754), was also a potent and selective inhibitor of *P. carinii* DHFR (50% inhibitory concentration, 0.082 μM). GR92754 is the first DHFR inhibitor described that exhibits greater potency, selectivity, and intracellular activity against both organisms than any of the DHFR agents used clinically, namely, trimethoprim, pyrimethamine, and trimetrexate. This information could provide the starting point for examination of the pharmacokinetic and therapeutic potential of GR92754 and related chemical entities with animal models.

The antifolate combination trimethoprim (TMP)-sulfamethoxazole (SMX) remains a widely used first-line drug for prophylaxis and treatment of *Pneumocystis carinii* pneumonia in immunocompromised patients. Trimethoprim (TMP), however, is a relatively weak inhibitor of both endogenous and heterologously expressed rat-derived *P. carinii* dihydrofolate reductase (DHFR) (2, 6) and is ineffective in preventing pneumocystosis when administered as a single agent to glucocorticoid-immunosuppressed rats (17). The anti-*P. carinii* contribution of the sulfonamide moiety is also unclear. While relatively high doses of SMX alone are efficacious in rat pneumocystosis (18), the published 50% inhibitory concentration (IC_{50}) of SMX for rat-derived *P. carinii* dihydropteroate synthase is high (440 μM) (12). The high doses required to generate anti-*P. carinii* efficacy in patients also suggest that this drug combination does not operate as a synergistic antifolate. The recommended therapeutic dose of 7 g of SMX daily for 21 days may well be a contributory factor in provoking the higher-than-average incidence of adverse reactions in patients with AIDS (8, 11).

The discovery of a more potent *P. carinii* DHFR inhibitor may obviate the need for combination with the sulfonamide. This principle has been demonstrated both experimentally and clinically with trimetrexate glucuronate (TMX), which is effective when administered alone (1, 9). Unfortunately, this potent inhibitor of *P. carinii* DHFR exhibits greater selectivity for the human homolog and despite the use of concurrent leucovorin rescue, TMX is not a practical first-line therapy or a candidate for prophylactic use. Nevertheless, the search for similarly potent but more selective inhibitors of *P. carinii* DHFR should continue since although recent studies have identified significantly more potent and selective antimicrobial DHFR inhibitors, none of these have been demonstrated to possess antimicrobial activity against intact *P. carinii* at physiologically achievable concentrations (3).

The Glaxo Research and Development compound files were searched for chemical entities (for example, pteridine ring-based structures) which might be expected to inhibit DHFR. Approximately 30 entities were selected and screened for activity against rat-derived *P. carinii*. Entities with activity against this organism that also exhibited antibacterial properties and/or were cytotoxic to mammalian cells at any concentration of <100 μM were eliminated. This process generated five structurally related leads, one of which (GR92754) exhibited anti-*P. carinii* activity comparable to that of TMX. The aim of these studies was to confirm the mode of action of the compounds by demonstrating their ability to selectively inhibit *P. carinii* DHFR. The potential of these entities to inhibit *Toxoplasma gondii* DHFR and replication of this organism in culture was also examined, since toxoplasmosis represents another difficult-to-treat condition for which novel antimicrobial agents with greater selectivity are sought. Furthermore, potent and selective DHFR inhibitors with activity against both *P. carinii* and *T. gondii* could provide a practical solution to the management of these endogenous pathogens in human immunodeficiency virus-positive individuals.

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MATERIALS AND METHODS

Putative DHFR inhibitors GR92754, AH10639, AH2503, AH2501, AH2504, AH2497, and AH2498 were synthesized at Glaxo Research and Development Ltd. (Ware, Hertfordshire, United Kingdom). Purity was confirmed by crystalization and microanalysis. TMP was obtained from Sigma (Poole, United Kingdom), TMX was obtained from Parke-Davis Research Laboratories (Hampshire, United Kingdom), and pyrimethamine sulfate was obtained from The Wellcome Foundation Ltd. (Beckenham, Kent, United Kingdom).

Stock solutions (10 mM) of all chemical entities were prepared in dimethyl sulfoxide, except pyrimethamine, which was dissolved in distilled water. Stock solutions were subsequently diluted in buffer or culture media as described below. In each case, vehicle controls were prepared at the highest concentration of dimethyl sulfoxide or distilled water used.

Generation of *P. carinii* organisms. *P. carinii* organisms were generated in glucocorticoid-immunosuppressed rats. Male Sprague-Dawley rats (initial weight, 160 to 180 g; PHLS, Colindale, United Kingdom) received a 2.25-mg/liter solution of dexamethasone sodium phosphate (Oradexon; Organon Laboratories Ltd.) continuously in their drinking water (this equates to a dose of approximately 240 μg/kg/day) for at least 8 weeks prior to necropsy. Coadministration of antibacterial prophylaxis, 500 mg of tetracycline hydrochloride (Achromycin; Lederle) per liter, and a low (8%)-protein diet (Teklad; BP Nutrition) was also carried out.

Isolation of *P. carinii*. Lungs, dissected free of other tissue, were homogenized in ice-cold Hanks balanced salt solution in a stomacher blender (Seward Medical) and filtered through a 40-mesh sieve (Sigma) prior to separation on a Ficoll-Paque (Pharmacia) gradient at 4°C. *P. carinii* was aspirated from the Ficoll-saline interface following centrifugation at 450 × *g* for 20 min and washed twice in ice-cold Hanks balanced salt solution (by centrifugation at 850 × *g* for 10 min). The resulting pellet was resuspended in 10 ml of ice-cold incubation medium (see below). Rigorous checking for any possible concurrent bacterial or fungal infection contaminating the primary isolate was carried out. Serial dilutions of the primary isolate used in the assays (see below) were plated on both horse blood and Sabouraud agar and incubated in alveolar air (5% CO_2–95% air) for 48 h. Only uptake data generated from *P. carinii* isolates from which there was no microbial outgrowth are presented here (the microbial detection limit was ≥20 CFU/ml).

Quantification of the *P. carinii* inoculum. Enumeration of *P. carinii* organisms in the primary isolate was carried out by direct counting of *P. carinii* nuclei in serial dilutions of the resuspended pellet described above; this takes into account the noncystic stages, cysts, and intermediate forms of the life cycle, all of which may be represented in primary isolates. Two 2-μl aliquots at each dilution were spotted evenly onto glass microscope slides, air dried, fixed in 70% methanol, and stained with eosin and methylene blue (Haema Gurr; BDH, Poole, United Kingdom). The number of *P. carinii* nuclei was counted in 20 microscope fields under oil immersion at a magnification of ×1,000. The mean count was expressed as the density of nuclei per oil immersion field.

Evaluation of anti-*P. carinii* activity. Anti-*P. carinii* activity was assessed by measuring the potential of the compounds to antagonize the uptake of a labelled folate precursor, [*para*-^{3}H]aminobenzoic acid (PABA), during incubation overnight (18 h) at 35°C in alveolar air (5% CO_2 and 95% air). Stock solutions of the compounds were serially diluted in 96-well plates (Nunclon) in sterile, deionized water (see below) to which the *P. carinii* inoculum was added to achieve a density of approximately 5×10^7 *P. carinii* nuclei per ml in the wells. The label, PABA (specific activity, 50 Ci/mmol; Moravek Biochemical Inc.), was added to give a final concentration of 5 μCi/ml.

Pentamidine isethionate, final concentration, 10 μg/ml (Sigma) was included as a potent anti-*P. carinii* agent (positive compound control). TMP and TMX were included as comparators for the putative novel antifolates synthesized at Glaxo. An aliquot of the same primary *P. carinii* inoculum boiled for 10 min was also included to assess any background association of PABA with the inoculum which did not reflect active uptake of the label.

The incubation medium used was Glasgow's modification of Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (Gibco, BRI), 1% nonessential amino acids, 0.66 mM L-glutamine, 100 μM 7,8-dihydro-D-neopterin (B. Schirks Laboratories), penicillin (100 IU/ml), and streptomycin (100 μg/ml). All components of the incubation medium were obtained from Flow Laboratories (Irvine, United Kingdom) unless otherwise stated.

Following incubation with the label, *P. carinii* was aspirated onto double-thickness glass fiber filters (Wallac) with a cell harvester (Tomtec Inc.). Filters were washed five times with phosphate-buffered saline (pH 7.2) to remove unincorporated PABA prior to scintillation counting in an LKB Betaplate liquid scintillation counter. Assays were performed in duplicate with three different primary isolates of *P. carinii* (n = 6). After subtraction of the background, the number of counts incorporated at each concentration of a compound was used to calculate the mean IC_{50} relative to the mean number of counts incorporated by the compound-free primary isolate in the same plate.

Evaluation of activity against *T. gondii*. *T. gondii* RH was maintained by twice-weekly intraperitoneal passage in adult, female outbred mice (Tucks, Battlesbridge, Essex, United Kingdom). Confluent monolayers of Madin-Darby bovine kidney cells grown on glass coverslips in 24-well tissue culture plates were infected with tachyzoites of *T. gondii* 4 h prior to exposure to the novel pteridine analogs. Cell growth was supported with Hams F-12 medium (Flow Laboratories) supplemented with 10% fetal calf serum (Globepharm UK Ltd.). The relative densities were 10^6 tachyzoites and 2.5×10^5 MDBK cells per well, and all incubations were carried out at 37°C in 5% CO_2–95% air. The pteridine analogs and a standard antifolate with known anti-*T. gondii* activity (pyrimethamine) were added to the cultures to give final concentrations in the wells ranging from 10 μM to 1 nM and incubated for a further 24 h: four replicate wells were examined for each concentration of a compound. Infected monolayers were then rinsed in phosphate-buffered saline, fixed in Bouins solution, dehydrated through graded alcohols, and stained in Giemsa prior to enumeration of parasites. Monolayers were examined by light microscopy and evaluated by scoring 200 cells and recording the number infected and the total number of parasites. Significant differences in the mean numbers of parasites per cell in cultures exposed to antifolates compared with nonexposed cultures were identified by analysis of variance. A significant anti-*T. gondii* effect was considered to be the minimum concentration at which a significant ($P < 0.05$) decrease in the mean number of parasites per infected cell.

Evaluation of potency and specificity of DHFR inhibition. DHFR activity was assayed in cytosolic fractions of rat liver, *T. gondii*, and rat-derived *P. carinii*. The mammalian enzyme was partially purified from rat liver homogenates by centrifugation at 100,000 × *g* for 1 h; DHFR activity was contained in the 50 to 90% ammonium sulfate fraction prepared from the supernatant. This preparation was dialyzed and stored in aliquots at −80°C for use as a reference in all assays. DHFR in crude cell lysates of purified rat-derived *P. carinii* contained little, if any, cross-contaminating rat DHFR, as demonstrated by an insensitivity to added KCl and a unique pattern of inhibition by TMP and TMX (3). Concerns about cross-contamination of *T. gondii* DHFR with mammalian DHFR were eliminated by growing *T. gondii* RH on a Chinese hamster ovary cell line that lacks DHFR (American Type Culture Collection 3952 CL, CHO/dhfr−) (4).

The catalytic activity assay used is based on the spectrophotometric measurement of NADPH oxidation in the presence of dihydrofolic acid (3, 4). The standard assay for *P. carinii* DHFR contained 40.7 mM sodium phosphate buffer (pH 7.4), 8.9 mM 2-mercaptoethanol, 0.117 mM NADPH, 1 to 3.7 IU of enzyme (1 IU = 0.005 optical density U/min), and 0.092 mM dihydrofolic acid. The first three reagents were combined in a disposable cuvette and brought to 37°C. Drug dilutions or diluent were added to adjust volume. Enzyme was added 30 s before the reaction was initiated with the substrate, dihydrofolic acid. Assays for rat liver DHFR and *T. gondii* DHFR were the same, except that 150 mM KCl was included in the assay mixture, because both of these forms of DHFR are activated by KCl. Activity with these assays was linear with enzyme concentration over at least a fourfold range.

Determination of IC_{50}s was done essentially as previously described (3, 4). DHFR was assayed with no inhibitor and with a series of concentrations of the test compound sufficient to produce a range of inhibition from 10 to 90%. At least three concentrations in this range were required for a calculation. The IC_{50} of each inhibitor for rat liver DHFR was determined on the day of assay to eliminate day-to-day variability. The selectivity ratio for a test compound was the IC_{50} for rat liver DHFR divided by the IC_{50} for *T. gondii* or *P. carinii* DHFR.

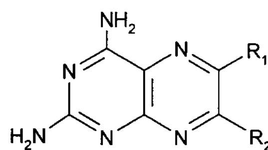
RESULTS

Five of the seven pteridine analogs tested were more potent inhibitors of *P. carinii* DHFR than TMP was (Table 1). Two compounds (AH2497 and AH2498) had poor aqueous solubilities, which precluded accurate IC_{50} determination: at concentrations at which these compounds were soluble, 4.54 μM AH2497 and 0.67 μM AH2498, *P. carinii* DHFR was inhibited to 72 and 92% of control values, respectively. Three compounds (GR92754, AH2503, and AH10639) exhibited modest selectivity for the *P. carinii* enzyme; however, none of them were more selective than TMP. The dibutyl-substituted pteridine (GR92754) was the most potent inhibitor of the *P. carinii* DHFR (IC_{50}, 0.082 μM) and also exhibited selectivity for the microbial enzyme (Table 1).

TMP did not antagonize the uptake of PABA by *P. carinii* at any concentration (<300 μM) (Table 2). In contrast, six of the seven Glaxo-synthesized *P. carinii* DHFR inhibitors did antagonize the uptake of PABA. GR92754 was the most potent antagonist (IC_{50} for PABA, 4.5 μM), and this correlates with the potent inhibitory activity of this entity at the enzyme level. The ranking of the remaining *P. carinii* DHFR inhibitors did not correlate absolutely with their activity at the enzyme level, indicating that other factors influenced intracellular potency.

The pattern of activity against *T. gondii* DHFR was distinct from that observed for the *P. carinii* enzyme; all of the Glaxo-

TABLE 1. Relationships between 6,7-disubstituted pteridine structures and inhibition of DHFR



Compound	R_1	R_2	IC_{50} (μM) for DHFR from:			Selectivity ratio	
			P. carinii	Rat liver	*T. gondii*	Rat liver/*P. carinii*	Rat liver/*T. gondii*
GR92754	*i*-Butyl	*i*-Butyl	0.082	0.32	0.028	3.9	11.4
AH10639	*i*-Propyl	*i*-Propyl	0.2	1.1	0.033	5.5	33.3
AH2503	Ethyl	Ethyl	0.62	2.2	1.0	3.5	2.2
AH2501	*n*-Pentyl	*n*-Pentyl	1.5	0.61	0.17	0.4	4.4
AH2504	*o*-Methoxyphenyl	Ethyl	2.0	0.41	0.018	0.2	22.8
AH2497	Cyclohexylmethyl	Cyclohexylmethyl	—[a]	—[a]	0.46[a]		
AH2498	*n*-Hexyl	*n*-Hexyl	—[a]	—[a]	46[a]		
TMP			12[b]	130[b]	2.7[c]	10.8	48.1
TMX			0.042[b]	0.003[b]	0.010[c]	0.07	0.3
Pyrimethamine			3.8[b]	2.3[c]	0.39[c]	0.6	6.9

[a] Insoluble under conditions of the assay at 5 μM.
[b] Previously published data (3).
[c] Previously published data (4).

synthesized pteridine analogs were potent and selective inhibitors of *T. gondii* DHFR (Table 1). GR92754 (IC_{50} for *T. gondii* DHFR, 0.028 μM), AH1063 (IC_{50} for *T. gondii* DHFR, 0.033 μM), and AH2504 (IC_{50} for *T. gondii* DHFR, 0.018 μM) are on the order of 10× more potent than pyrimethamine (published IC_{50} for *T. gondii* DHFR, 0.39 μM) (4). Furthermore, AH1063 and AH2504 exhibited markedly greater selectivity for the microbial enzyme (compared with the mammalian homolog) than did the standard inhibitor pyrimethamine (Table 1).

Four of the five *T. gondii* DHFR inhibitors examined significantly reduced the mean number of *T. gondii* tachyzoites per infected cell, and the most inhibitory, GR92754, was at least 100× more active than pyrimethamine in the same assay (Table 2). The potency rankings of the compounds were identical whether based on activity against the isolated enzyme or that against the intact microorganism.

TABLE 2. Activity against *P. carinii* and *T. gondii*

Compound	Mean IC_{50} (μM) for *P. carinii* PABA uptake ± SD[a]	MSI (μM) for *T. gondii*[b]
GR92754	4.5 ± 0.86	0.1 (30)
AH10639	16.2 ± 5.4	1 (44)
AH2503	15.7 ± 75	>10 (20)
AH2501	5.29 ± 3.5	10 (41)
AH2504	25.9 ± 11.9	0.1 (17)
AH2497	18.1 ± 14.6	NT[c]
AH2498	345[d]	NT
TMP	>300 ± 23	NT
TMX	50 ± 23	NT
Pyrimethamine	NT	10 (44)

[a] Mean concentration required to inhibit uptake of PABA to 50% of the control level (six determinations per concentration).
[b] MSI, mean significant inhibitory concentration, i.e., the concentration required to induce a significant ($P < 0.05$) decrease in the mean number of tachyzoites per infected cell (four determinations per concentration). Each value in parentheses is the mean percent reduction from the control at the concentration specified.
[c] NT, not tested.
[d] No standard deviation was calculated, as four of six determinations gave no inhibition at <350 μM.

DISCUSSION

Antifolates have proved to be valuable drugs in the management of infections caused by *P. carinii* and the coccidian protozoa, *T. gondii* and *Plasmodium* spp. The identification of more potent inhibitors with greater selectivity for the microbial enzymes, however, could allow significant improvements to be made. In particular, the management of pneumocystosis and toxoplasmosis in human immunodeficiency virus-positive individuals would be significantly improved if more potent and selective inhibitors of *P. carinii* DHFR and *T. gondii* DHFR were available: this would enable administration of lower doses and might obviate the need for concurrent administration of high sulfonamide doses. Tolerance of TMP-SMX in AIDS patients is a significant issue in the management of both pneumocystosis and toxoplasmosis, since this drug combination must be taken for the lifetime of a patient when the $CD4^+$ cell count falls below 200/ml (8, 13). Combinations of dapsone and pyrimethamine may also be of benefit in preventing toxoplasmic encephalitis, but tolerance is still a problem for around 40 to 50% of patients (13).

These studies provide further evidence that TMP exerts little or no anti-*P. carinii* effect, since there was no indication that concentrations of this inhibitor of <300 μM antagonized the uptake of a precursor of folate biosynthesis, PABA. Previously published data have demonstrated limited antagonism (a 30% reduction in PABA uptake) at a concentration of 100 μM, however (9). The lack of antagonism correlates with reports that TMP is a weak inhibitor of *P. carinii* DHFR: TMP IC_{50}s of 12 to 39 μM and 20 μM have been determined by using DHFR activity in crude cell lysates (2, 3) and the purified recombinant enzyme (6), respectively. Given that peak levels of TMP in plasma following oral administration of 160 mg to humans are reported to range from 4.4 to 6.4 μM (16), it is unlikely that TMP contributes to the anti-*P. carinii* effects observed following daily administration of 160 mg of TMP plus 800 mg of SMX; this is the recommended dose for secondary prophylaxis against *P. carinii* pneumonia (5). Furthermore, daily oral administration of 50 mg of TMP per kg for 7 to 9 weeks to concurrently immunocompromised rats has failed to demonstrate any prophylactic effect (5): SMX, however, exhib-

its a significant prophylactic effect when administered at 3 mg/kg/day by using the same protocol (17).

In contrast to TMP, all of the Glaxo-synthesized pteridine analogs antagonized PABA uptake: IC_{50}s for PABA uptake ranged from 4.5 to 345 μM. Furthermore, with the exception of AH2497, which was insoluble under the conditions of the DHFR assay, all of these analogs were inhibitors of *P. carinii* DHFR at $<$2 μM. GR92754 was the most potent inhibitor in both the enzyme assay and the functional assay (IC_{50} for *P. carinii* DHFR, 0.082 μM; IC_{50} for PABA uptake, 4.5 μM). At the enzyme level, the potency of this compound is comparable to those published for TMX (IC_{50} for *P. carinii* DHFR, 0.026 and 0.042 μM) (3, 6). GR92754, however, is markedly more active than TMX in the functional assay: the TMX IC_{50} for PABA uptake was 50 μM in these studies, and this compares favorably with the value of 70% inhibition of PABA uptake at 100 μM observed by Kovacs and coworkers, who used similar methods (9). This could indicate that AH92754 penetrates *P. carinii* more efficiently or possesses greater intracellular stability than TMX. Furthermore, GR92754 is significantly less toxic to mammalian DHFR than is TMX. Recently, novel chloroquinazoline analogs of TMX have also been synthesized which show potencies against *P. carinii* DHFR that are similar to or better than that of TMX itself, but like TMX, they exhibit little or no selectivity for the microbial enzyme (15). TMX (when administered parenterally) has demonstrated efficacy both in rodent models of pneumocystosis (10) and, with concurrent leucovorin rescue, in the human disease (1). Resynthesis of GR92754 to permit evaluation of its stability under physiological conditions and its pharmacokinetic profile and therapeutic efficacy after oral administration to rodents would be valuable.

Three of the Glaxo-synthesized compounds were more potent inhibitors of *T. gondii* replication than was pyrimethamine: GR92754, AH10639, and AH2504. All of these compounds were submicromolar inhibitors of *T. gondii* DHFR (IC_{50} for *T. gondii* DHFR, 0.028, 0.033, and 0.018 μM, respectively) and, on the basis of published data, are more potent and selective inhibitors of this enzyme than is pyrimethamine (IC_{50} for *T. gondii* DHFR, 0.24 to 0.39 μM) (4). The enhanced selectivity of the Glaxo DHFR inhibitors for the *T. gondii* enzyme is a key issue, since this organism replicates intracellularly and these three compounds efficiently penetrate both mammalian and microbial cells. The National Cancer Institute repository of compounds originally synthesized as potential inhibitors of DHFR for either antimicrobial or anticancer projects was recently screened to search for better inhibitors of *T. gondii* DHFR: several potent and selective *T. gondii* DHFR inhibitors were identified among the 130 entities examined (4). In contrast to the inhibitors in the Glaxo repository, however, none of the National Cancer Institute collection compounds exerted a significant inhibitory effect against the intact organism in cell culture at submicromolar concentrations. It is worth noting that no studies to date (including this one) have examined whether DHFR inhibitors can inhibit the replication of the encysted bradyzoite stages, which also contributes to the neuropathology of toxoplasmosis.

Studies published to date, which have evaluated a total of around 200 entities against *P. carinii* DHFR and *T. gondii* DHFR, suggest that while the relationship between structure and activity against the two enzymes is distinct, there is a possibility that the identification of entities with potent activity against both enzymes is achievable (7, 14, 15). A series of 15 6-substituted 2,4-diaminopyrimidines, which are closely related to the structures described in this study, have been shown to contain nanomolar inhibitors of both *P. carinii* DHFR and *T. gondii* DHFR (7). Several demonstrated selectivity for *T. gondii* DHFR but not for *P. carinii* DHFR. Although four of the compounds also demonstrated anti-*T. gondii* effects at micromolar concentrations in culture, cytotoxicity was evident at similar concentrations following exposure of a panel of tumor cell lines.

In conclusion, three 6,7-disubstituted pteridines have been identified which are potent and selective inhibitors of *T. gondii* DHFR and which inhibit replication of this organism in cell culture at submicromolar concentrations. One of the three entities, GR92754, is also a potent inhibitor of *P. carinii* DHFR and an antagonist of folate biosynthesis in this organism. On the basis of these data, the in vitro activity of these entities is superior to that of both TMP and pyrimethamine, which are used clinically in combination with sulfonamides in the management of pneumocystosis and toxoplasmic encephalitis. Although a number of groups have examined a range of potential folate antagonists, none has identified a structure that combines selectivity, potency at the enzyme level, and the ability to compromise the viability of both *T. gondii* and *P. carinii*. Resynthesis of GR92754 would enable its pharmacokinetic profile and metabolic stability to be established: effective penetration of the blood-brain barrier and the alveolar spaces is a property required for efficient chemotherapy of both infections. Studies of synergy with sulfonamide drugs would also be valuable and would suggest whether further resynthesis for efficacy testing in rodent models of pneumocystosis and toxoplasmosis is warranted. The data presented here could provide the basis for a chemical program to further optimize selectivity for and potency against either organism.

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