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% inhibitor concentration (IC50) 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.
MATERIALS AND METHODS

Putative DHFR inhibitors GR92754, AH10639, AH2503, AH2501, 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 (Hampshire, United Kingdom), and pyrimethamine sulfate was obtained from The Wellcome Foundation Ltd. (Buckingham, 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 the vehicle (10 mM dimethyl sulfoxide) for distilled water use.

Generation of P. carinii organisms. P. carinii organisms were generated in glucoctocid-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. Coadmixture of antibacterial prophylaxis, 500 mg of tetracycline hydrochloride (Achromycin; Lederle) per liter, and a low (8%) protein diet (Tolllip Limited) was continued in their drinking water (this equates to a dose of approximately 200 mg/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 (Tolllip Limited) was also continued.

Isolation of P. carinii. Lungs, dissected free of other tissue, were homogenized in ice-cold Hank’s 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 Hank’s 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 horse blood and Sabouraud agar and incubated in air at 35°C (5% CO₂–95% air) for 48 h. Only 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 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-ml 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). Spots were evenly stained with eosin and methylene blue (Haema Gurr; BDH, Poole, United Kingdom) and allowed to air dry for 48 h. Only uptake data generated from P. carinii nuclei were included as comparators for the putative novel antifolates synthesized at Glaxo.

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 (5-Hydroxyiminoamide), penicillin (100 IU/ml), and streptomycin (100 µg/ml). All components of the incubation medium were obtained from Flow (Flow Laboratories, Irvine, United Kingdom) unless otherwise stated.

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 as 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 10⁶ tachyzoites and 2.5 × 10⁶ MDBK cells per well, and all incubations were carried out at 37°C in 5% CO₂–95% 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 mM 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 in Boins solution, dehydrated through graded alcohol, 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 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 was observed.

Evaluation of potency and specificity of DHFR inhibition. DHFR activity was assessed 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-contamination of 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 39522, GA). The catalytic activity assay used is based on the spectrophotometric measurement of NADPH oxidation in the presence of dihydrofolinic acid (3, 4). The standard assay for P. carinii DHFR contained 40.7 nM sodium phosphate buffer (pH 7.4), 6.9 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 dihydrofolinic 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 either substrate, dihydrofolate, or dihydrostreptarotidin (TMX). Assays for P. carinii 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.

The IC₅₀ for 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 IC₅₀ for rat liver DHFR divided by the IC₅₀ 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 IC₅₀ 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 (IC₅₀ 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 (IC₅₀ for PABA, 4.5 µM), and this correlates with the potent inhibitory activity of this entity at the enzyme level. The rank order 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-
synthesized pteridine analogs were potent and selective inhibitors of *T. gondii* DHFR (Table 1). GR92754 (*IC₅₀* for *T. gondii* DHFR, 0.028 µM), AH1063 (IC₅₀ for *T. gondii* DHFR, 0.033 µM), and AH2504 (IC₅₀ for *T. gondii* DHFR, 0.018 µM) are on the order of 10× more potent than pyrimethamine (published IC₅₀ 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.

### 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 IC₅₀ 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, exhibit-
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: IC₅₀ₛ 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 (IC₅₀ for P. carinii DHFR, 0.082 μM; IC₅₀ for PABA uptake, 4.5 μM). At the enzyme level, the potency of this compound is comparable to those published for TMX (IC₅₀ 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 IC₅₀ for DHFR assay, all of these analogs were inhibitors of T. gondii 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 toxoplastic 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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