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

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Four novel, disubstituted diamonopteridines 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 (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.

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POTENTIAL OF THE COMPOUNDS TO ANTAGONIZE THE UPTAKE OF A LABELLED 3

The potential of the compounds to antagonize the uptake of a labelled 3

under oil immersion at a magnification of 800x was examined for each compound. Infected monolayers were then rinsed in phosphate-buffered saline (PBS) and stained with eosin and methylene blue (Haema Gurr; BDH, Poole, United Kingdom) before being washed five times with PBS to remove the stain.

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 analogue (5-hydroxymethyl)pterin), which 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 air at 5°C, 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 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 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.

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>IC₅₀ (µM) for DHFR from:</th>
<th>Selectivity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>P. carinii</td>
<td>Rat liver</td>
</tr>
<tr>
<td>GR92754</td>
<td>i-Butyl</td>
<td>i-Butyl</td>
<td>0.082</td>
<td>0.32</td>
</tr>
<tr>
<td>AH10639</td>
<td>i-Propyl</td>
<td>i-Propyl</td>
<td>0.2</td>
<td>1.1</td>
</tr>
<tr>
<td>AH2503</td>
<td>Ethyl</td>
<td>Ethyl</td>
<td>0.62</td>
<td>2.2</td>
</tr>
<tr>
<td>AH2501</td>
<td>n-Pentyl</td>
<td>n-Pentyl</td>
<td>1.5</td>
<td>0.61</td>
</tr>
<tr>
<td>AH2504</td>
<td>o-Methoxyphenyl</td>
<td>Ethyl</td>
<td>2.0</td>
<td>0.41</td>
</tr>
<tr>
<td>AH2497</td>
<td>Cyclohexylmethyl</td>
<td>Cyclohexylmethyl</td>
<td>— *</td>
<td>— *</td>
</tr>
<tr>
<td>AH2498</td>
<td>n-Hexyl</td>
<td>n-Hexyl</td>
<td>— *</td>
<td>— *</td>
</tr>
<tr>
<td>TMP</td>
<td></td>
<td></td>
<td>12 b</td>
<td>130 b</td>
</tr>
<tr>
<td>TMX</td>
<td></td>
<td></td>
<td>0.042 b</td>
<td>0.003 b</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td></td>
<td></td>
<td>3.8 b</td>
<td>2.3 c</td>
</tr>
</tbody>
</table>

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 (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.

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean IC₅₀ (µM) for P. carinii</th>
<th>MSI (µM) for T. gondii</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PABA uptake ± SD *</td>
<td></td>
</tr>
<tr>
<td>GR92754</td>
<td>4.5 ± 0.86</td>
<td>0.1 (30)</td>
</tr>
<tr>
<td>AH10639</td>
<td>16.2 ± 5.4</td>
<td>1 (44)</td>
</tr>
<tr>
<td>AH2503</td>
<td>15.7 ± 75</td>
<td>&gt;10 (20)</td>
</tr>
<tr>
<td>AH2501</td>
<td>5.29 ± 3.5</td>
<td>10 (41)</td>
</tr>
<tr>
<td>AH2504</td>
<td>25.9 ± 11.9</td>
<td>0.1 (17)</td>
</tr>
<tr>
<td>AH2497</td>
<td>18.1 ± 14.6</td>
<td>NT</td>
</tr>
<tr>
<td>AH2498</td>
<td>345 b</td>
<td>NT</td>
</tr>
<tr>
<td>TMP</td>
<td>&gt;300 ± 23</td>
<td>NT</td>
</tr>
<tr>
<td>TMX</td>
<td>50 ± 23</td>
<td>NT</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>10 (44)</td>
<td></td>
</tr>
</tbody>
</table>

* Mean concentration required to inhibit uptake of PABA to 50% of the control level (six determinations per concentration).
* 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.
* NT, not tested.
* 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 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, 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} 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 AH29754 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 antitumor 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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