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Inhibition of Recombinant *Pneumocystis carinii* Dihydropteroate Synthetase by Sulfa Drugs

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Forty-four sulfa drugs were screened against crude preparations of recombinant *Pneumocystis carinii* dihydropteroate synthetase. The apparent Michaelis-Menten constants (K_m) for *p*-aminobenzoic acid and 7,8-dihydro-6-hydroxymethylpterin pyrophosphate were 0.34 ± 0.02 and 2.50 ± 0.71 μM, respectively. Several sulfa drugs, including sulfathiazole, sulfachlorpyridazine, sulfamethoxypyridazine, and sulfathiourea, inhibited dihydropteroate synthetase approximately as well as sulfamethoxazole, as determined by the concentrations which cause 50% inhibition and/or by K_i. For all sulfones and sulfonamides tested, unsubstituted *p*-amino groups were necessary for activity, and sulfonamides containing an N^1-heterocyclic substituent were found to be the most effective inhibitors. Folate biosynthesis in isolated intact *P. carinii* was approximately equally sensitive to inhibition by sulfamethoxazole, sulfachlorpyridazine, sulfamethoxypyridazine, sulfisoxazole, and sulfathiazole. Two of these drugs, sulfamethoxypyridazine and sulfisoxazole, are known to be less toxic than sulfamethoxazole and should be further evaluated for the treatment of *P. carinii* pneumonia.

Pneumocystis carinii pneumonia (PCP) is a major cause of morbidity and mortality in AIDS patients. Even though the PCP incidence in AIDS patients has declined in recent years, PCP remains the most common AIDS-defining opportunistic infection (18).

Sulfa drugs are among the most effective agents used in the treatment of PCP. Co-trimoxazole, which is sulfamethoxazole in combination with trimethoprim, is the most effective of the currently available agents for both the prophylaxis and treatment of PCP. Unfortunately, approximately 30% of AIDS patients experience side effects, necessitating the use of alternative agents. Other sulfa drugs, including dapsone and sulfadoxine, are also in use, but they are all also limited by a high incidence of drug intolerance (17).

Sulfa drugs act by inhibiting the enzyme dihydropteroate synthetase (DHPS), the folate biosynthesis enzyme that catalyzes the linkage of 7,8-dihydro-6-hydroxymethylpterin pyrophosphate (H_2PtCH_2OPP) with *p*-aminobenzoic acid (PABA) to form dihydropteroate. The *P. carinii* DHPS has recently been cloned by Volpe and coworkers and expressed in a baculovirus vector (15, 16). The *P. carinii* DHPS is located on a multifunctional protein along with two other folate biosynthesis enzymes, dihydroneopterin aldolase and hydroxymethyldihydropterin pyrophosphokinase (15).

Sulfa drugs are known to vary in the frequency of their association with adverse effects (2). Thus, it is possible that other sulfa drugs might be as effective as sulfamethoxazole without eliciting adverse effects. Very few of the approximately 15,000 sulfa drugs that have been synthesized (8) have ever been tested against *P. carinii* in vitro (9, 14) or in vivo (5, 6, 20). This low rate of testing is due, in part, to the inability to culture these organisms continuously in vitro (12) and to the complexities of the various animal models (19). Therefore, an enzyme-based screen represents a more practical approach to the identification of effective sulfa drugs. Two small enzyme-based screens measuring DHPS activity in homogenates of isolated organisms have been carried out (9, 14). Both studies were limited by difficulties in obtaining large amounts of organisms. There are no such limitations when a recombinant enzyme is used. In this study, the recombinant DHPS enzyme was used to compare the relative efficacies of 44 sulfa drugs.

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MATERIALS AND METHODS

Chemicals and drugs. [^{3}H]PABA (50 Ci/mmol) was purchased from Moravek Biochemicals (Brea, Calif.). Tris-HCl, EDTA, PABA, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride, dimethyl sulfoxide, 1,4-dithiothreitol (DTT), dapsone, sulfamoxole, sulfadiazine, sulfamethoxazole, sulfaquinoxaline, and tetracycline were all purchased from Sigma Chemical Co. (St. Louis, Mo.). Sulfisomidine and sulfisoxazole were purchased from Nutritional Biochemicals Corporation (Cleveland, Ohio). *p,p′*-Difluoro, *m,m′*-dinitrodiphenyl sulfone was purchased from General Biochemicals (Chagrin Falls, Ohio). Sulfathiazole was provided by the University of Michigan Hospital (Ann Arbor). Ro 4-4393 (sulfadoxine), Ro 7-8307, Ro 5-2928, Ro 21-1182, Ro 5-5615, Ro 1-3354, Ro 1-4303 (sulfapyridine), Ro 1-9194, Ro 7-2844, Ro 4-0517 (sulfamethoxine), Ro 1-9623, Ro 5-0529 (sulfachlorpyridazine), Ro 02-0445, and Ro 04-3476 were provided by Hoffmann-La Roche Inc. (Nutley, N.J.), and diformyldapsone was provided by Jacobus Pharmaceutical (Princeton, N.J.). Sulfaguanidine, sulfamethoxypyridazine, sulfathiourea, and sulfamerazine were provided by Rhône-Poulenc Rorer (Centre de Recherche de Vitry-Alfortville, France). Sulfa drugs NSC 14652-J, NSC 39345-X, NSC 45751-M, NSC 52105-S, NSC 56605-K, NSC 74428-I, NSC 74587-G, NSC 78438-Q, NSC 107328-T, NSC 142456-T, NSC 163977-T, NSC 205491-S, NSC 229583-G, NSC 270146-U, NSC 279282-Z, NSC 303757-F, NSC 355394-H, NSC 403439-F, NSC 308795-G, and 6-hydroxymethylpterin pyrophosphate ($PtCH_2OPP$) were kindly provided by Mohamed Nasr, Division of AIDS, National Institutes of Health, Sf900-II serum-free medium, fetal bovine serum (FBS), gentamicin and amphotericin B (Fungizone) were purchased from Gibco Life Technologies (Grand Island, N.Y.).

Expression of recombinant *P. carinii* DHPS. *Spodoptera furgiperda* 9 cells and wild-type baculovirus were obtained from Richard Jove, Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor. They were routinely cultured as monolayers at 27°C in Sf900-II serum-free medium containing 10% FBS, 50 μg of gentamicin per ml, and 2.5 μg of amphotericin B per ml. Recombinant virus expressing fas 740 was obtained from Chris Delves, Wellcome Research Laboratories, Beckenham, Kent, United Kingdom. *S. furgiperda* 9 cells were seeded in 150-cm^2 flasks at 1.8×10^7 cells per flask and infected at a multiplicity of infection of 10 (15). Infected cells were harvested at 96 h postinfection by rapidly pipetting medium across the monolayer and were pelleted by centrifugation at 4°C at 1,100 × *g* for 10 min. The cell pellet was then washed three times with ice-cold lysis buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 μg of leupeptin per ml, 1 μg of pepstatin A per ml, 2 mM phenylmethylsulfonyl fluoride), resuspended in 3 volumes of lysis buffer, and sonicated with a microprobe (Sonifier Cell Disruptor; Heat Systems-

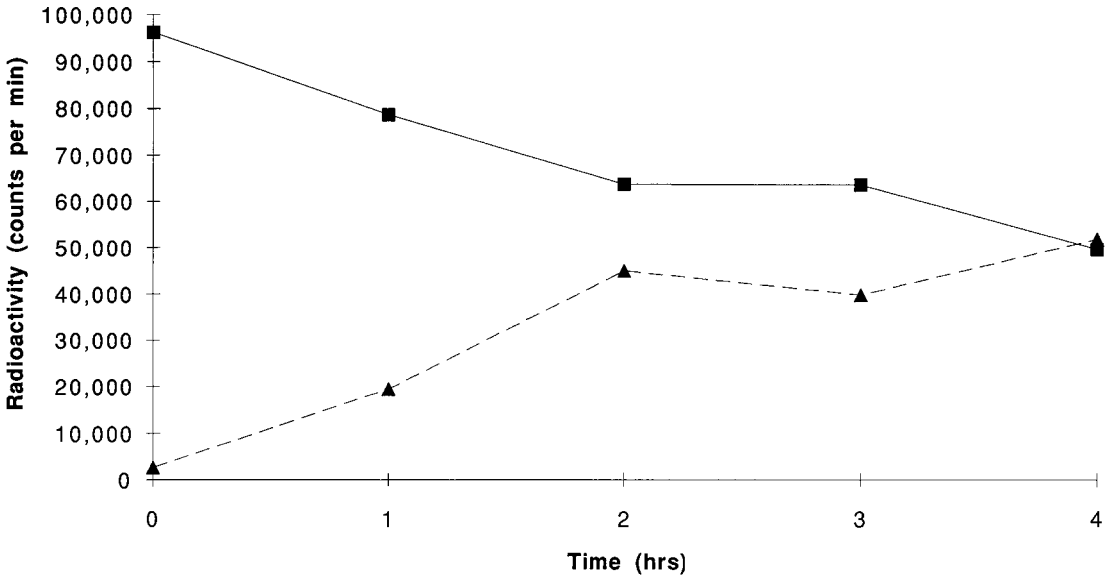


FIG. 1. Changes in the concentrations of H_2Pte (▲) and PABA (■) with time. Aliquots of a recombinant DHPS-containing lysate (40 μg) were incubated in 100 μl of assay buffer for 0 to 4 h at 37°C and then extracted with diethyl ether as described in Materials and Methods. Data represent single measurements.

Ultrasonics, Long Island, N.Y.) three times at maximum intensity for 5 s. After centrifugation at 12,000 × *g* at 4°C for 10 min, the pellet containing cell membrane and debris was discarded. The protein concentration in the supernatant was determined by the method of Bradford (3) (Bio-Rad Laboratories, Richmond, Calif.). The lysates were then stored in aliquots at −70°C.

DHPS assay. $PtCH_2OPP$ (10 mM in Tris-HCl, pH 8.0) was stored as 10-μl aliquots at −70°C. H_2PtCH_2OPP was prepared freshly by incubating 10 μl of 10 mM $PtCH_2OPP$ with 90 μl of a mixture of sodium dithionite (1.0 mg/ml) and potassium ascorbate (1.0 mg/ml, pH 8.0) at room temperature for at least 15 min immediately before use (11).

The enzyme assay buffer contained 40 mM Tris-HCl (pH 8.2), 5 mM $MgCl_2$, 10 mM DTT, 66 nM PABA (made up as a mixture of 16 nM [^{3}H]PABA and 50 nM unlabelled PABA), and 100 μM H_2PtCH_2OPP. The reaction was initiated by the addition of *S. furgiperda* 9 cell lysates (containing 4 U of enzyme) in a final volume of 100 μl at 37°C. Reaction mixtures without cell lysate or H_2PtCH_2OPP served as blanks. The amount of enzyme used was chosen because it is at the upper end of the range over which the reaction rate is proportional to the enzyme concentration.

After 1-h incubations, the reactions were stopped by adding of 300 μl of 1 M citrate-phosphate buffer, pH 3.8. The radioactive 7,8-dihydropteroate (H_2Pte) formed was separated from unreacted [^{3}H]PABA by using a modified ether extraction method (13). At pH 3.8, PABA, but not H_2Pte or folates, is extracted into the ether layer. Two hundred microliters of the pH 3.8 mixture was extracted with four 0.6-ml portions of anhydrous diethyl ether. An aliquot of the extracted water layer and the combined evaporated ether layers, redissolved in 0.2 ml of 0.1 N NaOH, were put into scintillation vials containing 15 ml of Scintiverse BD scintillation fluid (Fisher Scientific, Fair Lawn, N.J.). The radioactivity in the vials was counted in a Beckman LS 7000 scintillation counter. One unit of activity is defined as the amount of enzyme required to catalyze the production of 1 pmol of H_2Pte per h at 37°C.

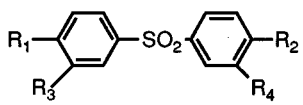
Kinetic studies of recombinant DHPS. The apparent K_m for PABA was determined by incubating the extract in mixtures of a constant concentration of [^{3}H]PABA (16 nM) and eight concentrations of unlabelled PABA varying from 1 nM to 5 μM. An apparent K_m for PABA was also determined with a lysate that had been dialyzed by three passages through a Centricon 30 (Amicon, Inc., Beverly, Mass.). The apparent K_m for the other substrate, H_2PtCH_2OPP, was determined by using six concentrations varying from 1 to 40 μM, with the PABA concentration kept at 66 nM (a mixture of 16 nM [^{3}H]PABA and 50 nM unlabelled PABA). Two independent experiments were performed for each determination. The apparent K_ms were calculated from Lineweaver-Burk double-reciprocal plots.

Determination of IC_{50}s and K_is. Stock solutions of each sulfa drug were prepared in dimethyl sulfoxide and then diluted in water to provide a range of concentrations appropriate for testing the DHPS activity. The final concentration of dimethyl sulfoxide in the assay mixture was ≤1%, a concentration which had no effect on enzyme activity. For determinations of 50% inhibitory concentrations (IC_{50}s), DHPS was assayed in the absence of inhibitor and in the presence of four inhibitor concentrations of up to 10 μM. The logarithm of drug concentration was plotted against percent inhibition, and the line was then drawn by linear regression. In all cases, r^2 was greater than 0.9.

The K_is of sulfa drugs were determined by Dixon plots (10). Two concentrations of [^{3}H]PABA (16 and 80 nM) and four concentrations of drug (10, 40, 70, and 100 nM) were used.

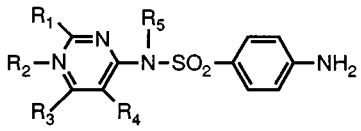
Isolation of *P. carinii* from rat lungs. The rat model for PCP was maintained as described previously (7). Male Sprague-Dawley rats (150 to 200 g) were immunosuppressed by the administration of dexamethasone (2 mg/liter) and tetracycline (0.5 mg/ml) in their drinking water. Within 6 to 8 weeks, rats showing signs of severe pneumocystis pneumonia were sacrificed. The lungs were quickly removed, weighed, and kept on ice. Touch preparations were made from the lungs and stained with Gram, Giemsa, and crystal violet stains. The experiment

TABLE 1. Structure-activity relationships of sulfones



Compounds	R_1	R_2	R_3	R_4	IC_{50}(μM)
Dapsone	$-NH_2$	$-NH_2$			0.15
Diformyldapsone	-NHCOH	-NHCOH			>10
Difluorodinitrophenylsulfone	-F	-F	$-NO_2$	$-NO_2$	>10

TABLE 2. Structure-activity relationships of 4-pyrimidinyl derivatives

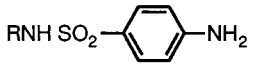


Compound	R1	R2	R3	R4	R5	IC50(μM)
Sulfadoxine	-H	-H	$-CH_3O$	$-CH_3O$	-H	0.740
Sulfisomidine	$-CH_3$	-H	$-CH_3$	-H	-H	0.370
Sulfadimethoxine	$-CH_3O$	-H	$-CH_3O$	-H	-H	0.025
Ro 43476	-H	-H	$-CH_3O$	-H	-H	0.054
Ro 55615	-H	-H	$-CH_3O$	-H	$-CH_3O$	>10
Ro 78307	=O	-H	$-CH_3O$	-H	-H	0.094

was continued if no fungi or bacteria were present. The lungs were then minced and incubated with collagenase (20 mg per lung) at 37°C for 30 min. After the incubation, the mixture was filtered through a wire mesh filter to remove lung tissue and rinsed with minimum essential medium (MEM) supplemented with 20% FBS (MEM-FBS) (4). The filtrate was centrifuged at 1,100 × *g* for 10 min. The supernatant was discarded, and the pellet was resuspended in 30 ml of MEM-FBS containing 100 mM DTT and centrifuged at 1,100 × *g* for 10 min. The resulting pellet was then resuspended in 15 ml of 0.86% NH_4Cl, incubated at 37°C for 15 min, and then repelleted. The pellet was once again resuspended in MEM-FBS, filtered through an 8-μm-pore-size filter (Millipore Corporation, Marlborough, Mass.), and washed with 2 to 5 ml of MEM-FBS. The filtrate was centrifuged at 1,100 × *g* for 10 min, and the pellet was then resuspended in an appropriate amount of MEM-FBS plus 100 μM dihydroneopterin.

[^{3}H]folate biosynthesis in *P. carinii*. The *P. carinii* suspension obtained as described above was incubated with 0.2 μCi of [^{3}H]PABA in the presence or absence of drug at 37°C. After the incubation, the parasites were spun down and washed three times with fresh MEM. In all cases at least three incubations were performed in parallel: one with drug for 2 h, one without drug for 2 h, and a third in which the organisms were mixed with [^{3}H]PABA and then immediately centrifuged and washed to measure nonspecific adsorption. In all cases, this incorporation of label at zero time was <3% of that found after 2 h of incubation. The parasite pellet was then resuspended in 200 μl of Tris-HCl buffer (pH 8) containing 3% β-mercaptoethanol and was lysed by sonication. The membranes and debris were discarded. One milliliter of 1 M citrate–1 M phosphate buffer (pH 3.8) was then added to the cytosol, and the pH of the solution was adjusted to 3.8 if necessary. This aqueous suspension was then extracted with diethyl ether four

TABLE 3. Structure-activity relationships of pyridinyl, 3-pyridazinyl, and 2,5-pyrimidinyl derivatives



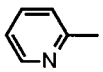
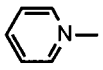
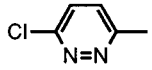
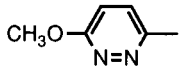
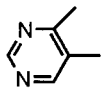
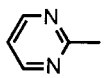
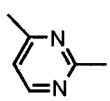
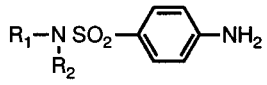
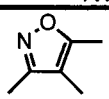
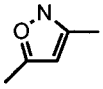
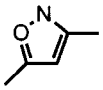
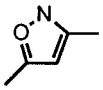
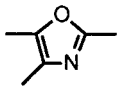
Compound	R1	IC50(μM)
Sulfapyridine		0.180
Ro 211182		>10
Sulfachlorpyridazine		0.018
Sulfamethoxypyridazine		0.017
Ro 19194		2.7
Sulfadiazine		0.19
Sulfamerazine		0.078

TABLE 4. Structure-activity relationships of heterocyclic derivatives



Compound	R1	R2	IC_{50}(μM)
Sulfisoxazole		H	0.040
Ro 72844		2-propoxyacetyl	0.023
Ro 52928		acetyl	0.020
Sulfamethoxazole		H	0.023
Sulfamoxole		H	0.089
Sulfathiazole		H	0.013

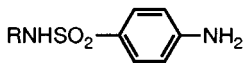
times to remove the unreacted PABA. This extraction procedure was found to remove >99% of free [^{3}H]PABA. The radioactivity in the aqueous layer, which contains H_2Pte and various forms of folic acid, was then counted.

Statistics. Standard deviations were calculated and two-tailed Student *t* tests were performed with Microsoft Excel version 4.0.

RESULTS

DHPS activity was detected in the cytosol of recombinant baculovirus-infected *S. furgiperda* 9 cells. The specific activity of DHPS in the crude preparation was 4.04 ± 0.07 pmol/mg of protein per h (n = 3) at 37°C. On the other hand, the cytosol of wild-type-baculovirus-infected *S. furgiperda* 9 cells had no detectable activity (<0.3 pmol/mg of protein per h). An *Escherichia coli* lysate was used as positive control and was found to have a specific activity of 5.1 ± 0.52 pmol/mg of protein per h (n = 2). The amount of DHPS protein expressed in the insect cells was so little that no band with the expected apparent

TABLE 5. Structure-activity relationships of phenyl derivatives



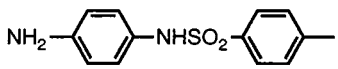
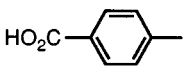
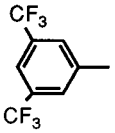
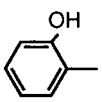
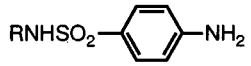
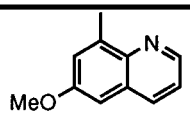
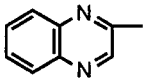
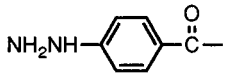
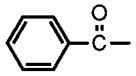
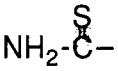
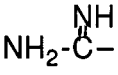
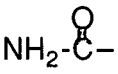
Compound	R	IC_{50}(μM)
NSC 14652-J		0.18
NSC 39345-X		0.26
NSC 56605-K		0.80
NSC 229583-G		0.23

TABLE 6. Structure-activity relationships of miscellaneous derivatives



Compound	R	IC_{50}(μM)
NSC 403439-F		0.38
Sulfaquinoxaline		0.030
NSC 74428-I		>10
NSC 74587-G		0.32
Ro 13354	H	4.2
Sulfathiourea		0.022
Sulfaguanidine		3.200
NSC 78438-Q		0.30

molecular mass of 71.5 kDa (15) could be seen by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

In the presence of a DHPS-containing lysate, the amount of the product (H_2Pte) increases with incubation time, while the substrate (PABA) disappears at approximately the same rate. Data from one of two equivalent experiments are shown in Fig. 1. In these experiments, the reaction was linear for the first hour, indicating that the 1-h incubations used in the experiments described below are valid for enzyme kinetic studies.

The K_ms for both substrates were calculated by Lineweaver-Burk plots. The apparent K_ms for the PABA and H_2PtCH_2 OPP substrates were 0.34 ± 0.02 and 2.50 ± 0.71 μM, respectively. The apparent K_m for PABA when determined in dialyzed lysates was not significantly different (0.15 μM).

After assay conditions had been established, 44 sulfa drugs, including dapsone and sulfamethoxazole, were screened. Under the assay conditions used, sulfa drugs appear to inhibit the enzyme in a linear fashion. One micromolar sulfamethoxazole caused complete inhibition at 20, 40, and 60 min (data not shown).

The IC_{50}s of these compounds, grouped according to the chemical structure, are listed in Tables 1 to 7. Of the compound tested, sulfamethoxazole was one of the most potent inhibitors, with an IC_{50} of 23 nM (Table 4). The IC_{50} of dapsone was much higher (150 nM) (Table 1). Seven of the drugs tested had IC_{50}s similar to that of sulfamethoxazole: sulfathiazole (13 nM) (Table 4), sulfachlorpyridazine (18 nM) (Table 3), sulfamethoxypyridazine (17 nM) (Table 3), sulfathiourea (22 nM) (Table 6), sulfadimethoxine (25 nM) (Table 3), sulfisoxazole (40 nM) (Table 4), and sulfaquinoxaline (30 nM) (Table 6).

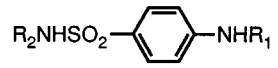
The K_is of sulfamethoxazole and four other sulfa drugs, determined by Dixon plots, are shown in Table 8. The K_i of sulfamethoxazole, 7.5 nM, is not appreciably different from those of sulfathiazole, sulfachlorpyridazine, sulfamethoxypyridazine, and sulfathiourea.

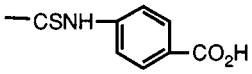
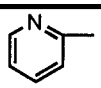
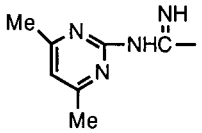
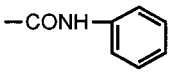
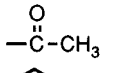
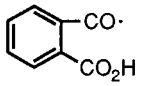
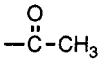
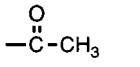
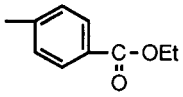
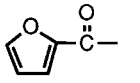
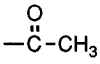
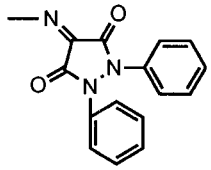
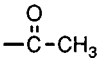
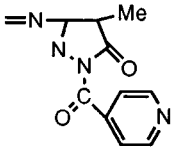
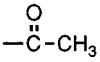
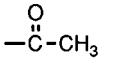
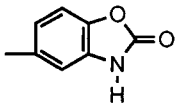
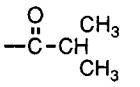
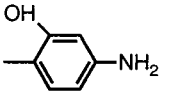
In order for a sulfa drug to be effective, it must not only inhibit DHPS but also be taken up by intact organisms. To determine this, we measured the effects of selected sulfa drugs on folate metabolism in intact, isolated *P. carinii*. The rate of folate synthesis in *P. carinii* was 23.8 ± 7.5 fmol/mg of protein per h ($n = 9$) in the absence of sulfa drugs. At 50 nM, sulfamethoxazole inhibited folate biosynthesis in situ by approximately half. Sulfachlorpyridazine, sulfamethoxypyradazine, sulfisoxazole, and sulfathiazole all inhibited folate biosynthesis to a similar extent at this concentration (Table 9). One drug, sulfamethoxypyridazine, was significantly more effective than sulfamethoxazole as determined by Student's t test ($P < 0.05$).

DISCUSSION

In this study, a crude preparation of recombinant DHPS was used to screen the inhibitory activities of 44 sulfa drugs. Many of these compounds had IC_{50}s similar to or lower than that of sulfamethoxazole. At least four of them inhibited folate metabolism in intact organisms to an extent similar to that of sulfamethoxazole.

TABLE 7. Structure-activity relationships of substituted *p*-amino derivatives



Compound	R_1	R_2	IC_{50}(μM)
NSC 52105-S			5.9
NSC 107328-T			>10
NSC 142656-T		$-CONHCH_2CH_2CHMe_2$	>10
NSC 163977-T			>10
NSC 205491-S			>10
NSC 270146-U			>10
NSC 279282-Z			>10
NSC 303757-F			>10
NSC 355394-H			>10
NSC 308795-G			>10

The K_ms reported here for PABA (0.34 ± 0.02 μM) and H_2PtCH_2OPP (2.50 ± 0.71 μM) are lower than those previously reported with *P. carinii* (9, 14). The K_m for H_2PtCH_2OPP is similar to that found in one study (3.0 μM) (14) but 40-fold lower than that found in the other (81 μM) (9); the K_m for PABA is >25-fold lower than those found in either study (8.7 μM [14] and 71 μM [9]). There are several factors that might contribute to these differences. First, all three studies used different enzyme assay methods. Second, the assays performed in the present study were carried out with extracts containing recombinant DHPS, whereas the other determinations were made with extracts from isolated *P. carinii*. Since all three studies measured DHPS activities in crude extracts, it is possible that other components of the extracts could have competed for binding with one or both of the substrates. Thus, the true K_ms of this enzyme will not be known until they are measured with purified enzyme.

The goal of this project was to develop a simple and rapid method to screen large numbers of sulfa drugs. In order to accomplish this, enzyme assays were performed on crude extracts from infected *S. furgiperda* 9 cells rather than on purified enzyme. This was done under the assumption that the critical factor was the relative potencies of the various drugs and not the absolute inhibitory efficacy. In addition, we utilized a simple and rapid ether extraction enzyme assay method. Since the [^{3}H]PABA was of high specific activity, only 0.1 μCi was needed for each enzyme assay, giving measurements of around 10,000 cpm after subtracting the value of the blank. Further-

TABLE 8. K_is of most-effective sulfonamides

Compound	K_i (nM)	IC_{50} (nM)
Sulfamethoxazole	7.5	23[a]
Sulfathiazole	10.5	13[a]
Sulfachlorpyridazine	10.0	18[b]
Sulfamethoxypyridazine	12.5	17[b]
Sulfathiourea	16.5	22[c]

[a] Data from Table 4.
[b] Data from Table 3.
[c] Data from Table 6.

more, the background radioactivity for this assay was never very high. For every assay performed, the radioactive counts in the aqueous layer after incubation in the presence of extract were always approximately four times higher than those found in the aqueous layers after control incubations. Thus, this procedure can potentially be applied to screening large numbers of drugs.

The chemical structures of the compounds affect their inhibitory abilities in several obvious ways. First, unsubstituted *p*-amino groups are essential for antipneumocystis activity for both the sulfones and sulfonamides. For example, dapsone, with two free *p*-amino groups, has an IC_{50} of 0.15 μM. In contrast, diformyldapsone, which has both of its amino groups blocked, has an IC_{50} of >10 μM. Also, NSC 52105-S (Table 7) differs from sulfapyridine (Table 3) by the substituent on the *p*-amino group; its IC_{50}, is 30-fold higher (5.9 vs 0.18 μM). Furthermore, all of the *p*-amino-substituted derivatives in Table 7 have very low inhibitory activities, with IC_{50}s higher than 6 μM. These results suggest that if the sulfa drugs are structural analogs of PABA, then the *p*-amino groups of both PABA and the sulfa drugs are involved in binding to the active site of DHPS.

Of all the classes of sulfa drugs tested, those with heterocyclic substitutions on N^1 are the most potent. The phenyl derivatives (Table 5) have IC_{50}s in a range of from 0.18 to 0.80 μM, while most of the heterocyclic sulfonamides have IC_{50}s lower than 0.1 μM (Tables 2 to 4). Four of the five most-effective drugs (Table 8) belong to these groups.

Changes or substitutions in the heterocycle ring affect the inhibitory activity. For example, sulfadimethoxine and sulfadoxine are identical except that they contain a single methoxy substituent in different positions (Table 2). Yet, sulfadoxine has an IC_{50} which is 30-fold higher than that of sulfadimethoxine. Sulfapyridine and Ro 211182 (Table 3) both have pyridinyl substituents but in different orientations, and their IC_{50}s differ by more than 56-fold. Similarly, Ro 19194 and sulfamerazine (Table 3) also have the same methylpyrimidinyl substituent but in different orientations. Their IC_{50}s are different by 35-fold.

A second substituent on N^1 does not have a consistent effect on inhibitory activity. Ro 55615 is identical to Ro 43476 except for an extra methoxyl group on N^1, yet its activity is more than 185-fold lower (Table 2). In other cases, the N^1 substituent has no effect. For example, sulfamethoxazole, Ro 72844, and Ro 52928 (Table 4) have almost the same IC_{50}s regardless of the presence or absence of an N^1 substituent.

TABLE 9. Inhibition of folate biosynthesis in situ

Compound	% Inhibition (mean ± SD)	n[a]
Sulfamethoxazole	48.6 ± 11.9	4
Sulfachlorpyridazine	29.5 ± 16.6	3
Sulfamethoxypyridazine	75.0 ± 8.1	3
Sulfisoxazole	39.2 ± 36.7	4
Sulfathiazole	60.9 ± 25.1	4

[a] Number of experiments.

Sulfachlorpyridazine, sulfamethoxypyridazine, sulfisoxazole, and sulfathiazole compare well with sulfamethoxazole not only in their abilities to inhibit recombinant DHPS but also in their abilities to inhibit de novo folate biosynthesis in *P. carinii*. Sulfamethoxypyridazine may even be a better inhibitor than sulfamethoxazole in intact cells.

Sulfamethoxazole is probably responsible for the adverse effects that occur during co-trimoxazole administration (1). A sulfa drug which is less toxic than sulfamethoxazole would be of great use. Two of the drugs which are equivalent to sulfamethoxazole in potency, sulfisoxazole and sulfamethoxypyridazine, have been shown to be less toxic. In a large study carried out in Sweden, the rates of serious adverse reactions to co-trimoxazole (sulfamethoxazole plus trimethoprim), sulfafurazole (sulfisoxazole), and sulfapral (sulfamethoxypyridazine plus sulfamethizole) were found to be 33, 8, and 6 per 100,000 users, respectively (2). Thus, sulfamethoxypyridazine and sulfisoxazole should be further evaluated for the treatment and prophylaxis for PCP.

In the present work, a small number of available sulfa drugs were tested. However, this in vitro screening system can now be used to screen a large number of compounds.

ACKNOWLEDGMENTS

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