In Vitro Activities of 2,4-Diaminoquinazoline and 2,4-Diaminopteridine Derivatives against *Plasmodium falciparum*

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The activities of 28 6-substituted 2,4-diaminoquinazolines, 2,4-diamino-5,6,7,8-tetrahydroquinazolines, and 2,4-diaminopteridines against *Plasmodium falciparum* were tested. The 50% inhibitory concentrations (IC_{50}s) of six compounds were <50 nM, and the most potent compound was 2,4-diamino-5-chloro-6-[*N*-(2,5-dimethoxybenzyl)amino]quinazoline (compound 1), with an IC_{50} of 9 nM. The activity of compound 1 was potentiated by the dihydropteroate synthase inhibitor dapsone, an indication that these compounds are inhibitors of dihydrofolate reductase. Further studies are warranted to assess the therapeutic potential of this combination in vivo.

Inhibition of the synthesis of folate derivatives has been exploited for the development of drugs with activities against *Plasmodium falciparum* infection. The enzyme target of antifolates is dihydrofolate reductase (DHFR), which catalyzes the reduction of dihydrofolate to tetrahydrofolate. The latter is an essential cofactor in the synthesis of thymidine, purines, and methionine (10). Inhibitors of DHFR, such as pyrimethamine and chlorcycloguanil, are used in combination with inhibitors of dihydropteroate synthase (DHPS), a second key enzyme of folate metabolism in *P. falciparum*. A number of sulfa drugs (inhibitors of DHPS), including sulfadoxine and dapsone (DDS), are known to potentiate the activities of DHFR inhibitors or to act synergistically with them. One combination already used for a number of years for the mass treatment of malaria in Africa is pyrimethamine-sulfadoxine (SP). However, the therapeutic value of this combination has been significantly diminished recently as a result of drug resistance (6–9, 11, 12, 15). A new antimalarial combination consisting of chlorproguanil (whose active metabolite is chlorcycloguanil) and DDS has been developed as an alternative to pyrimethamine-sulfadoxine (4, 7, 16). Because of the intrinsic ability of *P. falciparum* to quickly develop different mechanisms of drug resistance, newer agents are urgently needed.

As part of an effort to discover potent new antifolates that potentially may be drugs with activities against multidrug-resistant strains of *P. falciparum*, we have assessed the activities of a library of 2,4-diaminoquinazolines, 2,4-diamino-5,6,7,8-tetrahydroquinazolines, and 2,4-diaminopteridines against the highly resistant V1S strain of *P. falciparum*. The compounds were chosen on the basis of the findings presented in a recent paper (5) documenting their activities against *Saccharomyces cerevisiae* yeast cells whose DHFR genes were replaced with the *P. falciparum* DHFR gene.

MATERIALS AND METHODS

The compounds were synthesized at the Dana-Farber Cancer Institute, Boston, Mass., by procedures described elsewhere (5) and are listed by name in Table 1. Pyrimethamine and DDS were purchased from Sigma Aldrich Co., St. Louis, Mo. Chlorcycloguanil was a gift from AstraZeneca, Cheshire, United Kingdom.

The highly pyrimethamine-resistant V1S isolate that was used in the assays has four mutations in its DHFR gene: Asn-51-Ile (a mutation from asparagine to isoleucine at codon 51), Cys-59-Arg, Ser-108-Asn, and Ile-164-Le. This DHFR genotype is associated with a substantial decrease in the affinity of binding of pyrimethamine to the enzyme, explaining why parasites that carry these mutations are highly resistant to pyrimethamine (13). In vitro analyses of growth inhibition were performed in RPMI 1640 medium (GIBCO) containing physiological concentrations of *para*-aminobenzoic acid (0.5 μg/liter) and folic acid (10 μg/liter), 10% (vol/vol) normal human serum, 25 mM bicarbonate, and 25 mM HEPES buffer. Antimalarial activities in the presence of various concentrations of each test compound were determined by the radioisotope incorporation method (14). Results were expressed as the drug concentration required for 50% inhibition of [³H]hypoxanthine incorporation into parasite nucleic acid (IC_{50}) by regression analysis of the dose-response curve.
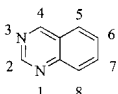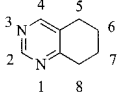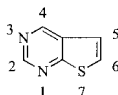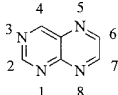
Synergy was analyzed by calculating the sum of the minimum fractional inhibitory concentrations (sFICs) (1). Synergy is demonstrated when the sFIC is <0.5. An sFIC >4.0 denotes an antagonistic effect, and an sFIC between 0.5 and 4 indicates either a nonsynergistic or a nonantagonistic interaction.

RESULTS AND DISCUSSION

The in vitro activities of the test compounds against pyrimethamine-resistant isolate V1S are shown in Table 1. Quinazoline compounds 1, 2, and 3 and tetrahydroquinazoline

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TABLE 1. In vitro activities of 2,4-diaminoquinazoline, 2,4-diamino-5,6,7,8-tetrahydroquinazoline, 2,4-diaminothieno[2,3-d]pyrimidine, and 2,4-diaminopteridine derivatives against multidrug-resistant isolate *P. falciparum* V1S[a]

Drug group and compound no.	Name	$IC_{50} \pm SD$ (nM)
Reference drugs		
	Pyrimethamine	4,200 ± 860
	Chlorcycloguanil	70 ± 7.4
	WR92210	2.7 ± 1.7
Quinazolines		
1	**2,4-Diamino-5-chloro-6-[N-(2,5-dimethoxybenzyl)aminoquinazoline**	**8.9 ± 3.3**
2	**2,4-Diamino-5-chloro-6-[N-(3,4,5-trimethoxybenzyl)amino]quinazoline**	**32 ± 7.4**
3	**2,4-Diamino-5-chloro-6-[N-(3,4,5-trimethoxybenzyl)-N-methylamino]quinazoline**	**31 ± 3.0**
4	2,4-Diamino-5-chloro-6-(2-bromo-3,4,5-trimethoxyanilino)methylquinazoline	220 ± 120
5	2,4-Diamino-5-chloro-6-[2-(3,4,5-trimethoxyphenyl)ethyl]aminoquinazoline	1,400 ± 70
6	2,4-Diamino-5-(2,5-dimethoxy-N-methylanilino)methylquinazoline	1,700 ± 110
7	2,4-Diamino-5-(3,4,5-trimethoxy-N-methylanilino)methylquinazoline	>10,000
Tetrahydroquinazolines		
8	**(6R,6S)-2,4-Diamino-6-(3-methoxybenzyl)-5,6,7,8-tetrahydroquinazoline**	**29 ± 5.8**
9	**(6R,6S)-2,4-Diamino-6-(2-methoxybenzyl)-5,6,7,8-tetrahydroquinazoline**	**36 ± 4.9**
10	**(6R,6S)-2,4-Diamino-6-(2-methylbenzyl)-5,6,7,8-tetrahydroquinazoline**	**50 ± 25**
11	(6R,6S)-2,4-Diamino-6-(4-trifluoromethoxybenzyl)-5,6,7,8-tetrahydroquinazoline	140 ± 4
12	(E/Z)-2,4-Diamino-6-(4-methoxybenzylidene)-5,6,7,8-tetrahydroquinazoline	170 ± 61
Thieno[2,3-d]pyrimidines		
13	2,4-Diamino-5-[2-(2-bromo-3,4,5-trimethoxy)phenyl]ethylthieno[2,3-d]pyrimidine	>10,000
14	2,4-Diamino-6-[2-(2,5-dimethoxyphenyl)ethyl]-5-methylthieno[2,3-d]pyrimidine	>10,000
Pteridines		
15	D,L-2-[N-(4-Amino-4-deoxy-N^{10}-methylpteroyl)aminopentane	1,700 ± 230
16	D,L-2-[N-(4-Amino-4-deoxy-N^{10}-methylpteroylaminopentane-1,5-diol	2,800 ± 900
17	N-(4-Amino-4-deoxy-N^{10}-methylpteroyl)aminocyclohexane	1,300 ± 570
18	1-[N-(4-Amino-4-deoxy-N^{10}-methylpteroyl)amino]adamantane	1,800 ± 890
19	1-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-4-carbethoxypiperazine	1,000 ± 670
20	Methotrexate α,γ-di(cyclopentylamide)	630 ± 160
21	Methotrexate α,γ-di(benzylamide)	1,200 ± 180
22	Methotrexate α,γ-di(n-propylamide)	1,400 ± 610
23	Methotrexate α,γ-di[3-(N,N-dimethylamino)propyl]amide	>10,000
24	2,4-Diamino-6-methylpteridine	>10,000
25	2,4-Diamino-7-methylpteridine	>10,000
26	2,4-Diamino-6,7-di(4-chlorobenzyl)pteridine	>10,000
27	2,4-Diamino-6,7-di(3,4-dichlorobenzyl)pteridine	>10,000
28	2,4-Diamino-6,7-di(2-naphthylmethyl)pteridine	>10,000

[a] IC_{50} represent the nanomolar drug concentration ± standard deviation for 50% inhibition of [^{3}H]hypoxanthine incorporation. Compounds with IC_{50}s <50 nM are indicated in boldface. See the work of Lau et al. (5) for a comprehensive list of papers describing the chemical synthesis of the compounds tested.

compounds 8 and 9 were the most active, with IC_{50}s of <50 nM; compound 1 had the lowest IC_{50} (ca. 9 nM). The potency of compound 1 in our assay was greater than those of pyrimethamine (IC_{50}, 4,200 nM) and chlorcycloguanil (IC_{50}, 81 nM) and, to our pleasant surprise, approached that of WR99210 (IC_{50}, 3 nM), the most potent antifolate described to date against *P. falciparum*. Interestingly, compound 2, which differed structurally from compound 1 only by the fact that it is

a 3,4,5-trimethoxy derivative as opposed to a 2,5-dimethoxy derivative, showed a ca. threefold loss in potency relative to that of compound 1. The *N*-methyl derivative (compound 3) was equipotent with compound 2, whereas the 2-bromo-3,4,5-trimethoxy analogue (compound 4) was sevenfold less potent than compound 2, suggesting greater tolerance for substitution on the bridge nitrogen than at the *ortho* position of the benzyl group. Elongation of the bridge by one carbon, as in compound 5, led to 157-fold less potency relative to that of compound 1. Three of the tetrahydroquinazoline analogues (compounds 8, 9, and 10) were reasonably active, with IC_{50}s <50 nM. However, the thienopyrimidine and pteridine analogues proved to be very weak inhibitors, with IC_{50}s >1,000 nM in the majority of cases, and thus, they were clearly of less interest than the quinazolines.

Although the compounds in Table 1 were not tested for their effects on mammalian cells as part of this study, it may be noted that compounds 1 and 2 had IC_{50}s of 85 ± 8.0 and 22 ± 4.0 nM, respectively, when they were tested in vitro at the Dana-Farber Cancer Institute against CCRF-CEM human leukemic lymphoblasts grown for 72 h in standard RPMI 1640 medium supplemented with 10% fetal bovine serum (unpublished results). Thus, while compound 1 was found to be more potent than compound 2 against *P. falciparum* in the present work, the opposite appears to be the case with regard to human cells, presumably reflecting subtle species-specific differences in the three-dimensional structure of the active site of DHFR in *P. falciparum* versus that in humans. However, bearing in mind that the antimalarial assays were based on [^{3}H]purine (from hypoxanthine) incorporation into nucleic acids, whereas the assays of activities against human cells were based on cell growth, this conclusion would have to be verified by directly comparing the activities of these compounds against purified enzymes.

It has been known for more than 50 years that the combination of a DHPS inhibitor and a DHFR inhibitor can synergistically block de novo folate synthesis in *P. falciparum* and the other microorganisms in which this pathway is essential for growth (2, 3). Pyrimethamine-sulfadoxine and chlorproguanil-DDS are examples of drug combinations that take advantage of this effect. Because a number of the quinazolines tested in this study had previously been found to inhibit the *P. falciparum* DHFR gene expressed in yeast (5), we postulated that these dicyclic compounds, too, would likewise act synergistically with DHPS inhibitors in retarding the growth of intact *P. falciparum* organisms in culture. Accordingly, the most potent compound in Table 1, compound 1, was tested in culture in the presence of various concentrations of DDS. The results are presented in Table 2. The IC_{50}s of compound 1 and DDS alone were 9 and 184,300 nM, respectively. In the presence of 9,200, 6,100, and 4,600 nM DDS, compound 1 IC_{50}s were reduced to 0.08, 0.09, and 0.12 nM, respectively; sFICs were between 0.037 and 0.061, a clear indication that DDS acts in synergy with compound 1. In comparison, we have included data on the activity of chlorcycloguanil, a well-established DHFR inhibitor, in combination with DDS (Table 2). DDS increased the activity of chlorcycloguanil; however, the range of chlorcycloguanil-DDS sFICs was higher (0.26 to 0.38) than that for compound 1-DDS, an indication that the latter combination is more synergistic. All this information supports our hypothesis that com-

pound 1 and, presumably, the other active compounds in Table 1 are inhibitors of DHFR.

In summary, this paper reports that several 6-substituted 2,4-diaminoquinazolines and 2,4-diamino-5,6,7,8-tetrahydroquinazolines are potent inhibitors of the growth of the highly pyrimethamine-resistant strain V1S, with IC_{50}s of <50 nM. One compound, compound 1 (IC_{50}, 9 nM), was more potent against this strain than pyrimethamine by 2 orders of magnitude and was nearly as potent as WR99210. These results point to compound 1 as a promising lead for further structure-activity optimization with the goal of designing new drugs of the quinazoline family, and further studies are warranted to assess the therapeutic potential of the combination compound 1-DDS in vivo, in an animal model, for the treatment of pyrimethamine-resistant human malaria.

TABLE 2. In vitro activities of the combinations of compound 1 and-DDS, and chlorcyloguanil-DDS against *P. falciparum* V1S

Drug name[a]	Drug IC_{50} (nM) in presence of DDS	sFIC
Compound 1	0.08 (9,200)[b]	0.060
	0.09 (6,100)	0.043
	0.12 (4,600)	0.038
CCG	15.40 (9,200)	0.27
	17.90 (6,100)	0.29
	24.40 (4,600)	0.37

[a] The IC_{50}s of compound 1, CCG, and DDS alone were 9, 71, and 184,300 nM, respectively.
[b] Values in parentheses represent the concentrations of DDS (in nanomolar).

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