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TRICYCLIC 2,4-DIAMINOPYRIMIDINES WITH BROAD ANTIFOLATE ACTIVITY AND THE ABILITY TO INHIBIT *PNEUMOCYSTIS CARINII* GROWTH IN CULTURED HUMAN LUNG FIBROBLASTS IN THE PRESENCE OF LEUCOVORIN

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Abstract—A selected number of 1,3-diaminobenzo[*f*]quinazolines and 1,3-diamino-5,6-dihydrobenzo[*f*]quinazolines, which may be viewed as tricyclic analogues of the lipid-soluble antifolates pyrimethamine (PM), metoprine (DDMP), and etoprine (DDEP), were tested as inhibitors of purified dihydrofolate reductase (DHFR) from WI-L2 lymphoblasts, and as inhibitors of the growth of *Streptococcus faecium* ATCC 8043 and L1210 murine leukemia cells in culture. In addition, these tricyclic compounds were tested for antimalarial activity against *Plasmodium berghei* in mice, and for the ability to inhibit the growth of *Pneumocystis carinii* trophozoites in WI-38 human lung fibroblast cultures in the presence of leucovorin (LV). The most potent analogues were those with chlorine substitution in the ring distal to the 2,4-diaminopyrimidine moiety. Fully aromatic compounds tended to be more active than those in which the 5,6-bond was reduced, suggesting that planarity favors binding to the DHFR active site and may be favorable for cellular uptake. Several of the 2,4-diaminopyrimidine analogues showed greater potency than PM, DDMP or DDEP, and were more nearly comparable to the bicyclic 2,4-diaminopyrimidine antifolates trimetrexate (TMQ) or piritrexim (BW301U), which are known to be selectively toxic to *P. carinii* in the presence of LV. Two of the tricyclic compounds, 1,3-diamino-8-chlorobenzo[*f*]quinazoline and 1,3-diamino-9-chlorobenzo[*f*]quinazoline, proved to have activity similar to TMQ and BW301U in this system.

Lipophilic inhibitors of dihydrofolate reductase (DHFR) have been studied for many years as antibacterial, antiparasitic and antineoplastic agents. Examples of this class of compounds used in clinical medicine include the antimalarial pyrimethamine (PM) [1] and the antibacterial trimethoprim [2]. Several PM analogues, such as metoprine (DDMP) and etoprine (DDEP) [3], and more recently 5-(1-adamantyl)-2,4-diaminopyrimidine [4], also showed promise as antitumor agents. These compounds were attractive because they diffused readily across the cell membrane and therefore represented a rational approach to overcoming transport-based resistance to methotrexate (MTX) and other classical antifolates [5–8]. Because of their lipophilicity, they could penetrate the central nervous system (CNS) more easily than classical antifolates and therefore were viewed as candidates for use against metastatic disease in the brain [9]. However, the persistence of simple 5-substituted or 5,6-disubstituted diaminopyrimidine derivatives in the CNS led to neurological side effects. For this reason, new types of lipophilic diaminopyrimidine derivatives with a broader spectrum of antitumor activity and minimal CNS toxicity were sought. Emphasis was placed on bicyclic diaminopyrimidine ring systems, leading to development of the quinazoline derivative trimetrexate (TMQ) [10] and the pyrido[2,3-*d*]pyrimidine derivative piritrexim (BW301U) [11]. These compounds were less neurotoxic than the monocyclic diaminopyrimidines and possessed sufficient activity in experimental antitumor assays to warrant Phase I clinical trial [12, 13].

The major difference between lipid-soluble antifolates and "classical" antifolates with a glutamic acid side-chain, such as MTX, is in their mode of uptake into cells. While MTX and other classical antifolates are taken up by a carrier-mediated pathway which is used also by reduced folates, the uptake of lipid-soluble antifolates is thought to occur by diffusion. These compounds thus have the ability to penetrate cells that are impermeable to classical antifolates and reduced folates. This property has stimulated interest in lipid-soluble antifolates as drugs against *Pneumocystis carinii* pneumonia, a life-threatening opportunistic infection observed in patients with acquired immunodeficiency syndrome (AIDS) [14]. Since an active transport pathway for antifolates and reduced folates appears to be lacking

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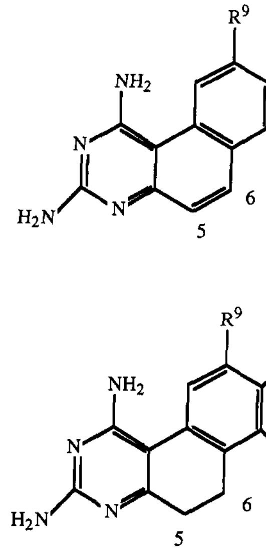

Fig. 1. Structures of 1,3-diaminobenzo[*f*]quinazolines.

in *P. carinii*, it has been proposed [15] that a rational chemotherapeutic strategy against this organism would be to combine lipid-soluble antifolates with leucovorin (LV), which would protect sensitive host tissues, but not *P. carinii* cells, from antifolate toxicity. TMQ and BW301U were identified recently as promising agents in this connection in preclinical models [15–18] as well as in patients [19].

1,3-Diaminobenzo[*f*]quinazolines (Fig. 1) were synthesized by us some years ago [20, 21] as part of a larger effort [22–25] directed toward the biological evaluation of rotationally restricted tricyclic analogues of PM and DDMP. It was hoped that these studies might provide insight into the relationship between molecular geometry and biological activity in small-molecule diaminopyrimidine antifolates. Because of the current interest in lipophilic antifolates as drugs against opportunistic parasitic infections in AIDS patients, we chose to test a group of our tricyclic analogues as inhibitors of purified human DHFR, as inhibitors of mammalian cell growth in culture, and as inhibitors of *P. carinii* proliferation in human lung fibroblast culture in the presence of LV. The results of these experiments, along with selected data from antibacterial and antimalarial assays, are reported in this paper. Our findings indicate that the diaminobenzo[*f*]quinazolines possess broad antifolate activity, and that their potency is comparable to that of structurally similar compounds of the diaminopyrimidine and diaminoquinazoline type.

MATERIALS AND METHODS

Chemical synthesis. Compounds were prepared as previously described [20, 21]. Briefly, the 1,3-diaminobenzo[*f*]quinazolines (Fig. 1, top) were prepared by thermal cyclization of N^1,N^5-bis(2-naphthylbiguanides) or by dehydrogenation of 1,3-diamino-5,6-dihydrobenzo[*f*]quinazolines (Fig. 1, bottom) with selenium dioxide. The biguanides were prepared from ring-substituted 2-naphthylamines [26]. The 5,6-dihydro compounds were obtained from appropriately substituted 2-tetralones [27] by condensation with cyanoguanidine, either in dimethyl sulfoxide (DMSO) solution or by fusion without solvent.

Inhibition of DHFR activity. DHFR activity was assayed spectrophotometrically at 340 nm using homogeneous enzyme from WI-L2/M4 cells, an MTX-resistant subline derived from human WI-L2 lymphoblasts by continuous culture in the presence of increasing concentrations of MTX [28]. The WI-L2/M4 cells produce *ca.* 150 times more DHFR than the parent line, but the kinetics of dihydrofolate reduction by the enzyme are in the normal range [28]. The assay mixture contained DHFR (0.0076 μM), dihydrofolate (9 μM), NADPH (30 μM, and KCl (0.15 M) in 0.05 M Tris buffer (pH 7.4) in a final volume of 1 ml. The assay was performed at 22° after a preincubation period of 2 min. The amount of DHFR added was determined by MTX titration.

Antibacterial activity. Compounds were tested as inhibitors of *Streptococcus faecium* (ATCC 8043) growth in the presence of 1 ng/ml (2.2 nM) folic acid as described earlier [29]. PM and MTX were used as positive controls.

Mammalian cell growth inhibition. L1210 and L1210/R81 murine leukemia cells were grown in suspension culture under 8% CO_2 in RPMI 1640 medium containing 5% fetal bovine serum and antibiotics [30]. The cells were plated as described earlier [31] in 96-well plates at an initial density of 1.2×10^5 per ml, and were stained after 48 hr with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), which is converted to dark blue formazan crystals by mitochondrial dehydrogenases in viable cells. The IC_{50}, defined as the concentration of drug required to inhibit cell growth by 50% relative to untreated controls, was determined from a concentration–response curve. MTX was used as a positive control.

Antimalarial activity. Compounds were tested for activity against *Plasmodium berghei* in the mouse according to the Rane protocol [32]. Groups of five ICR/Ha mice were treated subcutaneously with a single 40, 160, or 640 mg/kg dose of compound in peanut oil 3 days after intraperitoneal injection of 10^7 parasitized blood cells from infected donor animals. Active compounds in this assay are defined as those giving an increase in mean survival of at least 100% relative to untreated controls, while compounds are considered curative if they produce at least one animal surviving 60 days. Cured animals were excluded from the calculation of mean survival.

Inhibition of P. carinii *growth in culture.* Assays of the ability of compounds to inhibit the growth of *P. carinii* trophozoites on feeder layers of confluent mammalian cells were performed as described earlier [17]. Human lung fibroblasts (WI-38) were cultured in 12-well plates in minimal essential growth medium containing 10% fetal calf serum [33], with LV at a final concentration of 10 μM. Confluent cultures were inoculated with homogenates from lungs of immunosuppressed rats infected with *P. carinii*. The final concentration of trophozoites in the inoculum was 3 to 7×10^5 per ml. Plates were incubated at 35°

Table 1. Inhibition of human DHFR by 1,3-diaminobenzo[*f*]quinazolines

Entry	R^7	R^8	R^9	5,6-Bond	IC_{50} (nM)
1	H	H	H	CH═CH	46
2	H	Br	H	CH═CH	29
3	H	Cl	H	CH═CH	21
4	Cl	H	Cl	CH═CH	3.1
5	H	Cl	Cl	CH═CH	5.2
6	H	H	Me	CH═CH	7.1
7	H	H	MeO	CH═CH	12
8	H	Cl	H	CH_2CH_2	520
9	H	H	Cl	CH_2CH_2	610
10	H	Cl	Cl	CH_2CH_2	9.3
11	Me	H	H	CH_2CH_2	140
12	H	Me	H	CH_2CH_2	460
13	H	Et	H	CH_2CH_2	290
14	H	Me	Me	CH_2CH_2	29

in 5% O_2 and 5–10% CO_2, with the balance as N_2. Aliquots of culture supernatant fraction were removed on days 1, 3, 5, 7, and 10, and each aliquot was air-dried, fixed with methanol, and Giemsa-stained for counting with a microscope (×1000). The test compounds were added in DMSO to give a final concentration of 0.2 or 1 μg/ml. The final concentration of DMSO (1 μl/ml) was shown in control experiment to have no significant effect on growth. PM was used as a positive control. Each assay was performed in quadruplicate, and results were plotted as means.

RESULTS

DHFR inhibition. As shown in Table 1, the IC_{50} values of the seven 1,3-diaminobenzo[*f*]quinazolines that were tested as inhibitors of purified DHFR from WI-L2/M4 cells ranged from 3.1 nM ($R^7 = R^9 =$ Cl, $R^8 =$ H) to 46 nM ($R^7 = R^8 = R^9 =$ H), while those of the seven 5,6-dihydro derivatives ranged from 9.3 nM ($R^7 =$ H, $R^8 = R^9 =$ Cl) to 610 nM ($R^7 =$ H, $R^9 =$ Cl). The IC_{50} for MTX was 3.8 nM. Thus, three of the fully aromatic analogues were approximately as active as MTX (<2-fold difference in IC_{50}), while the others were 3- to 12-fold less active. In the 5,6-dihydro series, only one compound showed activity comparable to MTX, while the others were 7- to as much as 160-fold less active.

Binding to the enzyme appeared to be related to both the nature of the substituent and its location. Thus, entries 2 and 3 of Table 1 show that substitution at position 8 (equivalent to the *para* position in the phenyl ring of PM or DDMP) increased binding, though the effect of a Br substituent was less than that of the smaller, more electron-withdrawing Cl substituent. Introduction of two Cl atoms, at positions 7 and 9 (entry 4) or positions 8 and 9 (entry 5), was highly favorable. Introduction of an electron-donating MeO group at position 9 was detrimental (entry 7); however, 9-Me substitution (entry 6) was tolerated better than 9-MeO substitution.

In the 5,6-dihydro series, moving the Cl substituent from postion 8 to position 9 (entries 8 and 9) did not have the same favorable effect as it did in the fully aromatic series. On the other hand, it appeared that 8,9-dichloro substitution was more favorable than either 8- or 9-monochloro substitution (entry 10 vs entries 8 and 9). Moving the Me substituent from position 8 to position 7 increased activity (entries 11 and 12), but introduction of a second Me group at position 9 (entry 14 vs entries 11 and 12) had an even more favorable effect. Replacement of the Me group at position 8 by the more hydrophobic Et group (compare entries 12 and 13) appeared to increase binding. Where a direct comparison of identically substituted aromatic and 5,6-dihydro compounds could be made (entries 3 and 8, entries 5 and 10), the former proved more active, suggesting that coplanarity of the three rings favored binding. However, substituent effects were not identical in the two series. This can probably be explained on the basis that the electronic effects of substituents, and (at least where 7- and 9-substituents are concerned) their location in space, are likely to vary depending on whether the X-bridge is CH_2CH_2 or CH═CH. The phenyl and pyrimidine rings in the 5,6-dihydro compounds are colinear but not coplanar [21], which would diminish resonance and thus lessen any effect of phenyl ring substituents on the basicity of the 2,4-diaminopyrimidine moiety. In contrast, when the X-bridge is CH═CH, the three rings are fully conjugated and the electronic effects of substituents located on the terminal ring ought to be felt across all three rings.

Antibacterial activity. As shown in Table 2, the IC_{50} values of the 39 compounds from which candidates were subsequently selected for further evaluation ranged from 5.7 nM ($R^7 = R^9 =$ Cl, $R^8 =$ H) to 280 nM ($R^7 = R^8 =$ H, $R^9 =$ MeO) for the fully aromatic analogues and from 8.8 nM ($R^7 =$ H, $R^8 = R^9 =$ Me) to 2600 nM ($R^7 = R^9 =$ H, $R^8 =$ *t*-Bu) for the 5,6-dihydro derivatives. By comparison, the IC_{50} of PM in these assays was 64 nM, while that of MTX was 4.4 nM (data not shown). Overall, therefore, the aromatic tricyclic compounds were at least as active as PM and in some instances were substantially more active. Several members of the group showed activity approaching that of MTX.

In the aromatic series (X: CH═CH), Br and Cl substitution at position 8 (entries 2 and 4 vs entry 1) appeared to have a qualitatively similar effect on antibacterial activity as it did on DHFR binding, even though the DHFR was of human origin. Moreover, 9-Cl substitution was again more favorable than 8-Cl substitution (entries 4 and 5). Interestingly, 7-Cl and 7,9-Cl_2 substitutions (entries 3 and 7) were also well tolerated, whereas 8-Cl substitution was unfavorable in the presence of either 7- or 9-Cl substitution(entries 3 vs 6, and 5 vs 8). The effect of Me and MeO groups was somewhat different from that of a Cl atom, in that activity followed the order 8-Me > 9-Me (entries 10 and 11) and 8-MeO > 9-MeO (entries 21 and 22). When the straight-chain alkyl group at position 8 was lengthened from one to three carbons, activity increased (entry 10 vs entries 14 and 15); however, a further increase to seven carbons became progressively less favorable (entries 16, 18 and 19). Chain-branching was likewise detrimental (entry 16 vs 17). Where it was possible

Table 2. Inhibition of *S. faecium* (ATCC 8043) growth by 1,3-diaminobenzo[*f*]quinazolines

	5,6-Bond: CH=CH					5,6-Bond: CH_2CH_2			
Entry	R^7	R^8	R^9	IC_{50} (nM)	Entry	R^7	R^8	R^9	IC_{50} (nM)
1	H	H	H	100	23	H	H	H	120
2	H	Br	H	45	24	H	Cl	H	160
3	Cl	H	H	6.9	25	Cl	H	Cl	12
4	H	Cl	H	45	26	H	Cl	Cl	78
5	H	H	Cl	6.1	27	Me	H	H	110
6	Cl	Cl	H	30	28	H	Me	H	440
7	Cl	H	Cl	5.7	29	H	H	Me	710
8	H	Cl	Cl	10	30	Me	Me	H	1200
9	Me	H	H	13	31	H	Me	Me	8.8
10	H	Me	H	18	32	H	Et	H	630
11	H	H	Me	80	33	H	*n*-Pr	H	390
12	Me	Me	H	68	34	H	*n*-Bu	H	190
13	H	Me	Me	120	35	H	*t*-Bu	H	2600
14	H	Et	H	13	36	H	*n*-Hex	H	840
15	H	*n*-Pr	H	8.7	37	MeO	H	H	58
16	H	*n*-Bu	H	9.4	38	H	MeO	H	700
17	H	*t*-Bu	H	53	39	H	H	MeO	1000
18	H	*n*-Hex	H	48					
19	H	*n*-Hep	H	98					
20	MeO	H	H	25					
21	H	MeO	H	83					
22	H	H	MeO	280					

to compare identically substituted analogues, Cl substitution was more favorable than Me or MeO substitution, but only when the substituents were at positions 7 (entries 3, 9, and 20) and 9 (entries 5, 11, and 22). When position 8 was examined (entries 4, 10, and 21), the order of activity followed the order Me > Cl > MeO.

In the 5,6-dihydro series, 7-Cl and 9-Cl substitution was once again favorable (entries 25 and 26 vs entry 24). The second most active 5,6-dihydro compound (entry 25) and the most active aromatic compound (entry 7) were, in fact, both of the 7,9-Cl_2 type. Activity among the Me as well as MeO analogues followed the order 7 > 8 > 9, with Me substitution being superior to MeO substitution in each instance. Among the 8-alkyl-5,6-dihydro derivatives (entries 28 and 32–36) there was again a relationship betwen chain length and activity, with a maximum at R^8 = *n*-Bu (versus *n*-Pr in the fully aromatic series) and a decrease associated with chain-branching (entry 35).

Mammalian cell growth inhibition. The results presented in Table 3 show that all the 1,3-diaminobenzo[*f*]quinazolines were potent inhibitors of the growth of L1210 murine leukemia cells in culture, with IC_{50} values ranging from 2.0 nM (entry 3) to 78 nM (entry 1). The IC_{50} of MTX against these cells was 4.6 nM (data not shown). There appeared to be a correlation between growth inhibition and DHFR inhibition, as illustrated for example by the very active 8,9-dichloro and 9-methyl derivatives (entries 4 and 5) and the less active 8-bromo and 8,9-dimethyl-5,6-dihydro derivatives (entries 1 and 8). 8-Chloro substitution was more favorable than 8-bromo substitution (entries 1 and 2), 7-chloro substitution more favorable than 8-chloro substitution (entries 3 and 4), and 9-methyl substitution more favorable than 9-methoxy substitution (entries 5 and 6). Finally, in the single instance where a comparison of identically ring-substituted compounds could be made (entries 4 vs 7), there was little difference in potency between pairs of CH=CH and CH_2CH_2 analogues.

The activity of the 1,3-diaminobenzo[*f*]quinazolines was found to be considerably lower against MTX-resistant L1210/R81 cells than against wild-type L1210 cells. However, while L1210/R81 cells are >40,000-fold resistant to MTX [30], the level of resistance to all the tricyclic diaminopyrimidines was <200-fold, and in some instances was <30-fold. The resistance of the L1210/R81 cells has been shown to be due mainly to a severe defect in active transport, but is also associated with a 35-fold increase in content of DHFR with normal binding characteristics [30]. Thus, unless a freely diffusing small-molecule antifolate were a better DHFR inhibitor than MTX, complete absence of cross-resistance in L1210/R81 would be unlikely. In other cells in which resistance is based solely on a transport defect, lack of cross-resistance and even collateral sensitivity to small-molecule antifolates are well-known [5–8].

Antimalarial activity. As shown in Table 4, several of the 1,3-diaminobenzo[*f*]quinazolines were active (>100% increases in mean survival) and two (entries 3 and 10) were curative (at least one 60-day survivor). Activity was seen at 160 as well as 640 mg/kg, but not at 40 mg/kg (data not shown). The most effective compound was 9-chlorobenzo[*f*]quinazoline (entry 3). Moving the chlorine from the position 9 to position 8 (entry 2) caused profound loss of activity, even though the 8-chloro derivative more nearly resembles PM, which is *para*- rather than *meta*-substituted. Adding a chlorine at position 8 (entry 6)

Table 3. Inhibition of L1210 and L1210/R81 murine leukemia cell growth in culture by 1,3-diaminobenzo[*f*]quinazolines

					IC_{50} (nM)		
Entry	5,6-Bond	R^7	R^8	R^9	L1210	L1210/R81	IC_{50} Ratio*
1	CH=CH	H	Br	H	78	810	10
2	CH=CH	H	Cl	H	11	710	65
3	CH=CH	Cl	H	Cl	2.0	190	96
4	CH=CH	H	Cl	Cl	9.6	860	90
5	CH=CH	H	H	Me	5.5	160	29
6	CH=CH	H	H	MeO	14	200	14
7	CH_2CH_2	H	Cl	Cl	13	2500	190
8	CH_2CH_2	H	Me	Me	52	340	6.5

* IC_{50} Ratio = IC_{50} against L1210/R81 cells divided by IC_{50} against L1210 cells.

Table 4. Activity of 1,3-diaminobenzo[*f*]quinazolines against *P. berghei* in mice

Entry	5,6-Bond	R^7	R^8	R^9	Dose (mg/kg)	Mean ILS (%)	Evaluation
1	CH=CH	H	Br	H	160	57	
					640	146*	Active
2	CH=CH	H	Cl	H	160	<20	
					640	<20	
3	CH=CH	H	H	Cl	160	143	Active
					640	228	Curative
4	CH=CH	Cl	Cl	H	160	103	Active
					640	190	Active
5	CH=CH	Cl	H	Cl	160	<20	
					640	<20	
6	CH=CH	H	Cl	Cl	160	51	
					640	158†	Active
7	CH=CH	H	H	Me	160	<20	
					640	106	Active
8	CH=CH	H	H	MeO	160	<20	
					640	<20	
9	CH_2CH_2	H	Cl	H	160	116	Active
					640	192	Active
10	CH_2CH_2	H	Cl	Cl	160	110	Active
					640	113	Curative
11	CH_2CH_2	H	Me	Me	160	<20	
					640	<20	
12	CH_2CH_2	H	Et	H	160	<20	
					640	148	Active

* Four out of five toxic deaths.
† Two out of five toxic deaths.

was likewise detrimental but did not abolish activity completely. Adding a chlorine at position 7 (entry 5), on the other hand, completely abolished activity. Interestingly, addition of chlorine at position 7 of the 8-chloro and 9-chloro analogues had markedly different effects, activity being restored in the former instance and abolished in the latter. In the absence of additional data, e.g. on the binding to *P. berghei* DHFR, the reason for such erratic structure–activity relationships is unclear. Variations in activity observed in these *in vivo* studies may have resulted from differences in pharmacokinetics and tissue distribution.

Inhibition of P. carinii *proliferation.* The activities of three of the 1,3-diaminobenzo[*f*]quinazolines and of PM as inhibitors of the proliferation of *P. carinii* trophozoites in human lung fibroblast monolayer cultures containing 10 μM LV are shown in Fig. 2 A and B. As discussed above, LV was included with the aim of protecting the fibroblasts from the toxic effects of the antifolates. At 10 μM, LV did not influence *P. carinii* trophozoite growth in this system (data not shown). With no drugs present, the number of parasites in the culture supernatant fraction increased linearly for the first 5 days, and thereafter declined. The peak of parasite growth in the presence of 1 μg/ml of the diamino compounds and 10 μM LV similarly occurred between days 3 and 7. At 10 days, the number of parasites in the culture supernatant fraction after treatment with 1 μg/ml of PM and 10 μM LV was 32% lower than in controls, whereas with the tricyclic analogues this decrease was 41%

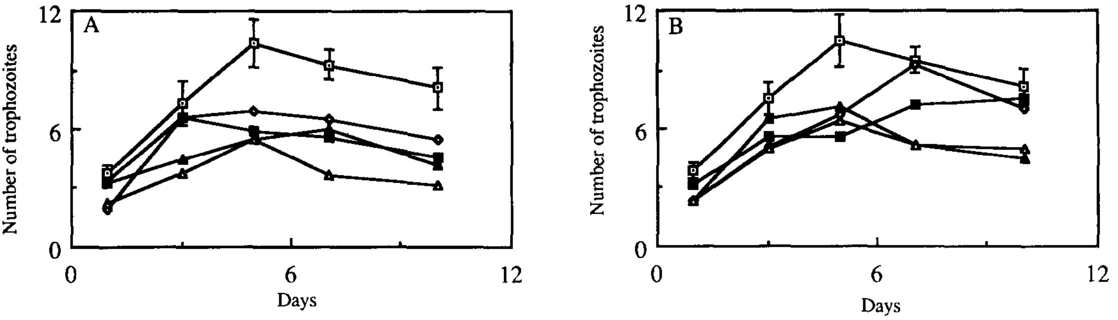

Fig. 2. Effects of 1,3-diaminobenzo[*f*]quinazolines and PM on the growth of *P. carinii* in human WI-38 lung fibroblast cultures in the presence of LV. Vertical axis depicts the number of trophozoites per cm^3 field at ×1000 magnification. Multiplying these numbers by 4×10^5 gives the number of trophozoites per ml of culture supernatant (see Ref. 17). Key: (□) 10 μM LV alone; (◇) PM + 10 μM LV; (▲) 1,3-diamino-8-bromobenzo[*f*]quinazoline + 10 μM LV; (△) 1,3-diamino-8-chlorobenzo[*f*]quinazoline + 10 μM LV; and (■) 1,3-diamino-9-chlorobenzo[*f*]quinazoline + 10 μM LV. Concentrations of diamino compounds were 1.0 μg/ml (panel A) or 0.2 μg/ml (panel B).

for the 9-chloro compound, 48% for the 8-bromo compound, and 60% for the 8-chloro compound. When the concentration of diamino compounds was reduced to 0.2 μg/ml (Fig. 2B), PM and 1,3-diamino-9-chlorobenzo[*f*]quinazoline were essentially inactive, whereas 1,3-diamino-8-bromobenzo[*f*]quinazoline and 1,3-diamino-8-chlorobenzo[*f*]quinazoline still decreased growth by 37 and 44% respectively. It thus appeared that, at the lower drug concentration, the 8-halogenated tricyclic compounds were somewhat more active than PM as inhibitors of *P. carinii* proliferation. Several other 1,3-diaminobenzo[*f*]quinazolines were also tested at a concentration of 1 μg/ml, but were inactive (data not shown). These included two fully aromatic compounds (8,9-Cl_2 and 9-MeO) and two 5,6-dihydro derivatives (8,9-Cl_2 and 8,9-Me_2).

DISCUSSION

Our DHFR assay data (Table 1) showed 1,3-diaminobenzo[*f*]quinazolines to be excellent inhibitors of this enzyme, with IC_{50} values in the 1–10 nM range in several instances. In fact, the inhibitory potency of several of the compounds was close to that of MTX, which had an IC_{50} of 3.8 nM. Moreover, the potency of these compounds substantially exceeded that of DDMP and more closely resembled that of TMQ and BW301U, the two non-classical lipophilic antifolates currently of greatest clinical interest as antineoplastic agents and as candidate antiparasitic agents for use in AIDS patients. The IC_{50} of TMQ as an inhibitor of DHFR from human leukemic cells has been reported to be 1.3 nM, as compared with 0.9 nM for MTX [34], while the IC_{50} values of BW301U and DDMP have been found by other investigators to be 5 and 95 nM, respectively, as compared with 7 nM for MTX [11]. In another study comparing 2,4-diamino-5-arylpyrimidines and MTX as inhibitors of human DHFR, the IC_{50} values of PM, DDMP, and DDEP were found to be 260, 50, and 10 nM as compared with 1 nM for MTX [9]. The present work suggests that tricyclic analogues can bind at least as tightly as those with two fused rings, although binding depends on the nature and location of substituents on the ring distal to the 2,4-diaminopyrimidine moiety. Also, 5,6-dihydro compounds tend to be less inhibitory than those in which the central ring is aromatic, suggesting that the planarity of the tricyclic system favors binding to the active site. An exception, however, was 1,3-diamino-8,9-dichloro-5,6-dihydrobenzo[*f*]quinazoline, which proved to be almost as active as its non-reduced analogue. It is of interest to note that this compound can be viewed as a ring-closed (and therefore rotationally restricted) structural analogue of DDEP.

Several of the planar 1,3-diaminobenzo-[*f*]quinazolines with a non-reduced 5,6-bond showed excellent antibacterial activity against *S. faecium*, with IC_{50} values in the 5–10 nM range. Activity was substantially greater among the non-reduced compounds than among the 5,6-dihydro derivatives, suggesting that coplanarity of the tricyclic system contributes to uptake as well as enzyme inhibition. The potency of the best members of the series exceeded that of PM and compared favorably with that of MTX.

As might be expected from the high potency of several of the 1,3-diaminobenzo[*f*]quinazolines as human DHFR inhibitors, these compounds also proved to be very active inhibitors of mammalian (L1210) cell growth in culture, with IC_{50} values of <20 nM for the majority of the compounds tested. 1,3-Diamino-7,9-dichlorobenzo[*f*]quinazoline, the most active compound in this group, was approximately twice as potent as MTX. Reported IC_{50} values for TMQ, BW301U, and DDMP against cultured L1210 cells are 7 nM [34], 22 nM [11], and 220 nM [11] respectively. From a comparison of our results and the published data, we conclude that the growth-inhibitory activity of the tricyclic analogues in cell culture, like their antibacterial activity, more nearly resembles that of MTX, TMQ, and BW301U than that of DDMP.

A member of the 1,3-diaminobenzo[*f*]quinazolines with *in vitro* antibacterial and antineoplastic activity also proved to be active *in vivo* against *P. berghei* malaria in mice, though none was as effective as the established drugs PM and cycloguanil [35]. The best compound in the series was the 9-chloro analogue, which gave a 143% increase in lifespan at 160 mg/kg and was curative at 640 mg/kg. Interestingly, the 8-chloro-5,6-dihydro analogue, which is the one most closely related in structure to PM, was also active at 160 mg/kg and curative at 640 mg/kg. The corresponding non-reduced 8-chloro compound, however, was completely inactive. The fact that this compound binds roughly 25 times less tightly to mammalian DHFR than the fully aromatic analogue may be important in this regard, since the observed absence of therapeutic effect could have been due to host toxicity.*

Three fully aromatic halogen-substituted 1,3-diaminobenzo[*f*]quinazolines were found to inhibit the growth of *P. carinii* trophozoites in monolayer cultures of human lung fibroblasts in the presence of 10 μM LV. When numbers of trophozoites in cultures grown in the presence of 1 μg/ml (*ca.* 4 μM) concentrations of drugs were compared (Fig. 2A), greater inhibition of growth was observed with all three tricyclic analogues than with PM. Fibroblasts were protected by 10 μM LV, whereas trophozoites were not protected even though the concentration of LV was ten times higher than that of the antifolates. This was consistent with the working model based on lack of a reduced folate transport in *P. carinii* [14, 15, 17]. To further compare the activity of the tricyclic compounds with that of PM, assays were also performed at 0.2 μg/ml (Fig. 2B). At this lower concentration, PM and 1,3-diamino-8-bromobenzo[*f*]quinazoline decreased growth of the organisms only for the first 5 days, whereas 1,3-diamino-8-chlorobenzo[*f*]quinazoline and 1,3-diamino-9-chlorobenzo[*f*]quinazoline continued to inhibit growth until day 10. The benzo[*f*]quinazolines at 1 μg/ml produced inhibition similar to that reported for TMQ and BW301U at concentrations of 0.1 to 1.0 μg/ml [17]. Moreover, the two compounds with the best activity against *P. carinii* were 10-fold less potent as inhibitors of human DHFR than TMQ or BW301U, which could prove advantageous in terms of therapeutic selectivity. *In vitro* activity has proven to be a useful guide to *in vivo* activity for several classes of compounds with activity against *P. carinii* [36–38], allowing rapid and economical screening of agents for further study *in vivo*. The *in vitro* screen similarly had the advantage in the present study of allowing structure–activity relationships to be tested with small amounts of compounds without the complicating factors of host absorption and metabolism.

It is of interest to note that, apart from their *in vitro* activity against *P. carinii*, several of our tricyclic analogues have been found to also be active *in vitro* against *Toxoplama gondii*, another major source of opportunistic infections in AIDS patients [18]; in preliminary assays carried out at the National Cancer Institute,† 1,3-diamino-9-chlorobenzo[*f*]quinazoline and 1,3-diamino-8,9-dichlorobenzo[*f*]quinazoline inhibited the growth of *T. gondii* in human fibroblast cultures by 50% at concentrations of 0.3 and 0.5 μM respectively. From these results it would appear that the potential of 1,3-diaminobenzo[*f*]quinazolines as agents for the treatment of *P. carinii* and *T. gondii* infections in patients with AIDS merits further exploration.

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* Similar incidence of dose-limiting toxicity has also been noted with several of the tricyclic diaminopyrimidines in preliminary assays against L1210 leukemia and other tumors in mice ([25] and unpublished results). This toxicity, which is similar to that of DDMP, suggests that use of these tricyclic compounds as antitumor agents may require leucovorin rescue for optimal results.

† Personal communication, 1988. With permission of Carmen J. Allegra.

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