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2,4-DIAMINO-5-METHYL-6-[(3,4,5-TRIMETHOXYANILINO)METHYL]QUINAZOLINE (TMQ), A POTENT NON-CLASSICAL FOLATE ANTAGONIST INHIBITOR—I

EFFECT ON DIHYDROFOLATE REDUCTASE AND GROWTH OF RODENT TUMORS *IN VITRO* AND *IN VIVO**

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Abstract—Seventeen non-classical 2,4-diamino-6-[(anilino)methyl]quinazoline antifolates were tested as inhibitors of dihydrofolate reductase from L1210 leukemia cells and from human leukemia cells (acute lymphocytic leukemia). Several potent inhibitors of this enzyme were found, some with I_{50} values of 10^{-9} M, thus displaying activity comparable to that of methotrexate. In general, the potency of dihydrofolate reductase inhibition correlated with the inhibition of cell growth *in vitro* against L1210 cells. Two of these compounds, compound 14 (2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl]quinazoline; TMQ, JB-11, NSC 249008) and compound 3 (2,4-diamino-5-chloro-6-[(3,4-dichloroanilino)methyl]quinazoline; NSC 208652), were further evaluated against murine tumors *in vivo* and both showed a broad spectrum of antitumor effects. The results of these studies encourage further evaluation of these compounds, in particular compound 14, as possible anti-neoplastic agents in the treatment of human disease.

The synthesis of an array of classical and non-classical quinazoline analogs of folic acid, aminopterin and methotrexate has been described recently [1, 2]. The absence of N-5 in the reduced forms of these 5,8-dideazapteridines obviously precludes the formation and interconversion of various 1-carbon units analogous to the known tetrahydrofolate coenzymes [2, 3]. It has been postulated that such quinazoline antifolates would function not only as reductase inhibitors, but also might inhibit enzymatic reactions at other sites in the folate metabolic pathway [2].

Among them, various classical 2,4-diamino- and 2-amino-4-hydroxyquinazoline Glu and Asp analogs [1, 2] (Fig. 1, x = 1 or 2; R = H or CH_3; X = OH or NH_2; Z = H, CH_3, or Cl) exhibited potent inhibitory effects against *Streptococcus faecalis* R (ATCC 8043) [4], *Strep. faecium* var. *durans* (SF/O) [5, 6], thymidylate synthetase from *Escherichia coli* [4] and C1300 mouse neuroblastoma [7], and dihydrofolate reductase from sensitive (SF/R) [4, 6] and methotrexate-resistant (SF/A_k [5, 8] *Strep. faecium*, L1210 mouse leukemia [5], C1300 mouse neuroblastoma [7], canine lymphosarcoma [9], and human leukemia cells [10]. Several of these quinazolines also proved to be more active than methotrexate against certain sublines of the L1210 mouse leukemia in culture [6] and in mice [6, 11–13].

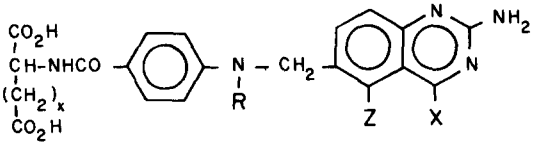

Fig. 1. Quinazoline antifolates (see text for discussion).

Moreover, chlorasquin (Fig. 1, x = 1; R = H; $X = NH_2$; Z = Cl) and methasquin (Fig. 1, x = 1; R = H; $X = NH_2$; $Z = CH_3$) retained anti-leukemic activity against several methotrexate-resistant L1210 lines, and resistance to methasquin and quinespar (Fig. 1, x = 1; R = H; $X = NH_2$; Z = H) developed more slowly than did resistance to methotrexate [6, 11]. In addition, representative Glu and Asp quinazoline antimetabolites inhibited the growth of both sensitive and drug-resistant cell lines of the mouse neuroblastoma C1300 tumor in culture [7].

As predicted, the Glu and Asp quinazoline antifolates (Fig. 1) lacked appreciable antiparasitic activity, presumably due to the lack of an active folate transport mechanism in such organisms [2, 14]. The hypothesis that replacement of the highly polar -COAsp (Glu) moiety of the classical quinazoline antifolates by more lipophilic substituents might confer antiparasitic properties spurred the preparation of various 2,4-diamino-6-[[(halo-, trifluoromethyl-, and alkoxy)anilino]methyl]-quinazolines (see Table 1) [2, ‡]. Strategy was oriented toward the introduction of substituents (R = NO, alkyl, or acyl and $Z = CH_3$ or Cl) at key positions

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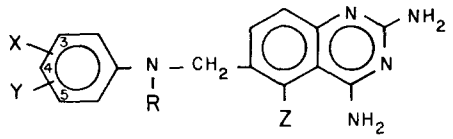
Table 1. *In vitro* inhibitory effects of 2,4-diamino-6-[(anilino)-methyl]quinazolines against purified dihydrofolate reductases from human acute lymphocytic leukemia (ALL) and a methotrexate-resistant subline of the L1210 murine leukemia (L1210R)*



Compound No.*	NSC No.	X, Y	R	Z	ID_{50} (M × 10^9) ALL	L1210R
1	250412	3,4-Cl_2	H	H	3.8	9
2	250413	3,4-Cl_2	H	CH_3	2.6	2.9
3	208652	3,4-Cl_2	H	Cl	1.1	2.0
4	250414	3,4-Cl_2	NO	H	10	10
5	250415	3,4-Cl_2	CH_3	H	5	
6	250416	3,4-Cl_2	$COCH_3$	H	50	10
7	250417	3,4-Cl_2	NO	CH_3	10	
8	250657	3-Cl	H	H	10	10
9	250418	4-Cl	H	H	6	5
10	250419	3-Br	H	H	2.2	2.3
11	250420	3-Br	H	CH_3	1.5	1.5
12	250421	4-Cl, 3-CF_3	H	H	4	
13	250422	3,4,5-$(OCH_3)_3$	H	H	6	
14	249008	3,4,5-$(OCH_3)_3$	H	CH_3	1.3	1.2
15		2-Cl, 4-CH_3	CH_3	H	60	
16		3-Br	H	Cl	4.0	
17		4-F	CH_3	H	30	
Methotrexate					0.9	0.9

* All compounds were tested as the acetate salts with the exception of compounds 5–7 which were tested as the free base.

corresponding to transfer groups in the tetrahydrofolate coenzymes [2, *]. As a class, the 2,4-diamino-6-[(anilino)methyl]quinazolines (see Table 1) displayed striking antimalarial and antifolate effects [2, *]. Furthermore, 2,4-diamino-5-chloro-6-[(3,4-dichloroanilino)-methyl]quinazoline (X, Y = 3,4-Cl_2; R = H; Z = Cl) (compound 3) proved to be a potent inhibitor (ID_{50} = 5.8 × 10^{-9} M) of dihydrofolate reductase from L1210 mouse leukemia cells [15]. These observations prompted an investigation of the inhibitory effects of such non-classical 5,8-dideazapteridine antimetabolites against dihydrofolate reductases from human and murine leukemia cells, and the effect of some of these compounds on the growth of murine tumors *in vitro* and *in vivo*.

EXPERIMENTAL

Chemicals. NADPH was purchased from Sigma (St. Louis, MO). Dihydrofolate (FH_2) was prepared by dithionite reduction of folic acid as described by Blakley [16].

The synthesis of the 2,4-diaminoquinazoline folate antagonists employed for these studies has been described [2, *].

Enzyme source. Dihydrofolate reductase (DHFR), purified to homogeneity from a methotrexate-resistant subline of the L1210 murine lymphoma, and from blast cells from a patient with acute lymphocytic leukemia, was used for the inhibition studies [17–19].

Enzyme assay. DHFR was assayed spectrophotometrically as described [18]. I_{50} values were obtained by inhibition assays in which the compound was added at various concentrations to a mix containing in a final volume of 1.0 ml: Tris–HCl buffer, pH 7.0, 100 μM; KCl, 150 μM; NADPH, 0.08 μM; and water. After a 2-min incubation at 37°, 0.02 μM FH_2 containing 10 μM 2-mercaptoethanol was added to start the reaction. In order to compare the I_{50} values for these compounds, care was taken to use the same amount of enzyme activity for each assay (Δ O.D. 0.020/min/37°). Controls without inhibitor and without FH_2 were run with each inhibitor. The quinazoline inhibitors were dissolved in dimethylsulfoxide (DMSO) at a concentration of 10^{-4} M, and then diluted appropriately in water to give the final concentration desired.

Cell culture studies. Logarithmically growing cells (L5178Y, S-180, W-256 or L1210) in Fischer's medium with 10% horse serum, at a concentration of approximately 3 × 10^5 cells/ml, were diluted with medium plus serum to approximately 5 × 10^4 cells/ml. The cells were then distributed to 15 ml culture tubes, in duplicate, containing either no inhibitor or various amounts of the inhibitor and incubated at 37°. At 48 hr the cells were counted by means of a Coulter counter. The concentration of drug required to decrease the cell count to 50 per cent of control (E_{50}) was determined by plotting the cell number versus the drug concentration.

In vivo studies. Growth inhibition properties of compound 14 and compound 3 in mice bearing various

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Table 2. Sensitivity of 2,4-diamino-6-[(anilino)methyl]quinazolines against L1210 cells grown *in vitro*

Compound	I_{50} for DHFR from L1210 ($M \times 10^9$)	ED_{50} for L1210* (tissue culture) ($M \times 10^8$)
14	1.2	0.7
11	1.5	1.0
9	5	2.5
1	9	4.0
8	10	4.8
6	10	6.0
10	2.3	6.0
4	10	9.0
MTX	0.9	0.8

* The ED_{50} values were measured as described in the Experimental section.

transplanted tumors were obtained through the antitumor screening program of the Drug Evaluation Branch of the National Cancer Institute. The protocols used for the testing of the compounds in B-16 melanoma and P-388 leukemia were as described [20]. For the two mouse colon tumor systems, testing was conducted according to protocols established by the Drug Evaluation Branch, NIH. Details of these experiments are given in Tables 4, 5 and 6.

RESULTS

Enzyme inhibition studies. Seventeen 2,4-diamino-6-[(anilino)methyl]quinazoline antifolates were tested as inhibitors of DHFR which had been purified to homogeneity from human leukemia cells (acute lymphocytic leukemia, ALL) and from a methotrexate-resistant subline of the L1210 mouse leukemia (L1210R). The reference drug methotrexate (MTX) was used as a standard. The 2,4-diaminoquinazolines were all potent inhibitors of these enzymes, with ID_{50} values ranging from 6×10^{-8} M to 1.1×10^{-9} M (Table 1).

Three of the 2,4-diamino-6-[(anilino)methyl]quinazolines [3, 11, 14] inhibited both the L1210R and the human leukemia enzyme at concentrations of $1.1–2.0 \times 10^{-9}$ M and thus were nearly as potent as MTX ($ID_{50} = 0.9 \times 10^{-9}$ M), a stoichiometric or "pseudo-irreversible" inhibitor of DHFR [21, 22].

Tissue culture studies. Eight compounds varying in potency as inhibitors of DHFR were tested further as inhibitors of the growth of L1210 cells propagated *in vitro* (Table 2). Two of these compounds (14 and 11) were potent inhibitors (as potent as MTX). In general, the potency of inhibition of cell growth correlated well with the inhibition of DHFR (except for compound 10).

Table 3. Comparison of compound 14 (TMQ) and MTX against four rodent tumors grown *in vitro*

	MTX	TMQ
	ED_{50}* ($M \times 10^8$)	
L1210	0.8	0.7
L5178Y	0.3	0.2
S-180	4.2	1.8
W-256	0.9	0.5

* The ED_{50} values were obtained as described in the Experimental section.

Table 4. Comparison of MTX (NSC 740) and TMQ (NSC 249008) against the murine tumors colon carcinoma 26 and 38*

Drug	Dose (mg/kg)	Colon 26 % T/C (surv.)†	Colon 38 % T/C (surv.)†
MTX	40	113 (10/10)	76 (10/10)
	20	117 (10/10)	117 (10/10)
	10	99 (10/10)	111 (10/10)
	5	120 (10/10)	122 (7/10)
	2.5	108 (10/10)	170 (10/10)
	1.25	122 (10/10)	105 (9/10)
TMQ	60	136‡ (10/10)	15‡ (8/10)
	30	139‡ (10/10)	37‡ (10/10)
	15	123 (10/10)	110 (10/10)
	7.5	130‡ (10/10)	150 (10/10)
	3.75	135‡ (10/10)	152 (10/10)
	1.88	112 (10/10)	152 (10/10)

* Mice (groups of ten) were injected with an i.p. homogenate (colon 26), or an s.c. fragment (colon 38). Treatment at the daily dose levels indicated was given at days 1 and 5 (colon 26), and days 2 and 9 (colon 38). Mice were evaluated for tumor weight vs control on day 20 (colon 38 mammary tumor), or median survival (colon 26).

† Toxicity day survivors.

‡ Active test.

Table 5. Comparison of MTX (NSC 740) and TMQ (NSC 249008) against the B-16 melanoma and L1210 leukemia*

Drug	Dose (mg/kg)	B-16 melanoma % T/C (surv.)†	L1210 leukemia % T/C (surv.)†
MTX	12	(10/10)	105 (6/6)
	6	(10/10)	156‡ (6/6)
	3	94 (10/10)	155‡ (6/6)
	1.5	98 (10/10)	144‡ (6/6)
	0.75	104 (10/10)	122 (6/6)
	0.37	103 (10/10)	105 (6/6)
TMQ	20	150‡ (10/10)	156‡ (5/6)
	10	128 (10/10)	136‡ (6/6)
	5	124 (10/10)	110 (6/6)
	2.5	107 (10/10)	110 (6/6)
	1.25	101 (10/10)	102 (6/6)
	0.62	104 (10/10)	102 (6/6)

* Mice (groups of ten or six) were implanted with 10^5 cells i.p. (L1210 leukemia) or a 1110 homogenate (B-16 melanoma). Treatment was given on days 1–9 with the daily dose levels indicated. Survival of the control groups and the treated groups was then recorded (% T/C).

† Toxicity day survivors.

‡ Active test.

Table 6. Effect of compound 3 (NSC 208652) against the murine tumors P-388, L1210 and the B-16 melanoma*

Dose (mg/kg)	Schedule (days)	P-388 leukemia % T/C (surv.)†	L1210 leukemia % T/C (surv.)†	B-16 melanoma % T/C (surv.)†
50	1–9	88 (5/6)		Toxic (10/10)
25	1–9	203‡ (6/6)		Toxic (10/10)
12.5	1–9	179‡ (5/6)		137‡ (10/10)
6.25	1–9	123 (6/6)		125 (10/10)
400	1,5,9		147‡ (6/6)	
200	1,5,9		152‡ (6/6)	
100	1,5,9		147‡ (6/6)	

* Groups of six (P-388 and L1210 leukemia) or ten mice (B-16 melanoma) were injected with 10^5 cells (P-388 and L1210 leukemia) or a 1:10 homogenate (melanoma), i.p. Treatment was given in the schedules indicated, and survival of the treated and control groups was recorded (T/C).

† Toxicity day survivors.

‡ Positive test.

In view of the potent *in vitro* effects of compound 14 (2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)-methyl]quinazoline; TMQ, (NSC 249008), it was selected for further study and the effect of this compound was compared with that of MTX on the growth of three other rodent lines (L5178Y, S-180 and W-256) propagated *in vitro*. Table 3 indicates that this compound was more potent than MTX in all the lines tested; against the L1210 and the L5178Y it appeared to be only slightly more effective, on a molar basis, than MTX.

In vivo *studies*. TMQ and compound 3 (NSC 208652) were tested further against mice bearing transplanted murine tumors, and their effects were again compared to that of MTX (Tables 4–6). TMQ had a broad spectrum of activity against these tumors, and was active against all four of the tumors tested (B-16 melanoma, colon carcinoma 26, colon carcinoma 38 and the L1210 tumor). In contrast, MTX was effective only against the L1210 tumor. Compound 3 was tested against the P-388 and L1210 leukemia, and the B-16 melanoma. This drug also showed activity against these three tumor screens.

DISCUSSION

An analysis of structure–activity relationships suggests the following trends: (1) the inhibitory effects of a given diaminoquinazoline against dihydrofolate reductases from either L1210R or human leukemia are of a similar order of magnitude; (2) potency is usually increased when a CH_3 or Cl group is introduced at position 5 (Z) of the quinazoline nucleus (1 vs 2 and 3; 10 vs 11; 13 vs 14); (3) substitution on the distal nitrogen (R) decreases activity (1 vs 4–7); and (4) among the parent members of the series, where R and Z are H, inhibitory potency progressively decreases from 2.2×10^{-9} M to 1×10^{-8} M as the benzene ring in the side chain is substituted with 3-Br; 3,4-Cl_2; 4-Cl, 3-CF_3; 4-Cl; 3,4,5-$(OCH_3)_3$; and 3-Cl. A recent multivariate computer analysis of these data together with other 2,4-diaminoquinazoline compounds has confirmed and extended these conclusions.*

TMQ is a potent inhibitor of DHFR and of the growth of certain rodent tumors *in vitro* and *in vivo*. The effect against murine tumors noted in the *in vivo* studies suggests that this substance is transported more readily in these lines than is MTX, since this compound is not as potent an inhibitor of dihydrofolate reductase as is MTX. Additional studies from this laboratory confirm that in the L5178Y cell, which is more sensitive to TMQ *in vitro*, the transport of the TMQ is greater than MTX.† Additional studies on the mechanism of transport are in progress. The finding that human leukemia DHFR is also potently inhibited by TMQ has encouraged studies of this compound in human neoplastic cells, and a remarkable degree of sensitivity of human leukemia cells as measured by inhibition of DNA synthesis has been found.‡ The reasons for the larger doses of TMQ than of MTX being tolerated by mice bearing the tumors are not known. TMQ could be metabolized more rapidly than MTX, or there may be a decreased sensitivity of normal replicating tissues to the drug. Marsh§ has studied the effect of TMQ on normal mouse and dog marrow stem cells, and found that in this assay TMQ is not as potent as MTX. These results encourage the further evaluation of "nonclassical" folate antagonists as cancer chemotherapeutic agents, and in particular, further trials with TMQ.

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