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Quinazoline Analogues of Folic Acid as Inhibitors of Thymidylate Synthetase from Bacterial and Mammalian Sources

K. J. SCANLON[1], B. A. MOROSON, J. R. BERTINO

Departments of Pharmacology and Medicine, Yale University School of Medicine, New Haven, Connecticut 06510

AND

J. B. HYNES

Department of Pharmaceutical Chemistry, College of Pharmacy, Medical University of South Carolina, Charleston, South Carolina 29401

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SUMMARY

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Three groups of quinazoline analogues of folic acid were studied as inhibitors of thymidylate synthetase (TMP synthetase) from *L. casei* and the mouse leukemia cell line, L1210. Several of these compounds were also tested as inhibitors of cell growth using selected mammalian tumor lines. The three groups of compounds studied were classical 2-amino-4-hydroxyquinazolines, classical 2,4-diaminoquinazolines, and 5,8-dideazapteroic acid derivatives. Of the 2-amino-4-hydroxyquinazolines, N^{10}-methyl-5,8-dideazafolic acid (compound *4*) was found to be the most potent inhibitor, in terms of ID_{50} values for both mammalian and bacterial TMP synthetase. In general, both the 2-amino-4-hydroxyquinazolines and the 2,4-diaminoquinazolines had lower ID_{50} values for the mammalian TMP synthetase than for the bacterial enzyme. The 2,4-diaminoquinazoline derivatives were better inhibitors of cell growth than the 4-hydroxy-counterparts. This effect presumably relates to the fact that they are potent inhibitors of dihydrofolate reductase. Inhibition of TMP synthetase by *4* and *5* (N^{10}-formyl-5,8-dideazafolic acid) was competitive with respect to 5,10-CH_2-H_4PteGlu, the substrate; methotrexate and the compound *21*, (5,8-deazaisoaminopterin) were non-competitive inhibitors with respect to 5,10-CH_2-H_4PteGlu.

INTRODUCTION

TMP synthetase (E.C. 2.1.1.45) catalyzes the conversion of 2'-deoxyuridylate (dUMP) to thymidylate (dTMP), with the concomitant transfer and reduction of a one carbon unit from 5,10-CH_2-H_4PteGlu.[2] This enzyme has been a target for antineoplastic agents because of its key role in DNA synthesis (1, 2).

The synthesis of an effective analogue of the substrate, 5,10-CH_2-H_4PteGlu may provide a useful antineoplastic or antibacterial agent. Previous studies have shown that certain quinazoline analogues of folic acid are effective inhibitors of TMP synthetase

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[1] To whom correspondence should be addressed.

[2] The abbreviations used are: H_4PteGlu, (±)-5,6,7,8-tetrahydrofolate: 5,10-CH_2-H_4PteGlu, (±)-5,10-methylenetetrahydrofolate; MTX, methotrexate.

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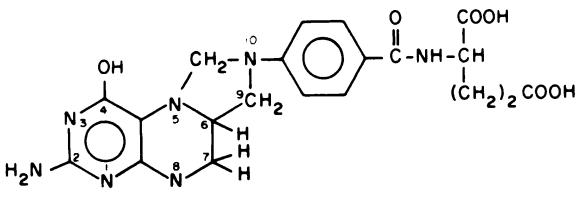
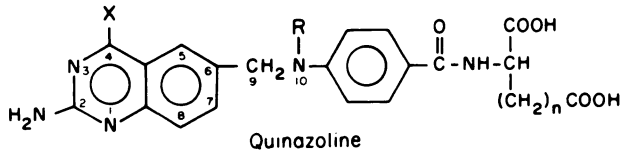


L-5,10-Methylenetetrahydrofolate



Quinazoline

X = OH, NH_2

R = H, CH_3

n = 1, 2

FIG. 1. *Structural relationship of quinazoline analogue of folic acid to the natural substrate N-5,10-methylenetetrahydrofolate*

(Fig. 1), and are substantially more potent than the corresponding pteridine analogues (3–5). However, the structure-activity patterns necessary for the design of more potent inhibitors which can be derived from these data are equivocal since only a limited number of compounds were evaluated (3–5). In this report we describe the inhibition of TMP synthetase from both bacterial and mammalian cells employing a wide variety of quinazoline analogues of folic acid. The bacterial source for the enzyme was *Lactobacillus casei* and the mammalian source was the mouse leukemia cell line, L1210.

EXPERIMENTAL

Materials. (±)-L-5,6,7,8-tetrahydrofolate was prepared according to Zakrzewski *et al.* (6). dUMP was obtained from Sigma Chemical Co. [5-^{3}H]dUMP (12.7 Ci/mmol) was purchased from Amersham.

Sources of TMP synthetase. TMP synthetase from L1210 mouse leukemia cells was highly purified by affinity chromatography (specific activity 0.75 μm/hr/mg protein) as previously described (7). Purified TMP synthetase from *L. casei* (specific activity 39.5 μm/hr/mg protein) was obtained from New England Enzyme Center, Boston, Mass.

TMP synthetase assay. TMP synthetase was assayed using the procedure of Roberts (8). The standard reaction mixture contained, in a final volume of 40 μl: 1.8 nmoles [5-^{3}H]-dUMP (ca. 3×10^7 cpm/μmole), 5.2 nmoles (±)-L-5,6,7,8-tetrahydrofolate, 0.1 μmole of formaldehyde, 0.4 μmole 2-mercaptoethanol, 2 μmoles NaF, 2 μmoles phosphate buffer pH 7.5 and the enzyme. The assay mix for *L. casei* contained 22 mM $MgCl_2$ for optimal enzyme activity. The quinazolines were dissolved in DMSO to a final concentration of 1% or less DMSO, and 4 λ of this solution were used in each assay. The enzyme was pre-incubated at 37° in the presence of the inhibitor for 15 minutes and the reaction was initiated by the addition of the mix. The samples were incubated for 60 min at 37° unless otherwise indicated. The reaction was linear for at least 90 min. One unit of enzyme is defined as the amount of enzyme required to form 1 nmole of TMP/min at 37° under our assay conditions. The reaction was terminated by the addition of 200 μl of a charcoal suspension (Norit, 100/mg/ml) in

2% trichloroacetic acid. The mixture was centrifuged at 1000 × *g* for 5 min. A 100 μl aliquot of the supernatant was added to 3.5 ml aqueous counting scintillant (Amersham/Searle) and measured in a Beckman LS230 liquid scintillation counter. All assays were performed in duplicate. I_{50} values were determined by plotting the enzyme activity versus inhibitor concentration, and extrapolating to 50% inhibition. A minimum of 8 concentrations in duplicate of inhibitor were used for each determination.

Cell culture conditions. The cytocidal effect of several quinazolines was tested using the L5178Y, L1210, S-180 and W-256 rodent tumors propagated *in vitro*. These lines were grown in Fischer's medium containing 10% horse serum (9). As an example of a human cell line an acute myelocytic leukemia line (K562) maintained in RMPI1640 (320 mg/l glutamine) containing 10% fetal calf serum (10), was also used to evaluate the quinazoline cytocidal effects. Cells in logarithmic growth were used for ED_{50} determinations. ED_{50} values were determined for each of the cell lines following 72 hours exposure to the drug being tested (9).

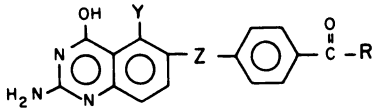
Inhibitors tested against thymidylate synthetase. The preparation of each of the quinazoline derivatives *1–31* has been described previously (11–15). The chemistry involving the synthesis of compounds *32–35* will be presented in a forthcoming publication. MTX was determined to be better than 95% pure by assay with high pressure liquid chromatography. The quinazolines were tested for purity on thin layer chromatography (11–15) and with high pressure liquid chromatography. The molar concentration of the drugs was determined on a Cary spectrophotometer. Compounds *3, 7, 9, 12, 16, 17, 20, 21* and *22*, have been assigned NCS numbers 289520, 289523, 289524, 289517, 529860, 289521, 289518, 289576 and 289575, respectively.

RESULTS

Inhibition of TMP synthetase: structure-activity relationships. The results obtained from *L. casei* and L1210 for 35 quinazoline derivatives as inhibitors of TMP synthetase are presented in Tables 1–3. Each of the compounds in Table 1 contains a 4-OH group and a terminal glutamyl residue. Structural differences include substitutions at positions 5 and 10 as well as modifications having a reversed configuration at

TABLE 1

Inhibition of L. casei and L1210 TMP synthetase by 2-amino-4-hydroxyquinazolines



Group I	Y	Z	R	ID_{50}	
				L. casei	L1210
				μM	μM
1	H	CH_2NH	$Glu(Et)_2$	70.0	0.25
2	H	CH_2NH	Glu	5.0	0.10
3	H	$CH_2N(CH_3)$	$Glu(Et)_2$	75.0	7.50
4	H	$CH_2N(CH_3)$	Glu	0.4	0.05
5	H	$CH_2N(CHO)$	Glu	3.0	0.24
6	H	CH_2S	$Glu(Et)_2$	8.0	0.10
7	H	CH_2S	Glu	2.5	1.00
8	H	CH_2O	Glu	>100.0	10.00
9	H	CH_2CH_2	Glu	7.5	5.00
10	CH_3	CH_2NH	Glu	12.5	0.10
11	H	$NHCH_2$	$Glu(Et)_2$	35.0	1.0
12	H	$NHCH_2$	Glu	10.0	0.5
13	CH_3	$NHCH_2$	$Glu(Et)_2$	>100.0	7.5
14	CH_3	$NHCH_2$	Glu	50.0	7.5

positions 9 and 10 (analogues of isofolic acid). In addition, several diethyl esters are included in order to evaluate the effect of this modification on inhibitory potency. The parent compound in this series, 5,8-dideazafolic acid, compound *2*, is an effective inhibitor of L1210 TMP synthetase. However, it is 50-fold less inhibitory toward the *L. casei* enzyme. This higher affinity for the mammalian enzyme as compared to the *L. casei* enzyme is observed with all of the compounds containing a glutamyl moiety although to widely varying degrees. The presence of a methyl group at position 5 (compound *10*) causes a slight reduction in potency against the bacterial enzyme but inhibition of the L1210 enzyme is unchanged. Compound *4*, on the other hand, which is methylated at position 10, is the most potent inhibitor of both enzymes in this series. The diethyl esters, *1* and *3*, are considerably less inhibitory than are the corresponding free glutamates, *2* and *4*.

Of interest is the series of compounds in which the nitrogen atom at position 10 is replaced by sulfur, oxygen, or a methylene group (*7, 8,* and *9*). Compound *7* is twofold more potent than compound *2* against the bacterial enzyme. For the L1210 enzyme, the level of inhibition decreases or remains about the same, *7* being the most effective of the three compounds. The diethyl ester of *7* (compound *6*) is less inhibitory toward the *L. casei* enzyme but is 10-fold more potent than *7* toward the tumor enzyme. This is the only example encountered in this study where a diethyl ester is significantly more potent than its corresponding free acid.

When the isofolic acid derivatives were examined (compounds *11–14*, reversal of the 9–10 bridge) compound *12* was a poorer inhibitor than its counterpart *2*, which has the normal folate configuration. Furthermore, the presence of a methyl group at position 5, resulting in compound *14*, affords an even weaker inhibitor of the enzyme from either source.

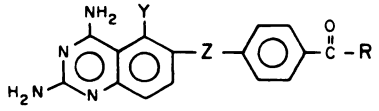
The results obtained with the 2,4-diaminoquinazolines containing a glutamyl residue are shown in Table 2. 5,8-Deazaminopterin, *16*, is 100-fold less inhibitory toward the mammalian TMP synthetase and 20-fold less effective against the bacterial enzyme than its 4-OH counterpart, *2*. The 10-formyl modification 17 is also a poor inhibitor of both enzymes, while the addition of a 5-methyl group resulting in compound *19*, enhances activity 10-fold, with respect to compound *16*.

While reversal of the 9-10 bridge is detrimental to activity for 4-OH derivatives, the 4-NH_2 derivative *21* is 40-fold more effective than *16* against the tumor enzyme. In fact, *21* and its 5-methyl derivative *22* are even better inhibitors of TMP synthetase than their 4-OH counterparts *12* and *14*.

The compounds listed in Table 3 are all devoid of a terminal glutamate, and as such

TABLE 2

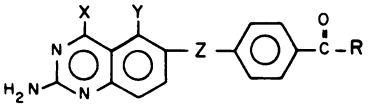
Inhibition of L. casei and L1210 TMP synthetase by 2,4-diaminoquinazolines



Group II	Y	Z	R	ID_{50}	
				L. casei	L1210
				μM	μM
15	H	CH_2NH	$Glu(Et)_2$	>100	>100.0
16	H	CH_2NH	Glu	>100	10.0
17	H	$CH_2N(CH)$	Glu	50	50.0
18	CH_3	CH_2NH	$Glu(Et)_2$	50	7.5
19	CH_3	CH_2NH	Glu	10	1.0
20	H	$NHCH_2$	$Glu(Et)_2$	10	1.0
21	H	$NHCH_2$	Glu	5	0.2.5
22	CH_3	$NHCH_2$	Glu	2.5	0.25

TABLE 3

Inhibition of L. casei and L1210 TMP synthetase by analogues of 5,8-dideazapteroic acid



Group III	X	Y	Z	R	ID_{50}	
					L. casei	L1210
					μM	μM
23	OH	H	CH_2NH	OH	>100	>100
24	OH	H	CH_2NH	OC_2H_5	>100	>100
25	OH	H	NHCH	OC_2H_5	50	>100
26	OH	H	NHCH	OH	>100	>100
27	OH	CH_3	NHCH	H	90	27
28	OH	CH_3	NHCH	OC_2H_5	90	>100
29	NH_2	CH_3	CH_2NH	OC_2H_5	>100	>100
30	NH_2	CH_3	NHCH	OC_2H_5	10	>100
31	NH_2	H	CH_2NH	OC_2H_5	>100	>100
32	NH_2	H	$CH_2N(CHO)$	OH	75	>100
33	OH	H	$CH_2N(CH_3)$	OH	75	20
34	SH	H	CH_2NH	OH	>100	>100
35	SH	H	$CH_2N(CH_3)$	OH	75	7.5

TABLE 4

Inhibition of cell growth by 2-amino-4-hydroxyquinazoline analogues of folic acid

Cells were incubated in the presence and absence of the quinazoline indicated at various concentrations (see EXPERIMENTAL).

	ED_{50}				
	L5178Y	L1210	S180	W-256	K562
	μM	μM	μM	μM	μM
2	1.4	3.5	11	>50	1.4
4	3.0	2.2	>50	6.2	2.3
5	>50	>50	>50	>50	>50
6	>50	>50	>50	>50	20
7	>50	>50	>50	>50	>50
8	>50	>50	>50	>50	>50
9	>50	>50	>50	>50	>50
11	>50	>50	>50	>50	>50
12	>50	>50	>50	>50	>50
14	11	14	>50	20	6

may be considered as analogues of pteroid acid or its ethyl ester. They are weak inhibitors of the bacterial enzyme and are moderately successful against the mammalian enzyme. In the absence of a terminal glutamate, the 10-methyl group is important for inhibition of the L1210 enzyme. This is demonstrated by comparing compounds *33* with *23* and compounds *35* with *34*. Interestingly, the introduction of a 4-SH group enhances the activity only if the 10-methyl group is present as in compound *35*, which is the best inhibitor.

Effects of quinazolines on cell growth in vitro. In order to correlate the enzyme inhibition with effects on cell growth, certain of the compounds from Tables 1 and 2 were tested as inhibitors of growth of various cell tumors in culture.

The 2-amino-4-hydroxyquinazoline inhibitors which were tested, with the exception of compounds *2*, *4* and *14*, are not potent inhibitors of cell growth (Table 4). Compounds *2* and *4*, the most potent inhibitors of TMP synthetase tested, were also the most potent inhibitors of cell growth *in vitro*. Of interest is the difference in potency with respect to cell type. The L5178Y, L1210 and the human line K562 were inhibited to the greatest degree by *2*, while the S-180 line was less sensitive; the W-256 was relatively insensitive. Compound *4* was equally inhibitory to the L5178Y, L1210 and K562 sublines. However, the S-180 was relatively insensitive to this inhibitor, while the W-256 was only slightly less sensitive than the L5178Y, L1210 and K562 cell lines. Presumably these differences relate to transport differences between the cell lines although other reasons may exist (e.g., me-

tabolism). Compound *14*, a relatively poor inhibitor of TMP synthetase, was a moderately good inhibitor of cell growth in all of the cell lines except the S-180 line. The 2,4-diaminoquinazoline analogues, especially *21* and *22* were modest inhibitors of cell growth (Table 5). However, compounds *16*, *18* and *19* were also potent inhibitors of dihydrofolate reductase (unpublished data).

Although compounds *5*, *6*, *10*, *21* and *22* were good inhibitors of TMP synthetase, they were not potent against cell growth as compared to group II. This limited cell killing effect may be due to the inability of the cells to transport these compounds.

Further inhibition studies of the inhibition of L1210 TMP synthetase by selected quinazolines. Initial velocity studies of the L1210 TMP synthetase suggest that the enzyme follows an ordered and sequential mechanism with Michaelis constants to be 28 μm for 5,10-CH_2-H_4PteGlu and 3 μm for dUMP.

Kinetic studies, graphed by the method of Lineweaver and Burk (16) indicated that both compounds *4* and *5* were competitive inhibitors with respect to 5,10-CH_2-H_4PteGlu with K_i values of 0.07 μm and 0.77 μm, respectively (Table 6 and Fig. 2). Two other inhibitors of TMP synthetase, 5,8-deazaisoaminopterin (*21*) and methotrexate, both of which contain a 4-amino group were found to be non-competitive inhibitors of the enzyme with respect to 5,10-CH_2-H_4PteGlu. The approximate K_i values of these inhibitors were 0.45 μm and 36 μm, respectively (17).

TABLE 5

Inhibition of cell growth by 2,4-diaminoquinazoline analogs of folic acid

	ED_{50}				
	L5178Y	L1210	S180	W-256	K562
	μM	μM	μM	μM	μM
16	.013	.006	.054	.013	N.D.
18	.007	.008	.310	.021	.001
19	.007	.010	.200	.035	9.5
20	.640	>1.000	>1.000	.120	N.D.
21	.350	.170	>1.000	.290	N.D.
22	.120	.160	>1.000	>1.000	N.D.
MTX	.003	.006	.009	.420	.022

N.D. = not determined.

TABLE 6

K_i values and mechanism of inhibition by certain folate antagonists against L1210 TMP synthetase

	Variable substrate[a]: 5,10-CH_2-H_4PteGlu[b]	
	K_i	Inhibition
	μM	
Compound 4	0.07	competitive
Compound 5	0.76	competitive
Compound 21	0.45	non-competitive
MTX	36.0	non-competitive

[a] The concentration of 5-^{3}H dUMP was held constant at 46 μm.

[b] The concentration of 5,10-CH_2-H_4PteGlu was varied between 8 to 160 μm. Incubation was for 30 min. Enzyme concentration was 0.5 units/ml.

DISCUSSION

Several quinazoline analogues of folic acid have been shown to be effective inhibitors of TMP synthetase from both bacterial and mammalian cells (5, 6, 9, 18). The 4-hydroxy compounds were more inhibitory than the 4-amino compounds against TMP synthetase from several sources; *E. coli* (3), *Diplococcus pneumoniae* (4), *L. casei* (5), neuroblastoma (5) and human AML cells (19). The results of the present study indicate also that the 2-amino-4-hydroxyquinazolines are significantly more potent inhibitors of TMP synthetase than their 2,4-diaminoquinazoline counterparts, a pattern which is the reverse of that obtained for the inhibition of dihydrofolate reductase (unpublished data). Our results confirm the inhibitory potency of compound *4* (3–5), but also show that most of the compounds studied are significantly more inhibitory toward the mammalian tumor enzyme than the *L. casei* enzyme. In addition, several other changes such as the 10-formyl (*5*) and 5-methyl (*10*) modifications result in a similar level of inhibition as *4*. As a result of this observation of tight binding of compound *5*, it has been utilized to prepare an affinity column which has been extremely useful for isolating TMP synthetase from L1210 leukemia cells (7). The reduced inhibitory activity of the 4-NH_2 modification with respect to their 4-OH counterparts on

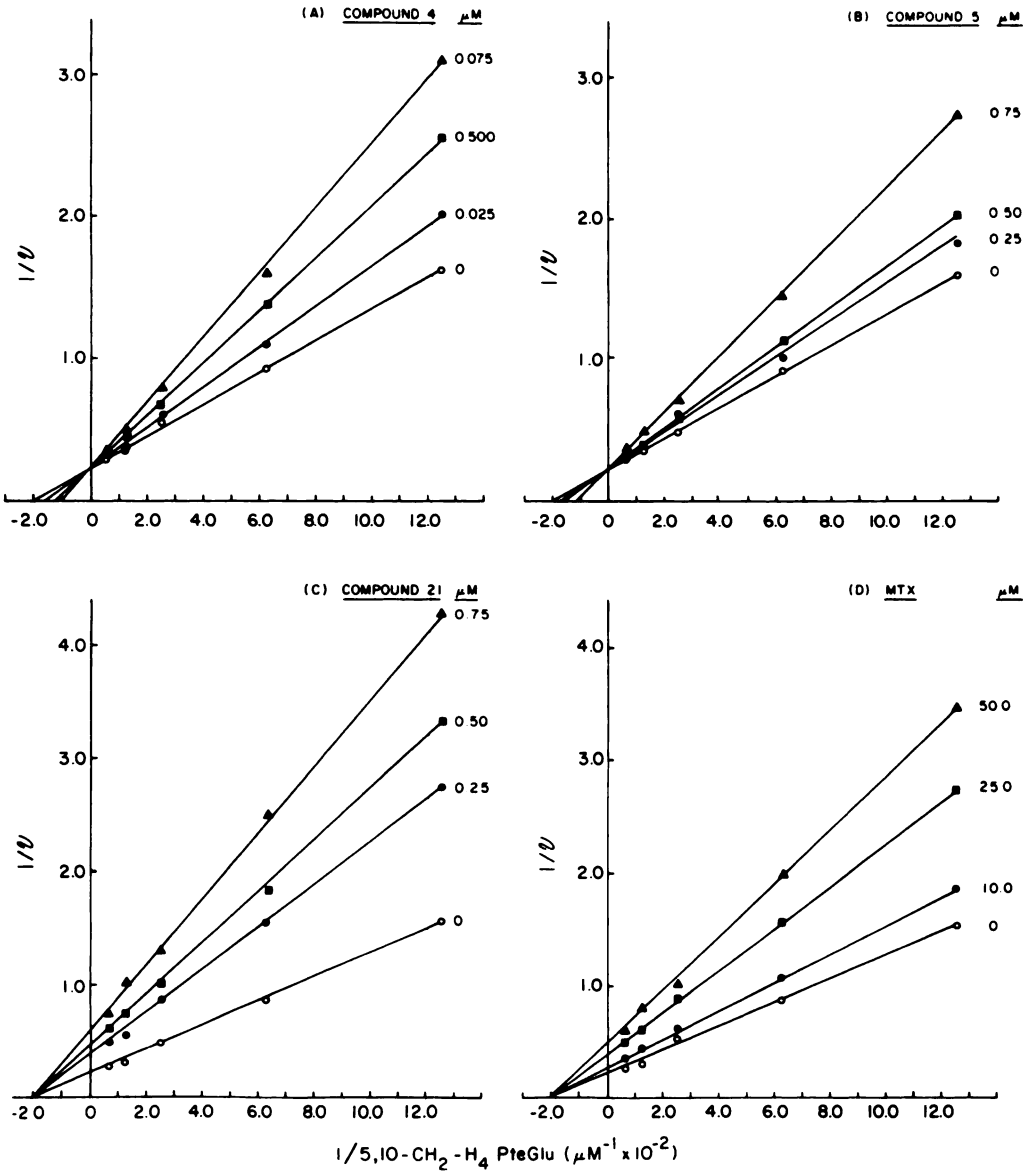


FIG. 2. *Inhibition of TMP synthetase by quinazolines and methotrexate with respect to 5,10-CH_2-H_4PteGlu*
Incubation was for 30 minutes. The enzyme concentration was 0.5 units/ml. The variable concentrations of the substrate were 160, 80, 40, 16 and 8 μm for 5,10-CH_2-H_4PteGlu and the concentration of [5-^{3}H]dUMP (ca. 3×10^7 cpm/μmole). 1/2 is expressed as nmoles of TMP formed/min/ml of enzyme.

TMP synthetase is obtained for all compounds having a normal folate bridge between the quinazoline and phenyl rings. However, the reverse relationship appears to apply for analogues of isofolic acid.

The data obtained for derivatives devoid of a glutamate residue in Table 3 indicate that the presence of this glutamate residue is necessary for potent inhibition of TMP synthetase. The fact that most of the diethyl ester derivatives studied have reduced affinity as compared to the corresponding glutamates also supports this contention. The anomalous result observed with the diethyl ester derivative *6* warrants further study and other esters of 10-thia-

5,8-dideazafolic acid should be prepared. It should be noted that in early studies modifications containing an aspartate group in place of glutamate resulted in decreased potency toward the *E. coli* enzyme but not in the case of the *D. pneumoniae* enzyme (3, 4).

The enhancement in potency obtained with the 4-SH derivative *35* as compared to *33* suggests that this modification may be a means of achieving activity enhancement for certain of the more potent inhibitors. Toward this end, the synthesis of the 4-SH analogues of compounds *2*, *4* and *5* is currently under study.

Perhaps not unexpected are the potent inhibitory effects of certain of the 2,4-diaminoquinazolines on cell growth; this effect is probably mediated by inhibition of DHFR (unpublished data). In contrast, the 2-amino-4-hydroxyquinazolines are relatively weak inhibitors of growth of L1210 cells; whether this is related to the relatively weak inhibition of TMP synthetase, or insufficient transport of the compound intracellularly has not yet been determined. Inhibition of cell growth of L1210 cells by these compounds (*2*, *4* and *14*) correlated with TMP synthetase inhibition.

The initial velocity studies for the L1210 TMP synthetase indicate K_m values for 5,10-CH_2-H_4PteGlu and dUMP of 28 and 3 μm, respectively. These values are similar to what has been reported for the human lymphoblast cell line, 4265 (20) and human leukemia cells (18).

Inhibition studies with 5,10-CH_2-H_4PteGlu as the variable substrate demonstrated that the 2-amino-4-hydroxyquinazoline compounds were competitive inhibitors of this enzyme with respect to 5,10-CH_2-H_4PteGlu. The 2,4-diaminoquinazoline compound *21* and MTX were found to be non-competitive inhibitors with respect to 5,10-CH_2-H_4PteGlu; thus compound *21* is both an effective inhibitor of dihydrofolate reductase (unpublished data) and is a potent inhibitor of the L1210 TMP synthetase (K_i = .45 μm). Further studies with this compound are warranted. Of interest, however, is the relative lack of potency of this compound against the rodent lines *in vitro*, presumably due to poor transport or intracellular metabolism. Non-competitive inhibition of MTX to TMP synthetase with respect to 5,10-CH_2-H_4PteGlu has been reported also for the human lymphoblast cell line 4265 (20) and human leukemia (19); the results obtained with compound *18* also warrant further investigation because of its inhibitory potency and cytocidal effects.

These investigations provide a basis for further structural modifications of quinazoline folate antagonists that could result in more effective inhibitors of TMP synthetase, better cell killing and/or better synergism with other cancer chemotherapy agents. The differences observed between inhibition of the *L. casei* enzyme and the L1210 enzyme by certain of these inhibitors also encourage the hope that inhibitors of bacterial TMP synthetase may be found that do not affect the mammalian enzyme, thus resulting in an anti-bacterial chemotherapeutic agent. It is also clear that the *L. casei* enzyme may not be a valid test system for screening potential inhibitors of TMP synthetase as cancer chemotherapy agents.

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