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FURTHER STUDIES ON SUBSTITUTED QUINAZOLINES AND TRIAZINES AS INHIBITORS OF A METHOTREXATE-INSENSITIVE MURINE DIHYDROFOLATE REDUCTASE

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Abstract—Data are presented on the systematic analysis of thirty-five quinazoline and substituted triazine compounds as inhibitors of a methotrexate-insensitive form of dihydrofolate reductase purified from methotrexate-resistant L5178Y murine leukemia cells. Several of the compounds were found to be more potent inhibitors of this enzyme activity than was methotrexate. Two of the triazine compounds had IC_{50} values approaching 10 nM, which is close to that of methotrexate for the normal drug-sensitive dihydrofolate reductase. In addition, some of these compounds, especially the triazines, exhibit a specificity of inhibition for the methotrexate-insensitive enzyme as compared to the normal methotrexate-sensitive dihydrofolate reductase derived from the same cell line. These compounds may, therefore, be potentially useful in the treatment of those methotrexate-resistant tumours which express an altered, methotrexate-insensitive dihydrofolate reductase.

Acquired resistance to methotrexate (MTX¶), a widely used anticancer agent, poses a major obstacle in the effective treatment of cancer with this compound. Studies with experimental tumour systems have delineated several biochemical mechanisms of resistance: impaired uptake of MTX [1]; overproduction of the intracellular target enzyme, dihydrofolate reductase (DHFR) [2], due to gene amplification [3]; decreased polyglutamation of MTX [4] expression of a catalytically more active DHFR [5]; and the expression of MTX-insensitive DHFR [6, 7].

We have described previously an MTX-resistant subline of murine leukemia L5178Y cells that expresses two forms of DHFR, one of which (form 2) is greater than 10,000-fold more resistant to MTX inhibition as compared to the wild type (form 1) DHFR [6, 8]. To determine if this form of DHFR could be inhibited by other compounds, we had examined previously several classical as well as non-classical folate antagonist drugs for their inhibitory potency against form 2 DHFR [9]. These preliminary studies indicated that certain substituted quinazolines and triazines can be significantly more potent inhibitors of this form of DHFR than MTX.

To obtain additional information concerning structural requirements for inhibition of the MTX-insensitive (form 2) DHFR, we have undertaken a more systematic analysis of substituted quinazoline as well as triazine compounds. The compounds examined differ at single positions, thus allowing for an analysis of the types of substituents that result in a greater or lesser inhibition of the enzyme. In addition, some of these compounds were also tested against the normal, MTX-sensitive (form 1) DHFR present in the same cells, in order to identify those compounds which may exhibit specificity for the MTX-insensitive (form 2) DHFR.

MATERIALS AND METHODS

Cells. The origin, maintenance and characteristics of MTX-resistant L5178Y (R_4) cells have been described previously [6, 9].

Assay for dihydrofolate reductase activity. DHFR was assayed using radiolabeled dihydrofolic acid as the substrate. This method of assaying for DHFR activity is at least 10- to 20-fold more sensitive than the spectrophotometric method described previously [9]. [^{3}H]Dihydrofolic acid was synthesized by dithionite reduction of [G-^{3}H]folic acid (5 Ci/mmole) (Amersham Corp., Oakville, Ont.) according to the method of Hayman *et al.* [10]. The radiopurity of each preparation of [^{3}H]dihydrofolic acid was determined by paper chromatography as described in Ref. 10 and was found routinely to be 90% or greater. The [^{3}H]dihydrofolic acid was stored in aliquots as dry powder at −70°, When needed, aliquots were dissolved in 1 ml of 20 mM Tris–HCl, pH 7.3, and the concentration and specific activity were determined for each aliquot by measuring the

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¶ Abbreviations: MTX, methotrexate, 4-amino-10-methyl-4-deoxyfolic acid; DHFR, dihydrofolate reductase; DMSO, dimethyl sulfoxide; IC_{50}, concentration of drug required to inhibit enzyme activity by 50% and IC_{100}, concentration of drug required to inhibit enzyme activity by 100%.

Table 1. Inhibition of form 2 DHFR by quinazolines (Type I)

Compound*	R_1	R_2	R_3	R_4	IC_{50} (μM)	IC_{50} (μM)	% Inh. by 1 μM	$\frac{IC_{50}\ MTX}{IC_{50}\ Quinazoline}$
MTX†					0.45	5.0	68%	1.0
A	NH_2	H	CH_2NH	Glu	0.026	1.0	98	17.3
B	NH_2	H	$CH_2N(CH_3)$	Glu	0.032	1.0	90	14.0
C	NH_2	H	$CH_2N(CHO)$	Glu	0.24	2.0	83	1.9
D	NH_2	H	$NHCH_2$	Glu	1.1	>10.0	48	0.41
E	NH_2	H	$N(CH_3)CH_2$	Glu	0.06	0.7	100	7.5
F	NH_2	H	$N(CHO)CH_2$	Glu	0.54	8.0	66	0.83
G	OH	H	$NHCH_2$	Glu	>1.0	>10.0	23	<0.5
H	OH	H	$N(CH_3)CH_2$	Glu	>1.0	>10.0	23	<0.5
I	OH	H	$N(CHO)CH_2$	Glu	>1.0	>10.0	32	<0.5
J	OH	H	CH_2NH	Glu	>1.0	>10.0	32	<0.5
K	OH	H	$CH_2N(CH_3)$	Glu	1.7	>10.0	45	0.26
L	OH	H	$CH_2N(CHO)$	Glu	0.2	4.0	75	2.25
M	OH	CH_3	$NHCH_2$	Glu	>1.0	>10.0	20	<0.5
N	OH	CH_3	$N(CH_3)CH_2$	Glu	>1.0	>10.0	10	<0.5
O	NH_2	CH_3	$NHCH_2$	OH	0.28	9.0	68	1.6
MTX‡					10.0	>10.0	43	1.0
P (H-334)	NH_2	Cl	CH_2NH	OH	0.12	5.0	85	83.0
Q	NH_2	Cl	CH_2NH	Glu	0.125	>10.0	79	80.0
R	NH_2	Cl	CH_2NH	OC_4H_9	ND§	ND	50	ND
S	NH_2	CH_3	CH_2NH	Glu	0.13	ND	65	76.0
T	NH_2	Cl	$NHCH_2$	Glu	0.3	ND	72	33.0
U	OH	Cl	$NHCH_2$	Glu	>100.0	ND	0	

* Compounds were evaluated using the spectrophotometric assay for DHFR; the assay as well as the inhibition of form 2 DHFR by these compounds has been described previously [8].

† The enzyme concentration was 0.6 nM for MTX and for compounds A to O.

‡ The enzyme concentration was 0.1 nM for MTX and for compounds R to U.

§ Not determined.

absorbance at 282 nm in 20 mM Tris–HCl, pH 7.3 (molar extinction coefficient at 282 nm = 28,400 M^{-1} cm^{-1} [11]), and scintillation counting.

Dihydrofolate reductase was assayed in 12.5 mM Tris-HCl, pH 7.5, 0.2 M KCl, 0.8 mM NADPH, and 0.1 mM [^{3}H]dihydrofolic acid in a total volume of 200 μl. Controls were performed in the absence of NADPH. To measure inhibition, the enzyme preparations were preincubated with each compound in the presence of NADPH for 2 min at room temperature. The reaction was started by the addition of [^{3}H]dihydrofolic acid and carried out at 37° for 20 min. The reaction was stopped by placing the tubes in ice, and 30 μl of unlabeled folic acid (0.027 M) was added to each tube. Unreduced dihydrofolic acid and folic acid were precipitated by the addition of 30 μl $ZnSO_4$ solution (0.17 M) and 10 μl glacial acetic acid. After centrifugation at 1000 *g* for 1 hr at 4°, 100 μl of the supernatant fraction was added to PCS liquid scintillation mixture (Amersham Corp.) and counted in a liquid scintillation counter. Each drug concentration was assayed in triplicate. The IC_{50} and IC_{100} values were determined from enzyme activity versus drug concentration titration curves using a drug concentration range of 1 nM to 10 μM with half-log increments. IC_{100} values were determined from the intersections of the titration curves with the x-axis and represent the concentration of the drugs at which the radioactive counts in the supernatant fraction were equal to or less than the blanks.

Stock solutions of the quinazolines were made up in 1% DMSO in 0.05 M Tris–HCl, pH 7.5. Substituted triazines were dissolved in 1% *N,N*-dimethylformamide in water. Neither DMSO nor *N,N*-dimethylformamide had any effect on the enzyme activities at concentrations used to prepare the stock solutions.

Protein concentrations were determined by the Bio-Rad protein assay method utilizing Coomassie blue (Bio-Rad Laboratories, Richmond, CA).

Purification of form 1 (MTX-sensitive) and form 2 (MTX-insensitive) DHFRs from L5178Y (R_4) cells. The details of the purification of these two forms of DHFR have been published elsewhere [12].

RESULTS AND DISCUSSION

Two general types of quinazoline compounds were used in these studies: those with a *p*-aminobenzoic acid moiety (type I) [13–17, *], and similar compounds lacking this side chain (type II)†. The results of the inhibition of purified MTX-insensitive (form 2) DHFR by these compounds are shown in Tables 1–3. Table 1 shows the results of the inhibition of

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† J. B. Hynes, A. Kumar and J. H. Freisheim, 189th ASC National Meeting, Miami Beach, FL, April 28–May 3, 1985, Abstracts MEDI 20.

DHFR by type I quinazolines. Although we have reported previously on some of these compounds (compounds P to U) [8], we have included them here since we present additional data pertaining to their inhibitory properties, and also in order to facilitate the comparison of the data. The ratio of IC_{90} MTX/IC_{50} quinazoline is a reflection of the potency of the compound as an inhibitor as compared to MTX. In addition, the IC_{100} values are also important in evaluating the potency of a particular compound since this value gives the concentration of the drug required to totally inhibit tetrahydrofolate synthesis. The type I quinazolines tested can be divided into several groups: those with an amino group position at 4 (R_1) (compounds A to F and O to T) and those with a hydroxyl group at this position (compounds G to N and compound U). These main groups can then be subdivided further depending on whether the bridge connecting the quinazoline moiety to the benzoic acid side chain is in the normal configuration (i.e. CH_2NH, compounds A to C and P to S), or the reverse configuration (i.e. $NHCH_2$, compounds D to I, M to O, T and U). In addition, compounds P to U have a chlorine at position 5 (R_2) and compounds M to O have a methyl group at this position.

The results clearly indicate that the most potent inhibitors from the type I quinazoline group were those with a chlorine at the R_2 position (compare compounds A and Q), those with the bridge in the normal configuration (compare compounds A and D) and those in which this bridge is less polar (compare compounds A and C). In addition, a chlorine at the R_2 position was much more effective than a methyl group (compare compounds O and T), and those compounds with a hydroxyl group at the R_1 position were far less effective than those with an amino group at this position. The presence or absence of the side chain glutamate moiety did not appear to affect the inhibitory potency of these compounds (compare compounds P and Q) and, therefore, one may infer that this part of the molecule does not play a direct role in the inhibition of the form 2 enzyme by these analogues. This contrasts with the important role of the glutamate moiety and, in particular, the α-carboxyl group in binding to the form 1 enzyme.

The most potent of the type I quinazolines were compounds P and Q, i.e. the ones with an amino group at position R_1, chlorine at position R_2 and a normally oriented bridge to the benzoic acid side chain. These are the compounds that we had shown previously to be quite potent inhibitors of this enzyme [8] and remain so after analyzing an additional nineteen of these types of compounds with various substituent groups.

Table 2 shows the results of the inhibition of DFHR activity by type II quinazolines. All of these compounds have an amino group at position 4, differ at the R_1, R_2 and R_3 positions, and lack the benzoyl glutamate side chains. Clearly, these compounds are either similar to or less potent than MTX in terms of inhibition of the MTX-insensitive DHFR. It would appear, therefore, that the bridge configuration and the benzoic acid moiety must be important in achieving proper binding of quinazolines to the active site, since the presence of a chlorine adjacent to the amino group position at 4 (compounds D and F, Table 2) was not sufficient, in the absence of a side chain, in enhancing the inhibitory potency.

The substituted triazines, one of which (compound G, Table 3) has been shown to bind covalently via tyrosine-31 to chicken liver DHFR [18], appeared to be more potent than the quinazolines as inhibitors of the MTX-insensitive (form 2) DHFR (Table 3). Of the eight compounds tested, the most potent were compounds A and B which, although only 55-fold more effective than MTX when IC_{50} values were considered, resulted in 100% inhibition of enzyme activity by 1 μM drug concentration. Triazines A and B are quite hydrophobic molecules, suggesting that the enzyme active site may have more hydrophobic in the MTX-insensitive DHFR.

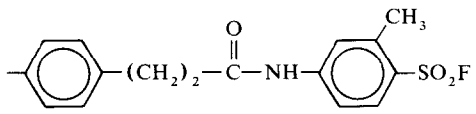
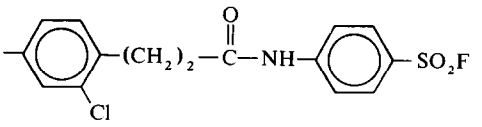
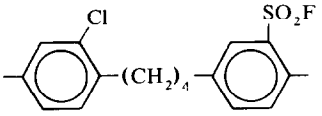
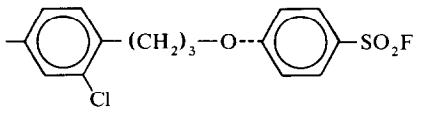
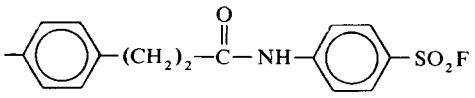
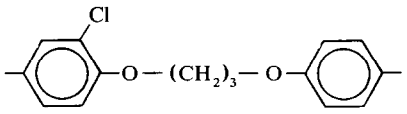
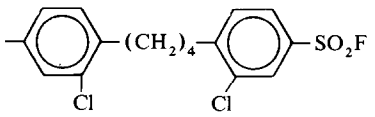
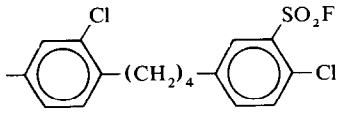
To determine if any of the compounds that appeared to be significantly better inhibitors of form 2 (MTX-insensitive) DHFR showed specificity towards this form of the enzyme as compared to form 1 (MTX-sensitive) DHFR, the IC_{50} values of these compounds for form 1 and form 2 DHFR were compared. These results are shown in Table 4. It is apparent that most of these compounds had a greater affinity for form 2 DHFR relative to MTX, whereas they had a lesser or an equal affinity for form 1 DHFR, relative to MTX. The quinazolines A and B are clearly more effective than MTX, at least in terms of their IC_{50} values. The compounds appear to have a higher selective specificity for form 2 DHFR than for form 1 DHFR, and the degree of the specificity is shown in Table 4 (last column). The two

Table 2. Inhibition of form 2 DHFR by type II quinazolines

Compound	R_1	R_2	R_3	IC_{50} (μM)	IC_{100} (μM)	% Inh. by 1 μM	$\frac{IC_{50}\,MTX}{IC_{50}\,Quinazoline}$
MTX*				0.7	1.2	90	1.0
A	CH_3	Cl	H	0.8	10.0	56	0.88
B	CH_3	Br	H	0.42	4.0	69	1.67
C	CH_3	H	Br	>10.0	>10.0		
D	Cl	Br	H	0.85	3.4	56	0.82
E	CH_3	H	Cl	>10.0	>10.0	0	
F	Cl	Cl	H	>1.0	>10.0	33	>0.5

* The enzyme concentration used was 0.056 nM.

Table 3. Inhibition of form 2 DHFR by substituted diaminotriazines

Compound		IC_{50} (μM)	IC_{100} (μM)	% Inh. by 1 μM	$\frac{IC_{50}\ MTX}{IC_{50}\ Triazine}$
MTX†	R =	1.0	3.0	50	1
A		0.018	1.0	100	55
B		0.018	1.0	100	55
C		0.12	1.0	100	8
D		0.35	2.0	80	2.8
E		0.8	8.0	55	1.25
F		0.1	1.0	100	10
MTX‡		10.0	>10.0	43	1.0
G (127755)		0.32	10.0	73	31
H		0.35	ND§	67	29

* Inhibition of form 2 DHFR by compounds G and H has been described previously [8].
† Enzyme concentration was 0.057 nM for MTX and compounds A to F.
‡ Enzyme concentration was 0.1 nM for MTX and compounds G and H.
§ Not determined.

most hydrophobic triazines had the highest selective specificity for form 2 DHFR.

These data suggest that such compounds may be of value in eradicating MTX-resistant tumour cells which express altered MTX-insensitive DHFRs, even in the presence of normal MTX-sensitive DHFRs.

We have found recently that the blast cells from some acute myelogeneous leukemia patients express more than one form of DHFR, and some of these forms are MTX-insensitive [19]. The compounds discussed above may, therefore, be of value in the treatment of MTX-resistant malignancies such as AML, in certain cases.

Although the *in vivo* effectiveness of these compounds has not yet been determined, these data show that the MTX-insensitive DHFR can be inhibited quite effectively at pharmacologically achievable levels of some of these compounds, and reinforce the idea that a systematic synthesis and analysis of

Table 4. A comparison of the inhibition of form 1 and form 2 DHFRs by some of the quinazoline and substituted triazines

Compound*	Form 2 DHFR† IC_{50} (μM)	Form 1 DHFR† IC_{50} (μM)	IC_{50}s Form 2 / Form 1	Ratios of IC_{50}s MTX/Drug		
				Form 2[a]	Form 1[b]	a/b
MTX	1.0	0.0014	~1000			
Triazine-G	0.032	0.0023	14	31	0.61	51
Triazine-A	0.018	0.01	2	55	0.14	393
Triazine-B	0.018	0.0035	5	55	0.4	138
Triazine-C	0.12	0.003	40	8	0.47	17
Quinazoline-P	0.012	0.0015	8	83	0.93	89
Quinazoline-A	0.026	0.0005	52	38	2.8	14
Quinazoline-B	0.032	0.0005	64	31	2.8	11

* Triazine compounds are given in Table 3; Type I quinazoline compounds are given in Table 2.

† The enzyme concentrations used were 0.06 nM except for compounds Triazine-G and Quinazoline-P (0.1 nM).

other compounds may lead to even more potent inhibitors of such altered forms of DHFR.

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