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Inhibition of a methotrexate-insensitive dihydrofolate reductase from L5178Y cells by substituted triazines and quinazolines

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We have recently demonstrated [1] the presence of two forms of dihydrofolate reductase (DHFR*; 5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) in two methotrexate (MTX)-resistant sublines of L5178Y mouse leukemia cells (L5178Y R3 and R4). The total dihydrofolate reductase activity was elevated approximately 8-fold in these resistant cells as compared to the parent sensitive cells. The principal form of the enzyme was found to be similar to that present in MTX-sensitive cells in terms of its affinity for MTX-Sepharose and sensitivity to MTX. The second form, present in a lower amount, was characterized by poor binding to MTX-Sepharose and marked insensitivity to MTX. Both forms have been partially purified and their properties characterized [2].

The presence of significant amounts of MTX-resistant dihydrofolate reductase in MTX-resistant L5178Y cells is likely a significant factor in producing phenotypic drug resistance in these cells. At extracellular drug concentrations sufficient to inhibit the sensitive reductase, the insensitive enzyme (K_i for MTX, 7.5×10^{-4} M) should be almost totally active. This could maintain cell viability in the face of continuous exposure to high concentrations of MTX. To kill such resistant cells would require the simultaneous inactivation of both forms of dihydrofolate reductase. Thus, if a potent inhibitor for the MTX-insensitive enzyme could be found, it should, in combination with MTX, prove to be an effective cytotoxic agent for these cells, provided that it is effectively transported into the cells. In attempts to inhibit this MTX-insensitive dihydrofolate reductase, several classical as well as non-classical folate antagonist drugs were examined.

Baker and his colleagues have synthesized a host of substituted triazines as potential irreversible inhibitors of dihydrofolate reductase. For example, a series of 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(phenylalkylphenyl)-*s*-triazines bearing a terminal sulfonylfluoride were evaluated as irreversible inhibitors of the reductase [3]. Rapid rates of irreversible inactivation of this enzyme have been reported with this series of substituted *s*-triazines, times for 50% inactivation being in many cases less than 2 min at 37° [4]. DTBSF (structure shown in Fig. 1), a substituted 4,6-diaminodihydrotriazine containing a terminal benzenesulfonylfluoride, binds stoichiometrically and covalently to tyrosine-31 of chicken liver dihydrofolate reductase [4]. Thus, this compound is an active site-directed irreversible inhibitor of avian dihydrofolate reductase.

Selected substituted triazines (including DTBSF), pyrimidines and quinazolines were examined as inhibitors of the MTX-insensitive dihydrofolate reductase from L5178Y R4 cells. The results presented in this communication demonstrate that selected substitutions on the quinazoline and triazine molecules should facilitate stronger inhibition of the MTX-insensitive dihydrofolate reductase, and that, provided these drugs are able to enter the target cells, the simultaneous exposure of the MTX-resistant cells to MTX and the quinazoline or triazine analog should result in significantly better growth inhibition.

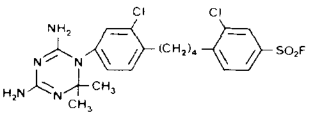
Materials and methods

Cell culture. The origin and maintenance of these L5178Y cells have been described previously [1]. The parent, MTX-sensitive cell line is designated S, and the resistant line, from which the MTX-insensitive dihydrofolate reductase was isolated, is designated R4. The IC$_{50}$ (defined as the concentration of MTX required to inhibit cell proliferation by 50%) was 3×10^{-8} M for S cells and 10^{-3} M for R4 cells.

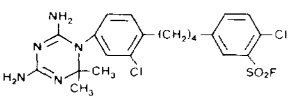
Assay for dihydrofolate reductase activity. Dihydrofolate reductase activity was measured spectrophotometrically at 35° essentially as described by Osborn and Huennekens [5]. Initial rates were derived from a change in absorbance at 340 nm continuously recorded with a Cary model 219 spectrophotometric system. The assay was carried out in a total reaction volume of 1.0 ml in 0.05 M Tris–Cl, pH 7.5. The final concentrations of dihydrofolic acid and NADPH were 100 μM each. The absorbance was corrected for the dihydrofolate-independent blank rate, and the resulting value was used in conjunction with a differential extinction coefficient of 12.3×10^3 M^{-1} cm^{-1} [6], to calculate the enzymatic rate. One unit of activity is defined as that amount required to reduce 1 μmole dihydrofolate/min.

To measure the inhibition of dihydrofolate reductase activity by MTX and other folate antagonists, the inhibitors and the enzyme were incubated in the presence of 100 μM NADPH for 5 min at 35°. The reaction was then started by the addition of dihydrofolate.

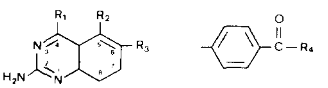
Stock solutions of quinazolines were made up in 1% DMSO in 0.05 M Tris–Cl, pH 7.5. DTBSF was dissolved in 1% *N,N*-dimethylformamide in water.



NSC 127755 (DTBSF)



NSC 128571



QUINAZOLINE

Fig. 1. Structural formulae of the triazines, DTBSF (NSC 127755) and (top) NSC 128571 (middle), and of the common core of the quinazoline compounds tested (bottom). The various substitutions are shown in Table 1.

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* Abbreviations: DHFR, dihydrofolate reductase; MTX, methotrexate, 4-amino-10-methyl-4-deoxyfolic acid; DTBSF, 3-chloro-4[4-[2-chloro-4-(4,6-diamino-2,2-dimethyl-*s*-triazin-1-(2*H*),yl)phenyl]-butyl]-benzenesulfonylfluoride, NSC 127755; DDMP, 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine; and DMSO, dimethyl sulfoxide.

Partial purification of MTX-insensitive dihydrofolate reductase from L5178Y R4 cells. The methods used for purification of the two forms of dihydrofolate reductase present in L5178Y R4 cells have been described in detail [2]. Dihydrofolate reductase activity, recovered after chromatography through a MTX-Sepharose affinity column and filtered through Sephadex G-100, was used in the inhibitor studies.

Methotrexate, dichloromethotrexate and 10-methyl folic acid were gifts from Lederle Laboratories, Montreal, Quebec. Quinazoline compounds **8** and **9** were gifts from Warner–Lambert/Parke–Davis, Pharmaceutical Research Division, Ann Arbor, MI, U.S.A. DTBSF (NSC 127755) and NSC 128571 (see Fig. 1) were provided through the courtesy of Dr. John A. R. Mead, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A. DDMP [2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine] was a gift from Burroughs Wellcome, Research Triangle Park, NC, U.S.A.

Results and discussion

Figure 2 (A through C) shows the inhibition of the MTX-insensitive dihydrofolate reductase by MTX, DTBSF, and quinazoline compounds **1** and **2** from Table 1 (for structural formulae see Fig. 1). In each figure, the inhibition of dihydrofolate reductase activity by one of the folate analogs is compared with the inhibition by MTX. The IC_{50} (i.e. the concentration of MTX required to inhibit enzyme activity by 50%) can be determined from these figures. All three folate antagonists were significantly more potent than MTX as inhibitors of the dihydrofolate reductase. Quinazoline (**1**) (Table 1) inhibited the enzyme with an IC_{50} of 1.2×10^{-7} M as compared to MTX ($\mathrm{IC}_{50} = 1.0 \times 10^{-5}$ M, Fig. 2B). In addition, a concentration of 5×10^{-6} M quinazoline (**1**) resulted in the total inhibition of the enzyme as opposed to less than 50% inhibition with MTX at the same concentration.

DTBSF was also more effective than MTX, but not as effective as quinazoline (**1**) (Fig. 2A). The IC_{50} for DTBSF was 3.2×10^{-7} M (Table 1), and approximately 8% of the original enzyme activity remained after incubation with 10^{-5} M DTBSF. Quinazoline (**2**), in which glutamic acid is substituted for hydroxyl at the terminal end of the molecule (position R4) (Table 1), was almost as effective as quinazoline (**1**) in terms of its IC_{50} (1.25×10^{-7} M); however 18% activity still remained after incubation with 5×10^{-6} M quinazoline (**2**).

Other quinazoline analogs of folic acid with alterations in different parts of the molecule were found to vary in their potencies as inhibitors of this dihydrofolate reductase (Table 1). DDMP and dichloro-MTX were found to be very weak inhibitors with less than 50% inhibition of enzyme activity at a concentration of 10^{-4} M (data not shown). Clearly, DTBSF and quinazolines (**1**) and (**2**) were *ca.* 100-fold more potent than MTX in inhibiting the altered dihydrofolate reductase (Table 1). In the 2,4-diamino-5-chloroquinazoline series, the presence or absence of the L-glutamic acid moiety had little effect on inhibition (**1** vs **2**), whereas the butylester derivative (**3**) was less effective (Table 1). The reversed bridge compound at positions 9 and 10 (compound **5**) was reasonably potent, whereas compounds **6**, **8** and **9** were virtually ineffective as inhibitors.

The IC_{50} values for inhibition by the substituted triazines (DTBSF and NSC 128571) of the MTX-sensitive DHFR from the same cells were found to be 2.6×10^{-8} M for DTBSF and 7.0×10^{-8} M for NSC 128571. These values constitute 12- and 5-fold differences between the inhibition of the MTX-sensitive and the insensitive DHFRs by these compounds.

Summary and conclusions

Inhibition by a variety of substituted triazines and quinazolines of a methotrexate-insensitive form of dihydrofolate reductase from highly MTX-resistant L5178Y mouse leukemia cells was examined. Some of these compounds were significantly more potent than MTX (up to 100-fold). Two triazenes, terminally substituted with benzenesulfonylfluoride residues, were approximately 30-fold more potent than MTX. Quinazoline analogs of folic acid with alterations in different parts of the molecule varied in their potencies as inhibitors. Although none of the compounds tested was as potent as MTX against MTX-sensitive dihydrofolate reductases, these studies show that some types of folate antagonists have increased specificity against this MTX-insensitive dihydrofolate reductase. This finding increases the likelihood that it may be possible to produce compounds with marked specificity for the insensitive reductase. Such compounds might have utility in antifolate combinations designed to overcome methotrexate resistance.

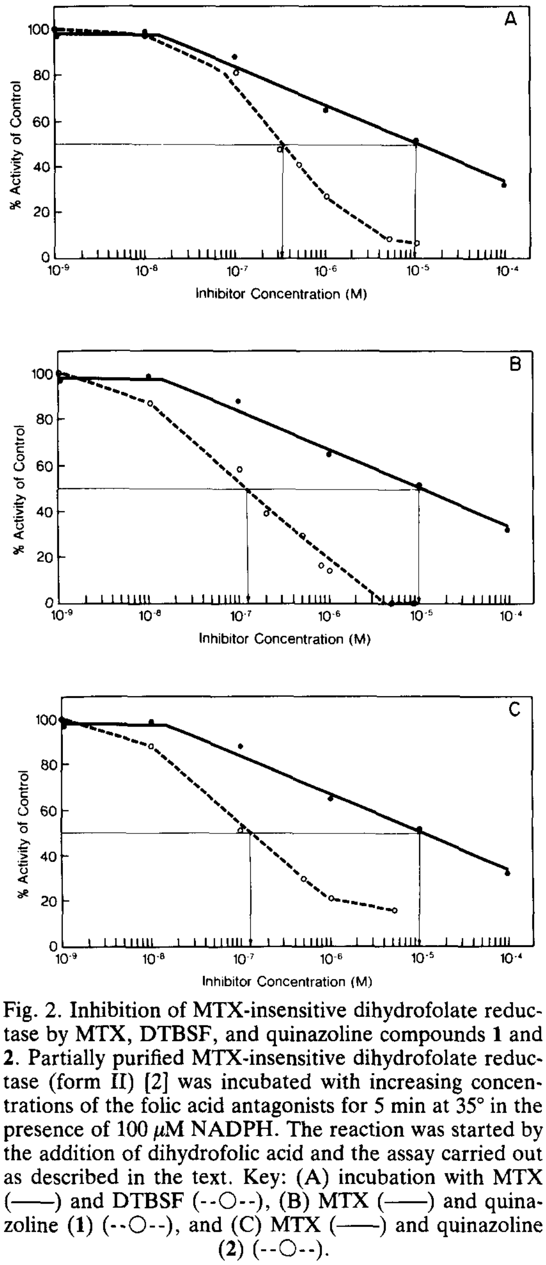

Fig. 2. Inhibition of MTX-insensitive dihydrofolate reductase by MTX, DTBSF, and quinazoline compounds **1** and **2**. Partially purified MTX-insensitive dihydrofolate reductase (form II) [2] was incubated with increasing concentrations of the folic acid antagonists for 5 min at 35° in the presence of 100 μM NADPH. The reaction was started by the addition of dihydrofolic acid and the assay carried out as described in the text. Key: (A) incubation with MTX (——) and DTBSF (--O--), (B) MTX (——) and quinazoline (**1**) (--O--), and (C) MTX (——) and quinazoline (**2**) (--O--).

Table 1. Inhibition of MTX-insensitive dihydrofolate reductase by folic acid antagonists*

Compounds					% Inhibition of DHFR activity†	IC_{50}‡ (M)
Methotrexate					43	1.0×10^{-5}
Triazines§						
DTBSF (NSC 127755)					73	3.2×10^{-7}
NSC 128571					67	3.5×10^{-7}
Quinazolines§						
	R1	R2	R3	R4		
(1)	NH_2	Cl	CH_2NH	OH	85	1.2×10^{-7}
(2)	NH_2	Cl	CH_2NH	GLU	79	1.25×10^{-7}
(3)	NH_2	Cl	CH_2NH	OC_4H_9	50	
(4)	NH_2	CH_3	CH_2NH	GLU	65	1.3×10^{-7}
(5)	NH_2	Cl	$NHCH_2$	GLU	72	3.0×10^{-7}
(6)	OH	Cl	$NHCH_2$	GLU	0	$> 1 \times 10^{-4}$
(7)	OH	H	CH_2N-CHO	GLU	64	5.0×10^{-7}
(8) 2,4-Diamino-6-[3,4-dichlorobenzyl)methylamino]quinazoline					<43	$> 1 \times 10^{-5}$
(9) 2,4-Diamino-6-[2-(3,4-dichlorophenyl)-1-pyrrolidinyl]quinazoline					<43	$> 1 \times 10^{-5}$
10-Methyl folic acid					0	$> 1 \times 10^{-5}$

* The inhibition by folic acid antagonists of the reduction of dihydrofolic acid to tetrahydrofolic acid was measured as described in the text.

† Percent inhibition of DHFR activity by 10^{-6} M inhibitor.

‡ Concentration of inhibitor required to inhibit activity by 50%.

§ Structural formulae of these compounds are shown in Fig. 1. Compounds **1** and **3** were synthesized as described by Hynes *et al.* [7]. The synthesis of compounds **2**, **4** and **7** is described in Hynes *et al.* [8], compound **8** in Elslager *et al.* [9] and compound **9** in Elslager *et al.* [10].

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SHOUKAT DEDHAR*
JAMES H. FREISHEIM†
JOHN B. HYNES‡
JAMES H. GOLDIE*§||

**Division of Advanced Therapeutics*
Cancer Control Agency of British Columbia
Vancouver, British Columbia
Canada V5Z 3J3

‡Department of Biological Chemistry
University of Cincinnati College of Medicine
Cincinnati, OH 45267, U.S.A.

‡Department of Pharmaceutical Sciences
Medical University of South Carolina
College of Pharmacy
Charleston, SC 29425, U.S.A.

§Department of Medicine
University of British Columbia
Vancouver, British Columbia
Canada V5Z 3J3

|| To whom correspondence should be sent: Cancer Control Agency of B.C., 2656 Heather St., Vancouver, B.C., Canada V5Z 3J3.

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