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Patterns of Cross-Resistance to the Antifolate Drugs Trimetrexate, Metoprine, Homofolate, and CB3717 in Human Lymphoma and Osteosarcoma Cells Resistant to Methotrexate[1]

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ABSTRACT

Methotrexate (MTX)-resistant sublines of malignant human cells were selected *in vitro* by stepwise increase in drug concentration in the medium. By this procedure a subline of Burkitt's lymphoma cells (RAJI) was made 290-fold resistant (RAJI/MTX-R), T-cell leukemia cells (CCRF-CEM) were obtained 210-fold resistant (CEM/MTX-R), and 3 MTX-resistant human osteosarcoma lines were selected: TE-85/MTX-R (19-fold resistant; relative to wild-type); MG-63/MTX-R (8-fold resistant); and SAOS-2/MTX-R (200-fold resistant). We also studied a B-cell lymphoblastoid line, WI-L2/m4, that was 13,000-fold resistant.

Assay of cellular dihydrofolate reductase (DHFR) showed the following pattern of activity in resistant cell lines, relative to parental cell activity: RAJI/MTX-R, 550-fold increased; CEM/MTX-R, unchanged; TE-85/MTX-R, 4-fold increased; MG-63/MTX-R, 6-fold increased; SAOS-2/MTX-R, unchanged; and WI-L2/m4, 110-fold increased. Measurement of MTX membrane transport showed decreased uptake in CEM/MTX-R and SAOS-2/MTX-R, relative to parental cell lines. The other DHFR-overproducing cells all gave normal initial MTX uptake rates but increased total uptake.

The DHFR-overproducing lines all had significant cross-resistance to both metoprine and trimetrexate; the two lines with defective MTX transport were not cross-resistant, and the CEM/MTX-R cells showed collateral sensitivity to these agents. Only minor cross-resistance to homofolic acid was found in all MTX-resistant lines. The highly MTX-resistant RAJI/MTX-R and WI-L2/m4 cells showed minor cross-resistance to the dual inhibitor of thymidylate synthetase and DHFR, CB3717 (5- and 15-fold, respectively). These studies demonstrated that, depending upon the mechanism of resistance, MTX-resistant human tumor cells may be effectively killed by antifolates with different routes of uptake into cells, or with a different enzyme target. Thus, there are at least three functionally distinct classes of folate antagonist with antitumor activity.

INTRODUCTION

Despite over 30 years of synthesis and evaluation of folate antagonists as anticancer drugs, MTX,[3] one of the earliest such agents (50), remains the only antifolate in established clinical use for cancer chemotherapy. Apart from its use in maintenance therapy of acute lymphoblastic leukemia, MTX has found applications in treatment of several solid tumors, both at conventional doses and at high doses in combination with folinic acid rescue (41). A number of interesting alternative antifolate compounds have been developed in recent years; it is particularly important to know the extent to which tumors resistant to MTX are cross-resistant to these new agents. Tumor cell resistance to MTX has been studied primarily in rodent cells, but several human studies have been reported; although drug transport defects may explain most examples of MTX resistance in human cells, DHFR-overproducing cells have also been described (8, 28, 33, 37, 39).

TMQ (9) is a tight-binding quinazoline inhibitor of DHFR, and a more potent inhibitor of DNA synthesis in human leukemia cells than MTX (2). TMQ has activity against several transplanted rodent tumors *in vivo* (3, 4).

Metoprine (DDMP) is another lipophilic antifolate that is known to penetrate the central nervous system more readily than MTX (5, 20). Because metoprine does not enter cells by the reduced folate carrier-facilitated route, it may be active against cells that are MTX-resistant because of a drug transport defect (25).

Our interest in homofolate (6) was based on the possibility that, since this agent requires metabolic activation through reduction by DHFR, MTX-resistant cells that overproduce DHFR may show collateral sensitivity to homofolate (34). CB3717 (31) is a quinazoline analogue of folic acid with a 10-propargyl substituent. It acts as an inhibitor of both thymidylate synthetase and dihydrofolate reductase; the thymidylate synthetase inhibition appears to be the primary site of action of CB3717 (25, 27).

Because of the clinical importance of MTX in treatment of T-cell leukemia, osteogenic sarcoma, and Burkitt's lymphoma, and the potential value of alternative antifolates that would be active against MTX-resistant cells, we have examined the sensitivity of 6 MTX-resistant human lines to TMQ, DDMP, homofolate, and CB3717.

MATERIALS AND METHODS

Chemicals. [^{3}H]MTX was obtained from the Radiochemical Centre, Amersham, England. MTX, NADPH, and dihydrofolate were purchased from Sigma Chemical Co., St. Louis, Mo. Trimetrexate (glucuronate salt) was a gift from Dr. L. M. Werbel, Warner-Lambert Co., Ann Arbor, Mich. Metoprine was provided by Dr. C. A. Nichol, Burroughs Wellcome Co.,

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[3] The abbreviations used are: MTX, methotrexate (amethopterin, NSC 740); DHFR, dihydrofolate reductase (EC 1.5.1.3); TMQ, 2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl] quinazoline glucuronic acid salt (trimetrexate, NSC 328564); DDMP, 2,4-diamino-5-(3,4-dichlorophenyl)-6-methylpyrimidine (metoprine; NSC 19494); homofolate, *N*-{[*p*-[2-(2-amino-4-hydroxy-6-pteridinyl)ethyl]amino]benzoyl}-L-glutamic acid (NSC 79249); CB3717, *N*-{[4-[*N*-[(2-amino-4-hydroxy-6-quinazolinyl)methyl]prop-2-ynylamino]benzoyl}-L-glutamic acid (NSC 327182); ID_{50}, drug concentration required for 50% inhibition of growth compared to that of the untreated control.

Research Triangle Park, N. C.; and CB3717 was from Dr. T. A. Jones, Institute of Cancer Research, London, United Kingdom. Homofolic acid was supplied by Vega Biochemicals, Tucson, Ariz. Tissue culture supplies were purchased from Grand Island Biological Co., Grand Island, N. Y.; from Flow Laboratories, Rockville, Md.; and from Seromed, München, West Germany.

Cell Culture. The B-cell lymphoblastoid line WI-L2 and the subline WI-L2/m4, resistant to a concentration of 5 μM MTX, were kindly donated by Dr. S. S. Susten and Dr. J. H. Freisheim, Department of Biological Chemistry, University of Cincinnati College of Medicine, Cincinnati, Ohio. The Burkitt's lymphoma line RAJI, the T-cell lymphoblast line CCRF-CEM, and the osteosarcoma line MG-63 were obtained from the American Type Culture Collection, Rockville, Md. Dr. J. Fogh, Sloan-Kettering Institute, New York, contributed the osteosarcoma lines SAOS-2 and TE-85. All cells in this study are of human origin. The lymphoblastoid lines were grown in suspension culture in Roswell Park Memorial Institute Medium 1640, and the osteosarcoma lines were propagated as monolayers in Eagle's minimum essential medium. Media were supplemented with 10% fetal calf serum, penicillin G (100 units/ml), and streptomycin (100 μg/ml). Cultures were incubated at 37°, 95% humidity, in a 5% CO_2 atmosphere. Resistant sublines were selected by stepwise increases in the concentration of MTX added to the growth medium of the parental cell lines as described previously (26, 40). The resistant sublines, RAJI/MTX-R and CCRF-CEM/MTX-R, were maintained in the continuous presence of 1 μM MTX, and the resistant osteosarcoma sublines grew continuously in medium containing 0.1 μM MTX. Prior to measurement of their transport and enzymatic characteristics, and for determinations of drug inhibition studies, the sublines were maintained for at least 3 transfer generations in the absence of MTX. For all studies, cells were harvested in mid-log phase. For growth inhibition experiments, 10-ml cultures of the lymphoblastoid lines were initiated in plastic flasks (25 sq cm) at an initial cell density of 1×10^5/ml. Drugs were added at the time of initiation of cultures and were present for the duration of the experiment. Cell counts were done 72 hr after initiation on a Model ZBI Coulter Counter. Inhibition experiments with the osteosarcoma lines were performed using 35-mm 6-well culture plates at initial cell densities of 2×10^4 cells/well for TE-85 and MG-63 and 8×10^4 cells/well for SAOS-2, in a final volume of 4 ml/well. Drug was added at the time of initiation of cultures, and removed after 24 hr. Seven days after initiation, cultures were harvested by trypsinization and were counted. The growth-inhibitory effect of drugs on the cell lines was determined by comparison on the cell count of drug-treated and control cultures. The uninhibited mean log-phase doubling times were: RAJI, 20 hr; RAJI/MTX-R, 24 hr; CCRF-CEM, 24 hr; CCRF-CEM/MTX-R, 24 hr; WI-L2, 17 hr; WI-L2/m4, 18 hr; MG-63, 43 hr; MG-63/MTX-R, 48 hr; TE-85, 31 hr; TE-85/MTX-R, 48 hr; SAOS-2, 64 hr; and SAOS-2/MTX-R, 66 hr.

Assay of Dihydrofolate Reductase. About 10^8 cells were suspended in 2 ml of Tris-chloride buffer, pH 7.2; lysed by 3 cycles of freezing and thawing; and centrifuged at 105,000 × *g* for 30 min at 4°. DHFR activity was present in the supernatant fractions. It was assayed spectrophotometrically as follows. A 3-ml quartz cuvet, containing 2.5 ml of 0.05 M Tris chloride buffer (pH 7.2), 0.02 ml of 6 mM NADPH, and 0 to 0.1 ml of enzyme extract, was equilibrated to 37° for about 7 min. The reaction was started by addition of 0.05 ml of 2 mM dihydrofolate, and decrease in absorbance was monitored at 340 nm. Results were calculated assuming a molar absorbance change for the reaction of 1.2×10^4 cm^{-1}.

MTX Uptake Studies. Measurements of MTX transport into the lymphoblastoid cell lines were carried out as described previously (39). For osteosarcoma cells, the procedure was modified as follows. Exponentially growing cells, in 100-mm plastic Petri dishes (about 10^6 cells/dish) were exposed to 0.25 μCi of [^{3}H]MTX (2 μM) in medium without serum at 37° or 0°. At the indicated times, cells were scraped off of the dishes using a rubber policeman, and uptake was stopped by transferring the cells into 10 ml of ice-cold 0.9% NaCl solution. After centrifugation (2000 × *g* for 5 min), the cell pellets were washed twice in ice-cold 0.9% NaCl solution and were then digested overnight in 1 ml of Protosol (New England Nuclear, Boston, Mass.) at 37°. After addition of 0.2 ml of 7 N H_2SO_4 and 10 ml of Aquasol (New England Nuclear), samples were counted in a Beckman LS-9000 scintillation counter at 45% counting efficiency.

RESULTS

Derivation of MTX-resistant Sublines. To select for MTX resistance, cultured human lymphoma and osteosarcoma cell lines were exposed to progressively increasing concentrations of the drug added to the growth medium. A total of 26 sublines with varying degrees of resistance were derived in this way. The studies described below were done with the most resistant subline derived from each of the parental lines. The maintenance MTX concentrations used for each subline are noted in Table 1. Growth-inhibitory effects of MTX on the 6 parental lines and the resistant sublines were assessed by adding the drug at early log phase, and the cell counts of control and treated cultures were compared as described in "Materials and Methods." The dose-response curves obtained in this way are depicted in Chart 1. The ID_{50} values of MTX that reduced the cell counts under the indicated conditions are listed in Table 1, which also shows the degree of resistance relative to the parental cells for the various sublines. Based on the ID_{50} values, the RAJI/MTX-R cells were made 294-fold resistant, and the CCRF-CEM/MTX-R cells were 213-fold resistant. The B-cell lymphoblastoid line WI-L2/m4 (7) was 13,000-fold resistant to MTX, relative to parental WI-L2 cells. The degree of resistance of the sublines derived from osteosarcoma cells ranged from 8- to 200-fold.

Activity of DHFR in MTX-resistant Lymphoblast and Osteosarcoma Cells. Since resistance to MTX is frequently associated with elevation of the target enzyme DHFR (10, 29, 36, 39), the activity of DHFR was measured in cytosol preparations from each cell line under study (Table 2). Both of the lymphoblastoid lines, RAJI/MTX-R and WI-L2/m4, showed a pronounced increase in DHFR activity, 550- and 110-fold, respectively, relative to the parental lines, whereas DHFR activity in the CCRF-CEM/MTX-R line was unchanged. In the osteosarcoma lines, MG-63/

Table 1

Growth inhibition by MTX in drug-sensitive and resistant human cell lines

Values for the resistant sublines were significantly different from values for the corresponding sensitive line in each case ($p < 0.05$; 2-tailed Student's *t* test).

Cell line	Maintenance concentration of MTX (μM)	ID_{50} (μM)	Resistance (-fold)
RAJI		0.017 ± 0.001[a, b]	
RAJI/MTX-R	1.0	5.0 ± 0.2[b]	294
WI-L2		0.015 ± 0.002[b]	
WI-L2/m4	5.0	200.0 ± 35.0[b]	13,000
CCRF-CEM		0.013 ± 0.001[b]	
CCRF-CEM/MTX-R	1.0	2.8 ± 0.7[b]	213
MG-63		0.17 ± 0.02[c]	
MG-63/MTX-R	0.1	1.4 ± 0.2[c]	8
TE-85		0.059 ± 0.003[c]	
TE-85/MTX-R	0.1	1.1 ± 0.2[c]	19
SAOS-2		0.004 ± 0.001[c]	
SAOS-2/MTX-R	0.1	0.80 ± 0.03[c]	200

[a] Mean ± S.D. for triplicate experiments.
[b] Drug was present for the duration of the experiment, and cultures were counted at 72 hr after initiation.
[c] Drug was removed after 24 hr, and cultures were counted at 7 days after initiation.

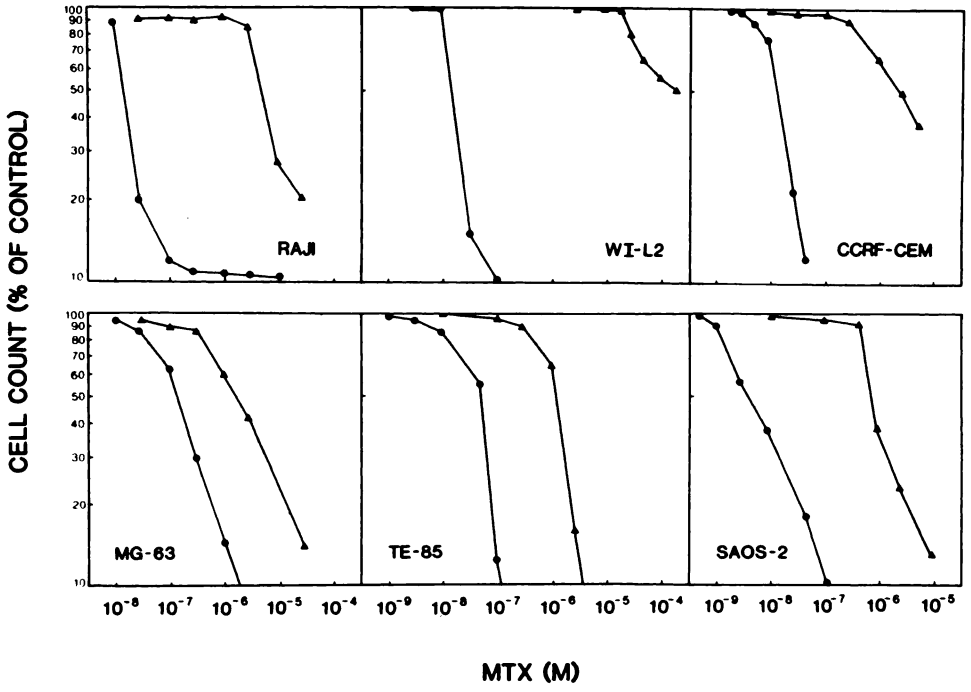

Chart 1. Dose-response curves for growth inhibition by MTX in 6 human cell lines sensitive (●) and resistant (▲) to MTX. *Points*, means of replicate determinations from at least 2 separate experiments.

Table 2

Dihydrofolate reductase activity in MTX-sensitive and -resistant human cell lines

Cell line	No. of samples	IU/10⁹ cells	Overproduction (-fold)
RAJI	5	0.025 ± 0.002[a]	
RAJI/MTX-R	5	13.7 ± 2.1	550
WI-L2	5	0.199 ± 0.062	
WI-L2/m4	5	22.0 ± 3.6	110
CCRF-CEM	4	0.047 ± 0.016	
CCRF-CEM/MTX-R	6	0.037 ± 0.009	
MG-63	4	0.172 ± 0.005	
MG-63/MTX-R	4	1.090 ± 0.020	6.3
TE-85	6	0.152 ± 0.030	
TE-85/MTX-R	5	0.657 ± 0.104	4.3
SAOS-2	6	0.205 ± 0.040	
SAOS-2/MTX-R	5	0.185 ± 0.044	

[a] Mean ± S.E.

Table 3

MTX transport in drug-sensitive and -resistant human cell lines

Cell line	Initial uptake rate[a] (pmol/min/10⁷ cells)	Steady-state drug level[a] (pmol/10⁷ cells)
RAJI	0.83	3.38[c]
RAJI/MTX-R	0.61 (73)[b]	
WI-L2	0.69	4.90[c]
WI-L2/m4	0.87 (126)	
CCRF-CEM	0.77	4.49
CCRF-CEM/MTX-R	0.24 (32)[d]	2.96
MG-63	15.8	36.3[c]
MG-63/MTX-R	20.3 (128)	
TE-85	11.2	30.9[c]
TE-85/MTX-R	10.8 (96)	
SAOS-2	8.01	52.6
SAOS-2/MTX-R	2.56 (32)[d]	40.0

[a] Measured at 37°; extracellular MTX, at 1 μM. Values are means from 2 to 4 replicate experiments.

[b] Numbers in parentheses, transport rate in resistant sublines as percentage of the rate in the corresponding parental lines.

[c] In dihydrofolate reductase-overproducing cell lines, the steady state was not reached during the time of the experiment.

[d] Significantly different from rate in corresponding parental line ($p < 0.05$; 2-tailed Student's t test).

MTX-R and TE-85/MTX-R, which were 8- and 19-fold resistant, the DHFR levels were elevated 6.3- and 4.3-fold, respectively, relative to activity in the parental lines. Assay of cellular DHFR in the osteosarcoma line SAOS-2 and its MTX-resistant subline SAOS-2/MTX-R showed no significant difference in activity. DHFR activities in cytosol extracts of the parental cells of all 3 osteosarcoma lines and the WI-L2 lymphoblastoid line were in the same range as those described in other mammalian cells (26, 39), while the activity in the lymphoblast lines RAJI and CCRF-CEM was found to be 5- to 10-fold lower than in the other cells. Preliminary karyotype studies with the DHFR-overproducing RAJI/MTX-R subline showed a large homogeneously staining region on chromosome 5 that was not present in sensitive cells, but the WI-L2/m4 cells did not contain either a homogeneously staining region or double minute chromosomes; a detailed report of these karyotype studies is in preparation.

Drug Transport into MTX-resistant Cell Lines. MTX resistance has often been attributed to decreased cellular uptake of the drug (1, 11, 13, 23, 33, 39, 45, 49), so transport of [^{3}H]MTX was measured in all sensitive and MTX-resistant lines under study. Table 3 summarizes the initial uptake rates and shows the relative rates of MTX transport in the resistant sublines as percentages of the rate in the corresponding parental lines, together with the steady-state MTX levels obtained. The lymphoblastoid subline CCRF-CEM/MTX-R and the osteosarcoma line SAOS-2/MTX-R showed marked impairment of uptake, while all other sublines had approximately normal initial rates of MTX transport. As illustrated in Charts 2 to 4, the pattern of drug uptake in both lymphoma and osteosarcoma cells was characterized by an early rapid association of the drug with the cells and proceeded at a linear rate for about 2 to 5 min. In the parental strains, the initial rapid rate of uptake was followed by a slower rate until a plateau was reached. Drug uptake in the

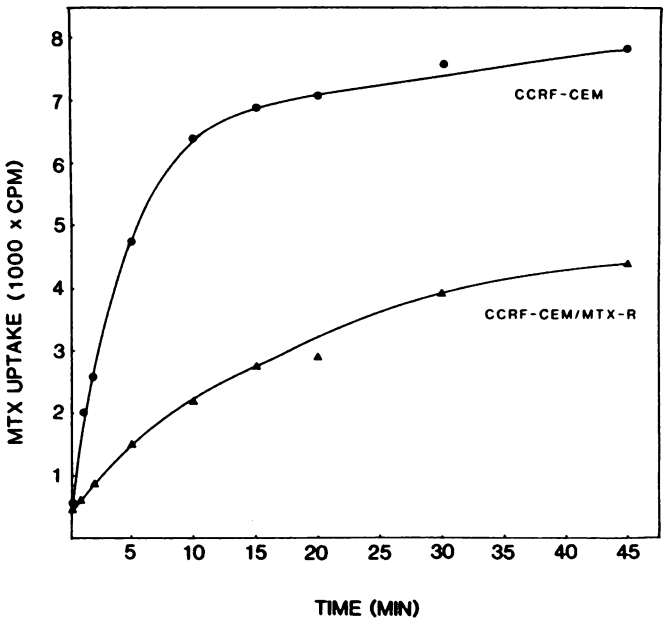

Chart 2. Time course for uptake of [^{3}H]MTX in MTX-sensitive (●) and MTX-resistant (▲) cells of CCRF-CEM human T-cell lymphoma. Cells (5 × 10^6/ml) were incubated at 37° with 2 μM [^{3}H]MTX (0.25 μCi). At the indicated times, aliquots of the cell suspension were removed for processing as described in "Materials and Methods."

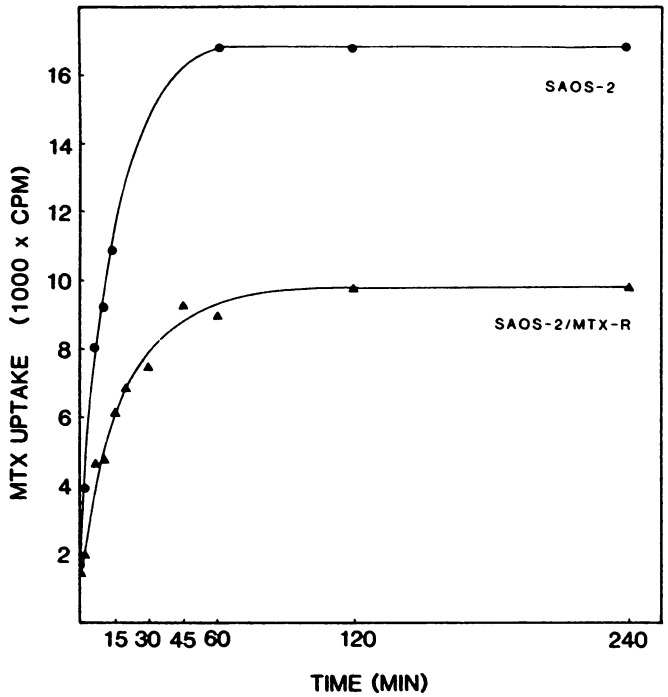

Chart 3. Time course for uptake of [^{3}H]MTX in MTX-sensitive (●) and MTX-resistant (▲) cells of human osteosarcoma SAOS-2. Cells (10^6/dish) were incubated at 37° with 2 μM [^{3}H]MTX (0.25 μCi). At the indicated times, cells were scraped off of the dishes and processed as described in "Materials and Methods."

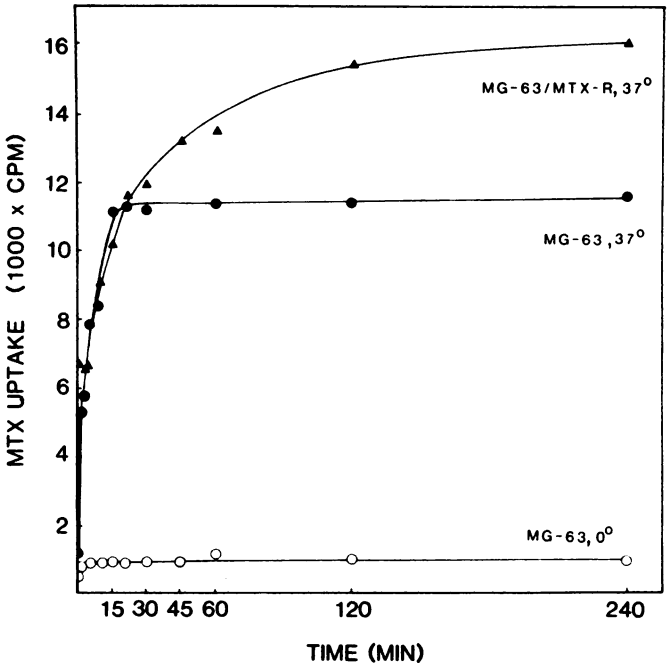

Chart 4. Time course for uptake of [^{3}H]MTX in MTX-sensitive cells at 37° (●) and 0° (○) and in MTX-resistant cells at 37° (▲) in the MG-63 human osteosarcoma line. Cells (10^6/dish) were incubated at the appropriate temperature with 2 μM [^{3}H]MTX (0.25 μCi). At the times indicated, cells were scraped off of the dishes for processing.

parental lines ceased after about 15 min (lymphoblastic lines) or 30 to 40 min (osteosarcomas), at which times rates of uptake and efflux presumably became equal. However, in all DHFR-overproducing sublines, which were normal with respect to initial uptake rates, the steady state was not reached during the time course of our experiments. This may be attributed to the elevated DHFR levels in these resistant cells, since the intracellular-bound MTX content at the steady state is determined by the number of drug binding sites present in the cell (16, 17). Under the same experimental conditions, the 2 MTX-resistant sublines, CCRF-CEM/MTX-R and SAOS-2/MTX-R, which showed no elevations in the cellular DHFR activity, took up the drug at a significantly reduced rate. Charts 2 and 3 show progress curves of transport experiments in such drug-sensitive lines and in the corresponding drug-resistant sublines.

As shown for other cell lines (1, 46), MTX uptake in osteosarcoma cells was shown to be temperature dependent (Chart 4). At 0°, although the drug uptake was reduced by 91%, there was still a measurable amount of [^{3}H]MTX associated with the cells, which was not removed by further washing procedures with ice-cold 0.9% NaCl solution. This amount of MTX did not increase during the time of the experiment, and may represent nonspecific binding to the cell surface (44).

Cross-Resistance to Other Antifolates. In an attempt to circumvent the major mechanisms of MTX resistance, we studied the response of the MTX-resistant cell lines to various nonclassical antifolates. Growth inhibition measurements were carried out as described in "Materials and Methods" for response of each of the sublines to trimetrexate, metoprine, homofolic acid, and CB3717. The ID_{50} values and measured degrees of resistance are summarized in Tables 4 to 8. Those MTX-resistant sublines that had elevated DHFR activities all showed significant cross-resistance to the lipophilic antifolates metoprine and trimetrexate (Tables 4 and 5). In several cases, the degree of resistance to TMQ or DDMP was similar to that for MTX, but the lipophilic antifols were proportionately more effective against the highly MTX-resistant WI-L2/m4 line. The 2 lines with defective MTX transport, CCRF-CEM/MTX-R and SAOS-2/MTX-R, were not cross-resistant to trimetrexate or metoprine and, in some cases,

Table 4

Growth inhibition by metoprine in MTX-sensitive and -resistant human cell lines

Cell line	ID_{50} (μM)	Resistance (-fold)
RAJI	0.11 ± 0.01[a, b]	
RAJI/MTX-R	50 ± 3	460
WI-L2	0.012 ± 0.004[a, b]	
WI-L2/m4	0.62 ± 0.01	52
CCRF-CEM	0.17 ± 0.02[a, b]	
CCRF-CEM/MTX-R	0.035 ± 0.001	0.21
MG-63	1.2 ± 0.3[b, c]	
MG-63/MTX-R	10.0 ± 1.5	8.3
TE-85	0.020 ± 0.004[b, c]	
TE-85/MTX-R	8.0 ± 0.48	400
SAOS-2	0.035 ± 0.005[c]	
SAOS-2/MTX-R	0.040 ± 0.002	1.3

[a] Drug was present for the duration of the experiment, and cultures were counted at 72 hr after initiation.
[b] ID_{50} for resistant subline significantly different from value for the corresponding sensitive line ($p < 0.05$; 2-tailed Student's t test).
[c] Drug was removed after 24 hr, and cultures were counted after 7 days.

Table 5

Growth inhibition by trimetrexate in MTX-sensitive and -resistant human cell lines

Cell line	ID_{50}[a] (nM)	Resistance (-fold)
RAJI	1.5 ± 0.1[b, c]	
RAJI/MTX-R	520 ± 77	347
WI-L2	0.45 ± 0.01[c]	
WI-L2/m4	120 ± 2	267
CCRF-CEM	1.4 ± 0.03[c]	
CCRF-CEM/MTX-R	1.0 ± 0.08	0.71
MG-63	16.0 ± 2.5[d]	
MG-63/MTX-R	2020 ± 310	126
TE-85	25.5 ± 3.7[d]	
TE-85/MTX-R	630 ± 69	25
SAOS-2	80.2 ± 3.3[d]	
SAOS-2/MTX-R	44.6 ± 1.3	0.56

[a] ID_{50} values for the resistant sublines were significantly different from values for the corresponding sensitive line in each case ($p < 0.05$; Student's t test).
[b] Mean ± S.D. for triplicate or quadruplicate experiments.
[c] Drug was present for the duration of the experiment, and cultures were counted at 72 hr after initiation.
[d] Drug was removed after 24 hr, and cultures were counted at 7 days after initiation.

collateral sensitivity was seen. Growth inhibition by the thymidylate synthetase inhibitor CB3717 in MTX-sensitive and -resistant cells is shown in Table 6. In general, none of the lines was highly cross-resistant to CB3717, but a minor degree of cross-resistance was observed in the highly DHFR-overproducing sublines RAJI/MTX-R and WI-L2/m4 (5- and 15-fold, respectively), and the transport-deficient CCRF-CEM/MTX-R cells showed 5-fold resistance to CB3717, compared with 213-fold resistance to MTX. As can be seen in Table 7, none of the sublines showed a significant degree of cross-resistance to homofolic acid, which, following intracellular metabolism, is believed to act as an inhibitor of purine biosynthesis. Table 8 summarizes the degree of resistance of each of the cell lines to MTX and the extent of cross-resistance of each line to all 4 experimental agents.

Table 6

Growth inhibition by CB3717 in MTX-sensitive and -resistant human cell lines

Cell line	ID_{50}[a] (μM)	Resistance (-fold)
RAJI	1.0 ± 0.2[b, c]	
RAJI/MTX-R	5.1 ± 0.1	5.1
WI-L2	1.2 ± 0.1[c]	
WI-L2/m4	18 ± 1.2	15
CCRF-CEM	1.2 ± 0.1[c]	
CCRF-CEM/MTX-R	5.5 ± 0.3	4.6
MG-63	22 ± 1.1[d]	
MG-63/MTX-R	40 ± 1.6	1.8
TE-85	4.5 ± 0.1[d]	
TE-85/MTX-R	11 ± 1.1	2.4
SAOS-2	4.0 ± 0.1[d]	
SAOS-2/MTX-R	5.6 ± 0.9	1.4

[a] ID_{50} values for the resistant sublines were significantly different from values for the corresponding sensitive line in each case ($p < 0.05$; 2-tailed Student's t test).
[b] Mean ± S.D. for triplicate or quadruplicate experiments.
[c] Drug was present for the duration of the experiment, and cultures were counted at 72 hr after initiation.
[d] Drug was removed after 24 hr, and cultures were counted at 7 days after initiation.

Table 7

Growth inhibition by homofolate in MTX-sensitive and -resistant human cell lines

Cell line	ID_{50}[a] (μM)	Resistance (-fold)
RAJI	34 ± 2.3[b, c]	
RAJI/MTX-R	140 ± 25	4.1
WI-L2	33 ± 1.7[c]	
WI-L2/m4	71 ± 7.4	2.2
CCRF-CEM	67 ± 6.0[c]	
CCRF-CEM/MTX-R	96 ± 2.6	1.4
MG-63	120 ± 10[d]	
MG-63/MTX-R	480 ± 94	4.0
TE-85	170 ± 11[d]	
TE-85/MTX-R	350 ± 22	2.1
SAOS-2	200 ± 13[d]	
SAOS-2/MTX-R	240 ± 18	1.2

[a] ID_{50} values for the resistant sublines were significantly different from values for the corresponding sensitive line in each case ($p < 0.05$; 2-tailed Student's t test).
[b] Mean ± S.D.
[c] Drug was present for the duration of the experiment, and cultures were counted at 72 hr after initiation.
[d] Drug was removed after 24 hr, and cultures were counted at 7 days after initiation.

Table 8

Summary of relative resistance of 6 drug-resistant human cell lines to MTX and other antifolates

The degree of resistance is expressed as the ratio of ID_{50} of the resistant subline to ID_{50} of the MTX-sensitive line.

	Drug				
Cell line	MTX	CB3717	DDMP	TMQ	Homofolate
RAJI/MTX-R	294	5.1	460	347	4.1
WI-L2/m4	13,000	15	52	267	2.2
CCRF-CEM/MTX-R	213	4.6	0.21	0.71	1.4
MG-63/MTX-R	8	1.8	8.3	126	4.0
TE-85/MTX-R	19	2.4	400	25	2.1
SAOS-2/MTX-R	200	1.4	1.3	0.56	1.2

DISCUSSION

Culture of human cells in the presence of escalating MTX concentrations resulted in selection of a variety of sublines with different degrees of drug resistance. For both lymphoblasts and osteosarcoma cells, both major classes of MTX resistance were observed, *i.e.*, DHFR-overproducers and cells with impaired membrane transport of drug. This appears to be the first report of acquired MTX resistance in osteosarcoma cells. In some cell lines, simultaneous alterations of both transport and DHFR activity have been reported (29, 39, 49), and it has been suggested that such doubly altered lines emerge by sequential selection, with the enzymatic alteration occurring within a transport-defective resistant population (12, 47). None of the sublines in the present study, however, exhibited such double alterations.

There are 2 main stratagems to overcome MTX resistance.

One is the use of high-dose MTX followed by folinic acid rescue; the other possibility is the use of folate analogues with different enzymatic targets or different routes of entry into the cells. The rational basis for high-dose MTX regimens is that high blood levels of the drug, achieved over long intervals, may overcome impaired membrane transport, high DHFR levels, or high inhibition constants of the enzyme. High-dose MTX therapy followed by folinic acid rescue is used extensively in clinical treatment of osteosarcoma as an adjuvant to surgery (30, 42). Since this tumor was found not to respond to conventional low-dose MTX regimens, it has been assumed that osteosarcoma cells do not possess an active transport system for MTX. However, no studies have been reported concerning the molecular basis of MTX transport or resistance in osteosarcoma. Further work on the biochemical effects of MTX in osteosarcoma may help to clarify the efficacy of high-dose regimens (38). The studies described here show that MTX uptake in osteosarcoma cells is highly temperature-dependent and reaches a steady-state concentration. Since passive diffusion exhibits little temperature dependence, this may be indicative of carrier-mediated uptake.

To investigate possibilities of overcoming MTX resistance by the use of other antifolates, we compared the antiproliferative effects of the 4 folate analogues, TMQ, DDMP, homofolate, and CB3717, against the MTX-resistant lymphoma and osteosarcoma lines. All sublines with raised DHFR levels were significantly cross-resistant to both of the lipophilic inhibitors, TMQ and DDMP. In some cases, differences in relative drug response (Table 8) could not be entirely explained by the DHFR increase. For example, the WI-L2/m4 cells, although resistant to TMQ and DDMP, were relatively less resistant than to MTX. Conversely, the MG-63/MTX-R and TE-85/MTX-R cells were disproportionately resistant to TMQ and DDMP, respectively. Perhaps additional biochemical alterations may be partly responsible for these differences, such as altered levels of folate or nucleotide pools (51), or an altered dissociation constant for DHFR-inhibitor complexes, as described previously by Jackson and Niethammer (28), Goldie *et al.* (15), and Haber *et al.* (18).

The 2 MTX-resistant mutants, CCRF-CEM/MTX-R and SAOS-2/MTX-R, having normal DHFR activity, showed decreased initial drug uptake compared with those of the parental cells. Neither of these transport mutants was cross-resistant to TMQ or DDMP; they were, in fact, found to be up to 5-fold more sensitive to these lipophilic antifolates than were the wild-type strains. A reduced affinity for natural folates by the membrane carrier could account for this observation. Thus, impaired MTX transport in such mutants will be paralleled by decreased uptake of 5-methyltetrahydrofolate in the cells, decreasing the ability of the cells to sustain tetrahydrofolate-dependent reactions in the presence of a DHFR blockade, and reducing the extent of competition with DHFR inhibitors by accumulated dihydrofolates (17, 26). As folinic acid is also transported by the MTX carrier, in cells with impaired MTX transport, the efficacy of folinic acid rescue may be decreased, resulting in selective protection of normal cells from lipophilic antifolates (14, 22, 24). Our findings confirm and extend previous studies on MTX-resistant mouse and human leukemia cells and M5076 murine tumor (21, 32, 40, 43, 48), where collateral sensitivity of transport-deficient MTX-resistant cells to lipophilic antifolates was observed and suggested to be related to partial folate starvation. In general, the new lipophilic antifolate, TMQ, had similar properties to DDMP. An advantage of TMQ is its greater dose potency. TMQ was more potent than MTX in 5 of 6 of the parental lines, and also against 5 of 6 of the MTX-resistant lines.

For the quinazoline antifolate CB3717, an inhibitor of thymidylate synthetase and of DHFR, only a slight degree of cross-resistance could be observed in sublines with highly increased levels of DHFR, consistent with the suggestion that thymidylate synthetase is the primary site of action of CB3717 in WI-L2 cells (27). It is interesting that the transport mutants did not show marked cross-resistance to CB3717, although Jones *et al.* (31) suggested that this agent might be transported by the MTX carrier. Although CB3717 was less dose-potent than MTX in most cases, its different biochemical mechanism and the relative lack of cross-resistance in DHFR-overproducing cells make this an agent of great interest.

Neither the DHFR-overproducing lines nor the transport mutants were markedly cross-resistant to homofolic acid, an inhibitor of purine biosynthesis (19). Since homofolic acid requires intracellular reduction by DHFR to its active tetrahydro form (35), it has been suggested that this compound might be more active against tumor cells with high levels of DHFR (34). However, no collateral sensitivity of the DHFR-overproducing cells to homofolate was observed in our experiments, suggesting that some other reaction (possibly polyglutamylation) may be rate-limiting for the bioactivation of this agent. The lack of cross-resistance of MTX-resistant lines to homofolate may be valuable, but the efficacy of this agent is limited by its low potency.

The antifolates, and MTX in particular, are useful antitumor compounds whose applications are sometimes limited by emergence of acquired resistance. The development of new antifolates with different transport properties, or different enzymatic targets, should extend the spectrum of activity of this important class of agents. Studies with drug-resistant cell lines provide a valuable means of evaluating these new antifolates.

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