Cytotoxic Effects of Folate Antagonists against Methotrexate-resistant Human Leukemic Lymphoblast CCRF-CEM Cell Lines

Enrico Mini, Barbara A. Moroson, Christine T. Franco, and Joseph R. Bertino

ABSTRACT

A human T-lymphoblast cell line, CCRF-CEM/R1, resistant to methotrexate by virtue of increased dihydrofolate reductase activity, was grown in stepwise increasing concentrations of methotrexate. This additional selection pressure resulted in a cell line, CCRF-CEM/R2, resistant to methotrexate by virtue of both an elevation of dihydrofolate reductase activity and a marked decrease in methotrexate transport. The R1 and R2 cells were approximately 70- and 350-fold more resistant to methotrexate than were the parent cells. The effects of three folate antagonists were studied on these cell lines and also on CCRF-CEM/R3 cells, characterized by impaired methotrexate transport but normal levels of dihydrofolate reductase.

INTRODUCTION

MTX, a classical folate antagonist, is used in the treatment of several human hematological and solid tumors (26). Its effectiveness, however, is limited in tumors which are intrinsically resistant or become resistant, despite initial sensitivity, following continued administration of the drug. Four mechanisms of acquired resistance to MTX have been documented in cultured tumor cells: (a) impairment of transport; (b) increase in the activity of the target enzyme, DHFR; (c) alteration in DHFR resulting in decreased binding of MTX (reviewed in Ref. 6); and (d) reduced polyglutamylation of MTX (15). These mechanisms might also play a role in the occurrence of natural resistance.

In order to circumvent resistance to MTX due to impaired transport, several approaches have been proposed: (a) the use of high doses of MTX with leucovorin rescue, which permit the entry of the drug into tumor cells by passive diffusion (23); (b) the use of agents bypassing MTX transport, i.e., liposomes (32, 49), poly-lysine or albumin conjugates of MTX (13, 45), lipophilic esters of MTX (17, 27, 44); and (c) the use of nonclassical folate antagonists that do not use the reduced folate transport system as does MTX, i.e., diaminopyrimidines (24, 38), triazines (48), and 2,4-diaminoquinazolines (8, 31, 39).

In order to overcome resistance to MTX due to elevated DHFR activity, the use of high-dose MTX with leucovorin rescue has been proposed, but has been shown to be of limited effectiveness (12). In treating this type of resistant cells, the use of nonclassical inhibitors of DHFR that accumulate intracellularly to higher levels than MTX [i.e., triazines (48), 2,4-diaminoquinazolines (31)] may be of value. The use of folate antagonists which are inhibitors of thymidylate synthase, rather than DHFR, (i.e., 2-amino-4-hydroxy-quinazolines that perhaps use alternate transport mechanisms) is also a strategy that is under investigation (21, 28).

The development of a human lymphoblast leukemia cell subline, CCRF-CEM/R1 resistant to MTX by virtue of increased DHFR activity has been described previously (36). By further exposing this subline to increasing concentrations of MTX, a resistant subline CCRF-CEM/R2, characterized by elevation of reductase activity and marked decrease in MTX transport, has been established. Using these cell lines as well as CCRF-CEM/R3, a resistant subline characterized by impaired MTX transport but normal levels of DHFR (44), the ability of 3 folate antagonists to overcome MTX resistance of these lines has been evaluated.

MATERIALS AND METHODS

Chemicals. The sources of the drugs used were as follows: MTX and TTT, National Cancer Institute, Bethesda, MD; trimetrexate, Warner-Lambert/Parks-Davis, Ann Arbor, MI; Doxorubicin, Adria Laboratories, Columbus, OH; actinomycin D, Merck, Sharpe and Dohme, West Point, NY; bleomycin, Bristol Laboratories, Syracuse, NY; vinblastine, Eli Lilly & Co., Indianapolis, IN; etoposide [4’-dimethylpipopophyllotoxin 9-(4,6-O-ethylene-d-0-glucopyranoside)], Bristol Italiana Sud, Latina, Italy; 2-methyl-9-hydroxy ellipicinium [2,5,11-trimethyl-8H-pyrido-(4,3-b)-carbazolium], Midy, Milan, Italy; and FdUr and FdUr, Sigma Chemical Co., St. Louis, MO. IAHQ was a gift of Dr. J. B. Hynes, Medical University of South Carolina, Charleston, SC. Trimetrexate, Doxorubicin, bleomycin, and MTX were a gift of Hoffmann-La Roche, Nutley, NJ.
actinomycin D, vinblastine, 2-methyl-9-hydroxy ellipticinium, FLRs, and 
FdUrd were dissolved in water. Etoposide was supplied in solution in an 
organic carrier containing 10 mg of citric acid, 150 mg of benzyl alcohol, 
400 mg of Tween 80, 3.25 g of polyethylene glycol-absolute alcohol in a 
total volume of 5 ml. TzT was dissolved in dilute acid. IAHO was 
dissolved in phosphate-buffered saline and the pH adjusted to 7.0. 
Insoluble material was removed by centrifugation. The concentration 
was determined using the formula: $c = \frac{\text{MTX concentration}}{\text{MTX volume}} \times \text{MTX absorbance}$.

MTX was dissolved in base, the pH adjusted to pH 7.0. The concentration 
was determined using the formula: $c = \frac{\text{MTX concentration}}{\text{MTX volume}} \times \text{MTX absorbance}$.

Dihydrofolate was synthesized by the method of Blakley (10). [5-3H]- 
Deoxyuridine monophosphate (12 Ci/mmole) and [3',5',7-3H]MTX (20 Ci/ 
mmole) were purchased from Moravek Biochemicals, City of Industry, CA. 
Tritiated MTX was purified by high-performance liquid chromatography 
or DEAE-cellulose as described previously (30) and was >99% pure. 
NADPH, deoxyuridine monophosphate, and Tris-HCl were obtained from 
Sigma Chemical Co., St. Louis, MO. Media, sera, and antibiotics for 
tissue culture were purchased from Grand Island Biological Co., Grand 
Island, NY, and plasticware was fromComing Glass Works, Corning, 
NY. Noble agar was purchased from Difco Laboratories, Detroit, MI. All 
other chemicals were the highest purity available and were obtained from 
standard commercial sources.

Cell Lines. A clones subline of the human T-lymphoblast cell line, 
CCRF-CEM, was used in this investigation (22). The MTX-resistant 
subline, CCRF-CEM/R, characterized by impaired transport but normal 
levels of DHFR, was obtained from Dr. H. Lazarus, Sidney Farber Cancer 
Institute, Boston, MA (44). Cells were grown as suspension culture in 
RPMI Medium 1640 supplemented with 10% horse serum, penicillin (100 units/ml), and streptomycin (100 µg/ml) at 37° in a 5% CO₂ 

tablet and subcultured twice weekly.

Selection Procedure. MTX-resistant cells, CCRF-CEM/R, growing 
in 0.2 MMTX, were transferred in progressively higher concentrations 
of MTX, starting with 0.4 µM. The cells were maintained in the same 
concentration of MTX for 2 to 3 transfers, until the cells recovered normal 
growth; the MTX dose was then increased 1.5- to 2-fold. This stepwise 
procedure was continued over a period of months yielding a resistant 
subline able to grow in 2 MMTX. This cell line was designated CCRF- 
CEM/R.

Cell Growth Inhibition Studies. Logarithmically growing cells (CCRF-
CEM, CCRF-CEM/R, CCRF-CEM/R, CCRF-CEM/R), CCRF-CEM/R) were prepared at a density of 5 x 10⁴ cells/ml and distributed in duplicate 5-ml portions 
to tissue culture tubes, to which 0.05 ml of drug solutions at various 
concentrations was added. The cells were incubated at 37°. After 72 hr, 
the number of cells was counted using a Coulter Counter Model B 
(Coulter Electronics, Inc., Hialeah, FL). ED₅₀ was determined by plotting 
the cell number versus the drug concentration.

Cell Viability Studies. Cell viability was measured by the soft agar 
cloning technique of Chu and Fischer (14). The number of cells cloned 
varying from 100 to 20,000/plastic tube depending upon the expected 
seed kill. Maximum cell inoculum used (2 x 10⁵) allowed quantitation of 
seed kill in the in vitro clonogenic assay ≤4 logs. The average cloning 
efficiency for control cells was 63, 65, 55, and 21% for CCRF- 
CEM, CCRF-CEM/R, CCRF-CEM/R, and CCRF-CEM/R, respectively. 
These values were normalized to 100%. Each condition was cloned 
in quadruplicate, and the experiments were repeated at least twice.

Preparation of Cell Extracts. The cells were harvested at midloga-
rithmic growth, centrifuged at 500 x g, and washed 3 times with 
phosphate-buffered saline (containing 140 mM NaCl, 3 mM KCl, 8 mM 
N₄HPO₄, 1.5 mM KH₂PO₄). The cells were then resuspended (1:4, v/v) 
in 50 mM Tris-HCl buffer (pH 7.5) containing 100 mM KCl, 10% glycrol, 
and 10 mM 2-mercaptoethanol. The cells were then disrupted by soni-
cation at 4° using a standard micropipet on a Branson Sonifier (Branson 
Sonic Power Co., Danbury, CT) set at 40 watts in 20-sec bursts. The 
preparation was checked microscopically for complete lysis. After cen-
trifugation at 37,000 x g for 30 min in a Sorvall RC-5B refrigerated 
centrifuge (DuPont Co., Wilmington, DE), the supernatant was used to 
determine enzyme activity.

Enzymes. DHFR (EC 1.5.1.3) activity was determined spectrophotomet-
ically as described by Bertino et al. (7). Briefly, the decrease in 
absorbance at 340 nm, which occurs when NADPH and dihydrofolate 
are converted to NADP and tetrahydrofolate, respectively, at 37°, was 
measured using a Gilford Model 2000 recorder (Gilford Instrumental, 
Oberlin, OH), attached to a Beckman DU spectrophotometer (Beckman 
Instruments, Fullerton, CA). The standard assay mixture contained in 
a final volume of 1.0 ml: 100 µmol of Tris-HCl buffer (pH 7.0 or 8.5); 150 
µmol of KCl; 0.1 µmol of NADPH enzyme; and deionized water. 
The reaction was initiated by the addition of 0.05 µmol of dihydrofolate 
containing 0.5 µmol of 2-mercaptoethanol.

ED₅₀ values for MTX were obtained by inhibition assays in which the 
drug was added at various concentrations to the standard assay mixture 
described above, which contained enzyme (activity Δ A 0.030/min at 
37°). After a 2-min incubation at 37°, 0.02 µmol of dihydrofolate contain-
ing 0.2 µmol of 2-mercaptoethanol was added.

Thymidylate synthase (EC 2.1.1.45) activity was determined by the 
isotopic method of Roberts (42) as modified by Rode et al. (43). Poly-
folylpolygutamate synthetase was assayed as described by McGuire et al. 
(55).

Protein Determination. Protein concentrations were determined by 
the semimicrobiuret method.

MTX Uptake. Logarithmically growing cells (CCRF-CEM, CCRF-CEM/R, 
2 x 10⁴ cells/ml) were harvested by centrifugation, washed twice, 
resuspended in folate-free RPMI Medium 1640 supplemented with 10% 
horse serum at a concentration of about 5.0 x 10⁵ cells/ml, and incubated 
at 37° in the presence of 2 µM [³H]MTX (specific activity, 254 µCi/µmol). 
At selected times, the cells were assayed for radioactivity as described 
previously by Nahas et al. (37).

RESULTS

Development of the MTX-resistant Cell Subline CCRF- 
CEM/R. A stepwise selection of a MTX-resistant subline, CCRF- 
CEM/R, in increasing concentrations of MTX, as described in 
"Materials and Materials," over a period of about 4 months, 
resulted in the establishment of a resistant cell subline, CCRF-
CEM/R, able to grow in 2 MMTX.

The resistant cell lines, CCRF-CEM/R, and CCRF-CEM/R₂, 
were selected on an ED₅₀ basis, the CCRF-CEM/R, and R₂ cells were approximately 70- 
and 350-fold more resistant to MTX (ED₅₀ = 0.7 and 3.5 µM, re- 
spectively) than were the parent cells (ED₅₀ = 0.01 µM), while the 
CCRF-CEM/R₂ cells were about 300-fold more resistant (ED₅₀ = 
3.1 µM). Little or no evidence of a plateau effect in the dose-
response curves for MTX cell kill was found, presumably due to the 
limits of detection of cell kill in the clonogenic assay (≤4 logs).

Enzyme Activity. DHFR specific activity and ED₅₀ values for 
MTX of crude extracts from parent and MTX-resistant cell lines,
ANTIFOLATE EFFECTS ON MTX-RESISTANT CCRF-CEM CELLS

Chart 1. Inhibitory effects of MTX on the colony formation of CCRF-CEM parent (○) and MTX-resistant lines, CCRF-CEM/R1 (△) and CCRF-CEM/R2 (●), and CCRF-CEM/Ra (▲). Cells were cloned in soft agar in the presence of the indicated concentrations of MTX. Numbers of visible colonies were counted after 16 days of incubation. Points, means of a representative experiment done in quadruplicate.

Table 1
Dihydrofolate reductase specific activity in CCRF-CEM parent and MTX-resistant cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>DHFR specific activity (µmol/hr/mg protein)</th>
<th>IC₅₀ of MTX (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCRF-CEM</td>
<td>0.3</td>
<td>0.62</td>
</tr>
<tr>
<td>CCRF-CEM/R₁</td>
<td>6.5</td>
<td>0.80</td>
</tr>
<tr>
<td>CCRF-CEM/R₂</td>
<td>5.3</td>
<td>0.66</td>
</tr>
<tr>
<td>CCRF-CEM/R₃</td>
<td>0.2</td>
<td>0.79</td>
</tr>
</tbody>
</table>

As shown in Table 1, the activities of these 2 folate enzymes were similar in the resistant lines and in the parent line.

[³H]MTX Uptake. The uptake of [³H]MTX by CCRF-CEM parent by MTX-resistant cells, R₁ and R₂, is shown in Chart 2. For comparison, the uptake of MTX by CCRF-CEM/R₃ cells is also shown. At an external concentration of 2 µM, the uptake of MTX in the parent line was linear for 15 min, thereafter reaching a steady-state level. In the MTX-resistant subline CCRF-CEM/R₁, the initial rate of uptake was more rapid, and MTX accumulated to a higher level, as compared to that of the parent cells. In the resistant subline R₂, a marked decrease in both rate and extent of MTX uptake was observed under the same conditions. CCRF-CEM/R₃ cells also were found to have a marked impairment of MTX uptake, confirming the studies of Rosowsky et al. (44).

Effects of Antifolates on the MTX-resistant Cell Lines. The growth-inhibitory effects of TZT, trimetrexate, and IAHQ were evaluated in the parent CCRF-CEM cells and in MTX-resistant R₁, R₂, and R₃ cells. The results are shown in Table 3. The ED₅₀ values for MTX were 0.015, 1.5, 5.0, and 3.4 µM for parent cells.

Table 2
Thymidylate synthase and folypolyglutamate synthetase-specific activities in CCRF-CEM parent and MTX-resistant cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Thymidylate synthase (nmol/hr/mg protein)</th>
<th>Folypolyglutamate synthetase (nmol/hr/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCRF-CEM</td>
<td>31.9</td>
<td>1.5</td>
</tr>
<tr>
<td>CCRF-CEM/R₁</td>
<td>27.6</td>
<td>1.1</td>
</tr>
<tr>
<td>CCRF-CEM/R₂</td>
<td>29.8</td>
<td>0.9</td>
</tr>
<tr>
<td>CCRF-CEM/R₃</td>
<td>27.8</td>
<td>2.0</td>
</tr>
</tbody>
</table>

The activities of thymidylate synthase and folypolyglutamate synthetase were also determined in resistant and sensitive lines.
ANTIFOLATE EFFECTS ON MTX-RESISTANT CCRF-CEM CELLS

R1, R2, and R3, respectively. Trimetrexate was about a 3-fold more potent inhibitor of the growth of CCRF-CEM parent cells than MTX; TZT and IAHQ were about 8- and 100-fold less potent. Trimetrexate and TZT were at least as potent against the MTX transport defective line, CCRF-CEM/R1, as compared to the parent cells. Trimetrexate and TZT were about 30- and 28-fold less potent against the DHFR overproducing line, CCRF-CEM/R1, than against the parent line. CCRF-CEM/R3 cells resistant to MTX by virtue of both increased DHFR levels and impaired MTX transport, showed about the same degree of cross-resistance to trimetrexate and TZT as compared to CCRF-CEM/R1 cells resistant by virtue of DHFR elevation. All 3 MTX-resistant lines showed some cross-resistance to IAHQ (18-, 15-, and 12-fold increase in ED50 for R1, R2, and R3 as compared to parent CCRF-CEM cells).

Effects of Fluoropyrimidines, Cytotoxic Antibiotics, and Alkaloids on the MTX-resistant Cell Lines. The sensitivity to FUra, FdUrd, and various cytotoxic antibiotics and alkaloids of parent and MTX-resistant CCRF-CEM cell lines was tested. The results are shown in Table 4. In all the MTX-resistant CCRF-CEM lines, there was no change in sensitivity to FUra and FdUrd as compared to the parent cells. MTX transport-defective CCRF-CEM/R3 and CCRF-CEM/R3 cells did not demonstrate cross-resistance to Doxorubicin, bleomycin, vinblastine, etoposide, and 2-methyl-3-hydroxyellipticinium. Minor cross-resistance (3- to 5-fold) to actinomycin D was noted in all the MTX-resistant lines.

### Table 3

**Inhibitory effects of TMQ, TZT, and IAHQ on the growth of CCRF-CEM parent and MTX-resistant cell lines**

The cells were incubated in the presence of drugs at various concentrations; numbers of cells were counted after 72 hr of incubation. The data represent the mean of duplicate samples from single experiments, which were repeated either once or twice.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MTX</th>
<th>TMQ</th>
<th>TZT</th>
<th>IAHQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCRF-CEM</td>
<td>0.015</td>
<td>0.005</td>
<td>0.125</td>
<td>1.45</td>
</tr>
<tr>
<td>CCRF-CEM/R1</td>
<td>1.500</td>
<td>0.150</td>
<td>3.500</td>
<td>27.00(16.6)</td>
</tr>
<tr>
<td>CCRF-CEM/R2</td>
<td>5.000</td>
<td>0.050</td>
<td>1.500</td>
<td>21.00(14.5)</td>
</tr>
<tr>
<td>CCRF-CEM/R3</td>
<td>3.400</td>
<td>0.003</td>
<td>0.180</td>
<td>17.50(12.1)</td>
</tr>
</tbody>
</table>

* Concentration of drug required to inhibit cell growth by 50% compared with untreated control cells.

### Table 4

**Inhibitory effects of FUra, FdUrd, antibiotics, and alkaloids on the growth of CCRF-CEM parent and MTX-resistant cell lines**

The cells were incubated in the presence of drug at various concentrations; numbers of cells were counted after 72 hr of incubation. The data represent the mean of duplicate samples from single experiments, which were repeated either once or twice.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>FUra (μM)</th>
<th>FdUrd (μM)</th>
<th>ADM (μM)</th>
<th>ACT D (μM)</th>
<th>BLM (μM)</th>
<th>VLB (μM)</th>
<th>VP-16 (μM)</th>
<th>NMHE (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCRF-CEM</td>
<td>3.9</td>
<td>4.5</td>
<td>165</td>
<td>1.9</td>
<td>1.3</td>
<td>5.2</td>
<td>150</td>
<td>0.6</td>
</tr>
<tr>
<td>CCRF-CEM/R1</td>
<td>4.6(1.2)</td>
<td>4.7(1.0)</td>
<td>165(1.0)</td>
<td>8.0(4.2)</td>
<td>1.5(1.2)</td>
<td>4.3(0.8)</td>
<td>330(2.3)</td>
<td>0.8(1.3)</td>
</tr>
<tr>
<td>CCRF-CEM/R2</td>
<td>4.6(1.2)</td>
<td>5.8(1.3)</td>
<td>230(1.4)</td>
<td>10.0(5.3)</td>
<td>2.2(1.7)</td>
<td>5.2(1.0)</td>
<td>400(2.7)</td>
<td>1.1(1.8)</td>
</tr>
<tr>
<td>CCRF-CEM/R3</td>
<td>5.1(1.3)</td>
<td>5.0(1.1)</td>
<td>155(0.9)</td>
<td>6.5(3.4)</td>
<td>2.0(1.5)</td>
<td>7.0(1.3)</td>
<td>110(0.7)</td>
<td>0.8(1.0)</td>
</tr>
</tbody>
</table>

* Concentration of drug required to inhibit cell growth by 50% compared with untreated control cells.

### DISCUSSION

Drug resistance continues to be a major problem in the chemotherapeutic treatment of human neoplastic diseases. The use of MTX-resistant tumor cells in culture offers a valuable approach for the study of the mechanisms of natural and acquired resistance to this drug, as well as for the development of strategies to overcome resistance.

In the human lymphoblast T-cell line, CCRF-CEM, resistance to MTX was induced by growing cells in gradually increasing drug concentrations. Resistance was associated with elevation of DHFR activity, due to gene amplification, occurring in cells growing in 0.2 μM MTX (CCRF-CEM/R1) (36). When the concentration of MTX was gradually further increased to 2 μM, following a stepwise procedure, CCRF-CEM/R1 cells became more resistant to MTX by virtue of impaired transport of this drug, while maintaining elevated levels of DHFR (CCRF-CEM/R2). Binding affinity of MTX to DHFR from resistant cell lines did not vary significantly as compared to that of the enzyme from the parent cells.

The same evolution of MTX resistance (increase in DHFR followed by impaired MTX transport) has been observed previously in other leukemia cell lines (1, 33). The reverse sequence in the development of MTX resistance has also been reported (1, 34). It has been proposed that the mechanism by which cells become resistant to MTX might depend on the extent of cell kill obtained by the first treatment with the drug (1).

The possibility that the activities of certain folate enzymes other than DHFR would be varied in MTX-resistant cell lines was investigated, since both an increase in thymidylate synthase (16) and a decrease in polyglutamate synthesis (15) have been reported to be associated with MTX resistance in prokaryotic and eukaryotic cells, respectively. No significant differences in levels of thymidylate synthase and folylpolyglutamate synthetase were observed between the parent and the 3 resistant lines.

A potentially important approach to overcome MTX resistance is the use of drugs able to selectively kill tumor cells resistant by virtue of different mechanisms (5). In particular, the use of nonclassical folate antagonist inhibitors of DHFR (e.g., 2,4-diaminoquinazolines, triazines), that accumulate intracellularly to higher concentrations than MTX by entering cells via passive diffusion or via transport systems other than that used by reduced folates and MTX, has been proposed for treating tumor...
cells resistant to MTX as a result of impaired transport and/or increased DHFR activity (20, 31, 39, 48). The use of folate antagonist inhibitors of enzymes other than DHFR has been proposed for the treatment of DHFR overproducing tumor cells (21, 28). Folate antagonist inhibitors of thymidylate synthase, (e.g., 2-aminopteroylglutamic acid) are potentially useful agents for this type of resistant tumor cells.

CCRF-CEM/R3 cells were at least as sensitive to trimetrexate and TZT as the parent cells. These data are consistent with previous observations (20, 39, 48) and demonstrate that the growth of human MTX transport defective resistant cells may be effectively inhibited by folate antagonists with different route of entry into cells. Minor cross-resistance to trimetrexate and TZT was observed in CCRF-CEM/R1 cells. These data demonstrate that these drugs are only partially able to overcome MTX resistance occurring as a result of increased levels of DHFR. Prolonged intracellular accumulation of these drugs at levels high enough to exceed DHFR binding capacity and thus to completely inhibit tetrahydrofolate formation might in fact not occur possibly due to rapid efflux. In CCRF-CEM/R3 cells, resistant to MTX by virtue of both mechanisms, trimetrexate and TZT were equipotent as toward CCRF-CEM/R1 cells, confirming that these compounds circumvented the impaired transport component of resistance. IAHQ, a folate antagonist inhibitor of thymidylate synthase, is a less potent inhibitor of CCRF-CEM cell growth than MTX. All 3 MTX-resistant lines showed minor cross-resistance to IAHQ. These results suggest that IAHQ and MTX might share a common transport mechanism. However, the decreased sensitivity of the R1 line remains unexplained by these results.

In mammalian cells, MTX shares the transport systems of folates, both reduced and oxidized (25). Human leukemic MTX-resistant cells with impaired transport of the drug may have a higher folate requirement than sensitive cells for optimal growth (40). These cells may contain lower levels of tetrahydrofolate coenzymes, in particular, 5,10-methylenetetrahydrofolate. Since the coexistence of fluoropyrimidines to thymidylate synthase is dependent on the intracellular levels of 5,10-methylenetetrahydrofolate, the coenzyme necessary for ternary complex formation (47), we evaluated the sensitivity to fluoropyrimidines in CCRF-CEM MTX-resistant cell lines. Sensitivity to these drugs did not vary in the resistant cell lines as compared to the sensitive line, regardless of the mechanisms of their resistance.

Cross-resistance to cytotoxic antibiotics and alkaloids has been demonstrated in several mammalian tumor cells (2, 9, 18, 19, 46, 50). A common alteration of membrane component involved in drug transport has been proposed as a possible explanation (4, 9, 29, 41). MTX transport-defective CCRF-CEM/ R2 and CCRF-CEM/R3 cells were not cross-resistant to various cytotoxic antibiotics and alkaloids, with the possible exception of actinomycin D. However, cross-resistance to actinomycin D was present also in DHFR overproducing CCRF-CEM/R0 cells, suggesting a mechanism of cross-resistance not related to a common alteration of the membrane. Similarly, the multi-drug-resistant phenotype lines described do not show cross-resistance to MTX (3, 9, 18, 19). These findings allow the suggestion that the alteration of the membrane transport carrier in MTX-resistant cells is different than that observed in cells resistant to antibiotics and alkaloids.

These lines will be useful in further elucidating the mechanisms of gene amplification and impaired MTX transport, as well as for further experimental studies with folate antagonists.

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