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Cytotoxicity of Trimetrexate against Antifolate-resistant Human T-Cell Leukemia Cell Lines Developed in Oxidized or Reduced Folate

Hayato Miyachi,[1,3] Yuzuru Takemura,[2] Hiroyuki Kobayashi[2] and Yasuhiko Ando[1]

[1]*Department of Clinical Pathology, Tokai University School of Medicine, Bohseidai, Isehara 259-11 and*
[2]*Department of Laboratory Medicine, National Defense Medical College, 3-2 Namiki, Tokorozawa 359*

Cytotoxicity of trimetrexate (TMQ), a lipophilic dihydrofolate reductase inhibitor, was examined in antifolate-resistant human T-cell leukemia cell lines developed in oxidized or reduced folate. An approximately 60-fold methotrexate (MTX)-resistant subline was developed in oxidized folate (pteroylglutamic acid: PGA) (CCRF-CEM/MTX_{60}-PGA) from human T-cell leukemia cell line CCRF-CEM; this line exhibited impaired membrane transport of the drug. Further enhancement of MTX resistance resulted in selection of an approximately 5000-fold MTX-resistant subline (CCRF-CEM/MTX_{5000}-PGA), which showed increased dihydrofolate reductase activity due to gene amplification in addition to further impairment of MTX transport. An approximately 140-fold MTX-resistant subline, and then a 1500-fold MTX-resistant subline were developed in reduced folate (10 n*M* leucovorin) (CCRF-CEM/MTX_{140}-LV and CCRF-CEM/MTX_{1500}-LV); they exhibited increased dihydrofolate reductase due to gene amplification accompanied by increased intracellular drug accumulation of MTX. While CCRF-CEM/MTX_{140}-LV and CCRF-CEM/MTX_{1500}-LV cells showed cross-resistance to TMQ, CCRF-CEM/MTX_{60}-PGA and CCRF-CEM/MTX_{5000}-PGA cells were at least as sensitive to TMQ as the parent cells. TMQ was more potent against approximately 200-fold N^{10}-propargyl-5,8-dideazafolic-acid (CB3717)-resistant human T-cell leukemia MOLT-3 sublines developed in PGA (MOLT-3/$CB3717_{200}$-PGA) or leucovorin (MOLT-3/$CB3717_{200}$-LV), as compared to the parent cells; MOLT-3/$CB3717_{200}$-PGA and MOLT-3/$CB3717_{200}$-LV cells were resistant to CB3717 by virtue of impaired transport, only the former possessing gene amplification of thymidylate synthase. The cytotoxicity of TMQ in both MOLT-3/$CB3717_{200}$-PGA and MOLT-3/$CB3717_{200}$-LV cells was reduced by addition of leucovorin in a dose-dependent manner, suggesting intracellular folate deficiency as a cause of TMQ sensitivity. These results demonstrate that TMQ overcomes transport-impaired antifolate resistance, irrespective of gene amplification of dihydrofolate reductase or thymidylate synthase. Types of folate used during the development of antifolate resistance seem to be important in relation to the mechanism of TMQ responsiveness as well as that of antifolate resistance.

Key words: Trimetrexate — Methotrexate — Folate — Transport — Gene amplification

Methotrexate (MTX), a classical folate antagonist, is used in the treatment of various kinds of human hematological and solid tumors.[1] Frequently, tumors that originally appeared to be responsive to MTX become increasingly resistant to the compound.[1] The mechanisms of development of resistance to MTX have been explored in cultured cells. Combinations of an elevated level of the target enzyme, dihydrofolate reductase (DHFR), due to gene amplification, impaired membrane transport of the drug, alteration in DHFR resulting in decreased binding of MTX, and reduced polyglutamylation of MTX, have been suggested to be major mechanisms of resistance.[1] In order to circumvent MTX resistance, various approaches have been explored. New antifolate agents have been designed to inhibit a different target or to use a different transport system than MTX.[1] Trimetrexate (TMQ) is a tight-binding quinazoline inhibitor of DHFR,[2,3] which has been undergoing clinical trial in the USA.[1] This lipophilic antifolate enters the cell through a route distinct from the classical reduced folate/MTX transport system.[3] Thus, TMQ has been demonstrated to have activity against MTX resistance arising from transport impairment in cultured cell lines.[4,5] However, the mechanisms of resistance demonstrated *in vitro* can not always explain the *in vivo* resistance, since the *in vivo* conditions, such as cell milieu, magnitude of resistance and drug exposure schedules are not always relevant to those *in vivo*.[6,7] In particular, previous studies in our laboratory have demonstrated that the type of folate used during development of resistance plays an important role in the mechanisms of resistance to MTX,[7] as is also the case for a potent thymidylate synthase (TS) inhibitor, N^{10}-propargyl-5,8-dideazafolic acid (CB3717).[8,9] In the standard culture medium, which contained an excess of oxidized folate (pteroylglutamic acid: PGA) as a source of folate, an MTX-resistant K562 subline emerged with impaired transport. In contrast, in a physiological level of reduced folate (10 n*M* leucovorin, 5-formyl tetrahydrofolate), an MTX-resistant K562 subline emerged with gene amplifi-

[3] To whom reprint requests should be addressed.

cation of DHFR.[7] Moreover, a CB3717-resistant human T-cell leukemia MOLT-3 subline developed in PGA had gene amplification of TS and impaired transport, while a CB3717-resistant MOLT-3 subline developed in leucovorin showed only impaired transport.[9] We have also demonstrated that an MTX-resistant human T-cell leukemia CCRF-CEM subline established in leucovorin emerged with a different cross-resistance pattern to fluoropyrimidines and azidothymidine than cells grown in PGA.[10] These findings emphasize the important role of the type of folate used during the development of antifolate resistance in determining both the cross-resistance pattern and the mechanisms of resistance. In this study, we developed MTX-resistant CCRF-CEM sublines in either PGA or leucovorin, and examined TMQ cytotoxicity and its correlation with the mechanisms of resistance in these sublines. TMQ cytotoxicity was also studied in CB3717-resistant MOLT-3 sublines developed in PGA or leucovorin. TMQ was effective against antifolate-resistant human T-cell leukemia cell lines that were characterized by impaired transport, irrespective of gene amplification or mRNA overexpression of DHFR or TS.

MATERIALS AND METHODS

Chemicals MTX and leucovorin (LV) were purchased from Lederle (Japan) Ltd., Tokyo, TMQ was generously provided by Dr. T. Ohnuma, Mount Sinai School of Medicine, New York, NY; [3′,5′,7′-^{3}H]MTX (444 GBq/mmol) was obtained from Amersham International plc (Buckinghamshire, UK); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) was purchased from Sigma Chemical Co., St. Louis, MO.

Cell lines The human T-cell leukemia cell line CCRF-CEM was obtained from Dr. J. R. Bertino, at Memorial Sloan-Kettering, New York, NY. The cells (CCRF-CEM/S) were maintained in either RPMI 1640 medium (Gibco, Grand Island, NY) with 10% undialyzed fetal calf serum (FCS) (Gibco) or RPMI 1640 medium (folic-acid-free) with 10% undialyzed FCS containing a physiological level of reduced folate, 10 n*M* leucovorin. Leucovorin was used as a reduced folate source, because 5-methyltetrahydrofolate was unstable in the culture medium, preventing CCRF-CEM cell growth. CCRF-CEM cells were continuously exposed to increasing concentrations of MTX to establish MTX-resistant sublines by soft agar cloning. After having been established, these cells were maintained in PGA.

Approximately 200-fold CB3717-resistant human T-cell leukemia MOLT-3 sublines were previously established in PGA (MOLT-3/CB3717$_{200}$-PGA) or leucovorin (MOLT-3/CB3717$_{200}$-LV)[9] and characterized in our laboratory: the former subline was resistant to CB3717 by virtue of impaired membrane transport and gene amplification of TS, and the latter, by virtue of only impaired membrane transport.

Cell growth inhibition studies For growth inhibition and cytotoxicity studies, suspensions of logarithmically growing cells with at least 95% viability were seeded at a density of 5×10^4 cells/ml. Cells were exposed to agents at 37°C in a 5% CO_2, 95% air atmosphere for 3 days in 96-well tissue culture plates (Corning Glass Works, Corning, NY). Viable cell number was measured by MTT assay as previously described.[7] After incubation, 10 μl of MTT solution (4 mg/ml in PBS) and 10 μl of 0.1 *M* sodium succinate were added to all wells in each assay, and plates were incubated at 37°C for 4 h. Then the plates were centrifuged at 650*g* for 5 min, and the supernatants were discarded. The dark-blue crystals of formazan were dissolved by adding 150 μl of dimethyl sulfoxide (Wako Pure Chemical Industries Ltd., Osaka), and the absorbance was read on a multi-well spectrophotometer (Bio-Rad, Richmond, CA), using a test wavelength of 540 nm. Drug concentration versus cell growth inhibition (dose/response) curves were obtained by plotting the percentage viable cell number measured in terms of the absorbance in drug-treated wells against that in control wells without drug. The 50%-inhibitory concentration for cell growth (IC_{50} value) was determined from the dose/response curve, and the relative resistance for each drug was determined as a ratio of IC_{50} of the resistant cells to that of the parent cells.

Nucleic acid isolation and blotting The cDNA for the human DHFR gene (0.7 kb) (Pst I digestion) was obtained from Dr. G. Attardi, the California Institute of Technology, Pasadena, CA.[11] Total cellular RNA was isolated by guanidinium isothiocyanate CsCl gradient centrifugation. RNA was electrophoresed on an agarose gel, which was then stained with ethidium bromide to verify that equal amounts of RNA had been loaded per lane. RNA was transferred onto Zeta-Probe nylon membranes (Bio-Rad) as previously described and then hybridized as previously described.[7] Intensities of blots were quantified with a scanning densitometer and normalized as the ratio of CCRF-CEM mRNA to those of the sublines. DNA was purified by the proteinase K/sodium dodecyl sulfate method. For Southern hybridization, DNA was digested with restriction endonuclease *Eco*R I. The DNA was fractionated by electrophoresis on a 0.8% agarose gel, transferred to a Zeta-Probe nylon membrane and then hybridized as previously described.[7] Intensities of blots were quantified with a scanning densitometer, and the DHFR gene was normalized to DHFR pseudogene in the same cells and expressed as the ratio of CCRF-CEM gene to that of the sublines.

Enzyme activity assay for DHFR The DHFR enzyme activity was measured spectrophotometrically, as described by Bertino *et al.*,[12] with minor modifications.

Briefly, the reaction mixture contained 100 μmoles of Tris, pH 7.5, 150 μmoles of KCl, 0.06 μmole of NADPH and 0.05 ml of enzyme in a total volume of 1.0 ml. The substrate, 0.07 μmole of dihydrofolate (FH_2), together with 4 μmoles of 2-mercaptoethanol, was added last to initiate the reaction. The decrease in absorbance at 340 nm was recorded for 3 min, using the DU-64 spectrophotometer series (Beckman, Fullerton, CA). Relative enzyme activity was expressed as a ratio of the activity measured in the resistant cells to that of the parent cells.

Transport studies CCRF-CEM cells were washed twice and resuspended in Eagle's minimum balanced salt solution (Gibco), and 2×10^5 cells were incubated at 37°C in the presence of 0.5 μM [^{3}H] MTX (0.025 μCi). The cells were assayed for radioactivity at six time points from 0 to 60 min, as described previously, with minor modifications.[7]

RESULTS

Establishment of MTX-resistant human leukemia cell lines The CCRF-CEM (CCRF-CEM/S) cells were made resistant to MTX by continuous exposure to the drug and soft agar cloning. An approximately 60-fold MTX-resistant subline was established in RPMI-1640 medium with 10% undialyzed FCS at the MTX concentration of 0.2 μM (CCRF-CEM/MTX_{60}-PGA), and an approximately 5000-fold MTX-resistant subline was further developed at the MTX concentration of 20 μM (CCRF-CEM/MTX_{5000}-PGA). An approximately 140-fold MTX-resistant subline was established in RPMI-1640 medium (PGA-free) with 10% undialyzed FCS containing 10 nM leucovorin at the MTX concentration of 0.2 μM (CCRF-CEM/MTX_{140}-LV), and an approximately 1500-fold MTX-resistant subline was further developed at the MTX concentration of 2.0 μM (CCRF-CEM/MTX_{1500}-LV). The results of cytotoxicity study in the MTX-resistant sublines are shown in Table I.

Cytotoxicity of trimetrexate on antifolate-resistant sublines The MTX-resistant CCRF-CEM sublines developed in PGA were at least as sensitive to TMQ as the parent cells. In contrast, the MTX-resistant sublines developed in leucovorin showed cross resistance to TMQ (11- and 31-fold). TMQ was more potent against MOLT-3/$CB3717_{200}$-PGA and MOLT-3/$CB3717_{200}$-LV cells as compared to the parent cells, while these sublines showed cross-resistance to MTX (Table I). Addition of

Table II. Effects of Addition of Leucovorin on Cytotoxicity of Trimetrexate (TMQ) in CB3717-resistant MOLT-3 sublines

Leucovorin added (M)	IC_{50} (nM) for TMQ in MOLT-3 sublines		
	MOLT-3/S	MOLT-3/$CB3717_{200}$-PGA	MOLT-3/$CB3717_{200}$-LV
0	4.0±0.5	1.1±0.1	1.2±0.2
10^{-8}	6.1±0.2	2.3±0.1	3.1±0.8
10^{-7}	39.5±4.5	7.8±2.6	8.6±2.2
10^{-6}	77.5±5.5	48.0±19.0	58.3±15.6
10^{-5}	873±105	230±60	230±80

Various concentrations of leucovorin (10^{-8} to 10^{-5} nM) were added to the culture medium, and cells were treated continuously with TMQ. The IC_{50} values were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described in "Materials and Methods." All assays were performed in triplicate. Results are means±SD.
PGA, pteroylglutamic acid; LV, leucovorin.

Table I. Cytotoxicity of Methotrexate (MTX) or Trimetrexate (TMQ) in MTX-resistant CCRF-CEM and CB3717-resistant MOLT-3 Sublines[a]

Sublines	IC_{50} (nM) (relative resistance)	
	Agents	
	MTX	TMQ
CCRF-CEM/S	11±0.5 (−)	4.5±0.4 (−)
CCRF-CEM/MTX_{60}-PGA	620±130 (×56)[b]	3.4±0.4 (×0.76)[b]
CCRF-CEM/MTX_{5000}-PGA	56000±4300 (×5091)[b]	4.1±0.5 (×0.91)
CCRF-CEM/MTX_{140}-LV	153±120 (×139)[b]	50±1 (×11)[b]
CCRF-CEM/MTX_{1500}-LV	17000±1400 (×1545)[b]	140±20 (×31)[b]
MOLT-3/S	22.8±0.6 (−)	4.0±0.6 (−)
MOLT-3/$CB3717_{200}$-PGA	62.8±1.0 (×2.8)[b]	1.1±0.1 (×0.3)[b]
MOLT-3/$CB3717_{200}$-LV	64.7±14 (×2.8)[b]	1.2±0.2 (×0.3)[b]

a) Cells were treated continuously with the agent and IC_{50} values were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described in "Materials and Methods." All assays were performed in triplicate. Results are means±SD.
b) IC_{50} values for the resistant sublines were significantly different from values for the corresponding parent line in each case ($P<0.05$ by the Student's t test).
PGA, pteroylglutamic acid; LV, leucovorin.

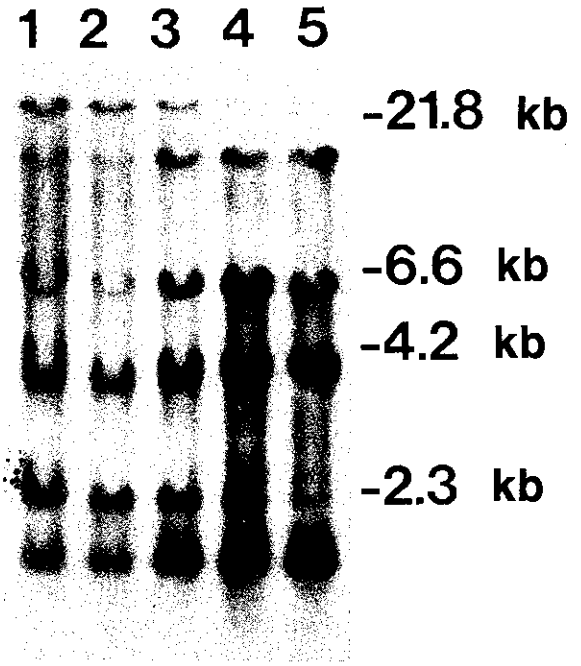

Fig. 1. Southern blot analysis of dihydrofolate reductase (DHFR) gene in methotrexate (MTX)-resistant CCRF-CEM sublines. Six micrograms of DNA was analyzed. Lane 1 CCRF-CEM/S, Lane 2 CCRF-CEM/MTX_{60}-PGA, Lane 3 CCRF-CEM/MTX_{5000}-PGA, Lane 4 CCRF-CEM/MTX_{140}-LV, Lane 5 CCRF-CEM/MTX_{1500}-LV. The values for intensities of Southern blots for lanes 1–5, respectively, are 1.0, 1.0, 2.0, 7.4 and 7.9.

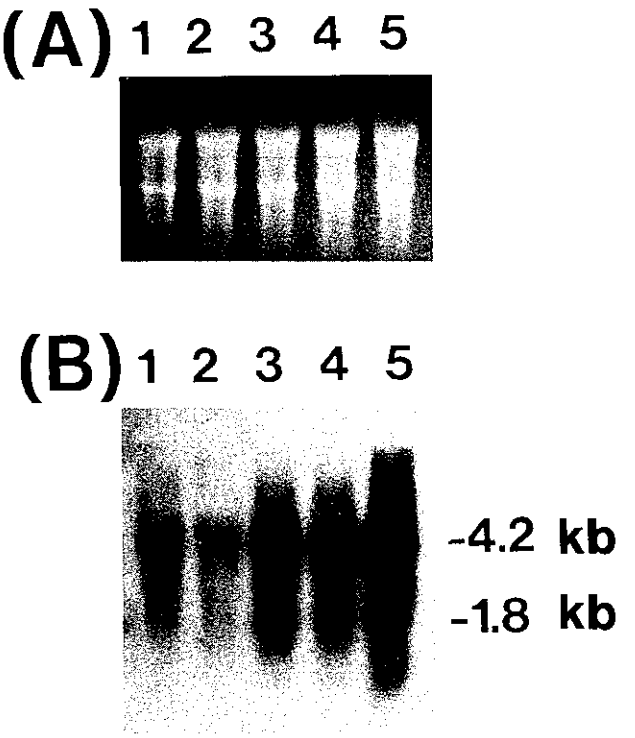

Fig. 2. Northern blot analysis of dihydrofolate reductase (DHFR) mRNA in methotrexate (MTX)-resistant CCRF-CEM sublines. Ten micrograms of total RNA was analyzed. All lanes were equally loaded, as confirmed by staining the gel with ethidium bromide (A). Lane 1 CCRF-CEM/S, Lane 2 CCRF-CEM/MTX_{60}-PGA, Lane 3 CCRF-CEM/MTX_{5000}-PGA, Lane 4 CCRF-CEM/MTX_{140}-LV, Lane 5 CCRF-CEM/MTX_{1500}-LV. The values for intensities of northern blots for lanes 1–5, respectively, are 1.0, 0.8, 2.6, 2.8 and 4.1.

leucovorin to the culture medium reduced the TMQ cytotoxicity in both MOLT-3/$CB3717_{200}$-PGA and MOLT-3/$CB3717_{200}$-LV cells in a dose-dependent manner (Table II).

Nucleic acid analysis of CCRF-CEM cells sensitive and resistant to MTX Southern blot analysis of the *DHFR* gene in CCRF-CEM/MTX_{140}-LV cells showed amplification of the gene, and CCRF-CEM/MTX_{1500}-LV cells showed further enhanced amplification (Fig. 1). CCRF-CEM/MTX_{60}-PGA cells lacked amplification of the *DHFR* gene, while CCRF-CEM/MTX_{5000}-PGA cells showed amplification of the gene. Northern blot analysis for *DHFR* gene expression in MTX-resistant CCRF-CEM sublines showed increases in DHFR mRNA expression levels that corresponded to the degree of amplification of the gene (Fig. 2).

Enzyme activity of DHFR in CCRF-CEM sublines Enzyme activity assay for DHFR in MTX-resistant CCRF-CEM sublines showed increases in DHFR activity that corresponded to the levels of DHFR mRNA expression (Table III).

Table III. Enzyme Activity of DHFR in MTX-resistant CCRF-CEM Sublines Established in PGA or LV

CCRF-CEM subline	Enzyme activity
CCRF-CEM/S	1
CCRF-CEM/MTX_{60}-PGA	0.92
CCRF-CEM/MTX_{5000}-PGA	5.3
CCRF-CEM/MTX_{140}-LV	35
CCRF-CEM/MTX_{1500}-LV	28

Crude cell lysate was assayed for DHFR activity as described in "Materials and Methods." Relative enzyme activity was expressed as a ratio of the activity measured in the resistant cells to that of the parent cells. Results are means of triplicate assays.

PGA, pteroylglutamic acid; LV, leucovorin.

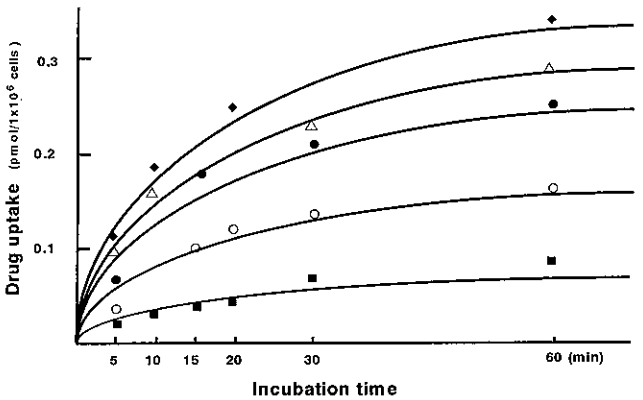

Fig. 3. Methotrexate (MTX) uptake in MTX-resistant CCRF-CEM sublines. Cells (2×10^5) were incubated with 0.5 μM [^{3}H]MTX. At the indicated times, cells were processed as described in "Materials and Methods." Results are means of duplicate assays. ◆ CCRF-CEM/MTX_{1500}-LV, △ CCRF-CEM/MTX_{140}-LV, ● CCRF-CEM/S, ○ CCRF-CEM/MTX_{60}-PGA, ■ CCRF-CEM/MTX_{5000}-PGA.
PGA, pteroylglutamic acid; LV, leucovorin.

Transport studies As shown in Fig. 3, [^{3}H]MTX (0.5 μM) uptake (0–60 min) was measured in CCRF-CEM cells sensitive and resistant to MTX. Cellular MTX accumulation was decreased to approximately 70% and 30% at 60 min in CCRF-CEM/MTX_{60}-PGA and CCRF-CEM/MTX_{5000} cells, respectively, as compared to CCRF-CEM/S cells. In contrast, the MTX accumulation was increased by 15% and 30% in CCRF-CEM/MTX_{140}-LV and CCRF-CEM/MTX_{1500}-LV cells, respectively.

DISCUSSION

Human leukemia CCRF-CEM cells were cultured with different types of folate in the presence of escalating MTX concentrations, which resulted in selection of MTX-resistant sublines through different mechanisms. MTX-resistant CCRF-CEM cells developed in leucovorin showed gene amplification of DHFR and increased MTX accumulation. In contrast, MTX-resistant CCRF-CEM cells developed in PGA exhibited impaired MTX transport. The differential influence of folate types on the mechanisms of MTX resistance was confirmed in human lymphoblastic leukemia cell line CCRF-CEM in addition to erythroblastic leukemia cell line K562.[7] The significance of this finding lies in the fact that MTX is more often used for, and resistance to it is more problematic in, the treatment of leukemia patients with lymphoblast surface markers than in those with non-lymphoblast, including erythroblast, markers.[1]

CCRF-CEM/MTX_{60}-PGA cells had impaired MTX transport, and further enhancement of resistance resulted in selection of a subline with an increase of DHFR, in addition to further impairment of MTX transport. This finding is in agreement with the observation that, in cultured cells, increase in DHFR due to gene amplification as a mechanism of MTX resistance is usually seen only in highly resistant cells.[13] Commercially available culture media contain a high concentration of oxidized folate, PGA.[14] In PGA, MTX-resistant cells with decreased membrane transport can take up folate to survive, since PGA uses a membrane transport route distinct from that of the reduced folate/MTX carrier. When cells become highly resistant to MTX as a result of exposure to a high concentration of MTX, the cells would need to have increased DHFR as an additional mechanism of MTX resistance to overcome cytotoxicity.[15] In contrast, MTX-resistant CCRF-CEM cells developed in reduced folate at a physiological concentration (10 nM leucovorin) exhibited increased DHFR without impaired transport. These data suggest the significance of the magnitude of resistance in relation to the mechanisms of resistance, in addition to the types of folate in the medium used during the development of the resistance.

Impairment in MTX transport was followed by an increase in DHFR during the evolution of MTX resistance in PGA. This sequence has been described in many cultured cells.[15,16] The reverse sequence has also been reported in an MTX-resistant CCRF-CEM subline.[17] 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.[18] Types of folate may also play a role.

Cytotoxicity of TMQ was examined in MTX- or CB3717-resistant human T-cell leukemia sublines developed in PGA or leucovorin. TMQ was as potent against CCRF-CEM/MTX_{60}-PGA cells and CCRF-CEM/MTX_{5000}-PGA cells as against the parent cells. This is in agreement with findings that transport-impaired MTX-resistant cells are efficiently killed by TMQ.[5,18] The collateral sensitivity to lipophilic antifolate in MTX-resistant cells is probably attributable to relatively low levels of intracellular folate by virtue of impaired transport of folate.[19,20] In addition, TMQ achieves intracellular drug concentrations 30- to 60-fold higher than MTX at comparable extracellular drug concentrations.[3,21] In contrast, CCRF-CEM/MTX_{140}-LV and CCRF-CEM/MTX_{1500}-LV cells exhibited cross resistance to TMQ. This observation is consistent with a report that lipophilic antifolates are not active on MTX-resistant cells with elevated DHFR.[18,22] By contrast, in spite of DHFR overproduction, CCRF-CEM/MTX_{5000}-PGA cells did not show cross resistance to TMQ. Thus, it seems that TMQ responsiveness in CCRF-CEM/MTX_{5000}-PGA

cells is more dependent on transport impairment than DHFR levels. These data suggest a possible role of the type of folates used during the development of the resistance in TMQ responsiveness, as well as in the mechanisms of resistance.

The CB3717-resistant MOLT-3 sublines developed in PGA and leucovorin were both cross resistant to MTX, reflecting both impaired transport of MTX and a minimal increase in the target enzyme DHFR.[9] In contrast, TMQ was more potent against CB3717-resistant MOLT-3 sublines than against the parent cells. The cytotoxicity of TMQ was reduced by adding leucovorin in a dose-dependent manner. This finding suggests intracellular folate deficiency by virtue of impaired membrane transport as a cause of the potent cytotoxicity of TMQ in CB3717-resistant MOLT-3 sublines, as in the case of transport-impaired MTX-resistant cells.

A major difference in CB3717-resistant MOLT-3 sublines is the TS mRNA overexpression as a result of gene amplification in MOLT-3/$CB3717_{200}$-PGA cells, but not in MOLT-3/$CB3717_{200}$-LV cells. The primary mechanism of cytotoxicity induced by DHFR inhibitors is thought to be the depletion of reduced folates as a consequence of DHFR inhibition with resultant cessation of purines, thymidylate, and protein synthesis.[1] Since cells with increased TS need more reduced folates as a cofactor to synthesize thymidine, cells with increased TS are susceptible to DHFR inhibitors.[18] However, not only MOLT-3/$CB3717_{200}$-PGA cells, but also MOLT-3/$CB3717_{200}$-LV cells that have no TS mRNA overexpression showed collateral sensitivity to TMQ. Therefore, the TMQ responsiveness in CB3717-resistant MOLT-3 cells appears to be dependent more on transport impairment than on *TS* gene amplification.

In conclusion, TMQ was active against MTX- or CB3717-resistant human T-cell leukemia cell lines that were characterized by impaired membrane transport, irrespective of gene amplification or mRNA overexpression of the target enzyme, DHFR or TS. MTX-resistant CCRF-CEM cells developed in leucovorin showed cross-resistance to TMQ. This may partly account for some cases with acute lymphoblastic leukemia that are resistant to therapy with TMQ, since folate used by cells *in vivo* consists preferentially of reduced-type folates rather than oxidized folate. In order to understand TMQ responsiveness in antifolate resistance, types of folates have to be taken account into consideration.

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