**Multidrug Resistance in a Human Leukemic Cell Line Selected for Resistance to Trimetrexate**

Hadara Arkin, Takao Ohnuma, Barton A. Kamen, James F. Holland, and Shanker Vallabhajosula

**ABSTRACT**

Trimetrexate (TMQ) is a lipophilic antifolate shown to have antitumor activity in humans. TMQ-resistant sublines of the MOLT-3 human acute lymphoblastic leukemia cell line were developed and designated as MOLT-3/TMQres, MOLT-3/TMQ100, and MOLT-3/TMQ200 based on degrees of resistance to TMQ. The TMQ resistance was accompanied by 5- to 7-fold increases in dihydrofolate reductase activity and markedly reduced cellular TMQ accumulation. Methotrexate accumulation was not impaired in TMQ-resistant cells. TMQ retention (efflux) was unchanged in these TMQ-resistant cells. Verapamil enhanced the TMQ accumulation in the resistant cells to the level seen in the parent cells but had no effects on the TMQ retention.

These sublines were cross-resistant not only to methotrexate but also to vincristine, doxorubicin, daunoorubicin, and mitoxantrone. There was no cross-resistance to bleomycin or cisplatin. Resistance to vincristine, doxorubicin, daunoorubicin, and mitoxantrone was reversed by verapamil. TMQ resistance was only minimally reversed by verapamil and methotrexate resistance not affected at all. Both cellular accumulation and retention of vincristine and daunoorubicin in the TMQ-resistant cells were markedly decreased. Verapamil enhanced both accumulation and retention of the drug.

Plasma membrane fractions of the TMQ-resistant cells analyzed by urea-sodium dodecyl sulfate-polycrylamide gel electrophoresis followed by staining with Coomassie Blue revealed the presence of a distinct band. This band was not seen in parental cells. These results show that the TMQ-resistant cells displayed not only decreased TMQ uptake and increased dihydrofolate reductase but also characteristics associated with a classical multidrug-resistant phenotype. Multidrug resistance includes lipophilic antifolate.

**INTRODUCTION**

The development of drug resistance is a major limiting factor to successful chemotherapy of cancer in humans. Cells in culture have served as an useful tool for the study of the mechanism(s) of drug resistance and a number of drug-resistant sublines were developed by exposing them to various chemotherapeutic agents.

In MTX-resistant sublines derived from MOLT-3 human lymphoblastic leukemia cells, we found that the initial step of MTX resistance was impaired transport, which was followed by increased activity of DHFR (1, 2). In reviewing published articles, we observed that in MTX-resistant cells developed in vitro the most common mechanism of resistance was impaired MTX transport (3). Increases in DHFR activity were seen only in highly resistant cell lines as an additional mechanism of resistance. In contrast, virtually all MTX-resistant cells developed in vivo had increases in DHFR. If transport was impaired in these latter cells, the impairment was only minimal. MTX-resistant cells isolated from clinical samples had mainly elevated DHFR activity as the mechanism of acquired MTX resistance (4-9). In these cells, impaired transport has not been recognized. Further studies in our laboratory revealed that the differences in the mechanism of acquired MTX resistance developed in vitro versus in vivo could be explained by the fact that the folate used in vitro was in the oxidized form, PGA, whereas in vivo it was mainly in the reduced form, 5-methyl-THF (10, 11). Since MTX shares the same carrier-mediated transport system with the reduced folate to enter cells, the transport of the reduced folate is decreased in transport-impaired MTX-resistant cell lines (2, 12-14). On the other hand, PGA does not use reduced-folate-MTX transport system to enter cells (13, 15, 16). We have shown that transport-related MTX-resistant cells could grow well in vitro in the presence of PGA, whereas they were unable to take up sufficient quantities of 5-methyl-THF for optimal cell growth (17). This observation is in contrast to an earlier report demonstrating a close relationship between MTX transport and de novo natural resistance to MTX in case of acute leukemia (18). From these findings, we considered that the cells which acquired resistance to MTX in vitro might serve as a model for natural resistance in vivo because in both cases decreased MTX transport appears to be the major (or an initial) mechanism.

TMQ is a new antitumor agent which was recently shown to have activity against human cancer (19-22). This lipophilic antifolate enters the cells by a route distinct from the classical reduced folate transport system (23-27). We hypothesized that TMQ-resistant cells developed in vitro may resemble MTX-resistant cells acquired in vivo because highly transport-impaired cells would be eliminated by TMQ and the increase in DHFR activity would become the major mechanism of resistance.

To this end, we developed TMQ-resistant sublines from MOLT-3 cells in vitro. Characterization of the TMQ-resistant cells revealed that as expected, increases in DHFR activity occurred in the early phase of the resistance. There was no impairment of MTX transport. Unexpectedly, however, we found that these cells displayed classical MDR phenotype. Portions of this work have appeared in abstract forms elsewhere (28-30).

**MATERIALS AND METHODS**

TMQ was supplied from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Stock solution was prepared by dissolving the drug with a small amount of dimethyl sulfoxide.
amount of absolute ethanol. The solution was stable for at least 1 year at 4°C. Subsequent dilutions were made in RPMI 1640 medium (GIBCO, Grand Island, NY) with 10% (v/v) heat-inactivated fetal bovine serum (GIBCO). MTX was purchased from Lederle Laboratories (Wayne, NJ); DXR from Adria Laboratories (Columbus, OH); bleomycin sulfate and cisplatin from Bristol Laboratories (New York, NY); DNR from Ives Laboratories (New York, NY); VCR from Eli Lilly Laboratories (Indianapolis, IN); and verapamil from Knoll Pharmaceutical (Whippany, NJ). Mitoxantrone hydrochloride was obtained through the National Cancer Institute. Each drug was reconstituted initially according to the company’s instructions and further dilution was made in RPMI 1640 medium. For accumulation and retention studies, each drug was diluted in HBSS. All drug dilutions were freshly prepared for each experiment.

[14C]TMQ (specific activity, 13.1 mCi/mmol) was made available from the Research Triangle Institute (Research Triangle Park, NC) through the National Cancer Institute; [14H]MTX (specific activity, 10 Ci/mmoll, [14H]VCR (specific activity, 4.4 Ci/mmoll), and Na23H (15 mCi/mg) were purchased from Amersham Radiochemicals (Arlington Heights, IL). [14H]DNR (2.1 mCi/mmoll) was purchased from New England Nuclear (Boston, MA). Acrylamide, SDS, urea, sucrose, Coomassie Brilliant Blue R-250, pyronin Y, high and low molecular weight standards, and other reagents for SDS-PAGE and electro-blotting were purchased from Bio-Rad (Richmond, CA). Bovine serum albumin (fatty acid-free) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN); protein assay reagent and albumin standard were purchased from Pierce Chemical (Rockford, IL). All other chemicals used for the experiments were reagent grade and purchased from Fisher (Fairlawn, NJ) or Sigma (St. Louis, MO).

Details on the development and characterization of MOLT-3 cell line and its MTX-resistant sublines have been described (1, 2). MOLT-3 cells and the resistant sublines were grown in suspension in culture flasks (Corning Glass Work, Corning, NY) containing RPMI 1640 medium supplemented with 10% fetal bovine serum. All the cell lines in the study were maintained periodically with Mycoplasma Plate (GIBCO) and/or Gen-Probe (Fisher Scientific, Pittsburg, PA) to assure the lack of contamination with Mycoplasma species.

For the development of TMQ-resistant sublines, MOLT-3 cells were initially exposed to TMQ at a concentration of 10−8 M, which corresponded to the ID50 concentration. Cells were maintained at this drug concentration until their growth rate approached that of the untreated parent cells. The TMQ concentration was then increased 2 to 3 times; usually 8 to 10 weeks were required to establish adequate growth at each TMQ concentration.

All the characterization studies were performed on cells grown in the medium free of drug for at least 14 days, usually for 3 weeks or longer. Cells in exponential growth with >90% viability, determined by Trypan blue dye exclusion, were used for all experiments. The degree of resistance to TMQ and other drugs of the parent and resistant sublines was determined by growth inhibition assay (2). Aliquots of cells in suspension in a final concentration of 106 cell/ml were added to individual cell wells (24-cell well; Corning Glass Work), to which 0.1 ml of graded concentrations of drug solutions in the medium were added. For controls, medium without drug was added. Cells were then incubated in 5% CO2/95% humidified air at 37°C for 3 days and the viable cell number was determined. Drug concentration versus cell growth inhibition (dose-response) curves were obtained by calculating the percentage of viable cell number in drug-treated wells as compared to that in control wells without drug and the ID50 was determined from the dose-response curve. The relative resistance for each drug was determined as a ratio of the ID50 of the resistant cells to the ID50 of parent sensitive MOLT-3 cells.

For the determination of reversal effects of verapamil, 0.1 ml each of drug solution and 10−4 M (final concentration, nontoxic) verapamil was added to 106 cells in a final volume of 1 ml. After 3 days of incubation, viable cells were enumerated and the reversal effects were determined by comparing the dose-response curves with and without verapamil.

Cell colony formation and determination of cloning efficiency were carried out by the method described by Kuroki (31).

Accumulation and retention of radiolabeled drugs were studied as described previously (32). For accumulation studies, cells were washed twice and resuspended in HBSS (GIBCO) that was supplemented with 1 mg/ml bovine serum albumin, at a cell concentration of 107 cell/ml for TMQ and VCR, 2 × 106 cells/ml for MTX, and 2 × 108 cells/ml for DNR. After preincubation for 10 min at 37°C or 2°C, radioisotopes with or without 10−5 M verapamil were added to the cell suspension to initiate the uptake. After predetermined time intervals, 0.5-ml aliquots of cell suspension were removed and layered on a mixture of mineral oil (Invenex Pharmaceutical, Charing Falls, OH) and silicone oil (Dow Corning, New Bedford, MA) (16/84, v/v) with the final density of 1.028. The cells were separated from the supernatant by centrifugation at 12,000 × g for 30 s using an Eppendorf microcentrifuge (model 5412; Brinkmann, Westbury, NY). After removal of the cell-free medium and oil, the cell pellets were suspended with 1 ml deionized water, mixed, and transferred to counting vials containing 10 ml ACS (Amersham). The radioactivity in the cell pellets was determined by a liquid scintillation counter (model LS-355; Beckman). Counting efficiency was 70% for 4C and 40% for 3H. The net accumulation of each drug was obtained by subtracting corresponding 2°C values from 37°C values.

For retention studies, cells were preloaded with radiolabeled TMQ, VCR, or DNR for an indicated period of time (10 min for TMQ, 60 min for VCR or DNR) at 37°C with or without 10−5 M verapamil. At the end of the incubation period, the cells were suspended in 15 volumes of HBSS at 2°C, washed twice with cold HBSS, and resuspended to the original volume. The cell suspension was divided into four portions and reincubated at 37°C or 2°C with or without 10−5 M verapamil. At specific time intervals, 0.5-ml aliquots were withdrawn and layered on oil as described above. Percentage of retention was calculated from zero time value as control.

For the determination of DHFR activity, cells (2 × 105) were washed twice with phosphate-buffered saline (GIBCO) and the binding capacity of MTX was determined as described previously (1, 2, 33).

For the determination of P-glycoprotein, plasma membrane vesicles were isolated from cell homogenates by isopycnic centrifugation on discontinuous sucrose gradients according to the method of Riordan and Ling (34). Membrane protein was assayed with Pierce reagent according to the method of Bradford (35). Membrane vesicles were concentrated by centrifugation in a Beckman airfluage at 130,000 × g for 15 min. SDS-PAGE (Bio-Rad Protean II, Palo Alto, CA) was performed according to the method of Fairbanks et al. (36) with a modification (37). Protein profiles were stained with Coomassie Blue or replica electroblotted (Western blotted) onto nitrocellulose filter paper, as described by Towbin et al. (38), which was then probed with a 125I-labeled monoclonal antibody (C219), as described by Kurnter et al. (39). The film, X-Omat AR (Kodak), was developed by automatic processing and examined visually. The molecular weight was estimated using standard molecular weight protein solution.

**RESULTS**

Continuous exposure of MOLT-3 cells to increasing concentrations of TMQ resulted in the development of progressively drug-resistant sublines. At TMQ concentrations of 5 × 10−8, 3 × 10−7, 3 × 10−6, and 10−5 M, cells became 30-, 200-, 800-, and 2500-fold resistant, respectively, as compared to the parent cells and these sublines were designated as MOLT-3/TMQ10, MOLT-3/TMQ200, MOLT-3/TMQ1000, and MOLT-3/TMQ2500 (Fig. 14).

The population doubling time of MOLT-3 cells was 18 h and that for each of the four resistant sublines was approximately 22 h. MOLT-3 cells grew as a single-cell suspension without clumping or aggregates, whereas TMQ-resistant cells tended to clump in easily dispersible aggregates. MOLT-3 cells produced colonies (20-50-cell aggregates) on an agar plate in approximately 7 days, whereas it took approximately 10 days for resistant sublines. Cloning efficacy was similar for all the cell sublines and was 10−20%. Giemsa-Wright staining of TMQ-resistant cells showed no morphological changes as compared to the
The TMQ-resistant sublines were able to maintain the resistance for over 2 years in the drug-free medium. Parent cells. The TMQ-resistant cells were able to maintain the stable resistance for over 2 years in the drug-free medium.

The TMQ-resistant sublines exhibited only a modest degree of cross-resistance to MTX and the dose-response curves of three TMQ-resistant sublines tended to overlap one another (Fig. 2B).

DHFR activities in the parent and TMQ- and MTX-resistant sublines are shown in Table 1. The TMQ-resistant sublines showed 5- to 7-fold increases in DHFR activity. In contrast to MTX-resistant sublines (2,3,4), the increases in DHFR activity were seen during the early phase of resistance development but higher levels of TMQ resistance did not show any parallel increases in DHFR activity.

The patterns of TMQ and MTX accumulation differed in that the former was more rapid and reached steady state in minutes (Fig. 2A). Progressive increase in TMQ resistance was accompanied by progressive decrease in the accumulation and steady state levels of TMQ. The uptake of TMQ was not impaired in MOLT-3/MTX_{10,000} cells. MTX uptake was, as described previously (2), greatly reduced in MOLT-3/MTX_{10,000} cells, whereas MTX uptake was not impaired in the TMQ-resistant sublines (Fig. 2B). The uptake of both TMQ and MTX was temperature dependent. At 2°C there was almost no intracellular accumulation of TMQ and MTX.

TMQ-resistant cells were found to be cross-resistant to VCR and DXR (Fig. 3, A and B). Thus, progressive increases in TMQ resistance were associated with similar increases in VCR or DXR resistance. MTX-resistant cells did not show cross-resistance to VCR or DXR. The sensitivity of TMQ-resistant cells to several other agents and the effects of verapamil (10^{-5} M) are summarized in Table 2. In growth inhibition assay, verapamil partially reversed TMQ resistance but had no effect on the sensitivity to MTX. Verapamil diminished the cross-resistance to VCR, DXR, DNR, and mitoxantrone by slight enhancement of the effects of these agents on the parent cells and marked increases in sensitivity in the TMQ-resistant cells. TMQ-resistant cells were not cross-resistant to bleomycin or cisplatin.

TMQ accumulation into TMQ-resistant cells was markedly diminished and simultaneous exposure to 10^{-5} M verapamil enhanced the TMQ accumulation to the level seen in the parent cells (Fig. 4). Verapamil did not alter the level of the TMQ accumulation in the parent cells. The percentage of TMQ retained was identical for the parent and TMQ-resistant cells, although the absolute amount of TMQ retained was significantly lower for the resistant cells (Fig. 5). Co-incubation of verapamil had no effect on TMQ retention. The TMQ retention was again temperature dependent. Thus, in an identical experiment at 2°C, both resistant and sensitive cells retained 95% of TMQ after 10 min of incubation in drug-free medium.

Similar studies with VCR revealed that the TMQ-resistant cells had decreased VCR accumulation reaching only 25% of control at 60 min (Fig. 6). The resistant cells were also unable to retain VCR as effectively as the parent cells and the VCR level decreased to 20% by 60 min (Fig. 7). At 2°C the sensitive and resistant cells were able to retain 95% of VCR by 60 min. In the presence of verapamil, cells restored both uptake and retention of VCR in the resistant cells to about 75% of the sensitive cells.

In order to test whether verapamil influenced the efflux of only the unbound (or exchangeable) fraction of drug, the retained percentage of unbound drug was calculated by subtracting the 10-min value for TMQ and the 60-min value for VCR from the respective total at each time point. Such an analysis gave an identical conclusion: TMQ retention in TMQ-resistant cells was virtually identical to that of the parent cells and

### Table 1 DHFR activity of MOLT-3 human lymphoblastic leukemia cell line and its TMQ-resistant sublines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>DHFR activity*</th>
<th>Fold increase</th>
</tr>
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<tbody>
<tr>
<td>MOLT-3</td>
<td>5.66 ± 2.4^a</td>
<td></td>
</tr>
<tr>
<td>MOLT-3/TMQ_{200}</td>
<td>33.8 ± 15.1</td>
<td>6.0</td>
</tr>
<tr>
<td>MOLT-3/TMQ_{500}</td>
<td>41.8 ± 6.8</td>
<td>7.4</td>
</tr>
<tr>
<td>MOLT-3/TMX_{200}</td>
<td>30.7 ± 13.0</td>
<td>5.4</td>
</tr>
<tr>
<td>MOLT-3/TMX_{10,000}</td>
<td>190.0 ± 50.1</td>
<td>33.6</td>
</tr>
</tbody>
</table>

*pmol [3H]MTX bound/10^6 cells.
^a Mean ± SD of ≥3 assays done in triplicate.
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Table 2  ID₉₀ values and relative resistance of various drugs tested, with or without 10⁻⁵ M verapamil

<table>
<thead>
<tr>
<th>Drugs</th>
<th>ID₉₀ (mM) MOLT-3</th>
<th>ID₉₀ (mM) MOLT-3/TMQ₈₀₀</th>
<th>ID₉₀ (mM) MOLT-3/TMQ₂₅₀₀</th>
<th>Relative resistance MOLT-3/TMQ₈₀₀</th>
<th>Relative resistance MOLT-3/TMQ₂₅₀₀</th>
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</thead>
<tbody>
<tr>
<td>TMQ</td>
<td>1.2 × 10⁻⁶</td>
<td>9.6 × 10⁻⁴</td>
<td>3.0 × 10⁻⁴</td>
<td>800</td>
<td>2500</td>
</tr>
<tr>
<td>TMQ + verapamil</td>
<td>1.2 × 10⁻⁶</td>
<td>2.5 × 10⁻⁴</td>
<td>1.0 × 10⁻⁴</td>
<td>200</td>
<td>833</td>
</tr>
<tr>
<td>MTX</td>
<td>9.0 × 10⁻⁹</td>
<td>7.0 × 10⁻⁴</td>
<td>ND</td>
<td>7</td>
<td>ND</td>
</tr>
<tr>
<td>MTX + verapamil</td>
<td>9.0 × 10⁻⁹</td>
<td>7.0 × 10⁻⁴</td>
<td>ND</td>
<td>7</td>
<td>ND</td>
</tr>
<tr>
<td>VCR</td>
<td>9.0 × 10⁻¹⁰</td>
<td>2.5 × 10⁻⁷</td>
<td>5.0 × 10⁻⁷</td>
<td>280</td>
<td>560</td>
</tr>
<tr>
<td>VCR + verapamil</td>
<td>3.0 × 10⁻¹⁰</td>
<td>3.0 × 10⁻⁴</td>
<td>5.0 × 10⁻⁴</td>
<td>10</td>
<td>17</td>
</tr>
<tr>
<td>DNR</td>
<td>1.2 × 10⁻⁸</td>
<td>1.8 × 10⁻⁷</td>
<td>2.0 × 10⁻⁷</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td>DNR + verapamil</td>
<td>9.0 × 10⁻⁹</td>
<td>1.2 × 10⁻⁴</td>
<td>1.2 × 10⁻⁴</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>DXR</td>
<td>2.2 × 10⁻⁹</td>
<td>3.0 × 10⁻⁷</td>
<td>4.0 × 10⁻⁷</td>
<td>14</td>
<td>18</td>
</tr>
<tr>
<td>DXR + verapamil</td>
<td>1.2 × 10⁻⁹</td>
<td>3.0 × 10⁻⁴</td>
<td>3.0 × 10⁻⁴</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>6.0 × 10⁻⁹</td>
<td>6.0 × 10⁻⁹</td>
<td>1.5 × 10⁻⁷</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>Mitoxantrone + verapamil</td>
<td>3.5 × 10⁻⁹</td>
<td>5.0 × 10⁻⁹</td>
<td>6.0 × 10⁻⁹</td>
<td>1.4</td>
<td>1.7</td>
</tr>
<tr>
<td>Bleomycin*</td>
<td>4.0</td>
<td>4.0</td>
<td>3.4</td>
<td>1</td>
<td>0.85</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>8.0 × 10⁻⁷</td>
<td>1.0 × 10⁻⁴</td>
<td>8.0 × 10⁻⁷</td>
<td>1.2</td>
<td>1</td>
</tr>
</tbody>
</table>

* ND, Not done.

Bleomycin in milliunits. 10⁻¹⁵ B.

Studies with DNR showed that the pattern of accumulation and retention were similar to that of VCR, although the accumulation of DNR was somewhat faster and approached the steady state levels in 45 min. Verapamil completely reversed the DNR accumulation and retention to the levels seen in the sensitive cells (data not shown).

Protein profiles of enriched plasma membrane vesicles of TMQ-resistant cells analyzed by urea-SDS-PAGE revealed the presence of a distinct band with a molecular weight of 170,000 that was not seen in the parent MOLT-3 or in MOLT-3/TMX₁₀₀₀ cells (Fig. 8A). Replica electroblotting of the protein profiles from the SDS-PAGE and probing with an ¹²⁵I-labeled monoclonal antibody against P-glycoprotein revealed an increased expression of the glycoprotein in the TMQ-resistant cells (Fig. 8B). Increasing TMQ resistance from 200- to 800- and 2500-fold resulted in progressive increases in P-glycopro-
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Fig. 8. Detection of P-glycoprotein in TMQ-resistant cells. Fifty-μg aliquots of membrane protein were loaded per lane. A, Staining of membrane fraction with Coomassie Blue. B, Immunochemical detection of P-glycoprotein. The blot was probed with 125I-monoclonal antibody C219. Lane a, MOLT-3 parent cell; lane b, MOLT-3/TMQ800; lane c, MOLT-3/TMQ800/lanp; lane d, MOLT-3/TMQ1,000; lane e, MOLT-3/MTX1,000.

The membrane preparation from the parent or MTX-resistant cells did not react with the antibody.

DISCUSSION

The present study demonstrates that exposure of MOLT-3 human leukemic cells in vitro to TMQ resulted in the development of resistance associated with decreased TMQ accumulation and increased DHFR activity. In addition, these cells displayed a stable classical MDR phenotype (40-43), which included cross-resistance to such structurally diverse agents as Vinca alkaloids and anthracyclines, reversal of resistance by calcium channel blockers, e.g., verapamil, decreased drug accumulation, and increased expression of P-glycoprotein on the cell membrane.

Since cellular accumulation of MTX was not impaired in TMQ-resistant cells (Fig. 2B), cross-resistance to MTX may be explained by increases in DHFR alone. It is of interest to note that DHFR activities were 5- to 7-fold higher in the TMQ-resistant cells than in parent cells, irrespective of the degrees of TMQ resistance. This observation is consistent with the 7-fold cross-resistance to MTX and the clustering of dose-response curves of TMQ-resistant cells to MTX (Fig. 1). The reason for the progressive increases in TMQ resistance without accompanying further increases in DHFR activity is unknown. It is possible that the development of the membrane-associated mechanism (P-glycoprotein overexpression) is a more efficient way for cells to adapt to the cytotoxic environment.

In TMQ-resistant cells, the TMQ accumulation inversely correlated with the degree of resistance to TMQ, whereas that of MTX was not impaired. Conversely, TMQ accumulation was not impaired in MTX-resistant cells (Fig. 2). These data suggest that there may be important differences in how these structurally related antifolates enter and exit cells. Furthermore, higher levels of resistance to TMQ are likely to be a result of disturbances in drug trafficking at the membrane level.

In view of the hypothesis that P-glycoprotein functions as a drug efflux pump (41-45), it is possible to relate the reduced VCR and DNR accumulation to concomitant increases in efflux (Fig. 7). In the case of TMQ, cellular accumulation was decreased in the TMQ-resistant cells but the retention was unchanged (Figs. 4 and 5). Verapamil enhanced TMQ accumulation but had no effect on the drug retention. Reanalysis of retention data with the concept of an exchangeable fraction again failed to show increased TMQ efflux or the effect of verapamil on TMQ retention. This phenomenon cannot be explained by the known hypothesis of the P-glycoprotein as an efflux pump. Studies are in progress in our laboratory to elucidate the relationship between TMQ transport and that of drugs related to MDR.

It is of note that, while verapamil completely reversed TMQ accumulation in MOLT-3/TMQ800 to the level observed for the parent cells (Fig. 4), its ability to modulate the TMQ resistance in the cell growth inhibition assay was far less than expected (Table 2). This may be related to the concomitant increase in DHFR activity.

The TMQ-resistant sublines demonstrated various degrees of cross-resistance to unrelated drugs such as VCR and DXR (Fig. 3). As cells were subjected to more stringent selective conditions, the absolute levels of cross-resistance varied but a general pattern of increasing cross-resistance was observed. Thus, the degrees of cross-resistance to VCR or anthracyclines are substantially less than that to the selecting agent, TMQ. This observation is consistent with that observed by others indicating that the greatest resistance occurs against the selecting agent and the cross-resistance to other classes of drugs is less (41, 42, 46). The variable and complex pattern of differential cross-resistance in the MDR phenotypes appears to be due to more than one mechanism of resistance involved; P-glycoprotein expression is a common mechanism of resistance and there may be a second mechanism(s) specific to each drug or group of drugs. Increases in DHFR activity and development of MDR in TMQ-resistant cells is a case in point.

Since MTX-resistant cells are sensitive to DNR and VCR and DXR-resistant cells were sensitive to MTX, antifolates were not considered to be a part of the MDR phenotype (47). The idea that TMQ-resistant cells may be multidrug resistant is, however, intuitively conceivable because P388 cells resistant to DNR were found to be cross-resistant to dimethylesters of MTX, TMQ, and BW301U (47-49) and the resistance was partially reversed by verapamil (49). The association of TMQ and MDR may be due to the drug's lipophilic property. WI-L2 human lymphoblastic cells made resistant to TMQ glucuronate were recently reported to be sensitive to DXR and VCR, implying that this was not a form of MDR (50). It is not clear at this time if the difference in MDR expression between our TMQ-resistant MOLT-3 cells and cells described in Ref. 50 is related to degrees of TMQ resistance, differences in type of cells used, or the fact that TMQ glucuronate is different from TMQ in induction of MDR. Since TMQ is dissolved in ethanol and TMQ glucuronate is water soluble, we tested whether the presence of ethanol per se could have caused MDR. MOLT-3 cells exposed to 0.5% ethanol for 6 months failed to produce MDR phenotype.4

TMQ may have an advantage over MTX in that the former enters cells through a route distinct from reduced folate transport. Its effectiveness against MTX-resistant cells by virtue of impaired transport is an example (1). In contrast, TMQ produces MDR in vitro and a similar phenomenon is expected to occur in a more realistic in vivo system. Our TMQ-resistant cells may serve as a useful tool to understand the complexity associated with drug transport and resistance.

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