Pergamon

Biochemical Pharmacology, Vol. 49, No. 8, pp. 1069–1078, 1995.
Elsevier Science Ltd
Printed in Great Britain.

0006-2952(95)00062-3

CHARACTERIZATION OF CROSS-RESISTANCE TO METHOTREXATE IN A HUMAN BREAST CANCER CELL LINE SELECTED FOR RESISTANCE TO MELPHALAN

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(*Received* 11 *July* 1994; *accepted* 28 *November* 1994)

Abstract—We have demonstrated previously decreased melphalan accumulation in a human breast cancer cell line selected for resistance to melphalan (MelR MCF-7). Cross-resistance studies of MelR MCF-7 cells revealed that this cell line was 6.7-fold cross-resistant to methotrexate, but only 2-fold resistant to trimetrexate. Methotrexate transport studies in MelR MCF-7 cells showed a 2-fold decrease in initial methotrexate uptake and a 2-fold decrease in the V_{max} for methotrexate uptake in the resistant cells. Methotrexate resistance in MelR MCF-7 cells was also associated with a decrease in non-effluxable methotrexate following incubation with radiolabeled drug for 24 hr. Characterization of intracellular methotrexate after accumulation for 24 hr demonstrated decreased levels of free methotrexate in MelR MCF-7 cells. Analysis of methotrexate polyglutamate formation in MelR MCF-7 cells indicated that the decrease in non-effluxable, non-protein-bound methotrexate was associated with a 3-fold decrease in higher order methotrexate polyglutamate formation. No difference was noted in folylpolyglutamate synthetase activity between the resistant and parental cell lines. Therefore, the observed decrease in methotrexate polyglutamate formation in MelR MCF-7 cells appeared to result from decreased availability of substrate. There was no evidence of any alteration in the amount of the catalytic activity of dihydrofolate reductase in MelR MCF-7 cells compared with parental MCF-7 (WT MCF-7) cells; moreover, the binding affinity of dihydrofolate reductase for methotrexate and the percentage of protein-bound methotrexate were similar in both cell lines. In addition, the total amounts of thymidylate synthase protein and thymidylate synthase catalytic activity in MelR MCF-7 cells were unchanged. Thus, acquired methotrexate resistance in MCF-7 cells selected for resistance to melphalan appears to result from down-regulation of methotrexate uptake.

Key words: methotrexate; melphalan; drug resistance; breast cancer

We have reported previously the isolation of a melphalan-resistant MCF-7 human breast cancer cell line by serial incubation of drug-sensitive MCF-7 cells in increasing concentrations of melphalan [1]. The resulting cell line, MelR MCF-7, was 30-fold resistant to melphalan at the IC_{50}‖ level, and characterization of MelR MCF-7 cells revealed that these cells were defective in melphalan accumulation, largely resulting from an altered V_{max} for initial melphalan uptake [516 vs 2110 amol/cell/min for MelR MCF-7 and the parental MCF-7 (WT MCF-7) cells, respectively]. Melphalan uptake competition studies with BCH, a specific inhibitor of system L amino acid transport [2], indicated that the decrease in melphalan uptake in MelR MCF-7 cells was due to down-regulation of the system L amino acid transporter. In addition, no differences in glutathione metabolism were identified between MelR MCF-7 and WT MCF-7 cell lines.

Characterization of the cross-resistance pattern of MelR MCF-7 cells unexpectedly revealed that this cell line was significantly cross-resistant to MTX. To investigate the development of cross-resistance between these very dissimilar chemotherapeutic agents, we have characterized the mechanism of MTX resistance of MelR MCF-7 cells.

MATERIALS AND METHODS

Cytotoxicity assay. MelR MCF-7 cells were non-clonally selected from WT MCF-7 cells by serial incubation in increasing concentrations of melphalan as previously described [1] and passaged in IMEM with 5% fetal bovine serum containing 20 μM melphalan. MelR MCF-7 cells were removed from drug 2 weeks prior to experiments. MelR MCF-7 and WT MCF-7 cells were plated in triplicate in 96-well microtiter plates in IMEM with 5% fetal bovine serum at densities of 4000 and 2000 cells/well, respectively, for 4-day assays and 750 and 250 cells per well, respectively, for 7-day assays. After 24 hr, serial dilutions of drugs were added to the cells.

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‖ Abbreviations: IC_{50}, concentration of drug required to decrease cell growth by 50% relative to untreated cells; MTX or $MTXGlu_1$, methotrexate parent drug; $MTXGlu_n$, higher order MTX polyglutamates; TMQ, trimetrexate; DHFR, dihydrofolate reductase; TS, thymidylate synthase; FPGS, folylpolyglutamate synthase; d-PBS, Dulbecco's phosphate-buffered saline; IMEM, Improved Minimum Essential Medium; and BCH, 2-amino-bicyclo[2,2,1]-heptane-2-carboxylic acid.

After another 3 or 6 days, the cells were fixed in 10% tricarboxylic acid, rinsed with water and dried. The cells were then stained with sulforhodamine in 1% acetic acid, washed in 1% acetic acid, and dried again as described [3]. The stained cells were solubilized in 1 M Tris base, and the absorbance at 540 nm was determined on a microplate reader. The IC_{50} value was calculated from the concentration–response curve as the concentration of drug that produced a 50% decrease in the mean absorbance compared with the untreated wells, and reported as the average of at least three independent determinations performed in triplicate.

MTX transport studies. WT MCF-7 and MelR MCF-7 cells were plated in either 6- or 12-well Linbro dishes. Approximately 48 hr after plating, during the exponential growth phase, the cells were washed three times with transport medium (folate-free serum-free IMEM) pre-warmed to 37°. Transport medium containing [^{3}H]MTX (Moravek) was then added to the cells and incubated at 37° for the specified time. At the end of the uptake period, the medium was aspirated quickly, and the plates were immersed in four consecutive baths of ice-cold d-PBS in rapid succession. The plates were blotted dry, and the cells were solubilized by overnight incubation in 0.2 N NaOH at room temperature. The cell lysates were neutralized with 0.2 N HCl, and the radioactivity was determined by liquid scintillation counting. The total number of cells was determined in replicate plates, in which cells were trypsinized, resuspended in medium, passed several times through a 19-gauge needle to make a single cell suspension, diluted in isotonic buffered saline, and counted in a Coulter counter. For determination of initial uptake in 1 μM MTX at 2 min, total uptake at 0 sec and uptake in the presence of unlabeled excess MTX (100 μM) at 2 min were both subtracted from total MTX accumulation in the absence of unlabeled excess MTX at 2 min. For determination of methotrexate efflux, MelR MCF-7 cells were incubated for 2 or 24 hr in 1 μM MTX. Transport was stopped by immersion in cold d-PBS either immediately (for total MTX uptake) or after a 1-hr washout period in folate-free IMEM at 37°.

Enzyme assays. WT MCF-7 and MelR MCF-7 cells were harvested during the logarithmic growth phase by washing the adherent cells in ice-cold PBS three times followed by scraping the cells into ice-cold PBS. An aliquot of cells was counted in a Coulter counter after five passages through an 18-gauge needle. The cells were pelleted in a table top centrifuge and frozen on dry-ice after aspiration of the PBS. The cell pellets were stored at −80° until thawed for enzyme assays.

The FdUMP binding assay for TS was adapted from a previously published method [4]. Equivalent volumes of cytosolic extracts (50 μL) were assayed in duplicate. The assay was performed in a total volume of 200 μL, containing 75 μM 5,10-methylene tetrahydrofolate, 3 pmol [^{3}H]FdUMP, 100 mM 2-mercaptoethanol, and 50 mM KH_2PO_4, pH 7.4, and the results were expressed in picomoles per milligram of cytosolic protein.

TS catalytic activity was assayed using a modification of a previously published method [5]. Equivalent volumes of cytosolic extracts (50 μL) were assayed in duplicate. The assay was performed in a final volume of 200 μL, containing 50 μL of cellular extracts in 100 μM [5-^{3}H]dUMP, 100 mM 2-mercaptoethanol, 50 mM KH_2PO_4, pH 7.4. After incubation at room temperature for 60 min, the reaction was stopped and an albumin-coated activated charcoal solution was added to the reaction mixture. After centrifugation, a 200-μL sample of the supernatant was assayed for ^3H_2O by liquid scintillation counting.

The DHFR binding affinity assay was adapted from a modification of a previously published method by Myers *et al.* [6]. Equivalent volumes of cytosolic extracts (50 μL) were assayed in duplicate. The assay was performed in a total volume of 250 μL containing 100 mM Tris–HCl buffer (pH 7.5), various concentrations of [^{3}H]MTX, 50 mM NADPH and 0.2 to 0.5 μg of cytosolic protein. Scatchard analysis was used to determine the dissociation constant (K_d) [7].

For the DHFR catalytic activity assay, the cells were resuspended in 200 μL of 100 mM Tris–HCl buffer (pH 7.5), and then sonicated with four 2-sec bursts from a Vibra Cell sonifier from Sonics & Materials Inc. (Danbury, CT). After centrifugation at 8000 *g* for 30 min, the cytosolic DHFR catalytic activity was calculated using the previously described spectrophotometric assay [6]. Protein concentrations were determined using the Bio-Rad protein assay [8].

FPGS activity was measured by the method of Cichowitz and Shane [9] using tetrahydrofolate as substrate, and assayed in duplicate. Cells in logarithmic growth phase were washed three times in ice-cold PBS, and then harvested in cold PBS. The cell pellet was resuspended in ice-cold buffer of 100 mM Tris and 50 mM glycine, pH 9.3, with 100 mM 2-mercaptoethanol. Cytosolic protein was added to a reaction buffer of 100 mM Tris with 50 mM glycine, pH 9.3, 10 mM $MgCl_2$, 20 mM KCl, 100 μg/mL bovine serum albumin, 5 mM ATP, 100 mM 2-mercaptoethanol, 240 μM sodium glutamate, 1.25 μCi [^{14}C]glutamate and 50 μM tetrahydrofolate, and the reaction was incubated at 37° for 2 hr. The reaction was stopped with ice-cold 25 mM 2-mercaptoethanol containing 5 mM sodium glutamate, and was then loaded onto a Whatman DE52 column, washed three times with Tris buffer, pH 7.3, containing 80 mM NaCl and 5 mM sodium glutamate. The column was eluted with 0.1 N HCl, and the eluted radioactivity was determined by scintigraphy.

MTX polyglutamate formation. HPLC separation of MTX polyglutamates was performed by the method of Jolivet *et al.* [10]. Cells were incubated in folate-free IMEM 24 hr prior to the study and then exposed to 1 μM [^{3}H]MTX in folate-free IMEM supplemented with 25 μM hypoxanthine, glycine and thymidine. At the times indicated, cells were washed three times in ice-cold PBS, and scraped off the flask surface with a rubber policeman in the presence of ice-cold 0.15 M $KHPO_4$, pH 6.2. Cells were lysed by sonication, and tricarboxylic acid was added to a final concentration of 10%. Cellular debris was pelleted by centrifugation at 10,000 *g* for 15 min,

and the cell extract was injected onto a Sep-Pak C18 cartridge (Waters Associates) that had been prepared by prior injection with 2 mL of 100% methanol followed by 5 mL of water. Then the cartridge was washed by injecting 5 mL of water followed by 2 mL of methanol. The sample was evaporated to dryness under N_2 and resuspended in the initial HPLC mobile phase that consisted of 25% acetonitrile and 75% 10 mM $KHPO_4$, with 5 mM tetrabutylammonium phosphate, pH 5.5. MTX and MTX polyglutamates were separated using a 4 μm C18 column in an RCM-100 radial compression module (Waters Associates) at 2 mL/min along a gradient of 25–35% acetonitrile. Individual polyglutamate species were determined by a measure of radioactivity under each peak, which co-eluted with an authentic MTX standard as detected by an in-line radioactive flow detector (Flo-One Beta, Radiomatic Inst., Downers Grove, IL).

Determination of bound versus free MTX. Separation of bound and free MTX was performed as described [11]. Cells were incubated in folate-free IMEM 24 hr prior to the study and then exposed to 1 μM [^{3}H]MTX in folate-free IMEM supplemented with 25 μM hypoxanthine, glycine and thymidine. At the times indicated, cells were washed three times in ice-cold PBS, and scraped off the flask surface with a rubber policeman in the presence of ice-cold 0.15 M $KHPO_4$, pH 6.2. The cellular debris was removed by centrifugation at 10,000 *g* for 5 min, and 1 mL of lysate was applied to a 0.8 × 2.5 cm DEAE-Sephacel minicolumn equilibrated with 0.15 M $KHPO_4$, pH 6.2, at 4°. Protein-bound drug was eluted with 4 mL of the equilibration buffer, following which unbound radiolabeled drug was eluted with 5 mL of 1 M NH_4CO_3. Results were expressed as a percentage of MTX bound and free determined from scintillation counts of material recovered from the minicolumn eluates.

Western blot analyses. Lysates of WT MCF-7 and MelR MCF-7 cells were prepared by sonication in buffer followed by centrifugation of cellular debris. For detection of TS, equivalent amounts of protein (200 μg) from each cellular lysate were resolved by polyacrylamide gel electrophoresis using 12.5% acrylamide, according to the method of Laemmli [12]. The gels were electroblotted onto a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) in transfer buffer containing 48 mM Tris, 39 mM glycine, 0.5 M EDTA in 20% methanol for 2 hr. Next, the nitrocellulose membrane was incubated with a blocking solution (Blotto 5% Carnation nonfat milk, 10 mM Tris, 0.01% Thimerosol) for 2 hr and then incubated with the TS 106 monoclonal antibody (10 μg/mL) for 2 hr at room temperature [13, 14]. After the overnight incubation, a horseradish peroxidase conjugated goat anti-mouse immunoglobin was added and incubated at 1/500 dilution for 2 hr. Chromogenic substrate 4-chloro-1-naphthol (Sigma) was subsequently added, and the positive TS bands were identified. Protein concentrations were determined by the Bio-Rad protein assay [8]. For detection of DHFR, 30 μg cytosolic protein was size-fractionated on a 12.5% Daiichi gel (Integrated Separation Systems) and transferred to nitrocellulose using a semi-dry electroblotter. The blot was blocked in Tris-buffered saline containing 5% skim milk and 0.15% Tween-20. Then the membrane was incubated with a rabbit polyclonal antibody for DHFR (Johnston PG, unpublished data), washed with Tris-buffered saline containing 0.1% Tween-20, and developed using a phosphorescence detection kit according to the manufacturer's instructions (Amersham).

Northern blot analysis. Total RNA was isolated from WT MCF-7 and MelR MCF-7 by a standard guanidinium isothiocyanate-cesium chloride gradient centrifugation method [15]. Total RNA was size fractionated on a 1% agarose gel in 20 mM 3-(*N*-morpholino)propanesulfonic acid buffer containing 2% formaldehyde, 1 mM EDTA and 5 mM sodium acetate. The size-fractionated RNA was transferred to a Nytran membrane (Schleicher & Schuell), hybridized overnight with ^{32}P-labeled probe for the folate binding protein [16], washed at a final stringency of 0.1 × standard saline citrate and 0.1% sodium dodecyl sulfate at 65°, and detected by autoradiography.

RESULTS

Cytotoxicity assays. The relative resistance of MelR MCF-7 cells to antifolates was affected by the length of incubation of the cells in drug. In 4-day cytotoxicity assays (1 day for cells to adhere followed by 3 days of drug exposure), the resistance of MelR MCF-7 cells to MTX was 60-fold (IC_{50} values for MelR MCF-7 and WT MCF-7 of 9.0 and 0.15 μM, respectively, Fig. 1A). However, a 6-day drug exposure in the 7-day assay reduced the IC_{50} of MelR MCF-7 cells to 1.0 μM, while leaving the WT MCF-7 IC_{50} unchanged, which resulted in a relative resistance of only 6.7-fold. This phenomenon may be related to the slower doubling time of MelR MCF-7 cells of 47 hr compared with 27 hr for WT MCF-7 cells [1]. The fact that the concentration–response curves fell to less than 10% of control growth after 7 days at high drug concentrations, compared with the higher plateaus seen with shorter drug exposure, further supports the idea that a certain number of cell doublings may be required for cell death after MTX exposure. In comparison, the difference between 4- and 7-day cytotoxicity assays in the relative resistance of the cell lines to melphalan changed only from 28- to 20-fold, with a decrease in the IC_{50} of melphalan in both WT MCF-7 and MelR MCF-7 cells in response to the increased incubation time (Fig. 1B).

Using the 7-day cytotoxicity assay, we also examined the relative resistance of MelR MCF-7 cells to TMQ, an antifolate, which is neither subject to carrier-mediated uptake nor polyglutamation by FPGS. As shown in Fig. 1C, the IC_{50} of MelR MCF-7 cells to TMQ was 1.2 compared with 0.6 μM for WT-MCF-7 cells, a relative resistance of 2-fold, or less than one-third of the relative resistance of MelR MCF-7 cells to MTX. This suggested that the MTX resistance seen in MelR MCF-7 was due largely to changes in MTX uptake or polyglutamation. We also examined the resistance of MelR MCF-7 cells to fluorouracil and found, surprisingly, that MelR MCF-7 cells were collaterally sensitive to fluorouracil

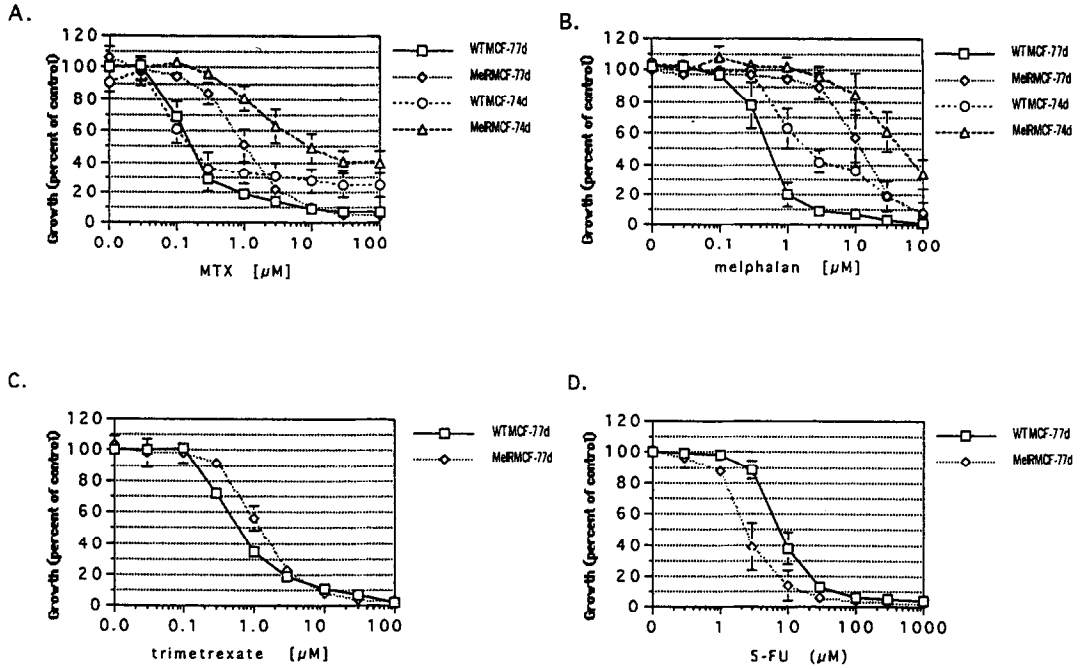

Fig. 1. Cytotoxicity assays of MTX (panel A), melphalan (panel B), TMQ (panel C) and fluorouracil (panel D) in WT MCF-7 and MelR MCF-7 cells. Cell growth after 1 day of incubation in medium without drug followed by continuous exposure to drug for either 3 or 6 days (for panels A and B) or 6 days only (for panels C and D) was determined relative to untreated controls using a sulforhodamine dye assay as described in Materials and Methods. The graphs indicate the mean ± SD of three separate determinations performed in triplicate.

(Fig. 1D), with an IC_{50} of 2.2 vs 7.5 μM for WT MCF-7 cells, giving a relative resistance ratio of 0.29.

MTX uptake studies. Comparison of MTX uptake in WT MCF-7 and MelR MCF-7 cells demonstrated decreased MTX accumulation in MelR MCF-7 cells (Fig. 2A). After 30 min, MTX accumulation was 1.96 ± 0.06 pmol/10^6 cells for MelR MCF-7 cells and 3.45 ± 0.27 pmol/10^6 cells for WT MCF-7 cells. At 2 hr, total MTX accumulation in MelR MCF-7 cells was 2.8 ± 0.6 pmol/10^6 cells vs 5.0 ± 0.8 pmol/10^6 cells for WT MCF-7 cells. Following 24 hr of incubation in radiolabeled drug, the total accumulation of MTX was 3-fold less in MelR MCF-7 cells than in the parental cells (4.9 vs 15.3 pmol/10^6 cells, respectively).

To determine the initial rate of MTX uptake, the accumulation of MTX at 2 min was measured over a range of drug concentrations. An inverse reciprocal plot of initial MTX uptake (Fig. 2B) revealed a V_{max} of 1.5 ± 0.1 vs 2.0 ± 0.2 pmol/10^6 cells/min for MelR MCF-7 and WT MCF-7 cells, respectively, and K_t values of 0.92 ± 0.08 vs 0.97 ± 0.08 μM for MelR MCF-7 and WT MCF-7 cells, respectively. Therefore, the V_{max} in MelR MCF-7 cells was 50% of the V_{max} seen in WT MCF-7 cells, while the relative affinity of the receptor for MTX remained unchanged.

Determination of non-effluxable MTX. The pool of non-effluxable MTX was determined following incubation of cells for 2 or 24 hr in 1 μM MTX, as described in Materials and Methods. No difference was noted between the two cell lines in non-effluxable MTX following 2 hr of incubation with radiolabeled MTX (Fig. 3), suggesting that there was no difference in the intracellular pool of protein-bound drug between the two cell lines. However, following 24 hr incubation in [^{3}H]MTX, the total amount of non-effluxable MTX (now consisting of both protein-bound and polyglutamated MTX) was 4-fold less in the resistant cells (2.1 vs 8.6 pmol/10^6 cells for MelR MCF-7 and WT MCF-7 cells, respectively) (Fig. 3). Thus, MelR MCF-7 cells appeared to have decreased formation of polyglutamated MTX.

MTX polyglutamate formation. To determine directly whether the decrease in effluxable MTX in MelR MCF-7 cells after a 24-hr exposure to MTX was the result of a decrease in polyglutamate formation, we examined MTX polyglutamate formation in WT MCF-7 and MelR MCF-7 cells following incubation with 1 μM MTX over a time-course from 2 to 48 hr by HPLC. Figure 4A displays $MTXGlu_{1-2}$ formation and Fig. 4B depicts $MTXGlu_{3-5}$ formation in the two cell lines. Analysis of intracellular MTX polyglutamate formation after a 24-hr incubation demonstrated a decrease in the formation of $MTXGlu_{3-5}$ relative to total MTX in MelR MCF-7 vs WT MCF-7 cells (43 vs 62%, respectively). The total $MTXGlu_{3-5}$ in MelR MCF-7 cells was 29% of WT MCF-7 cell levels at both the 24- and 48-hr time points.

Protein-bound versus free MTX. In addition to

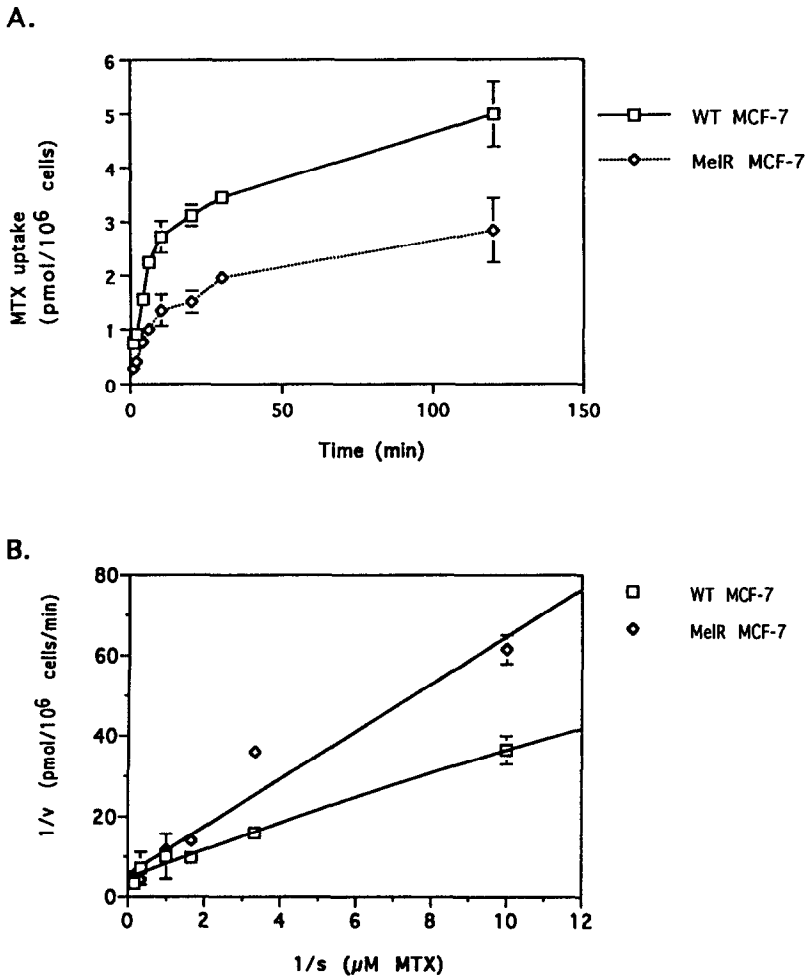

Fig. 2. MTX accumulation in WT MCF-7 and MelR MCF-7 cells. In panel A, uptake of 1 μM MTX was determined from 1 to 30 min at 37° as described in Materials and Methods. The graph indicates the mean ± SD of three separate determinations performed in duplicate. In panel B, an inverse reciprocal plot of MTX uptake over a concentration range of 0.1 to 6 μM was determined over 2 min as described in Materials and Methods. The graph indicates the mean ± SD of three separate determinations performed in duplicate or triplicate.

the differences in polyglutamation between the two cell lines, changes in non-effluxable MTX may also result from differences in protein-bound versus free MTX. However, as shown in Fig. 5, no difference in the total amount of protein-bound MTX was found between the two cell lines. In contrast, the amount of free MTX was 2–4 times less in MelR MCF-7 cells, depending on the time point, consistent with the observed decrease in MTX polyglutamation in this cell line.

Folate pathway enzymes. The lack of difference between MelR MCF-7 and WT MCF-7 cells in the amount of protein-bound MTX suggested that there was no difference between the two cell lines in the expression of DHFR. This was confirmed in two ways, by direct measurement of DHFR catalytic activity (Table 1) and by western blot analysis (Fig. 6B); both of these studies showed that DHFR levels in the two cell lines were essentially identical. Furthermore, the affinity of DHFR for MTX (Table 1) was also equivalent in the two cell lines. Thus, there was no observable quantitative or qualitative difference in DHFR between WT MCF-7 and MelR MCF-7 cells.

The difference in the amounts of polyglutamated MTX between the two cell lines may result from decreased substrate availability, decreased FPGS activity, or both. Therefore, we determined FPGS activity in both cell lines, and, as shown in Table 1, found no difference in FPGS activity between the two cell lines.

Since MelR MCF-7 cells were collaterally sensitive to fluorouracil, we also examined the two cell lines for differential expression of TS. We found that FdUMP binding was decreased in MelR MCF-7 cells to 60% of the level found in WT MCF-7 cells (Table 1). In addition, a western blot of TS appeared to show a slight decrease in TS protein (Fig. 6A).

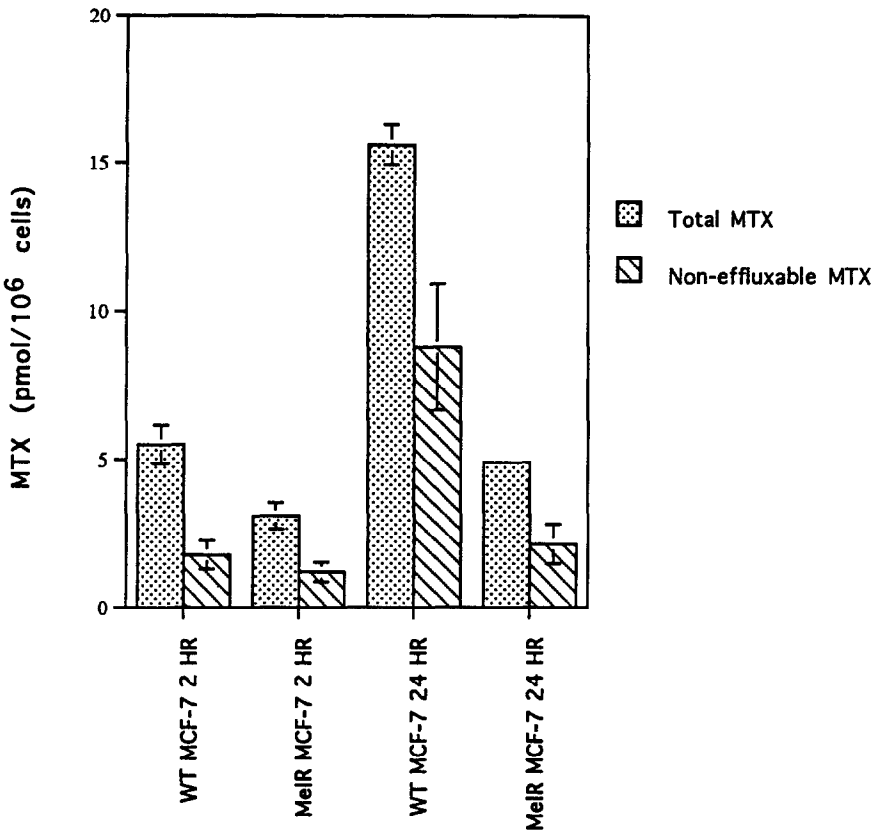

Fig. 3. Non-effluxable MTX pool in WT MCF-7 and MelR MCF-7 cells. After incubation in 1 μM MTX for 2 or 24 hr, total MTX was measured either immediately (total cellular MTX) or after a 1-hr wash-out period (non-effluxable MTX). The results (mean ± SD) are the averages of two independent experiments performed in duplicate.

However, there was no significant difference in TS catalytic activity between the two cell lines (Table 1). Thus, it is not clear whether these changes could account for the difference in sensitivity to fluorouracil, nor is it clear how these changes could contribute to MTX resistance.

Folate receptor expression. There was no detectable expression of folate receptor RNA in either WT MCF-7 or MelR MCF-7 cells (Fig. 6C). This is consistent with previous studies that have found no detectable folate receptor expression in WT MCF-7 cells [16, 17].

DISCUSSION

We report and characterize MTX cross-resistance in a human MCF-7 breast cancer cell line selected for resistance to melphalan by serial incubation in increasing concentrations of melphalan. These cells were never exposed to antifolates during the selection procedure. Therefore, these cells exhibited an unexpected pattern of pleiotropic resistance. To identify the mechanism(s) underlying this novel pattern of multiple drug resistance, we sought to characterize the mechanism of MTX resistance in these cells.

The concentration–response curves in Fig. 1 demonstrate that the degree of resistance of MelR MCF-7 cells to MTX varied greatly with the length of exposure to the drug. In the 4-day cytotoxicity assay, the degree of resistance of MelR MCF-7 cells to MTX (60-fold) actually exceeded the degree of resistance of the cells to the selecting agent melphalan (28-fold). However, the data concerning the mechanism of resistance of MelR MCF-7 cells to MTX are more consistent with the lower level of MTX resistance (6.7-fold). This illustrates the importance of controlling for the number of cell doublings when measuring the relative sensitivity of different cell lines towards antimetabolites, and indicates that IC_{50} values computed on concentration–response curves with high terminal plateaus may not reflect complete cytotoxic activity of the drug exposure.

The data shown in Fig. 1 also demonstrate that MelR MCF-7 cells are cross-resistant to MTX to a greater degree than they are cross-resistant to TMQ. This pattern of resistance suggested that the major cause of resistance of MelR MCF-7 cells to MTX was related to either MTX uptake, MTX polyglutamation or both. The studies summarized in Fig. 2 showed that MelR MCF-7 cells have

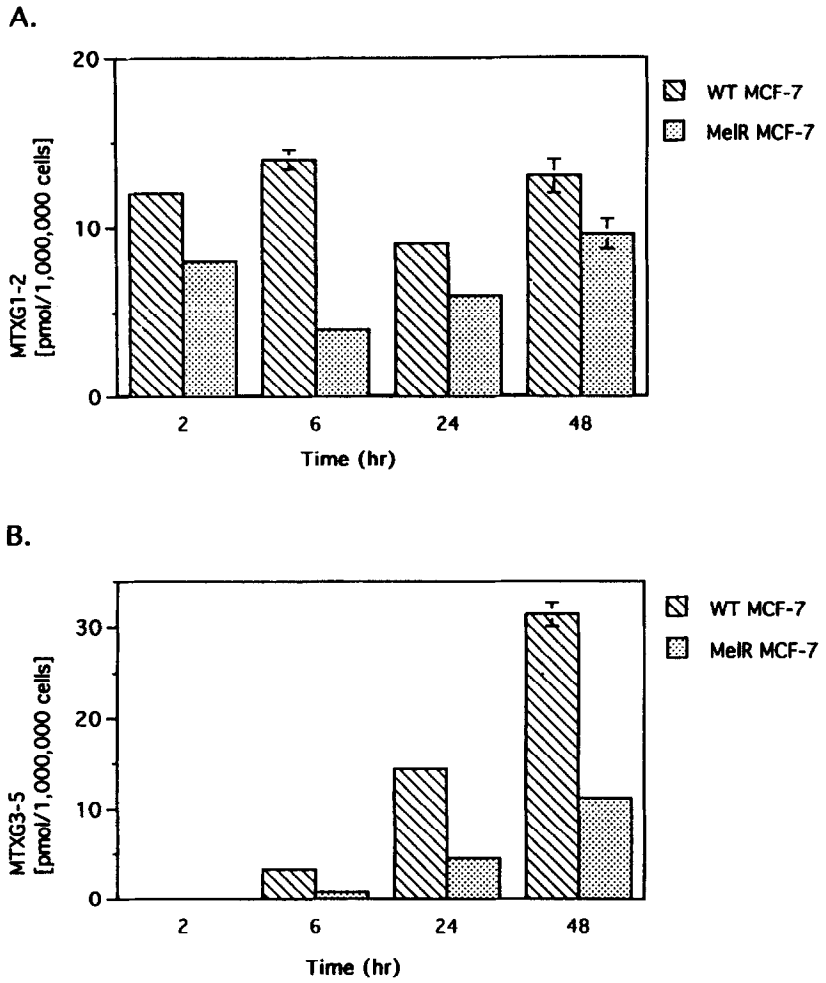

Fig. 4. MTX polyglutamate formation in MelR MCF-7 and WT MCF-7 cells. Cells were incubated with labeled MTX in folate-free medium for the times indicated, and MTX polyglutamate formation was determined by HPLC as described in Materials and Methods. The results (average ± range, N = 2) represent the averages of replicate samples.

decreased uptake and retention of MTX. The decreased uptake was associated with a decrease in the V_{max} for MTX uptake. The apparent K_t for MTX transport in WT MCF-7 cells was 0.97 μM, which is within the range for MTX uptake seen in other cell lines [18–20] but lower than a previous report of MTX uptake in MCF-7 cells (8.2 μM) [21]. The apparent V_{max} was 2.9 pmol/10^6 cells/min or 36.2 pmol/min/mg protein, which compares to 12.2 pmol/min/mg protein observed in a previous study of MCF-7 cells [21].

In contrast to the equivalent pools of non-effluxable MTX seen after a 2-hr incubation in drug, a decrease in non-effluxable MTX was observed in MelR MCF-7 cells at 24 hr (Fig. 3). We demonstrated that the decrease in MTX retention at this time point was associated with decreased MTX polyglutamate formation in the resistant cells (Fig. 4). Decreased MTX polyglutamate formation did not appear to be due to changes in FPGS activity (Table 1). Thus, the resulting 4-fold decrease in the level of MTX polyglutamates (Fig. 3) may be the result of decreased substrate availability. Since MTX polyglutamates are more potent inhibitors of DHFR than the parent drug, the 4-fold decrease in MTX polyglutamate formation may explain most, if not all, of the 7-fold level of MTX resistance observed in these cells.

Neither DHFR nor TS appears to play a role in the MTX resistance of MelR MCF-7 cells (Fig. 6, panels A and B, and Table 1). Reduced affinity of an altered DHFR enzyme has been observed in MTX-resistant cell lines with evidence of DHFR overexpression and gene amplification [22, 23]. However, neither overexpression nor altered affinity of DHFR appears to play a role in MTX resistance in MelR MCF-7 cells.

Therefore, MelR MCF-7 cells are resistant to two drugs with completely different mechanisms of action on the basis of decreased uptake of each drug. In fact, the antifolate MTX and the alkylating agent melphalan have little in common except for the fact that, as analogues of endogenous substrates, uptake

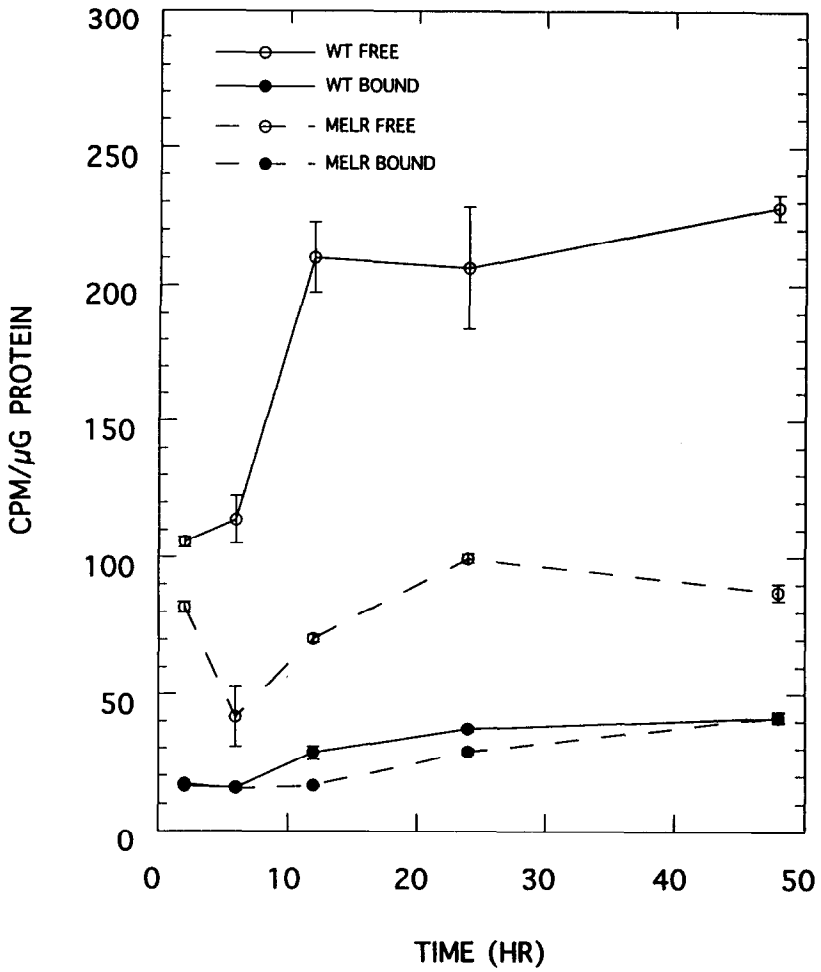

Fig. 5. Free versus bound MTX in MelR MCF-7 and WT MCF-7 cells. Cells were incubated with labeled MTX in folate-free medium for the times indicated, and free versus bound MTX was determined as described in Materials and Methods. The results (average ± range, N = 2) represent the averages of replicate samples.

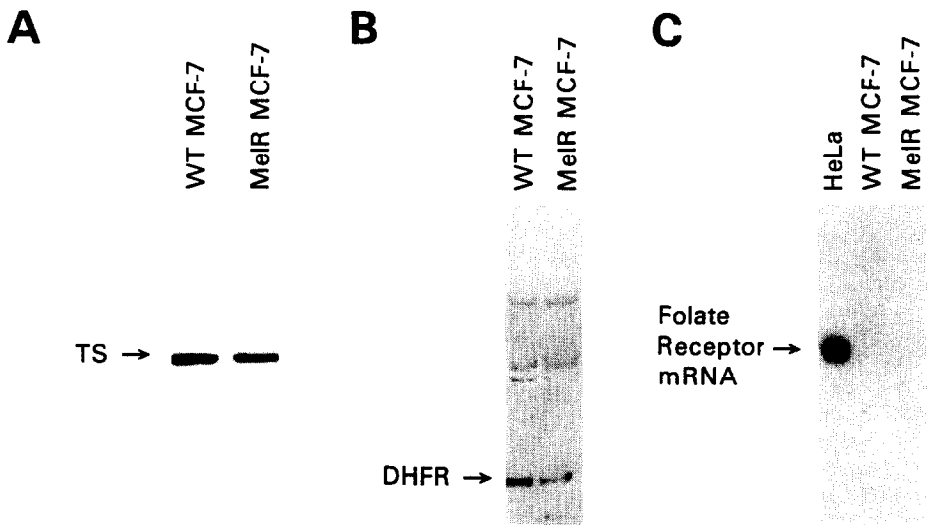

Fig. 6. (A) A western blot of TS protein expression in WT MCF-7 and MelR MCF-7 cells. (B) A western blot showing DHFR protein expression in WT and MCF-7 cells. (C) A northern blot of folate binding protein mRNA expression in WT MCF-7 and MelR MCF-7 cells. HeLa cell RNA was used as a positive control.

Table 1. Folate pathway characteristics of MelR MCF-7 and WT MCF-7 cells

Enzyme	WT MCF-7	MelR MCF-7
FdUMP binding*	$3.54 \pm 0.43 \times 10^{-14}$	$2.1 \pm 0.38 \times 10^{-14}$
Thymidylate synthase catalytic activity †	$1.64 \pm 0.22 \times 10^{-12}$	$1.49 \pm 0.31 \times 10^{-12}$
Dihydrofolate reductase catalytic activity†	$4.06 \pm 0.67 \times 10^{-9}$	$3.63 \pm 0.38 \times 10^{-9}$
Dihydrofolate reductase MTX affinity K_d (M)	$35.6 \pm 12.3 \times 10^{-12}$	$49.7 \pm 19.4 \times 10^{-12}$
Folylpolyglutamate synthase activity‡	$34.8 \pm 1.3 \times 10^{-12}$	$34.1 \pm 4.6 \times 10^{-12}$

* Expressed in mol/mg cytosolic protein.
† Expressed in mol/mg protein/min.
‡ Expressed in mol/mg protein/hr.

of both drugs is mediated by specific high-affinity transporters. In both cases, MelR MCF-7 cells demonstrated a decreased V_{max} for drug uptake.

The mechanisms of uptake for melphalan and MTX are distinctly different. Melphalan uptake is mediated by two amino acid carriers, system L and system ASC [24]. System L is a sodium-independent transporter of leucine and phenylalanine, which is inhibited by the synthetic inert amino acid BCH. System ASC (for alanine, serine and cysteine) is sodium-dependent and unaffected by BCH. In MelR MCF-7 cells, decreased melphalan uptake is the result of down-regulation of system L [1]. There is no evidence that systems L or ASC can transport reduced folates.

There are also two mechanisms of MTX uptake, the folate receptor and the reduced folate carrier. The folate receptor has a higher affinity for folic acid in comparison to its affinity for folinic acid and MTX. Transfection of folate receptor cDNA into breast cancer cells has yielded mixed results with respect to alteration of MTX sensitivity. Transfection of WT and MTX-resistant ZR-75B breast cancer cells with an expression vector for the folate receptor does not alter the sensitivity of the transfected cells to MTX [18]. However, similar transfection of WT MCF-7 cells results in increased sensitivity of the cells to MTX [17]. Down-regulation of the reduced folate carrier has also been implicated as a mechanism of acquired MTX resistance in several independent cell lines selected for resistance *in vitro* [25–28] and *in vivo* [29]. Recently, our laboratory has cloned a mouse cDNA that has characteristics of the reduced folate carrier, in that it restored reduced folate uptake and MTX sensitivity to the transport-deficient ZR-75B human breast cancer cell line [30]. A similar gene has been isolated from Chinese hamster ovary cells [31]. MTX uptake competition experiments with both WT MCF-7 and MelR MCF-7 cells has suggested that the reduced folate carrier is the active transporter in both cell lines, since reduced folates competed more effectively for MTX uptake than folic acid (data not shown). In addition, northern blot analysis of RNA from WT MCF-7 and MelR MCF-7 cells could not detect folate binding protein expression (Fig. 6C). Therefore, the decreased V_{max} for MTX uptake in MelR MCF-7 cells is likely due to down-regulation of the reduced folate carrier.

Cross-resistance to MTX has been described previously in cells selected for resistance to cisplatinum. Rosowsky *et al.* [32] described a cisplatinum-resistant head and neck carcinoma cell line in which cross-resistance to MTX was associated with decreased MTX uptake and polyglutamate formation and decreased DHFR activity. Scanlon and co-workers [33] described a cisplatinum-resistant human ovarian carcinoma cell line in which cross-resistance to MTX was associated with an increase in TS activity and collateral resistance to fluorouracil. However, another ovarian cell line (TR170) selected for cisplatinum resistance has been described which has the opposite antimetabolite cross-resistance pattern of MelR MCF-7 cells, in that the cisplatinum-resistant TR170 cells were cross-resistant to fluorouracil but collaterally sensitive to MTX [34]. Increased MTX sensitivity in these cells was associated with increased MTX uptake, and DHFR expression was unaffected.

Thus, several cell lines have been described in which selection for resistance to different DNA-damaging agents results in altered sensitivity to antifolates. Taken together, these reports suggest that there is a common mechanism affecting sensitivity of cells to all of these agents. Characterization of MelR MCF-7 cells suggests that this pleiotropic pattern of resistance could result from alteration of the efficiency of multiple transporters. MelR MCF-7 cells, therefore, may be a useful cell line in which to study the mechanism by which pleiotropic drug resistance results from multiple changes in transport-mediated drug uptake.

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