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Investigations on the mechanisms of methotrexate resistance in a cisplatin-resistant L1210 murine leukemia cell subline

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Abstract We report a murine leukemia cell variant (L1210/DDP), selected for cisplatin (DDP) resistance, to be cross-resistant to methotrexate (MTX). Cross-resistance of L1210 cells to DDP and MTX has been observed by others, and has also been recorded in P388 murine leukemia and SSC-25 human squamous carcinoma cells. We demonstrated that MTX resistance is not due to dihydrofolate reductase (DHFR) gene amplification, increased DHFR enzyme activity or decreased MTX binding to the target enzyme. Of the mechanisms commonly proposed for MTX resistance, only differences in transport were observed when comparing sensitive (L1210/0) and resistant (L1210/DDP) cells. Our results suggest that MTX resistance in L1210/DDP cells is due to altered methotrexate uptake.

Key words Methotrexate · Cisplatin · Drug resistance

Introduction

Cisplatin (cisdiamminedichloroplatinum (II), DDP) is one of the most widely used agents in combination therapy with demonstrated efficacy in the treatment of testicular, bladder, ovarian, and head and neck cancers [1–3]. As with other commonly used drugs, the development of DDP resistance is considered a major limitation in the successful treatment of malignant tumors. Our studies have demonstrated that a subline of L1210 murine leukemia cells selected for resistance to DDP, (L1210/DDP cells), a drug which exerts its toxic effects by binding to DNA [4,5], is cross-resistant to methotrexate (MTX), an antifolate [6]. Cross-resistance to MTX has been reported in other DDP-resistant cell lines [7–9], which suggests a possible functional relationship between DDP resistance and folate resistance.

Several mechanisms have been proposed for MTX resistance including: (a) dihydrofolate reductase (DHFR) gene amplification [10], (b) increased DHFR enzyme activity [11], (c) biochemical alterations in (DHFR), resulting in decreased methotrexate binding [12], and (d) defective MTX transport [13,14]. Each of these parameters was investigated as possible causes of MTX resistance in L1210/DDP cells. Because the mechanisms of DDP resistance in L1210/DDP cells are well characterized [15], our studies were directed towards identifying factors which contribute to MTX resistance in L1210/DDP cells and to determine if an association exists between these factors and DDP resistance.

Materials and methods

Cell culture

Wild-type (L1210/0) and L1210/DDP murine leukemia cells were maintained in continuous suspension in McCoy's 5A medium (Gibco, Grand Island, N.Y.), supplemented with 5% donor horse serum (Hazelton, Lenexa, Kan.), and 5% fetal bovine serum (Hyclone, Logan, Utah), respectively, at 37°C in a humidified atmosphere containing 10% CO_2. CHOC 400 cells were grown to confluence in DMEM medium, (Gibco), supplemented with 10% fetal bovine serum (Gibco). Stock cultures were split on a biweekly basis and were maintained at 37°C in a humidified incubator in an atmosphere containing 5% CO_2. The cell cultures were tested for mycoplasma contamination regularly (Bionique Testing Laboratories, Saranac Lake, N.Y.).

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Chemicals

DDP, NADPH, dihydrofolic acid and *L.casei* DHFR were purchased from Sigma Chemical Co. (St. Louis, Mo.). MTX was generously provided by Dr. N. Heintz, (University of Vermont, Burlington). [L-glutamyl-3, 4-^{3}H]-MTX (41.0 Ci/mmol) was purchased from New England Nuclear Research Products (Boston, Mass.). DDP and MTX were dissolved in 0.9% NaCl prior to use in each experiment.

Growth inhibition

Sensitivity to DDP and to MTX was measured by determination of growth inhibition. The L1210/0 and L1210/DDP cells in the mid-exponential phase of growth ($0.5–1.0 \times 10^6$ cells/ml) were diluted to 5×10^4 cells/ml in their respective media. Cells were treated with various concentrations of drugs and incubated for 72 h at 37°C in a humidified atmosphere containing 10% CO_2. Cell numbers were determined using a model ZB_F Coulter Counter (Coulter Electronic, Hialeah, Fl.). Results are expressed as ID_{50}, the drug concentration necessary to inhibit cell growth by 50%, relative to untreated controls. The resistance factor, RF, is defined as the ratio of the ID_{50} in L1210/DDP cells to that in L1210/0 cells.

Southern blot analysis

L1210/0 and L1210/DDP cells were harvested at the mid-exponential phase of growth by centrifugation (8000 *g*, 4°C, 10 min) and resuspended in 2 ml lysis buffer (50 m*M* Tris, 20 m*M* EDTA, 100 m*M* NaCl, 0.1% SDS, 70 μg/ml proteinase K) for 12 h at 37°C. Total genomic DNA was isolated by phenol/chloroform extraction, precipitated with $2\frac{1}{2}$ volumes 100% ethanol and NaCl at a final concentration of 0.3 *M*, followed by digestion with restriction enzyme ECoR1 (Promega, Wis.) at 37°C. The DNA concentration was determined using a fluorimetric method utilizing Hoechst 33258, and 10 μg DNA was digested again in ECoR1 enzyme for 4 h at 37°C. Genomic DNA was extracted from CHOC 400 cells, which contain 600–800 copies of the DHFR gene [16], to be used as a positive control. Digested DNA was resolved using a 0.7% agarose 1x TAE (0.04 *M* Tris-acetate, 2 m*M* EDTA) gel at 40 mA then transferred to nitrocellulose by the method of Southern [17]. The membrane was baked under vacuum for $2\frac{1}{2}$ h at 80°C, then prehybridized for 20 h (42°C) in 20 ml modified Starks solution (50% formamide, 1x Denhardt's solution, 0.3 mg/ml sonicated salmon sperm DNA and 4x SSC, which is 3 *M* sodium chloride/0.3 *M* sodium citrate, pH 7.0). A cDNA clone for mouse DHFR was obtained from plasmid △ VapMT2 [16] and labelled with ^{32}P-dATP (New England Nuclear, Mass.) via a random primer extension method as described elsewhere [18]. The membrane was hybridized for 20 h at 42°C with 1.0×10^6 cpm/ml labelled cDHFR mini-gene in 20 ml modified Starks solution. After washing, once with 2x SSC/0.1% SDS for 30 min at 65°C and twice with 0.2x SSC/0.1% SDS for 45 min at 65°C, the radioactivity bound to the membrane was detected by autoradiography.

Dihydrofolate reductase assay

Approximately 10^8 exponentially growing L1210/0 and L1210/DDP cells were washed twice with cold PBS, harvested by centrifugation (400 *g*, 15 min, 4°C), and resuspended in 3 ml 50 m*M* Tris-HCl buffer (pH 6.0). Cells were sonicated for 20 s (setting 7) and centrifuged at 25,000 *g* for 20 min at 4°C. DHFR activity in L1210/0 and L1210/DDP cells was determined in a reaction mixture (0.4 ml) containing 1.25 μ*M* dihydrofolic acid, 0.22 m*M* NADPH, 20 m*M* mercaptoethanol and 20 m*M* phosphate buffer, pH 7.0. This reaction mixture was inverted several times at room temperature for 1 min and the reaction was started by the addition of 50 μl of the dihydrofolate solution. DHFR activity was measured by monitoring the decrease in absorbance associated with the concomitant oxidation of NADPH to NADP at 340 nm at 37°C [19]. No activity was seen in NADPH-depleted reaction mixtures. To measure and compare the inhibitory effects of MTX on enzyme activity in L1210/0 and L1210/DDP cell extracts, increasing concentrations of MTX were added to the reaction mixture in the presence and absence of NADPH for 3 min, prior to the activation of the reaction by the addition of dihydrofolate, until no change in absorbance was detected [19].

Folylpolyglutamate synthetase assay

Folypolyglutamate synthetase (FPGS) was assayed following a method developed by McGuire et al. (19). Briefly, a typical assay mixture (0.25 ml, pH 8.4), containing Tris-HCl (0.1 *M*), ATP (10 m*M*), $MgCl_2$ (20 m*M*), KCl (20 m*M*), 2-mercaptoethanol (100 m*M*), methotrexate (50 μ*M*), 4 m*M* [^{3}H]-glutamate ($4X10^6$ cpm/ μmol), and crude extracts, was incubated at 37°C. At the end of the incubation period, the reaction mixture was diluted with 1 ml ice-cold buffer (10 m*M* Tris-HCl, 110 m*M* NaCl, and 25 m*M* 2-mercaptoethanol). Each crude extract was loaded on to a DE52 column (Whatman, Ore.). The columns were packed with DE52 which was extensively washed with 1 M NaCl and equilibrated with 10 m*M* Tris-HCl (pH 7.5) and 110 m*M* NaCl. Less than 30 min before use, the columns were washed with 3 ml of this buffer containing 25 m*M* 2-mercaptoethanol (column buffer). After loading of the reaction mixture, the columns were washed with column buffer until a total of 14.75 ml was collected. All polyglutamates were eluted with 3 ml 0.1 *N* HCl following the wash. Part of the eluate (2.5 ml) was counted using a Packard Scintillation counter.

Cellular association of MTX

Association measurements were carried out by a method descibed elsewhere [20].

MTX association

Exponentially growing L1210/0 and L1210/DDP cells were washed three times with PBS by centrifugation (400 *g*, 15 min, 4°C) and resuspended in Hanks' balanced salt solution (HBSS) at 37°C at a cell density of approximately 2×10^7 cells/ml (protein concentrations were similar in both the cell lines) containing [^{3}H]-MTX to initiate MTX association. The reaction was terminated by the addition of 0.5-ml aliquots of cell suspension to 10 ml ice-cold PBS at selected time intervals. Cells were washed three times with cold PBS and cell pellets digested in 0.5 *M* NaOH. The resultant mixture was analyzed for radioactivity in 10 ml Universol (ICN Radiochemicals, Irvine, Calif.) in a Packard 2500TR Liquid Scintillation Analyzer.

MTX dissociation

Exponentially growing cells were harvested as described above. [^{3}H]-MTX efflux was measured by preincubating L1210 and L1210/DDP cells (1×10^7 cell/ml) in 2 or 10 μ*M* [^{3}H]-MTX, respectively, at 37°C for 20 min. Cells were centrifuged, washed twice with PBS and resuspended in drug-free HBSS and incubated at 37°C and 4°C for selected time periods. Analysis of radioactivity was determined identically as outlined above for influx measurements.

Results

Growth inhibition studies

The results in Table 1 demonstrate that cells of the experimentally derived DDP-resistant subline of L1210 murine leukemia cells (L1210/DDP), which exhibit a 38-fold greater resistance to DDP than parental L1210/0 cells, were more than 25,000 times more resistant to MTX. It should be noted that L1210/DDP cells were sensitive to MTX but maximal inhibition of growth (38%) of the L1210/DDP cells by MTX was achieved at 1.0 μg/ml, with no further toxicity noted despite measuring MTX concentrations as high as 1 mg/ml (Fig.1a, 1b).

Table 1 ID_{50} values (μg/ml) and relative resistance of L1210 cells to DDP and MTX (*RF* resistance factor, defined as the ratio of ID_{50} in L1210/DDP cells to that in L1210/0 cells)

Drug	L1210/0	L1210/DDP	RF
DDP	0.1	3.8	38
MTX	0.04	> 1000	> 25,000

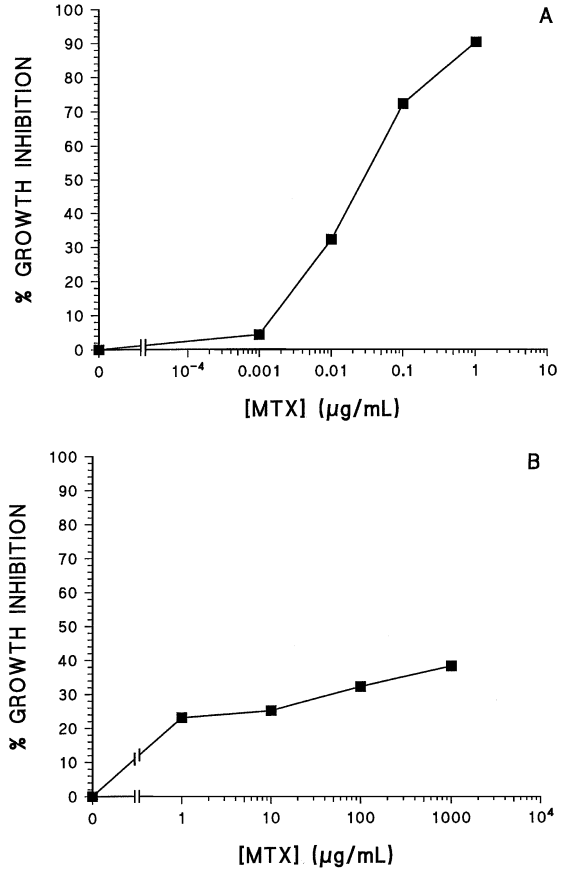

Fig. 1 Growth inhibition of L1210/0 (***A***) and L1210/DDP (***B***) cells by methotrexate

Southern blot analysis

Since resistance to MTX is often associated with an increased cellular content of DHFR, due to DHFR gene amplification, we tested this possibility in L1210/DDP cells. Southern blot analysis of ECoR1 digests of genomic DNA (Fig. 2) extracted from L1210/0 (lane 1) and L1210/DDP (lane 2) cells revealed that MTX resistance in L1210/DDP cells was not due to DHFR gene amplification. In contrast, DNA extracted from CHOC 400 cells, which reportedly contain 600–800 copies of the DHFR gene, demonstrated a protein pattern similar to that previously reported for that cell line (lane 3) [21]. The pattern of bands in lane 3 was different from that in lanes 1 and 2 due to different species (Chinese hamster and mouse). Southern blot analysis of Hind III digests gave similar results (data not shown).

Dihydrofolate reductase activity

Although no DHFR gene amplification was observed, it was possible that increased DHFR activity rather than total DHFR protein could account for the MTX resistance. NADPH acts as a coenzyme in the conversion of dihydrofolate (H_2-folate) to tetrahydrofolate (H_4-folate). Therefore DHFR activity in L1210/0 and L1210/DDP cell extracts was determined and compared by measuring the change in absorbance at 370 nm, which is associated with the concomitant oxidation of NADPH during the reaction. Spectro-

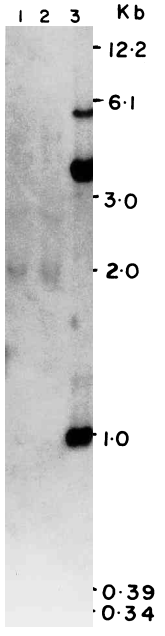

Fig. 2 Southern blot analysis of ECoR1 digests of genomic DNA from L1210/0 (*lane 1*), L1210/DDP (*lane 2*) and CHOC 400 (*lane 3*) cells which reportedly contain 600–800 copies of the DHFR gene

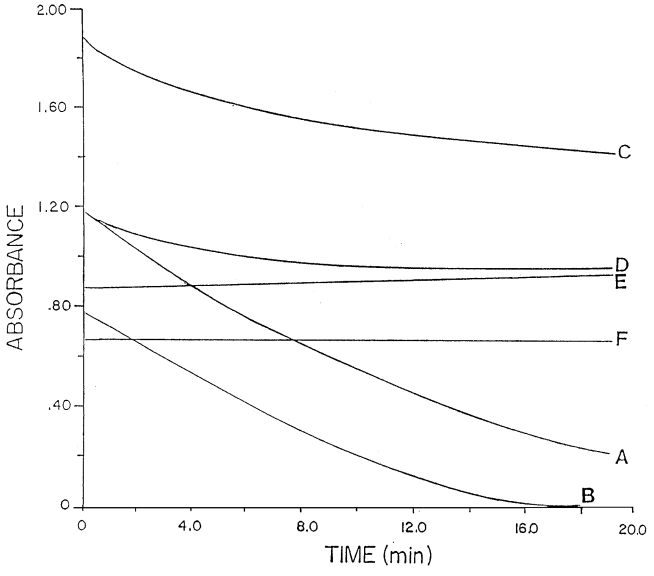

Fig. 3 DHFR enzyme activity in L1210/0 and L1210/DDP cell extracts. DHFR activity in cell extracts was measured by monitoring the oxidation of NADPH to NADP spectrophotometrically at 340 nm (*curve A* L1210/0 cells, *curve B* L1210/DDP cells, *curve C* L1210/0 cells with 0.18 μg/ml MTX, *curve D* L1210/DDP cells with 0.18 μg/ml MTX, *curve E* L1210/0 cells with 1.8 μg/ml MTX, *curve F* L1210/DDP cells with 1.8 μg/ml MTX)

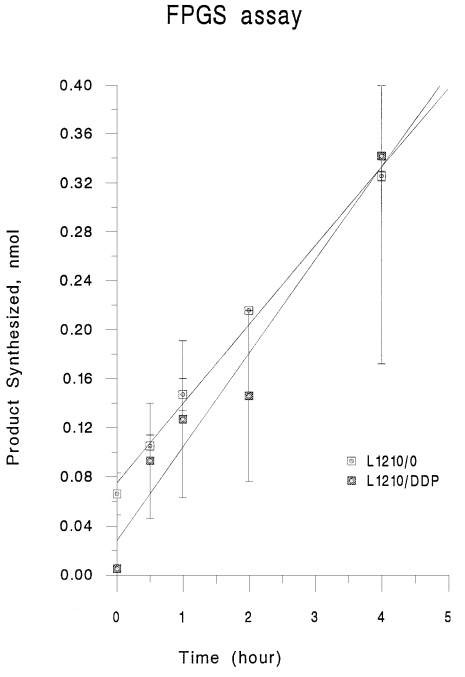

Fig. 4 Time course for MTX polyglutamation. Assay conditions used at 50 μ*M* MTX in a total volume of 0.25 ml. At each time point, duplicate samples were diluted by 1 ml ice-cold buffer and loaded to their respective columns. After elution radioactivity was determined in the eluate and the enzyme activity calculated

photometric analysis of L1210/0 and L1210/DDP cell extracts demonstrated similar DHFR activities in both cell lines as indicated by similar slopes (Fig. 3).

The inhibitory effects of MTX on DHFR activity in resistant cells may be impaired by biochemical alterations in the enzyme resulting in reduced affinity for MTX. To address this possibility, the inhibitory effects of MTX on DHFR activity in L1210/0 and L1210/DDP cell extracts were measured and compared by adding increasing concentrations of MTX to the reaction mixture until no change in absorbance was detected. As shown in Fig. 3, inhibition of DHFR by MTX was similar for both the sensitive and the resistant cell lines. These results suggest that neither DHFR activity nor its affinity to MTX are contributors to the MTX resistance in L1210/DDP cells.

Folylpolyglutamate synthetase activity

FPGS mediates the formation of polyglutamates of MTX and biologically important folates [22, 23] which enhances their cellular retention. Further, it has been shown that impaired polyglutamation alone can cause resistance to MTX [24]. Thus, cell extracts from L1210/0 and L1210/DDP were assayed for FPGS activity using MTX (50 μ*M*) as substrate, as shown in Fig. 4. FPGS activity in L1210/DDP cells was not significantly different than that in sensitive cells ($P = 0.159$, $n = 4$). In kinetic studies, as shown in Fig. 5, the enzymatic activities in both cell lines were quite similar.

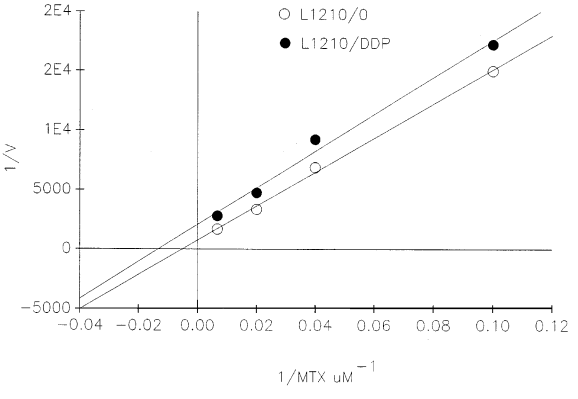

Fig. 5 Folylpolyglutamate assay. The substrate-velocity curves for L1210/0 and L1210/DDP cells. Cell extract was incubated for 2 h with the indicated concentrations of MTX

Cell association studies

Effective intracellular transport is a critical factor in successful chemotherapy with MTX and utilizes an active folate transport system [12]. The fact that L1210/DDP cells were appreciably less resistant to trimetrexate, a lipophilic MTX analogue which enters the cells through passive diffusion, suggests the possibility of defective transport playing a role in MTX resistance in L1210/DDP cells. To test this, cell-associated MTX was measured using radiolabelled MTX.

In both cell lines, [^{3}H]-MTX association was found to approach equilibrium within the first 10 min. The steady-state levels of MTX were higher in L1210/0 cells than in L1210/DDP cells (Fig. 6). In contrast, efflux of [^{3}H]-MTX was approximately equal in both cell lines (Fig. 7). Kinetic analysis of the [^{3}H]-MTX association

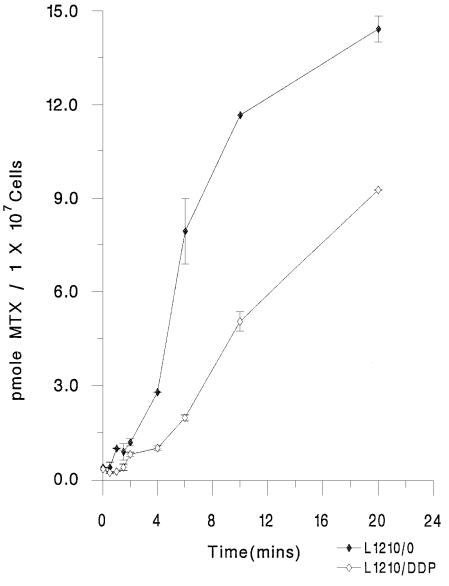

Fig. 6 MTX association. The MTX association in L1210/0 and L1210/MTX cells was determined by incubating 10^7 cells with 0.5 μM [^{3}H]-MTX. Aliquots were removed at various intervals and association terminated by diluting cells 20-fold with ice-cold PBS. The cells were washed three times with PBS and radioactivity determined in the cell pellets. The results are the mean $\pm$ 1 S.D. for 3 experiments

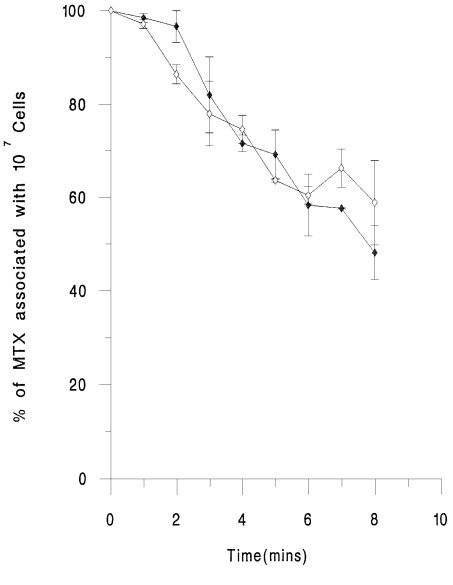

Fig. 7 MTX dissociation. The MTX dissociation was determined by incubating L1210/0 and L1210/DDP cells with 2 and 10 μM [^{3}H]-MTX at 37°C for 20 min. The cells were washed three times and the efflux determined by incubating cells in drug-free HBSS. Aliquots were removed every minute and dissociation terminated as explained in the text. The results are the mean $\pm$ 1 S.D. for 3 experiments

Table 2 Kinetics of MTX association. Values are averages of four experiments

Cell line	MTX influx (K_t) μM	V_{max} (pmol/10^7cells)
L1210/0	2.02 $\pm$ 0.05	1.19 $\pm$ 0.06
L1210/DDP	2.05 $\pm$ 0.29	0.76 $\pm$ 0.09

in the two cell lines was done by Lineweaver-Burke analysis of initial association at 37°C measured during 2 min at varying concentrations of MTX (0.5–6 μM). The results summarized in Table 2 show similar K_t values but a lower V_{max} in the resistant cells.

Discussion

The acquisition of cross-resistance to functionally unrelated drugs represents one of the major obstacles in the treatment of human cancers. The development of drug resistance in malignant tumors is most likely multimodal, and may result from both selection pressure and the induction of random mutations. Thus, numerous mutational events could occur at different loci, the result of which may inadvertently lead to a spectrum of change in other cellular characteristics. Consequently, a cell selected for resistance to one oncolytic drug may express altered sensitivity to other non-biochemically related chemotherapeutic agents. Whether such molecular mechanisms are responsible for the development of L1210/DDP cell cross-resistance to MTX remains unclear.

Most reports indicate that DDP cytotoxicity is related to the formation of platinum adducts on nuclear DNA [4, 5]. Therefore, mutations in DDP-resistant cells most likely involve alterations in the characteristics of DNA repair and related processes.This in turn allows for increased capacity for the removal of platinum adducts permitting cell survival in otherwise lethal concentrations of DDP. MTX is a 2-diamino-10-methyl analogue of folic acid whose mechanism of cytotoxicity is thought to be the intracellular depletion of reduced folate cofactors, as a result of the stoichiometric inhibition of DHFR, the enzyme that reduces dihydrofolate to tetrahydrofolate in the presence of NADPH [25]. Since tetrahydrofolate is converted to a variety of folate coenzymes required for reactions involved in the synthesis of methionine, glycine, thymidylate and purines, the inhibition of DHFR inevitably can lead to the inhibition of DNA, RNA and protein synthesis. Although it is evident from this discussion that the primary mechanisms of MTX and DDP cytotoxicity are unrelated, our findings, together with those of other published studies [7–9], suggest a possible association between DDP resistance and folate metabolism.

We have demonstrated that DHFR gene copy number and enzyme characteristics remained unaltered in

L1210/DDP cells (Figs. 2 and 3). Further, no differences were observed in the FPGS activity (Fig. 5). However, differences in MTX transport were observed between the sensitive and resistant cells (Fig. 6). It is generally accepted that MTX uptake is mediated by an energy-dependent membrane transport complex which primarily mediates the intracellular transport of reduced folates. Since DDP-resistant cells have been shown (a) to have higher intracellular levels of reduced folates [26], (b) to be less dependent on exogenous methionine supplies [27], and (c) to have a more efficient folate metabolism [28], it appears that L1210/DDP cells may be related to a reduced dependence of these cells on exogenous folates and a decreased uptake of both folates and antifolates. How a twofold difference in MTX uptake can account for the significant MTX resistance seen in L1210/DDP cells is uncertain. Several laboratories have reported MTX resistance due to altered drug influx [13, 14]. Without an intact reduced folate transport complex, sufficient levels of MTX required for cytotoxicity cannot reach its intracellular targets. Hence, if the L1210/DDP cells have a faulty transport mechanism, the actual amount of MTX reaching DHFR could be dramatically less than the amount of cell-associated drug measured in our studies. Our laboratory is currently investigating whether L1210/DDP cells have an altered folate transport.

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