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MECHANISMS OF CROSS-RESISTANCE TO METHOTREXATE AND 5-FLUOROURACIL IN AN A2780 HUMAN OVARIAN CARCINOMA CELL SUBLINE RESISTANT TO CISPLATIN*

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Abstract—Some properties of the human ovarian carcinoma line A2780 and a subline three times more resistant than the parent line to cisplatin are compared in this report. The rates of uptake and release of cisplatin were similar in the two cell lines. Resistance to cisplatin was associated with: (a) cross-resistance to 5-fluorouracil and methotrexate; (b) a 2.5-fold increase in thymidylate synthase, as measured by both enzyme activity and the capacity to complex 5-fluorodeoxyuridylate; and (c) an increase in the intracellular pools of 5,10-methylenetetrahydrofolate and tetrahydrofolate. These data suggest that cross-resistance to 5-fluorouracil and methotrexate in A2780 cells may be a consequence of increases in their respective target enzymes.

Acquisition of resistance to initially effective drugs and cross-resistance to seemingly unrelated drugs has plagued the treatment of ovarian carcinoma, as it has the treatment of most solid tumors. The efficacy of cisplatin in this disease fits into this same general pattern [1, 2]. Several different mechanisms for resistance to cisplatin have been demonstrated, e.g. decreased cross-linking of DNA [3], increased rates of DNA repair [4], and increased levels of intracellular thiols [5]. However, no one mechanism has been uniformly present. For example, sensitivity to cisplatin appears to correlate with glutathione levels in A2780 human ovarian carcinoma cells [5] but not in other cell lines [6]. Likewise, decreased cellular accumulation of cisplatin occurs in cisplatin-resistant sublines of a human squamous carcinoma cell line SCC-25 [7] and a human ovarian carcinoma cell line 2008 [8] but is not associated with resistance to cisplatin in two leukemic cell lines [9, 10].

Recent studies have implicated an interaction between cisplatin and folate metabolism in the mechanism of resistance. Resistance to cisplatin is associated with cross-resistance to the folate antagonist methotrexate (MTX¶) in P388 murine leukemia [11] and in SSC-25 human carcinoma [12]. A MTX-resistant subline of L1210 murine leukemia is also cross-resistant to cisplatin [13]. In A2780 cells, cisplatin treatment causes a rise in 5,10-methylenetetrahydrofolate (CH_2-H_4-folate) and tetrahydrofolate (H_4-folate) levels, which is associated with increased sensitivity to 5-fluorouracil (5-FUra) [14]. These results led us to investigate the possibility of collateral resistance or sensitivity to MTX and 5-FUra in a subline of A2780 resistant to cisplatin.

MATERIALS AND METHODS

The following chemicals were obtained from the companies indicated: 5-FUra, Hoffmann-La Roche, Nutley, NJ; cisplatin and MTX, Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD; [5-^{3}H]dUMP (22 Ci/mmol), [6-^{3}H]5-fluorodeoxyuridine (FdUrd) (20 Ci/mmol) and [6-^{3}H]5-fluorodeoxyuridylate (FdUMP) (20 Ci/mmol), Moravek Biochemicals, Inc., Brea, CA; and [^{195m}Pt]cisplatin (145 mCi/mmol), Oak Ridge National Laboratories, Oak Ridge, TN. (6*S*)-H_4-Folate was a gift from Dr. R. Moran, Childrens' Hospital of Los Angeles.

Cell culture. Both the original A2780 human ovarian cancer cell line [15] and a platinum-resistant subline selected *in vitro* by stepwise increases in the concentration of cisplatin in the medium [16] were provided by Dr. R. Ozols, National Cancer Institute. The methodology for cell culture and cell proliferation studies has been described [14]. The generation times for A2780S and A2780DDP cells were 26 ± 2 and 25 ± 2 hr, respectively. Although the resistance to cisplatin in A2780DDP was only 3-fold using our assay conditions, whereas it was 7-fold in a colony formation assay [16], this degree of resistance was stable.

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¶ Abbreviations: CH_2-H_4-folate, 5,10-methylenetetrahydrofolate; DHFR, dihydrofolate reductase; 5-FUra, 5-fluorouracil; FdUrd, 5-fluorodeoxyuridine; FdUMP, 5-fluorodeoxyuridylate; H_4-folate, tetrahydrofolate; and MTX, methotrexate.

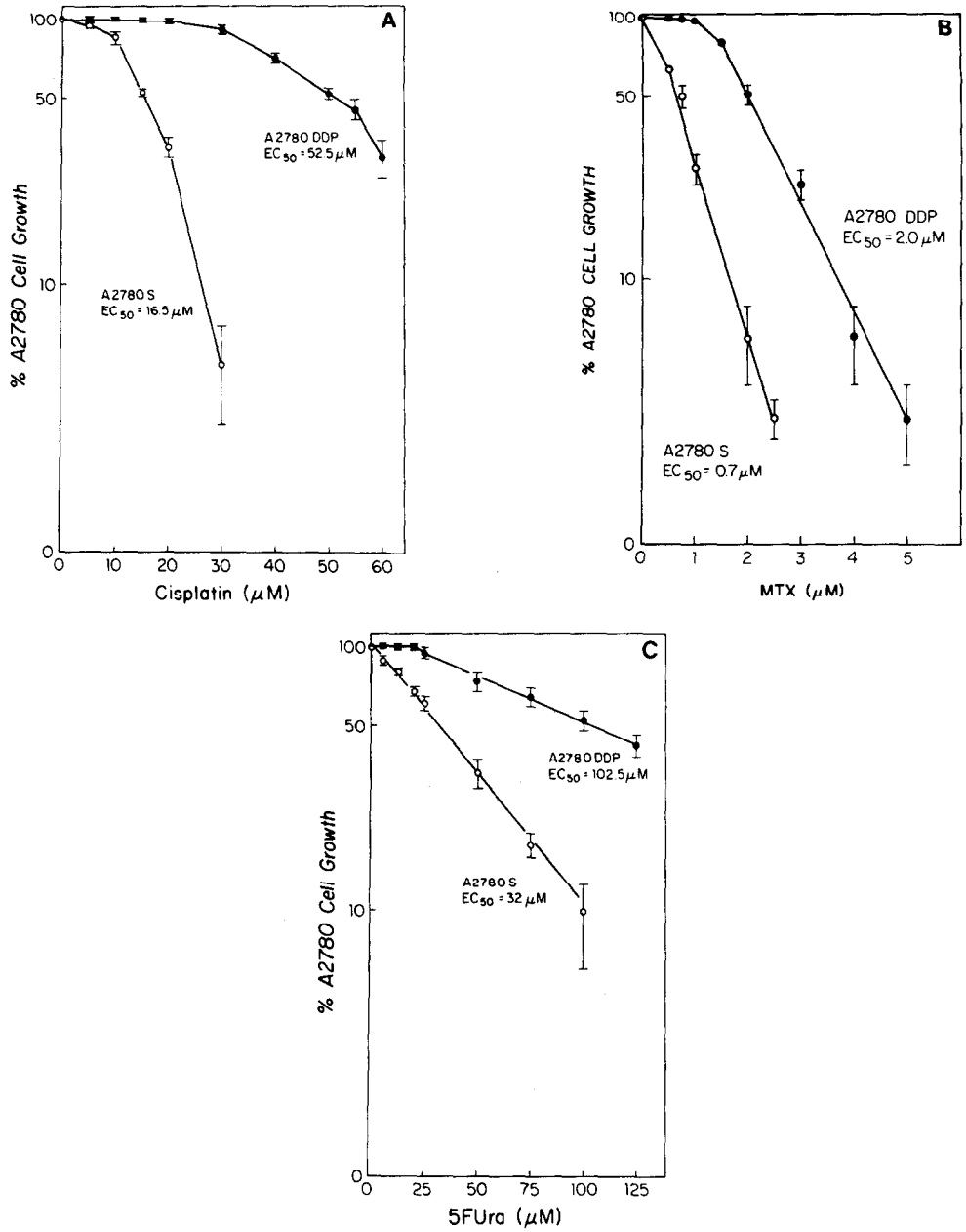

Fig. 1. Cell growth in the presence of chemotherapeutic agents. A2780S (○) and A2780DDP (●) cells were exposed to cisplatin for 1 hr (A), or MTX for 2 hr (B) or 5-FUra for 2 hr (C). The cultures were rinsed and refed with drug-free medium and returned to the incubator. The cells were counted 6 days later. Cultures not exposed to the drug grew from 5×10^3 cells/30 mm dish to approximately 1×10^5 cells/30 mm dish during this interval.

Transport studies. The accumulated radioactivity was measured after incubation with radioactive cisplatin at concentrations of 2, 10 or 25 μM (145 mCi/mmol, dissolved in Earle's balanced salt solution) as described [14]. For the 0-time accumulation, the radioactive cisplatin was added to the flasks containing the cells; the flasks were immediately placed on ice; and the cells were washed and processed as for the later time points. The data are presented graphically without subtracting this 0-time accumulation.

dTMP Synthase, enzyme activity and capacity to complex FdUMP. The activity of dTMP synthase, as determined by both the release of tritium from [5-^{3}H]dUMP [17] and the formation of a complex with [6-^{3}H]FdUMP in the presence of excess CH_2-H_4-folate [18], was measured in the freshly prepared 12,000 *g* supernatant fraction from A2780 cells as described [14].

Reduced folate pools. Approximately 10^7 cells were removed from flasks with trypsin, counted, resuspended in buffer (10 mM Tris–HCl, 1 mM EDTA, 250 mM sucrose, 1% ascorbic acid) and frozen at −70°. H_4-Folate and CH_2-H_4-folate con-

centrations in cell lysates were estimated by their incorporation into a covalent ternary complex with *Lactobacillus casei* dTMP synthase and [^{3}H]FdUMP in the presence (H_4-folate + CH_2-H_4-folate) and the absence (CH_2-H_4-folate only) of formaldehyde using the method of Priest *et al.* [19].

Incorporation of [^{3}H]*FdUrd into dTMP synthase ternary complex in intact cells.* The amount of FdUMP-CH_2-H_4-folate-dTMP synthase in the cells after incubation of intact cells with [6-^{3}H]FdUrd was determined as the difference in the amount of acid-insoluble radioactive material with and without pre-incubation at 65° [20], as described [14], except that the concentration of [^{3}H]FdUrd was increased to 25 nM for the current experiments.

HPLC identification of material released at 65°. Cells were incubated with [^{3}H]FdUrd, trypsinized, and lysed as for the determination of dTMP synthase ternary complex in intact cells. At that point free nucleotides were removed by treatment with activated charcoal prepared as described for the FdUMP-binding assay [18]. Following centrifugation small charcoal particles remaining in the supernatant cytosol were removed by filtration through Whatman GF-C paper. One aliquot of the cytosol was precipitated with trichloroacetic acid immediately, and a second aliquot was first heated to 65° for 15 min. Care was taken to keep the samples at 0–4°, except when specifically heated to 65°. After neutralization [21], non-radioactive FdUMP was added as an internal standard, and FdUrd metabolites were separated using reverse-phase ion-pair HPLC. The column (4.6 × 150 mm) was packed with 5 μm C_{18}-bonded silica gel (ultrasphere-ODS). Flowing at 1.2 ml/min, a 20-min linear gradient was generated from 5 mM tetrabutylammonium sulfate, 5 mM potassium phosphate, pH 6, to the same buffer in 18.5% methanol. The pertinent retention times were: FdUrd, 7.0 min; 5-fluorouridylate, 11.1 min; and FdUMP, 14.4 min. The effluent was collected in 0.6-ml fractions, in which radioactivity was measured by scintillation spectrometry. Prior to injecting each sample, the column was washed with buffer containing 40% methanol and then re-equilibrated with the starting buffer.

All data are expressed as means ± SD of at least duplicate determinations in at least two experiments. Error bars in the figures also represent SD.

RESULTS

Tumor cell growth in the presence of cancer chemotherapeutic agents. The concentration of cisplatin that reduced by one-half cell proliferation during 6 days subsequent to a 1-hr incubation with the drug was 16.5 μM for A2780S cells and 52.5 μM for A2780DDP cells (Fig. 1A). A2780DDP cells were collaterally resistant to MTX and 5-FUra, 2.9 and 3.2-fold, respectively (Fig. 1, B and C).

Uptake and release of [^{19m}Pt]*cisplatin.* The 0-time point uptake of cisplatin in the A2780DDP cells exposed to 10 μM cisplatin was 22% less than in the A2780S cells, and the rates of uptake of cisplatin during the next 75 min were 2.0 ± 0.1 and 1.9 ± 0.1 pmol/min/mg protein for A2780S and A2780DDP cells respectively (Fig. 2). This similarity

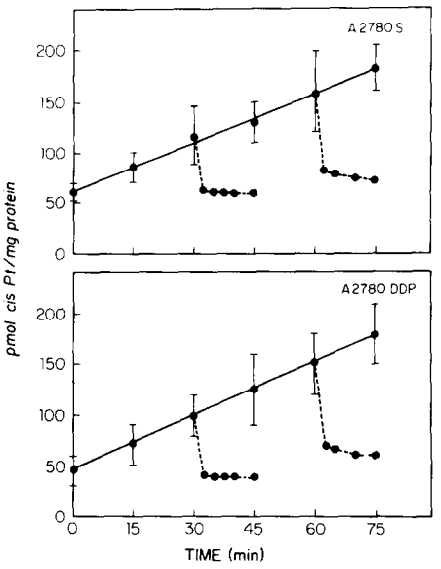

Fig. 2. Uptake and release of cisplatin in A2780 cells. A2780S cells (upper panel) and A2780DDP cells (lower panel) were exposed to cisplatin (10 μM). The uptake (——) was measured over 75 min. The release (– – – –) of cisplatin was measured at 30 and 60 min by incubating the A2780 cells in drug-free medium at 37°.

of uptake was also evident in samples taken between 2 and 8 min and at concentrations of cisplatin as low as 2 μM (data not shown). At 25 μM cisplatin, a concentration above the EC_{50} in the sensitive line, the rate of uptake was 18% lower in the A2780DDP cells than in the A2780S cells (data not shown).

The release of cisplatin at 37° was virtually complete in 5 min in both cell lines (Fig. 2). After 60 min of uptake at an extracellular concentration of 10 μM cisplatin followed by 15 min of release, the amount of radioactivity remaining was only 5% greater than the cisplatin taken up at the 0-time point.

dTMP Synthase. dTMP Synthase activity as measured by the tritium release assay was increased 2.5-fold in cytosols from A2780DDP cells in comparison with cytosols from A2780S cells. Associated with this increase in enzyme activity were increases in the capacity to complex [^{3}H]FdUMP *in vitro* in the presence of added folate cofactor and the levels of CH_2-H_4-folate and H_4-folate (Table 1).

FdUMP complexed with dTMP synthase in intact cells. Incorporation of [^{3}H]FdUrd into the dTMP synthase–FdUMP complex was 1.7 times higher in the resistant line than in the sensitive line (Table 1). HPLC of the acid-soluble material released by heating to 65° for 15 min confirmed that it was FdUMP; 97 ± 7% of the radioactivity injected onto the HPLC column was recovered in the fractions of effluent collected, and 94 ± 2% of the material recovered coeluted with the nonradioactive FdUMP standard.

DISCUSSION

There were small differences between the 0-time point uptakes of cisplatin in the sensitive and resistant cell lines. The rate of uptake of cisplatin at an

Table 1. Properties of extracts of A2780S and A2780DDP cells related to dTMP synthase activity

Properties of A2780 cells*	A2780S	A2780DDP
dTMP synthase (pmol/min/10^7 cells)	170 ± 6†	420 ± 20
FdUMP complex formed *in vitro* (pmol/10^7 cells)	1.6 ± 0.5	4.1 ± 0.5
5,10-CH_2-H_4-folate (pmol/10^7 cells)	2.9 ± 0.4†	7.2 ± 0.5
H_4-folate (pmol/10^7 cells)	2.2 ± 0.3†	4.8 ± 0.5
FdUMP complex formed in intact cells (pmol/10^7 cells)	1.8 ± 0.5	3.1 ± 0.2

* All analyses were performed as described in Materials and Methods using approximately 10^7 cells for each sample.
† Ref. 14.

extracellular concentration of 25 μM was also 18% less in the resistant cells, although this phenomenon was not seen at lower concentrations of cisplatin. These differences in drug accumulation seem unlikely, in themselves, to explain the 3-fold difference in the sensitivity of the two cell lines to cisplatin. Other investigators have described a subline of the 2008 ovarian carcinoma cell line made 4-fold resistant to cisplatin which accumulates the drug at one-half of the rate of the parent line [8] and a 20-fold resistant subline of the SCC-25 squamous carcinoma cell line in which the rate of uptake of cisplatin is only 24% lower than in the parent line [7]. However, these data do not rule out the possibility that there are differences in the accumulation of cisplatin into some critical intracellular compartment or in the accumulation of some critical form of cisplatin, e.g. cisplatin bound to thiols.

The observed resistance to MTX in A2780DPP cells (Fig. 1B) is consistent with previously reported cross-resistance in unrelated cell lines [11, 12]. Recent results regarding the activity of dihydrofolate reductase (DHFR) in this cell line [22] suggest a plausible mechanism for resistance to MTX. DHFR activity was higher by a factor of 2.5 in the A2780DDP cells compared to the A2780S cells. The ratio of the methotrexate-binding capacities was 2.8, also indicating increased DHFR.

Increased intracellular levels of H_4-folate and CH_2-H_4-folate in the cisplatin-resistant subline (Table 1) are consistent with the hypothesis that there is an interaction between resistance to cisplatin and folate metabolism in this cell line, although the causal link between the two is not yet completely understood. Short-term treatment of the parental A2780 cell line with cisplatin also led to increased levels of these two folates, as well as increased formation of the FdUMP–dTMP synthase complex and *increased* sensitivity to 5-FUra [14]. In contrast, the A2780DDP subline was less sensitive to 5-FUra (Fig. 1C). The resolution of this paradox apparently lies in the fact that in the cisplatin-resistant subline the total amount of dTMP synthase available was increased to a greater extent than was the FdUMP–dTMP synthase complex formation in intact cells (Table 1).

The potential for collateral sensitivity or resistance between drugs and the potential for synergism or antagonism when those same drugs are used in rapid sequence are distinct phenomena. The results reported here should not be interpreted to mean that cisplatin should not be used in combination with MTX or 5-FUra. However, the cross-resistance between cisplatin and two antimetabolites demonstrated in this model system may have clinical implications in regard to the treatment of patients whose tumors have become resistant to cisplatin. The possibility of such cross-resistance warrants further study with samples of resistant tumors taken directly from patients.

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