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Biochemical basis for cisplatin and 5-fluorouracil synergism in human ovarian carcinoma cells

(folates/thymidylate synthase ternary complex/combination chemotherapy)

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ABSTRACT **The human ovarian cell line A2780 was exposed to either cisplatin (10 μM) or 5-fluorouracil (5FUra) (5 μM) for 1 hr. Cytotoxicity was less than 14% with either agent alone. Cisplatin (10 μM) and 5FUra (5 μM) in combination for 1 hr caused a 76% reduction in cell growth. Thymidine (dThd, 10 μM), if given concomitantly with the combination of cisplatin and 5FUra, completely protected the tumor cells. A 30-min exposure to cisplatin increased the intracellular pools of 5,10-methylenetetrahydrofolate and tetrahydrofolate 2.5-fold. The capacity of intact cells to form 5-fluorodeoxyuridylate (FdUMP)–thymidylate (dTMP) synthase complex when incubated with fluorodeoxyuridine (FdUrd) was enhanced 2.5-fold when the cells were pretreated with cisplatin. These experiments demonstrate that cisplatin can increase the availability of the reduced folate necessary for tight binding of FdUMP to dTMP synthase, thus enhancing the cytotoxicity of the cisplatin and 5FUra combination.**

5-Fluorouracil (5FUra) and *cis*-diamminedichloroplatinum-(II) (cisplatin) in combination have been shown to have synergistic cytotoxicity against both murine and human neoplasms (1, 2). The mechanism of this synergy has not been elucidated. 5FUra is metabolized within cells to 5-fluorodeoxyuridine monophosphate (FdUMP). FdUMP can covalently bind to dTMP synthase in the presence of 5,10-methylenetetrahydrofolate (CH_2-H_4folate) and inhibit DNA synthesis by depleting the cells of dTMP. 5FUra is also metabolized to 5-fluorouridine triphosphate (FUTP), which is incorporated into RNA. One or both of these 5FUra metabolites account for the antineoplastic activity of 5FUra in experimental models (3). The antitumor activity of the second agent, cisplatin, may be explained in part by DNA cross-linking (4, 5), interactions with the cell-surface nucleic acids (6) or with the plasma membrane (7–9). Also, cisplatin can inhibit methionine uptake into tumor cells and cause perturbation of the methionine pools (7–9). Cells may respond by increasing methionine biosynthesis and increasing the pools of folate cofactors (10). The interaction of these two chemotherapeutic drugs was studied in patients with human ovarian cancer (11). Data presented in this report suggest that cisplatin treatment does increase the CH_2-H_4folate pool and the binding of FdUMP to dTMP synthase. The resultant inhibition of dTMP synthesis appears to be responsible for the cytotoxicity of the 5FUra and cisplatin combination.

MATERIALS AND METHODS

The following chemicals were obtained from these companies: thymidine, Sigma; 5FUra, Hoffmann–La Roche; deoxyuridine, P-L Biochemicals; cisplatin and 5-fluorodeoxyuridine (FdUrd), Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD; 5F[6-^{3}H]dUrd (20 Ci/mmol; 1 Ci = 37 GBq), F[6-^{3}H]dUMP (20 Ci/mmol), [5-^{3}H]dUMP (20 Ci/mmol), and 5F[6-^{3}H]Ura (20 Ci/mmol), Moravek Biochemicals, Brea, CA; and (^{195m}Pt) cisplatin (145 mCi/mmol), Oak Ridge National Laboratories, Oak Ridge, TN.

Cell Culture. The human ovarian cancer cell line A2780 was obtained from R. Ozols, National Cancer Institute (12) and maintained in RPMI 1640 with 10% (vol/vol) fetal calf serum, penicillin (62.9 mg/liter) and streptomycin (100 mg/liter) (GIBCO). Cells were exposed to chemotherapeutic agents for only 60 min in RPMI 1640 medium and 10% (vol/vol) fetal calf serum and then washed three times with drug-free RPMI 1640 medium with 10% (vol/vol) fetal calf serum. Cell growth studies were determined by the increases in cell number after 7 days. Cells were trypsinized and counted on a Coulter Counter ZBI as described (8).

Transport Studies. The uptake of radioactive cisplatin (10 μM; 145 mCi/mmol; dissolved in Earle's balanced salt solution) into A2780 cells (cultured in 30-mm diameter Petri dishes) was measured as described (8). [^{3}H]5FUra (5 μM, 2 × 10^6 dpm/μmol) uptake over 60 min was quantified as follows. After the 60-min incubation, the A2780 cells were washed three times with ice-cold Dulbecco's phosphate-buffered saline (PBS, without $CaCl_2$ and $MgCl_2$, GIBCO). The radioactive material associated with these cells was solubilized by incubating the cells overnight in 1 M NaOH. Aliquots were saved for protein determination, and the remainder was neutralized with 1 M HCl. The radioactivity was determined by scintillation spectrometry.

dTMP Synthase Assay. dTMP synthase activity was measured in extracts of A2780 cells by the amount of tritium released to H_2O from [5-^{3}H]dUMP as described (13), except that 1% Triton X-100 was included in the buffer to extract the cells.

FdUMP Binding Assay. The number of FdUMP binding sites was determined in A2780 extracts by incubation with tetrahydrofolate-H_4folate; (500 μM), formaldehyde (0.22%), and [^{3}H]FdUMP (200 nM) for 15 min at 30°C (14). Free [^{3}H]FdUMP was removed from the incubation mixture by the addition of activated charcoal (10 mg/ml) in bovine serum albumin (2.5 mg/ml) and Dextran T-70 (0.25 mg/ml) and subsequent centrifugation. dTMP synthase–FdUMP–CH_2-H_4folate complex (TS-ternary complex) was precipitated from the resulting supernatant by the addition of trichloroacetic acid. The acid-insoluble material was washed three times, and the radioactivity was counted by scintillation spectrometry. A blank reaction mixture including all but the supernatant was processed in the same manner. The radio-

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Abbreviations: CH_2-H_4folate, 5,10-methylenetetrahydrofolate; H_4-folate, tetrahydrofolate; TS-ternary complex, dTMP synthase–FdUMP–CH_2-H_4folate complex.

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activity measured in any sample was at least two-fold greater than the blank radioactivity.

Reduced Folate Pools. H_4folate and CH_2-H_4folate concentrations were estimated by their incorporation into a covalent ternary complex with *Lactobacillus casei* dTMP synthase and [^{3}H]FdUMP in the presence of (H_4folate and CH_2-H_4folate) and the absence of formaldehyde (CH_2-H_4folate only) (15). Although high concentrations of other folates may also promote limited binding of FdUMP to dTMP synthase, the binding of CH_2-H_4folate should predominate in these reactions.

Incorporation of [^{3}H]FdUrd into TS-Ternary Complex in Intact Cells. The following method is based on the observation by Washtien and Santi (16) that the FdUMP–CH_2-H_4folate–dTMP synthase complex dissociates at 65°C. The A2780 cells in Earle's balanced salt solution were incubated with [^{3}H]FdUrd (5 nM) for 60 min. The cells were washed four times with ice-cold PBS and removed from the flask by trypsinization. Aliquots were taken for cell number and protein determination, the remaining cells were lysed with water, then either precipitated with trichloroacetic acid immediately or heated to 65°C for 15 min prior to precipitation. The acid-insoluble material was collected on Whatman glass-fiber filters that were washed four times with ice-cold trichloroacetic acid and once with 70% (vol/vol) ethanol. Then the filters were dried, and radioactivity was determined by scintillation spectrometry.

Determination of Protein. Cells were extracted with trichloroacetic acid as described above, and the precipitated protein was collected by centrifugation. The protein pellet was dissolved in 0.1 M NaOH, and the protein concentration was determined by the method of Bradford (17), using the Bio-Rad protein assay kit. Bovine serum was used as the standard.

RESULTS

Exposure of A2780 cells for 60 min to the sequence of cisplatin (10 μM) for 30 min and then of 5FUra (5 μM) for 30 min reduced cell growth to 24 ± 5.1% (Table 1). In contrast, at these concentrations neither of the two drugs alone reduced cell growth below 86% of the control. The sequencing of 5FUra (5 μM) 30 min prior to the addition of cisplatin (10 μM) for 30 min resulted in 41 ± 6.3% cell growth. The ED_{50}s for cisplatin for 1 hr and 5FUra for 2 hr were 16.5 μM and 32 μM, respectively. If inhibition of dTMP synthase is critical for the cytotoxicity of this drug combination, then the addition of dThd to circumvent the need for *de novo* dTMP synthesis should protect the tumor cells from the synergistic action of the drugs. dThd alone for 1 hr at a concentration of 10 μM was not cytotoxic to the A2780 cells (Table 1). When 10 μM dThd was given in combination with cisplatin (10 μM) and 5FUra (5 μM), the cells were completely protected from the cytotoxic action of the cisplatin/5FUra combination as cell growth reverted to control levels.

Table 1. Inhibition of A2780 cell growth by cisplatin and 5FUra

Concentration, μM			
Cisplatin	5FUra	dThd	% control growth
1	—	—	96
2	—	—	91
10	—	—	86
—	5	—	106
—	10	—	93
—	—	10	103
2	5	—	30
2	5	10	110
10	5	—	24
10	5	10	103

A2780 cells (5×10^3 cells) were exposed to cisplatin, dThd, and/or 5FUra under various conditions. The cells were pretreated for 30 min with cisplatin alone or with dThd, then 5FUra was added for 30 min. Thus, the A2780 cells were preincubated for a total of 60 min with the drugs, then incubated for 7 days in a drug-free medium. These experiments were performed in triplicate. The final cell number was $0.5–1.0 \times 10^5$ cells per culture in untreated cultures (100%).

Table 2. [^{3}H]FdUMP binding assay in A2780 cell supernatants

Exogenous CH_2-H_4folate	[^{3}H]FdUMP binding sites, pmol per 10^7 cells
−	0.42 ± 0.02
+	1.06 ± 0.05

The number of FdUMP binding sites was determined in the freshly prepared 12,000 × *g* supernatant fraction from A2780 cells as described (14). +, H_4folate and formaldehyde were added to the 12,000 × *g* supernatant to generate CH_2-H_4folate for maximum FdUMP binding. −, No exogenous CH_2-H_4folate was added.

Drug uptake studies were done to determine if altered accumulation of one or both of the drugs could explain the observed synergy. The uptake of radioactive cisplatin (10 μM) in the absence and presence of 5FUra (5 μM) was 70.4 ± 8.3 and 75.12 ± 9.2 pmol/mg of protein per 60 min, respectively. The uptake of radioactive 5FUra (5 μM) in the absence of cisplatin was 13.95 ± 1.74 and in the presence of cisplatin 14.4 ± 2.46 pmol/mg of protein per 60 min. The uptake of both drugs was linear over 60 min for three time points.

The incorporation of [6-^{3}H]dUrd (5 μM) into DNA was 230 pmol/mg of protein per 60 min. Cisplatin (10 μM) or 5FUra (5 μM) alone only slightly inhibited [^{3}H]dUrd incorporation into DNA, 21.1% and 7.5%, respectively. However, the combination of these drugs at the above concentrations inhibited the incorporation of [^{3}H]dUrd into DNA by 70.4%. At nanomolar concentrations of dUrd, this combination completely inhibited DNA synthesis (unpublished data).

Since cisplatin can react with proteins, a direct effect of cisplatin on dTMP synthase was considered. The addition of 10 μM cisplatin *in vitro* to the 12,000 × *g* supernatant fraction from A2780 cells did not alter dTMP synthase activity as measured by tritium release from [5-^{3}H]dUMP. The dTMP synthase activity was 170 pmol per 10^7 cells per min with or without cisplatin (10 μM). Cisplatin (10 μM) did not alter the number of [^{3}H]FdUMP binding sites in this fraction. Maximum FdUMP binding *in vitro* was achieved only when H_4folate and formaldehyde were added to the A2780 supernatant fraction (Table 2).

Therefore, the CH_2-H_4folate pool might limit the ability of the 5FUra metabolite, 5FdUMP, to kill cells by inhibiting dTMP synthase. This was examined directly by measuring the folate pools in cell-free extracts. The A2780 cells contained CH_2-H_4folate and H_4folate pools of 0.29 ± 0.04 and 0.22 ± 0.03 pmol per 10^6 cells, respectively. Pretreatment for 30 min with 10 μM cisplatin elevated the reduced folate pools by 60% (Table 3). Since the folate pools may be compartmentalized, the extent of FdUMP binding in intact cells was also measured using FdUrd as a precursor of FdUMP capable of crossing the cell membrane. [^{3}H]FdUrd (5 nM) incorpo-

Table 3. Changes in the folate pools after treatment with cisplatin in A2780 cells

Treatment	CH_2-H_4folate, pmol per 10^6 cells	H_4folate, pmol per 10^6 cells
Control	0.29 ± 0.04	0.22 ± 0.03
Cisplatin treated	0.49 ± 0.04	0.42 ± 0.05

A2780 cells ($1–2 \times 10^7$ cells) were incubated at 37°C for 30 min in the presence or absence of 10 μM cisplatin. Cells were then analyzed for folates.

ration into heat-labile acid-insoluble material was 0.55 pmol per 10^7 cells after 90 min. Pretreatment with cisplatin produced a 2.5-fold increase in the amount of heat-labile, acid-insoluble material (Table 4).

DISCUSSION

Cisplatin and 5FUra are synergistically cytotoxic in L1210 cells (1). This combination has also been used clinically in the treatment of solid tumors (2). Using the human ovarian carcinoma line A2780 in culture; we have shown that low concentrations of cisplatin followed by 5FUra are more cytotoxic than the reverse sequence or either drug alone. The duration of drug exposure in these studies was based on the serum half-lives of these agents in patients (4). Thus, clinically achievable exposures to cisplatin and 5FUra produced synergistic toxicity toward these ovarian carcinoma cells. At these concentrations, cisplatin uptake was not influenced by 5FUra and, conversely, 5FUra uptake was not influenced by cisplatin. Thymidine nullified the effects of this drug combination, suggesting that dTMP synthase could be the site of action. *In vivo* studies with 5 μM [^{3}H]dUrd demonstrated that the combination of cisplatin and 5FUra inhibited dTMP synthase as measured by the incorporation of the radioactivity into DNA. At lower concentrations of [^{3}H]dUrd (5 nM), cisplatin and 5FUra completely inhibited incorporation, but cisplatin partially inhibited the transport of the radioactive deoxynucleoside. Cisplatin did not have a direct effect on dTMP synthase in cell-free extracts, confirming results obtained with the bacterial enzyme (18). Cisplatin also did not affect the metabolism of fluoropyrimidines to FdUMP (unpublished data). Pretreatment of A2780 cells with cisplatin increased the pools of CH_2-H_4folate and H_4folate and caused a 2.5-fold increase in the FdUMP binding to dTMP synthase in intact cells. The CH_2-H_4folate and H_4folate pools are less in the untreated A2780 cells than in the mouse leukemia L1210 cell line by a factor of 10 (19). It has been suggested (20) that in some human solid tumors the availability of reduced folates might be rate limiting for maximum FdUMP binding to dTMP synthase. Our data suggest that in the A2780 cell line the CH_2-H_4folate pool limited the binding of FdUMP to dTMP synthase. We have shown that cisplatin interacts selectively at the tumor membrane and inhibits methionine uptake in other tumor cells (8, 9). Changes in the methionine pools have been shown to alter the intracellular concentrations of reduced folates (10). Perhaps this is the mechanism by which sublethal doses of cisplatin can increase CH_2-H_4folate and make the tumor cells more sensitive to fluoropyrimidines.

Table 4. Formation of TS-ternary complex in intact A2780 cells

Treatment	Cisplatin, μM	Net acid-soluble ^{3}H-labeled material, pmol of [^{3}H]FdUMP per 10^7 cells
Control	—	0.55
Cisplatin	10	1.36

Cells were pretreated with cisplatin for 30 min prior to the addition of 5 nM [^{3}H]FdUrd for 60 min, and the amount of TS-ternary complex formed was determined.

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