0026-895X/95/051072-08$3.00/0

MOLECULAR PHARMACOLOGY, 47:1072–1079 (1995).

Arabinosyl-2-fluoroadenine Augments Cisplatin Cytotoxicity and Inhibits Cisplatin-DNA Cross-link Repair

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Received December 8, 1994; Accepted February 21, 1995

SUMMARY

Cytotoxicity was increased significantly when arabinosyl-2-fluoroadenine (F-ara-A) was administered in simultaneous combination with cisplatin (CDDP) to human colon tumor cell lines relatively sensitive (LoVo) or resistant (CP2.0) to CDDP. Because the mechanism of action of F-ara-A indicates that it may be an effective inhibitor of DNA repair, we hypothesized that F-ara-A induces cytotoxic augmentation by suppressing cellular repair in CDDP-damaged DNA lesions. To test this, we compared the repair of CDDP-induced DNA interstrand cross-links in the total genome and in *ERCC1* gene-specific sequences of LoVo and CP2.0 cells for treatments with CDDP and CDDP plus F-ara-A. We determined the DNA repair by measuring the rate of removal of the cross-links, using two methods, i.e., an ethidium bromide fluorescence binding assay, which detects the DNA lesion in the total genome, and a method combining denaturation/renaturation neutral agarose gel electrophoresis and Southern hybridization to detect gene-specific lesions. When F-ara-A (15 μM) was coadministered with CDDP (15 μg/ml for LoVo cells and 30 μg/ml for CP2.0 cells) for 4 hr, the initial cross-link index for the total genome was increased 67% (4.5 versus 2.7 with CDDP alone) in LoVo cells and 93% (2.9 versus 1.5 with CDDP alone) in resistant CP2.0 cells. At 10 hr after the treatment, only 5% of the cross-links had been removed in combination-treated LoVo cells, compared with 40% in CDDP-treated LoVo cells; in CP2.0 cells, F-ara-A inhibited the removal of cross-links from 95% to 45%. Similar results were obtained for *ERCC1* gene-specific DNA sequences. These data suggest that F-ara-A enhances the accumulation of CDDP-induced cross-links in LoVo and CP2.0 cells by suppressing the repair of such lesions, thereby enhancing the cytotoxicity of CDDP in combination treatment.

CDDP is an effective antitumor agent for a broad spectrum of human tumors. However, therapeutic responses vary among patients, and the emergence of resistance to this drug is frequently encountered (1). Therefore, identification of strategies for enhancing tumor sensitivity to this drug by circumventing intrinsic and acquired CDDP resistance is quite desirable, to improve the therapeutic index of CDDP-based regimens.

DNA cross-linking is believed to be a primary cytotoxic mechanism of CDDP-based chemotherapies. CDDP most often disrupts DNA by inducing intrastrand cross-links between two adjacent purines and, to a lesser extent, interstrand cross-links and monoadducts (2). Although inter- and intrastrand cross-links are potentially lethal, differences in the extent of their repair should affect the magnitude of cell kill. In fact, evidence indicates that an inverse relationship exists between cellular repair capacity and cytotoxic sensitivity (3).

With *in vitro* studies, we and others showed that CDDP produced fewer DNA interstrand cross-links in CDDP-resistant cells, compared with sensitive parental cells (4, 5). Among the several proposed mechanisms that can contribute to cellular resistance to DNA damage (6–8), increased cellular capacity for DNA repair has been under active investigation (9, 10). It is known that CDDP-resistant cells often express elevated levels of mRNA for the genes encoding the enzymes that are directly (e.g., DNA polymerases, DNA ligase I, and human excision nuclease *ERCC1*) or indirectly (e.g., dihydrofolate reductase and thymidine kinase) involved in DNA repair (4, 11–14).

We previously reported that ara-C produced a synergistic cytotoxicity with CDDP in LoVo human colon cancer cells, possibly in part by increasing the formation of CDDP-induced DNA interstrand cross-links. However, a relatively high dose of ara-C was required for the synergistic effect, and these actions were tissue restricted (15, 16). F-ara-A, the

This work was supported by Grant CA28596 from the National Cancer Institute, Grant DHP-1 from the American Cancer Society, and a Physician Referral Services Clinical Research Grant from The University of Texas M. D. Anderson Cancer Center.

ABBREVIATIONS: CDDP, cisplatin [*cis*-diamminedichloroplatinum(II)]; ara-C, 1-β-D-arabinofuranosylcytosine; F-ara-A, arabinosyl-2-fluoroadenine; EBFA, ethidium bromide fluorescence binding assay; fludarabine, arabinosyl-2-fluoroadenine monophosphate; CI, combination index; DRI, dose-reduction index; CLI, cross-link index; kb, kilobase(s); F-ara-ATP, arabinosyl-2-fluoroadenine triphosphate.

structurally related arabinosyl nucleoside of the clinically active drug fludarabine (17), was metabolically more stable than ara-C and produced cytotoxic effects by other mechanisms. For instance, in addition to its inhibition of DNA strand elongation by DNA polymerases (18–22), F-ara-ATP inhibits other key enzymes involved in DNA metabolism, e.g., ribonucleotide reductase (21, 22), DNA primase (23, 24), the polymerase ϵ-associated 3′ to 5′ proofreading exonuclease (25), and DNA ligase I (26). These actions imply that F-ara-A is potentially an effective inhibitor of DNA repair. Herein we report that F-ara-A synergistically enhances the cytotoxicity of CDDP and the synergy is associated with the inhibition of cellular repair of the CDDP-induced interstrand cross-links by F-ara-A.

Materials and Methods

Drugs. F-ara-A was prepared by dephosphorylation of fludarabine (kindly provided by Berlex Biosciences, Alameda, CA) with alkaline phosphatase (*Escherichia coli* type III; Sigma Chemical Co.). A 500 mM solution of fludarabine in 50 mM Tris·HCl, pH 8.5, was incubated with alkaline phosphatase (10 units/ml) at 37° for 1 hr. The reaction mixture was stored at 4° overnight, and F-ara-A crystals were washed on a hardened Whatman filter (no. 50), dried under vacuum, and stored at −20°. CDDP was obtained in liquid form from Bristol-Myers Squibb (Princeton, NJ). Immediately before use, each drug was diluted with growth medium to the appropriate working concentrations.

Cell lines. The LoVo human colon carcinoma cell line and its 5-fold more CDDP-resistant counterpart CP2.0 were used in this study; their characteristics have been detailed previously (6, 16). The resistant CP2.0 line was selected from the parental LoVo cells with CDDP, by the conventional method of continuous drug exposure. Both cell lines were maintained in Ham's F-10 medium supplemented with 10% fetal bovine serum, in the presence (CP2.0) or absence (LoVo) of CDDP (2 μg/ml). Before the experiments, CP2.0 cells were grown without CDDP for one cell cycle (about 50 hr).

Cytotoxicity assay. Cytotoxicity was evaluated by clonogenic assay, as described previously (16). Briefly, the dose-response relationship for cell survival was determined by exposing LoVo or CP2.0 cells to increasing concentrations of F-ara-A at 37° for 2 hr. In time course experiments, the cells were incubated with various doses of F-ara-A (1, 5, 7.5, and 15 μM for LoVo cells and 1, 5, 15, and 45 μM for CP2.0 cells) for 4–48 hr. At the end of the drug treatment, the cells were harvested and plated into 60-mm Petri dishes in Ham's F-10 medium, at various cell densities so that 50–100 colonies/dish would be obtained after a 2-week incubation. The absolute plating efficiencies were 41 ± 11% for LoVo cells and 20 ± 7% for CP2.0 cells. The survival fractions were calculated by setting the plating efficiency of untreated control cells as 100%.

To study the cytotoxic effects of the two agents in combination, the cells were treated for 4 hr with increasing concentrations of F-ara-A (2.5, 5.0, 7.5, 10, and 15 μM for LoVo cells and 2.5, 5.0, 7.5, 15, and 30 μM for CP2.0 cells) or CDDP (1, 2, 3, 4, and 6 μg/ml for LoVo cells and 2.5, 5.0, 7.5, 15, and 30 μg/ml for CP2.0 cells) as single agents or with a combination of the two agents at concentrations in a fixed ratio (molar ratio of F-ara-A to CDDP of 1.5:2 for LoVo cells and 1.5:5 for CP2.0 cells). The interaction between F-ara-A and CDDP was analyzed by the median-effect method described by Chou and Talalay (27).

Analysis of interactions. Using the median-effect method, the dose-effect curve was plotted for each agent and for multiple dilutions of a fixed-ratio combination by using the equation

$$f_a/f_u = (D/D_m)^m$$

In this equation, D is the dose administered, D_m is the dose required for 50% inhibition of growth, f_a is the fraction affected by dose D, f_u is the unaffected fraction, and m is a coefficient denoting the sigmoidicity of the dose-effect curve. The dose-effect curve was plotted by using a logarithmic conversion of the equation to determine the m and D_m values, and the dose D_x required for $x$percent effect $(f_a)_x$ was then calculated as

$$D_x = D_m[(f_a)_x/(f_u)_x]^{1/m}$$

Thus, the CI can be defined by the isobologram equation

$$CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2 + \alpha[(D)_1 \cdot (D)_2/(D_x)_1 \cdot (D_x)_2]$$

where $(D_x)_1$ is the dose of CDDP required to produce x percent effect alone and $(D)_1$ is the dose of CDDP required to produce the same x percent effect in combination with F-ara-A; similarly, $(D_x)_2$ is the dose of F-ara-A required to produce x percent effect alone and $(D)_2$ is the dose of F-ara-A required to produce the same x percent effect in combination with CDDP. Theoretically, CI is the ratio of the combination dose to the sum of the single-agent doses at an isoeffective level. Consequently, CI values of <1 indicate synergism, values of >1 show antagonism, and values of 1 indicate additive effects. The CI values obtained from both the classical ($\alpha = 0$) and conservative ($\alpha = 1$) isobologram equations are presented in this report.

The DRI (28), which describes the fold dose reduction at a given degree of inhibition (x percent) in the combination, compared with the drug as a single agent, was calculated using the equation DRI = $(D_x)/(D)$.

Quantification of DNA interstrand cross-linking and repair in the whole genome. CDDP-induced DNA interstrand cross-links were measured after the cells were treated for 4 hr with CDDP (15 μg/ml for LoVo cells and 30 μg/ml for CP2.0 cells) as a single agent or in combination with F-ara-A (15 μM), and the cross-link frequency was expressed as CLI. For quantification of the cross-links, an EBFA was adapted from the procedure of Brent (29), as modified by Sriram and Ali-Osman (30). Briefly, cells were lysed with a lysing solution (10 mM Tris·HCl buffer containing 100 mM NaCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate, and 0.5 mg/ml proteinase K, pH 8.0) after a 3-hr incubation at 50°. Genomic DNA was extracted from cell lysates with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with 0.5 volume of 7.5 M ammonium acetate and 2 volumes of 95% ice-cold ethanol, air dried, and resuspended in TE buffer (10 mM Tris·HCl containing 1 mM EDTA, pH 8.0). RNA was removed by treatment with 100 μg/ml DNase-free RNase (Sigma) at 37° for 1 hr, followed by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. The purified DNA was diluted to a final concentration of 100 μg/ml with potassium phosphate buffer (2 mM EDTA in 20 mM potassium phosphate, pH 11.8). A 10-μg DNA sample was transferred to a borosilicate glass tube, heated at 100° for 10 min, and then cooled at 23° for 10 min. At the end of the heating/cooling cycle, 3 ml of potassium phosphate buffer containing 1.0 μg/ml ethidium bromide were added to each sample, and the intensity of fluorescence was measured immediately with a fluorometer (model LS-50; Perkin-Elmer, Norwalk, CT) at 305-nm excitation and 590-nm emission wavelengths. In each assay, salmon sperm DNA (Sigma) with a known number of interstrand cross-links, formed by treatment with CDDP in TE buffer at 37° for 4 hr, was included as a reference, to monitor the consistency of the assay. The relative CLI for drug-treated cells was calculated as follows: CLI = $(N_d - N_c)/N_c$, with $N = -\ln X$, where N is the number of cross-links per DNA molecule, X is the fraction of fluorescence representing non-cross-linked DNA after the heating/cooling cycle, N_d is the N value for drug-treated cells, and N_c is the N value for the control cells.

To examine the DNA repair, cells were first treated with CDDP alone or CDDP and F-ara-A in combination to induce the cross-links, as described above. At the end of the treatment, the cells were washed to remove the drug and incubated for additional periods of 0–24 hr with fresh growth medium containing no drugs. The repair efficiency, determined by the rate of removal of the cross-links, was calculated as the percentage of initial cross-links that remained after

drug removal. In some experiments, immediately after the cells were washed free of CDDP and F-ara-A they were exposed to 1 mM thiourea for 1 hr, to block the conversion of monoadducts to cross-links during the repair, and CLI was then determined at 0, 3, 6, 10, and 24 hr after the removal of CDDP and F-ara-A.

Quantification of cross-links and repair in the *ERCC1* gene. Effects of F-ara-A on the formation and repair of CDDP-induced cross-links in the *ERCC1* gene were examined after the cells were exposed for 4 hr to CDDP at increasing concentrations (5, 15, 45, and 120 μg/ml) or to CDDP (at the same series of concentrations) in combination with F-ara-A (15 μM). A denaturation/renaturation procedure, adapted from the method described by Vos and Hanawalt (31), was used to quantify the DNA interstrand cross-links in the *ERCC1* gene. Briefly, a 10-μg aliquot of genomic DNA extracted from the drug-treated cells was digested with *Kpn*I and then denatured with 0.2 N NaOH by heating and immediate chilling. The alkaline-denatured DNAs were loaded on a 0.5% neutral agarose gel in a loading buffer containing 1 mM Tris, 10 mM EDTA, 4% sucrose, and 0.1% bromphenol blue. Electrophoresis was carried out in TBE buffer (0.089 M Tris base, 0.089 M boric acid, 0.002 M EDTA, pH 8.0) at 30 V for 16–19 hr. DNAs were transferred onto GeneScreen Plus membranes (New England Nuclear, Boston, MA), and the membranes were prehybridized and then hybridized with a gel-purified, ^{32}P-labeled, *ERCC1* cDNA (kindly provided by Dr. J. Hoeijmakers, Erasmus University, The Netherlands). The intensity of the signal was quantified with a Betascope 603 blot analyzer (Betagen Corp., Waltham, MA).

For the repair experiments, cells were prelabeled with [^{3}H]thymidine (0.5 μCi/ml; specific activity, 80 Ci/mmol; Amersham Corporation, Arlington Heights, IL) for 48 hr, followed by an overnight chase period. The cells were then treated for 4 hr with CDDP (15 μg/ml for LoVo cells and 30 μg/ml for CP2.0 cells) in the presence or absence of F-ara-A (15 μM). The amount of cross-links found in the cells immediately after the drugs were washed off was taken for the calculation for CLI at 0 hr of repair. To determine the CLI for the repair at other time points, cells were incubated with bromodeoxyuridine (10 μM) and fluorodeoxyuridine (1 μM) for an additional 14 or 24 hr after removal of the drugs, to density label the newly synthesized DNA during the post-drug treatment period. The genomic DNA was isolated and digested with *Kpn*I, and the parental DNA was then separated from daughter DNA by CsCl equilibrium gradient centrifugation (32). The positions of the replicated (heavy-light chain) and parental (light-light chain) DNAs were identified by the tritium labeling in the gradient fractions. Fractions containing the parental DNA were pooled, dialyzed overnight against TE buffer, concentrated by ethanol precipitation, and then subjected to denaturation/renaturation gel electrophoresis as described above.

Northern blot analysis. Total RNA, isolated from exponentially growing cells by the acid guanidium thiocyanate phenol/chloroform method (33), was denatured with glyoxal and dimethylsulfoxide, fractionated on a 1.2% agarose gel, transferred to a nylon membrane, and probed with a ^{32}P-labeled *ERCC1* cDNA probe under stringent conditions, as described previously (6).

Statistical analysis. Statistical significance was tested using a two-tailed paired-sample *t* test.

Results

Cytotoxicities of F-ara-A as a single agent and of F-ara-A and CDDP combined. The cytotoxicity of F-ara-A as a function of either dose or exposure duration was assessed in LoVo and CP2.0 cells by a clonogenic assay. Biphasic dose-survival curves, with an initial exponential decrease in survival followed by a second phase of a much slower loss of viability, as is typical of a cell cycle phase-specific drug, were observed after a 2-hr exposure (Fig. 1, A and B). The IC_{50} values (the drug concentrations that kill 50% of the cell

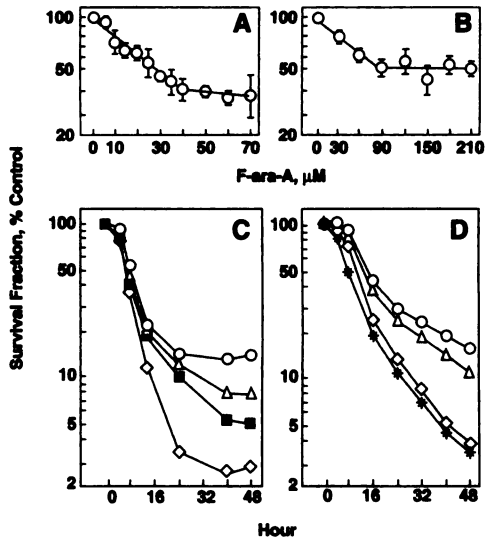

Fig. 1. Dose- and time-dependent survival curves for LoVo and CP2.0 cells after treatment with F-ara-A. A and B, Dose-response relationships for cell survival were determined after LoVo (A) and CP2.0 (B) cells were exposed to increasing concentrations of F-ara-A for 2 hr. C and D, The time course of survival was assessed after the cells were exposed continuously to F-ara-A at 1 (○), 5 (△), 7.5 (■), and 15 (◇) μM (for LoVo cells) (C) or 1, 5, 15, and 45 (*) μM (for CP2.0 cells) (D). *Points*, means of six determinations from at least two independent experiments. *Bars*, standard deviations.

population) were 25 ± 3.0 μM and 82 ± 6.8 μM for LoVo and CP2.0 cells, respectively. Also, there was a dose-dependent exponential loss of viability in both LoVo (Fig. 1C) and CP2.0 (Fig. 1D) cells as the time of exposure to F-ara-A was increased.

To analyze the cytotoxic interaction of CDDP and F-ara-A, we used the median-effect method of Chou and Talalay (27) to determine the degree of synergism and the dose and level of inhibition required for synergism to occur. The parameters of the median-effect plot are summarized in Table 1. To calculate the CI values, both the classical and conservative isobologram equations were used, and the CI values at 25%, 50%, 75%, and 90% levels of inhibition are also shown in Table 1. Fig. 2 shows the affected fraction-CI plots constructed by computer analysis using the conservative isobologram equation. In both LoVo cells (at 15–99% inhibition levels) and CP2.0 cells (at 1–99% inhibition levels), the CI values were all <1, indicating that F-ara-A synergistically enhanced CDDP cytotoxicity in both the parental LoVo and resistant CP2.0 cell lines at a wide range of inhibition levels. In LoVo cells, D_m values for CDDP were 9.4 μM (2.8 μg/ml) as a single agent and 4.5 μM (1.4 μg/ml) when combined with F-ara-A. Similarly, in CP2.0 cells, D_m values were 54.1 μM (16.2 μg/ml) and 15.3 μM (4.6 μg/ml) for CDDP as a single agent and in combination with F-ara-A, respectively. Thus, the DRI values were 2.09 for LoVo cells and 3.54 for CP2.0 cells, suggesting that the enhancement of CDDP cytotoxicity was somewhat greater in the resistant cells.

Effects of F-ara-A on the formation and repair of CDDP-induced DNA cross-links in the overall genome. To quantify the cross-links formed in the overall genome as a result of the drug treatment, genomic DNA was extracted from the cells immediately after the drug treatment and measured by EBFA, and the results were expressed as the

TABLE 1
Analysis of the synergy of F-ara-A and CDDP in LoVo and CP2.0 cells by the median-effect method

Cell line	Drug	Median-effect plot parameter			CI			
		D_m	m[a]	r[b]	25%[c]	50%	75%	90%
		μM						
LoVo	F-ara-A	30.2	1.11	0.99				
	CDDP	9.4	1.93	0.99				
	F-ara-A + CDDP	3.4 + 4.5	2.49	0.95	0.84[d] (0.74)[e]	0.64 (0.59)	0.51 (0.48)	0.37 (0.36)
CP2.0	F-ara-A	90.5	1.17	0.95				
	CDDP	54.1	1.73	0.99				
	F-ara-A + CDDP	4.6 + 15.3	2.06	0.97	0.41 (0.39)	0.35 (0.33)	0.30 (0.29)	0.26 (0.25)

[a] m, slope of the median-effect plot.
[b] r, correlation coefficient of the median-effect plot.
[c] Level of inhibition.
[d] CI value calculated by using the conservative isobologram equation.
[e] CI value calculated by using the classical isobologram equation.

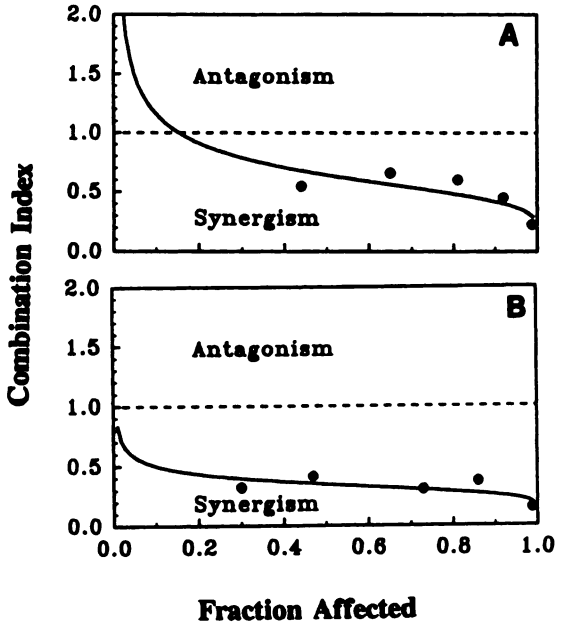

Fig. 2. Cytotoxic synergy of combined F-ara-A and CDDP in LoVo and CP2.0 cells. The cytotoxicity was determined by clonogenic assays. Cells were treated with F-ara-A (2.5, 5.0, 7.5, 10, and 15 μM for LoVo cells and 2.5, 5.0, 7.5, 15, and 30 μM for CP2.0 cells) or CDDP (1, 2, 3, 4, and 6 μg/ml for LoVo cells and 2.5, 5.0, 7.5, 15, and 30 μg/ml for CP2.0 cells) as single agents or with a combination of the two agents at a fixed concentration ratio (molar ratio of F-ara-A to CDDP of 1.5:2 for LoVo cells and 1.5:5 for CP2.0 cells) for 4 hr. Using the conservative isobologram equation, the affected fraction-CI plots for LoVo (A) and CP2.0 (B) cells were constructed by computer analysis of the data generated from the median-effect analysis. The CI values of <1 occurred at a wide range of inhibition levels, indicating synergism produced by the combination.

TABLE 2
DNA cross-linking induced by CDDP in LoVo and CP2.0 cells and salmon sperm DNA
Values are mean ± standard deviation.

	CDDP	Cross-link formation	
		N[a]	CLI
	μg/ml		
LoVo cells	0	0.06 ± 0.01	
	2.5	0.09 ± 0.01	0.55 ± 0.08
	5.0	0.10 ± 0.01	0.71 ± 0.10
	10.0	0.19 ± 0.01	2.16 ± 0.24
	15.0	0.22 ± 0.01	2.72 ± 0.51
CP2.0 cells	0	0.08 ± 0.02	
	5.0	0.10 ± 0.01	0.26 ± 0.06
	10.0	0.13 ± 0.01	0.64 ± 0.14
	20.0	0.16 ± 0.01	1.06 ± 0.17
	30.0	0.20 ± 0.02	1.51 ± 0.28
SSDNA[b]	0	0.09 ± 0.003	
	0.5	0.13 ± 0.003	0.44 ± 0.07
	1.0	0.16 ± 0.006	0.78 ± 0.12
	2.5	0.22 ± 0.006	1.44 ± 0.13
	5.0	0.41 ± 0.006	3.56 ± 0.10

[a] N, number of cross-links/molecule of DNA.
[b] SSDNA, salmon sperm DNA, an external control.

CLI. Table 2 shows dose-dependent increases, after exposure to CDDP, in the CLI values for LoVo and CP2.0 cells, as well as those for salmon sperm DNA, which served as an external control to monitor assay consistency. When the cells were treated simultaneously with F-ara-A (15 μM) and CDDP (15 μg/ml for LoVo cells and 30 μg/ml for CP2.0 cells), the CLI was increased from 2.72 ± 0.51 (with CDDP alone) to 4.51 ± 0.29 in LoVo cells and from 1.51 ± 0.28 to 2.93 ± 0.37 in CP2.0 cells, indicating that concomitant treatment with F-ara-A results in a significantly enhanced accumulation of CDDP-induced cross-links in the overall genome of both cell lines ($p < 0.05$). Next, the rate of removal of DNA cross-links was quantified to determine whether F-ara-A treatment affected this process. When the cells were treated with CDDP alone, the rate of removal of DNA cross-links was greater in CP2.0 cells than in LoVo cells. For example, 10 hr after washing of cells into fresh drug-free medium, the cross-links in CP2.0 cells were reduced to 5% of the initial level, compared with 60% in LoVo cells (Fig. 3). When F-ara-A was given simultaneously with CDDP, the removal of the cross-links was greatly inhibited in both cell lines. At 10 hr after treatment, the removal of the cross-links was reduced from 40% to 5% in LoVo cells and from 95% to 45% in CP2.0 cells (Fig. 3).

Effects of F-ara-A on the formation and removal of cross-links in *ERCC1* DNA sequences. Because DNA damage and repair are believed to be intragenomically heterogeneous (5, 34), we next determined the effect of F-ara-A on the formation and repair of the CDDP-induced cross-links in a specific gene and compared the results with those in the total genome. *ERCC1* is a human DNA excision repair gene that confers resistance to CDDP in UV repair-deficient Chinese hamster ovary cells of complementation group 1 (35). A recent report indicated that transfection of cells with the

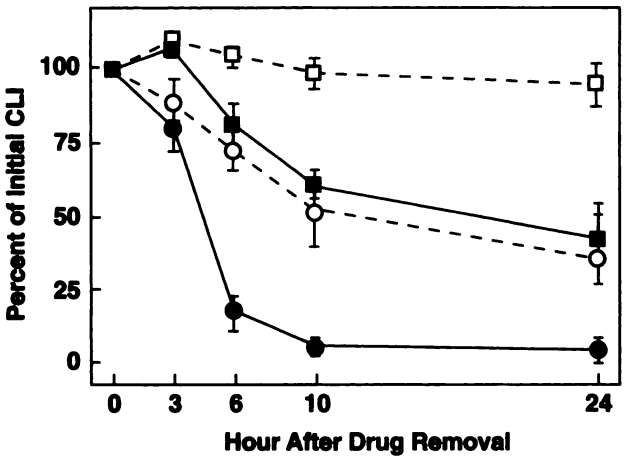

Fig. 3. Effects of F-ara-A on the repair of CDDP-induced DNA interstrand cross-links. LoVo (■, □) and CP2.0 (●, ○) cells were treated for 4 hr with CDDP (15 μg/ml for LoVo cells and 30 μg/ml for CP2.0 cells) as a single agent (■, ●) or combined with 15 μM F-ara-A (□, ○). The cross-links were measured by EBFA at the indicated times after drug removal. *Points*, percentage of initial CLI (mean ± standard deviation). The initial CLI values were 2.72 ± 0.51 (LoVo) and 1.51 ± 0.28 (CP2.0) for cells treated with CDDP alone and 4.51 ± 0.29 (LoVo) and 2.93 ± 0.37 (CP2.0) for cells treated with CDDP plus F-ara-A.

ERCC1 gene resulted in enhanced repair of gene-specific CDDP-DNA interstrand cross-links (12). In CP2.0 cells, the mature *ERCC1* transcript was present at twice the level found in LoVo cells (Fig. 4), suggesting that this may be an actively transcribed gene contributing in part to the CDDP resistance phenotype of the CP2.0 line. The formation of *ERCC1*-specific cross-links was measured by denaturation/renaturation gel electrophoresis, followed by Southern blot analysis, and the results were compared for the relative intensity of the double-stranded bands, i.e., the ratio of the intensity of the double-stranded band to the sum of the intensities of the double- and single-stranded bands. The relative intensity of the double-stranded bands from both cell lines increased as the CDDP concentration was increased, indicating a dose-dependent formation of cross-links in the *ERCC1* gene (Fig. 5). However, at the same drug concentration, CDDP as a single agent induced a greater relative

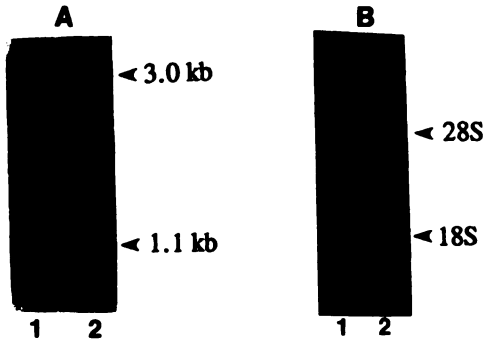

Fig. 4. A, Northern blot analysis of *ERCC1* RNA. Total RNA (30 μg) from LoVo (*lane 1*) and CP2.0 (*lane 2*) cells was isolated, fractionated on a 1.2% agarose gel, and transferred to a nylon membrane. The blot was hybridized with *ERCC1* cDNA. The 1.1-kb RNA is the mature *ERCC1* transcript, whereas the 3.0-kb band is the precursor RNA species. B, Ethidium bromide staining of the 28 S and 18 S bands, which served as loading controls. The relative levels of 1.1-kb *ERCC1* RNA, as assessed with a Betascope blot analyzer, were 1.0 and 2.0 for LoVo and CP2.0 cells, respectively.

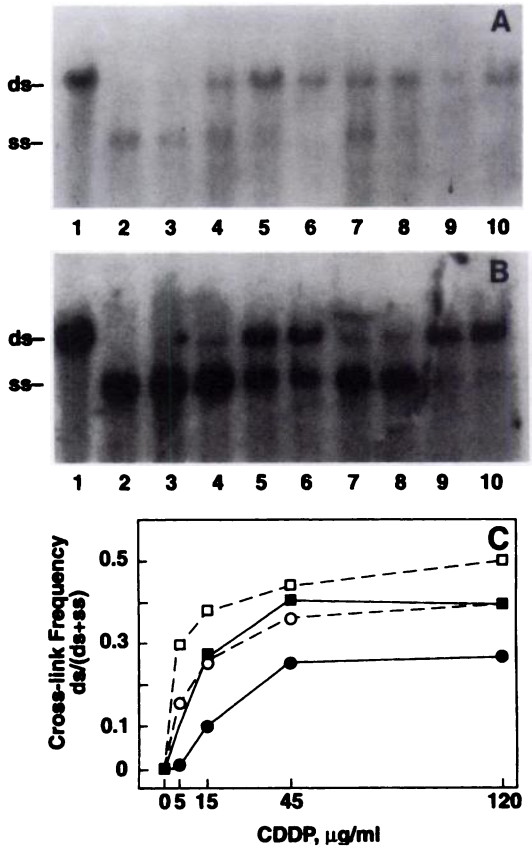

Fig. 5. Effects of F-ara-A on the CDDP-induced DNA interstrand cross-links in specific DNA sequences. A and B, The cross-links in LoVo (A) and CP2.0 (B) cells were measured by Southern blotting after denaturation/renaturation agarose gel electrophoresis. Cells were exposed to CDDP alone at increasing concentrations (5, 15, 45, and 120 μg/ml for *lanes 3, 4, 5,* and *6*, respectively) or to CDDP at the same concentrations in combination with 15 μM F-ara-A (*lanes 7-10*, respectively), for 4 hr. The blot was probed with radiolabeled *ERCC1* cDNA. Also shown are controls from nondenatured (*lane 1*) and denatured (*lane 2*) DNAs from cells without exposure to the drugs. C, Quantification of the band intensities in A and B, with a Betascope blot analyzer, is shown. ■, LoVo cells, CDDP alone; □, LoVo cells, CDDP plus F-ara-A; ●, CP2.0 cells, CDDP alone; ○, CP2.0 cells, CDDP plus F-ara-A. *ds*, double-stranded; *ss*, single-stranded.

intensity of the double-stranded band in LoVo cells than in CP2.0 cells, indicating that the *ERCC1* gene in LoVo cells was more susceptible to CDDP than was its resistant counterpart. Simultaneous addition of F-ara-A and CDDP significantly increased the relative intensity of the double-stranded band, compared with that induced by CDDP alone (Fig. 5, A and B, *lanes 7-10*), indicating that F-ara-A had a cooperative effect with CDDP on the formation of the cross-links in the *ERCC1* gene in both the parental line and its CDDP-resistant variant. Furthermore, F-ara-A, when used in combination with CDDP, also increased the susceptibility of the *ERCC1* gene in resistant CP2.0 cells to a level comparable to that seen in LoVo cells treated with CDDP alone (Fig. 5C).

Cellular repair of CDDP-induced DNA adducts in the *ERCC1* gene was next examined. In these experiments, the repair deficiency was determined as the rate of adduct removal in the cells during the post-CDDP treatment period, and the results were expressed as percentages of the initial content of cross-links remaining (Fig. 6). At 14 hr after treat-

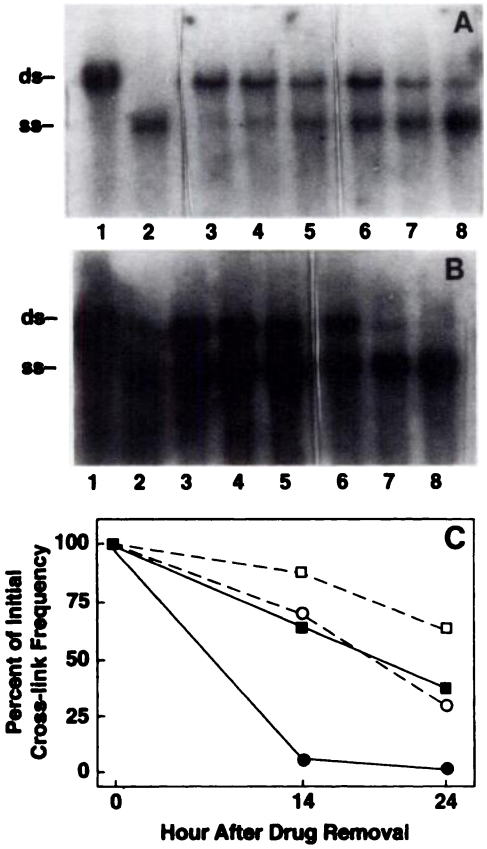

Fig. 6. Effects of F-ara-A on the repair of gene-specific DNA interstrand cross-links. A and B, LoVo (A) and CP2.0 (B) cells were treated with CDDP (15 μg/ml for LoVo cells and 30 μg/ml for CP2.0 cells) for 4 hr, in the presence (*lanes 3-5*) or absence (*lanes 6-8*) of F-ara-A (15 μM). The *ERCC1*-specific interstrand cross-links were measured by Southern hybridization of cellular DNA with an *ERCC1* cDNA probe after denaturation/renaturation agarose gel electrophoresis, at 0 hr (*lanes 3* and *6*), 14 hr (*lanes 4* and *7*), and 24 hr (*lanes 5* and *8*) after drug removal. Also shown are controls from nondenatured (*lane 1*) and denatured (*lane 2*) DNAs from cells without exposure to the drugs. C, Betascope quantification of the data in A and B shows the fractions of the cross-links that remained at the indicated times after drug removal. ■, LoVo cells, CDDP alone; □, LoVo cells, CDDP plus F-ara-A; ●, CP2.0 cells, CDDP alone; ○, CP2.0 cells, CDDP plus F-ara-A. *ds*, double-stranded; *ss*, single-stranded.

ment with CDDP, the cross-links were reduced 37% in LoVo cells and 94% in CP2.0 cells. When F-ara-A was added in combination with CDDP, the removal of the cross-links in LoVo cells was reduced to 14%, representing a 62% inhibition by F-ara-A, and the repair efficiency in CP2.0 cells was reduced to 32%, i.e., a 66% inhibition. The data indicate that F-ara-A suppressed the repair of CDDP-induced cross-links in gene-specific DNA sequences as well as in the nonspecific overall genome.

Effects of thiourea on the formation and removal of cross-links in the overall genome. Because the formation of the cross-links normally takes place in two stages, i.e., an initial formation of DNA monoadducts and a subsequent conversion into bidentate cross-links, any cross-links formed after the removal of CDDP and during the period of repair assessment may complicate the determination of repair activity. To clarify this possibility, we performed experiments in which thiourea was included to block the conversion of the monoadducts. In these experiments, the cells were treated with thiourea for 1 hr immediately after the completion of the treatment with CDDP, and the assessment of the cross-link content followed thereafter. When the cells were treated with CDDP as a single agent, treatments of LoVo cells with 1 mM thiourea resulted in a 33% reduction of CLI at 1 hr after the removal of CDDP (1.84 ± 0.50 versus 2.72 ± 0.51) (Fig. 7), whereas similar treatments of CP2.0 cells with thiourea had little effect (1.60 ± 0.50 and 1.51 ± 0.28 for the thiourea-treated and non-thiourea-treated cells, respectively). The reasons for the different responses of the two cell lines to thiourea are discussed below. At 10 hr after the completion of the CDDP treatment, only 4% of the initial cross-link content remained in CP2.0 cells, compared with 32% in LoVo cells, indicating a greater efficiency of the former in removing the cross-links. Combination treatment with F-ara-A and CDDP resulted in a drastic reduction not only of the efficiency but also of the capacity of both LoVo and CP2.0 cells for the removal of cross-links (Fig. 7), suggesting that the hampered repair resulted from the inhibitory effect of F-ara-A, rather than from the rearrangement of monoadducts. Taken together, the results from thiourea experiments confirmed that 1) resistant CP2.0 cells possessed a greater repair capacity than did their sensitive parental cells (LoVo cells) and 2) F-ara-A suppressed the removal of CDDP-induced cross-links in cells treated simultaneously with the combination of CDDP plus F-ara-A.

Discussion

We carried out the present investigation to evaluate the ability of F-ara-A to overcome resistance to CDDP in tumor cell lines that originated from an organ that is relatively resistant to chemotherapy, namely a parental line and its resistant variant, which exhibits an enhanced repair capacity. Our data demonstrate that, at clinically achievable concentrations, F-ara-A synergistically enhanced the cytotoxic-

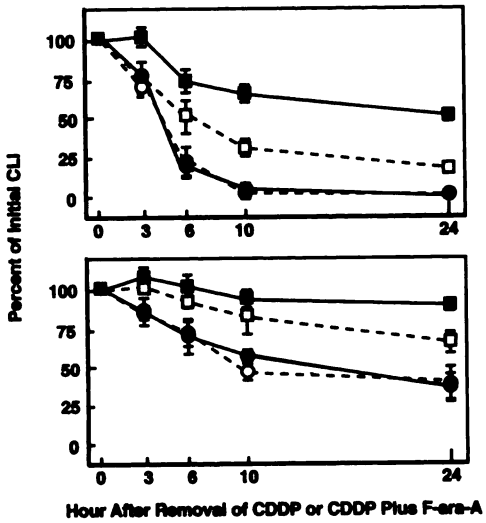

Fig. 7. Effects of thiourea on the removal of CDDP-induced DNA interstrand cross-links. LoVo (■, □) and CP2.0 (●, ○) cells were treated for 4 hr with CDDP alone (*upper*) or with CDDP and F-ara-A in combination (*lower*). The cross-linking was assessed without further treatment (■, ●) or after further treatment with 1 mM thiourea for 1 hr (□, ○). The cross-links were measured by EBFA at 0, 3, 6, 10, and 24 hr after CDDP and F-ara-A treatments. *Points*, percentage of initial CLI (mean ± standard deviation).

ity of CDDP both in cells with intrinsic resistance and in those with acquired resistance. Similar synergism of the CDDP/F-ara-A combination was also observed in other cell lines from different organs, e.g., F-ara-A-sensitive K-562 human chronic myelogenous leukemia and CDDP-sensitive SK-OV-3 human ovarian tumor cell lines.[1] Although the mechanisms responsible for such potentiation are presently unclear, evidence suggests that F-ara-A plays a major role in the inhibition of DNA repair. First, F-ara-ATP may act as a DNA elongation terminator when it is incorporated into DNA (19), especially when the incorporation occurs at the 3′ terminus; incorporation at this position makes its removal by DNA polymerase ϵ-associated 3′ to 5′ exonuclease difficult (25). Second, F-ara-ATP inhibits human ligase I by two mechanisms, i.e., direct interaction with the enzyme and incorporation at the 3′ terminus of the DNA strand, which becomes a poor substrate for ligation (26).

It has been reported that, after exposure to CDDP, the conversion of monofunctional adducts to their inter- and intrastrand cross-links can be inhibited by thiourea (36). We showed here that thiourea reduced the formation of the interstrand cross-links in LoVo cells, presumably because the monoadduct rearrangement was inhibited. Nevertheless, in our experiments thiourea did not suppress the cross-linking in CP2.0 cells (Fig. 7). The reason for this discrepancy is presently unclear. However, we previously showed that the glutathione level in CP2.0 cells was 8-fold higher than that in LoVo cells (10.3 versus 1.3 nmol/10^6 cells) (6). Considering that a higher level of glutathione can block conversion of monoadducts into bifunctional products (37), it may be logical to assume that in cells, such as CP2.0, that already contain very high levels of endogenous glutathione thiourea may not further diminish this conversion. Alternatively, had the monoadducts in CP2.0 cells been repaired rapidly, few would remain to be rearranged. Although the latter possibility is supported by the data showing an enhanced repair capability in the CP2.0 cells, additional studies are required. Regardless of the mechanism, the results from the thiourea experiments support the notion that the CLI in both cell lines was increased because F-ara-A suppressed repair.

In general, actively transcribed genes are more prone to damage but are also repaired more efficiently (5, 34). However, we found that in CP2.0 cells the repair was not more efficient in the *ERCC1* gene, compared with that in the whole genome. At least two possibilities may explain these findings. First, the *ERCC1* gene may not be actively transcribed in CP2.0 cells, and the observed elevation of the RNA level may have been caused by a prolonged half-life of the *ERCC1* message. Second, the method used in our experiments may not be sensitive enough to detect differences in activities over an already very efficient repair; 95% of the cross-links in CP2.0 cells had been removed 10 hr after the completion of the CDDP treatment. To this end, we demonstrated that F-ara-A similarly inhibited CDDP-induced DNA cross-link formation and repair in the total genome and the *ERCC1* gene and that the repair in the resistant CP2.0 cells was suppressed by F-ara-A to approximately the same level as in the parental LoVo cells treated with CDDP alone (Figs. 3 and 6). This result was consistent with a report by Zhen *et al.* (3), in which they showed a significant repair difference between parental and CDDP-resistant human ovarian tumor cells in removing interstrand cross-links from specific gene sequences. This result, together with our recent findings that F-ara-ATP also inhibited the repair of CDDP-DNA intrastrand adducts,[2] suggested that F-ara-A would be able to completely reverse CDDP resistance in CP2.0 cells. However, our data indicate that F-ara-A treatment produced only partial reversal of CDDP resistance. The discrepancy in magnitude between the F-ara-A-suppressed repair and the F-ara-A-potentiated CDDP cytotoxicity may be attributable to other resistance mechanisms that are not related to repair and are not affected by F-ara-A.

The data presented here indicate that F-ara-A enhances the formation of CDDP-induced DNA interstrand cross-links by inhibiting cellular removal of such lesions. However, our results do not exclude the importance of the contribution of CDDP-induced intrastrand adducts to CDDP cytotoxicity or the possibility of inhibition of adduct repair by F-ara-A. On the contrary, recent evidence has accumulated to indicate that CDDP adducts are an important DNA lesion for CDDP cytotoxicity. By using a cell-free system, we found that F-ara-ATP inhibited nucleotide excision repair of CDDP-DNA adducts by CP2.0 cell extracts.[2] It is not within the scope of this communication to determine the relative roles of CDDP-DNA interstrand cross-linking and intrastrand adduct formation in the lethal activity of CDDP. Nevertheless, our present data clearly indicate the synergistic antitumor effect of the CDDP/F-ara-A combination *in vitro* and demonstrate that the synergy is associated with inhibition of the repair of CDDP-DNA cross-links. These results have provided the rationale for clinical studies of CDDP and fludarabine combinations that are underway in our institution (38).

[1] K-562 cells were treated with either CDDP at concentrations of 0.3, 0.75, 1.5, 3.0, or 7.5 μM or F-ara-A at concentrations of 0.1, 0.25, 0.5, 1.0, or 2.5 μM and SK-OV-3 cells were treated with either CDDP at concentrations of 0.1, 0.25, 0.5, 1.0, or 2.5 μM or F-ara-A at concentrations of 2, 5, 10, 20, or 50 μM, for 4 hr. Both cell lines were also treated with CDDP and F-ara-A in combination at a fixed concentration ratio of 1:3 for K-562 cells and 1:20 for SK-OV-3 cells. Cytotoxic synergy of the combination treatment was analyzed by median-effect analysis. The CI values from the affected fraction-CI plots were all <1 for inhibition levels of 20% to 95%, indicating synergism (L.-Y. Yang, L. Li, X.-M. Liu, M. J. Keating, and W. Plunkett, unpublished observations).

[2] L.-Y. Yang, L. Li, X.-M. Liu, M. J. Keating, and W. Plunkett, unpublished observations.

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