

# Fludarabine-Mediated Repair Inhibition of Cisplatin-Induced DNA Lesions in Human Chronic Myelogenous Leukemia-Blast Crisis K562 Cells: Induction of Synergistic Cytotoxicity Independent of Reversal of Apoptosis Resistance

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## SUMMARY

We demonstrated previously that the nucleoside of fludarabine (F-ara-A), a clinically effective agent against chronic lymphocytic leukemia and low-grade lymphoma, produces synergistic cytotoxicity against cisplatin-resistant CP2.0 human colon tumor cells when administered in combination with cisplatin. The purpose of this study was 2-fold: (i) to determine whether the synergy occurs in K562 human chronic myelogenous leukemia cells, which, unlike CP2.0 cells, are relatively resistant to drug-induced apoptosis because they express P210<sup>bcr-abl</sup> and (ii) to study the underlying mechanism for the synergy if the enhancement of cytotoxicity occurs in K562 cells. When K562 cells were treated with fludarabine nucleoside and cisplatin as single agents for 4 hr, IC<sub>50</sub> values for fludarabine and cisplatin were 3.33 and 2.28  $\mu\text{M}$ , respectively, as measured by a clonogenic survival assay. The simultaneous treatment of K562 cells with the two agents resulted in synergistic cell killing as determined by median-effect analysis. Such synergistic cell killing by combined cisplatin and fludarabine could not be detected in repair-deficient human xeroderma pigmentosum cell lines. Within the range of cytotoxic concentrations, fludarabine (2.5–15  $\mu\text{M}$ ) and cisplatin (3–30  $\mu\text{M}$ ) as single agents produced no detectable internucleosomal DNA fragmentation as revealed by gel elec-

trophoresis, nor did the combination of the two drugs induce apoptotic DNA degradation. The effects of fludarabine on the repair of cisplatin-induced DNA adducts and interstrand cross-links in K562 cells were analyzed to determine their correlation with the cytotoxic synergy. The interstrand cross-links were measured by the ethidium bromide binding fluorescence assay and quantitative Southern blotting technique. Repair of the intrastrand adducts was detected with whole-cell extracts using a cisplatin-damaged plasmid as the substrate for the *in vitro* repair assay. Fludarabine at clinically achievable concentrations (1.5–4.5  $\mu\text{M}$  fludarabine nucleoside; 20–100  $\mu\text{M}$  fludarabine triphosphate) inhibited the repair of the DNA lesions induced by cisplatin in a dose-dependent fashion in K562 cells but not in xeroderma pigmentosum cells. Cotreatment with fludarabine preferentially increased the number of interstrand cross-links induced by cisplatin in actively transcribed genes in K562 cells. These data demonstrate the DNA-repair-inhibitory effect of fludarabine and suggest that this effect may contribute to the synergistic cytotoxicity of the fludarabine/cisplatin combination that resulted in decreased clonogenic survival of apoptosis-resistant K562 cells.

Fludarabine (9- $\beta$ -D-arabinofuranosyl-2-fluoroadenine-5'-monophosphate), a purine nucleotide analogue, is effective against B cell malignancies, particularly in patients with chronic lymphocytic leukemia and indolent lymphocytic diseases (1). Once it enters the bloodstream, fludarabine is

dephosphorylated to its nucleoside form (F-ara-A), which in turn is phosphorylated to F-ara-ATP intracellularly before entering the cell nucleus, where it interacts with DNA. The exact mechanisms through which fludarabine produces its tumoricidal effect are not yet totally understood, although *in vitro* studies have revealed that F-ara-ATP disrupts cellular deoxynucleotide pools (2), inhibits DNA replication (3) and repair (4), and induces apoptosis (5). Among these potential mechanisms, inhibition of DNA repair seems to be particularly interesting in view of the fact that fludarabine is spe-

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**ABBREVIATIONS:** F-ara-A, fludarabine nucleoside; F-ara-ATP, fludarabine triphosphate; CDDP, *cis*-diamminedichloroplatinum(II); NER, nucleotide excision repair; CML, chronic myelogenous leukemia; XP, xeroderma pigmentosum; *ERCC1*, excision repair-complementing group 1 gene; *DHFR*, dihydrofolate reductase gene; CI, combination index; EBFA, ethidium bromide binding fluorescence assay; CLI, cross-link index; D<sub>m</sub>, drug concentration that kills 50% of the population; HMG, high mobility group.

cifically effective against quiescent leukemia and lymphomas.

CDDP (cisplatin) causes lethal effects mainly by disrupting the cellular DNA structure, thereby preventing DNA replication and RNA transcription. The two most important CDDP-induced DNA lesions are intrastrand adducts, which account for >85% of the total lesions, and interstrand cross-links, which together with monoadducts represent the remaining lesions (6). The subsequent repair of these lesions plays an important role in the ability of the cells to survive genotoxic treatment. It is generally accepted that cells repair CDDP-induced DNA intrastrand cross-links through the NER mechanism (7). However, the pathways for the repair of DNA interstrand cross-links in mammalian cells are poorly defined; most of the study was performed in the bacterium *Escherichia coli*. A model for the repair of interstrand cross-links in *E. coli* was proposed that consisted of the sequential event of excision and recombination (8, 9).

It has been reported that CDDP and F-ara-A may exert their cytotoxic effects by inducing programmed cell death, or apoptosis (5, 10). Apoptosis is a physiological mode of cell death characterized by morphological changes and internucleosomal DNA fragmentation (11). We reported previously that F-ara-A and CDDP in combination produce synergistic cytotoxicity in CP2.0 human colon cancer cells (4). Further research, as reported here, revealed that these two agents in combination induced apoptosis in CP2.0 cells and prompted us to investigate whether such a combination produces similar cytotoxic synergy in cells that are relatively resistant to drug-induced apoptosis. Moreover, a fludarabine-plus-CDDP combination regimen is being evaluated in clinical trials in patients with refractory chronic lymphocytic leukemia, and the results so far show that the two-drug combination is moderately effective *in vivo* (12). However, it remains to be determined whether the fludarabine-plus-CDDP combination is effective against other hematological diseases, such as CML.

The K562 cell line is a candidate for this test of synergy; it was derived from a patient with CML. Like the majority of human CML, K562 cells possess the genotypic abnormality involving dysregulation of the p210 tyrosine kinase activity of the bcr-abl fusion oncoprotein (13). The expression of p210<sup>bcr-abl</sup> in K562 cells is mainly responsible for their resistance to differentiation and drug-induced apoptosis (13–15). In addition, the gene products of the antiapoptosis *Bcl-x<sub>L</sub>* and *Bcl-2* regulate drug-induced apoptosis (16). For example, enforced expression of bcl-x<sub>S</sub> in K562 cells significantly enhances the p210<sup>bcr-abl</sup>-mediated inhibition of apoptosis induced by antitumor agents (17).

In this study, we determined the cytotoxic interaction of fludarabine with CDDP in K562 cells. Although CDDP is not commonly used to treat hematological neoplasias, we chose K562 cells for this study based on the effectiveness of fludarabine in hematological malignancies. To investigate whether an intact repair system is important for the cytotoxic synergy, we also examined the interaction between these two agents in repair-deficient XP cells with defects in two different complementation groups. Complementation group A cells (e.g., XP20S and XP12BE) are generally considered to be the most deficient in repairing ultraviolet light-induced DNA lesions (18) because they lack NER activity (19). These cells also possess undetectable or very low activ-

ity in removing DNA interstrand cross-links (20). XP complementation group C cells (e.g., XP8BE) are also defective in the overall NER process, and the residual repair activity of the cells is confined to the transcribed strand of active genes (21, 22). The data in this report indicate that the combination of F-ara-A and CDDP within the range of clinically effective concentrations produced synergistic cytotoxicity in K562 cells without inducing apoptosis. Such a synergy could not be detected in repair-deficient human XP cell lines. The latter observation prompted us to investigate the effect of fludarabine on the repair of CDDP-induced DNA lesions. The results presented here provide direct evidence that F-ara-ATP suppresses the NER of CDDP-induced DNA adducts and inhibits the repair of interstrand cross-links in K562 human CML-blast crisis cells.

## Materials and Methods

**Chemicals and enzymes.** F-ara-A was prepared through dephosphorylation of fludarabine (kindly provided by Berlex Biosciences, Alameda, CA) with alkaline phosphatase as described previously (4). F-ara-ATP containing no detectable dATP contamination was synthesized chemically as described previously (23). CDDP (Sigma Chemical, St. Louis, MO) was dissolved in water (1 mg/ml) and stored at –80°. [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol) was purchased from Dupont-New England Nuclear (Boston, MA). All restriction endonucleases were obtained from GIBCO BRL (Gaithersburg, MD).

**Cell lines.** K562 leukemia cells were obtained from American Type Culture Collection (Rockville, MD). The XP cell lines, including XP20S (GM2345, group A), XP12BE (GM2250, group A), and XP8BE (GM2249, group C), were from Coriell Cell Repositories (Camden, NJ). All cell lines were grown in RPMI 1640 medium containing 1% glutamine and 10% fetal calf serum. CP2.0 cells, which are CDDP-resistant human colon tumor cells, were grown in Ham's F-10 medium as reported previously (24). All cells were determined to be free of mycoplasma contamination by testing with the MycoTect Kit (GIBCO BRL).

**DNA probes.** The DNA probes used in Southern hybridization were a 1.0-kb cDNA fragment of *ERCC1* (provided by Dr. J. Hoeijmakers, Emrasmus University, Rotterdam, The Netherlands), a 1.2-kb *Sam/HindIII* exon 6 fragment of the human *DHFR*, and a 2.5-kb *HindIII* fragment of  $\alpha$ -satellite sequences from human chromosome 8 (American Type Culture Collection). All three fragments were derived from plasmid constructs, gel purified, and <sup>32</sup>P-labeled using nick translation kits (Boehringer-Mannheim, Indianapolis, IN).

**Clonogenic assay.** The cytotoxicities of F-ara-A and CDDP as single agents and in combination were evaluated in K562 and XP cells by a clonogenic assay using 0.35% soft agar in growth medium containing 20% fetal calf serum. The growth medium for all three groups of XP cells was supplemented with 20% conditioned medium to aid colony formation. Cells grown in logarithmic phase were treated with F-ara-A (0.1–2.5  $\mu$ M for K562, 0.05–1.0  $\mu$ M for XP), CDDP (0.3–7.5  $\mu$ M for K562, 0.15–3.0  $\mu$ M for XP), or a combination of the two agents (in concentrations of a fixed CDDP/F-ara-A molar ratio of 3:1) for 4 hr. Aliquots of treated cells were suspended in the growth medium containing 0.35% soft agar, plated onto 60-mm Petri dishes containing 0.7% solidified soft agarose, and incubated for 2 weeks. The plating efficiencies were 43  $\pm$  15% for K562 cells and 11  $\pm$  2%, 9  $\pm$  3%, and 6  $\pm$  2%, for XP20S, XP12BE, and XP8BE cells, respectively.

**Analysis of cytotoxic interaction.** The cytotoxic interaction between F-ara-A and CDDP was analyzed by the median-effect method of Chou and Talalay (25); the method has been described in detail previously (4). The dose-effect curve was plotted for each agent and for multiple dilutions of a fixed-ratio combination. The CI was

then defined through computer analysis using a conservative isobologram. Theoretically, the 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 synergy, values of >1 show antagonism, and a value of 1 indicates additive effects.

**DNA fragmentation analysis.** DNA fragmentation was analyzed by agarose gel electrophoresis and quantified by diphenylamine reagent as described by McConkey *et al.* (26). Briefly, an aliquot of  $2.0 \times 10^6$  K562 cells was treated with CDDP (3.0–30  $\mu\text{M}$ ), F-ara-A (2.5–15  $\mu\text{M}$ ), or both for 4 hr. The drug-treated cells were washed and then incubated in drug-free medium for an additional 20 hr before being lysed in 25 mM Tris buffer, pH 8.0, containing 0.5% Triton X-100, 10 mM EGTA, and 10 mM EDTA. The supernatant of the lysate containing fragmented DNA was separated through centrifugation from the pellet containing intact chromatin. The DNA contents in both fractions were determined using the diphenylamine reagent. The results were expressed as the ratio of the content of DNA fragments in the supernatant to the total DNA content in both the supernatant and pellet fractions. To reveal the fragmentation pattern, DNA was precipitated from the supernatant with isopropanol and NaCl (0.5 M final concentration) and then treated with RNase A. The fragmented DNA was resolved by electrophoresis in 1.8% agarose gels containing ethidium bromide and visualized by transillumination of the gel with ultraviolet light.

CP2.0 cells were included in these experiments to serve as a positive control for the drug-induced DNA cleavage. CP2.0 cells were exposed to higher but equitoxic concentrations of CDDP (7.5–30  $\mu\text{M}$ ) and F-ara-A (15 or 30  $\mu\text{M}$ ) because compared with K562 cells, these cells are relatively resistant to both agents.

**Cell morphological assessment.** K562 and CP2.0 cells were treated with CDDP (7.5 or 15  $\mu\text{M}$ ), F-ara-A (2.5 or 15  $\mu\text{M}$ ), or both drugs (four combinations using the above concentrations of each drug) for 4 hr. At 0, 4, 10, and 20 hr after removal of the drug, cells were collected by cytospin, stained with Wright/Giemsa stain, and examined under a light microscope. Non-drug-treated K562 or CP2.0 cells were used as controls.

**EBFA.** The EBFA detects DNA interstrand cross-links on the basis of differential binding of ethidium bromide to double- and single-stranded DNA. To perform the assay, cells were treated with CDDP (1.5, 3.0, or 4.5  $\mu\text{M}$  for K562; 0.75, 1.5, or 3.0  $\mu\text{M}$  for the three XP lines) alone or in combination with F-ara-A (2.5  $\mu\text{M}$ ) for 4 hr, and DNA interstrand cross-links were then quantified by EBFA, as detailed previously (27). The cross-link frequency was expressed as the CLI, which was calculated as  $\text{CLI} = (Nd - Nc)/Nc$ ,  $n = -\ln X$ , 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,  $Nd$  is the number of drug-treated cells, and  $Nc$  (considered as the background measured) is the number of untreated control cells.

**Quantitative Southern blotting.** The formation of CDDP-induced interstrand cross-links in *ERCC1* and *DHFR* genes and  $\alpha$ -satellite DNA sequences was examined by a quantitative Southern blotting technique adapted from the method described by Vos and Hanawalt (28). Briefly, cells were exposed for 4 hr to CDDP (5, 10, 20, or 50  $\mu\text{M}$ ) alone or in combination with F-ara-A (2.5  $\mu\text{M}$ ). An aliquot of genomic DNA extracted from the drug-treated cells was digested with *KpnI* for the cross-linking in *ERCC1* and *DHFR* and digested with *EcoRI* for the  $\alpha$ -satellite fragments. DNA was denatured with 0.2 N NaOH, electrophoresed in a 0.5% neutral agarose gel, transferred onto the membrane, and hybridized with  $^{32}\text{P}$ -labeled probes. The intensity of the signal was quantified with a Betascope (Betagen, Waltham, MA), and the degree of cross-link formation was determined by the ratio of the intensity of the double-stranded band to the sum of the intensities of the single- and double-stranded bands. Controls (denatured and nondenatured DNA from cells not exposed to the drugs) were used to localize the gel migration positions of single- and double-stranded forms of a designated gene fragment.

**Detection of CDDP-induced DNA interstrand cross-link repair.** The cellular repair of genomic interstrand cross-links was detected with EBFA by measuring the kinetics of cross-link removal. Cells were first treated with CDDP (3.0  $\mu\text{M}$  for K562, 1.5  $\mu\text{M}$  for XP20S) alone or in combination with F-ara-A (2.5  $\mu\text{M}$ ). The cells were then washed to remove CDDP and F-ara-A and immediately exposed to thiourea (1 mM) for 1 hr to block the conversion of monoadducts to bifunctional cross-links during the measurement of repair (29). The CLI, assessed by EBFA as described above, was then determined at 0, 3, 6, 10, and 24 hr after the removal of CDDP and F-ara-A. The repair efficiency was expressed as the percentage of initial cross-links that remained after drug removal.

**Detection of CDDP-induced DNA intrastrand adduct repair.** The *in vitro* repair assay of Hansson and Wood (30) was adapted for these experiments. In this assay system, a CDDP-damaged plasmid served as the substrate for repair enzymes in whole-cell extracts. Cell extract-mediated NER was reflected by repair-patch synthesis activity, which was quantified by the specific incorporation of radioactive nucleotide into a damaged plasmid. The plasmids for the assay were the 2959-bp CDDP-modified pBluescript KS<sup>+</sup> plasmid (pBS; Stratagene, La Jolla, CA) and a 3738-bp pHM14 plasmid (pHM; a gift from Dr. R. D. Wood, Imperial Cancer Research Fund, South Mimms, Herts, UK). The pBS plasmid was platinated using a CDDP-to-nucleotide molar ratio of 0.005 (30). The total platinum content in the closed circular plasmid DNA was determined by flameless atomic absorption spectroscopy, and the number of interstrand cross-links per plasmid was assessed by EBFA as described above. The pHM plasmid DNA was used as the nondamaged control in the repair reaction for measuring background incorporation of radioactive nucleotide.

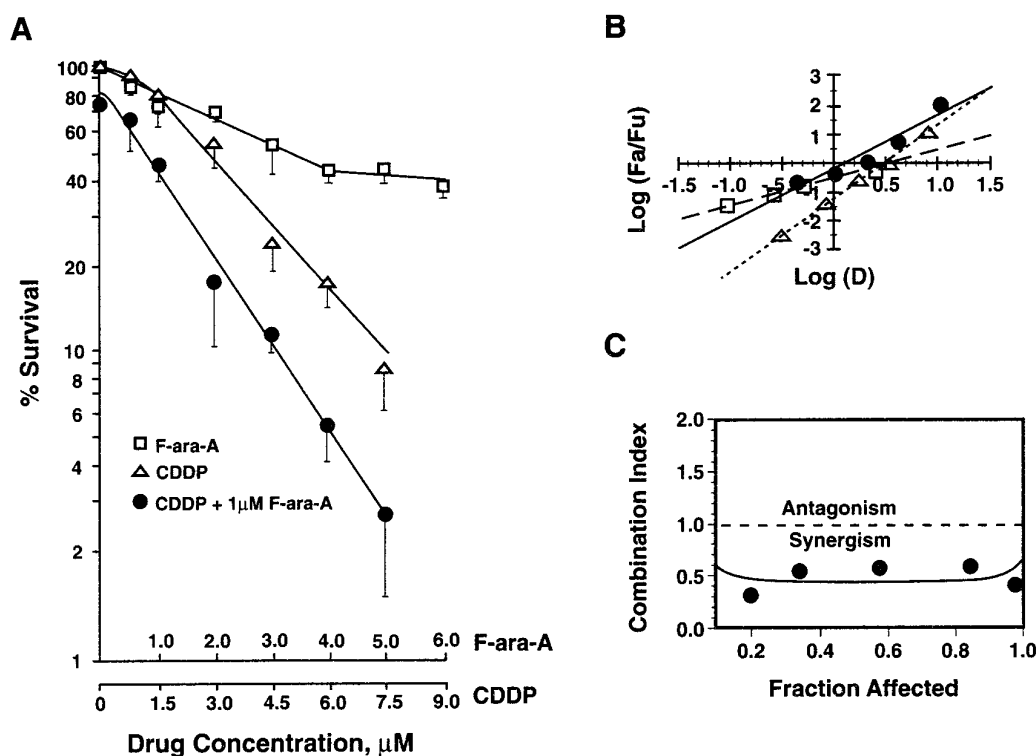
Whole-cell extracts were prepared according to the method of Manley *et al.* (31), with minor modifications as described by Shivji *et al.* (32). A standard 50- $\mu\text{l}$  reaction mixture containing 300 ng each of CDDP-damaged pBS and nondamaged pHM in 45 mM HEPES-KOH, pH 7.8, 70 mM KCl, 7.4 mM  $\text{MgCl}_2$ , 0.9 mM dithiothreitol, 0.4 mM EDTA, 2 mM ATP, 40 mM phosphocreatine, 2.5  $\mu\text{g}$  of creatine phosphokinase (Type I; Sigma), 3.4% glycerol, 18  $\mu\text{g}$  of bovine serum albumin, 20  $\mu\text{M}$  concentration each of dGTP and dTTP, 4  $\mu\text{M}$  concentration each of dATP and dCTP, and 2  $\mu\text{Ci}$  of [ $\alpha$ - $^{32}\text{P}$ ]dCTP was incubated with increasing concentrations of cell extracts from K562 or XP cells at 30° for 5 hr. After the reaction was stopped by the addition of 20 mM EDTA, treatment with proteinase K (0.5 mg/ml, 50° for 30 min) plasmid DNA was purified, linearized by *Bam*HI, and subjected to electrophoresis. The incorporation of radioactive nucleotides was detected by autoradiography and quantified with a Betascope (Betagen). The results were normalized for the DNA content in the corresponding lanes. DNA-repair synthesis, determined by specific incorporation of [ $\alpha$ - $^{32}\text{P}$ ]dCMP, was calculated by subtracting nonspecific incorporation measured in the nondamaged pHM control from the total incorporation measured in the CDDP-damaged pBS substrate.

The inhibition of repair activity by F-ara-ATP was measured by the repair assay using 100- $\mu\text{g}$  protein equivalent whole-cell extracts in the presence of increasing concentrations of F-ara-ATP (20, 40, 60, 80, or 100  $\mu\text{M}$ ) in the reaction mixture. The controls were samples that contained no F-ara-ATP. The inhibition was expressed as a percentage of control activity by assigning a value of 100% to the specific incorporation of dCMP by the control.

## Results

**Cytotoxicity interaction of F-ara-A with CDDP.** Fig. 1A shows the survival of K562 cells treated with various concentrations of F-ara-A and CDDP as single agents and in combination. The data indicate that the two drugs in combination produced a cooperative cytotoxic effect compared with the single agents. The precise mode of this cooperation was





**Fig. 1.** Cytotoxicity produced by F-ara-A and CDDP in K562 cells. A, Survival of K562 cells treated with F-ara-A and/or CDDP. The cytotoxicity was determined by a soft-agar clonogenic assay as described in Materials and Methods. The dose-response cell survival was assessed after the cells were exposed to various concentrations of F-ara-A (0.5–6.0  $\mu\text{M}$ ), CDDP (0.75–7.5  $\mu\text{M}$ ), or both (CDDP, 0.75–7.5  $\mu\text{M}$ ; F-ara-A, 1  $\mu\text{M}$ ) for 4 hr. B, Median-effect plot. The cells were treated for 4 hr with CDDP (0.3, 0.75, 1.5, 3.0, or 7.5  $\mu\text{M}$ ), F-ara-A (0.1, 0.25, 0.5, 1.0, or 2.5  $\mu\text{M}$ ), or both at a fixed molar ratio of 3:1. The median-effect plot was constructed as described in Materials and Methods. Fa, affected fraction; Fu, unaffected fraction; D, drug concentration;  $\Delta$ , CDDP;  $\square$ , F-ara-A;  $\bullet$ , CDDP plus F-ara-A. C, The fraction affected-CI plot was constructed by computer analysis of the data in B using the conservative isobologram. CI values of  $<1$  occurred at a wide range of inhibition levels, indicating synergy produced by the combination. The data are the means of two separate experiments with samples in duplicate. Bars, mean  $\pm$  standard deviation.

analyzed by the median-effect method (25). Fig. 1B shows that the slopes of the median-effect plots for the single drugs and the combination were not parallel, suggesting that the two agents had different modes of action and that their effects were mutually nonexclusive. Therefore, the equation for the conservative isobologram was used to calculate the CI values shown in the fraction affected-CI plot constructed by computer analysis. The CI values (at 1–99% inhibition levels) were all  $<0.62$  (Fig. 1C), indicating that combined F-ara-A and CDDP produced synergistic cytotoxic effects in K562 cells.

To investigate whether the repair deficiency also results in drug hypersensitivity, three XP cell lines were subjected to the same drug treatment used for K562 cells. Table 1 summarizes the  $D_m$  ( $IC_{50}$ ) values for F-ara-A and CDDP as single agents or in combination in different cell lines. Based on the  $D_m$  values, F-ara-A and CDDP both were more cytotoxic to XP than to K562 cells. This finding is consistent with previous studies showing that the drug sensitivity or resistance of a cell is related to its DNA-repair capacity (4). The two drugs in combination, however, produced no significant synergistic effects in XP cells, as indicated by the fact that the CI values (0.99–1.05, 1.09–1.24, and 1.14–1.22 for XP20S, XP12BE, and XP8BE cells, respectively) were all  $>0.99$ .

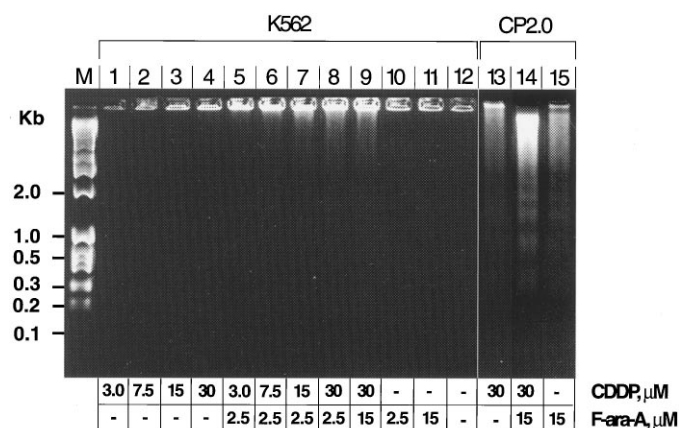
**Studies on DNA fragmentation and morphological characteristics of apoptosis.** To determine whether F-ara-A by itself or in combination with CDDP induced apoptosis in K562 cells, DNA fragmentation and morphological changes were examined in K562 cells treated with the drugs as single agents or in combination. The apoptosis-permissive CP2.0 human colon cancer cells were included in this experiment for comparison. DNA isolated from CP2.0 cells treated with F-ara-A or CDDP alone exhibited a ladder of internucleosomal DNA cleavage characteristic of apoptosis (Fig. 2, lanes 13 and 15). F-ara-A, when combined with CDDP, significantly enhanced CDDP-induced DNA fragmentation in CP2.0 cells, and the enhancement was greater than an additive effect (Fig. 2, lane 14) as assessed by the reaction with diphenylamine reagent: the fractions of fragmented DNA in the groups treated with F-ara-A, CDDP, and both drugs were  $7.1 \pm 1.7\%$ ,  $15.5 \pm 2.5\%$ , and  $37.3 \pm 4.0\%$ , respectively. Thus, in CP2.0 cells, the F-ara-A-mediated enhancement of apopto-

TABLE 1

**Relative cytotoxicities of F-ara-A and CDDP as single agents and in combination in K562 cells and three XP lines**

Cells were exposed to F-ara-A and CDDP alone or in combination for 4 hr; the  $D_m$  value was determined by the median-effect method as described in the text. Values in parentheses are standard deviation of the mean of two separate experiments with samples in duplicates.

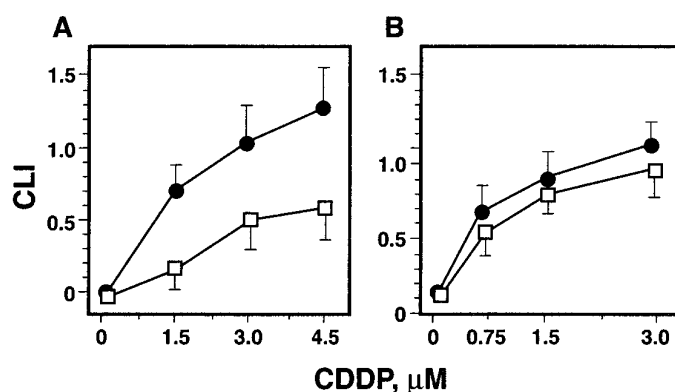
Drug	Cell line $D_m$			
	K562	XP20S	XP12BE	XP8BE
	$\mu\text{M}$			
F-ara-A	3.33 (0.52)	0.22 (0.05)	0.18 (0.05)	0.82 (0.12)
CDDP	2.28 (0.33)	0.17 (0.06)	0.15 (0.04)	0.52 (0.03)
F-ara-A + CDDP	0.36 $\pm$ 1.10	0.06 $\pm$ 0.16	0.05 $\pm$ 0.16	0.20 $\pm$ 0.67



**Fig. 2.** Effects of F-ara-A and/or CDDP on internucleosomal DNA fragmentation in K562 and CP2.0 cells shown is a representative result of agarose gel analysis of DNA fragmentation in K562 (lanes 1–12) and CP2.0 (lanes 13–15) cells after treatment with F-ara-A, CDDP, or both for 4 hr. At the end of the drug treatment, cells were incubated with drug-free medium for an additional 20 hr before being lysed for DNA isolation. The fragmented DNA was separated from the intact chromatin by centrifugation, precipitated, and resolved by electrophoresis in a 1.8% gel. M, DNA molecular weight markers.

sis correlated well with the previously reported cytotoxic synergy of combined F-ara-A and CDDP (4). In contrast, such nuclear DNA cleavage was not observed in K562 cells exposed to these agents (Fig. 2, lanes 1–12). In addition, although CP2.0 cells exhibited morphological changes consistent with apoptosis (i.e., cell shrinkage and condensation of nuclear chromatin), no such changes were observed in K562 cells (data not shown). Instead, at 24 hr after the onset of the drug treatment, the plasma membrane and chromatin of K562 cells were seen as small and ill-defined masses, providing additional evidence that K562 cells died by necrosis (11). These results therefore exclude the possibility that the synergistic cytotoxicity arose through the reversal of apoptosis resistance in K562 cells by the two agents in combination.

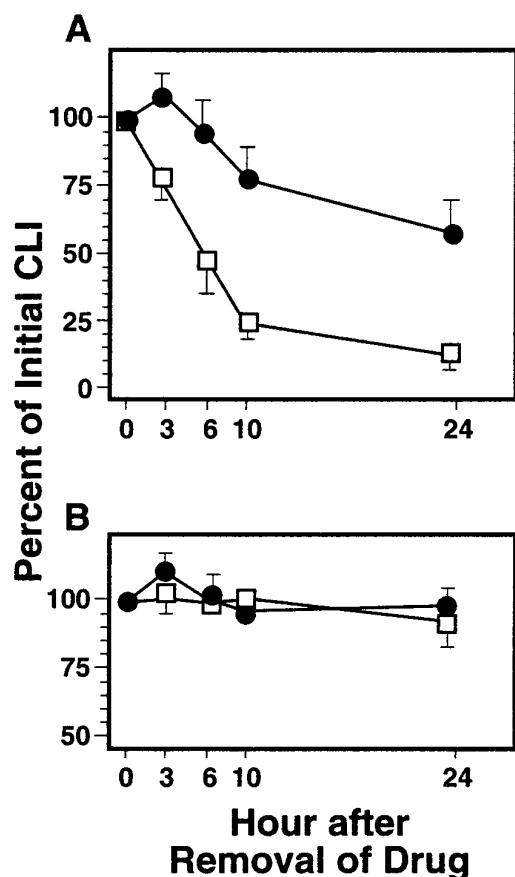
**Effects of F-ara-A on the formation and repair of DNA interstrand cross-links.** The observation that the synergy of F-ara-A and CDDP did not take place in NER-defective XP cells (Table 1) led us to speculate that the cytotoxic synergy in K562 cells occurred through the interference of F-ara-A with cellular repair of CDDP-induced DNA damage. To test this hypothesis, we first examine the formation of interstrand cross-links in the overall genome. CDDP produced interstrand cross-links, and the effect increased as a function of the concentration of the agent (Fig. 3A). When K562 cells were treated with F-ara-A and CDDP in combination, the cross-link formation increased significantly ( $p < 0.005$ ) over that with CDDP alone at all CDDP concentrations tested (Fig. 3A). A plausible interpretation of these results is that F-ara-ATP, the active metabolite of F-ara-A, enhanced the cross-link formation by inhibiting cellular removal of these lesions. To test this hypothesis, we compared the effect of the same treatment in XP cells, which lack the capacity to make repair incisions at the DNA damage sites (7, 33). In XP20S cells, CDDP alone induced a dose-dependent formation of DNA cross-links similar to that observed in K562 cells (Fig. 3B); however, the combination of CDDP and F-ara-A failed to significantly increase ( $p > 0.1$ ) cross-link formation, clearly indicating that integrity of re-



**Fig. 3.** Effects of F-ara-A on the formation of CDDP-induced DNA interstrand cross-links in the overall genome. K562 (A) and XP20S (B) cells were treated for 4 hr with various concentrations of CDDP (1.5, 3.0, or 4.5  $\mu$ M for K562; 0.75, 1.5, or 3.0  $\mu$ M for XP20S) alone ( $\square$ ) or in combination with 2.5  $\mu$ M F-ara-A ( $\bullet$ ). The cross-links were measured by EBFA after drug removal and expressed as the CLI calculated with the Poisson formula as described in Materials and Methods. Points, mean values of three separate experiments. Bars, mean  $\pm$  standard deviation.

pair capacity was essential for F-ara-A to augment the effects of CDDP. Furthermore, experiments in which the rate of removal of CDDP-induced cross-links from K562 cells was measured provided direct evidence for F-ara-A-induced repair inhibition. Fig. 4A shows that when F-ara-A was combined with CDDP, not only the rate of removal but also the capacity of the cells to remove the cross-links was greatly inhibited compared with those of cells treated with CDDP alone. At 10 hr after treatment, although  $\sim 77\%$  of the initially formed cross-links had been removed (linearly at a rate of 7.7%/hr) from the cells that were not exposed to F-ara-A, in cells treated with the agents in combination, only  $\sim 22\%$  (2.2%/hr) of the DNA lesions had been removed. Again, such effects of F-ara-A were not detected in repair-deficient XP20S cells (Fig. 4B).

Several groups (6) have shown that actively transcribing genes are more susceptible to assault by genotoxic agents than are silent genes and, likewise, that damage to actively transcribing genes may have more profound biological consequences. Therefore, we studied the effects of F-ara-A on the formation of cross-links in the actively transcribing *ERCC1* and *DHFR* genes and in the nontranscribed  $\alpha$ -satellite DNA sequences. *ERCC1* is a mammalian DNA excision repair gene that confers resistance to CDDP in Chinese hamster ovary cells of complementation group 1 that are deficient in their capacity to repair ultraviolet light-induced damage (34). Northern blot analysis indicated that this gene was actively expressed in K562 cells (data not shown). We found that CDDP induced cross-links in an 18-kb *KpnI* *ERCC1* fragment in a dose-dependent fashion (Fig. 5A). Simultaneous exposure to F-ara-A and CDDP (Fig. 5A, lanes 4–6, top) significantly increased the number of cross-links (lanes 1–3). The F-ara-A-mediated enhancement of CDDP-induced cross-linking was also observed in the *DHFR* housekeeping gene (in a 16-kb *KpnI* DNA fragment) (Fig. 5B). Such an effect, however, was not seen in the nontranscribed  $\alpha$ -satellite DNA sequence (in a 1.8-kb *EcoRI* DNA fragment) (Fig. 5C). These data indicate that F-ara-A inhibited DNA cross-linking in a gene-specific manner and that the effect on actively transcribing genes exceeded that on inactive genes.



**Fig. 4.** Effects of F-ara-A on the cellular removal of CDDP-induced DNA interstrand cross-links. K562 (A) and XP20S (B) cells were treated for 4 hr with CDDP (3.0  $\mu$ M for K562, 1.5  $\mu$ M for XP20S) alone (□) or in combination with 2.5  $\mu$ M F-ara-A (●). The initial mean CLI values measured by EBFA were  $0.55 \pm 0.14$  (K562) and  $0.75 \pm 0.26$  (XP20S) for cells treated with CDDP alone and  $1.09 \pm 0.20$  (K562) and  $0.83 \pm 0.08$  (XP20S) for cells treated with CDDP plus F-ara-A. After the cells were washed to remove the drugs, CLI values were determined again at the times indicated during the postwashing incubation. Data are the mean values of three separate experiments. Bars, mean  $\pm$  standard deviation.

#### Effect of F-ara-ATP on nucleotide excision repair.

We further examined the effect of F-ara-ATP on the repair of intrastrand adducts. A CDDP-modified pBS plasmid was used as the substrate in the *in vitro* repair assay, in which the repair activity was quantified by measuring the specific incorporation of [ $^{32}$ P]dCMP into CDDP-damaged plasmid DNA during repair synthesis. The platinated pBS contained 14–16 platinum molecules as measured by atomic absorption spectrophotometry. Only 5–7% of the platination product was interstrand cross-links as revealed by EBFA; therefore, the major platination product in the CDDP-damaged pBS substrate was presumably intrastrand adducts. Fig. 6 shows that the repair activity in K562 cell extracts increased linearly as the protein concentration increased and reached  $75.6 \pm 12.0$  fmol/100- $\mu$ g protein equivalent. F-ara-ATP at 75  $\mu$ M inhibited 50% of dCMP incorporation by K562 cell extracts (Fig. 7); the inhibition was dose dependent. In contrast, when we used XP20S cell extracts, which had a 12% basal activity in comparison with K562 extracts (Fig. 6), F-ara-ATP showed no significant inhibition of dCMP incorporation (data not shown).

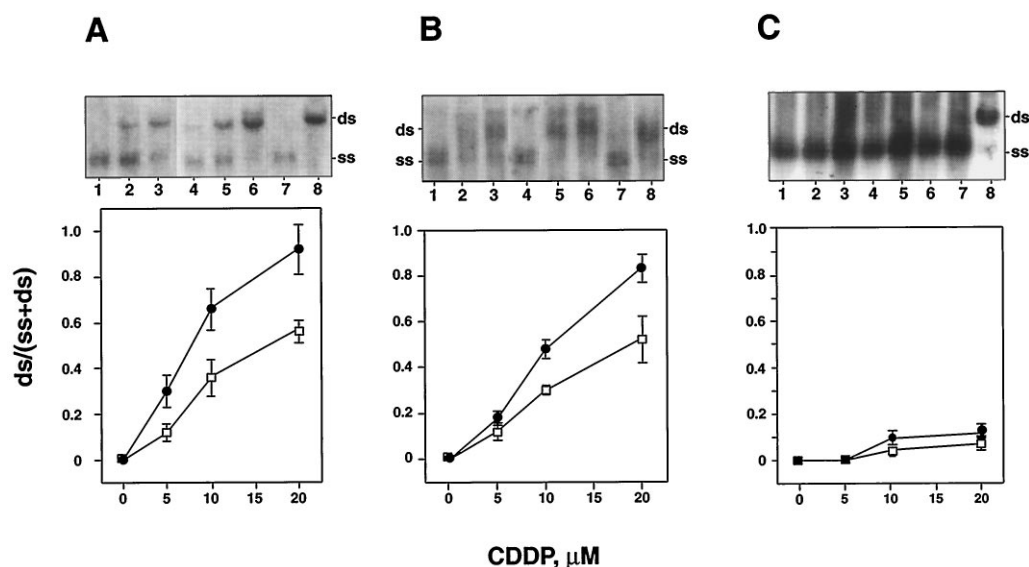
## Discussion

This study revealed synergy between F-ara-A and CDDP in apoptosis-resistant K562 cells and suggests that DNA-repair inhibition by F-ara-A is responsible for the cytotoxic synergy of the F-ara-A-plus-CDDP combination. These findings have important implications for cancer treatment: (i) the F-ara-A and CDDP combination may prove to be effective against tumors that are apoptosis permissive or resistant, and (ii) DNA-repair modulation could be a useful target for drug development.

Previously, data from this laboratory have demonstrated that combined F-ara-A and CDDP produced synergistic cytotoxicity in CP2.0 cells (4). Because it has been reported that F-ara-A and CDDP are apoptosis-inducing agents, we therefore assume that the synergy produced by these agents arises through the enhancement of apoptosis. Support for this contention comes from the data in Fig. 2, which show that F-ara-A significantly enhanced apoptotic DNA fragmentation induced by CDDP in CP2.0 cells. In applying this finding to K562 cells, we first speculated that the enhanced cytotoxicity produced by the combination treatment was a result of overcoming the apoptosis resistance in K562 cells. Such speculation was further encouraged by our initial observations in this study and by the findings of others (15, 17). Our observation that F-ara-A significantly increased CDDP-induced formation of DNA cross-links in a gene-specific manner raises the possibility that F-ara-A preferentially inhibits the repair of certain overexpressed or actively transcribed genes that are important to the leukemia biology of K562 cells. McGahon *et al.* (15) reported that treatment of K562 cells with an antisense oligonucleotide directed against the *Bcr-Abl* oncogene resulted in a down-regulation of the oncoprotein and sensitization of K562 cells to apoptosis caused by cytotoxic agents. Recently, Ray *et al.* (17) reported that inhibition of the antiapoptosis bcl- $x_L$  protein function in K562 cells by transfection with an expression vector encoding bcl- $x_S$ , a dominant inhibitor of bcl- $x_L$  and bcl-2, rendered the cells sensitive to cytarabine-induced differentiation and apoptosis. However, we could find no evidence of apoptosis in K562 cells treated with F-ara-A plus CDDP, suggesting that programmed cell death was not responsible for the reduced colony formation. On the basis of DNA electrophoresis and light microscopy, we suggest that the death of the drug-treated cells was due to necrosis rather than apoptosis.

The association of repair inhibition with cytotoxic synergy was first indicated by the restriction of the synergy to repair-proficient K562 cells. In all of three repair-deficient XP cell lines tested, F-ara-A was not synergistic with CDDP. Because F-ara-A and CDDP were active in XP cells, as shown by the fact that F-ara-A and CDDP as single agents were more cytotoxic to all XP lines than to K562 cells (Table 1), the lack of synergy in XP cells cannot be attributed to inactivation of these drugs in XP cells. Therefore, the likely explanation for the lack of a synergistic interaction would be a defect in the repair system of these cells. The association of repair and synergy was further demonstrated by the fact that the degree of synergy correlated with the cellular capacity to repair DNA damage, as determined by a comparison of the current results with those from our previous study (4). CP2.0 cells possess  $\sim 40\%$  greater DNA-repair activity than do K562 cells, as assessed by the *in vitro* repair assay ( $107 \pm 3.6$





**Fig. 5.** Effects of F-ara-A on the formation of CDDP-induced DNA interstrand cross-links in specific DNA sequences. The cross-links in K562 cells were measured by Southern blotting after denaturation/renaturation agarose gel electrophoresis. Cells were exposed for 4 hr to CDDP alone (5, 10, and 20  $\mu\text{M}$  for lanes 1–3, respectively) or to CDDP at the same concentrations in combination with 2.5  $\mu\text{M}$  F-ara-A (lanes 4–6). The blot was probed with radiolabeled *ERCC1* (A), *DHFR* (B), or  $\alpha$ -satellite (C) DNA. Also shown are controls in which cells were not exposed to the drugs (lane 7, denatured DNA; lane 8, nondenatured DNA). ds, double-stranded; ss, single-stranded. *Top*, autoradiographic results from quantitative Southern blotting. *Bottom*, quantification of the band intensities shown in top with a Betascope blot analyzer. Cross-link formation was expressed as the ratio of the intensity of the double-stranded band to the sum of the intensities of the single- and double-stranded bands. The minor bands of the single- and double-stranded in B are presumably derived from *KpnI* cleavage of exon 6 of *DHFR*.  $\square$ , CDDP alone;  $\bullet$ , CDDP plus F-ara-A. Points, mean values of three separate experiments. Bars, mean  $\pm$  standard deviation.

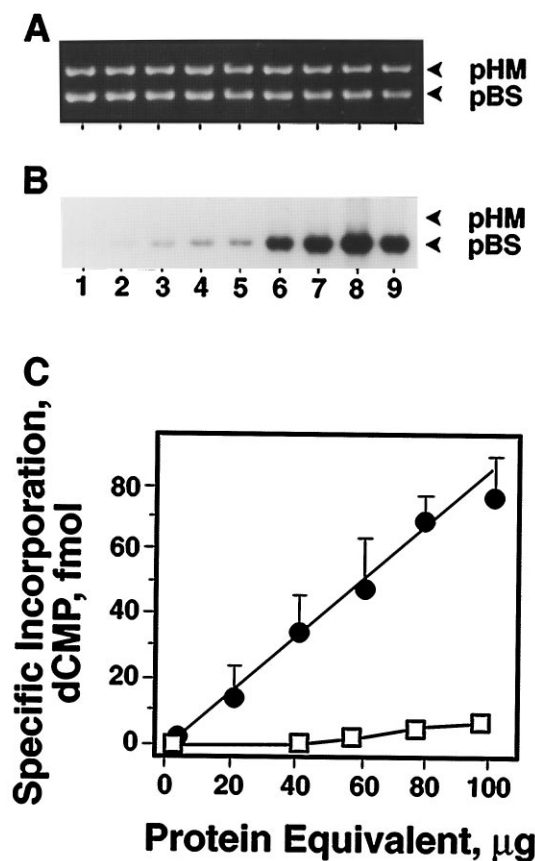
versus  $75.6 \pm 12.0$  fmol/100  $\mu\text{g}$  of protein). When the interactions between the two agents in the two cell lines were compared, F-ara-A produced greater cytotoxic synergy in CP2.0 cells than in K562 cells. These data imply not only that the cytotoxic synergy is restricted to repair-proficient cells but also that the degree of synergy correlates with the cellular competence in repairing DNA damage. The inhibitory effect of fludarabine on the repair of CDDP-induced DNA interstrand cross-links in K562 cells was demonstrated by the results from EBFA and quantitative Southern blot analysis. Treatment of K562 cells with F-ara-A enhanced formation of CDDP-induced interstrand cross-links (Figs. 3 and 5), and the accumulation of these CDDP-induced DNA lesions was concomitant with the inhibition of cross-link removal by fludarabine (Fig. 4). The inhibition of repair of CDDP-induced intrastrand adducts was indicated by the results of the *in vitro* repair assay, which showed that the NER of CDDP-induced DNA adducts by the K562 cell extracts was inhibited by F-ara-ATP (Fig. 7). Taken together, our results strongly suggest that the inhibition of DNA repair plays a primary role in the synergistic cytotoxicity produced by the two agents in combination. In apoptosis-permissive cells, such as the CP2.0 line, inhibition of repair presumably facilitated the apoptotic pathway and thereby led to increased cell kill, whereas in apoptosis-resistant K562 cells, repair inhibition enhanced the cell death through necrosis.

It should be noted that two types of non-NER proteins, hMutS $\alpha$  mismatch repair protein and HMG domain-containing proteins, have been reported to bind CDDP adducts. The former binds exclusively to CDDP 1,2-d(GpG) (35), whereas the latter binds to both d(GpG) and d(ApG) adducts (36, 37). Although their precise roles in binding to CDDP-DNA adducts are still under debate, there is evidence that they interfere with the repair process or block repair components

from access to the damage sites (38). Therefore, it is reasonable to question to what degree the observed fludarabine-mediated NER inhibition represents a secondary effect of fludarabine interaction with hMutS $\alpha$  and/or HMG proteins. Further experimentation would be required to explore these links. Nevertheless, previous studies have shown that fludarabine inhibited incision and repair synthesis in the NER pathway (23) and that hMutS $\alpha$  or HMG proteins poorly recognized CDDP 1,3-d(GpNpG) adducts and interstrand cross-links (35–37). These results, when considered together with the data presented in this report, argue favorably for the notion that fludarabine-mediated inhibition of DNA repair plays a primary role in increasing the chemosensitivity of K562 cells to CDDP.

In a separate *in vitro* repair synthesis assay using extracts from CP2.0 cells, which have an enhanced repair capacity (4), we compared the relative effectiveness of repair inhibition based on  $\text{IC}_{50}$  values among a group of nucleotide analogues that included F-ara-ATP, Cl-dATP, Cl-F-ara-ATP, d-F-dCTP, ara-CTP, and ara-UTP ( $\text{IC}_{50}$  values were 38, 28, 85, 70, 40, and 84  $\mu\text{M}$ , respectively).<sup>1</sup> The results suggest that fludarabine is a relatively potent repair modulator, although it is not the sole nucleotide analogue that inhibits NER. In the current report, a relatively high level of F-ara-ATP (75  $\mu\text{M}$ ) was required to elicit significant inhibition of NER in K562 cell extracts (Fig. 7). One may therefore question the potential of fludarabine for clinical application. It is known, however, that the active metabolites of fludarabine remain relatively stable inside the cell because of their resistance to adenosine deaminase (39) and that intracellular accumulation of relatively high levels of F-ara-ATP is readily achievable. For example, after a brief exposure of K562 cells to F-ara-A,

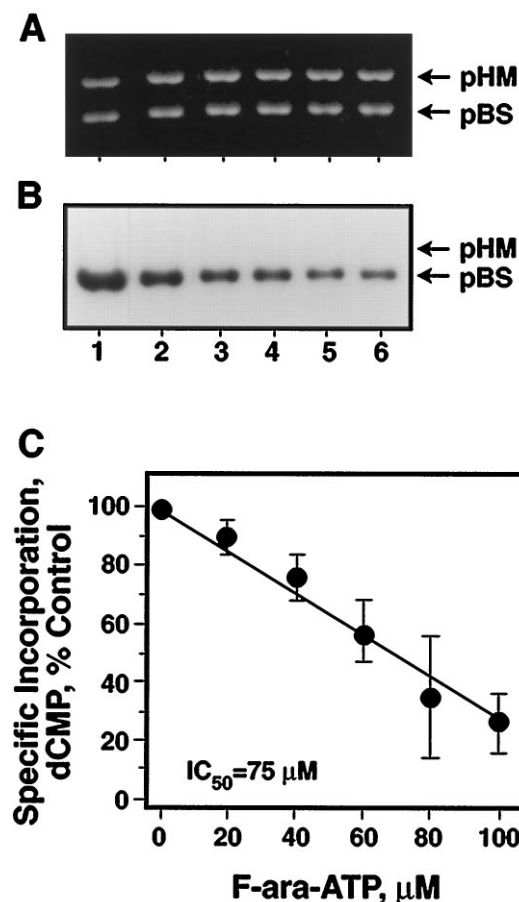
<sup>1</sup> M.-J. Li and L.-Y. Yang, unpublished observations.



**Fig. 6.** NER of CDDP-modified DNA with whole-cell extracts. CDDP-damaged pBS plasmid and a nondamaged pHM control were incubated with cell extracts from K562 or XP20S cells to measure the specific incorporation of [ $\alpha$ - $^{32}$ P]dCMP into the damaged pBS as detailed in Materials and Methods. A, Representative ethidium bromide-staining gel. B, Autoradiographic results of A. Lanes 1–4, extracts from XP20S cells. Lanes 5–9, extracts from K562 cell. C, Betascope quantification of the band intensity shown in B. The incorporation of dCMP is expressed as a function of the protein equivalent of the extract. ●, K562; □, XP20S. Points, mean values of two separate experiments with samples in duplicates. Bars, mean  $\pm$  standard deviation.

intracellular F-ara-ATP levels of  $>100 \mu\text{M}$  have been reported (2). In the clinic, F-ara-ATP concentrations of 50–100  $\mu\text{M}$  have often been measured in leukemic lymphocytes of patients undergoing fludarabine therapy (40). These results further support the continued clinical application of fludarabine in multidrug regimens.

In conclusion, this work demonstrated that fludarabine inhibited the repair of interstrand cross-links and intra-strand adducts induced by CDDP and that combined F-ara-A and CDDP produced synergistic cytotoxicity in apoptosis-resistant K562 cells that expressed the *Bcr-Abl* oncogene. The cytotoxic synergy was not only restricted to repair-competent cells but also correlated with the extent of cellular repair capacity, strongly suggesting that fludarabine-mediated repair inhibition is responsible for the synergy. In apoptosis-permissive CP2.0 cells, the synergistic interaction of F-ara-A and CDDP enhanced apoptotic cell death, whereas in apoptosis-resistant K562 cells, the same interaction did not induce apoptosis but instead led to an enhanced cell death by necrosis. These results have important implications for the clinical use of fludarabine; they suggest that fludarabine may have a role as a DNA-repair modulator. Fludarabine may



**Fig. 7.** Dose-dependent inhibition of NER by F-ara-ATP in K562 cell extracts. The NER activity was measured by the *in vitro* repair assay as described in Materials and Methods. A, Representative ethidium bromide staining gel. B, Autoradiographic results of A. C, Betascope quantification of the inhibition of the specific incorporation of [ $\alpha$ - $^{32}$ P]dCMP into damaged pBS in the presence of various concentrations of F-ara-ATP. A value of 100% was assigned to the incorporation in the absence of F-ara-ATP, and percentages of incorporation in the presence of F-ara-ATP were calculated to construct the inhibition curves. Points, mean values of three separate experiments. Bars, mean  $\pm$  standard deviation.

thus prove useful in combination with agents that induce DNA repair in repair-proficient tumor cells regardless of their sensitivity to drug-induced apoptosis.

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