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## Original Paper

# Fludarabine as a Modulator of Cisplatin Activity in Human Tumour Primary Cultures and Established Cell Lines

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**The potential of the purine analogue fludarabine (9-β-D-arabinofuranosyl-2-fluoroadenine-5′ monophosphate) as a modulator of cisplatin cytotoxicity was investigated in four established cell lines and 20 primary cultures of human melanoma and ovarian cancer. Tumour cells were exposed to fludarabine and cisplatin, alone or in combination, for 4 h. Fludarabine did not affect the growth of ovarian cancer cell lines, whereas it induced a marked and dose-dependent inhibition of proliferation in melanoma cell lines. In primary cultures of both histotypes, the purine analogue did not induce appreciable antiproliferative effects. Combined cisplatin–fludarabine treatment caused additive effects in all established cell lines. Conversely, a synergistic effect of the combination was seen in 5 of 10 melanoma and 4 of 10 ovarian cancer primary cultures, with a dose-modifying factor ranging from 2.1 to 3.9 for melanomas and from 4.0 to 7.5 for ovarian cancers, respectively. In the remaining cultures, the interaction between fludarabine and cisplatin was additive. The alkaline filter elution analysis performed on primary cultures showed that the synergistic interaction between the two drugs was paralleled by an increase in the extent and persistence of the cisplatin-induced DNA interstrand cross-links. Our results indicate that fludarabine can enhance cisplatin cytotoxic activity in human tumour primary cultures from ovarian cancer and malignant melanoma. Such an effect may be partially due to an interference by fludarabine on cisplatin-induced DNA adduct metabolism and repair.** 

**Key words: fludarabine, cisplatin, malanoma, ovarian cancer, cytotoxicity, DNA repair**
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## INTRODUCTION

CISPLATIN (CDDP) IS one of the most widely used compounds for the treatment of a wide spectrum of human malignancies [1, 2]. However, its clinical therapeutic efficacy is limited by the emergence of inherent or treatment-induced resistant tumour cell subpopulations [3].

CDDP is believed to exert its anticancer effect by DNA adduct formation, and more specifically, through the induction of DNA intrastrand, interstrand, and DNA-protein cross-links [4]. One of the most credited mechanisms for tumour cell resistance to CDDP seems to be an enhanced repair of DNA lesions [5]. As a consequence, much effort has been made in the search for modulators able to inhibit DNA repair processes. In particular, compounds such as aphidicolin, 1-β-arabinofuranosyl-cytosine (ara-C) and hydroxyurea (which interfere with the final step of DNA repair, i.e. DNA resynthesis) have demonstrated their ability to potentiate the cytotoxicity of some cross-linking agents including CDDP and nitrogen mustards [6–9]. Moreover, inhibitors of tumour cell energy metabolism, such as lonidamine [10], positively modulate CDDP cytotoxic activity through an enhancement of CDDP-induced interstrand cross-links [11].

Fludarabine (9-β-arabinofuranosyl-2-fluoroadenine-5′ monophosphate [F-ara-AMP]), is a fluorinated purine analogue, metabolically more stable than ara-C, successfully used in the clinical management of chronic lymphocytic leukaemia and other haematological malignancies [12]. In the active, triphosphate form, F-ara-ATP is incorporated into DNA and terminates the elongation of the DNA strand [13]. Moreover, the drug inhibits DNA synthesis through inhibition of DNA polymerases α and ε, DNA primase, ribonucleotide reductase and DNA ligase I [14–18]. Such a mechanism of action indicates F-ara-AMP may be a potential effective inhibitor of DNA repair. In fact, the drug has already been shown to reduce the repair of ionising radiation-induced chromosome damage in human peripheral blood cells [19] as well as to inhibit CDDP–DNA cross-link repair in human colon adenocarcinoma cell lines [20].

Based on these findings, in the present study we proposed

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to investigate the potential of F-ara-AMP as a modulator of CDDP cytotoxicity in human cutaneous melanoma and ovarian cancer cell lines as well as in primary cultures, directly obtained from clinical specimens. Moreover, the possible interference of the purine analogue on the accumulation and removal of CDDP-induced DNA interstrand cross-links (DNA-ISC) was investigated.

## MATERIALS AND METHODS

*Cell lines*

Two human melanoma cell lines (M14, JR8) and two ovarian carcinoma cell lines, one sensitive (A2780) and one with experimentally induced resistance CDDP (A2780/Cp8, with a resistance index of 5.8) were used. Their biological characteristics have been previously described [21–23]. Cell lines were maintained as a monolayer at 37°C in a 5% carbon dioxide humidified atmosphere in air, using RPMI-1640 medium supplemented with 10% fetal calf serum, 2 μM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 U/ml insulin (only for A2780 and A2780/Cp8).

*Primary cultures*

Solid tumour specimens were obtained from 20 previously untreated patients who underwent surgery at the National Cancer Institute of Milan. Samples included nine lymph node and one visceral metastases from cutaneous melanomas, and five primary lesions and five visceral metastases from ovarian cancers. Immediately following surgery, the samples were placed in cold Hank's balanced salt solution (HBSS, GIBCO Laboratories, Grand Island, New York, U.S.A.), trimmed of adipose and necrotic tissue, and cut into small pieces. Melanoma fragments were mechanically disaggregated, and ovarian cancer fragments, due to the low cell yield, were enzymatically digested to obtain a cell suspension [24]. Cell viability, determined by Trypan blue dye exclusion, was similar for the two tumour types and ranged from 13 to 87%, with a median value of 56%. Viable cells ($2–5 \times 10^4/cm^2$) were plated in 7 ml of serum-free Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12 (DME/F12, Sigma Chemical Co., St. Louis, Missouri, U.S.A.) and cultured as reported elsewhere [24]. Cytotoxicity assay and alkaline filter elution experiments were simultaneously performed on exponentially growth primary cultures at 6–10 days after plating. The nature of cells was assessed by using different monoclonal antibodies, specific against melanoma (anti-S100, Biogenex Laboratories, San Ramon, California, U.S.A.; and HMB45, Enzo Biochemicals, New York, New York, U.S.A.) and ovarian cancer cells (anti-CA125, Signet Laboratories, Dedham, Massachusetts, U.S.A.). Antibody binding to cells was evidenced by the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique according to Cordel and coworkers [25]. All analysed cultures were highly positive (>80% of cells) to at least one marker.

*Cell cycle distribution analysis*

Cells ($1 \times 10^6$) were stained in a solution containing 50 μg/ml propidium iodide, 50 mg/ml RNase and 0.05% Nonidet P-40 for 30 min at room temperature. Samples were filtered through a 30-μm pore polyester filter and analysed by a FACScan (Becton Dickinson, Sunnyvale, California, U.S.A.). At least $3 \times 10^4$ cells were collected for each sample. The percentage of cells in the different cycle phases were evaluated from DNA plots according to a rectangular model (RFIT) provided by Becton Dickinson.

*Drugs and treatment conditions*

Exponentially growing cell lines or primary cultures were exposed to CDDP (Platamine®, Farmitalia Carlo Erba, Nerviano, Italy) and F-ara-AMP (Fludara®, kindly supplied by Schering, Berlin, Germany), alone or in combination, for 4 h, according to treatment times used in previous studies [20, 26]. CDDP was dissolved in 0.9% sodium chloride and protected from light. F-ara-AMP was reconstituted in phosphate buffered saline (PBS). Both drugs were then diluted with fresh medium immediately before each experiment. Drug concentrations ranged from 1 to 60 μM for CDDP, and from 0.2 to 10 μM for F-ara-AMP. In a parallel set of experiments performed on JR8 and A2780 cell lines, we comparatively analysed the activity of F-ara-AMP, which is the clinically admistered drug, and of 9-β-D arabinofuranosyl-2-fluoroadenine (F-ara-A, Sigma, St. Louis, Missouri, U.S.A.), which is the compound taken up by cells, to assess to what extent the ability of cells to dephosphorylate F-ara-AMP influences *in vitro* sensitivity to this drug.

*Cytotoxicity assay*

*Cell lines.* After treatment, cells exponentially growing in 6-well plates were incubated with fresh medium for 4 days, then monolayers were trypsinised to obtain single cell suspension and cells were counted with a particle Counter (Coulter Electronics Limited, Luton, U.K.) as previously described [27]. The results were expressed as percentage differences in cell number of treated samples compared with controls.

*Primary cultures.* After treatment, monolayers were trypsinised and cells were cultured by the method of Tanigawa and colleagues [28], opportunely modified [29]. Briefly, cells were grown in double layer agarose in plastic petri dishes for 72 h, then labelled with 5 μCi of $^3$H-thymidine (specific activity 5 Ci/mmol, Amersham Int., Buckinghamshire, U.K.) and incubated for an additional 24 h. Agarose was then solubilised and removed, cells were treated with ice-cold 10% trichloroacetic acid (TCA) for 1 h at 4°C, then TCA-precipitable material was collected by centrifugation. Radioactivity was determined in a 1217 RackBeta scintillation counter (LKB, Turku, Finland). The results were expressed as percentage differences in cpm incorporated in treated samples compared with controls.

In both experimental systems, the activities of F-ara-AMP and CDDP, singly or in combination, were expressed in terms of concentrations able to inhibit cell proliferation by 50% ($IC_{50}$).

*Alkaline filter elution*

Exponentially growing monolayer primary cultures were labelled for 48 h with 0.1 μCi/ml $^{14}$C-thymidine (specific activity, 56 mCi/mmol, Amersham) and chased for 16 h in medium without $^{14}$C-thymidine. Cells were then treated with CDDP alone or in combination with F-ara-AMP for 4 h and incubated at 37°C in fresh medium for 24 or 48 h. Cultures were then trypsinised, washed, γ-irradiated (600 rad; 1000 rad/min) and processed by the alkaline elution technique, according to Kohn [30] to measure DNA-ISC. Data were plotted as the relative amount of DNA retained on the filter versus elution time (given in hours). DNA-ISC frequency in rad equivalents was calculated from the formula [31]:

$$ISC = \left[\left(\frac{1}{1-r}\right)^{\frac{1}{2}} - 1\right] \times 600 \text{ rad},$$

where $r_0$ is the fraction of [$^{14}$C]DNA remaining on the filter in irradiated control cells and $r$ is the fraction of [$^{14}$C]DNA remaining on the filter in drug-exposed, irradiated cells calculated after 12 h of elution.

*Data analysis*

The type of interaction between CDDP and F-ara-AMP in paired experiments was assessed by the method of Drewinko and colleagues [32]. For this evaluation, the agents were assumed to provide independent effects. The growth fraction of cells (with respect to controls) for a given dose of CDDP or F-ara-AMP was, respectively, SFa and SFb, and that for the drug combination was SFab. SFab = SFa × SFb indicates additivity, and any deviation from additivity is given by SFab-(SFa × SFb). Since the estimate of deviation from additivity divided by the square root of its variance was normally distributed, the normal variate $Z$ (whose mean value is 0 and S.D. is 1) was used as a score to quantify the deviations and to evaluate their statistical significance. Differences in the frequency of DNA-ISC in samples treated with CDDP alone or CDDP and F-ara-AMP were analysed by Student's $t$ test. All statistical tests were two-sided.

## RESULTS

*Cell lines*

The response to a 4-h treatment with 0.2–10 μM F-ara-AMP was markedly different in the various cell lines (Figure 1). Specifically, the drug did not affect cell growth of A2780 or A2780/cp8 ovarian cancer cell lines at any concentration tested, whereas a significant antiproliferative effect was achieved in both melanoma cell lines. In particular, biphasic dose-survival curves, with an initial exponential inhibition of cell survival at the lowest concentrations and a smoothed slope at the highest concentrations were observed. The $IC_{50}$ values were 0.25 μM for M14 and 0.8 μM for JR8. A 4-h exposure of A2780 and JR8 cells to the dephosphorylated compound F-ara-A produced cytotoxic effects very close to those induced by F-ara-AMP. Specifically, in A2780, a negligible effect of F-ara-A on cell growth was seen at all the concentrations tested, whereas in JR8 an $IC_{50}$ value of 0.45 μM was recorded. The different sensitivity of the two melanoma cell lines to F-ara-AMP, with respect to ovarian cancer cell lines, was not ascribable to differences in cell cycle distribution. In fact, the percentages of cells in the different cycle phases were superimposable for all cell lines (Table 1).

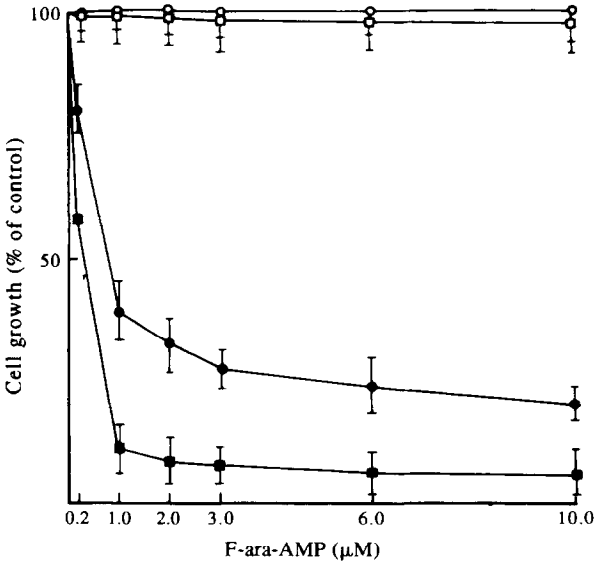


**Figure 1. Dose–response curves of A2780 (□), A2780/Cp8 (○), M14 (■) and JR8 (●) cell lines exposed to F-ara-AMP for 4 h. Each value is the average ± S.D. of three independent experiments.**

The cytotoxic interaction of F-ara-AMP and CDDP, given in combination for 4 h, was further investigated in the two ovarian cancer and in the JR8 melanoma cell lines (Figure 2). Since F-ara-AMP by itself induced an almost complete inhibition of M14 melanoma cell proliferation starting at very low concentrations, we did not use this cell line for F-ara-AMP/CDDP combination experiments. Due to the very different sensitivity of the cell lines to F-ara-AMP, the purine analogue was used at a concentration of 10 μM in A2780 and A2780/cp8 and 1 μM in JF8. F-ara-AMP slightly increased the cytotoxic activity of CDDP in CDDP-sensitive and CDDP-resistant ovarian cancer cell lines. Specifically, the CDDP $IC_{50}$ was lowered from 6.9 to 4.6 μM in A2780 cells and from 40.9 to 25.6 μM in A2780/Cp8 cells. Statistical analysis of the dose–effect plots indicated that the interaction between F-ara-AMP and CDDP was simply additive in both cell lines, with an effect by the combination very close to the product of the activity of individual agents. In a parallel set of experiments, we found that another scheme (CDDP for 1 h followed by F-ara-AMP for 24 h) produced a potentiating effect lower than that obtained with simultaneous exposure (data not shown). Similarly, in JR8 melanoma cells, F-ara-AMP did not significantly potentiate the cytotoxic effect of CDDP, and a simple additive effect by the drug combination was recorded.

*Primary cultures*

The cytotoxic activity of F-ara-AMP on primary cultures of 10 cutaneous melanoma and 10 ovarian cancers is shown in Table 2. Ten μM of F-ara-AMP for 4 h did not significantly affect cell growth of melanomas and produced only a modest cytotoxic effect on ovarian cancers. In fact, in only 1 (case 11) of 10 cultures, did F-ara-AMP produce a growth inhibition greater than 20%. The activity of F-ara-AMP was independent of the growth rate of control samples (Table 2). As regards CDDP, a variable degree of growth inhibition as a function of drug concentration was observed in the different cultures (Figure 3). $IC_{50}$ values ranged from 2.3 to 56 μM in melanomas, and from 2.0 to 15.0 μM in ovarian cancers. When the same tumours were simultaneously exposed for 4 h to CDDP and F-ara-AMP, the cytotoxicity of CDDP was enhanced in most of the tumours. Specifically, the concentration of CDDP that inhibited cell growth by 50% was reduced by 1.4- to 3.9-fold in melanomas and by 1.4- to 7.5-fold in ovarian cancers (Table 3). The extent of CDDP potentiation by F-ara-AMP was not dependent on the inherent sensitivity of tumours to CDDP as a single agent. Statistical analysis of CDDP dose–effect plots obtained in individual tumours indicated that the interaction between CDDP and F-ara-AMP was synergistic in 5 of 10 melanomas and 4 of 10 ovarian cancers (Table 3). An additive effect of the two agents was observed in the remaining 11 tumours.

To elucidate a mechanism of interaction between the two drugs, the effect of F-ara-AMP on the extent and persistence of CDDP-induced DNA-ISC after 24 and 48 h from drug removal was analysed in four melanoma and three ovarian

*Table 1. Cell cycle distribution in the different cell lines*

| Cell line | Percentage of cells $G_{0/1}$ | S | $G_2 + M$ | Mean doubling time (h) |
|---|---|---|---|---|
| Ovarian cancer | | | | |
| A2780 | 36 ± 5* | 46 ± 3 | 18 ± 4 | 25 |
| A2780/Cp8 | 37 ± 4 | 47 ± 4 | 16 ± 3 | 22 |
| Melanoma | | | | |
| M14 | 45 ± 7 | 43 ± 6 | 12 ± 4 | 24 |
| JR8 | 42 ± 5 | 46 ± 7 | 12 ± 5 | 24 |

* Mean ± S.D. from three independent experiments.

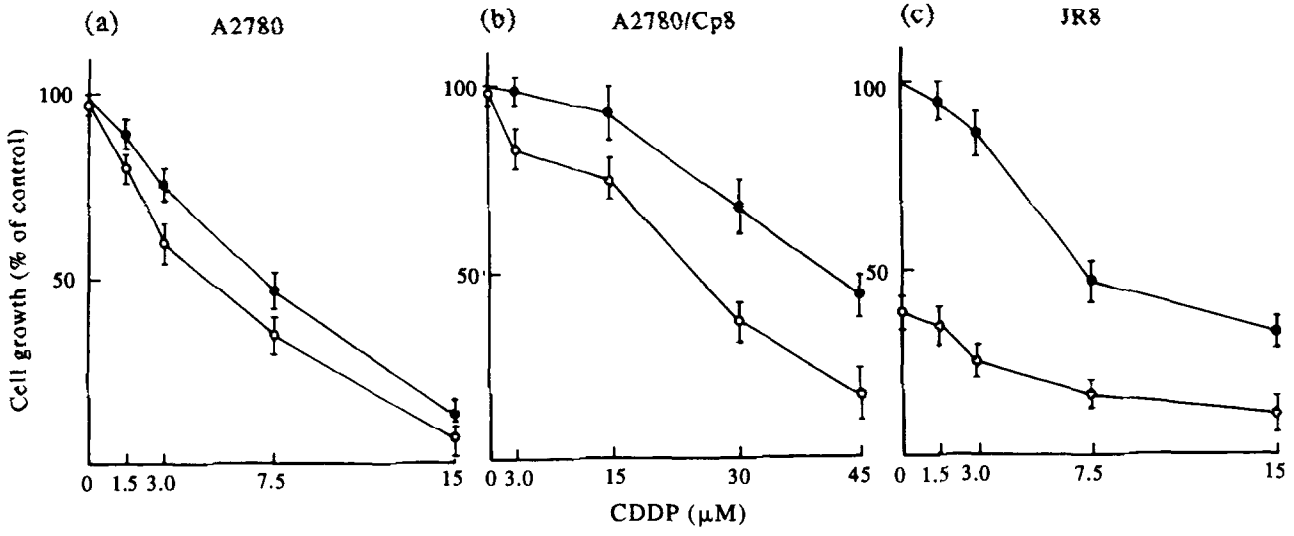


**Figure 2. Dose–response curves of A2780, A2780/Cp8 and JR8 cell lines exposed to CDDP alone for 4 h (●) or to CDDP and F-ara-AMP for 4 h (○). Each value is the average ± S.D. of three independent experiments. F-ara-AMP concentration was 10 μM in A2780 and A2780/Cp8 cells and 1.0 μM in JR8 cells.**

*Table 2. Cytotoxic activity of a 4-h exposure to 10 μM F-ara-AMP*

| Melanoma | | | Ovarian cancer | | |
|---|---|---|---|---|---|
| Case | Control growth rate* (cpm) | Growth inhibition† (%) | Case | Control growth rate* (cpm) | Growth inhibition† (%) |
| 1 | 3327 | 15 | 11 | 13872 | 29 |
| 2 | 6277 | 14 | 12 | 1293 | 20 |
| 3 | 386146 | 10 | 13 | 28288 | 18 |
| 4 | 8950 | 4 | 14 | 1293 | 18 |
| 5 | 76783 | 0 | 15 | 42121 | 13 |
| 6 | 57451 | 0 | 16 | 15821 | 7 |
| 7 | 147149 | 0 | 17 | 6220 | 3 |
| 8 | 5435 | 0 | 18 | 7610 | 0 |
| 9 | 2805 | 0 | 19 | 28084 | 0 |
| 10 | 359652 | 0 | 20 | 42211 | 0 |

* Expressed as cpm incorporated in control cells after 24 h labelling with 5 μCi [$^3$H]thymidine. † Percentage reduction with respect to control.

cancer primary cultures. Representative DNA elution curves are depicted in Figure 4. Irradiated untreated cells were used as controls for experiments with CDDP alone, and irradiated F-ara-AMP-treated cells were used as controls for CDDP/F-ara-AMP combination experiments. Thus, any possible strand breakage induced by F-ara-AMP was compensated for in the cross-linking index calculation. However, a negligible degree of F-ara-AMP-induced strand break accumulation was seen in all the cultures (data not shown).

Concomitant exposure to F-ara-AMP significantly increased the extent of CDDP-induced DNA-ISC 24 h after treatment in 2 of 7 tumours studied (melanomas, cases 2 and

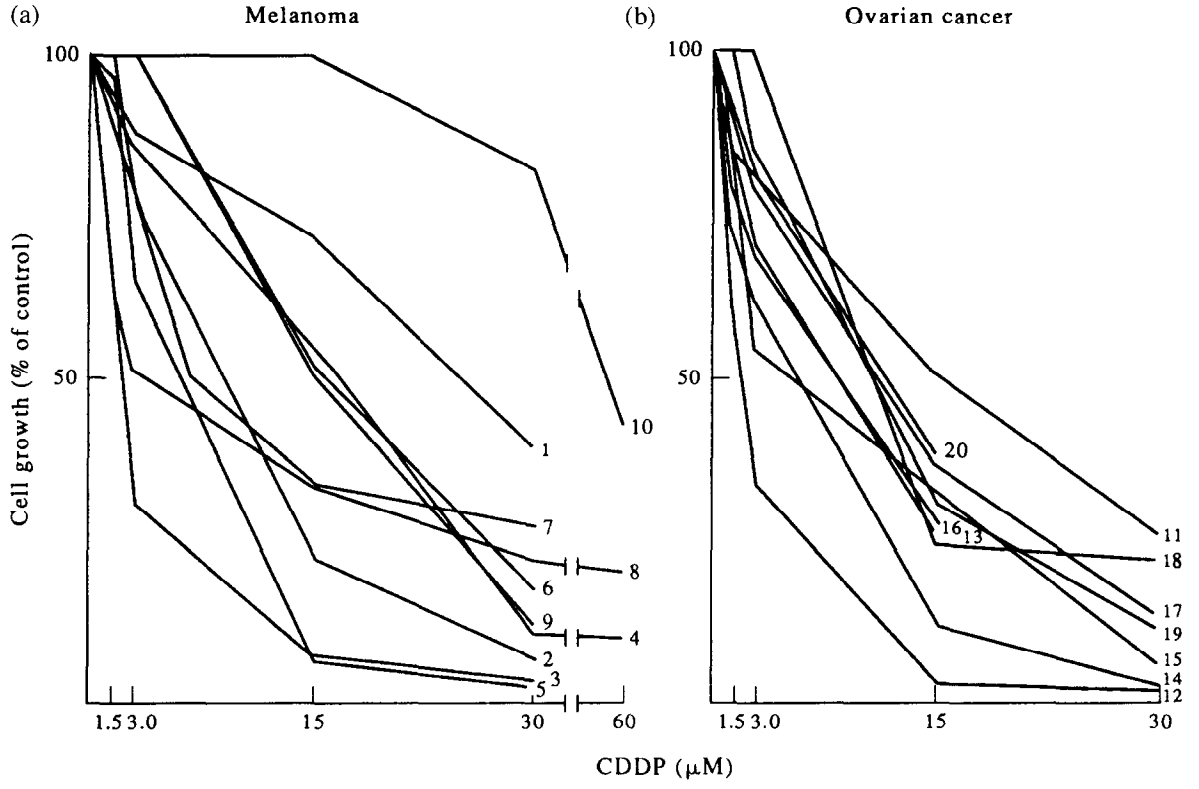


**Figure 3. Dose–response curves of 10 melanoma (a) and 10 ovarian cancer (b) primary cultures exposed to CDDP for 4 h. Each value represents the mean of six replicate samples.**

*Table 3. Cytotoxic activity of CDDP alone or in combination with F-ara-AMP**

| Tumour type | Case | $IC_{50}$ (μM) CDDP alone | $IC_{50}$ (μM) CDDP+ F-ara-AMP | DMF† | *P*‡ | Type of drug interaction |
|---|---|---|---|---|---|---|
| Melanoma | 3 | 2.3 | 1.5 | 1.5 | ns | Additive |
| | 8 | 3.3 | 2.3 | 1.4 | ns | Additive |
| | 5 | 6.3 | 3.0 | 2.1 | <0.05 | Synergy |
| | 7 | 6.7 | 2.3 | 2.9 | <0.05 | Synergy |
| | 2 | 9.0 | 2.3 | 3.9 | <0.01 | Synergy |
| | 9 | 15.0 | 6.7 | 2.2 | <0.05 | Synergy |
| | 6 | 15.3 | 11.0 | 1.4 | ns | Additive |
| | 4 | 16.7 | 11.7 | 1.4 | ns | Additive |
| | 1 | 25.0 | 16.7 | 1.4 | ns | Additive |
| | 10 | 56.0 | 25.0 | 2.2 | <0.05 | Synergy |
| Ovarian cancer | 12 | 2.0 | 0.5 | 4.0 | <0.01 | Synergy |
| | 15 | 4.3 | 3.1 | 1.4 | ns | Additive |
| | 14 | 6.0 | 3.2 | 1.9 | ns | Additive |
| | 16 | 8.3 | 1.7 | 4.9 | <0.01 | Synergy |
| | 13 | 8.3 | 1.8 | 4.6 | <0.01 | Synergy |
| | 19 | 10.7 | 7.6 | 1.4 | ns | Additive |
| | 17 | 10.8 | 7.5 | 1.4 | ns | Additive |
| | 18 | 11.3 | 7.0 | 1.6 | ns | Additive |
| | 20 | 11.4 | 7.6 | 1.5 | ns | Additive |
| | 11 | 15.0 | 2.0 | 7.5 | <0.01 | Synergy |

* 10 μM. † Dose-modifying factor: CDDP $IC_{50}$ in the absence of F-ara-AMP/CDDP $IC_{50}$ in the presence of F-ara-AMP. ‡ Calculated according to the method of Drewinko and associates (see Materials and Methods). ns, not significant.

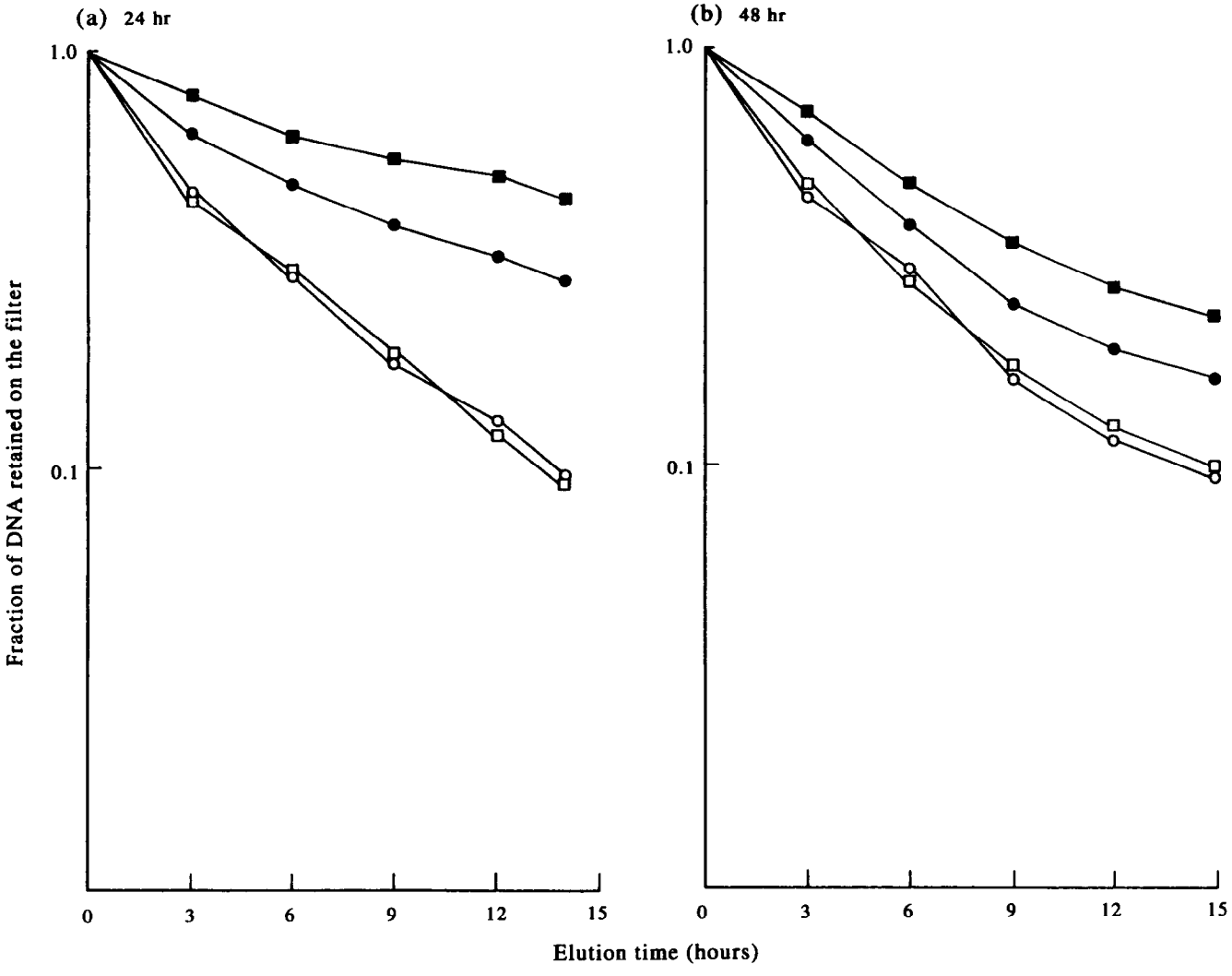


**Figure 4. Representative DNA elution curves at 24 h (a) and 48 h (b) following CDDP and/or F-ara-AMP exposure. Symbols: (●), 15 μM CDDP only; (■), 15 μM CDDP+10 μM F-ara-AMP; (○), untreated irradiated control; (□), 10 μM F-ara-AMP irradiated control. Each value represents the mean of three replicate samples.**

7) and determined a more marked persistence of residual DNA lesions 48 h after treatment in two (melanoma, case 7, and ovarian cancer, case 13) of the three tumours analysed. For these tumours, a synergistic interaction between CDDP and F-ara-AMP was demonstrated in the cytotoxicity assay (Table 4). Conversely, in the remaining tumours, for which an additive cytotoxic effect of the two-drug combination was observed, F-ara-AMP did not interfere with the accumulation and removal of CDDP-induced DNA-ISC.

## DISCUSSION

The present study was designed to evaluate the potential of the purine analogue F-ara-AMP as a modulator of CDDP cytotoxicity in human melanoma and ovarian cancer cells. The investigation was carried out on established cell lines and primary cultures from melanoma and ovarian cancer. Since a main criticism in using primary cultures is possible contamination of normal stromal cells or fibroblasts, we ascertained the tumoral nature of cells by monitoring their reactivity to specific monoclonal antibodies.

Results from established cell lines indicated that F-ara-AMP, at a wide range of concentrations and after an appropriate treatment time, did not influence cell growth of ovarian cancer lines, whereas it induced a marked inhibition of cell proliferation in both melanoma cell lines starting at very low concentrations. Such a different sensitivity was not ascribable to differences in cell distribution in the different cycle phases and in particular in the S phase, during which F-ara-ATP is incorporated into DNA [33]. Another possible factor that can influence cell response to F-ara-AMP is the efficiency of cells to dephosphorylate the compound to the nucleoside F-ara-A, even though we did not find appreciable differences in the cytotoxic activity of F-ara-AMP and F-ara A when comparatively tested on sensitive and resistant cell lines. Moreover, no significant differences were observed among cell lines in the catalytic activity of deoxycytidine kinase, the enzyme which converts F-ara-A into tF-ara-AMP (Silvestrini, unpublished observation). Therefore, a possible explanation for the large variability in sensitivity of the different cell lines to F-ara-AMP could be a different magnitude of the intracellular deoxynucleotide triphosphate pools.

Exposure to 10 μM of the purine analogue, a concentration already used in experimental studies [20, 33] and clinically achievable, induced a negligible effect on cell growth of primary cultures from both tumour histotypes. This finding is in agreement with the lack of activity of F-ara-AMP as a single

*Table 4. Effect of F-ara-AMP* on accumulation and removal of CDDP-induced† DNA-ISC*

| Tumour type | Case | DNA-ISC (rad equivalents) 24 h CDDP alone | 24 h CDDP+ F-ara-AMP | 48 h CDDP alone | 48 h CDDP+ F-ara-AMP | Type of interaction§ |
|---|---|---|---|---|---|---|
| Melanoma | 2 | 55 | 127‡ | nd | nd | Synergy |
| | 7 | 70 | 121‡ | 36 | 64‡ | Synergy |
| | 1 | 116 | 118 | nd | nd | Additive |
| | 8 | 217 | 209 | nd | nd | Additive |
| Ovarian cancer | 13 | 253 | 257 | 24 | 68‡ | Synergy |
| | 19 | 101 | 113 | nd | nd | Additive |
| | 20 | 126 | 130 | 35 | 68 | Additive |

* 10 μM. † 15 μM. ‡ $P < 0.05$, Student's $t$ test, compared with samples without F-ara-AMP. § Type of interaction between CDDP and F-ara-AMP observed in the cytotoxicity assay.
nd, not determined.

agent observed in a phase II clinical study on melanoma patients [34], and indicates that primary cultures as an experimental model are more appropriate for reproducing the drug-refractory nature of melanoma cells than established cell lines.

When used in combination with CDDP, F-ara-AMP slightly increased the alkylating agent's activity in CDDP-sensitive and resistant ovarian cancer cell lines and did not interfere with CDDP cytotoxicity in JR8 melanoma cells. Conversely, in primary cultures, concomitant exposure to F-ara-AMP increased the cytotoxic activity of CDDP in most of the tumours of both histotypes, with dose-modifying factors ranging from 1.4 to 7.5. Moreover, the type of interaction between the two drugs was synergistic in approximately half and additive in the remaining tumours. The occurrence of synergy was independent of the initial activity of CDDP as a single agent.

Since F-ara-AMP has been shown to inhibit enzyme DNA polymerases and DNA ligase (enzymes which are involved in DNA repair processes) in primary cultures, we tested the hypothesis that the enhancement of CDDP cytotoxicity by F-ara-AMP was mediated by an interference in the accumulation and/or removal of CDDP-induced DNA adducts. Among the different types of lesions induced by CDDP, we focused on DNA-ISC, since (although they represent only a minority of platinum adducts) a clear quantitative correlation between the degree of DNA-ISC and the cytotoxicity of CDDP [35] has been observed. The mechanism of CDDP-induced DNA-ISC formation involves initial binding to DNA as monofunctional adducts, followed by a gradual formation of bifunctional adducts, leading to a delayed formation of ISC [36]. A previous study on ovarian cancer primary cultures showed that cross-links peak between 9 and 24 h and are still present, to a lesser extent, 48 h after treatment [37]. Based on this finding, in the present study we analysed DNA-ISC 24 and 48 h after drug exposure.

The increased level and persistence of DNA-ISC observed in samples treated with CDDP and F-ara-AMP, compared with samples treated with CDDP alone, are consistent with a delay or inhibition of DNA adduct repair by F-ara-AMP. Such a repair inhibition could occur at the early monofunctional adduct stage, resulting in later bifunctional adducts and consequently enhanced cytotoxicity.

The results we obtained on primary cultures are in agreement with previous findings by Yang and coworkers [20], who used the same treatement conditions on the parental human colon adenocarcinoma cell line LoVo and its CDDP-resistant subline CP 2.0. The simultaneous treatment with F-ara-AMP and CDDP significantly increased CDDP cytotoxicity in both cell lines, and the potentiating effect was paralleled by an increased accumulation and a reduced repair of CDDP-induced DNA-ISC. Moreover, F-ara-AMP inhibited nucleotide excision repair of CDDP–DNA adducts by CP 2.0 cell extracts in a cell-free system [26].

The consistent enhancement of CDDP activity by F-ara-AMP, as a consequence of an interference with DNA adduct metabolism observed in different tumour models, indicates that the effect is not cell-type dependent. Overall, the results indicated F-ara-AMP as a possible candidate for combination therapies with CDDP in colon and ovarian cancers as well as in melanoma. It should be noted that repair inhibition by F-ara-AMP on CDDP-induced DNA lesions can also involve normal tissue and produce side toxicity. However, since DNA repair is an important limiting factor of treatment efficacy, particularly in very resistant tumours such as melanomas [38], a therapeutic gain with the combined treatment can be hypothesised. The perspective of a combined F-ara-AMP/CDDP therapy is particularly attractive from a clinical point of view, considering that the two agents only have partially overlapping toxicity on normal organs. In fact, F-ara-AMP primarily produces myelosuppression, haematopoietic suppression (neutropenia and thrombocytopenia) and immunosuppression [39], whereas CDDP dosage is generally limited by renal toxicity [2] (even though neutropenia and thrombocytopenia can be observed after continuous infusion). It should also be stressed that the synergistic effects of F-ara-AMP and CDDP in combination were seen at clinically achievable and tolerable drug concentrations.

1. Loehrer PJ, Einhorn LH. Cisplatin. *Ann Intern Med* 1984, **100**, 704–713.
2. Rosenberg B. Fundamental studies with cisplatin. *Cancer* 1985, **55**, 2303–2316.
3. Perez RP, Hamilton TC, Ozols RF, Young RC. Mechanisms and

modulation of resistance to chemotherapy in ovarian cancer. *Cancer* 1993, **71**, 1571–1580.
4. Eastman A. Characterization of the adduct produced in DNA by cis-diammine-dichloroplatinum and cis-dichloro (ethylenediamine) platinum (II). *Biochemistry* 1983, **22**, 3927–3933.
5. Zhen W, Link CJ, O'Connor PM, *et al.* Increased gene specific repair of cisplatin interstrand cross-links in cisplatin-resistant human ovarian cancer cell lines. *Mol Cell Biol* 1992, **12**, 3689–3698.
6. Masuda H, Ozols RF, Lai GM, Fojo A, Rothenberg M, Hamilton TC. Increased DNA repair as a mechanism of acquired resistance to cis-diamminedichloroplatinum (II) in human ovarian cancer cell lines. *Cancer Res* 1988, **48**, 5713–5716.
7. Swinnen LJ, Barnes DM, Fisher SG, Albain KS, Fisher RI, Erickson LC. 1-β-D-Arabinofuranosylcytosine and hydroxyurea production of cytotoxic synergy with cis-diamminedichloroplatinum (II) and modification of platinum-induced DNA interstrand cross-linking. *Cancer Res* 1989, **49**, 1383–1389.
8. Frankfurt OS, Seckinger D, Sugarbaker EV. Inhibition of DNA repair in cells treated with a combination of alkylating agents. *Anticancer Res* 1993, **13**, 947–952.
9. Alaoui-Jamali M, Loubaba B-B, Robyn S, Tapiero H, Batist G. Effect of DNA-repair-enzyme modulators on cytotoxicity of L-phenylalanine mustard and cis-diamminedichloroplatinum (II) in mammary carcinoma cells resistant to alkylating drugs. *Cancer Chemother Pharmacol* 1994, **34**, 153–158.
10. Floridi A, Paggi MG, Marcante ML. Lonidamine, a selective inhibitor of murine cancer cells. *J Natl Cancer Inst* 1981, **66**, 497–499.
11. Silvestrini R, Zaffaroni N, Villa R, Orlandi L, Costa A. Enhancement of cisplatin activity by lonidamine in human ovarian cancer cells. *Int J Cancer* 1992, **52**, 813–817.
12. Keating MJ, Kantarjian H, Talpaz M, *et al.* Fludarabine: a new agent with major activity against chronic lymphocytic leukemia. *Blood* 1989, **74**, 19–25.
13. Huang P, Chubb S, Plunkett W. Termination of DNA synthesis by 9-β-D-arabinofuranosyl-2-fluoroadenine. *J Biol Chem* 1990, **265**, 16617–16625.
14. Tseng WC, Derse D, Cheng YC, Brockman RW, Bennett LL. *In vitro* activity of 9-β-D-arabinofuranosyl-2-fluoroadenine and the biochemical actions of its triphosphate on DNA polymerases and ribonucleotide reductase from HeLa cells. *Mol Pharmacol* 1982, **21**, 474–477.
15. Parker VB, Bapat AR, Shen JX, Townsend AJ, Chen YC. Interaction of 2-halogenated dATP analogs (F, Cl, and Br) with human DNA primase, and ribonucleotide reductase. *Mol Pharmacol* 1988, **34**, 485–491.
16. Catapano C, Perrino FW, Fernandes DJ. Primer RNA chain termination induced by 9-β-arabinofuranosyl-2-fluoroadenine 5′-triphosphate: a mechanism of DNA synthesis inhibition. *J Biol Chem* 1993, **268**, 7179–7185.
17. Catapano CV, Chandler KB, Fernandes DJ. Inhibition of primer RNA formation in CCRF-CEM leukemia cells by fludarabine triphosphate. *Cancer Res* 1991, **51**, 1829–1835.
18. Kamiya D, Huang P, Plunkett W. Mechanism of inhibition of the 3′→5′ exonuclease activity of human DNA polymerase by DNA containing fludarabine nucleotide. *Proc Am Assoc Cancer Res* 1994, **35**, 393.
19. Jayanth RV, Hittelmann WN. 9-β-D-arabinofuranosyl-2-fluroadenine (F-ara-A) inhibits both the fast and slow components of chromosome repair (Abstract). In Chapman JD, Dewey WC, Whitmore GF, eds. *Radiation Research: A Twentieth Century Perspective*. San Diego, Academic Press, 1991, 411.
20. Yang LY, Li L, Keating MJ, Plunkett W. Arabinosyl-2-fluoroadenine augments cisplatin cytotoxicity and inhibits cisplatin–DNA cross-link repair. *Mol Pharmacol* 1995, **47**, 1072–1079.
21. Badaracco G, Corsi A, Maisto A, Natali PG, Starace G, Zupi G. Expression of tumor-associated antigens and kinetic profile of two melanoma cell lines. *Cytometry* 1981, **2**, 63–69.
22. Zupi G, Mauro F, Balduzzi MA, Pardini C, Cavaliere R, Greco C. Established melanoma cell lines from different metastatic nodules of a single patient. A useful model for cancer therapy. *Proc Am Assoc Cancer Res* 1985, **26**, 22.
23. Behrens BC, Hamilton TC, Masuda H, *et al.* Characterization of a cis-diammine-dichloroplatinum (II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. *Cancer Res* 1987, **47**, 414–418.
24. Villa R, Zaffaroni N, Orlandi L, *et al.* Reliability of a primary culture system to test cytotoxic drug activity in human malignant melanoma. *Int J Oncol* 1992, **1**, 619–624.
25. Cordell JL, Falini B, Erber W, *et al.* Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). *J Histochem Cytochem* 1984, **32**, 219–229.
26. Yang LY, Li L, Keating MJ, Plunkett W. Fludarabine triphosphate inhibition of DNA excision repair of cisplatin-induced DNA adducts in a cell-free system. *Proc Am Assoc Cancer Res* 1994, **35**, 396.
27. Villa R, Zaffaroni N, Orlandi L, Bearzatto A, Costa A, Silvestrini R. *In vitro* effect of lonidamine on the cytotoxicity of mitomycin c and BCNU in human colon adenocarcinoma cells. *Eur J Cancer* 1994, **30A**, 1534–1540.
28. Tanigawa N, Kern DH, Hikasa Y, Morton DL. Rapid assay for evaluating the chemosensitivity of human tumors in soft-agar culture. *Cancer Res* 1982, **42**, 2159–2164.
29. Zaffaroni N, Slivestrini R, Grignolio E, Villa R, De Marco C. Comparison of an antimetabolic assay and an antiproliferative assay, both using $^3$H-thymidine incorporation, to test drug sensitivity of human tumors. *Int J Cell Cloning* 1988, **6**, 392–403.
30. Kohn DW. DNA as a target in cancer chemotherapy: measurement of macromolecular DNA damage produced in mammalian cells by anticancer agents and carcinogens. *Meth Cancer Res* 1979, **16**, 291–298.
31. Kohn KW, Ewig RAG, Erickson LC, Zwelling LA. Measurement of strand breaks and cross-links by alkaline elution. In Freidberg EC, Hanawalt PC, eds. *DNA Repair. A Laboratory Manual of Research Procedures*. New York, Marcel Dekker, 1981, 379–401.
32. Drewinko B, Loo TL, Brown B, Gottlib JA, Freireich EJ. Combination chemotherapy *in vitro* with adriamycin. Observation of additive, antagonistic, and synergistic effects when used in two-drug combinations on cultured human lymphoma cells. *Cancer Biochem Biophys* 1976, **1**, 187–195.
33. Huang P, Plunkett W. Fludarabine- and gemcitabine-induced apoptosis: incorporation of analogs into DNA is a critical event. *Cancer Chemother Pharmacol* 1995, **36**, 181–188.
34. Kish JA, Kopecky K, Samson MK, *et al.* Evaluation of fludarabine phosphate in malignant melanoma. *Invest New Drugs* 1991, **9**, 105–108.
35. Zwelling LA, Michaels S, Schwartz H, Dobson PO, Kohn KW. DNA cross-linking as an indicator of sensitivity and resistance of mouse L1210 leukemia to cis-diamminedichloroplatinum (II) and L-phenylalanine mustard. *Cancer Res* 1981, **41**, 640–649.
36. Zwelling LA, Kohn RW. Effects of cisplatin on DNA and the possible relationships to cytotoxicity and mutagenicity in mammalian cells. In Prestayko AW, Crooke ST, Carter SK, eds. *Cisplatin, Current Status and New Developments*. New York, Academic Press Inc., 1980, 21–26.
37. Balconi G, Broggini M, Erba E, *et al.* Human ovarian tumors in primary culture: growth, characterization and initial evaluation of the response to cis platinum treatment *in vitro*. *Int J Cancer* 1988, **41**, 809–816.
38. Zaffaroni N, Orlandi L, Villa R, Bearzatto A, Rofstad EK, Silvestrini R. DNA double-strand break repair and radiation response in human tumor primary cultures. *Int J Radiat Biol* 1994, **66**, 279–285.
39. Cheson BD. The purine analogs—A therapeutic beauty contest. *J Clin Oncol* 1992, **10**, 352–355.

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