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EFFECTS OF *CIS*-DIAMMINEDICHLOROPLATINUM ON DNA INCORPORATION AND CYTOTOXICITY OF 1-β-D-ARABINOFURANOSYLCYTOSINE*

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Abstract—1-β-D-Arabinofuranosylcytosine (ara-C) incorporates into replicating cellular DNA and the extent of this incorporation correlates with loss of clonogenic survival. More recent findings have demonstrated that incorporation of ara-C into DNA undergoing repair of damage induced by u.v. light also results in cell lethality. On the basis of previous studies demonstrating a marked synergism between *cis*-diamminedichloroplatinum (CDDP) and ara-C in LoVo colon carcinoma cells, the present work has examined the interaction of these agents at a biochemical and cellular level in the MCF-7 human breast carcinoma line. The extent of ara-C incorporation into MCF-7 DNA correlated significantly with loss of clonogenic survival in a dose-dependent manner. The effects of CDDP on the formation of MCF-7 (ara-C)DNA were monitored using both cesium sulfate and cesium chloride density centrifugation. The results demonstrate that CDDP had little, if any, detectable effect on incorporation of ara-C into DNA. Furthermore, combinations of CDDP and low concentrations of ara-C (10^{-7} and 10^{-6} M) decreased MCF-7 clonogenic survival in an additive but not synergistic manner. Modest synergy was detectable with CDDP and higher ara-C concentrations (10^{-5} and 10^{-4} M). The interaction between CDDP and ara-C was apparently dependent on concentration, duration of exposure and cell type. There was no dramatic synergy between CDDP and ara-C in MCF-7 cells. These findings may be relevant to the design and interpretation of CDDP/ara-C clinical trials.

Ara-C§ is one of the most effective agents in the treatment of acute myelogenous leukemia in adults [1]. Clinically, it has been used both alone and in combination with anthracyclines to produce a high rate of remission induction in these patients. The precise molecular mechanism of ara-C and possible interactions with other antineoplastic agents remain controversial [2]. Ara-C is a known inhibitor of replicative DNA synthesis [3]. The active metabolite, ara-CTP, is a competitive inhibitor of DNA polymerase [3, 4]. Ara-C is incorporated into cellular DNA (but not RNA), and the extent of this incorporation correlates with inhibition of DNA synthesis and loss of clonogenic survival [5, 6]. Other studies which demonstrated ara-C residues in internucleotide linkage and at the 3-terminus have suggested that this agent acts as a relative chain terminator, especially at high concentrations [7]. The disruption of replicative DNA synthesis by ara-C results in DNA fragmentation and reinitiation of synthesis in previously replicated segments [8, 9].

Ara-C is also a potent inhibitor of DNA repair synthesis. This agent increases the frequency of DNA single-strand breaks, chromosomal abnormalities, and cytotoxicity induced by u.v. light, certain alkylating agents and X-irradiation [10–13]. Ara-C is incorporated into the DNA repair segment following treatment of growth-arrested fibroblasts with u.v. light [10]. The incorporation of ara-C during u.v.-induced DNA repair correlates with loss of clonogenic survival. Other studies have demonstrated that ara-C decreases the repair rate of single-strand breaks induced by X-irradiation [13]. This inhibition of DNA repair correlates with a corresponding loss of clonogenic survival [13]. However, in contrast to the formation of (ara-C)DNA during repair of u.v.-induced damage, ara-C incorporation into DNA undergoing repair is not detectable after X-irradiation-induced damage [11].

CDDP has assumed a central role in the treatment of many solid tumors, including carcinomas of the testes, ovary, lung and oropharynx [14]. The "*cis*" position of the two chloride ions is required for activity [15]. This bifunctional molecule binds to DNA with the formation of adducts, particularly intrastrand and interstrand cross-links between guanine bases [15, 16]. Repair of these lesions may be necessary for cell survival. Inhibitors of DNA excision repair processes, such as caffeine, markedly enhance the cytotoxic effects of CDDP *in vitro* [17]. Furthermore, cell lines with decreased ability for DNA damage excision and repair (derived from patients with xeroderma pigmentosum) have increased sensitivity to CDDP [17]. These studies

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§ Abbreviations: ara-C, 1-β-D-arabinofuranosylcytosine; ara-CTP, 1-β-D-arabinofuranosylcytosine triphosphate; and CDDP, *cis*-diamminedichloroplatinum.

have generated interest in combinations of CDDP with DNA repair inhibitors, including ara-C.

The combination of ara-C and CDDP has been studied previously in the LoVo human colon carcinoma cell line [18]. The LoVo cell line is resistant to brief exposures to ara-C alone, while the combination of ara-C and CDDP results in a synergistic loss of clonogenic survival. These findings have encouraged clinical trials combining CDDP and ara-C [19, 20]. Based on our previous studies of ara-C incorporation into DNA undergoing repair, we have investigated the effect of CDDP on (ara-C)DNA formation and cytotoxicity in the MCF-7 human mammary carcinoma cell line.

MATERIALS AND METHODS

Cell culture. MCF-7 human mammary carcinoma cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY), 1% L-glutamine, streptomycin (100 units/ml) and penicillin (100 μg/ml).

Clonogenic survival. MCF-7 cells were grown in 10 cm petri dishes for 16 hr prior to drug treatment. The cultures were then treated with various concentrations of ara-C in serum free medium for 1 hr prior to the addition of various concentrations of CDDP. After 1 hr, the CDDP was removed and the cells were exposed to ara-C for an additional 4 hr. The cells were then rinsed with phosphate-buffered saline (PBS) and incubated for 7 days in Dulbecco's medium with 10% fetal calf serum. Clonogenic survival was determined by scoring colonies of more than 20 cells. The percentage of colony formation was determined by the ratio of colonies formed by drug-treated cells to untreated control cells.

Incorporation of [^{3}H]ara-C into DNA. MCF-7 cells were incubated with various concentrations of CDDP and [^{3}H]ara-C (Moravek Biochemicals, Brea, CA) for periods of 1 and 6 hr, according to the schedule described for the clonogenic survival experiments. The cells were removed with trypsin-EDTA and rinsed with saline. The cells were lysed with 0.01 M Tris (pH 7.4), 0.001 M EDTA and 0.5% sodium dodecyl sulfate, and the protein was digested by incubation with 100 μg/ml proteinase K for 2 hr at 37°. The nucleic acids were precipitated with 4 M NaCl and ethanol, resuspended in 0.005 M EDTA, and the RNA was digested by incubation with 100 μg/ml RNase A (Sigma Chemical Co., St. Louis, MO). Salmon sperm DNA (Sigma; 50 μg/tube) was added, and the DNA was precipitated with ice-cold trichloroacetic acid. The precipitate was collected on glass fiber filters (Fisher Scientific, Pittsburgh, PA) and analyzed for radioactivity.

Cesium sulfate gradients. MCF-7 cell cultures were exposed to various concentrations of [^{32}P], [^{3}H]ara-C and CDDP according to the described schedule. After treatment, the cells were removed with trypsin-EDTA and washed with saline. The nucleic acids were extracted with phenol, denatured with formamide, and then separated on a Cs_2SO_4 density gradient [5]. Fractions (0.4 ml) were collected and analyzed for acid precipitable radioactivity.

High pressure liquid chromatography (HPLC). Purified DNA was digested to nucleosides using DNAse I, snake venom phosphodiesterase and alkaline phosphatase (Sigma) as described previously [6]. The enzymes were removed by perchloric acid precipitation, and the supernatant fraction was neutralized with potassium bicarbonate. The supernatant was then analyzed on a High Pressure Liquid Chromatograph (Waters Associates, Milford, MA) with a Z-module radial compression unit and an SCX strong cation exchange cartridge (Waters Associates, Milford, MA). An isocratic separation was performed using 0.1 M ammonium phosphate, pH 3.5. Fractions were collected and analyzed for radioactivity.

Ara-C incorporation during DNA repair. MCF-7 cells were incubated for 2 hr with 10^{-5} M BrdUrd and 10^{-6} M FdUrd. The cell cultures were then treated with various concentrations of CDDP and [^{3}H]ara-C as already described. BrdUrd and FdUrd were present throughout the treatment period. The cells were then harvested for purification of nucleic acids. The DNA was then separated on two sequential CsCl gradients as described [21]. The fractions were collected and analyzed for acid-insoluble radioactivity.

Statistical analysis. The incorporation of ara-C into DNA was analyzed by linear regression after logarithmic transformation to calculate the relationship between dose and loss of clonogenic survival. The calculations were performed using the SAS computer based statistical package [22].

The analysis of ara-C/CDDP interactions for ara-C incorporation was performed using a model I two-factor analysis of variance with four replicates [23]. The combination of ara-C and CDDP was evaluated for synergy using a modification of previously described methods [24]. The synergy ratio (R) was calculated by the equation:

$$\frac{[\text{ara-C}]}{[\text{ara-C}]_e} + \frac{[\text{CDDP}]}{[\text{CDDP}]_e} = \frac{1}{R}$$

In this equation, [ara-C] and [CDDP] are the experimental doses of ara-C and CDDP, while $[\text{ara-C}]_e$ and $[\text{CDDP}]_e$ are the equivalent single agent dose for the same cytotoxic effect. Thus, for $R > 1$, the combination will be synergistic, and antagonistic for $R < 1$. Additive combinations will have $R = 1$.

RESULTS

The incorporation of ara-C into MCF-7 nucleic acid was monitored by Cs_2SO_4 density gradient centrifugation. This technique allows the separation of RNA (density 1.62 to 1.68 g/ml) from DNA (density 1.42 to 1.48 g/ml). As shown in Fig. 1, tritium was detectable only in the DNA fractions. Moreover, the incorporation into DNA was dependent upon time exposure. The purified DNA from cells labeled with [^{3}H]ara-C was also collected for further analysis. Using DNAse I, phosphodiesterase and alkaline phosphatase, the DNA was enzymatically digested to nucleosides for analysis by HPLC. The chromatogram is shown in Fig. 2. The tritium comigrated with ara-C and not with deoxycytidine, ara-U or other nucleoside markers.

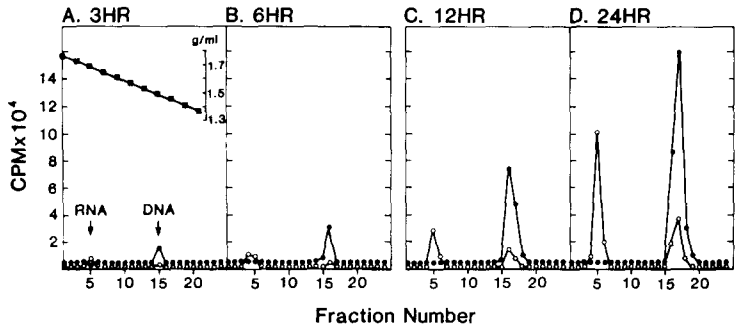

Fig. 1. Incorporation of [^{3}H]ara-C (●) and [^{32}P] (○) into MCF-7 nucleic acids. MCF-7 cells in logarithmic growth phase were incubated with 10^{-7} M [^{3}H]ara-C and 10 μCi/ml of [^{32}P] for 3, 6, 12 and 24 hr. The total nucleic acids were purified by cesium sulfate density centrifugation. The tritium and [^{32}P] counts banding in the RNA region (between density 1.62 and 1.68 g/ml) and DNA region (1.42 and 1.48 g/ml) of the gradients were determined and used as a measure of incorporation of ara-C into DNA and the relative rates of RNA and DNA synthesis. The density of each fraction (■) was determined by measurement of the refractive index.

The incorporation of ara-C into DNA has been shown previously to be highly correlated with loss of clonogenic survival [25]. The relationship between (ara-C)DNA formation and loss of clonogenic survival was therefore similarly investigated for MCF-7 cells. Cells were exposed to various concentrations of [^{3}H]ara-C for a period of 3–24 hr in order to monitor incorporation into DNA. Parallel experiments with unlabeled ara-C were performed to determine the time–concentration effects of ara-C on clonogenic survival. Figure 3 illustrates an analysis of these data. A significant correlation was found between ara-C incorporation into DNA (expressed on a logarithmic scale) and loss of clonogenic survival ($r = -0.86$, $P < 0.001$). These findings suggested that incorporation of ara-C into MCF-7 DNA is related to cytotoxicity.

The combination of ara-C and CDDP was next examined to determine if CDDP treatment would alter the metabolism of ara-C. MCF-7 cells were exposed to [^{3}H]ara-C for 1 hr, 10^{-6} M CDDP was added for a second hour, and then treatment was continued with [^{3}H]ara-C alone for an additional 4 hr. The DNA was then collected and analyzed by Cs_2SO_4 density gradient centrifugation. The results in Fig. 4 demonstrate that ara-C incorporated only into DNA under these experimental conditions. Furthermore, the tritium label detectable in DNA represented ara-C and not a metabolite (data not shown). More importantly, 10^{-6} M CDDP treatment had a minimal effect on the incorporation of 10^{-7} M or 10^{-5} M [^{3}H]ara-C.

The effect of CDDP on the incorporation of ara-C into DNA was further investigated at a variety of concentrations of each agent. Figure 5 illustrates the effect of CDDP and ara-C concentration on ara-C incorporation into MCF-7 DNA. A two-factor analysis of variance for this relationship was performed [23]. CDDP had no significant effect on ara-C incorporation at any dose level. The incorporation

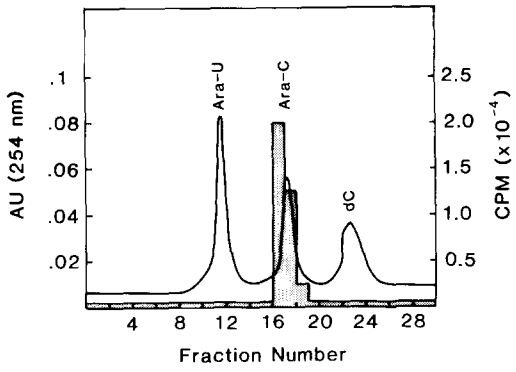

Fig. 2. Chromatogram of digested [^{3}H]ara-C-labeled MCF-7 DNA. MCF-7 cells were labeled with [^{3}H]ara-C, and the DNA fraction was purified and digested to nucleosides with DNAse I, snake venom phosphodiesterase and bacterial alkaline phosphatase. Nucleosides were purified and analyzed by high pressure liquid chromatography. Appropriate nucleoside markers were added, and fractions (0.4 ml) were collected during the elution for the assay of tritium counts. The shaded area is a measurement of tritium in each fraction.

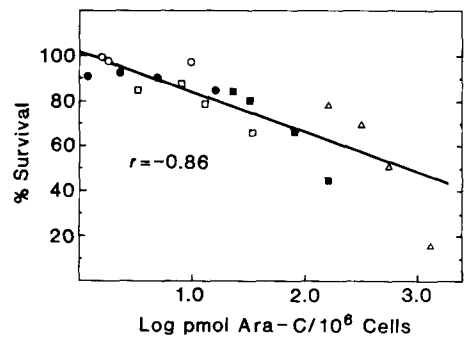

Fig. 3. Relationship between MCF-7 clonogenic survival and incorporation of ara-C into DNA. The relationship of MCF-7 clonogenic survival to [^{3}H]ara-C incorporation into DNA (log pmoles/10^6 cells) was determined at ara-C concentrations of 10^{-7} M (○), 10^{-6} M (●), 10^{-5} M (□), 10^{-4} M (■) and 10^{-3} M (△) during time periods ranging from 3 to 24 hr. All values are the mean of two determinations each performed in duplicate.

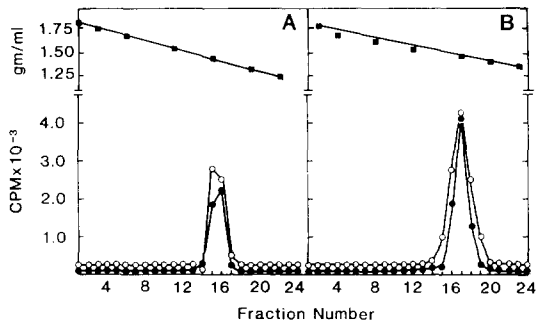

Fig. 4. Effect of CDDP on [^{3}H]ara-C incorporation into MCF-7 nucleic acids. MCF-7 cells in logarithmic growth phase were exposed to 10^{-7} M [^{3}H]ara-C for 1 hr prior to the addition of 10^{-6} M CDDP. After a 1-hr incubation with both agents, the CDDP was removed and the cells were exposed to the [^{3}H]ara-C for an additional 4 hr. (A) MCF-7 cells exposed to 10^{-7} M [^{3}H]ara-C alone (●) or in combination with 10^{-6} M CDDP (○). (B) MCF-7 cells exposed to 10^{-5} M [^{3}H]ara-C alone (●) or in combination with 10^{-6} M CDDP (○). The nucleic acids were extracted and analyzed by cesium sulfate density gradient centrifugation as described in the legend to Fig. 1. The density of each fraction (■) was determined by measurement of the refractive index.

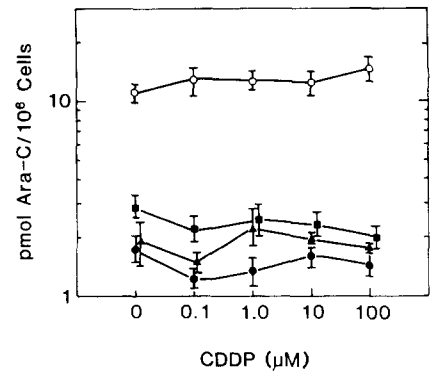

Fig. 5. Effect of various CDDP and ara-C concentrations on the formation of (ara-C)DNA. MCF-7 cells were incubated with 10^{-7} M (●), 10^{-6} M (▲), 10^{-5} M (■) and 10^{-4} M (○) [^{3}H]ara-C in combination with various CDDP concentrations (10^{-7} M to 10^{-4} M). The amount of ara-C incorporation into MCF-7 DNA was determined and expressed as the mean ± SEM for four determinations.

of ara-C into DNA was only dependent on the concentration of ara-C and was increased significantly for concentrations above 10^{-6} M ($P < 0.01$).

The initial studies thus demonstrated that the overall extent of (ara-C)DNA formation was not measurably changed by CDDP. However, the pattern of incorporation into DNA undergoing semiconservative and repair synthesis may have been altered by CDDP treatment. This possibility was investigated by CsCl density gradient centrifugation, which separates DNA undergoing semiconservative and repair replication. The results obtained are illustrated in Fig. 6. The addition of CDDP resulted in a modest increase in the incorporation of ara-C into the repair segment. Multiple replicates demonstrated that CDDP increased ara-C incorporation 2- to 3-fold ($N = 9$). This increase was small (less than 0.1 pmole) when compared with the extent of ara-C incorporation into DNA during seminconservative synthesis (Fig. 5).

The effect of CDDP and ara-C on cytotoxicity was also examined under similar experimental conditions. Each drug was tested at four concentrations: 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M, encompassing the concentrations achievable by most clinical regimens. We employed a 1-hr exposure for CDDP and a 6-hr exposure for ara-C. Up to a 90% loss of clonogenic survival was observed for each agent alone.

Each concentration pair was evaluated in six replicates, and the results are shown in Fig. 7. Clonogenic survival was decreased by exposure to higher concentrations of either agent. The synergy ratio was calculated for each concentration pair [24] to determine whether or not synergistic effects were present. Synergy is defined by a synergy ratio of greater than 1, simple additive effects by a ratio of 1.0 and competition by a ratio of less than 1.0. Because CDDP was so highly toxic at 10^{-4} M, no meaningful estimate of synergy could be made at that concentration. The other calculated synergy values are shown in Fig. 8. Significant synergy ($R > 1$, $P < 0.05$) was not detectable for low concentrations of ara-C. However, minimal but statistically significant synergy was detectable with: (1) 10^{-5} M ara-C and 10^{-5} M CDDP; and (2) 10^{-4} M ara-C and all CDDP concentrations.

DISCUSSION

The interaction between ara-C and other antineoplastic agents has been an area of interest. The

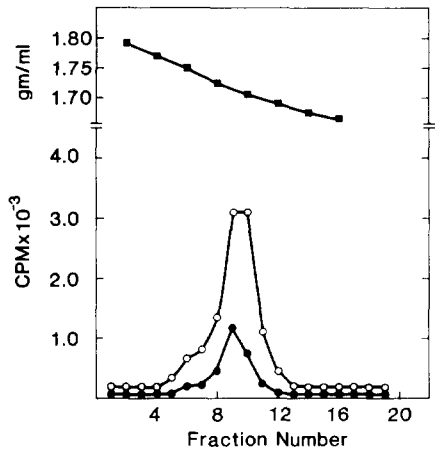

Fig. 6. Incorporation of [^{3}H]ara-C into MCF-7 DNA undergoing replicative and repair synthesis. MCF-7 cells were treated with 10^{-5} M BrdUrd and 10^{-6} M FdUrd for 2 hr and then exposed to [^{3}H]ara-C alone (●) or CDDP (10^{-6} M) and [^{3}H]ara-C (○). The DNA was then purified and analyzed by CsCl gradient centrifugation. DNA undergoing repair forms a band at a density of 1.70 g/ml and DNA undergoing semiconservative synthesis forms a band at 1.75 g/ml. The density of each fraction (■) was determined by measurement of the refractive index.

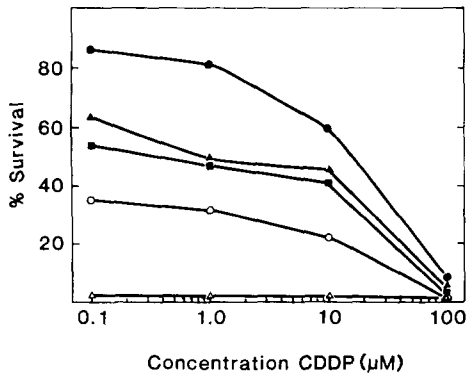

Fig. 7. Clonogenic survival of MCF-7 cells after exposure to ara-C and CDDP. MCF-7 cells in logarithmic growth phase were exposed to CDDP alone (●) or 10^{-7} M (▲), 10^{-6} M (■), 10^{-5} M (○) or 10^{-4} M (△) ara-C, in combination with various concentrations of CDDP. Colonies (>20 cells) were scored 10 days later, and all values are expressed as a percentage of untreated controls. The results are expressed as the mean ± SEM for six determinations.

combination of anthracycline compounds with ara-C has already been established in the treatment of AML [1]. This interaction may be explained by certain experimental findings. Adriamycin and ara-C have been shown to be additive in terms of inducing single-strand DNA breaks, while ara-C inhibits repair of 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA)-induced DNA damage [26, 27]. The combination of ara-C and other agents might also be useful in the treatment of solid tumors. Since alkylating agents and platinating agents cause significant DNA damage, the inhibitory effect of ara-C on DNA repair may be useful in potentiating the effects of these drugs.

The repair of DNA damage is a complex process. DNA damaging agents result in at least two different types of DNA repair. Ultraviolet light and *N*-methyl-*N*′-nitrosoguanidine result in a "long" patch of repaired DNA (60–100 bases), whereas other agents such as bleomycin and gamma-radiation result in "short" (3–4 bases) patch repair [28]. DNA polymerase alpha is involved in long patch repair and is inhibited by a number of drugs including ara-C [29]. In contrast, short patch repair synthesis probably involves DNA polymerase beta [29]. DNA polymerase beta is less sensitive to the inhibitory effects of ara-C and may result in displacement DNA repair synthesis when DNA polymerase alpha is inhibited by this agent [29, 30]. The repair of DNA damage is a multistep process postulated to include recognition, excision, resynthesis and ligation. Ara-C does not appear to affect excision since enzymatic single-strand breaks accumulate in the presence of this agent [31]. However, ara-C does inhibit resynthesis of damaged DNA. This inhibition is overcome by high intracellular levels of triphosphate nucleotides, suggesting that non-dividing cells with lower dNTP levels may be more sensitive to this particular effect [32].

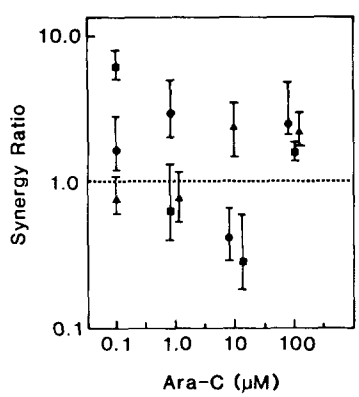

Fig. 8. Synergy ratio for combinations of ara-C and CDDP. Synergy ratios were calculated for 10^{-7} M (●), 10^{-6} M (■), and 10^{-5} M (▲) CDDP in combination with ara-C. Results are expressed as the harmonic mean ± SEM for six determinations.

In the present studies, the extent of ara-C incorporation into MCF-7 DNA was dependent on both duration of exposure and drug concentration. The formation of (ara-C)DNA correlated with the loss of MCF-7 clonogenic survival. The enhanced incorporation of ara-C into DNA during repair synthesis has been demonstrated following u.v. light exposure [10]. Furthermore, the extent of ara-C incorporation into this long repair patch correlated with loss of clonogenic survival. In contrast, CDDP did not cause a measurable increase in ara-C incorporation at any concentration of ara-C or CDDP. During treatment with combinations of ara-C and CDDP, the formation of (ara-c)DNA was dependent only on the concentration of ara-C. There was a small detectable increase in ara-C incorporation into DNA specifically undergoing repair as determined by CsCl density gradient centrifugation. The magnitude of this incorporation into repair segment DNA was small, representing less than 10% of the total ara-C incorporation into DNA. These findings may be related to a small repair patch with a low probability of ara-C misincorporation or a low level of DNA repair synthesis following CDDP-induced damage. In this regard, we were unable to detect a significant enhancement of [^{3}H]thymidine incorporation in repair sequence DNA after 10^{-6} M CDDP exposures (data not shown). The relative importance of this repair segment incorporation of ara-C may be greater for resting or non-dividing cells than for cells in logarithmic growth.

The cytotoxic interaction of CDDP and ara-C in MCF-7 cells was quantitated by a calculated synergy ratio. This calculation was based on the individual cytotoxicity of each agent derived from single agent dose–response curves. For these MCF-7 cells in logarithmic growth phase, there was no statistically significant synergy with 10^{-7} and 10^{-6} M ara-C. At higher ara-C concentrations (10^{-5} and 10^{-4} M), there was a modest but significant synergistic effect for the ara-C/CDDP combinations. Previous studies have demonstrated a dramatic synergy between CDDP and ara-C in LoVo cells [18]. The basis for the discrepancy between the findings in LoVo cells and those in MCF-7 cells is unclear. The results for both cell lines are in agreement, however, in that any synergy was observed only at high ara-C con-

centrations ($>10^{-5}$ M). These findings are consistent with early clinical results. A combination of CDDP and "standard" dose ara-C (60 mg/m^2/day) had only modest efficacy [19]. In contrast, a high dose intracavitary combination of CDDP and ara-C has had more promising results thus far [20]. We have recently completed a phase I study of high dose continuous infusion ara-C (250 mg/m^2/hr). With this schedule, ara-C plasma levels of more than 10^{-5} M can be maintained for up to 24 hr [33]. This ara-C dose schedule is being combined with CDDP for further clinical testing.

The mechanism and patch sizes for repair of CDDP-induced DNA damage in human cells are unclear. Ultraviolet-specific endonuclease which cleaves DNA strands for u.v.-irradiated Chinese hamster ovary cells has no effect on DNA from cells treated with CDDP [31]. The basis for the effect of ara-C on repair of CDDP-induced DNA damage will require further definition of the CDDP-induced repair process. The modest synergy observed in MCF-7 cells was not correlated with (ara-C)DNA formation, since CDDP had no detectable effect on the incorporation of ara-C into DNA. Other interactions such as increased number of DNA single-strand breaks, or persistent interstrand cross-links may be important in the CDDP/ara-C interaction. In this regard, the previous studies in LoVo cells demonstrated that ara-C inhibits repair of CDDP-induced DNA–DNA cross-links [18]. Since DNA cross-linking has been correlated with sensitivity to CDDP [32], the increase in CDDP-induced cross-links by ara-C could account for the synergistic cell lethality. What is required now, in part, are more incisive studies regarding the effect of ara-C on the excision process of CDDP-induced cross-links.

The interactions between ara-C and CDDP are thus probably quite complex. In MCF-7 cells, there was modest synergy seen only at high doses of ara-C. Synergistic interactions between ara-C and DNA damaging agents will probably be dependent on a variety of pharmacologic factors including dose, schedule of administration, and type of resultant long or short patch DNA repair. Synergy will also depend on tumor factors including growth fraction and intracellular nucleotide levels. Further studies are required to delineate these complex interactions.

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