Interactions of cis-Diaminedichloroplatinum(II) with 1-β-D-Arabinofuranosylcytosine in LoVo Colon Carcinoma Cells

Robert J. Fram, Norman Robichaud, Sheldon D. Bishov, and John M. Wilson

ABSTRACT

Prior reports demonstrated more than additive cytotoxic effects of cis-diaminedichloroplatinum(II) (CDDP) and 1-β-D-arabinofuranosylcytosine (ara-C) in LoVo colon carcinoma cells. We have extended these findings by analyzing mechanisms that may underlie the effect of ara-C on CDDP-induced cytotoxicity. In contrast to a previous study, ara-C neither enhances DNA interstrand cross-link formation by CDDP nor affects the excision of platinum from DNA. Features peculiar to ara-C, such as its misincorporation into DNA, probably contribute since more than additive cytotoxic effects do not occur by combinations of CDDP with inhibitors of DNA synthesis that are not incorporated into DNA. Also, while ara-C does not significantly enhance the degree of inhibition of DNA synthesis caused by CDDP, the recovery of DNA synthesis after drug removal is significantly slowed when cells are exposed to both drugs. These findings contrast with those obtained with CDDP and aphidicolin (the latter agent resembles ara-C in competing with dCTP for binding to DNA polymerase α but, unlike ara-C, is not incorporated into DNA). Lastly, ara-C is incorporated into LoVo cell DNA undergoing replicative synthesis as well as into DNA undergoing repair synthesis after CDDP-induced DNA damage.

INTRODUCTION

CDDP is an agent widely used in the treatment of solid tumors (1–4). ara-C, on the other hand, is almost exclusively used in the therapy of patients with acute myelogenous leukemia and, to a lesser extent, lymphoma (5, 6). While the former agent is not phase specific, the latter is highly S phase specific, perhaps explaining its lack of efficacy in the treatment of solid tumors (7, 8). In LoVo colon carcinoma cells, however, highly synergistic cytotoxic effects by this combination were reported previously (9, 10). Such effects by these two agents are not present in all tumor lines and also appear schedule dependent (11, 12). In this report we wished to analyze mechanisms underlying more than additive cytotoxic effects by this drug combination in LoVo cells.

Prior studies have shown that ara-C is incorporated into DNA during replicative DNA synthesis and that the extent of incorporation of this agent is significantly correlated with cytotoxicity in human myeloblasts, HL-60 promyelocytes, and L1210 murine leukemia cells (13, 14). We endeavored to evaluate whether the effect of ara-C on cis-platinum-induced cytotoxicity was simply related to inhibition of DNA synthesis by ara-C or whether other effects peculiar to ara-C, such as misincorporation into DNA, might contribute to cellular lethality. ara-C also inhibits excision repair of DNA damage induced by UV irradiation (15–18). In the classical model of excision repair of UV-induced pyrimidine dimers, damage-specific endonuclease incises DNA 5' to UV-induced dimers. Excision then occurs via a 5'-3' exonuclease followed by DNA repair synthesis and ligation (19, 20). The inhibition by ara-C of DNA repair following UV damage occurs after incision, since the breaks introduced enzymatically in the damaged DNA accumulate in the presence of drug. This accumulation does not occur in incision-deficient fibroblasts from patients with xeroderma pigmentosum (21, 22).

Inhibition of UV-induced excision repair by ara-C is associated with the incorporation of ara-C into DNA undergoing DNA repair synthesis, an event that is significantly correlated with enhanced cytotoxicity by ara-C in combination with UV irradiation in growth-arrested human fibroblasts (23).

As with UV-induced pyrimidine dimers, CDDP, after binding to DNA, is excised from DNA. The evidence for this conclusion is derived from analysis of the excision of total platinum from DNA as well as CDDP-induced DNA lesions, such as DNA interstrand cross-links (24, 25). Also consistent with the involvement of excision repair in the removal of cis-platinum adducts is the enhanced sensitivity of fibroblasts from patients with xeroderma pigmentosum to this agent (26). Escherichia coli mutants deficient in excision repair and which lack uvr endonuclease are also highly sensitive to cis-platinum as well as platinum analogues in a cis configuration (27).

In view of the effects of ara-C on excision repair, of the induction of an excision repair mechanism by cis-platinum bound to DNA, and because a prior report indicated that ara-C increased CDDP-induced DNA cross-links, we also wished to analyze whether ara-C altered either the extent or the kinetics of repair of CDDP-induced DNA damage.

MATERIALS AND METHODS

Cell Culture. LoVo colon cancer cells were obtained from the American Type Culture collection (Rockville, MD). Cells were routinely tested for Mycoplasma and maintained by serial passage in Ham's F-12 media with 15% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Reagents. CDDP was obtained from Sigma Chemical Co (St. Louis, MO) and also was provided by Bristol Laboratories (Syracuse, NY). ara-C, hydroxyurea, and methotrexate were obtained from Sigma. Aphidicolin was kindly provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Drugs were prepared in media without serum just prior to use except for aphidicolin which was dissolved in dimethyl sulfoxide.

Cytotoxicity. Flasks (25 cm²; Corning Glassworks, Corning, NY) were seeded with 10⁴ cells in 10 ml media and drug exposure was carried out on day 3. Cytotoxicity was analyzed by exposing nonconfluent cells to drug for 1 h at 37°C. Cells were washed twice, trypsinized, and plated on 100-mm Petri dishes (Corning). Colonies with at least 50 cells were counted after incubation for 14 days at 37°C and with 5% CO₂. Cytotoxicity was determined by counting 6 control plates and 3 plates for each drug treatment. Experiments were done at least twice and generally 3 times. Representative experiments are shown.

Alkaline Elution Analysis. The procedure for alkaline elution analysis is described elsewhere except as noted (28). LoVo carcinoma cells were labeled with 0.2 μCi/ml [methyl-³H]thymidine (specific activity, 53.2 mCi/mmol; New England Nuclear, Boston, MA) for 48 h, washed, and

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3 The abbreviations used are: CDDP, cis-diaminedichloroplatinum(II); ara-C, 1-β-D-arabinofuranosylcytosine; PBS, phosphate-buffered salt solution; SDS, sodium dodecyl sulfate; ara-CTP, 1-β-D-arabinofuranosylcytosine triphosphate.
were washed and trypsinized, and two neutral CsCl gradients were
with 5 x 10^-6 M CDDP or no drug for 1 h. After drug treatment, cells
were then exposed to 10^-6 M [3H]ara-C (28 Ci/mmol) in combination
(10^-6 M) and 5-fluorodeoxyuridine (10^-6 M) were added for 2 h. Cells
plates. Cells were grown to near confluence and then medium contain
approach used is described extensively elsewhere (30). Briefly, 2.5 x
was precipitated with 0.1 volume 4 M NaCl and 2 volumes absolute ethanol overnight at 4°C. Specimens were centrifuged at 2,800 rpm for
phase were exposed to 10^-7-10^-3 M [3H]ara-C (Amersham Interna
tional, Amersham, United Kingdom) in the presence or absence of 5 x10^-7 M CDDP for 1 h at 37°C. Cells were washed twice with cold PBS
and allowed to settle on 2 µm polyvinyl chloride filters (Millipore, New
Bedford, MA). [3H]-Labeled internal standard cells (5 x 10^6) also were
added. PBS was allowed to drain by gravity and cells were washed with an additional 10 ml cold PBS. Cells were lysed with 5 ml 0.2% Sarkosyl, 2 m NaCl, 0.04 M EDTA (pH 10.0), and 50 µg/ml proteinase K followed
by washing with 3 ml 0.02 M EDTA (pH 10). Tetrapropylammonium hydroxide with 0.1% SDS and 0.02 M EDTA (pH 12.1) was pumped at a rate of 0.04 ml/min over the filters. Fractions were collected hourly for 12-14 h and assayed for radioactivity after the addition of 14 ml of a toluene–scintillation fluid (National Diagnostics, Somerville, NJ).

The DNA-DNA interstrand cross-linking coefficient was calculated as described previously (28).

Platinum Content in DNA. Nonconfluent cells in logarithmic growth phase were exposed to CDDP in the presence or absence of 2 x 10^-2 M ara-C for 1 h, washed, and trypsinized. Cells were resuspended in PBS at 10^6 cells/ml, digested with proteinase K (50 µg/ml) for 12 h at 37°C, and incubated with 2 ml 0.01 M Tris-0.01 M EDTA-0.5% SDS, (pH 7.4), for 3 h. The solution was extracted with an equal volume of phenol, incubated with RNase (50 µg/ml) for 90 min at 37°C, extracted with phenol, and then DNA precipitated by adding 0.1 volume of 4 M NaCl and 2 volumes of absolute ethanol. DNA was hydrolyzed with 1 N HCl and quantitated spectrophotometrically at 260 and 280 nm.

Total platinum content was assessed with a Perkin-Elmer (Norwalk, CT) model 2380 atomic absorption spectrophotometer equipped with an HGA-400 graphite furnace. The approach is described extensively elsewhere (29).

Recovery of DNA Synthesis. Cells were exposed to drug for 1 h, washed, and then incubated with 5 µCi/ml 32P (New England Nuclear) for 0–24 h. After trypsinization, DNA was isolated as described previously, and 32P incorporation was measured following the addition of 5 ml scintillation fluid. Results are expressed as 32P cpm/10^6 cells. Values are the mean ± SD of duplicates. Experiments were repeated at least twice and representative experiments are shown.

Incorporation of ara-C into DNA. LoVo cells in logarithmic growth phase were exposed to 10^{-2}-10^{-3} M [3H]ara-C (Amersham International, Amersham, United Kingdom) in the presence or absence of 5 x 10^{-4} M CDDP for 1 h at 37°C. Cells were washed twice with cold PBS and cells were trypsinized. Cells (10^6) in 2 ml PBS were digested with 50 µg/ml proteinase K and 2 ml 0.01 M Tris-0.01 M EDTA-0.5% SDS, (pH 7.4) for 12 h at 37°C. The solution was extracted with phenol and the aqueous phase was treated with 50 µg/ml RNase (Sigma) for 90 min at 37°C. A second phenol extraction was performed and the DNA was precipitated with 0.1 volume 4 M NaCl and 2 volumes of absolute ethanol. DNA was hydrolyzed with 1 N HCl and quantitated spectrophotometrically at 280 and 260 nm for purity. Samples were then filtered on Whatman GF/A filters (Whatman, Maidstone, United Kingdom) and radioactivity was analyzed by filtering the tritiated cpm that band at the density of parental, [3H]-labeled DNA by the [3H] cpm measured in the same fractions. A representative experiment is shown.

RESULTS

To analyze the effect of ara-C on CDDP-induced lethality, nonconfluent LoVo colon carcinoma cells were exposed to either agent alone or both drugs simultaneously for 1 h. Fig. 1 demonstrates that ara-C, even at doses up to 4 x 10^{-2} M, was not toxic to these cells. When cells were exposed to CDDP at 5 x 10^{-3} M, a more than additive decrease in clonogenic survival was noted when cells were simultaneously treated with ara-C and CDDP. This effect also was present at 10^{-3} M and 5 x 10^{-5} M CDDP.

To evaluate whether inhibition of DNA synthesis or an effect specific to ara-C, such as misincorporation into DNA, underlies these results, cytotoxicity by aphidicolin in combination with cis-platinum was analyzed. Aphidicolin, like ara-C, competes with dCTP for binding to DNA polymerase α but is not incorporated into DNA (31). Effects by aphidicolin in combination with cis-platinum were less than additive (see Fig. 2). The range of concentrations of aphidicolin used in these experiments caused a comparable degree of inhibition of DNA synthesis as the range of ara-C concentrations used in the prior experiment (32). Further, other agents such as hydroxyurea and methotrexate, that inhibit DNA synthesis did not enhance CDDP-induced cytotoxicity (data not shown).

To ensure that neither the binding of CDDP to DNA nor the repair of CDDP-induced DNA lesions was affected by ara-C, total platinum content in DNA and the excision of platinum and CDDP-induced cross-links from DNA were analyzed by atomic absorption spectroscopy and by alkaline elution, respectively. Table 1 demonstrates that exposure of cells to 2 x 10^{-2}
CDDP INTERACTIONS WITH ara-C IN COLON CARCINOMA

Fig. 2. Effect of CDDP and aphidicolin on survival. Cells were incubated with CDDP and aphidicolin for 1 h at 37°C, washed twice, trypsinized, and plated. No CDDP, 0–10⁻⁶ M aphidicolin (○); 5 × 10⁻⁶ M CDDP, 0–10⁻³ M aphidicolin (□); 10⁻⁶ M CDDP, 0–10⁻³ M aphidicolin (△); 5 × 10⁻⁵ M CDDP, 0–10⁻³ M aphidicolin (■). Points, means; bars, SD.

Table 1 Effect of ara-C exposure on platinum content in DNA
Cells were exposed to CDDP in the presence of 2 × 10⁻³ M ara-C for 1 h at 37°C and washed twice, and DNA was isolated. Platinum content in DNA was analyzed by atomic absorption spectroscopy. Results are from a representative experiment and differences in the two treatment groups are not significant.

<table>
<thead>
<tr>
<th>CDDP concentration (M)</th>
<th>Platinum in DNA (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−ara-C</td>
<td>+ara-C</td>
</tr>
<tr>
<td>5 × 10⁻⁴</td>
<td>111</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>188</td>
</tr>
<tr>
<td>5 × 10⁻⁶</td>
<td>216</td>
</tr>
</tbody>
</table>

platinum from DNA was not altered by exposure of cells to 2 × 10⁻³ M ara-C for 1 h.

While ara-C did not alter the excision of platinum from DNA and had, in fact, less than additive effects on the inhibition of DNA synthesis by CDDP (data not shown), the recovery of DNA synthesis was markedly delayed after exposure to both drugs simultaneously than after treatment with either agent alone (see Fig. 5a). Thus, by 24 h, DNA synthesis by cells exposed concurrently to 5 × 10⁻⁵ M CDDP and 2 × 10⁻² M ara-C was 5–6-fold less than that of cells exposed to either agent alone or to no drug. In contrast, recovery of DNA synthesis was the same in cells exposed to both 2 × 10⁻² M aphidicolin and 5 × 10⁻⁵ M cis-platinum than to either agent alone (see Fig. 5). These findings suggest that delayed recovery of DNA synthesis after exposure to ara-C and CDDP might be related to incorporation of ara-C into DNA.

Fig. 6 demonstrates that proliferating LoVo cells incorporate ara-C into DNA in a dose-dependent fashion. Further, the extent of incorporation of [³H]ara-C into DNA was not significantly different in the presence or absence of 5 × 10⁻⁶ M CDDP during a 1-h incubation at 37°C.

Since CDDP-induced DNA damage may cause incorporation of ara-C into DNA undergoing repair synthesis, we analyzed the incorporation of [³H]ara-C into DNA undergoing repair synthesis. Fig. 7 demonstrates that exposure of cells concurrently to 5 × 10⁻⁶ M CDDP and to 10⁻⁶ M [³H]ara-C for 1 h caused a 1.9-fold enhancement in the ratio of tritiated ara-C cpm to [¹⁴C]cpm in parental DNA prelabeled with [¹⁴C]thymidine when compared to the [³H]/[¹⁴C] cpm ratio in DNA from cells exposed solely to [³H]ara-C.

m ara-C did not significantly increase binding of CDDP to DNA. Neither the induction nor the excision of CDDP-induced DNA interstrand cross-links was affected by exposure to ara-C (see Fig. 3). Lastly, as shown in Fig. 4, the excision of total DNA interstrand cross-links was not altered by exposure of cells to 2 × 10⁻³ M ara-C for 1 h.

Fig. 3. Effect of ara-C on the excision of cis-platinum-induced DNA-DNA interstrand cross-links. Cells were exposed to 5 × 10⁻⁵ M CDDP alone (○) or in combination with 2 × 10⁻² M ara-C (□) for 1 h at 37°C, washed twice, and further incubated in media. Cells were trypsinized and DNA-DNA interstrand cross-links were analyzed at 0, 3, 6, 12, and 24 h. Points, means; bars, SD.

Fig. 4. Effect of ara-C on excision of platinum from DNA. Cells were exposed to 5 × 10⁻⁵ M CDDP (○), or 5 × 10⁻⁵ M CDDP and 2 × 10⁻² M ara-C (□) for 1 h at 37°C. Cells were washed twice and incubated in media at 37°C for the indicated intervals. After trypsinization of cells, DNA was isolated and total platinum content was analyzed by atomic absorption spectroscopy (see "Materials and Methods"). Points, means; bars, SD.

HOURS AFTER DRUG REMOVAL

Fig. 5 demonstrates that proliferating LoVo cells incorporate ara-C into DNA in a dose-dependent fashion. Further, the extent of incorporation of [³H]ara-C into DNA was not significantly different in the presence or absence of 5 × 10⁻⁶ M CDDP during a 1-h incubation at 37°C.

Since CDDP-induced DNA damage may cause incorporation of ara-C into DNA undergoing repair synthesis, we analyzed the incorporation of [³H]ara-C into DNA undergoing repair synthesis. Fig. 7 demonstrates that exposure of cells concurrently to 5 × 10⁻⁶ M CDDP and to 10⁻⁶ M [³H]ara-C for 1 h caused a 1.9-fold enhancement in the ratio of tritiated ara-C cpm to [¹⁴C]cpm in parental DNA prelabeled with [¹⁴C]thymidine when compared to the [³H]/[¹⁴C] cpm ratio in DNA from cells exposed solely to [³H]ara-C.
**DISCUSSION**

We have demonstrated that ara-C in combination with CDDP causes more than additive cytotoxic effects in LoVo colon carcinoma cells. These results, while confirming prior observations in this cell line, were less marked. The basis for this variability is unclear, but it may be related to differences in cells as well as technical considerations. Also important, particularly with regard to clinical applications of this drug combination, is that this combination does not cause more than additive cytotoxic effects in all cell lines tested. For example, such effects were not present in a human ovarian cancer line (11). Duration and schedule of drug treatment also affect cytotoxicity by this drug combination in LoVo cells (12).

The basis for enhanced cytotoxicity by ara-C in combination with CDDP may result from incorporation of ara-C into DNA. In contrast to ara-C and CDDP, the combination of aphidicolin and CDDP does not cause more than additive cytotoxic effects. The former agent resembles ara-C in competing for binding to DNA polymerase α with dCTP but is not incorporated into DNA (31). Further, other agents, such as hydroxyurea and methotrexate, that inhibit DNA synthesis but are not incorporated into DNA cause less than additive cytotoxic effects in combination with CDDP. Lastly, exposure of cells to both ara-C and CDDP caused a marked delay in recovery of DNA synthesis than after exposure to either drug alone. In contrast, recovery of DNA synthesis after cells were incubated with both aphidicolin and CDDP was similar to that noted after exposure to either agent alone. An alternative explanation for the effects of ara-C in combination with CDDP on cytotoxicity and recovery of DNA synthesis is that ara-CTP pools persist after drug removal. Cytotoxic effects by ara-C might
then occur by the inhibition of DNA polymerase α and not by incorporation of ara-CTP into DNA. The persistence of elevated ara-CTP pools is not likely, however, because DNA synthesis in cells exposed to 2 × 10⁻⁹ M ara-C for 1 h recovers to the same level present in control cells by 3 h after drug removal.

Our results also demonstrate that ara-C is incorporated in LoVo cell DNA in a concentration-dependent fashion. Further, the incorporation of [³H]ara-C into DNA undergoing repair synthesis is enhanced almost 2-fold after concurrent exposure of cells to 5 × 10⁻⁶ M CDDP and 10⁻⁸ M ara-C. These results are similar to those obtained in MCF-7 cells by others (33). The effect of enhanced incorporation of ara-C into DNA undergoing repair synthesis after exposure to CDDP is unclear. Since the extent of DNA synthesis during repair is probably small compared to that during replication in proliferating cells, the evidence is far from conclusive that enhanced incorporation after CDDP exposure underlies increments in cytotoxicity. Such an effect could significantly enhance cytotoxicity by ara-C in growth-arrested cells and experiments analyzing this point are being pursued.

The inhibition in the recovery of DNA synthesis after exposure to both ara-C and CDDP may partly result from the effects of these agents on chain termination during DNA synthesis. CDDP causes DNA intrastrand cross-links, particularly at adjacent guanines (34). These lesions probably underlie effects on DNA synthesis, because chain termination occurs at these sites during in vitro DNA synthesis on single stranded DNA templates (35). ara-C, after incorporation into DNA, also causes chain termination during DNA synthesis (36–39). The site-specific insertion of ara-C at the 3' termini of DNA oligomers prevents further addition of bases by HeLa pol II holoenzyme while incorporation of ara-C in internucleotide linkage slows nucleotide insertion opposite the site of ara-C residues (39). Thus, the marked effect of ara-C and CDDP on inhibiting the recovery of DNA synthesis may result from their complimentary effects on chain termination during DNA synthesis.

Despite a significant level of misincorporation of ara-C into LoVo cell DNA, exposure of cells to ara-C was neither cytotoxic nor caused delays in the recovery of DNA synthesis by 12–24 hrs. Increased excision of ara-C from DNA or the ability of LoVo cell polymerase alpha to bypass ara-C lesions in DNA might plausibly underlie such findings. Longer incubations of LoVo cells with ara-C, however, are associated with enhanced misincorporation of ara-C into DNA as well as significant delays in the recovery of DNA synthesis and increased cytotoxicity (32). Thus, a critical number of ara-C residues in DNA may be necessary to cause irreversible delays in the recovery of DNA synthesis and lethal effects.

In contrast to a prior report, no effect of ara-C on the induction of interstrand DNA cross-links was noted (9). Further, ara-C neither enhanced the binding of CDDP to DNA nor affected the excision of total platinum from DNA. Thus, unlike the inhibition of UV-induced excision repair by ara-C where the removal of pyrimidine dimers is dramatically slowed, the excision of platinum adducts in DNA and of CDDP-induced DNA interstrand cross-links is not altered by this agent under our conditions (40). These findings suggest that mechanisms underlying excision repair of CDDP-induced DNA damage may differ from those involved in UV-induced excision repair.

Our findings do not exclude the possibility that ara-C may inhibit the excision of CDDP-induced DNA intrastrand cross-links. It is conceivable that the kinetics of excision of these lesions might be altered by ara-C and obscured by analyzing total platinum in DNA. Studies to examine this possibility would be of interest.

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