Synergistic Interaction between Cisplatin and Gemcitabine in Vitro

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ABSTRACT

2',2'-Difluorodeoxycytidine (dFdC; gemcitabine) is a new antineoplastic agent that is active against ovarian carcinoma, non-small-cell lung carcinoma, and head and neck squamous cell carcinoma. cis-Diamminedichloroplatinum (CDDP; cisplatin) is used commonly for the treatment of these tumors. Because the two drugs have mechanisms of action that might be complementary, we investigated a possible synergism between dFdC and CDDP on growth inhibition. The combination was tested in the human ovarian carcinoma cell line A2780, its CDDP-resistant variant ADDP and its dFdC-resistant variant AG6000, the human head and neck squamous cell carcinoma cell line UMSCC-22B, and the murine colon carcinoma cell line C26-10. The cells were exposed to dFdC and CDDP as single agents and to combinations in a molar ratio of 1:500 for 1, 4, 24, and 72 h with a total culture time of 72 h. Synergy was evaluated using the multiple drug effect analysis. In A2780 and ADDP cells, simultaneous exposure to the drugs for 24 and 72 h resulted in synergism, but shorter exposure times were antagonistic. No synergism was found in the UMSCC-22B and C26-10 cell lines at prolonged simultaneous exposure. However, a preincubation with CDDP for 4 h followed by a dFdC incubation for 1, 4, 24, and 72 h was synergistic in all cell lines except C26-10 cells. A 4-h preincubation with dFdC followed by an incubation with the combination for 20 and 68 h was synergistic in all cell lines. Initial studies of the mechanism of interaction concentrated on the effect of CDDP on dFdC accumulation and DNA strand break formation. In all cell lines, CDDP failed to increase dFdC accumulation at 4- or 24-h exposure to dFdC; in two cell lines, CDDP even tended to decrease dFdC accumulation. Neither dFdC nor CDDP caused more than 25% double strand break formation, whereas in the combination, CDDP even tended to decrease this type of DNA damage. The synergistic interaction between the two drugs is possibly the result of dFdC incorporation into DNA and/or CDDP-DNA adduct formation, which may be affected by each other.

INTRODUCTION

dFdC2 is a deoxycytidine analogue with two fluorine atoms substituted for the two hydrogens at the 2' position of the ribose ring. The compound has established activity against solid tumors, as described for several solid tumor models, including ovarian and head and neck cancer (1–3). In the clinical setting, dFdC has proven to be effective against ovarian cancer and non-small cell lung cancer and moderately effective against head and neck cancers (4–6). After entering the cell, the drug has to be phosphorylated by dCK to dFdCMP and subsequently to dFdCTP (7), which can be incorporated into both DNA and RNA (8, 9). After incorporation of dFdCTP, one more deoxynucleotide can be incorporated, after which DNA polymerization stops (9). Unlike ara-C, exonuclease activity is unable to excise dFdCMP from the DNA (9). dFdC can be inactivated by the action of deoxycytidine deaminase to 2',2'-difluorodeoxyuridine (7).

For the treatment of the above-mentioned tumors, CDDP, a square planar coordination compound, is an important agent (10). The antitumor activity of CDDP is the result of the formation of adducts within the DNA (11–17). The binding of the CDDP moiety with two adjacent guanines on the same DNA strand is the most abundant lesion and is held responsible for the antitumor activity (12, 13). This lesion is thought to introduce a distortion in the DNA that is large enough to stop the division of the cells without being recognized rapidly and, thus, removed efficiently by repair enzymes (18). There is a possible relation between CDDP-DNA adduct levels and cell growth inhibition in cultured cells (14, 15).

Synergistic interactions of CDDP with ara-C and DAC, two other deoxycytidine analogues, have been described previously. Studies on the mechanism of the synergism suggested differences in the interactions between ara-C and CDDP and DAC and CDDP (19, 20). CDDP-DNA adduct formation was enhanced by DAC incorporation, but ara-C did not affect the binding of CDDP to DNA. However, ara-C delayed the recovery of DNA synthesis inhibited by CDDP markedly. Because dFdC is related structurally to ara-C and DAC, it seemed appropriate to investigate the combination of CDDP with this compound too. One possible mechanism for synergy could be the inhibitory effect of CDDP on ribonucleotide reductase (21), possibly causing a depletion of dCTP pools, a potent feedback inhibitor of dCK. Subsequently, this could lead to increased dFdC phosphorylation. Alternatively, DNA might become more accessible to DNA adduct formation by distortions caused by

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3 The abbreviations used are: dFdC, 2',2'-difluorodeoxycytidine (gemcitabine); dCK, deoxycytidine kinase; ara-C, 1-beta-D-arabinofuranosyl cytosine; CDDP, cis-diamminedichloroplatinum (cisplatin); DAC, 2'-deoxy-5-azacytidine; 22B, UMSCC-22B; TCA, trichloroacetic acid; SRB, sulforhodamine B; IC50, 50% inhibitory concentration; CI, combination index; FA, fraction affected; DCR, dose required to produce a 50% growth inhibition; FADU, fluorometric analysis of DNA unwinding.
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Exposed to both drugs in different concentrations and schedules. CDDP. Three human ovarian carcinoma cell lines, the dFdC-resistant variants ADDP and AG6000, respectively, the human head and neck squamous cell carcinoma cell line 22B, and the dFdC-insensitive murine colon tumor cell line C26-10 were exposed to both drugs in different concentrations and schedules. To investigate possible mechanisms of synergism, several parameters known to be important in the action of dFdC were measured to clarify the mechanism of the synergistic interaction.

MATERIALS AND METHODS

Chemicals and Reagents. DMEM and RPMI 1640 were purchased from Flow Laboratories (Irvine, United Kingdom); FCS was from Gibco (Grand Island NY), TCA, glutamine, and gentamicin were from Merck (Darmstadt, Germany), trypsin, and SRB was from Sigma Chemical Co. (St. Louis, MO). dFdC was kindly supplied by Eli Lilly Research Labs (Indianapolis, USA). CDDP was purchased from Bristol-Myers Squibb (Weesp, the Netherlands). All other chemicals were of analytical grade and commercially available.

Cell Culture. The in vitro experiments were performed with five different cell lines: A2780, a human ovarian cancer cell line (22, 23), and two variants, ADDP, with a 50-fold induced resistance to CDDP (kindly provided by Dr. K. J. Scanlon City of Hope National Medical Center, Duarte, CA; Ref. 22), and AG6000, with a 150,000-fold induced resistance to dFdC (23); 22B, a human head and neck squamous cell carcinoma cell line (24); and the murine colon carcinoma cell line C26-10 (25). Doubling times of the cell lines were 21, 32, 37, 40, and 14 h, respectively. Cells were grown in monolayers in DMEM supplemented with 5% heat-inactivated FCS, 1 mM l-glutamine, and 250 ng/ml gentamicin, except for ADDP, which was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 1 mM l-glutamine, and 250 ng/ml gentamicin at 37°C at 5% CO2.

Chemosensitivity Testing. The determination of the IC50 was performed using the SRB assay (26, 27). The assay was performed using the National Cancer Institute protocol with some small modifications (27). Culture conditions were optimized for each cell line. The five cell lines were exposed to dFdC and CDDP as separate agents and to a combination of both. At day 1, the cells were plated in 96-well plates in different densities, depending on their doubling times. The optimal plating number was the highest number of cells possible to enable log linear growth for 72 h (A2780, 5,000 cells/well; ADDP, 12,000 cells/well; AG6000, 18,000 cells/well; C26-10, 1,500 cells/well; and 22B, 15,000 cells/well) in a volume of 100 µl/well. Of the 96-well plates, the upper and lower rows (rows A and H) and the last two columns at the right side (columns 11 and 12) were not used for cells, because we observed evaporation in these wells earlier. Thus, the first column contained only medium, the second column contained cells not exposed to drugs, and eight columns contained cells exposed to increasing concentrations of drugs. For each drug or drug combination, three wells were used.

On day 2, dFdC and CDDP were added in a volume of 100 µl, resulting in series of final concentrations of 2.10-11-1.10-6 M dFdC and 1.10-8-5.10-4 M of CDDP. The concentrations in the combination had the same range as the separate agents, with a dFdC:CDDP ratio of 1:500, which was based on the separate IC50 values in the various cell lines. Similar to the National Cancer Institute protocol, we chose the same culture time for all cell lines. The cells were exposed for 1, 4, 24, and 72 h. After the exposure, the medium in the control and the drug-containing wells was removed and replaced by fresh, drug-free medium after a washing step with 50 µl PBS (pH 7.4), and the cells were cultured until 72 h after the initial drug addition. Care was taken to ensure that cell loss was minimal in the procedure; furthermore, control wells were also washed. Final A values in washed and nonwashed wells were hardly different. At the end of the culture, the cells were precipitated with 50 µl ice-cold 50% (w/v) TCA and fixed for 60 min, after which the SRB assay was performed as described (27). Only proteins of intact cells are stained, because proteins from killed cells would be degraded to amino acids by endogenous proteases after lysis (27). Growth inhibition curves were made relative to control A of every SRB assay. Two control values were always included, that of the A of cells at the day of drug administration (day 0, set at 0%) and that of cells after 3 days (set at 100%). This enabled us to calculate IC50 values (relative A at 50%), total growth inhibition (A of drug-treated cells similar to the initial value, 0%) and cell killing (A of drug treated cells lower than the initial value 0%). The points were connected by straight lines, and the IC50 values were determined from the interpolated graph (28). Also, the method of Chou and Talalay (29) was used; for these cell lines similar values (Dm) were achieved. The cells were not only exposed to the combination simultaneously, but two sequential schedules were also used: (a) 4-h preincubation with CDDP, after which the cells were exposed to dFdC for 1, 4, 24, or 72 h; and (b) 4-h preincubation with dFdC, followed by an incubation with both dFdC and CDDP for 20 or 68 h (Fig. 1).

Dose-response interactions (antagonism, additivity, and synergism) between dFdC and CDDP were performed as a nonexclusive case CI for every FA, using the method of Chou and Talalay (29), processed by a computer program developed by Chou and Chou ( Biosoft, Cambridge, United Kingdom). This program was chosen because it provided one of the few objective computerized evaluation procedures (30). The data are supported by classic isobolograms made by the same computer program, which were based on the original method of Steel and Peckham (31). Other observations in favor for the choice of this program were the sigmoidal forms of the growth curves, ideal for median effect analysis. Thus, IC50 values (by interpolation) and Dm values (by the computer program) were usually similar. Measurable IC50 values for each drug separately are not required for computerized evaluation; Dm values would be calculated then by the program by extrapolation. For the separate drugs, we had to introduce their respective growth inhibition parameters, expressed as FA (e.g., a FA of 0.25 is a growth
inhibition of 25%). The program was unable to evaluate values of FA > 1, indicating cell killing or FA = 0, indicating no growth inhibition. Growth inhibition of 0% of each drug separately had to be introduced as a FA of 0.01, whereas total growth inhibition of 100% had a FA of 0.99. For the final evaluation, we excluded values of the combination for almost complete growth inhibition (FA > 0.90) and hardly any effect (FA < 0.10). The CI was calculated by the formula:

\[
CI = \frac{[(D)1/(D_2)] + [(D)2/(D_3)] + [\alpha(D)1,2/(D_3)1,2]}{[(D)1/(D_2)] + [(D)2/(D_3)] + [\alpha(D)1,2/(D_3)1,2]}
\]

Where \( \alpha = 1 \) for mutually nonexclusive drugs; \((D)1, (D)2, \) and \((D)1,2\) are the doses of the separate drugs and their combination in a fixed ratio of 1:500; and \((D_2)1, (D_3)2 \) and \((D_3)1,2\) are the doses resulting in a growth inhibition of x%. These doses are calculated by the formula:

\[
D = D_m \times \frac{[\alpha FA1 - FA)]m}{FA1}
\]

where \( D_m \) is the dose required to produce a 50% growth inhibition, \( FA \) is the fraction affected, and \( m \) is the slope of the median effect plot (a measure of sigmoidicity). A CI <1 indicates synergism, >1 indicates antagonism, and a CI of 1 indicates additivity. Results were also analyzed by isobologram analysis using the same program as well by simple calculations as comparisons of expected and observed growth inhibition or cell killing. All three methods led to comparable conclusions, underlining the significance of the data obtained with the multidrug effect analysis.

dFdCTP Accumulation. At day 1, the cells were plated in six-well plates in different densities, depending on their doubling times (A2780, 1.5 \( \times 10^5 \) cells/well; ADDP, 1.5 \( \times 10^5 \) cells/well; AG6000, 2.5 \( \times 10^5 \) cells/well; C26-10, 1 \( \times 10^5 \) cells/well; and 22B, 1.0 \( \times 10^5 \) cells/well) in a volume of 2 ml/well. At day 2, the cells were exposed to either 0.1 \( \mu M \) dFdC or 1 \( \mu M \) dFdC as single agents or to combinations of 0.1 \( \mu M \) dFdC with 20 \( \mu M \) CDDP or to 1 \( \mu M \) dFdC with 100 \( \mu M \) CDDP and incubated at 37°C. After 4 and 24 h of incubation, the cells were harvested by trypsinization, washed, and counted while kept on ice. Thereafter, the nucleotides were extracted using TCA precipitation and alamine and freon neutralization (32). Finally, the nucleotides were analyzed on high-performance liquid chromatography using a Partisphere SAX (Whatman, Clifton, NJ) column with a linear gradient between 5 mm NH_4H_2PO_4 (pH 2.8; buffer A) and 0.5 mm NH_4H_2PO_4/0.25 mm KCL (pH 3.0; buffer B; 35–100% B over 30 min) at a flow rate of 1.5 ml/min. Samples were detected at 254 and 280 nm (32).

**FADU Assay.** To measure the dFdC-induced DNA damage the FADU assay was used (33, 34). DNA damage was assessed by exposing lysed cells to an alkaline environment, allowing the DNA to unwind. The extent of strand breaks in the DNA determines the extent of DNA unwinding at the end of incubation. For this purpose, 3–5 \( \times 10^6 \) A2780 or 22B cells were incubated for 24 h at 37°C with either 1.5 nm dFdC as a single agent or the combination of 1.5 nm dFdC and 0.75 \( \mu M \) CDDP. For C26-10 cells, a higher drug concentration was chosen, because for the concentrations used for the A2780 and 22B cell lines, no DNA damage could be detected. Three to 5 \( \times 10^6 \) C26-10 cells were incubated for 24 h with either 20 nm dFdC as a single agent or the combination of 1.5 nm dFdC and 0.75 \( \mu M \) CDDP. Cells exposed to 50 \( \mu M \) VP-16 (Etoposide) for 1 h were used as a positive control; cells not exposed to drugs were used as a negative control.

After harvesting, using trypsinization, cells were counted and suspended in 0.25 mm meso-inositol, 10 mm sodium phosphate, and 1 mg MgCl_2 (pH 7.2) buffer (solution B; 1.5–2.5 \( \times 10^6 \) cells/ml). Every sample was distributed to three tubes (in duplicate, 0.2 ml/tube), in which the cells were lysed by 0.2 ml solution of 9 mm urea, 10 mm NaOH, 2.5 mm cyclohexanediami-netetraacetate, and 0.1% SDS (solution C). The cells were exposed to an alkaline environment by adding 0.1 ml solution D (45% solution C and 55% 0.2 mm NaOH) and 0.1 ml solution E (40% solution C and 60% 0.2 mm NaOH) gently to the tubes without mixing and then kept on ice for 30 min to achieve a slowly increasing pH (final pH, 12.8). One of the three tubes was protected against the alkaline environment by adding 0.4 ml solution of 1 mm glucose and 14 mm \( \beta \)-mercaptoethanol (solution F) before adding solutions D and E. In this tube, the DNA did not unwind (100% dsDNA; tube T). One of the two other tubes exposed to the alkaline environment was vortexed until no dsDNA was left (0% dsDNA; tube B), whereas the third tube represented the actual measurement (tube P). The cells were incubated at 15°C, allowing the DNA to unwind. For every cell line, an optimal unwinding time had to be determined; for the A2780 and 22B cells, an unwinding time of 60 min was chosen, and for the C26-10 cell line, the unwinding time was 30 min. The incubation was terminated by adding 0.4 ml solution F to tubes P and B, which were mixed subsequently and chilled on ice. The dsDNA was stained by adding 1.5 ml solution of 6.7 \( \mu g/ml \) etidium bromide and 13.3 mm NaOH. Fluorescence was measured on a SPEX Fluoromax fluorescence spectrometer (Perkin Elmer/Cetus, Norwalk, CT; excitation, 520 nm; analyzer, 590 nm). The percentage of dsDNA was calculated by:

\[
(P - B)(T - B) \times 100\%
\]
**Table 1** Summary of the sensitivity of the tested cells to dFdC and CDDP

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Exposure (h)</th>
<th>dFdC (μM)</th>
<th>CDDP (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>1</td>
<td>52.5 ± 8.4</td>
<td>10.6 ± 2.84</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>12.4 ± 2.7</td>
<td>2.86 ± 0.59</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>4.34 ± 2.16</td>
<td>1.20 ± 0.48</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>2.16 ± 1.01</td>
<td>1.96 ± 0.77</td>
</tr>
<tr>
<td>ADDP</td>
<td>1</td>
<td>730 ± 213</td>
<td>460 ± 220</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>239 ± 117</td>
<td>197 ± 82</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>193 ± 43</td>
<td>63 ± 15</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>625 ± 154</td>
<td>52 ± 13</td>
</tr>
<tr>
<td>AG6000</td>
<td>1</td>
<td>&gt;10^6</td>
<td>54.2 ± 13.8</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>&gt;10^6</td>
<td>17.1 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>&gt;10^6</td>
<td>4.70 ± 0.88</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>50.5 ± 20.2</td>
<td>4.51 ± 1.01</td>
</tr>
<tr>
<td>22B</td>
<td>1</td>
<td>62.3 ± 12.5</td>
<td>56.3 ± 15.6</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>14.0 ± 3.5</td>
<td>14.7 ± 5.8</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>4.50 ± 1.21</td>
<td>5.83 ± 0.52</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.86 ± 0.32</td>
<td>4.10 ± 0.10</td>
</tr>
<tr>
<td>C26-10</td>
<td>1</td>
<td>655 ± 141</td>
<td>23.8 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>254 ± 109</td>
<td>6.00 ± 1.08</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>24.7 ± 9.1</td>
<td>2.95 ± 0.67</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>22.6 ± 11.6</td>
<td>3.28 ± 0.74</td>
</tr>
</tbody>
</table>

**RESULTS**

**Growth Inhibition Tests.** The sensitivities of the five cell lines to dFdC and CDDP, expressed as IC_{50} values, are listed in Table 1. Drug effects were easily detectable after 72 h of drug exposure. The fast growing human ovarian cancer cell line A2780 and the slow growing human head and neck squamous cell carcinoma cell line 22B are the cell lines most sensitive to dFdC. However, 22B cells were less sensitive to CDDP than A2780 cells. As expected, ADDP was the least sensitive cell line to CDDP, and AG6000 was the least sensitive to dFdC. Furthermore, it seemed that AG6000 and ADDP cells were also cross-resistant to both drugs, AG6000 about 4-fold to CDDP and ADDP about 40-fold to dFdC (24-h exposure).

In most cell lines studied, the IC_{50} for 72-h exposure was less than for shorter incubation times, consistent with the known cell cycle specificity of dFdC. However, the IC_{50} value of ADDP cells for dFdC is higher at 72 h than at 24 h of incubation. The IC_{50} value of AG6000 cells for dFdC for the exposure times of 1, 4, and 24 h was greater than the highest concentration used, 10^{-3} M. The murine colon cancer cell line C26-10 seemed to have an intrinsic resistance to both drugs compared with A2780.

Fig. 2 shows representative relative growth inhibition curves of ADDP and AG6000 cells when exposed to dFdC and CDDP alone and to a combination of 1:500 for 24 h. The curve for dFdC in ADDP cells showed a rather flat pattern, in contrast to CDDP; the combination curve, however, was shaped similarly as that for CDDP, but with a shift to the lower concentration range. The AG6000 cells were exposed to the combination of dFdC and CDDP simultaneously. dFdC did not affect the growth of AG6000 cells. The growth inhibition curve of AG6000 cells exposed to CDDP was similar to that of dFdC and CDDP together, suggesting that the growth inhibition of the combination is only due to CDDP exposure. At concentrations of >30 μM, CDDP induced cell killing. In a multiple drug effect analysis, this effect is represented by a CI of about 1. Fig. 3 shows representative CI/FA plots of A2780 and ADDP cells exposed to dFdC and CDDP. In A2780 cells, the combination of dFdC and CDDP was antagonistic (CI > 1) at exposure times of 1 and 4 h. The CIs varied over a range of fractions affected studied; at a FA of 0.25, a high CI (CI = 5–10) was found, but lower values (CI = 2–4) were observed at a FA of 0.75 (Fig. 2).

Synergistic interaction (CI < 1) between dFdC and CDDP was found in A2780 cells at an exposure time of 24 h. In the ADDP cell line, CI/FA curves similar to those in A2780 cells were found. However, in ADDP cells, synergism was observed at 4, 24, and 72 h. Also, analysis of the combination of dFdC and CDDP with isobolagrams was performed. Fig. 4 shows a representative isobologram of ADDP cells exposed to dFdC and CDDP for 24 h. The observed position of the combination point on the left of the connecting line between the IC_{50} values of dFdC and CDDP indicates synergism. For every experiment, the isobologram analysis revealed the same dose-response interaction as the method of Chou and Talalay (29).

Table 2 summarizes the CIs of the five cell lines exposed to the simultaneous or sequential combination of dFdC and CDDP. Only values at a FA of 0.5 are represented. When the cells were exposed simultaneously to both drugs, very different patterns were observed between the cell lines, with antagonism in A2780 and ADDP cells but synergism for 22B cells at short exposure times. At exposure durations of 4, 24, and 72 h, however, the combination of dFdC and CDDP was not synergistic in the 22B cell line, with increasing CIs at longer exposure periods. In the C26-10 cell line, interaction between the drugs was modest, with only additive to slightly antagonistic effects.

In all three ovarian cancer cell lines, preincubation with CDDP for 4 h followed by a dFdC incubation for 1, 4, 24, and 72 h resulted in synergism or additivity for all combinations. This is in contrast to the simultaneous exposure schedule. Even in the AG6000 cell line, synergism was observed. In the 22B cell line, the pattern of the CIs per exposure time has changed compared with the simultaneous exposure. For this schedule, the 1- and 4-h exposure times were antagonistic, and the 24- and 72-h exposure times were synergistic. In C26-10 cells, all schedules of sequential CDDP followed by dFdC were antagonistic.

In all cell lines for all exposure times, dFdC preincubation for 4 h followed by exposure to the combination of dFdC and CDDP was synergistic. Using this schedule, the lowest CIs were found in the ADDP cell line. Again, in the dFdC-resistant AG6000 cells, synergism was observed. Also, for the colon cancer cell line C26-10, for which no synergism was observed in the other schedules, synergism was observed in the schedule of preincubation with dFdC followed by schedule of the combination of dFdC and CDDP. Surprisingly, in all cell lines, the 24-h exposure time revealed a lower CI than the 72-h exposure time.

**Effect of CDDP on dFdCTP Accumulation.** To determine whether CDDP might influence the metabolism of dFdC, we measured the accumulation of the main metabolite of dFdC, dFdCTP. For this purpose, cells were exposed to 0.1 μM and 1 μM dFdC as a single agent or in combination with 20 μM and
Fig. 2. Representative growth inhibition curves of the cell lines ADDP (A) and AG6000 (B). Cells were exposed to dFdC (+), CDDP (△), and a combination of both simultaneously (●) for 24 h. After drug exposure, both cell lines were cultured in fresh medium. Total culture time was 72 h. The lines had different sigmoidicity expressed as the m value, which was 1.315, 1.813, and 1.646 for the ADDP cells, respectively, and 0, 1.084, and 1.080 for the AG6000 cell line, respectively.

100 μM CDDP, respectively, for either 4 or 24 h. Results in four cell lines are summarized in Fig. 5. In AG6000 cells, no dFdCTP could be detected, consistent with its known dCK deficiency (23); therefore, this cell line was not included in these studies. Despite the lower sensitivity of ADDP cells compared with A2780 cells, this cell line showed at the 4-h exposure time similar or greater accumulation of dFdCTP, both at 0.1 μM and 1 μM dFdC. dFdCTP accumulation was similar in A2780 and...
22B cells, but in the murine colon carcinoma cell line C26-10, dFdC accumulation at 0.1 μM dFdC was less. Also, exposure to 1 μM dFdC resulted in similar accumulation of dFdCTP in A2780 and 22B cells. In contrast, at the 24-h incubation time, the A2780 cell line showed the highest accumulation of dFdCTP both at 0.1 and 1 μM dFdC, whereas in ADDP cells, the lowest accumulation was found. At 0.1 μM dFdC, the accumulation of dFdCTP was undetectable in C26-10 cells.

Fig. 3 Synergy analysis of the interaction between CDDP and dFdC in the cell lines A2780 (A) and ADDP (B). Cells were exposed to dFdC and CDDP simultaneously for 1 h (▲), 4 h (●), 24 h (▼), and 72 hr (+). The values of the CIs are: CI > 1, antagonism; CI = 1, additivity; and CI < 1, synergism. Values are representative of three to five independent experiments.
At an exposure time of 4 h, CDDP did not increase the dFdC-TP accumulation in any of the five cell lines, neither at 0.1 μM dFdC in combination with 20 μM CDDP nor at 1 μM dFdC in combination with 100 μM CDDP. However, C26-10 cells accumulated less dFdCTP in the presence of CDDP at 1 μM dFdC in combination with 100 μM CDDP compared with dFdC alone. Also, at an exposure time of 24 h, CDDP did not increase accumulation of dFdCTP in C26-10 cells. Under these conditions, not only in C26-10 cells but also in A2780 cells, CDDP decreased dFdCTP accumulation. In A2780 and 22B cells, dFdCTP concentrations could not be measured at the 24-h incubation time due to the toxicity of the combination causing cell death. In the more resistant ADDP and C26-10 cells, dFdCTP accumulation could be measured at the higher concentrations also, but in ADDP cells, no effect was observed, whereas in C26-10 cells, lower dFdCTP accumulation was found.

DNA Damage. Another interaction between CDDP and dFdC could be due to drug-induced DNA damage. This possibility was evaluated with a DNA unwinding assay. Table 3 shows the relative amounts of dsDNA in A2780, 22B, and C26-10 cells after a 24-h exposure to 1.5 nm dFdC, 0.75 μM CDDP, and a combination of both. Under these conditions, dFdC alone did not cause DNA strand breaks in the A2780 and C26-10 cells, but 20% DNA strand breaks were found in 22B cells. After CDDP exposure, 15–25% DNA strand break formation was found in all three cell lines. However, the combination of dFdC and CDDP did not result in an increase in DNA strand break formation, because the effect of the combination was less than could be expected based on an additive interaction.

**DISCUSSION**

In this study, we show a definite synergism between dFdC and CDDP on growth inhibition in a panel of five solid tumor cell lines. The synergy is dependent on exposure duration and sequence of the drugs. Exposure to the combination of dFdC and CDDP simultaneously was synergistic in A2780 and ADDP cells. Preincubation with CDDP followed by dFdC was synergistic in all cell lines except C26-10 cells. Incubation with dFdC followed 4 h later by the combination was synergistic in all cell lines.

Mechanistic studies concentrated on dFdC metabolism, with emphasis on accumulation of the active metabolite dFdCTP. No consistent pattern of the effect of CDDP on dFdCTP accumulation was observed. It is remarkable that in the dFdC-insensitive ADDP cell lines, dFdCTP accumulation was comparable to that of the parent cell line A2780. In contrast to other cell lines, ADDP cells accumulated less dFdCTP with 24-h exposure to the drug than with 4-h exposure. This early saturation may explain the lower IC50 value at 4 and 24 h than at 72 h in ADDP cells. CDDP at the 4-h exposure time decreased dFdCTP accumulation slightly in the ADDP cell line, whereas the combination of dFdC and CDDP on growth inhibition was synergistic. However, in C26-10 cells, CDDP decreased dFdCTP accumulation considerably at both the 4- and 24-h exposure times. The explanations for these phenomena are unclear, but possibly, CDDP inhibits dFdC uptake into cells and, eventually, subsequent phosphorylation. Also, it was found previously that in a combination of DAC and CDDP, CDDP decreased the cellular uptake of DAC (19, 35). We plan to explore this hypothesis further in future studies.

To investigate the mechanism of interaction further, we studied drug-induced DNA damage. In previous studies by others, CDDP did not affect the levels of incorporated DAC into DNA (35). Although CDDP did not inhibit the incorporation of ara-C into normally replicating DNA, CDDP enhanced the

**Table 2. Summary of the CIs of the combination of dFdC and CDDP**

<table>
<thead>
<tr>
<th>Drug administration</th>
<th>Incubation (h)</th>
<th>A2780</th>
<th>ADDP</th>
<th>AG6000</th>
<th>22B</th>
<th>C26-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDDP and dFdC</td>
<td>72</td>
<td>0.01</td>
<td>0.36</td>
<td>1.13</td>
<td>0.58</td>
<td>1.01</td>
</tr>
<tr>
<td>simultaneously</td>
<td>4</td>
<td>0.18</td>
<td>0.88</td>
<td>4.83</td>
<td>6.50</td>
<td>4.79</td>
</tr>
<tr>
<td>24</td>
<td>0.45</td>
<td>1.06</td>
<td>2.35</td>
<td>0.12</td>
<td>0.45</td>
<td>0.45</td>
</tr>
<tr>
<td>72</td>
<td>0.71</td>
<td>1.45</td>
<td>3.74</td>
<td>0.21</td>
<td>0.86</td>
<td>1.09</td>
</tr>
<tr>
<td>CDDP 4 h before dFdC</td>
<td>24</td>
<td>0.35</td>
<td>0.90</td>
<td>2.25</td>
<td>0.62</td>
<td>0.62</td>
</tr>
<tr>
<td>24</td>
<td>0.20</td>
<td>0.69</td>
<td>1.33</td>
<td>0.21</td>
<td>0.69</td>
<td>0.21</td>
</tr>
<tr>
<td>72</td>
<td>0.18</td>
<td>0.61</td>
<td>1.04</td>
<td>0.07</td>
<td>0.45</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Values represent CIs (non-mutually exclusive case) calculated at the fraction affected of 0.5 of the combination of dFdC and CDDP and are means of three to five experiments; SEs were less than 30%. Cells were exposed to the combination either simultaneously or preincubated with CDDP for 4 h followed by a 1-, 4-, 24-, or 72-h dFdC incubation or incubated with dFdC for 4 h followed by a combination of dFdC and CDDP for 20 or 68 h. The ratio dFdC/CDDP was 1:500. CI > 1 indicates antagonism; CI = 1 is additive; and CI < 1 is synergism.
Synergistic Interaction between Cisplatin and Gemcitabine

A

![Graph A]

B

![Graph B]

Table 3  Effect of dFdC and CDDP on cellular dsDNA

The effect of 1-h exposure to 50 μM VP-16 was always included as a positive control for the assay, because VP-16 is known to produce ds breaks.

DNA damage was evaluated as percentage of dsDNA. Values are means ± SE of percentages of dsDNA in treated compared with that in untreated cells, which was set at 100% in each experiment. The actual amounts of dsDNA of cells not exposed to drugs were: A2780, 86.0 ± 5.2; 22B, 88.1 ± 7.0; and C26-10, 73.0 ± 3.3% of fluorescence of samples not exposed to an alkaline environment.

Exposure was 24 h to dFdC (1.5 nm) and CDDP (0.75 μM) as single agents and a combination of both agents (1.5 nm + 0.75 μM). The unwinding time in the FADU assay was 60 min for A2780 and 22B, 30 min for C26-10.

<table>
<thead>
<tr>
<th>Drug</th>
<th>A2780</th>
<th>22B</th>
<th>C26-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>dFdC</td>
<td>102 ± 6.7</td>
<td>79.2 ± 4.6</td>
<td>97.5 ± 6.6</td>
</tr>
<tr>
<td>CDDP</td>
<td>89.3 ± 10.4</td>
<td>76.2 ± 6.7</td>
<td>85.8 ± 3.8</td>
</tr>
<tr>
<td>dFdC+CDDP</td>
<td>103 ± 8.2</td>
<td>83.5 ± 11.2</td>
<td>87.4 ± 6.5</td>
</tr>
<tr>
<td>VP-16</td>
<td>25.7 ± 15.4</td>
<td>32.3 ± 5.7</td>
<td>52.1 ± 27.3</td>
</tr>
</tbody>
</table>

* dFdC 20 nm and CDDP 2 μM as single agents and in combination.

Incorporation of ara-C into DNA may inhibit exonuclease activity, thereby preventing excision of misincorporated dFdCMP (9). Interestingly, in all cell lines, the two drugs did not cause any obvious additive DNA damage. Actually, in the combination, DNA damage was always less than that caused by CDDP alone, and in A2780 and 22B cells, damage was also less than that caused by dFdC alone. Not only might this be related to inhibition of dFdC-induced DNA damage, but dFdC incorporated into DNA also might result in inhibition of the repair of CDDP-DNA adducts, causing apparent stabilization of DNA. Thus, the synergistic interaction between dFdC and CDDP might be related to reduced DNA repair. Moreover, dFdCTP inhibits the excision repair of intrastrand and possibly interstrand cross-links of CDDP, which may result in synergy (37). These mechanisms might clarify why no increased DNA damage as a result of the combination of dFdC and CDDP was found in the FADU assay, although these events may enhance cytotoxicity. Further evidence that DNA repair might play a role in the interaction of cytidine analogues with CDDP was found in studies concentrating on the role of caffeine (38–40). This methylated xanthine is capable of sensitizing tumor cells to cytotoxic agents, probably by inhibiting DNA repair. In a nude mouse model with human pancreatic adenocarcinoma, the combination of ara-C and CDDP did not enhance the therapeutic effect. The addition of caffeine, however, to the combination of ara-C and CDDP resulted in an early tumor response followed by complete tumor regression. It is of interest that this action of caffeine was seen only in the triple combination ara-C, CDDP, and caffeine, but not when caffeine was combined only with either ara-C or CDDP. Probably, the addition of methylated xanthines can convert the ara-C-CDDP interaction to synergism, due to inhibition of DNA repair by caffeine. In contrast to dFdC, ara-C incorporation into DNA does not lead to inhibition of DNA repair (41). Therefore, it is likely that the observed interactions between dFdC and CDDP are due to reduced DNA repair, in a manner similar to the combination of ara-C, CDDP, and caffeine. The observation that the sequential exposure of dFdC followed by CDDP was synergistic in all cell lines supports this hypothesis. Pretreatment of

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Fig. 5  Effect of CDDP on the accumulation of dFdCTP in A2780, ADDP, 22B, and C26-10 cells after 4-h (A) and 24-h (B) exposures to dFdC with or without CDDP. Values are means ± SEM of three to five experiments. ■, 0.1 μM dFdC; □, 0.1 μM dFdC and 20 μM CDDP; ▣, 1 μM dFdC; □, 1 μM dFdC and 100 μM CDDP.
cells with dFdC will allow sufficient dFdCTP accumulation and incorporation into DNA, resulting in decreased repair when CDDP-DNA adducts are formed.

Other studies of the mechanism of synergistic interaction in the combination of cytidine analogues, including DAC, and ara-C, and CDDP, suggest that the synergy is due to the incorporation of DAC into the DNA. DNA hypomethylated by DAC bound greater amounts of CDDP than normally methylated DNA (19). Hypomethylation could produce conformational changes in DNA, which make potential sites of CDDP adduct formation more accessible. Like dFdCTP, ara-CTP does not hypomethylate DNA. It was found that ara-C had no effect on the binding of CDDP to DNA, the induction of CDDP-induced interstrand DNA cross-links, the excision of CDDP-induced interstrand DNA cross-links, or the excision of total platinum from DNA. However, ara-C delayed the recovery of DNA synthesis inhibited by CDDP markedly (20). Similar mechanisms may operate in the combination of dFdC and CDDP. It seems likely that one site of interaction between dFdC and CDDP is at the DNA level, because in AG6000 cells, which did not accumulate dFdCTP, no synergism is observed with simultaneous exposure. The sequential effects of the combination, not only in this cell line, but also in the other cell lines, suggest an additional type of interaction between dFdC and CDDP not necessarily requiring dFdC phosphorylation. The synergistic effect of both sequences suggests not only an effect of dFdC (or its metabolites) on CDDP uptake and intracellular distribution, but also an effect of CDDP on dFdC metabolism. One such interaction may be at the level of regulation of dFdC metabolism. CDDP is known to inhibit ribonucleotide reductase (21), which may lead to a decrease of dCTP and an imbalance in other deoxyribonucleotides. This cannot only influence dFdC metabolism; it also affects DNA repair. Furthermore, differences in enzyme parameters (V_{max} and K_{m} for ribonucleotide reductase and DNA repair enzymes) in different cell lines might clarify the difference in the interaction between dFdC and CDDP in the cell lines (42). It remains to be investigated whether dFdCCTP affects CDDP, including adduct formation, or whether CDDP affects dFdC. Both types of interaction may occur.

The combination of dFdC and CDDP is synergistic in vivo. This synergism is time and schedule dependent. The most effective sequence is 4-h dFdC preincubation followed by the combination of dFdC and CDDP, which was synergistic in all cell lines. This indicates an enhanced effect of dFdC on CDDP due to this preincubation. This sequential synergistic interaction, in addition to nonoverlapping toxic side effects, is of interest for future clinical studies, especially in tumor types such as non-small cell lung cancer, ovarian cancer, and head and neck cancer, in which both drugs have shown single agent activity.

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REFERENCES


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Synergistic interaction between cisplatin and gemcitabine in vitro.


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