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Combination Chemotherapy Studies with Gemcitabine and Etoposide in Non-small Cell Lung and Ovarian Cancer Cell Lines

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ABSTRACT. Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC) and etoposide (4′-demethylepipodo-phyllotoxin-9-4,6-*O*-ethylidene-β-D-glucopyranoside, VP-16) are antineoplastic agents with clinical activity against various types of solid tumors. Because of the low toxicity profile of dFdC and the differences in mechanisms of cytotoxicity, combinations of both drugs were studied *in vitro*. For this purpose, we used the human ovarian cancer cell line A2780, its *cis*-diammine-dichloroplatinum-resistant and VP-16 cross-resistant variant ADDP, and two non-small cell lung cancer cell lines, Lewis Lung (LL, murine) and H322 (human). The interaction between the drugs was determined with the multiple drug effect analysis (fixed molar ratio) and with a variable drug ratio. In the LL cell line, the combination of dFdC and VP-16 at a constant molar ratio (dFdC:VP-16 = 1:4 or 1:0.125 after 4- or 24-hr exposure, respectively) was synergistic (combination index [CI], calculated at 50% growth inhibition = 0.7 and 0.8, respectively; CI < 1 indicating synergism). After 24- and 72-hr exposure to both drugs at a constant ratio, additivity was found in the A2780, ADDP, and H322 cell lines (dFdC:VP-16 = 1:500 for both exposure times in these cell lines). When cells were exposed to a combination of dFdC and VP-16 for 24 or 72 hr, with VP-16 at its IC_{25} and dFdC in a concentration range, additivity was found in both the LL and H322 cells; synergism was observed in the A2780 and ADDP cells, which are the least sensitive to VP-16. Schedule dependency was found in the LL cell line; when cells were exposed to dFdC 4 hr prior to VP-16 (constant molar ratio, total exposure 24 hr), synergism was found (CI = 0.5), whereas additivity was found when cells were exposed to VP-16 prior to dFdC (CI = 1.6). The mechanism of interaction between the drugs was studied in more detail in the LL cell line; dFdCTP accumulation was 1.2-fold enhanced by co-incubation with VP-16, and was even more pronounced (1.4-fold) when cells were exposed to VP-16 prior to dFdC. dCTP levels were decreased by VP-16 alone as well as by the combination of both compounds, which may favor phosphorylation of dFdC, thereby increasing dFdCTP accumulation. DNA strand break (DSB) formation was increased for exposure to both compounds together compared to exposure to each compound separately, this effect being most pronounced when cells were exposed to VP-16 prior to dFdC (38% and 0% DSB for dFdC and VP-16 alone, respectively and 97% DSB for the combination). The potentiation in DSB formation might be a result of the inhibition of DNA repair by dFdC. Provided the right schedule is used, VP-16 is certainly a compound eligible for combination with dFdC. BIOCHEM PHARMACOL **57**;4:407–415, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. gemcitabine; etoposide; synergism; schedule dependency; DNA damage; DNA strand breaks

dFdC§ is a deoxycytidine analog [1], with clinical activity against several solid tumors, such as ovarian cancer, NSCLC, head and neck cancer, and pancreatic cancer [2]. After entering the cell, dFdC is phosphorylated to its triphosphate (dFdCTP) which can be incorporated into DNA, followed by one more deoxynucleotide after which DNA polymerization stops [3], which probably determines its cytotoxic effect. Besides this effect, the dFdC metabolite dFdCDP is also capable of inhibiting ribonucleotide reductase [4], an enzyme with a key role in DNA-repair mechanisms.

VP-16 is a widely used anticancer drug against small cell and non-small cell lung cancer, ovarian cancer, testicular cancer and leukemias. VP-16 inhibits topoisomerase II and

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§ *Abbreviations:* CDDP, *cis*-diammine-dichloroplatinum, cisplatin; CI, combination index; dCK, deoxycytidine kinase; dFdC, gemcitabine, 2′,2′-difluorodeoxycytidine; DSB, DNA strand break; FA, fraction affected; FBS, fetal bovine serum; LL, Lewis Lung; NSCLC, non-small cell lung cancer; OD, optical density; RT, room temperature; and VP-16, etoposide, 4′-demethylepipodo-phyllotoxin-9-4,6-*O*-ethylidene-β-D-glucopyranoside.

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subsequently causes DSBs [5]. VP-16 appears to be synergistic with many DNA-interacting drugs such as CDDP and the alkylating agents [6], but the mechanism of this interaction has not yet been elucidated. dFdC previously exhibited synergistic interactions with CDDP in ovarian, head and neck and colon cancer cell lines [7], likely due to an inhibition of DNA repair by dFdC. Furthermore, VP-16 was shown to increase the activity of dCK [8], an enzyme in the salvage pathway used for pyrimidine synthesis and important for the phosphorylation of dFdC [9].

The purpose of the present investigation was to study the interaction of dFdC and VP-16 by using different schedules of both compounds. Emphasis was on ovarian cancer cells and NSCLC cells. Furthermore, we determined the effect of VP-16 on the accumulation of dFdCTP, the effect on dCTP concentration, and the extent of DSBs caused by the combination. The synergism between the compounds is possibly due to an increase in DSB formation by both compounds.

MATERIALS AND METHODS

Drugs and Chemicals

dFdC was a kind gift from Eli Lilly Inc. and was solubilized with PBS to a concentration of 10 mM. VP-16 was solubilized with PBS to a concentration of 34 mM. Final dilutions of both drugs were made in culture medium. All other chemicals were of analytical grade and commercially available.

Cell Culture

Four different cell lines, with two major histological subtypes, were used. For human ovarian cancer, A2780 was the parental cell line and ADDP [10] the variant with induced resistance to CDDP. For NSCLC, we used the human H322 cell line (subtype BAC, NCI), and the murine LL tumor cell line (kindly provided by Dr. Lelieveld). A2780 cells were cultured in Dulbecco's medium with 5% heat-inactivated FBS. ADDP cells were cultured in RPMI medium with 5% heat-inactivated FBS. H322 and LL cells were cultured in RPMI medium with 10% heat-inactivated FBS. Gentamicin (225 μL/200 mL medium) was added to the media during culturing and experiments. All cell lines were grown as monolayers.

Growth Inhibition Experiments

Growth inhibition experiments were performed in triplicate in 96-well flat-bottom plates (Costar) essentially as described previously [11]. Cells were seeded in 100 μL of medium containing 5% FBS at different densities; 6000/well for A2780 cells, 12,000/well for ADDP cells, 20,000/well for H322 cells, and 5000/well for LL cells. After 24 hr, 100 μL of drug-containing medium was added and cells were cultured for another 72 hr. After 4 and 24 hr, the cells were washed and cultured in drug-free medium for 68 and 48 hr, respectively. Cells were exposed to dFdC and VP-16 alone or to a combination of both drugs at either a constant or variable molar ratio. The constant ratio was 1:500 (dFdC:VP-16) for all exposure times in the A2780, ADDP, and H322 cells, and 1:4 and 1:11 for 4- and 24-hr exposure in LL cells, respectively (based on the separate IC_{50} values for both drugs). LL cells were not only exposed to the combination simultaneously, but two sequential schedules were also used: (a) a 4-hr incubation with dFdC, followed (after washing the drug away) by a 20-hr incubation with VP-16, and (b) a 4-hr incubation with VP-16, followed by a 20-hr incubation with dFdC, both exposures followed by a 48-hr drug-free period. In the variable ratio method, cells were exposed to VP-16 at a concentration causing about 25% growth inhibition and to dFdC in a concentration range. The VP-16 concentrations in A2780, ADDP, H322, and LL cells for 24-hr exposure were 0.16, 6, 3, and 0.15 μM, respectively, and for 72-hr exposure 0.12, 3, 15, and 0.15 μM, respectively. The dFdC concentration range was 2 pM–0.2 μM in A2780 cells, and 0.2 nM–2 μM in ADDP, H322 and LL cells. Growth inhibitory effects were evaluated with the standard sulforhodamine B protein assay [12]. Growth of the cells was exponential during the whole incubation period. Relative growth was calculated as described previously [11]: $[(OD_{treated}/OD_{zero}) - 1]/[(OD_{control}/OD_{zero}) - 1)] \times 100\%$. The OD was read at 540 nm. The OD_{zero} depicts the cell number at the moment of drug addition, the $OD_{control}$ reflects the cell number of untreated wells, and the $OD_{treated}$ reflects the cell number in treated wells on the day of the assay.

We evaluated possible synergism using the median effect analysis method of Chou and Talalay [13], processed by a computer program developed by Chou and Chou (Biosoft). This program provides one of the few objective computerized evaluation procedures [14]. D_m values (IC_{50} values) are calculated by extrapolation. For the separate drugs, the respective growth inhibition parameters, expressed as FA (e.g., an FA of 0.25 is a growth inhibition of 25%), were introduced. The CI was calculated by the formula: $CI = [(D)_1/(D_x)_1] + [(D)_2/(D_x)_2] + [\alpha(D)_1(D)_2/(D_x)_1(D_x)_2]$. Where $\alpha = 1$ for mutually nonexclusive drugs and $(D)_1$, $(D)_2$, and $(D_1(D)_2$ and $(D_x)_1(D_x)_2$ are the doses resulting in a growth inhibition of x %. These doses are calculated by the formula: $D = D_m[FA/(1 - FA)]^{1/m}$ where D_m is the dose required to produce a 50% growth inhibition and m is the slope of the median effect plot. A CI < 1 indicates synergism, >1 indicates antagonism, and a CI of 1 indicates additivity. Results of the growth inhibition experiments with the variable molar ratio were analyzed both by median effect analysis and by comparison of the measured and expected growth inhibition curves. The expected growth curves were calculated by multiplying the relative growth inhibition caused by the drug given in a concentration range with that of the drug given at the IC_{25} (concentration that causes 25% cell growth inhibition). From this curve, the expected IC_{50}, IC_{75}, and IC_{100} (50, 75 and 100% growth inhibition) were determined. The measured IC values were

divided by the calculated values, with a ratio of 1 indicating no interaction between the drugs (additive effect), a ratio of <1 indicating synergy, and ratio >1 denoting antagonism.

dFdCTP Accumulation

The effect of VP-16 on the accumulation of dFdCTP was studied by exposing 2–4.10^5 cells, in 6-well plates in duplicate, to 1 μM dFdC alone or to 1 μM dFdC combined with 0.5 μM VP-16, either simultaneously for 24 hr or VP-16 for 4 hr prior to 24 hr of dFdC. As a control, nonexposed cells were cultured for the same period. At the end of the incubation, cells were washed in ice-cold PBS, harvested by rapid trypsinization (1 min at RT), and subsequently suspended in ice-cold culture medium with FBS, followed immediately by chilling on ice and cell counting. Nucleotides were extracted and analyzed by HPLC as described previously [7, 9]. Briefly, cells were suspended in 150 μL ice-cold PBS, followed by adding 50 μL of 40% trichloroacetic acid 5% final concentration) to precipitate proteins and nucleic acids, and then chilled on ice for 20 min. Subsequently, the mixture was centrifuged for 10 min at 10,000 *g* at 4°. A 2-fold excess (400μL) of a freshly prepared mixture of trioctylamine and 1,1,2-trichlorotrifluorethane (1:4) was added to neutralize the supernatant. After centrifugation for 1 min at 10,000 *g* at 4°, the cellular extract containing nucleotides (upper aqueous layer) was carefully taken off and stored at −20° until analysis. Separation and quantification of the normal ribonucleotides and of dFdCTP was achieved with a gradient HPLC system (Partisphere SAX anion exchange column) connected to a photodiode array detector, regularly set at 254 and 280 nm as described previously [9]. Peaks were quantitated by a data acquisition program.

dCTP Pool Determination

The assay used for the dCTP measurement was based on the original DNA polymerase assay for dNTP [15], which has been optimized subsequently by the use of 96-well plates [16] and most importantly the use of tailor-made oligonucleotides [17, 18]. The present assay combines several features of the various protocols. The effect of VP-16 and dFdC on the dCTP pools was studied by exposing 75-cm^2 culture flasks containing about 5.10^6 cells in duplicate to 1 μM dFdC or 0.5 μM VP-16 alone, or to 1 μM dFdC combined with 0.5 μM VP-16, either simultaneously for 24 hr or VP-16 for 4 hr prior to 24 hr of dFdC. As a control, nonexposed cells were cultured for the same period. At the end of the incubation, cells were washed in ice-cold PBS, harvested by rapid trypsinization (1 min at RT), and subsequently suspended in ice-cold culture medium with FBS, followed immediately by chilling on ice and cell counting. Nucleotides were extracted with triochloroacetic acid as described for the dFdCTP measurement, dried using a Speedvac drying system at RT, and stored at −20°. A primer–template mix was prepared as described by Sherman and Fyfe [17] by ligating a tailor-made oligo template (T; 5′P-TTTGTTTGTTTGTTTGTTTG-GGCGGTGGAG-GCGG-3′OH) with a 14-mer primer (P; 5′P-CCGCCTC-CACCGCC-3′OH)) at a ratio of 2:1 in buffer (50 mM Tris–HCl, 50 mM NaCl, pH 7) at 95° and slowly cooling it down to RT. The T-P mix was diluted to 12–6 μM and stored at −20° until use. On the day of analysis, dried samples were reconstituted in assay buffer (200 mM HEPES, 20 mM $MgCl_2$, pH 7.3) to a final concentration of 10^7 cells/mL. Standards of 0, 1, 2.5, and 5 pmol dCTP were added to a diethylaminoethyl filter plate (Millipore) in duplicate to prepare a standard curve for intrapolation of samples. Fifteen microliters of the samples was added to the filter plate in duplicate. Demi water was added to all wells up to 30 μL followed by 70 μL of a reaction mix, consisting of 10 μL [8-^{3}H]dATP (25 μM; 1.6 Ci/mmol; 0.4 μCi/μL), 5 μL T-P mix, 5 μL Klenow DNA pol 1, and 50 μL assay buffer. The filter plate was gently vortexed and allowed to incubate at RT for 2 hr. Each well was washed 4 times with washing buffer (0.25 M KH_2PO_4, 0.5 M KCl, pH 4.3) and the wet filters were punched out in LSC vials, using a Multiscreen™ Assay System as described by Van der Wilt *et al.* [19]. Five hundred microliters of 2M NaOH was added to each vial and shaken for 3 hr before 4.5 mL of LSC fluid (Ultima Gold, Packard) was added and the [^{3}H] content of all samples was counted in an LSC counter (Packard, 1900 TR). In order to determine whether dFdCTP would affect incorporation of [^{3}H]dATP into the oligonucleotides, a standard curve of dCTP was made with a range of dFdCTP concentrations (0–150 pmol dFdCTP) overlapping the values reached in the accumulation assay (486, 602, and 663 pmol dFdCTP/10^6 cells are comparable to concentrations of 73, 90, and 99 pmol dFdCTP in a 15 μL sample). This addition caused a significant consistent increase in [^{3}H]dATP incorporation into the template in this dCTP–dFdCTP concentration range of up to 60% at 0.5 pmol (the highest absolute value that was reached for the LL samples in the assay). Therefore, all data had to be corrected, with a standard curve including the amount of dFdCTP that was measured by HPLC as described above.

FADU DNA Damage Assay

The extent of DSB caused by dFdC and dFdC in combination with VP-16 was measured by the fluorometric analysis of DNA unwinding assay as described previously by Birnboim and Jevcak [20] and slightly modified by Bergman *et al.* [7]. This assay is based on the principle that the rate of unwinding of DNA under alkaline conditions depends on the presence of strand breaks; DNA with a high amount of strand breaks will unwind faster under alkaline conditions than DNA with no strand breaks. Double-stranded DNA (dsDNA) can be detected by ethidium bromide staining. One 75-cm^2 culture flask containing about 5.10^6 LL cells was incubated with VP-16 (0.5, 0.25, and 0.1 μM and 100, 80, and 50 nM for 4-hr and 20-hr exposure, respectively) alone or in combination with dFdC (1, 0.625, and 0.25 μM

TABLE 1. Sensitivity of non-small cell lung and ovarian tumor cell lines to gemcitabine and VP-16

	IC_{50} gemcitabine (nM)			IC_{50} VP-16 (μM)		
Cell line	4 hr	24 hr	72 hr	4 hr	24 hr	72 hr
A2780	14.4 ± 2.4	3.4 ± 1.0	1.8 ± 0.5	1.6 ± 0.2	0.27 ± 0.03	0.3 ± 0.1
ADDP	737 ± 280	193 ± 42.6	594 ± 116	15.4 ± 5.5	6.9 ± 2.6	3.2 ± 1.8
LL	800 ± 100	27.3 ± 6.7	12.8 ± 4.4	1.3 ± 0.5	0.3 ± 0.1	0.3 ± 0.1
H322	708 ± 333	420 ± 201	120 ± 54	76.0 ± 16.6	17.6 ± 6.4	25.0 ± 7.1

Sensitivity is expressed as the concentration of drug to achieve 50% growth inhibition (IC_{50}) as determined withe the SRB assay [12]. Values are means ± SEM of three to six separae experiments. Cells were exposed for 4, 24, or 72 hr, folloed by 68, 48 and 0 hr culture in drug-free medium, respectively.

and 40, 25, and 10 nM for 4-hr and 20-hr exposure, respectively) at 37°, either simultaneously or sequentially. A high concentration of VP-16 (50 μM) was used as a positive control drug, and added to the cells 1 hr before harvesting. Untreated cells were used as controls. Cells were briefly trypsinized, washed with ice-cold Dulbecco's modified Eagle's medium minus phenol red, harvested, kept on ice, and directly used in the assay. For this purpose, the cells were suspended in 2 mL of ice-cold 0.25 M mesoinositol, 10 mM NaH_2PO_4, and 1 mM $MgCl_2$ (pH 7.2), and the suspension was equally divided among three sets of tubes in duplicate: T-, B- and P-tubes. All tubes were incubated with 200 μL of buffer C (9 M urea, 10 mM NaOH, 2.5 mM cyclohexane-diammine-tetra-acetate, 0.1% SDS) for 10 min on ice to disrupt the chromatin. T-tubes (total fluorescence: fluorescence due to presence of double-stranded DNA and contaminants) were then treated with 400 μL of buffer F (1 M glucose, 14 mM β-mercaptoethanol) to stabilize DNA so that unwinding could not occur due to alkali. Subsequently, 100 μL of 0.45 volume buffer C in 2 M NaOH and 100 μL of 0.40 volume of buffer C in 2 M NaOH was added to the T-tubes which were incubated for 30 min at 0° to bring the reaction mixture to an alkaline pH. B-tubes (background fluorescence: correction for fluorescence of free dye and all components other than double-stranded DNA) were vortexed vigorously for at least 30 sec so that the double-stranded DNA was sheared. All tubes were incubated for 30 min at 15° so that the DNA would unwind and then put on ice. Buffer F (400 μL) was then added to the P-tubes (estimate of unwinding rate of the DNA by comparison with tubes T and B) and B-tubes. One point five milliliters of 6.7 mg/ml ethidium bromide, 13.2 mM NaOH was added to all tubes and all tubes were vortexed. The fluorescence was measured at excitation wavelength 520 nm, emission 590 nm on a SPEX Fluoromax fluorescence spectrometer. The extent of DSBs was calculated by: $(P\text{-}B)/(T\text{-}B) \times 100\%$.

RESULTS

Analysis of the Interaction between dFdC and VP-16

The IC_{50}s of dFdC and VP-16 alone in the A2780, ADDP, H322, and LL cell lines are summarized in Table 1. Clear differences were observed in the sensitivity to both drugs in these cell lines: at all exposure times, A2780 was the most sensitive cell line to both compounds, followed by LL. However, LL was resistant to dFdC when exposed for only 4 hr. The H322 cell line was very resistant to dFdC (>50-fold compared to A2780) and was the most resistant cell line to VP-16 (>45-fold resistant). ADDP, the CDDP-resistant variant of the A2780 cell line, was cross-resistant to VP-16 (>7.5-fold resistant).

Based on these sensitivity data, combination experiments were designed in which cells were exposed to the approximate IC_{25} of one drug and a concentration range of the other. From the separate growth inhibition data, expected curves could be calculated. Figure 1 shows representative growth inhibition curves for dFdC alone, for the combination of dFdC and VP-16, and the expected growth inhibition curves in the A2780, ADDP, H322, and LL cell lines. The ratios between the measured and expected IC_{50}s and IC_{100}s are given in Table 2. At 24-hr exposure, additivity was found in all cell lines at the IC_{50} concentration of dFdC. However, at 72-hr exposure, synergism was found in the A2780 cell line, while the combination was additive in all other cell lines at the IC_{50} level. Remarkably, the combination of dFdC and VP-16 was synergistic at the IC_{100} in the dFdC- and VP-16–resistant cell line ADDP after 24- and 72-hr exposures.

The interaction between dFdC and VP-16 at a constant ratio was studied with multiple drug effect analysis. Figure 2 shows the mean CI/FA plots of A2780, ADDP, H322, and

TABLE 2. Evaluation of the interaction between dFdC and VP-16 in ovarian and NSCLC cell lines

		Ratio measured/expected	
Cell line	Exposure time (hr)	IC_{50}	IC_{100}
A2780	24	0.6 ± 0.3	0.4 ± 0.3
	72	0.4 ± 0.2*	0.8 ± 0.3
ADDP	24	4.4 ± 4.4	0.4 ± 0.0*
	72	5.2 ± 2.4	0.4 ± 0.1*
H322	24	10 ± 7.9	0.8 ± 0.2
	72	4.4 ± 4.4	0.8 ± 0.2
LL	24	1.1 ± 0.3	1.1 ± 0.2
	72	1.4 ± 0.5	0.9 ± 0.2

Cells were exposed for 24 or 72 hr to VP-16 at the approximate IC_{25} and dFdC in a concentration range. Synergism/antagonism was evaluated by calculating ratios between measured IC_{50} values and expected IC_{50} values. Ratios are means ± SEM (n = 3), calculated for all cell lines at 4-, 24-, and 72-hr drug exposure. A ratio < 1 depicts synergism, a ratio > 1 antagonism, and a ratio = 1 additivity.

* = measured value significantly lower than expected value ($P < 0.05$) as determined by a paired *t*-test.

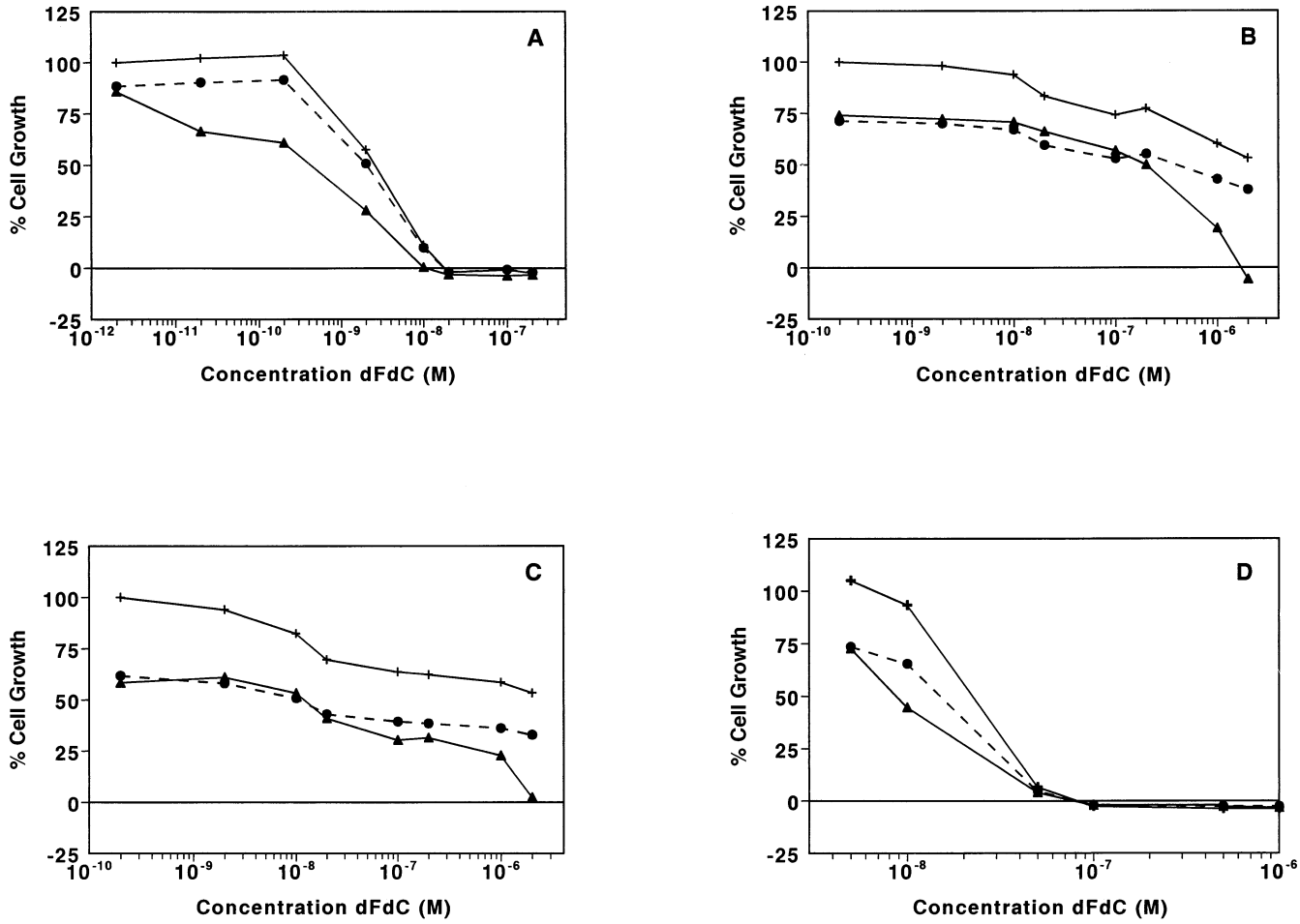

FIG. 1. Representative growth inhibition curves of the A2780 (A), ADDP (B), H322 (C), and LL (D) cell lines. Cells were exposed to dFdC alone (+) or in combination with VP-16 at an IC_{25} concentration (▲) for 72 hr. From the values of dFdC and VP-16 alone, the expected curve was calculated (●). After drug exposure, all cell lines were cultured in fresh medium. Total culture time was 72 hr.

LL cells. In the A2780 and H322 cells, the combination of dFdC and VP-16 was antagonistic (CI > 1) at 24-hr exposure. The CI's varied over a range of FAs studied; at an FA of 0.2, a high CI (>3) was found, but lower values were observed at an FA of 0.8 (±1). The combination was additive after 72-hr exposure in these cell lines. In the ADDP cell line, the combination was additive over the whole range of FAs (CI = 1), at 24- as well as 72-hr exposures. In the LL cell line, however, synergism was observed (CI < 1) after 4- and 24-hr exposures.

The schedule dependency of the interaction between dFdC and VP-16 was further studied in the LL cell line (Fig. 3). The simultaneous combination of the compounds was synergistic after 24-hr exposure, whereas when cells were exposed to VP-16 before dFdC, an antagonistic effect was observed. However, when cells were exposed to dFdC prior to VP-16, a higher level of synergism than in the simultaneous combination was seen.

Effects of VP-16 on dFdCTP Accumulation

To determine a possible role for dFdCTP in the interaction between dFdC and VP-16 in LL cells, we measured the accumulation of dFdCTP after 24-hr exposure to 1 μM dFdC alone or in combination with 0.5 μM VP-16. Cells were treated with the drugs either simultaneously for 24 hr or sequentially with VP-16 for 4 hr prior to 24 hr of dFdC (Fig. 4). VP-16 increased dFdCTP accumulation 1.2-fold compared to dFdC alone, but the sequential exposure increased dFdCTP accumulation 1.4-fold.

Effects of VP-16 and dFdC on dCTP Levels

In an attempt to account for the slight increase in dFdCTP accumulation by VP-16 in LL cells, we measured dCTP levels after exposure to the drugs. dCTP can affect dFdCTP formation since it is a feedback regulator of dCK and will compete with dFdC phosphorylation. Cells were treated with the drugs either simultaneously for 24 hr or sequentially with VP-16 for 4 hr prior to 24 hr of dFdC (Fig. 5). Exposure to dFdC alone for 24 hr did not decrease dCTP levels in LL cells. However, 24-hr exposure to VP-16 alone decreased dCTP 3-fold compared to control levels, this effect being time-dependent since it was not yet observable

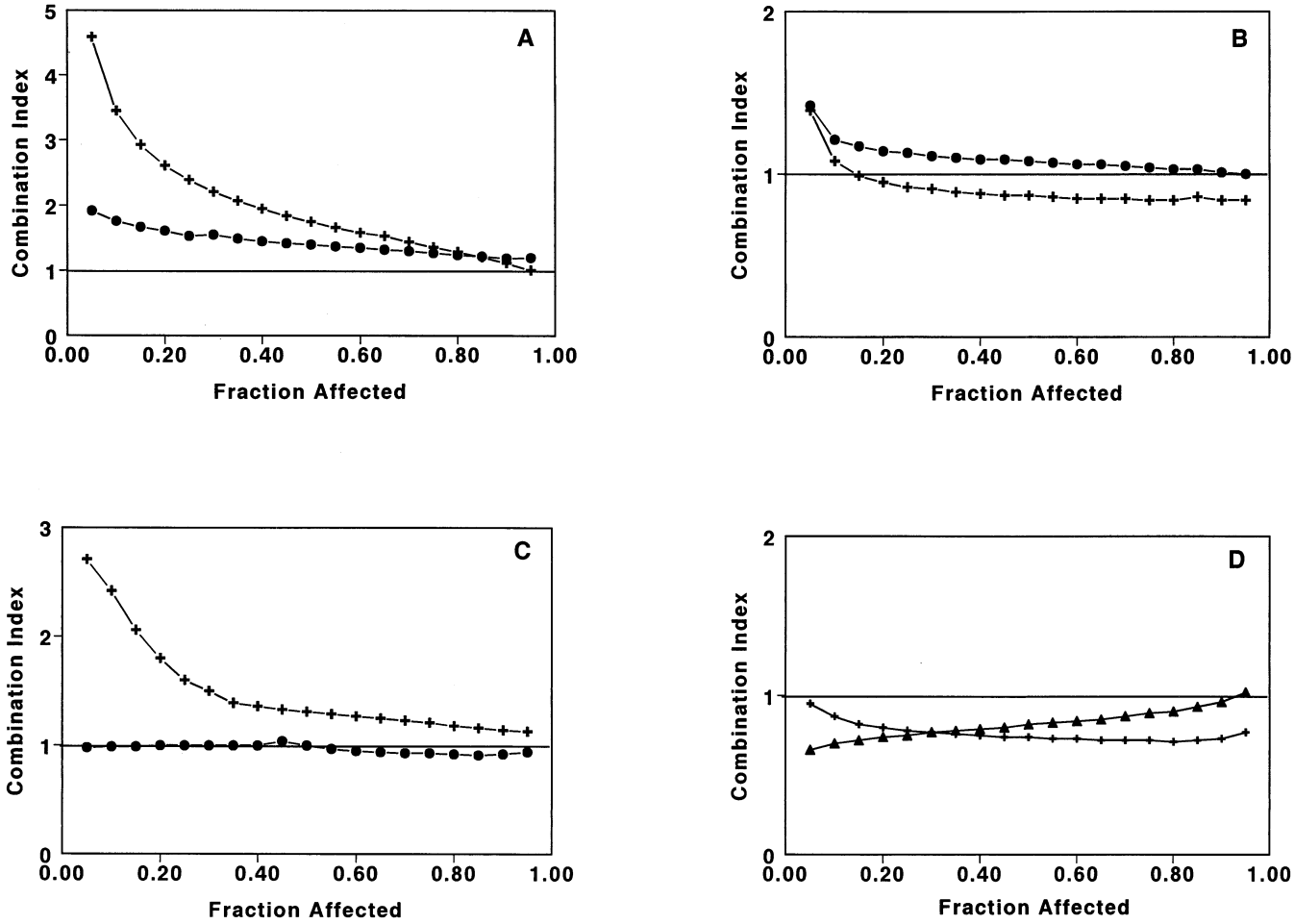

FIG. 2. Synergy analysis of the interaction between dFdC and VP-16 in the A2780 (A), ADDP (B), H322 (C), and LL (D) cell lines. Cells were exposed to dFdC and VP-16 simultaneously for 4 (only LL, ▲), 24 (+) and 72 (except for LL, ●) hr. The values of the CI's are: CI > 1, antagonism; CI = 1, additivity; CI < 1, synergism. Values are means of three to five independent experiments, except for the 72-hr exposure of LL (n = 1). SEM of FA 0.5 for A2780: 0.55 and 0.46; ADDP: 0.32 and 0.24; H322: 0.29 and 0.31 for 24 and 72 hr, respectively. For LL: 0.43 and 0.33 for 4 and 24 hr, respectively.

after 4-hr exposure. Simultaneous and sequential combination of the drugs tended to decrease dCTP pools, but not more than expected from both compounds alone.

DSB Formation

The extent of DSB formation after exposure to either VP-16 or dFdC alone or to a combination of the compounds was measured in LL cells to determine the possible contribution of this type of DNA damage to the interaction between the compounds (Fig. 6). Both dFdC and VP-16 formed DSB; this effect was time-dependent, more DSBs being formed after 20-hr incubation compared to 4-hr incubation. Treatment of cells with the compounds simultaneously for 4 hr did not increase DSB formation. Exposure to dFdC before VP-16 led to an increased DSB formation, although cells treated with VP-16 before dFdC formed a higher amount of DSB than could be expected from the DSB formed by the compounds alone.

DISCUSSION

In this study, we have shown that the combination of dFdC and VP-16 can be synergistic depending on exposure duration, ratio between drugs, and the sequence of drug addition.

Using median drug effect analysis, additivity and synergism between dFdC and VP-16 was found in a panel of four different cancer cell lines, depending on the level and duration of drug exposure. It was remarkable that in the most resistant cell lines, a better effect was observed at the IC_{100} concentration of dFdC, indicating that the combination can exert significant antitumor activity by killing cells. In LL cells, the interaction between the compounds was clearly time- and schedule-dependent, with the most pronounced synergism occurring when dFdC preceded VP-16 by 4 hr.

Mechanistic studies in the LL cell line concentrated on dFdCTP accumulation and DNA damage, which are important parameters of the action of dFdC and VP-16,

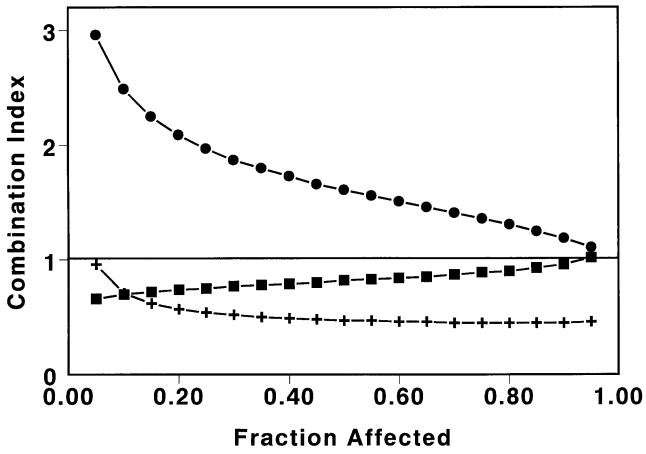

FIG. 3. Synergy analysis of the schedule-dependent interaction between dFdC and VP-16 in the LL cell line. Cells were exposed to dFdC and VP-16 either simultaneously for 24 hr (■) or sequentially: dFdC 4 hr before 20-hr VP-16 (+) or VP-16 4 hr before 20 hr of dFdC (●). The values of the CI's are: CI > 1, antagonism; CI = 1, additivity; CI < 1, synergism. Values are means of three to five independent experiments. SEM of FA 0.5: 0.33, 0.12, and 0.51 for 24-hr simultaneous exposure, dFdC before VP-16, and VP-16 before dFdC, respectively.

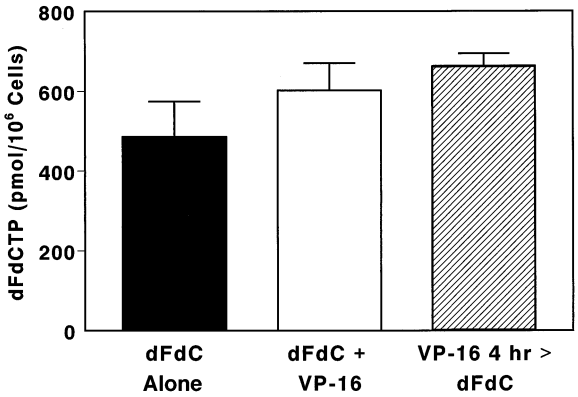

FIG. 4. Effect of VP-16 on the accumulation of dFdCTP. LL cells were exposed for 24 hr to either 1 μM dFdC alone (black bar), simultaneously with 0.5 μM VP-16 (white bar), or to 0.5 μM VP-16 4 hr prior to 24 hr of dFdC (hatched bar). Values are means ± SEM of three experiments (P value = 0.06 for both combinations compared to dFdC alone).

respectively. Co- or pre-incubation with VP-16 tended to increase dFdCTP accumulation. This might have been due to increased dCK activity by VP-16, as was previously shown in leukemic cells [8]. This increased dCK was also found for several other drugs that inhibit DNA synthesis such as aphidicolin and hydroxyurea [21]. The increase in dFdCTP accumulation is in contrast to the results we previously obtained with the combination of dFdC and CDDP [7, 22] with CDDP not increasing dFdCTP accumulation.

dFdCDP is known to inhibit ribonucleotide reductase, which can lead to a depletion of dCTP pools [23, 24]. However, dFdC did not decrease dCTP pools in the LL cell line. This might be related to the 3.3-fold increase in CTP concentration by dFdC as measured on HPLC (results not shown), since dCTP concentrations are also regulated by the concentration of its precursors. VP-16 decreased dCTP time dependently. Since a decrease in dCTP favors the phosphorylation of dFdC to dFdCTP by dCK, this might be one of the mechanisms contributing to the increase in dFdCTP accumulation in the LL cell line.

To study the possible interaction of the compounds with respect to DNA damage, we determined the effect of both drugs on DNA integrity. DNA damage tended to increase when cells were treated with dFdC and VP-16 simultaneously, compared to DSB formation of the compounds alone. The most pronounced effect was seen after sequential exposure to VP-16 before dFdC. This phenomenon might be explained by a decreased repair by dFdC of DNA damage induced by VP-16 [3, 4]. The increase in dFdCTP accumulation was higher when VP-16 preceeded dFdC than when cells were exposed to the drugs simultaneously; therefore, this might also contribute to the fact that the DNA damage was higher with this particular combination. The higher DNA damage could also be a result of a potentiation of dFdCTP incorporation into DNA by the

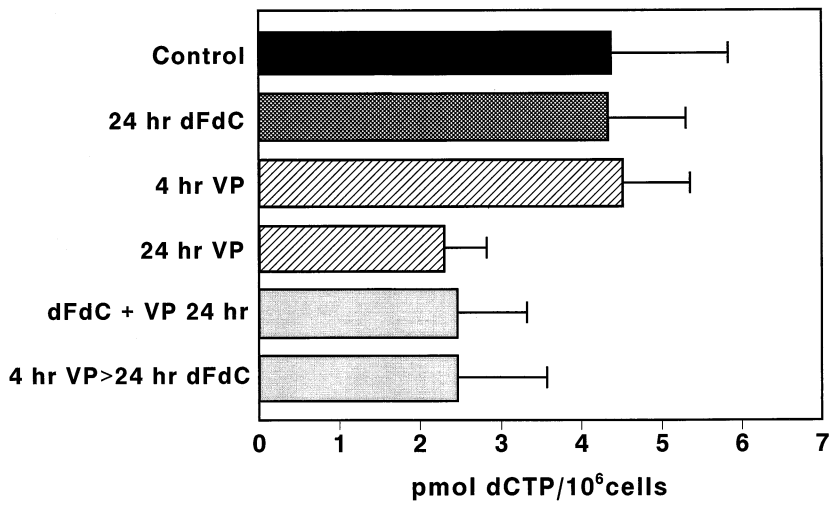

FIG. 5. Effect of 1 μM dFdC and 0.5 μM VP-16 (VP) alone, simultaneously, or as a sequential combination on dCTP concentrations in LL cells (P value = 0.08 for 24 hr VP-16 alone compared to control). dCTP values were corrected for interference of dFdCTP in the DNA polymerase assay.

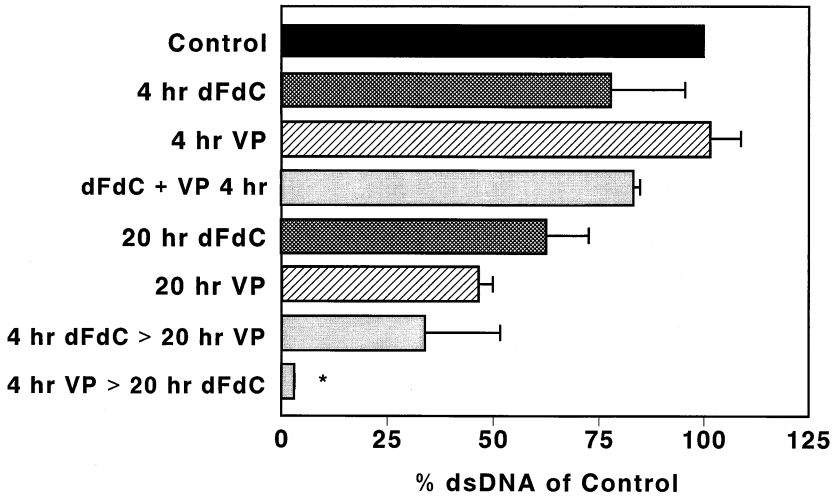

FIG. 6. Effect of the combination of dFdC and VP-16 (VP) on DSB formation in LL cells. Values (in % of double-stranded DNA of untreated cells) are means ± SEM of 3 separate experiments. Concentrations used: dFdC: 4 hr, 1000 nM; 20 hr, 40 nM; VP-16: 4 hr, 0.5 μM; 20 hr, 0.1 μM. The actual level of double-stranded DNA in untreated LL cells at the end of the unwinding time was 76.8 ± 10.6%, this value being subsequently set at 100% per experiment to calculate the relative double-stranded DNA values. *Significantly different at a level of $P = 0.001$; measured DSB to expected DSB (DSB of both drugs added together). Other P values: 0.12 for dFdC 4 hr before VP-16 and 0.06 for simultaneous exposure compared to the expected DSB.

interaction of VP-16 with topoisomerase II, whereby the enzyme's ability to religate cleaved DNA is inhibited [25]. However, the fact that DNA damage was most pronounced when VP-16 preceeded dFdC is in contrast with the antagonism this combination caused in LL cells. With the reverse synergistic combination, i.e., dFdC preceeding VP-16, DNA damage was only additive, indicating that a yet unknown mechanism and not DNA damage alone might be important for the synergistic cell killing.

The mechanism of interaction between gemcitabine and VP-16 seems to be different and less pronounced than that between gemcitabine and CDDP [7, 22]; however, both combinations show schedule dependency. In the LL cell line, the potentiation in DSB formation seems to be related to the inhibition of DNA repair by gemcitabine, although this does not appear to be the only mechanism. Further research is warranted. Provided the right schedule is used, eventually in combination with CDDP as well, VP-16 is a compound eligible for combination with gemcitabine, which may be of particular interest in lung cancer.

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