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Sequence-dependent growth-inhibitory effects of the in vitro combination of fluorouracil, cisplatin, and dipyridamole

M. Barberi-Heyob, G. Griffon, J. L. Merlin, B. Weber

Centre Alexis Vautrin, Avenue de Bourgogne, F-54511 Vandœuvre-Les-Nancy Cedex, France

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Abstract. The present study was designed to analyze the growth-inhibitory effects of the combination of fluorouracil (FUra), cisplatin (CDDP), and dipyridamole (DP). These toxic effects were assessed on the human breast-carcinoma cell line MCF-7 using the MTT (tetrazolium bromide) assay in 96-well culture dishes. Data were analyzed using the median-effect principle. The drug combinations tested included FUra concentrations ranging from 0.8 to 800 nmol/l, CDDP concentrations of 0.3–30 μmol/l, and DP concentrations of 2–200 μmol/l. A total of 189 different experimental conditions were tested, including different sequences of administration, with being DP applied before, simultaneously with, or after the two antitumor drugs. Synergistic cytotoxic interactions were found between FUra and CDDP, FUra and DP, and CDDP and DP as well as when the three drugs were combined. The sequence of exposure did not influence the growth-inhibitory activity of the combination FUra-CDDP but altered the effect of combinations of either FUra or CDDP with DP, since at lower concentrations the effect shifted from synergism to antagonism when DP was added simultaneously with CDDP and after the two antitumor drugs. However, the interaction was shown to be truly synergistic by median-effect analysis when the two antitumor drugs were simultaneously associated, with no change in the synergistic effect being observed for the three DP administration sequences.

Introduction

A variety of new therapeutic strategies continues to evolve from in vitro work attempting to manipulate pharmacologically the biochemical homeostasis of cancer cells. Among these novel strategies, two would appear promising: first, the use of particular drug combinations identified as being capable of developing in vitro synergistic efficacy, and second, the exploitation of a new class of drugs known as modulators. We report the results of an in vitro analysis that exploits the second of these chemotherapeutic strategies simultaneously: the use of a modulator (dipyridamole) to enhance the efficiency of the chemotherapeutic agents fluorouracil (FUra) and cisplatin (CDDP).

Dipyridamole (DP) is well known as an antithrombotic agent and is reported to be a potent inhibitor of facilitated transmembrane transport systems for purines and pyrimidines [9, 19, 26]. In addition, DP has been observed in vitro to be a modulator of a variety of cytotoxic agents, including antimetabolites and topoisomerase II-directed agents, and it has also been reported to reduce the cytotoxicity of cytarabine in normal and malignant myeloid cells [15] by altering the cellular uptake and retention of antimetabolite drugs. Other agents have been reported to be potentiated by DP, including mitoxantrone [7], etoposide [10], doxorubicin [11], and vinblastine [11]. Furthermore, DP has been reported to be an inducer of class I interferons [8] and to potentiate the inhibition of replication of human immunodeficiency virus in human monocyte-macrophages, suggesting that this drug may become important in antiviral therapy.

The evaluation of the ternary combination (FUra, CDDP, and DP) arises as a logical therapeutic strategy for several reasons. First, in HT-29 and MCF-7 (respectively, human colon and breast cancer) cell lines, we have demonstrated that inactive, low FUra concentrations can induce toxic effects in a dose-dependent manner when combined with DP [3]. Moreover, we recently reported that the in vivo toxicity of FUra was enhanced by DP in $B_6D_2F_1$ mice bearing P388 ascitic tumors and receiving a 4-day schedule of FUra following a 1-h exposure to DP. These data al-

Abbreviations: FUra, fluorouracil; CDDP, cisplatin, *cis*-diamminedichloroplatinum(II); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (azolyl blue); IC_{50}, concentration inducing 50% inhibition of cell growth; fa, fraction affected; fu, fraction unaffected; CI, combination index

Correspondence to: M. Barberi-Heyob

lowed a prospective evaluation of 4-day treatment with i.p. FUra and DP, revealing an increase in life span without toxic death [2]. Keane et al. [14] also recently reported that the in vivo cytotoxicity of CDDP against human tumor xenografts could be increased by DP.

The objectives of the present study were to analyze the interactions of FUra + CDDP, FUra + DP, CDDP + DP, and (FUra + CDDP) + DP in MCF-7 human breast-carcinoma cells using the multiple drug-effect analysis of Chou and Talalay [5, 6] and to compare three sequences of administration so as to obtain maximal synergistic growth inhibition [16].

Materials and methods

Drugs. CDDP (Cisplatyl) was obtained from Laboratoires Roger Bellon, France; FUra, from Produits Roche, Neuilly, France; and DP, from Laboratoires Boehringer, Ingelheim, France. All drugs were stored at –20° C and constituted the stock solutions. Dulbecco's modified Eagle's medium (DMEM), L-glutamine, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Seromed (Polylabo, Strasbourg, France). The MTT test was performed using 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sodium dodecyl sulfate (SDS) obtained from Sigma (La Verpillère, France).

Cell cultures. MCF-7 human breast-adenocarcinoma cells were maintained in DMEM supplemented with 10% FBS, penicillin (10,000 IU l^{-1}), streptomycin (86 mol/l) and L-glutamine (2 µmol/l) at 37° C in an atmosphere containing 5% CO_2. The cultures were proved to be mycoplasma-free by the European Organization for Research and Treatment of Cancer (EORTC) Clonogenic Assay Screening Study Group test procedure (Dr. L. Suardet, Institut Suisse de Recherches Expérimentales sur le Cancer, Lausanne, Switzerland). Exponentially growing cells were harvested by enzymatic disaggregation (trypsin-ethylenediaminetetraacetic acid) and assayed for cytotoxicity in 96-well dishes (2×10^4 cells/well).

The drug concentrations ranged between 0.8 and 800 nmol/l for FUra, between 0.3 and 30 µmol/l for CDDP, and between 0.2 and 200 µmol/l for DP. The duration of cell exposure to FUra, CDDP, and DP was 72 h; it was compatible with the cell-doubling time, as under the conditions described above the doubling time for the MCF-7 cell line was 36 h.

Evaluation of growth inhibition. The cytotoxicity of the drugs tested alone or in combination were assessed after 3 days using the MTT assay. This assay was carried out according to a procedure based on the assay of Tada et al. [24]. MTT was dissolved in phosphate-buffered saline (PBS), filter-sterilized, and stored at 4° C. After 72 h, 50 µl of a 0.5% MTT solution was added to each well and incubated for 3 h at 37° C to allow MTT metabolism. The crystals formed were dissolved within 15–30 min by the addition of 100 µl of a 25% SDS solution to each well and were vigorously pipetted to ensure the homogeneity of the solution prior to scanning. Absorbance was measured at 540 nm on an MCC/340 Titertek Multiskan (Flow, Les Ulis, France). Results were expressed as the relative percentage of absorbance as compared with untreated controls.

Before performing growth-inhibition assays, we examined the linearity of the MTT assay with increasing numbers of plated cells (between 10^4 and 10^6 cells/well) and obtained quite satisfactory results (r<0.995). Blank measurements were performed on wells containing no cell and no drug.

Each experimental point was determined as the mean value derived from eight wells. IC_{50} was defined as the concentration inhibiting 50% of the cell growth as compared with that in untreated controls. IC_{50} values were calculated by logarithmic analysis and were divided by the corresponding value obtained in the absence of drug to give an estimate of the cytotoxicity-potentiating effect.

Drug combinations and data analyses. In this study, cells were exposed to FUra, CDDP, and DP either alone or in combination for 72 h as follows: treatment 1, FUra + CDDP; treatment 2, FUra + DP; treatment 3, CDDP + DP; and treatment 4, (FUra + CDDP) + DP. Using these four combinations, we looked for sequence-dependent cytotoxic effects. Thereby, in treatment 1, CDDP was added either before (+1 h), during, or after (–1 h) exposure to FUra, corresponding to assays 1 A, 1 B, and 1 C, respectively. In treatment 2, DP was added either before (+1 h), during, or after (–1 h) exposure to FUra, corresponding to assays 2 A, 2 B, and 2 C, respectively. In treatment 3, DP was also added either before (+1 h), during, or after (–1 h) exposure to CDDP, corresponding to assays 3 A, 3 B, and 3 C, respectively. In treatment 4, DP was added either before (+1 h), during, or after (–1 h) exposure to FUra-CDDP, which were simultaneously tested, corresponding to assays 4 A, 4 B, and 4 C, respectively.

The surviving cell fraction was determined using the MTT assay as described above. The synergy of activity was analyzed according to the median-effect principle described by Chou and Talalay [5, 6]. This principle is described by the equation $fa/fu = (D/Dm)^m$, where *fa* is the fraction of MCF-7 cells affected by the dose *D*, *fu* is the unaffected fraction, *Dm* is the dose required for a 50% effect (e.g., 50% inhibition of cell growth at 72 h as compared with the control), and *m* is a Hill-type coefficient signifying the sigmoidicity of the dose-effect curve. A plot of y = log (fa/fu) versus x = log (dose), i.e., a median-effect plot, linearizes the dose-effect relationship with the slope m and the x-intercept at log (Dm). These values for FUra, CDDP, and DP were calculated for each experiment and for each drug combination at a fixed concentration ratio.

Interaction of the effects of the two drugs FUra and CDDP was quantitatively determined by the combination index (CI) according to the equation:

$$CI = (D_{FUra}/D_{FUrax}) + (D_{CDDP}/D_{CDDPx}) + (D_{FUrax} \times D_{FUra}/D_{CDDPx} \times D_{CDDP}).$$

The same equation was used to calculate the interactions of the effects of FUra and DP as well as CDDP and DP. D drug × (D_{FUrax}, D_{CDDPx}, or D_{DPx}) are the doses of each drug required to produce an effect of x% when the drugs are used alone, and D_{FUra}, D_{CDDP}, or D_{DP} are the cytotoxic contributions of FUra, CDDP, and DP, respectively, in the mixture as calculated from the known dose ratio of the three groups of drugs that also yield an effect of x% in combination.

The dose ratios were chosen such that we could conveniently explore a range of cytotoxicities and such that the different concentrations used would be lower than the IC_{50} value for each compound. Finally, a combination index (CI) versus fraction affected (fa-CI) plot was generated for cell fa values between 0.1 and 0.9. Thus, when CI<1, synergism was indicated; when CI = 1, an additive effect was indicated; and when CI>1, antagonism was indicated. The CI isobologram method of Chou and Talalay [5, 6] for quantitation of synergism and antagonism was also applied to three-drug combination studies in an equipotency combination ratio [16]. A computer program based on the above-mentioned equations was used in the present study for automated analysis of the dose-effect data [3].

Results

For all the experiments, the coefficients of variation ranged between 3% and 16%.

Dose-response curve of FUra

Figure 1A shows the results of the assay when the cells were exposed to FUra alone. The IC_{50} value for a 72-h exposure was 16 µmol/l.

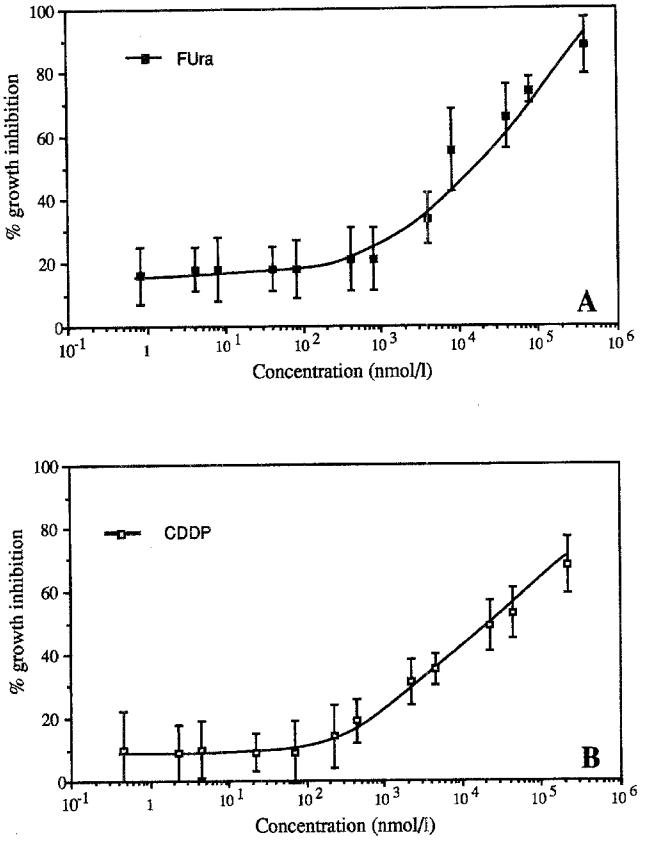

Fig. 1 A, B. Dose response plots for MCF-7 cells following treatment with **A** FUra and **B** CDDP alone. Exponentially growing cells were seeded in 15% serum-supplemented RPMI medium at 2×10^4 cells/well. After 72 h, cell survival was determined by the MTT assay, with absorbance being measured at 540 nm with an automated microplate reader, and expressed as the percentage of survival as compared with untreated controls. Points represent the mean values triplicate determinations (SE, <11% and 9% for FUra and CDDP, respectively) obtained in at least 3 independent experiments

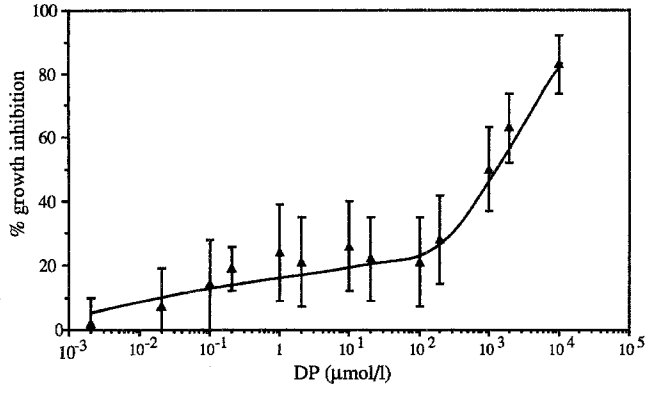

Fig. 2. Dose-response plot for MCF-7 cells following treatment with DP alone. Points represent the mean values of triplicate determinations (SE, <16%) obtained in at least 3 independent experiments

Dose-response curve of CDDP

Figure 1B shows the results of the assay when the cells were exposed to CDDP alone. The IC_{50} value for a 72-h exposure was 15 μmol/l.

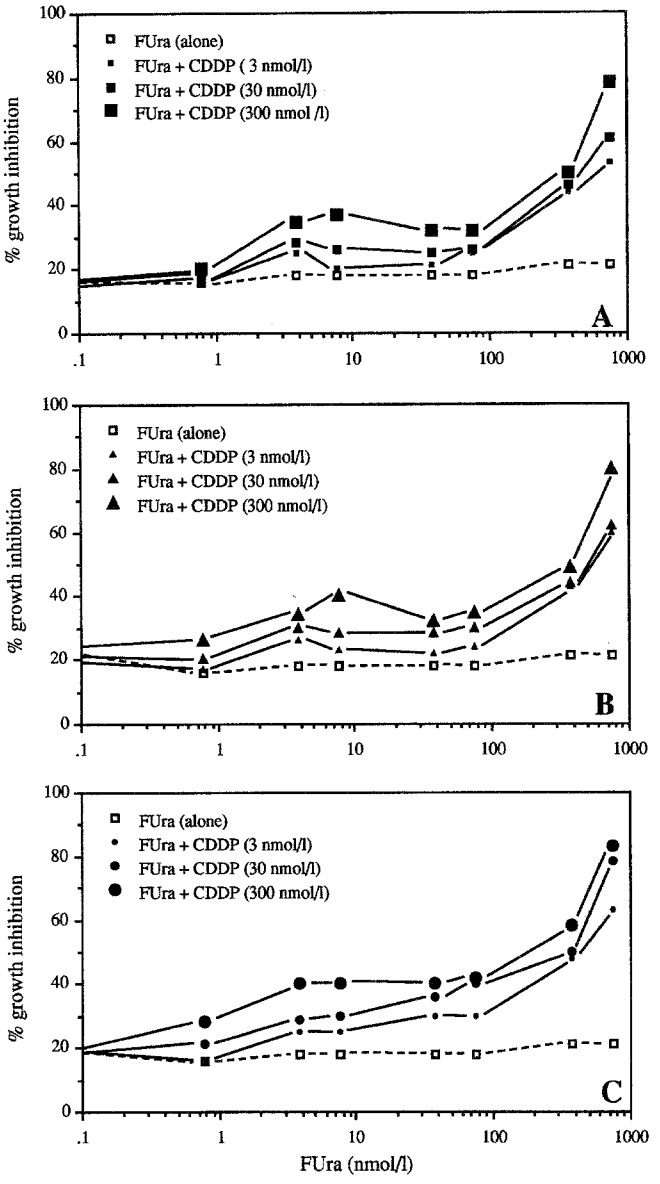

Fig. 3 A – C. Dose-response plots for MCF-7 cells exposed to combinations of FUra with nontoxic concentrations of CDDP (3, 30, or 300 nmol/l). CDDP was added either **A** 1 h before, **B** during, or **C** 1 h after exposure to FUra. Points represent the mean values of sextuplicate determinations (SE, <10%) obtained in 3 independent experiments

Dose-response curve of DP

Figure 2 shows the results of the assay when the cells were exposed to DP alone. The IC_{50} value for a 72-h exposure was 1000 μmol/l.

Potentiation of FUra growth inhibition by CDDP

The drug combinations tested included FUra concentrations of 0.8–800 nmol/l and CDDP concentrations of 3, 30, and 300 nmol/l. Figure 3 shows the dose-response curves for MCF-7 cells exposed to FUra in the presence or absence of nontoxic doses of CDDP (<IC_{50}). A total of 45 different experimental conditions were used. Cells were exposed to CDDP prior to treatment with FUra (Fig. 3A),

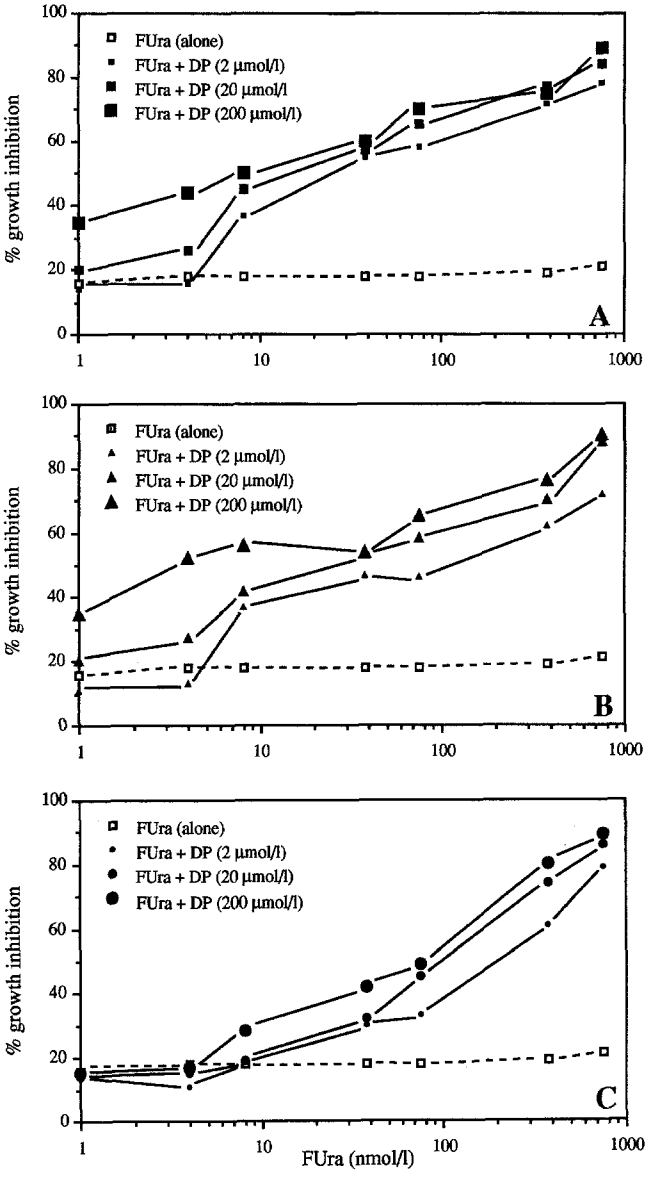

Fig. 4 A – C. Dose-response plots for MCF-7 cells exposed to combinations of FUra with nontoxic concentrations of DP (2, 20, or 200 µmol/l). DP was added either **A** 1 h before, **B** during, or **C** 1 h after exposure to FUra. Points represent the mean values of triplicate determinations (SE, <10%) obtained in 3 independent experiments

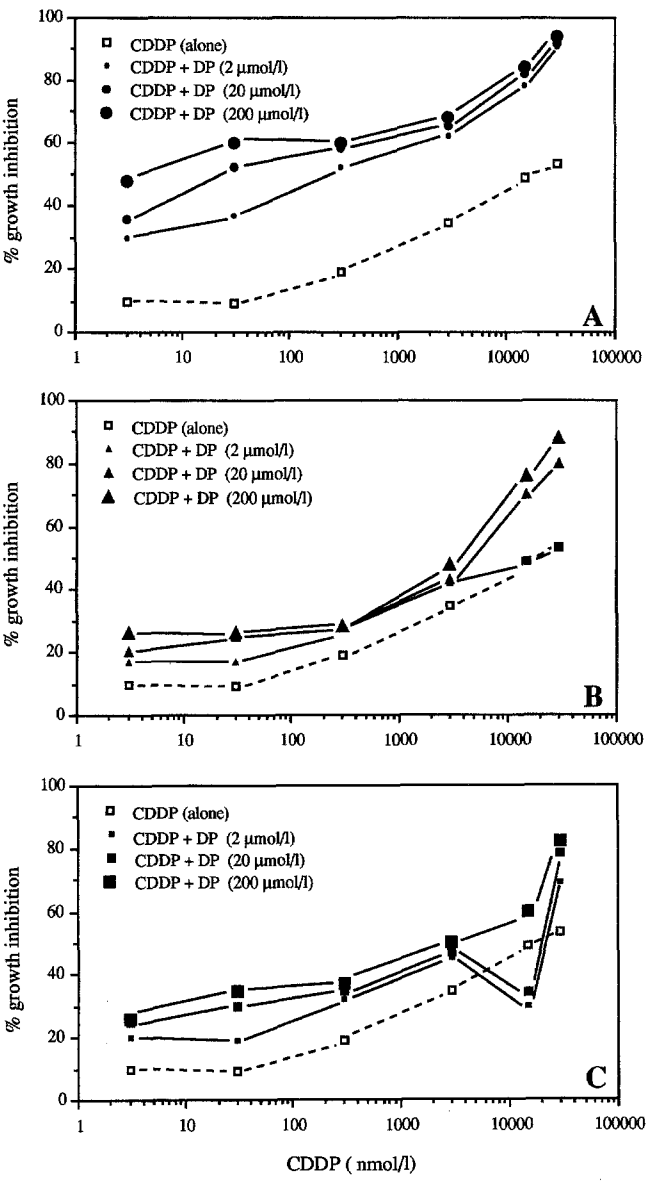

Fig. 5 A – C. Dose-response plots for MCF-7 cells exposed to combinations of CDDP with nontoxic concentrations of DP (2, 20, or 200 µmol/l). DP was added either **A** 1 h before, **B** during, or **C** 1 h after exposure to CDDP. Points represent the mean values of triplicate determinations (SE, <14%) obtained in 3 independent experiments

simultaneously with FUra (Fig. 3B), or after FUra treatment (Fig. 3C). The IC_{50} ratio for FUra in the presence or absence of 300 nmol/l CDDP averaged about 40, indicating a 40-fold enhancement of FUra cytotoxicity for the 3 sequences of administration.

Potentiation of FUra growth inhibition by DP

The effect of FUra at varying concentrations in the acceptably nontoxic range of 0.8–800 nmol/l was assessed with or without DP at concentrations of 2, 20, and 200 µmol/l. In this experiment (Fig. 4), the IC_{50} ratio for FUra in the presence or absence of 200 µmol/l DP was 2000, 400, and 200 for treatments A, B, and C, respectively. Thus, more intense effects were observed when cells were exposed to DP at 200 µmol/l at 1 h before FUra treatment (A), resulting in a very high ratio of 2000.

Potentiation of CDDP growth inhibition by DP

The effect of CDDP at varying concentrations in the nontoxic range of 0.3–30 µmol/l was assessed with or without DP at concentrations of 2, 20, and 200 µmol/l (Fig. 5). The IC_{50} ratio for CDDP in the presence or absence of 200 µmol/l DP averaged about 3750, 3.8, and 5 for treatment A, B, and C, respectively, indicating a 3750-fold enhancement of CDDP cytotoxicity when DP was applied 1 h prior to CDDP treatment.

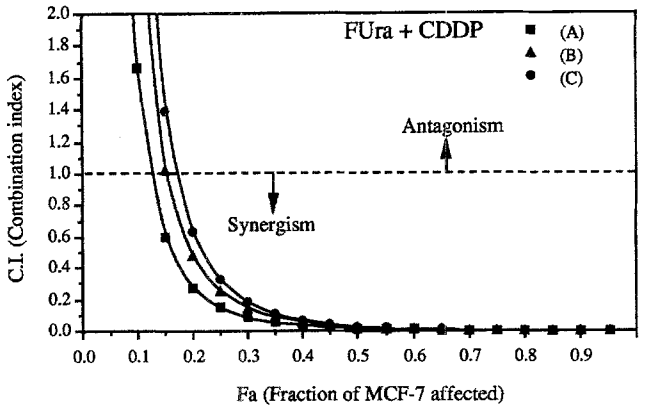

Fig. 6. Combined effect of a 72-h exposure of MCF-7 cells to FUra and CDDP, whereby CDDP was added (*A*) 1 h before, (*B*) simultaneously with, or (*C*) 1 h after FUra treatment. Computer-generated curves describe the combined effects of FUra : CDDP at fixed ratios of 1 : 0.375. Results are plotted as a function of the fraction of treated cells affected versus the combination index (fa-CI plot) under a mutually nonexclusive assumption. All points lying above a CI value of 1 are antagonistic and those lying on a CI value of 1 are additive. Interactions of FUra + CDDP were strongly synergistic (plot *below the horizontal dotted line*) over nearly the entire range of concentrations tested

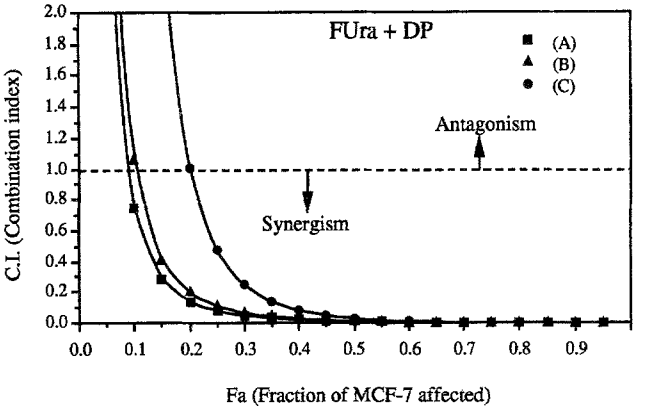

Fig. 7. Combined effect of a 72-h exposure of MCF-7 cells to FUra plus DP, whereby DP was added (*A*) 1 h before, (*B*) simultaneously with, or (*C*) 1 h after FUra treatment. Computer-generated curves describe the combined effects of FUra : DP at fixed ratios of 1 : 250. Results are plotted as a function of the fraction of treated cells affected versus the combination index (fa-CI plot) under a mutually nonexclusive assumption. All points lying above a CI value of 1 are antagonistic and those lying on a CI value of 1 are additive. Analysis of the data for FUra and DP combined at this ratio suggested that the two drugs acted synergistically over nearly the entire range of concentrations tested but were antagonistic at lower concentrations

Synergy between FUra and CDDP

To determine whether the interaction between FUra and CDDP was truly synergistic, MCF-7 cells were exposed to FUra and CDDP either alone or combined over a wide range of concentrations but at a fixed dose ratio for the three administration cases (A, B, and C). In this experiment (FUra : CDDP concentration ratio, 1 : 0.375), for the A, B, and C tests, respectively, the median-effect doses (Dm) were 258, 226, and 130; the correlation coefficients (r) were 0.883, 0.851, and 0.882; and the slopes (m) were 4.296 ± 1.317, 4.065 ± 0.447, and 4.092 ± 1.226.

The analysis of the data obtained with FUra and CDDP combined at a ratio of 1 : 0.375 suggested that the two drugs acted synergistically over nearly the entire range of concentrations tested but were antagonistic at lower concentrations (fa, $\leq .015$ for treatments A and B; <0.2 for treatment C). Figure 6 shows the results of the interaction of the effects of the two drugs FUra and CDDP as determined by CI analysis.

Synergy between FUra and DP

To determine whether the interaction between FUra and DP was really synergistic, MCF-7 cells were exposed to FUra and DP either alone or in combination over a wide range of doses but at a fixed dose ratio for the three administration cases (A, B, and C). In the experiments (FUra : DP concentration ratio, 1 : 250), for the A, B, and C tests, respectively, the median-effect doses (Dm) were 24.9, 34.8 and 81.3 nmol/l; the correlation coefficients (r) were 0.982, 0.931, and 0.977; and the slopes (m) were 4.258 ± 0.4704, 4.285 ± 0.973, and 6.105 ± 0.774. Figure 7 illustrates the cytotoxic effects observed when DP was added 1 h before (A), simultaneously with (B), or 1 h after FUra treatment (C). The DP and FUra concentrations and the sequence of administration (DP added after FUra) were determinant factors that changed the effects of a combination from antagonism (at low DP concentrations of ≤ 20 μmol/l) into synergism (at high DP concentrations of >100 μmol/l). Analysis of the data obtained for FUra and DP combined at a ratio of 1 : 250 suggested that the two drugs acted synergistically over nearly the entire range of concentrations tested but were antagonistic at lower concentrations (fa, ≤ 0.05 for treatment A; ≤ 0.1 for treatment B; <0.2 for treatment C). The best results were obtained with treatment A, when DP was applied 1 h before FUra. Figure 7 details the results of the interaction of the effects of the two drugs FUra and DP as determined by CI analysis.

Synergy between CDDP and DP

In this experiment (CDDP : DP concentration ratio, 1 : 6.6), for the A, B, and C tests, respectively, the median-effect doses (Dm) for CDDP were 19.6, 1663.4, and 4318.6 nmol/l; the correlation coefficients (r) were 0.993, 0.946, and 0.780; and the slopes (m) were 1.261 ± 0.280, 1.429 ± 0.283, and 0.916 ± 0.424. Analysis of the efficacy of CDDP in inhibiting the growth of MCF-7 cells were carried out in a way similar to those described above. The analysis of the data obtained for CDDP and DP combined at a ratio of 1 : 6.6 suggested that the two drugs acted synergistically over nearly the entire range of concentrations tested but were antagonistic at lower concentrations (Fig. 8; fa, <0.1 for treatment A; <0.3 for treatment B; <0.2 for treatment C). As in treatment 2 (FUra + DP), the best results were obtained with combination A, when DP was added 1 h before CDDP. The DP and CDDP concentrations and the sequence of administration (DP given prior to

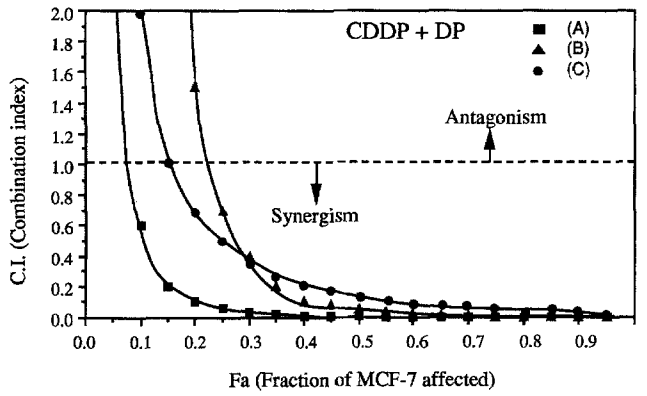

Fig. 8. Combined effect of a 72-h exposure of MCF-7 cells to CDDP plus DP, whereby DP was added (*A*) 1 h before, (*B*) simultaneously with, or (*C*) 1 h after CDDP treatment. Computer-generated curves describe the combined effects of CDDP : DP at fixed ratios of 1 : 6.6. Results are plotted as a function of the fraction of treated cells affected versus the combination index (fa-CI plot) under a mutually nonexclusive assumption. All points lying above a CI value of 1 are antagonistic and those lying on a CI value of 1 are additive. The DP and CDDP concentrations and the sequence of administration (DP added prior to CDDP) were determinant factors that modified the effects of a combination from antagonism into synergism

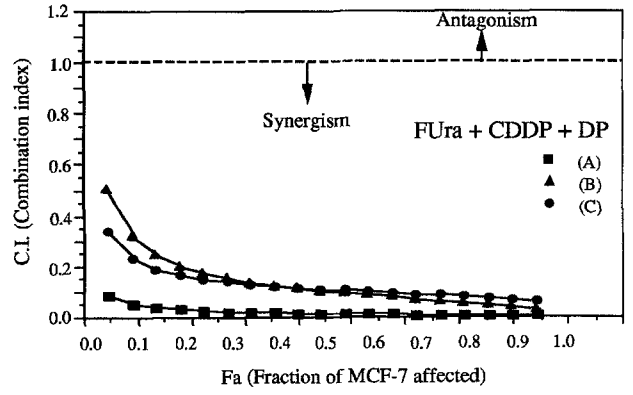

Fig. 9. Combined effect of a 72-h exposure of MCF-7 cells to (FUra + CDDP) plus DP whereby DP was added (*A*) 1 h before, (*B*) simultaneously with, or (*C*) 1 h after FUra + CDDP. Computer-generated curves describe the combined effects of FUra : CDDP : DP at fixed ratios of 1 : 0.325 : 250. Results are plotted as a function of the fraction of treated cells affected versus the combination index (fa-CI plot) under a mutually nonexclusive assumption. All points lying above a CI value of 1 are antagonistic and those lying on a CI value of 1 are additive. Interactions of FUra-CDDP + DP were strongly synergistic (plot *below the horizontal dotted line*) over nearly the entire range of concentrations tested

or simultaneously with CDDP) were determinant factors that modified the effects of a combination from antagonism into synergism. Figure 8 details the results of the interaction of the effects of the two drugs CDDP and DP as determined by CI analysis.

Synergy between (FUra + CDDP) and DP

In these experiments (FUra : CDDP : DP concentration ratio, 1 : 0.375 : 250), for the A, B, and C tests, respectively, the median-effect doses (Dm) were 4.65, 34.2, and 32.1; the correlation coefficients (*r*) were 0.951, 0.899, and 0.932; and the slopes (m) were 3.13 ± 0.45, 2.87 ± 0.62, and 3.11 ± 0.54. Analysis of the data obtained for FUra + CDDP and DP combined suggested that the three drugs acted synergistically over the entire range of concentrations tested. The composite fa-CI plot of the data from one such experiment is presented in Fig. 9.

Discussion

Biochemical modulation is now a well-established technique in cancer chemotherapy. A synergistic interaction has been demonstrated between FUra and DP in P388 tumor-bearing $B_6D_2F_1$ mice receiving a 4-day schedule of FUra (2 mg/kg daily) following a 1-h exposure to DP (100 mg/kg daily) [2]. DP was found to lower significantly ($P < 0.0001$) the mortality induced by FUra in tumor-bearing mice by nearly 2.5 times. These data allowed a prospective evaluation of 4-day treatment with i.p. FUra and DP, revealing an increase in life span without toxic death [2]. In HT-29 and MCF-7 (human colon and breast cancer, respectively) cell lines we used noncytotoxic FUra doses and demonstrated that FUra cytotoxicity was enhanced by DP in a dose-dependent manner in a soft agar colony-forming assay with continuous drug exposure [3]. The potentiation effect appeared to be higher in HT-29 cells than in MCF-7 cells (enhancement ratios, 1.1–2.7 and 1.1–1.9, respectively). Furthermore, recent in vitro studies with human cell lines derived from other organs such as the colon and ovary [14] have revealed a synergistic activity between CDDP and FUra.

The present study demonstrates several new observations. Although previous in vitro studies [14] disclosed that exposure of tumor cells to CDDP before administration of FUra was necessary to achieve maximal synergistic cytotoxicity over the entire range of concentrations tested, our results suggest that maximal cytotoxic effect and synergism are not so related to the sequence of exposure to FUra and CDDP (Fig. 6), since no substantial difference arose in terms of whether FUra was applied 1 h before or after CDDP. If the sequence of administration of FUra and CDDP has no influence, as our data suggest, the design of clinical trials could be vastly simplified.

The data presented in Figs. 4 and 5 indicate that at nontoxic doses, DP can enhance FUra- and CDDP-induced growth inhibition in a dose-dependent manner and that the growth-inhibitory effects of the combinations FUra + DP and CDDP + DP depend on the sequence of administration of DP (Figs. 7, 8). However, the DP, FUra, and CDDP concentrations and the sequence of administration are the main important parameters that modified the nature of the interaction from antagonism into synergism, and the highest cytotoxic effects were obtained when DP was applied 1 h before FUra or CDDP. Preexposure of MCF-7 cells to DP at 1 h before FUra-CDDP combination treatment (Fig. 9) did not appear necessary for the achievement of maximal synergistic cytotoxicity.

Several mechanisms have been advanced to explain the enhancing effect of DP on FUra in in vitro experiments [9,

19]. Two major mechanisms of action appear capable of inducing cell injury: (a) activation of fluorodeoxyuridine (FdUrd) to fluorodeoxyuridine monophosphate (FdUMP), a potent inhibitor of thymidylate synthetase, which can prevent the formation of deoxythymidine 5′-monophosphate and subsequently interfere with DNA synthesis; and (b) orotic acid phosphoribosyltransferase can directly transfer a ribose phosphate from 5-phosphoribosyl-1-pyrophosphate to form fluorouridine 5′-monophosphate (FUMP), or the sequential action of uridine kinase can generate fluorouridine 5′-monophosphate. After activation to the ribonucleotide level, fluorouridine 5′-triphosphate (FUTP) can be incorporated into RNA and subsequently cause alterations in RNA processing and function. Whereas DP does not affect the uptake of FUra, a nucleobase, it inhibits the efflux of FUrd (fluorouridine) and FdUrd in HCT-116 cells [9], leading to an increased retention of intracellular FdUMP. This pathway is important only when an external source for deoxyribose 1-phosphate is provided. The addition of sufficient thymidine (dThd) can prevent the increase in FUra cytotoxicity induced by DP, as dThd reduces FdUMP levels and inhibits the uptake and phosphorylation of FdUrd. Thus, DP could perturb FUra metabolism and cytotoxicity in cancer cells through several alternative mechanisms, and kinetic studies of drug influx and efflux have demonstrated that the transport of specific nucleosides across cell membranes is strongly influenced by DP, resulting in a higher, or at least longer, maintained intracellular concentration of the cytotoxic agent [20]. Since in the present study the best results were obtained with treatments A and B, when DP was given simultaneously with and 1 h before FUra, we could imagine that DP might preferentially block the salvage of physiological levels of dThd and therefore augment FUra toxicity by maintaining a state of thymidylate deprivation in cells dependent on salvage pathways. This explanation is supported by our evaluation in a human colon-adenocarcinoma cell line (HT-29) of the role played by thymidine (dThd) at 25 μ*M* in the cytotoxic action of FUra and in the potentiating action of DP. We demonstrated (data not shown) that the blockage of dThd by DP was dose-dependent and that the growth-inhibitory effect of the association FUra + DP was considerably modified by the addition of dThd (25 μ*M*) 1 h before FUra and DP.

The DP and CDDP concentrations and the sequence of administration (DP added before or simultaneously with CDDP) were determinant factors that modified the effects of a combination from antagonism into synergism. Thus, exposure to DP changes the cell such that it is more sensitive to the cytotoxic effect of CDDP or FUra. Moreover, the truly synergistic nature of the interaction bodes well for the possible clinical use of this combination because it may allow the use of relatively low doses of DP.

In the present study, enhancement of cellular sensitivity to CDDP and FUra was observed at the lowest concentrations tested (20 and 200 μmol/l) and may occur at even lower concentrations (2 μmol/l). Whereas 20 μmol/l is higher than the total DP concentration obtained by oral DP dosing [1], concentrations of 5–10 μmol/l can be achieved in plasma by continuous i.v. infusion, and concentrations of at least 100 μmol/l can be obtained in the peritoneal cavity by i.p. instillation [18]. However, as DP was 95%–99% protein-bound in plasma [1], it was hypothesized that the concentration of free DP achieved in plasma using nontoxic DP doses would not be sufficient to produce the expected effects on FUra and CDDP metabolism. On the basis of physiological characteristics and pharmacokinetic data collected following p.o. and i.v. administration of DP, Chan et al. [4] have predicted that i.p. infusion could be an alternative that would leave free DP levels of 30% in the peritoneal cavity, since only 70% of the molecules will reach the portal circulation and be metabolized in the liver, where glucoronidation is the primary mechanism of inactivation of DP in humans [18]. Thus, since appropriate concentrations of DP are attainable and since DP increases the antitumor efficacy of CDDP [14] and FUra [2] in animal models in vivo, the clinical exploitation of these double or triple combinations can be envisaged.

The mechanism(s) by which DP enhances sensitivity to CDDP remains undefined. Impaired CDDP uptake is a common characteristic of cells selected for CDDP resistance, and Jekumen et al. [13] have demonstrated that DP produces a concentration-dependent increase in the uptake of [^{195m}Pt]-CDDP. However, cellular sensitivity to CDDP is known to be influenced by numerous parameters, including increased glutathione content and enhancement of DNA-repair synthesis [22]. No information is available as to how DP itself causes cytotoxicity, but it must be remembered that the synergistic interaction may result from an effect of CDDP on cellular sensitivity to DP rather than the other way around. Regardless of whether a DP-induced increase in the uptake of CDDP might contribute to synergy, the observation that DP can increase CDDP uptake is independently of interest.

The mechanism(s) by which CDDP enters cells is not well characterized. There is strong evidence that some fraction of the drug enters by simple diffusion; however, the identification of inhibitors of uptake together with the recent demonstration that activation of the protein kinase A signal-transduction pathway can increase drug uptake argues in favor of a regulated route as well [12]. It is not clear whether an energy-dependent membrane pump is involved in either of these processes; some investigators have proposed that CDDP enters cells by passive diffusion [22]. Methionine uptake is different in CDDP-sensitive and -resistant cancer cell lines [13]. The ability of DP to increase CDDP uptake adds further support to the hypothesis that some component of drug uptake occurs via a regulated channel or transporter; other investigators have shown that CDDP can interact selectively at the tumor cell-membrane level. The effect of DP appears to be specific in that it is saturable and does not occur in the simple bilayer-lipid membranes of liposomes.

In summary, DP, which has no inherent growth-inhibitory effect at the doses used in these studies, can potentiate the cytotoxic effect of CDDP and FUra both alone and in combination. Administration of FUra and CDDP before DP produced marked antagonism in the resulting growth-inhibitory effects, whereas a majority of synergistic effects were observed for the inverse sequence. Further clinical investigation would appear indicated.

References

1. Barberi M, Merlin JL, Weber B (1991) Sensitive determination of free and plasma protein-bound dipyridamole by high-performance liquid chromatography. J Chromatogr Biomed Appl 565: 511–515
2. Barberi-Heyob M, Merlin JL, Weber B (1992) Intraperitoneal injection of dipyridamole increases the life span of tumor bearing mice treated with fluorouracil. In Vivo 6: 535–540
3. Barberi M, Merlin JL, Weber B (1990) Preclinical studies of fluorouracil-dipyridamole association in human cell lines and in mice. J Cancer Res Clin Oncol 116 [Suppl 1]: S620
4. Chan TCK, Gordon LC, Solomon Z, Stephen C, Stephen BH (1988) Pharmacokinetics of intraperitoneally asministered dipyridamole in cancer patients. Cancer Res 48: 215–218
5. Chou TC, Talalay P (1984) Quantitative analysis of dose effect relationship: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22: 27
6. Chou TC, Talalay P (1987) Application of the median-effect principle for the assessment of low dose risk of carcinogens and for the quantitation of synergism and antagonism of chemotherapeutic agents. In: Harrap KR, Connors TA (eds) New avenues in developmental cancer chemotherapy. Academic Press, New York, p 37
7. Desai PB, Sridhar R (1989) Effect of chemical modifiers on the toxicity of mitoxantrone towards CHO-K-a cells in vitro. Proc Am Assoc Cancer Res 30: 559
8. Galabov AS, Mastikova M (1991) Dipyridamole is an interferon inducer. Acta Virol 26: 137–147
9. Grem JL, Fischer PH (1986) Alteration of fluorouracil metabolism in human colon cancer cells by dipyridamole with a selective increase in fluorodeoxyuridine monophosphate levels. Cancer Res 46: 6192–6199
10. Howell SB, Hom DK, Sanga R, Vick JS, Chan TCK (1989) Dipyridamole enhancement of etoposide sensitivity. Cancer Res 49: 4147–4153
11. Howell SB, Hom DK, Sanga R, Vick JS, Abramson IS (1989) Comparison of the synergistic potentiation of etoposide, doxorubicin, and vinblastine cytotoxicity by dipyridamole. Cancer Res 49: 3178–3183
12. Howell SB, Isonishi S, Christen RC, Andrews PA, Mann SC (1991) Cellular pharmacology strategies for overcoming drug resistance: potential applications to regional therapy. Eur J Surg [Suppl] 561: 45–48
13. Jekumen A, Vick J, Sanga R, Chan TCK, Howell SB (1992) Synergism between dipyridamole and cisplatin in human carcinoma cells in vitro. Cancer Res 52: 3566–3571
14. Keane TE, Rosner GL, Gingrich JR, Poulton SHM, Walther PJ (1991) The therapeutic impact of dipyridamole chemopotentiation of the cytotoxic combination 5-fluorouracil/cisplatin in an animal model of human bladder cancer. J Urol 146: 1418–1424
15. King ME, Narporn A, Young B, Howell SB (1984) Modulation of cytarabine uptake and toxicity by dipyridamole. Cancer Treat Rep 68: 361–366
16. Kong XB, Chou JH, Kim H, Chou TC (1992) Computerized quantitation of synergism and antagonism in three drug combinations. Proc Am Assoc Cancer Res 33: 442
17. Konits PH, Aisner J, Van Echo DA (1981) Mitomycin C and vinblastine chemotherapy for advanced breast cancer. Cancer 48: 1295–1298
18. Mahony C, Wolfram KM, Bjornsson TD (1982) Dipyridamole kinetics. Clin Pharmacol Ther 31: 330–338
19. Miller EM, Willson JK, Fisher PH (1987) Folinic acid alters the mechanism by which dipyridamole increases the toxicity of fluorouracil in human colon cancer cells. Proc Am Assoc Cancer Res 28: 326
20. Perloff M, Hart RD, Holland JF (1978) Vinblastine, Adriamycin, thiotepa and halostestin (VATH): therapy for advanced breast cancer refractory to prior chemotherapy. Cancer 42: 2534–2537
21. Piper AA, Nott SE, Mackinnon WB, Tattersall MH (1983) Critical modulation by thymidine and hypoxanthine of sequential methotrexate-5-fluorouracil synergism in murine L1210 cells. Cancer Res 43: 5701–5706
22. Rosenberg B (1985) Fundamental studies with cisplatin. Cancer 55: 2302–2307
23. Shionoya S, Lu Y, Scanlon KJ (1986) Properties of amino acid transport systems in K562 cells sensitive and resistant to *cis*-diamminedichloroplatinum(II). Cancer Res 4: 3445–3448
24. Tada H, Shito O, Kuroshima K, Tsukamoto K (1986) An improved colorimetric assay for interleukin 2. J Immunol Methods 93: 157–165
25. Wilson AP, Ford CH, Newman CE, Howell A (1987) *cis*-Platinum and ovarian carcinoma. In vitro chemosensitivity of cultured tumor cells from patients receiving high dose *cis*-platinum as first line treatment. Br J Cancer 56: 763–773
26. Zhen Y, Lui MS, Weber G (1983) Effects of acivicin and dipyridamole on hepatoma 3924 A cells. Cancer Res 43: 1616–1619