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Report

Selective cell kill of the combination of gemcitabine and cisplatin in multilayered postconfluent tumor cell cultures

José M Padrón, Catharina JA van Moorsel, Andries M Bergman, Evelien Smitskamp-Wilms, Clasina L van der Wilt and Godefridus J Peters
Department of Medical Oncology, University Hospital Vrije Universiteit, 1007 MB Amsterdam, The Netherlands.

Both gemcitabine (2′,2′-difluorodeoxycytidine, dFdC) and cisplatin (*cis*-diammine-dichloroplatinum) have significant anticancer activity against ovarian, head and neck, and non-small cell lung cancer (NSCLC). dFdC can be incorporated into DNA and RNA, and inhibit DNA repair, while cisplatin can form Pt–DNA adducts. We previously observed schedule-dependent synergism of the combination of dFdC and cisplatin in monolayer cell cultures. We now evaluated whether the combination would also enable selective cell kill in multilayered postconfluent cell cultures, since each compound showed variable activity in multilayered cells. The combination was tested in multilayered cultures from cell lines with a different histological origin: the human head and neck squamous cell carcinoma cell line UMSCC-22B (22B), the human NSCLC cell line H322, and ADDP, a cisplatin-resistant variant of the human ovarian cancer cell line A2780. Sensitivity of the multilayered cells was dependent on exposure duration and sequence of the drug combinations, which were added in a constant molar ratio (dFdC:cisplatin 1:100), with a total exposure time of 96 h. The type of interaction was related to the degree of resistance of the cell lines to either dFdC or cisplatin. Thus, the very sensitive 22B cells only showed an additive effect when cells were preincubated for 24 h with dFdC prior to exposure to the combination. In contrast, in the resistant ADDP and H322 cells, synergism was the most common profile (three out of four schedules tested). This is of special relevance when we take into account that these drugs only show cytostatic effects when administered alone, whereas the combination produced cytotoxic cell killing. In conclusion, combining dFdC with cisplatin can be at least additive, but synergistic in multilayered postconfluent cells resistant to dFdC and cisplatin. [© 1999 Lippincott Williams & Wilkins.]

Key words: Cisplatin, gemcitabine, synergism, three-dimensional cultures.

Introduction

The antimetabolite gemcitabine (2′,2′-difluorodeoxycytidine, dFdC) is a deoxycytidine analog which exhibits good activity against several solid tumor models, including ovarian, head and neck cancer, and non-small cell lung cancer (NSCLC).[1–4] In clinical phase II studies, dFdC has shown remarkable activity against ovarian and NSCLC, but is moderately effective against head and neck cancers.[5–8] dFdC is now an established agent in the treatment of NSCLC and pancreatic cancer. dFdC needs extensive metabolism before it can exert its activity. After entering the cell, dFdC is phosphorylated to its active metabolite, dFdCTP, which can then be incorporated into DNA,[9] subsequently leading to inhibition of exonuclease and DNA repair.[10] dFdC can also be incorporated into RNA[9] and dFdCDP is capable of inhibiting ribonucleotide reductase, an enzyme with a key role in DNA repair mechanisms.[11] The latter effect also depletes the cell of deoxyribonucleotides and favors the incorporation of dFdC into DNA.

Cisplatin (*cis*-diammine-dichloroplatinum) is widely used for chemotherapy of a broad range of solid tumors like ovarian, head and neck, and NSCLC.[12] The antitumor activity of cisplatin appears to be to due to the binding of these drugs to cellular DNA. Cisplatin can form mono-adducts and various types of bifunctional adducts.[13] This type of DNA damage is generally believed to be the origin of the cytotoxic action of

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Correspondence to GJ Peters, University Hospital Vrije Universiteit, PO Box 7057, 1007 MB Amsterdam, The Netherlands.
Tel: (+31) 20 4442633; Fax: (+31) 20 4443844;
E-mail: gj.peters@azvu.nl

cisplatin, and a relation between drug sensitivity and poor cisplatin-DNA adduct repair has also been established for fibroblasts.[14,15]

dFdC and cisplatin can interact at several sites depending on cell line and scheduling, resulting in synergism.[16] Previously we described synergistic interactions of dFdC with cisplatin in various monolayer cell cultures,[17-19] while in the *in vivo* models variable extents of additivity were observed.[20-22] Therefore, it was questioned whether the monolayer model was suitable for predicting *in vivo* activity.

Three-dimensionally cultured tumor cells, in contrast with monolayers, offer the possibility to study the role of solid tumor-specific parameters such as drug penetration, hypoxia and cell-cell interactions.[23] Most three-dimensional model systems are time- and labor-consuming, and therefore are not extensively used as screening systems. We developed a system in which cells were cultured as multilayered postconfluent cultures in V-shaped wells with a pattern of organization and sensitivity profiles resembling tumors *in vivo*.[24,25] Furthermore, both growth inhibition and cell kill can be studied with the sulforhodamine B (SRB) assay.[26,27] In the multilayer model a selectivity of dFdC for ovarian cancer over colon cancer has been found, which was more significant than with monolayers.[28]

The objective of the present study was to assess whether dFdC and cisplatin would have a selective synergistic interaction in multilayered cells compared to monolayer cells. Three cell lines with different histologic origins, i.e. UMSCC-22B, H322 and ADDP, were used as a model system.

Materials and methods

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from Flow Laboratories (Irvine, UK). Fetal calf serum (FCS) was from Gibco (Grand Island, NY), trichloroacetic acid (TCA), glutamine and gentamicin were from Merck (Darmstadt, Germany), and trypsin and SRB were from Sigma (St Louis, MO). dFdC was a kind gift of Eli Lilly. (Indianapolis, IN) and was solubilized with phosphate-buffered saline (PBS) to a concentration of 10 mM. Cisplatin was purchased from Bristol-Myers Squibb (Weesp, The Netherlands) and was solubilized with PBS to a concentration of 3 mM. Final dilutions of both drugs were made in culture medium. All other chemicals were of analytical grade and commercially available.

Cell culture and plating

As a model system, the experiments *in vitro* were performed with three different cell lines: UMSCC-22B (22B), a human head and neck squamous cell carcinoma cell line;[29] H322, a human NSCLC cell line (subtype BAC, NCI); and ADDP, a cisplatin-resistant variant of the human ovarian cancer cell line A2780.[30]

Detailed description of routine cell culture and plating procedures used during these experiments were reported elsewhere.[24,28] Briefly, mycoplasma-negative cells were maintained without antibiotics in DMEM supplemented with 10% heat-inactivated FCS and 1 mM L-glutamine in a 37°C, 5% CO_2, 95% humidified air incubator. Exponentially growing cells were trypsinized and resuspended in antibiotic-containing medium (50 μg gentamicin/ml); single-cell suspensions displaying 90% or greater viability by Trypan blue dye exclusion were subsequently counted and seeded (20 000 cells/50 μl/well) in 96-well 'V'-bottomed plates (D0). The number of plated cells provided exponential growth during the first days, and thereafter the formation of multiple cell layers started and cultures became supraconfluent (after approximately 5 days, depending on the cell line).

Twenty-four hours after seeding (D1), 100 μl of medium was added to each well. From the second day after plating until the end of the experiments, culture medium was gently aspirated and once daily replaced by fresh medium (150 μl/well).[24]

Chemosensitivity testing

Chemosensitivity tests were performed using a slightly modified SRB assay.[26-28] The three cell lines were exposed to dFdC and cisplatin as separate agents, and to a combination of both drugs (either simultaneously or sequentially). On day 5 (D5) after plating, the cells received 150 μl of drug-containing medium in triplicate, resulting in a series of final concentrations of 0.5 nM to 1 μM of dFdC and 50 nM to 100 μM of cisplatin. Control cells received 150 μl of drug-free medium. The concentrations of dFdC and cisplatin in the combination had the same range as the separate agents, with a dFdC:cisplatin constant ratio of 1:100. Serial dilutions of the stock solutions were prepared in culture medium immediately before drug addition. The cells were exposed to the drugs for 24 or 96 h. After 24 h exposure, the medium was replaced by fresh drug-free medium after a washing step with 150 μl drug-free medium and the cells were cultured until 96 h (D9) after the initial drug addition. Care was taken to ensure that cell loss was minimal during the procedure.

At the end of the culture, the cells were precipitated with 25 μl ice-cold 50% (w/v) TCA and fixed for 60 min, after which the SRB assay was performed. The optical density (OD) of each well was measured at 450 nm (a suboptimal wavelength required to produce absorbance readings within the linearity range of the assay) using a microtiter plate reader (Titertek Multiscan MCC/340; Flow Laboratories). Data were expressed in terms of %T/C [(OD of treated cells/OD of control cells) $\times$ 100]. Values were corrected for background OD of wells only containing medium. The resulting dose-response curves allowed the extrapolation of EC_{50} values, defined as the effective drug concentration which yielded OD readings 50% lower than those of control wells (defined as 100%).

As part of the assay, additional plates were assessed for cellular density just before drug exposure (D5). This allowed us to define the effect as percentage of growth (PG) for 96 h (D5 to D9) when introducing the latter parameter in the calculations.[31] Thus, for the culture period from D5 to D9, IC_{50} represents 50% growth inhibition when OD is in the middle of that D5 and D9, TGI represents total growth inhibition when OD at D9 $\approx$ OD at D5, and LC_{50} represents 50% of cell killing, when the OD D9 is between the OD at D5 and the OD of control wells.

Values were calculated for these parameters if the level of effect was reached, but if the effect was not reached, then the value was expressed as greater than the maximum drug concentration tested.

Dose–effect analysis

Dose-response interactions (antagonism, additivity and synergism) between dFdC and cisplatin were evaluated using the median drug effect analysis method of Chou and Talalay,[32] processed by a computer program based on the original concept of Dr TC Chou. This program provides one of the few objective computerized evaluation procedures.[33] Effects were expressed as a mutually non-exclusive case combination index (CI) for every fraction affected (FA). In order to evaluate the interaction between the drugs, in multilayered postconfluent cells absorbance in control wells at D9 was set at FA=0, while background absorbance was set at FA=1, a FA of 0.25 is an effect 25% decrease in absorbance. For the final evaluation, we used values of FA in the range $0.05 < FA < 0.95$.

D_m values (comparable to EC_{50} values) were calculated by the program by extrapolation. For the separate drugs the respective effect parameters, expressed as FA were introduced. The CI (combination index) was calculated by the formula:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} + \alpha \frac{(D)_1}{(D_x)_1}\frac{(D)_2}{(D_x)_2}$$

where α=1 for mutually non-exclusive drugs; $(D)1$ and $(D)_2$ are the doses of the separate drugs and their combination in a fixed ratio; and $(D_x)_1$ and $(D_x)_2$ are the doses resulting in an effect of x%. These doses are calculated by the formula: $D=D_m \times [FA/(1-FA)]^{1/m}$, where D_m is the dose required to produce absorbance readings 50% lower than those of non-treated wells (EC_{50}), FA is the fraction affected and m is the slope of the median drug effect plot. Simplifying, a $CI < 1$ indicates synergism, $CI > 1$ indicates antagonism and CI=1 indicates additivity. Since CIs changed with FA, the averaged CIs at the FAs 0.5, 0.75, 0.9 and 0.95 were used, as described in more detail by Chou (CalcuSyn v 1.1.1; Biosoft 1996, Cambridge, UK).

Table 1. Chemosensitivity of mono- and multilayers to dFdC and Cisplatin

Cell line	Exposure (h)	Monolayers IC_{50}[a]		Multilayers EC_{50}		Ratio EC_{50}:IC_{50}[b]		Multilayers TGI	
		dFdC (nM)	Cisplatin (μM)	dFdC (nM)	Cisplatin (μM)	dFdC	Cisplatin	dFdC (nM)	Cisplatin (μM)
ADDP	24	193±43	63±15	>1000	>100	>5	>1.6	>1000	>100
	72 or 96[c]	625±154	52±13	>1000	96.5±4.2	>1.6	1.9	>1000	>100
H322	24	420±201	15.3±3.7	>1000	31.9±4.0	>2.4	2.1	>1000	46.0±14
	72 or 96	120±54	14.9±2.7	>1000	24.8±4.7	>8.3	1.7	>1000	24.5±5.0
22B	24	4.50±1.21	5.83±0.52	63.1±8.1	7.12±0.9	14	1.2	68.2±11	9.1±1.3
	72 or 96	0.86±0.32	4.10±0.10	33.3±9.6	3.01±0.9	39	0.7	25.9±10	6.7±2.8

IC_{50} and EC_{50} values are means ± SEM of at least three independent experiments.
[a]See Bergman *et al.*[17] and Van Moorsel *et al.*[19]
[b]Ratio EC_{50} multilayer versus IC_{50} monolayer.
[c]72 h for monolayers and 96 h for multilayers

Results

The cytotoxicity of dFdC and cisplatin on the multilayer system, EC_{50}, was expressed as the concentration of drug resulting in reduction in OD (reflecting the number of cells) by 50%, when compared to the number of cells in untreated wells. These calculations allowed us to evaluate the growth curves over the whole range.

The sensitivities of ADDP, H322 and 22B cell lines to dFdC and cisplatin, in both mono- ($D1\text{-}IC_{50}$) and multilayer ($D5\text{-}EC_{50}$) models are listed in Table 1. Similarly to previous results,[28] cells growing in multilayers were, in general, more resistant to dFdC and cisplatin than when cells were grown as monolayer cultures. For ADDP and H322 cells, EC_{50} values for dFdC were higher than the maximum drug concentration tested (1 mM). Against postconfluent 22B cells, dFdC was sufficiently cytotoxic to cause reduction in cell mass. This cell line was the most sensitive to dFdC. The cell lines ADDP and H322 are resistant to dFdC when compared to 22B or A2780.[28]

In Table 1, the effects of the drugs are expressed as a function of the $D5\text{-}EC_{50}$:$D1\text{-}IC_{50}$ ratios. For a given drug, a low ratio would indicate similar performances against mono- and multilayer cultures. Overall, the results showed lower ratios for cisplatin compared to dFdC in all three cell lines. Cisplatin was the least affected by the culture conditions, producing EC_{50}:IC_{50} ratios of 0.7–2.1. Ratios for dFdC could not be determined reliably in H322 and ADDP cells, because of the insensitivity of the multilayered cells.

Combination studies

When exposed to a combination of dFdC and cisplatin, the drugs were combined in a concentration ratio of 1:100 (dFdC:cisplatin). This constant ratio was based on the EC_{50} values for 22B cells, since the other two cell lines present resistance to dFdC and an EC_{50} could

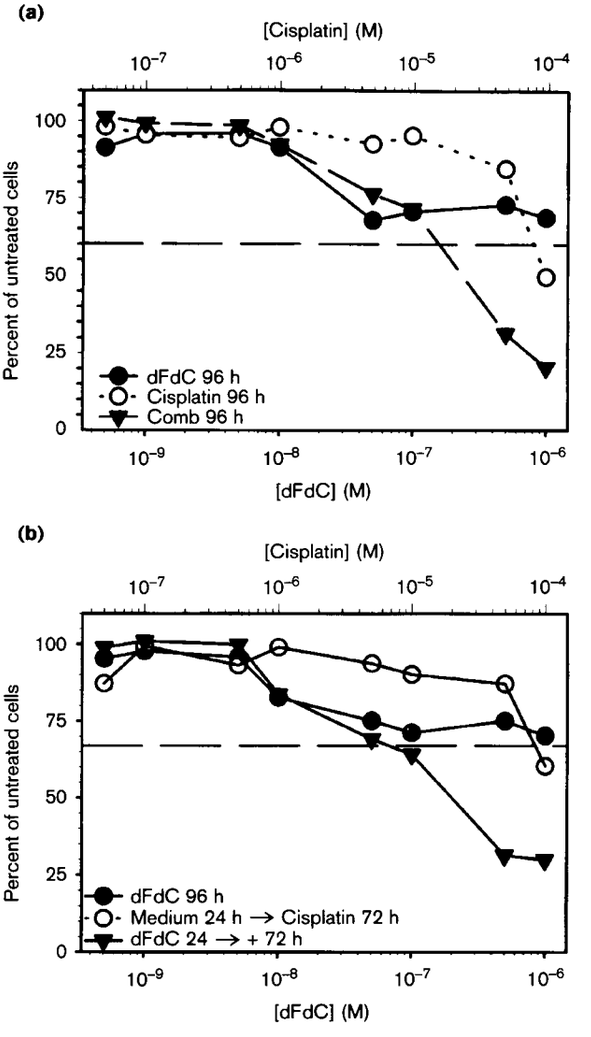

Figure 1. Representative dose–response curves for synergistic combinations of dFdC and cisplatin in ADDP (a) and H322 (b) cell lines. In each graph, the horizontal broken line indicates the relative amount of cells found in non-treated wells at D5. The region above this line represents growth inhibitory effects, while the region below it indicates the presence of cytotoxicity (cell kill).

Table 2. Summary of the median drug effect analysis for the combination of dFdC and cisplatin (1:100) in postconfluent multilayer cultures

Drug schedule	Incubation (h)	ADDP	H322	22B
dFdC and cisplatin simultaneous	24	0.37±0.16	0.48±0.12	1.53±0.32
	96	0.05±0.02	0.09±0.02	1.23±0.35
dFdC 24 h before combination	96	16.4±4.20	0.02±0.02	0.91±0.25
Cisplatin 24 h before combination	96	0.33±0.11	1.88±0.68	52.3±5.31

Values represent the averaged CI (non-exclusive case) at EC_{50}, EC_{75}, EC_{90} and EC_{95}, and are means of three to five experiments

not be reached. Thus, the drugs were combined at their approximate EC_{50} values, and a range of concentrations below and above were chosen in the same fixed ratio. In the same experiment, we always included controls with each drug alone. Figure 1 shows examples of ADDP (Figure 1a) and H322 (Figure 1b) cells when exposed to dFdC, cisplatin and the combination of these drugs.

This method would also allow us to use a computerized, objective evaluation of the interaction between both drugs. Table 2 summarizes the multidrug effect analysis of the three cell lines exposed to the simultaneous or sequential combination of dFdC and cisplatin in a constant ratio (dFdC:cisplatin, 1:100). For the combination of dFdC and cisplatin $\alpha=1$ (mutually non-exclusive case) was assumed, since these agents are known to act by different mechanisms. The CI is the mean of the CI values at EC_{50}, EC_{75}, EC_{90} and EC_{95}. According to the methodology described in the manual of CalcuSyn when any of these values were greater than 2, the antilog of the averaged log(CI) value was used to avoid significant biases of overestimating antagonism. In these combination studies the sensitivity to dFdC and cisplatin is dependent on exposure duration and sequence of the drugs. Scheduling and a certain extent of exposure to dFdC and cisplatin appeared to be important factors for the interaction of both drugs.

The exposure to the combination of dFdC and cisplatin simultaneously resulted in synergism in the multilayered cultures of ADDP (Figure 2a) and H322 (Figure 2b) cell lines, and moderate antagonism in the 22B cell line (Figure 2c). Furthermore, this synergistic effect was much more evident when ADDP and H322 cell lines were treated for 96 h to the combination (CI=0.05 and 0.09, respectively). In contrast, no significant difference in the multidrug effect analysis between exposure for 24 and 96 h was found for the 22B cell line. These results are similar to those obtained when cells were grown as monolayers. In that model system, synergism was observed for the ADDP and H322 cell lines, while antagonism was found for the 22B cell line.[17,19]

When the cells were preincubated for 24 h with one drug followed by a 72 h additional exposure to the combination, a different pattern was observed. In ADDP cells synergism (CI=0.33) was found when they were preincubated for 24 h with cisplatin and very strong antagonism (CI=16.4) in the case of 24 h of preincubation with dFdC. For H322 cells the pattern was the opposite, presenting moderate antagonism (CI=1.88) when cisplatin was administered 24 h prior to the combination and strong synergism (CI=0.02) when the preincubation for 24 h was with dFdC.

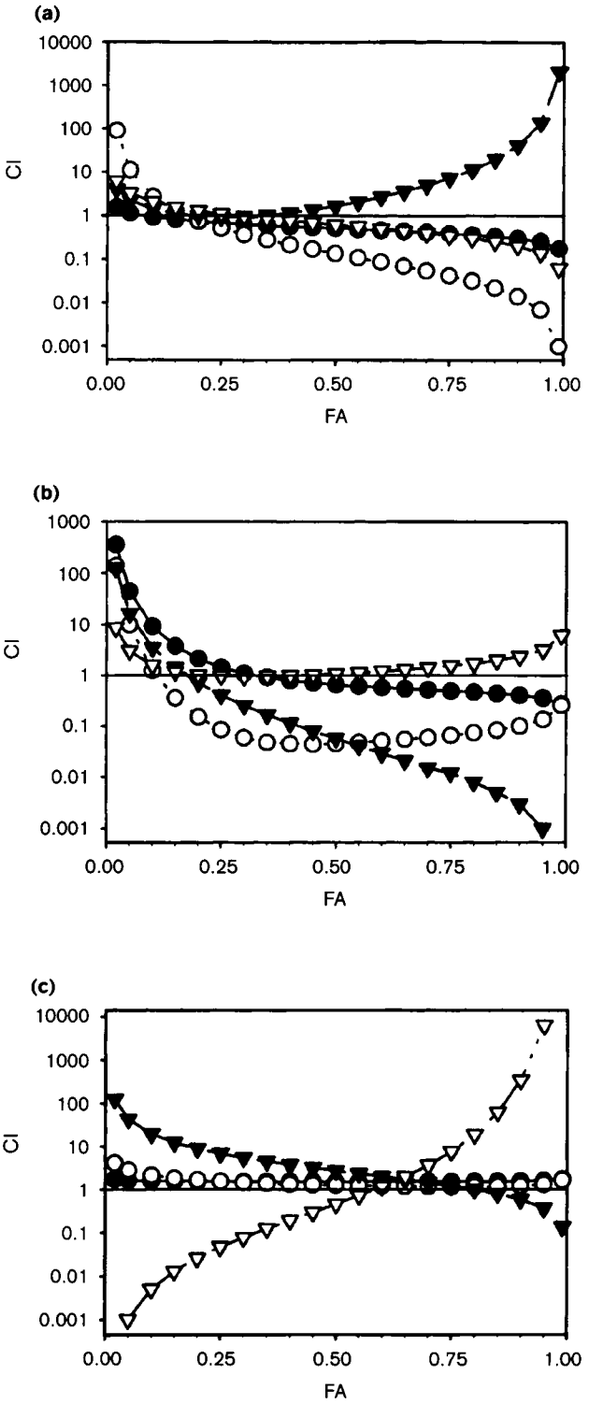

Figure 2. Dose–effect analysis of the interaction between dFdC and cisplatin in the multilayered cultures of ADDP (a), H322 (b) and 22B (c) cell lines. Values are representative of three to five experiments. Cells were exposed to dFdC and cisplatin simultaneously for 24 (●) and 96 (○) h, or preincubated for 24 h with dFdC (▼) or cisplatin (▽) before treatment to the combination for an additional 72 h.

Similar to previous studies in the monolayer model, synergism at simultaneous exposure was most pronounced in the drug-resistant cell lines ADDP and H322.[19] For 22B cells (Figure 2c) the dose-effect analysis showed clear antagonism in three of the four schedules tested. Furthermore, only an additive effect (CI=0.91) was achieved when cells were preincubated with dFdC for 24 h prior to exposure to the combination.

The growth inhibition parameters TGI and LC_{50} for the combination of dFdC and cisplatin are shown in Table 3. For ADDP cells, a TGI value was reached for the combination with 96 h simultaneous exposure and 24 h preincubation with cisplatin. By contrast, this effect could not be reached when the cells were treated with the drugs alone. In H322 cells, TGI was reached for the treatment with cisplatin alone, whereas the combination of the drugs produced TGI values in all the schedules tested. Furthermore, an LC_{50} value was reached showing a clear cell killing effect. Finally, in the sensitive cell line 22B, the combination produced an increase of TGI values compared to each drug alone with the exception of 24 h preincubation with dFdC.

Discussion

This study shows the potency of the postconfluent multilayered cell culture system to predict selective sensitivity of solid tumors for the combination of dFdC and cisplatin. Although we found large differences in sensitivity for dFdC between the mono- and multilayer model, with EC_{50}:IC_{50} ratios ranging from 1.6 to 39 (Table 1), the combination with cisplatin was less affected by the culture conditions and was even more selective than cisplatin alone.

In vitro synergy and antagonism were evaluated with the median drug effect analysis using EC values. The latter parameter was employed for the calculations since IC values did not allow us to evaluate the whole curve. In the median drug effect analysis a CI is calculated for the interaction between two or more drugs. In the present study we could observe a clear difference between the cell lines tested. For 22B cells, which present sensitivity to both drugs, the combination of dFdC and cisplatin mainly resulted in antagonism. Only an additive effect could be observed when cells were preincubated for 24 h with dFdC. Similarly, in monolayer cultures of 22B cells synergism was only found when dFdC was administered before the combination.[17] In previous *in vivo* studies with head and neck xenografts (HNX-22B and HNX-14C), the most active schedules were those in which dFdC was given before cisplatin.[21]

Of special interest was the fact that synergism was most pronounced and the most common effect in the drug-resistant cell lines ADDP and H322. Furthermore, when ADDP and H322 cells were treated with the combination of dFdC and cisplatin it was possible to observe cell kill (lower OD values than at D5), an effect that could not be reached with the exposure to dFdC alone (Figure 1).

From these results we can conclude that dFdC enhances the activity of cisplatin. For the moment the mechanism of synergistic interaction between these drugs has not been clarified completely, although it appears to be mainly due to an increase in Pt-DNA adduct formation possibly related to changes in DNA due to dFdC incorporation into DNA.[19] This study also indicates that the effect of dFdC on the cytotoxicity of cisplatin, presumably on Pt-DNA adduct levels, is not necessarily associated with cell division.

A number of clinical studies on the combination of cisplatin and dFdC have been initiated. The results are very promising, especially in NSCLC. Using various schedules, the activity of this combination was at least as great as what could be expected based on the antitumor effect of each drug alone.[34,35] However, in the study of Crinó *et al.*[36] in which dFdC was given

Table 3. Growth inhibition parameters[a] for the combination of dFdC and cisplatin

Drug schedule	Incubation (h)	ADDP		H322		22B	
		TGI (nM)	LC_{50} (nM)	TGI (nM)	LC_{50} (nM)	TGI (nM)	LC_{50} (nM)
dFdC and cisplatin simultaneous	24	>1000	>1000	401±228	728±136	61.9±15.4	208±25.3
	96	586±127	>1000	212±77.1	484±56.3	37.8±6.96	95.5±12.7
dFdC 24 h before combination	96	>1000	>1000	383±131	>1000	6.04±1.96	>1000
Cisplatin 24 h before combination	96	865±49.5	>1000	241±53.8	416±166	40.6±12.2	>1000

[a]TGI and LC_{50} are given for the concentration of dFdC in the combination. The concentration of cisplatin in the combination is 100 times the concentration of dFdC

24 h before cisplatin, an overall response rate of 54% was observed. The response rate with each of the two compounds alone has been reported to be approximately 20%.[37] Retrospectively, using the multilayer model we could have demonstrated a predictive value for solid tumor sensitivity. Thus this model may be used in the evaluation of new drugs to determine which drug should or should not be tested further *in vivo*.

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