

Genotoxicity and Cytotoxicity of the Anticancer Drugs Gemcitabine and Cisplatin, Separately and in Combination: *in vivo* Studies

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Abstract: 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. Because of differences in mechanisms of action and toxicity profiles, combination of the two drugs has enormous clinical potential. The combination of both is increasingly applied in clinical oncology. In this study, the genotoxic effects of cisplatin and gemcitabine separately and in combination were detected in the male mice bone marrow cells. Four doses were used for each drug. In CDDP experiments 6, 12, 24 and 36 mg kg⁻¹ body weight were used. For dFdC 40, 50, 60 and 80 mg kg⁻¹ body weight were used. Three drug combination doses were used: 4 mg kg⁻¹ body wt. CDDP, 20 mg kg⁻¹ body wt. dFdC; 6 mg kg⁻¹ body wt. CDDP, 20 mg kg⁻¹ body wt. dFdC; and 8 mg kg⁻¹ body wt. CDDP, 20 mg kg⁻¹ body wt. dFdC. In the drug combination experiments, CDDP were injected 4 hours prior to dFdC. Total chromosomal aberrations and sister chromatid exchanges (SCEs) frequencies were increased after exposure to combined drugs compared to exposure to each drug separately. Both single and combined drugs decreased the mitotic activity of the cells and induced a cell cycle delay with increasing the doses. In conclusion, the potentiation in chromosomal aberrations and sister chromatid exchanges formation might be a result of the inhibition of DNA repair by dFdC. The synergism between dFdC and CDDP 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.

Key words: Genotoxicity, Gemcitabine, cisplatin, cytotoxicity, micebone marrow cells

Introduction

The majority of antineoplastic drugs, besides their generic growth property, display genotoxic effects which in turn contribute to growth inhibition. These genotoxic effects may lead to initiation of unrelated tumors years after cessation of chemotherapy (Beretta, 1991). Therefore, the introduction of any new antineoplastic substance for anti-tumor therapy requires careful examination of its genotoxic properties in appropriately chosen *in vitro* and *in vivo* systems.

Cis-Diammine-dichloroplatinum (Cisplatin, CDDP) is an established anticancer drug with activity in a variety of solid tumor types such as ovarian cancer, non-small-cell lung carcinoma (NSCLC), head and neck cancer (HNC) both as a single agent and in combination with other agents (Muggia, 1984). CDDP is generally considered to exert its cytotoxic effect by binding to DNA, resulting in mutation induction (Fichtinger-Schepman *et al.*, 1984). The high mutagenic potency of CDDP raises the concern that its use in cancer chemotherapy may be responsible for secondary malignancies, which have been observed in animals and some cured patients treated with CDDP (Kempf and Ivankovic, 1986; Greene, 1992 and Pillaire *et al.*, 1994).

2',2'-Difluorodeoxycytidine (Gemcitabine, dFdC) is a relatively new antineoplastic drug with activity against several solid tumors including ovarian cancer, non-small-cell lung carcinoma (NSCLC), head and neck cancer (HNC) (Van Moorsel *et al.*, 1997). 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 (Huang *et al.*, 1991); which probably determines its cytotoxic effect. Besides this effect, dFdC is also capable of inhibiting ribonucleotide reductase (Heinemann *et al.*, 1988); an enzyme with a key role in DNA repair mechanisms. Some authors (Auer *et al.*, 1997) demonstrated that dFdC led to an increase of DNA single breaks using diploid, mortal low-passage fibroblasts (LPF cells) and the spontaneously transformed cell line V79. They also showed that it induce SCEs as well as chromosome breaks in V79 and not in LPF. Despite these *in vitro* data on the mutation frequency caused by dFdC (Auer *et al.*, 1997); no information is presently available on the genotoxic properties of Gemcitabine in the more relevant *in vivo* situation for either normal or malignant cells.

The combination of CDDP and dFdC has been studied quite extensively (Brankhuis *et al.*, 1995 and Bergman *et al.*, 1996). These two drugs differ completely in mechanism of action and toxic side effects, with CDDP inducing side effects, such as nephrotoxicity, neurotoxicity, ototoxicity and severe nausea and vomiting (Von Hoff *et al.*, 1979 and Vermorken and Pinedo, 1982) and dFdC being primarily mylotoxic (Abratt *et al.*, 1994). Theoretically, the two drugs might interact at different levels (Peters *et al.*, 1995). CDDP could influence dFdC accumulation, metabolism or the extent of DNA damage. On the other hand, dFdC might interact with the cellular accumulation of CDDP, DNA platination, or repair of DNA adducts (Peters *et al.*, 1995 and Van Moorsel *et al.*, 1999).

Because of the extensive and increasing use of cisplatin and gemcitabine in successful combination therapy regimens, an understanding of their mutagenic and carcinogenic properties is important.

In this study, we assessed several cytogenetic parameters of genotoxicity of dFdC and CDDP, separately and in combination, namely induction of chromosome aberrations, sister chromatid exchange (SCE), mitotic index and replicative index.

Materials and Methods

Random-bred, male mice (*Mus musculus*), aged 6-8 weeks were provided by the Egyptian Organization for Biological and Vaccine Production, Cairo, Egypt. Food and water were given *ad libitum*. CDDP and dFdC were dissolved in 0.9 NaCl solution in darkness 10-18 min before use.

Groups of mice, [5 animals/group] were treated intraperitoneally with four different doses of both CDDP [6, 12, 24 and 36 mg kg⁻¹] and dFdC [40, 50, 60 and 80 mg kg⁻¹]. In the drug combination experiments the doses used were 4 mg kg⁻¹ body wt. CDDP: 20 mg kg⁻¹ body wt. dFdC, 6 mg kg⁻¹ body wt. CDDP: 20 mg kg⁻¹ body wt. dFdC and 8 mg kg⁻¹ body wt. CDDP: 20 mg kg⁻¹ body wt. dFdC. When the dose of dFdC was increased than 20 mg kg⁻¹ body wt. in the drug combinations, it induced inhibitory effect, resulting in a delayed response (data not shown). In the drug combination experiments, CDDP were injected 4 hours prior to dFdC. The control groups received only the vehicle solution. All animals were sacrificed 24 hrs. after treatment.

Chromosome analysis and mitotic index

Bone marrow, from the femurs of control groups and of animals with different doses, was sampled 24 hrs. after treatment. Two hours before sacrifice, the mice were injected intraperitoneally with 0.2 ml colchicine (0.6 mg/ml). Bone marrow cells were processed according to standard cytogenetic methods for chromosome preparation and slides were prepared for cytogenetic analysis.

Sister chromatid exchanges

5-Bromodeoxyuridine (BrdU) tablets (55 mg) were implanted subcutaneously in mice. These tablets dissolve gradually following subcutaneous implantation, lasting for about 16 h (Allen *et al.*, 1978). Half an hour before implantation of BrdU, the groups of animals were treated with the different doses of the anticancer drugs and the control groups injected with 0.9% NaCl solution. Hypotonic treatment, fixation of the cells and chromosome preparation were done with the standard air-drying technique. The differential staining of sister chromatids was performed according to the fluorescence plus Giemsa method (Perry and Wolff, 1974).

Scoring

For chromosomal aberration analysis, at least 50 cells from each animal were examined for both structural and numerical aberrations. The total aberrant cells and their mean percentage were calculated.

The frequency of sister chromatid exchange was recorded for each animal in at least 25 second division cells. The frequency of SCEs/cell was determined.

Slides prepared for chromosome aberrations were used to determine the proliferative rate (mitotic index). The determinations of mitotic index were based on scoring of 1000 cells from each animal. The number of dividing cells, including prophases and metaphases was recorded. The estimate of changes in cell cycle kinetics was obtained by evaluating the replicative index (R.I) using slides prepared for SCE analysis, 100 metaphase cells from each animal were analyzed.

Statistical analysis

For chromosomal aberration analysis, the Chi-Square test (2 × 2 contingency table) was used, whereas the t-test of the difference between means was used for sister chromatid exchange, mitotic index and replicative index data analysis.

Results

To evaluate the mutagenic effect of both dFdC and CDDP, statistical analysis was carried out comparing the data obtained from exposed animals and those of control groups. The results were tabulated as the mean from all animals within the treatment plus or minus the standard error of the mean.

The number of cells with CA in animals treated with cisplatin and/or gemistabine are presented in Table 1 and Fig. 1. The increase in number of cells with CA was found to be dose dependent.

With single drugs, breaks constitute the main type of structural aberrations observed. Other aberrations were observed, such as fragments, deletions, chromosome breaks, centric fusions and centromeric attenuations. In the drug combination experiments, the number of cells with breaks, centric fusions, chromosome breaks, centric fusions and centromeric attenuations was decreased gradually with increasing the dose of CDDP in the combination. Only cells with fragments were observed to increase gradually with increasing the dose. In the single drug doses, the number of cells with one structural aberration and more appeared to increase gradually with dose increasing but with 80 mg kg⁻¹ dose of dFdC the number decreased, although it was still

Table 1: Chromosomal aberrations induced in mouse bone marrow cells by single and combined drug doses of cisplatin and gemcitabine

Dose mg kg ⁻¹	No. of examined cells	No. of Cells with different structural Chromosomal Aberrations							Cells with more than 1 St.Ab.	Cells with more than 1 St.Ab.	Total structural Aberrant Cells		Total Numerical Aberrant Cells	
		-----									-----		-----	
		Br	F	D	Ch Br	CF	CA	No.			%	No.	%	
Control														
	557	2	1	0	0	8	0	10	1	11	1.97	19	3.41	
CDDP 6	291	7**	1n.s	0	2*	10n.s	2*	19***	2n.s	21***	7.22	33***	11.34	
12	270	18***	13***	7***	3*	15***	6***	41***	12***	53***	19.63	22**	8.15	
24	267	20***	6**	4**	1n.s.	23***	10***	47***	8***	55***	20.59	53***	19.85	
36	261	15***	5**	1n.s	2*	36***	2*	49***	8***	57***	21.84	32***	12.26	
dfdC 40	282	9***	4*	3*	4**	25***	10***	36***	9***	45***	15.96	54***	19.15	
50	255	11***	3n.s	2*	2*	32***	18***	44***	17***	61***	23.92	39***	15.29	
60	257	23***	3n.s	1n.s	4**	33***	8***	54***	13***	67***	26.07	33***	12.84	
80	253	9***	0	2*	6***	13**	10***	25***	10***	35***	13.83	63***	24.90	
Combination														
1	275	23***	3n.s.	1n.s	4**	14**	7***	43***	5**	48***	17.45	33***	12.00	
2	270	22***	3n.s.	1n.s	2*	11**	4**	36***	3n.s	39***	14.44	31***	11.48	
3	264	9***	4*	1n.s	0	8n.s	0	14**	7***	21***	7.95	33***	12.50	

* p<0.05 ** p<0.01 *** p<0.001 n.s.= not significant.; Br.: Breaks, F.: Fragments, D.: Deletions, Ch. Br.: Chromosome Breaks, CF: Centric Fusions, CA: Centromeric Attenuations. (Combinations 1, 2 and 3 are 4, 6 and 8mg kg⁻¹ CDDP :20 mg kg⁻¹ dFdC respectively).

Table 2: Effect of different doses of cisplatin and gemcitabine on mitotic index, sister chromatid exchanges and replicative index of mouse bone marrow cells

Dose	Mean \pm SD		
	MI	SCEs/cell	RI
Control	30.3 \pm 2.16	3.49 \pm 0.60	1.88 \pm 0.05
6 mg kg ⁻¹ CDDP	22.6 \pm 1.14***	6.53 \pm 0.74***	1.84 \pm 0.07n.s
12 mg kg ⁻¹ CDDP	18.4 \pm 1.67***	9.57 \pm 0.75***	1.82 \pm 0.03*
24 mg kg ⁻¹ CDDP	16.0 \pm 0.71***	13.24 \pm 0.77***	1.46 \pm 0.06***
36 mg kg ⁻¹ CDDP	15.2 \pm 1.30***	13.75 \pm 0.99***	1.45 \pm 0.03***
40 mg kg ⁻¹ dfdC	26.2 \pm 1.64**	4.59 \pm 0.32**	1.84 \pm 0.01n.s
50 mg kg ⁻¹ dfdC	20.2 \pm 1.48***	4.64 \pm 0.30**	1.81 \pm 0.01*
60 mg kg ⁻¹ dfdC	17.2 \pm 1.48***	4.79 \pm 0.33***	1.71 \pm 0.07***
80 mg kg ⁻¹ dfdC	12.2 \pm 1.79***	4.95 \pm 0.18***	1.69 \pm 0.06***
Com. 1	19.6 \pm 1.67***	6.36 \pm 0.49***	1.77 \pm 0.01***
Com. 2	16.2 \pm 1.30***	5.25 \pm 0.52***	1.67 \pm 0.04***
Com. 3	10.6 \pm 2.07***	4.54 \pm 0.07**	1.62 \pm 0.02***

n.s.= not significant *p<0.05 ** p<0.01 *** p<0.001

highly significant. In the combination experiments, cells with one structural aberration were decreased significantly but cells with more than one aberration were increased.

Regarding SCE analysis, CDDP had induced-SCE following the same pattern as the CA. The increase in SCE frequency after treatment with different doses was also dose dependent. For dfdC, the mean value of the SCEs/cell was significant at p<0.01 for the doses 40 and 50 mg kg⁻¹ when compared with the control. This mean increased with the higher doses (p<0.001) (Table 2).

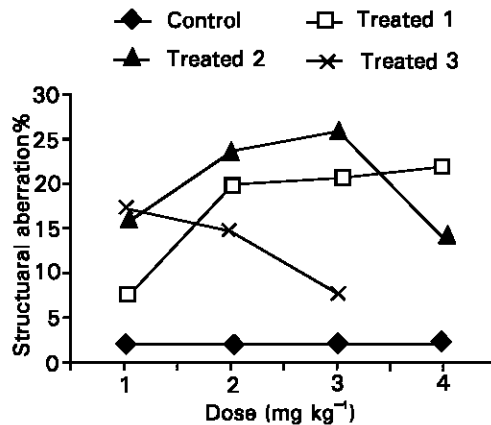


Fig. 1: Relationship between the percentage of the total structural chromosomal aberrations and different doses of cisplatin and gemcitabine (Treated 1 for cisplatin with doses of 6, 12, 24 and 36 mg kg⁻¹ body wt. Treated 2 for gemcitabine with doses of 40, 50, 60 and 80 mg kg⁻¹ body wt. Treated 3 for drug combinations, 4,6 and 8 mg kg⁻¹ CDDP: 20 mg kg⁻¹ dfdC)

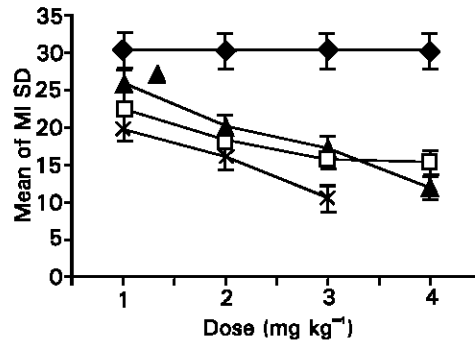


Fig. 2: Relationship between the means (\pm SD) of mitotic indices and different doses of cisplatin and gemcitabine in single and combinations (Treated 1 for cisplatin with doses of 6, 12, 24 and 36 mg kg⁻¹ body wt. Treated 2 for gemcitabine with doses of 40, 50, 60 and 80 mg kg⁻¹ body wt. Treated 3 for drug combinations, 4,6 and 8 mg kg⁻¹ CDDP: 20 mg kg⁻¹ dFdC)

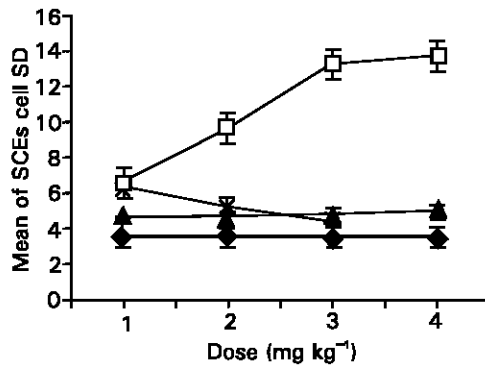


Fig. 3: Relationship between the means (\pm SD) of sister chromatid exchanges/cell and different doses of cisplatin and gemcitabine (Treated 1 for cisplatin with doses of 6, 12, 24 and 36 mg kg⁻¹ body wt. Treated 2 for gemcitabine with doses of 40, 50, 60 and 80 mg kg⁻¹ body wt. Treated 3 for drug combinations, 4,6 and 8 mg kg⁻¹ CDDP: 20 mg kg⁻¹ dFdC)

The mean values of SCEs/cell were found to be 6.36, 5.25 and 4.54 in the three drug combinations tested respectively. Although, the mean of SCEs/cell was found to decrease with increasing the dose of CDDP in the combination experiments, it was found that this mean was statistically significant at $p < 0.001$ in all the three drug combinations used (Table 2). Fig. (3) illustrates the relationship between SCEs frequencies and the different doses of CDDP and dFdC either alone or in combination.

The cytotoxic effects of cisplatin and gemcitabine were found to be dose related. Both drugs had decreased the rate of bone marrow proliferations as the dose was increased. This decrease was statistically significant ($p < 0.001$) in animals treated with the two higher doses of CDDP and at ($p < 0.01$) for the two higher doses of dFdC. In the three drug combinations it was

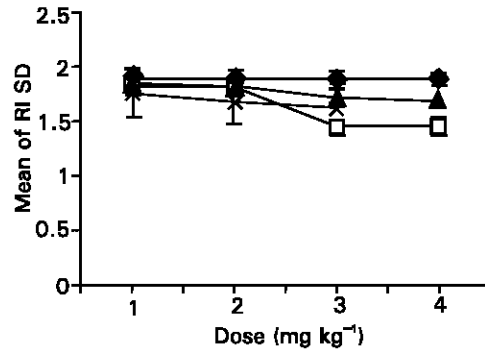


Fig. 4: Relationship between the means (\pm SD) of replicative indices and the different doses of cisplatin and gemcitabine (Treated 1 for cisplatin with doses of 6, 12, 24 and 36 mg kg⁻¹ body wt. Treated 2 for gemcitabine with doses of 40, 50, 60 and 80 mg kg⁻¹ body wt. Treated 3 for drug combinations, 4,6 and 8 mg kg⁻¹ CDDP: 20 mg kg⁻¹ dFdC)

significant at ($p < 0.001$). Fig. (2) demonstrated the negative correlation between increased drug doses and mitotic indices in both control and treated animals. Both drugs induced a decrease in RI in the bone marrow cells of mice (Table 2). This decrease was found to be dose related and significant at $p < 0.001$. In the drug combinations, the mean value of RI decreased as the dose of CDDP increased.

Discussion

Studies of genetic damage in somatic cells of patients receiving chemotherapy may not only improve our knowledge of the mutagenic potency of the treatment but also may provide important information for the evaluation of carcinogenic risk (Lambert *et al.*, 1978). dFdC and CDDP are potent antitumor agents used world wide against many forms of human cancers. CDDP has been demonstrated to have the potential for initiating genetic events in non-tumor cells in humans and in animal systems. However, to our knowledge this is the first report to demonstrate the genotoxic effects of dFdC *in vivo*.

Development of chromosomal aberrations (CA), sister chromatid exchanges (SCEs) have been commonly used as sensitive biological indicator in the mutagenic bioassays of a drug.

In the present study, the development of these mutagenic parameters were seen after cisplatin treatment *in vivo* and it supports earlier findings of its genotoxic properties (Pillaire *et al.*, 1994 and Overback *et al.*, 1996). The chromosomal aberration pattern revealed that chromatid breaks and fragments occurred more frequently. The total number of aberrant metaphases was noticed to be increased with increasing the dose. This clastogenic effect of CDDP was observed in other studies carried out on mice bone marrow cells (Tandon and Sodhi, 1985).

The inhibition of mitotic activity with increasing the dose indicates that, when cisplatin is injected into the body it binds to DNA, blocks and prolongs the division cells in the G2 phase of the cell cycle. The blockage of cells in G2 phase is related to the inhibition of chromatin

condensation (Tandon and Sodhi, 1985). Our results disagree with the study of Choudhury *et al.* (Choudhury *et al.*, 2000) who indicated that CDDP was non mitotoxic and this may be attributed for using small doses of CDDP (2, 3 and 5 mg kg⁻¹ body wt.).

Sister chromatid exchanges were increased with increasing the doses of CDDP. This significant increase of SCEs agrees well with the results of other different studies such as *in vivo* studies on patients with lung cancer (Tominaga *et al.*, 1984); testicular tumor patients (Gundy *et al.*, 1989); nonseminomatous testicular cancer patients (Gundy *et al.*, 1990) and in other samples of cancer patients (Perera *et al.*, 1990). Also agrees with *in vitro* studies on Chinese Hamster (V79) cells (Pleskova *et al.*, 1984; Solana *et al.*, 1986; Solana *et al.*, 1987 and Chibber and Ord, 1989); rabbit lymphocytes (Morrison *et al.*, 1981); human lymphocytes Tominaga *et al.*, 1984; Morrison *et al.*, 1981; Blasko *et al.*, 1987, Ohe *et al.*, 1990 and Kojima *et al.*, 1993); human malignant glioma biopsy cell line specimens (Aida and Bodell, 1987) and human salivary gland adenocarcinoma cell lines (Yoshida *et al.*, 1988). The increase in SCE frequencies observed in the present work seems to support our previous conclusion that CDDP exerts its action after the G₁ stage of the cell cycle.

Decreasing of the RI, i.e. cell cycle delay and induction of cell killing in our study agrees well with the study of Krishnaswamy and Dewey (Krishnaswamy and Dewey, 1993). They demonstrated that the cell cycle delay in Chinese hamster ovary cells treated with CDDP occurred during G₁ or late S phase.

The data presented in this paper described for the first time the *in vivo* genotoxic effects of dFdC. Clearly, the molecular mechanisms underlying the different behavior of normal cells after treatment with dFdC still warrant further investigation.

Chromosomal aberrations induced by different doses of dFdC shown in our study, was observed in the fibroblast cell lines (V79: the spontaneously transformed fibroblast cell line) previously described by Auer *et al.* (1997). It was also shown that dFdC induced cytotoxicity and DNA fragmentation in human colon cancer cells (Ren *et al.*, 1998).

In the present study, dFdC significantly decreased the mitotic index. This significant decrease in mitotic index was observed previously in *in vitro* study performed by Auer *et al.* (1997) in the cell lines V79. It was found also that dFdC has the ability to induce growth inhibitory effect on the ovarian cancer cells growing *in vitro* (Cappella *et al.*, 2001). The frequency of SCEs was increased gradually with increasing the dose of dFdC. This significant increase of SCEs was demonstrated previously with the transformed V79 cells in which up to eight-fold increase was induced by dFdC (Auer *et al.*, 1997).

Our results demonstrated that the RI was decreased gradually with increasing the dose of dFdC.

The controversy still exists about the genetic effect of the combination between CDDP and dFdC. In the present study, the CA induced were decreased with increasing the dose of CDDP in the combination although it was highly significant (p<0.001). This was agreed with other previous studies (Bergman *et al.*, 1996 and Peters *et al.*, 1995) which reported that CDDP caused a marginal decrease of the number of double strand breaks in the DNA caused by dFdC. On the contrary, the combination between CDDP and dFdC induced more than 25% DNA strand breaks

than each drug alone (Van Moorsel *et al.*, 1999). In the drug combinations studies between CDDP and other compounds, abnormal chromosome count distribution and increased incidence of structural changes were observed in peripheral lymphocytic cultures from SCLC (small cell lung cancer) patients treated with CDDP and etoposide VP-16 (PVP) (Tominaga *et al.*, 1986).

The mitotic activity of the cells decreased gradually as the dose of CDDP was increased. These results were supported by the synergistic interaction between dFdC and CDDP in inducing growth inhibitory effect in the solid tumor cell lines (Bergman *et al.*, 1996 and Peters *et al.*, 1995). The combination of dFdC and CDDP proved to produce selective cell kill in H322 cells (NSCLC cell line), although neither of the drugs was independently able to produce similar effects (Van Moorsel, 1999 and Padron *et al.*, 2000). Also, it was found that the drug combination of dFdC and CDDP has the ability to induce growth inhibitory effect on the ovarian cancer cells growing *in vitro* (Cappella *et al.*, 2001).

Regarding SCE study, the frequency of the mean SCEs/cell was decreased gradually as the dose of drug combinations increased. However, the present study disagrees with previous studies (Tominaga *et al.*, 1986 and Shinkai *et al.*, 1989). They detected a significant increase of SCE frequency in peripheral lymphocytic cultures from SCLC patients treated with CDDP and etoposide VP-16 (PVP). Shinkai *et al.* (1989), also, demonstrated a significant increase in the SCE frequencies in NSCLC patients treated with the combination of mitomycin C, vindesine and CDDP. The present study indicated that the replicative index (RI) was decreased significantly ($p < 0.001$) even with the lowest dose of the drug combination.

In conclusion, the potentiation in chromosomal aberrations and sister chromatid exchanges formation might be a result of the inhibition of DNA repair by dFdC. The synergism between dFdC and CDDP 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.

The results reported here for the bone marrow tests indicate that further observations are necessary to find out the mechanism(s) by which gemcitabine separately and/or in combination induce genetic damage.

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