Cancer Chemother Pharmacol (2001) 48: 488–492
DOI 10.1007/s002800100370

ORIGINAL ARTICLE

**Patricia Hernández · Primitivo Olivera
Alfonso Dueñas-Gonzalez · Miguel A. Pérez-Pastenes
Adolfo Zárate · Vilma Maldonado
Jorge Meléndez-Zajgla**

Gemcitabine activity in cervical cancer cell lines

Received: 20 March 2001 / Accepted: 14 July 2001 / Published online: 27 September 2001

Abstract *Purpose*: Gemcitabine (2′,2′-difluorodeoxycytidine) is an antineoplastic agent with activity against a variety of solid tumors. To investigate its in vitro activity toward cervical cancer, we exposed six cervical cancer cell lines to gemcitabine. *Methods*: Combinational cytotoxic studies using viability tests and clonogenicity assays. *Results*: Gemcitabine was cytostatic and cytotoxic in some of the lines at peak plasma concentrations similar to those achieved in clinical trials. Gemcitabine was also found to effectively synergize with cisplatin and showed a radiosensitizing effect in these cells. The cytotoxicity observed in sensitive cell lines was due to apoptosis, as demonstrated by DNA fragmentation assays. *Conclusions*: We recommend performing additional in vitro experimentation so that these results can be confirmed to support clinical trials of gemcitabine in cervical cancer patients both as first-line therapy and with concomitant radiation.

Keywords Gemcitabine · Cervical cancer · In vitro · Synergy · Radiosensitization

Introduction

Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC) is an antineoplastic agent with activity against a variety of solid tumors. Several of its metabolites are responsible for the cellular actions of the drug. This drug is phosphorylated intracellularly to produce di- and triphosphate active forms which exert their cytotoxic effects by inhibition of DNA replication and repair. Also, the triphosphate form inhibits DNA polymerase and terminates DNA chain elongation after the addition of a last nucleotide. The diphosphate form is a potent inhibitor of the enzyme ribonucleotide reductase, an action that reduces deoxynucleotide pools. Decreased cellular concentrations of deoxycytidine triphosphate permit more rapid phosphorylation of gemcitabine and reduce the metabolic clearance of gemcitabine nucleotides by deoxycytidine monophosphate deaminase [1].

Gemcitabine is a potent radiosensitizer in a variety of human tumor cell lines [2, 3, 4, 5, 6]. This effect may be due to an effective redistribution into the S phase of the cell cycle, and to depletion of the deoxyadenosine triphosphate pool, because under these conditions, it is unlikely that the DNA damage produced by the radiation can be repaired [5]. Due to its mechanism of action, gemcitabine is an attractive agent to combine with a wide range of anticancer drugs. In fact, combination studies have been performed that have demonstrated synergy with cisplatin in non-small-cell lung, ovarian, and head and neck cancer cell lines [7, 8].

This study was performed to investigate the in vitro effects of gemcitabine in cervical cancer cell lines in order to gauge its possible relevance in the clinical setting.

P. Hernández · P. Olivera · A. Dueñas-Gonzalez
V. Maldonado · J. Meléndez-Zajgla (✉)
Molecular Biology Laboratory,
Basic Research Division. Instituto Nacional de Cancerología,
Av. San Fernando no. 22, Tlalpan 14000,
Mexico City, Mexico
E-mail: jorgezajgla@hotmail.com
Tel.: +52-5-6280425
Fax: +52-5-6280426

M.A. Pérez-Pastenes · A. Zárate
Medical Physics Department,
Instituto Nacional de Cancerología,
Av. San Fernando no. 22,
Tlalpan 14000, Mexico City, Mexico

Materials and methods

Cell culture

The following human cervical cancer cells lines were used: HeLa, CasKi, SiHa and C33A (obtained from the American Type Culture Collection); and CaLo and InBl (established from Mexican cervical cancer patients [9]). The cell lines were maintained as a monolayer at 37ºC, and cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum in a humidified atmosphere comprising 5% (v/v) carbon dioxide in air. Dulbecco's modified Eagle's medium and fetal bovine serum were obtained

from GIBCO (Gaithersburg, Md.), gemcitabine was purchased from Eli Lilly (Mexico), and all other chemicals were obtained from Sigma (St. Louis, Mo.).

Cellular viability and combinational cytotoxic effects

Cells were seeded in 24- or 96-well chamber dishes and exposed to several concentrations of gemcitabine (1×10^{-4} to 1×10^{-10} *M*) for 24 h The medium was then changed and, at 24, 48 or 72 h, the cells were fixed in 70% ethanol at –20°C, washed in PBS and stained with crystal violet (1% in water). After washing, the stain was solubilized in 33% acetic acid and the absorbance determined in an ELISA reader at 570 nm [10, 11]. The analysis was performed at least in triplicate in four independent experiments. For synergism analysis, the strategy devised by Tsai et al. [12] was used. The concentrations of cisplatin tested were 5, 10, 20, 40 and 80 μ*M*, and of gemcitabine were 1×10^{-9} to 1×10^{-4} *M*. All possible combinations were tested. Cervical cancer cells were exposed to gemcitabine for 24 h, the medium in the dishes was changed and cisplatin in fresh medium added for an additional 24 h. In HeLa cells a combination assay was performed using a clonogenicity assay to ensure that reproductive death had occurred (data not shown). The combination index (CI) was defined as the sum of the relative doses (e.g. IC_{50} units) of each drug that yielded an isoeffect (e.g. 30% inhibition of cell viability) when added together.

$$\mathrm{CI} = \frac{\text{Dose of cisplatin}}{\text{IC value of cisplatin}} + \frac{\text{dose of gemcitabine}}{\text{IC value of gemcitabine}}$$

Since there were multiple drug concentrations that achieved the same isoeffect, each experiment generated a set of CI values for a particular effect level. The CI values obtained from four replicate experiments (each data point repeated eight times) were averaged to produce a single data set for a particular cell line, drug combination and effect level. The mean CI value for this set is reported as the summary measure (Table 1). Upper and lower bounds of 1.05 and 0.95 were selected for interpreting the results, so CI values lower than 0.95 or higher than 1.05 were assigned as suggestive of synergy and antagonism, respectively. Values in this range were interpreted as additive. To determine whether there were significant differences in the cell lines means between CIs and a null hypothesized CI of 1 and also between effect levels, Wilcoxon's Signed Ranks test was performed.

Clonogenicity assay and radiation enhancement ratios

Clonogenicity assays were performed in 100-mm Petri dishes. The cells to be tested were seeded and exposed for 24 h to gemcitabine. The dishes were washed twice with PBS before adding fresh culture medium. When applicable, the medium was aspirated to leave only a 1-mm film and the cells exposed to 1, 3 and 5 G doses of gamma radiation. Medium was added to the plates, which were then placed for 14 days in an incubator under an atmosphere containing 7% CO_2 at 37°C. The cells were then fixed and stained with crystal violet, and colonies counted using an inverted microscope. Enhancement ratios for radiosensitization were calculated as described previously [13]. The area under the curve (mean inactivation dose) for the irradiated cells (gemcitabine- and vehicle-treated) was calculated. The enhancement ratio was defined as the mean inactivation dose for the control cells divided by that for the drug-treated cells. An enhancement ratio greater than 1 indicated that the drug was acting as a radiosensitizer.

DNA fragmentation assays

Assays were performed using an ELISA DNA fragmentation analysis kit (Roche, Manheim Germany) as recommended by the manufacturer. Briefly, cells were seeded in 96-well chamber dishes labeled with bromodeoxyuridine for 24 h and exposed to gemcitabine (1×10^{-4} *M*) or vehicle for another 24 h. In order to detect small DNA fragments, cytosols and supernatants were obtained and subjected to an ELISA capture assay using anti-DNA antibodies for capture, and anti-bromodeoxyuridine antibodies were conjugated with peroxidase to detect binding.

Results

Exposure of HeLa cells to gemcitabine resulted in a concentration- and time-dependent decrease in cell viability as measured by a crystal violet assay (Fig. 1A). This effect occurred at concentrations as low as 1×10^{-6} *M*. With the more sensitive clonogenic assay, susceptibility to gemcitabine increased by an order of magnitude (Fig. 1B). Activity was also demonstrated in the other cervical cancer cell lines tested, although with differential sensitivity (Table 2): C33A and CasKi cells lines were the most sensitive, while SiHa cells were resistant. It is noteworthy that in the two most sensitive cell lines short-term exposure to gemcitabine induced apoptosis as demonstrated by DNA fragmentation analysis (Fig. 2), and morphological and fluorescent nuclear staining. Although the latter data are not shown, kinetic analysis

Table 1 Combinational effects of cisplatin and gemcitabine in cervical cancer cells lines (*CI* combination index, *N* = number of data points; – additivity, *S* synergy)

Cell line	Effect level								
	30%			50%			70%		
	Mean CI	*N*	Interval	Mean CI	*N*	Interval	Mean CI	*N*	Interval
SiHa	0.86	9	S	0.7	9	S	1.05	9	–
CaLo	0.87	9	S	0.57	9	S	0.23	9	S
InBl	0.97	9	–	0.91	9	S	0.30	9	S
HeLa	0.29	8	S	0.26	9	S	0.84	9	S
C33A	0.92	9	S	1.02	9	–	0.28	8	S
CasKi	0.96	9	–	0.93	8	S	0.19	9	S
Mean ± SEM	0.81 ± 0.10			0.73 ± 0.11			0.48 ± 0.15		
P-value[a]	0.027			0.046			0.046		

[a]Wilcoxon's Signed Ranks test

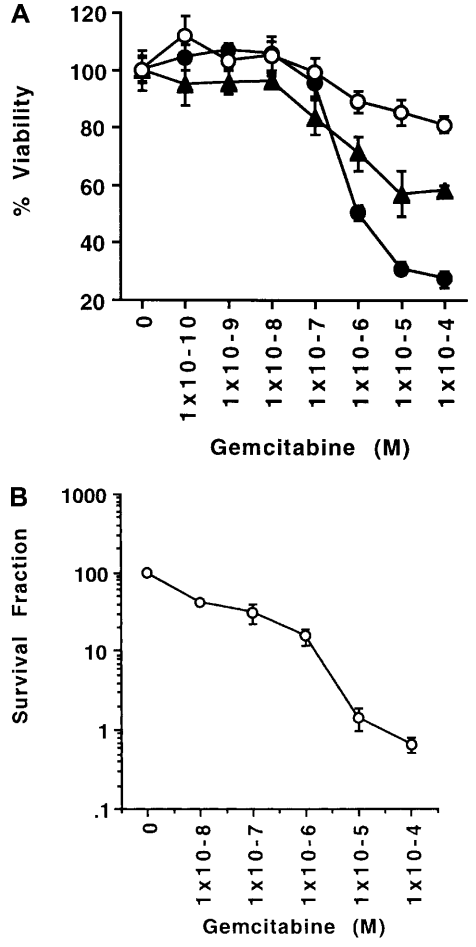

Fig. 1 **A** HeLa cells exposed to gemcitabine for 24 h. Viability, expressed as percentage of control, was measured at 24 h *(open circles)*, 48 h (*black triangles*), or 72 h (*black circles*) after the gemcitabine-containing medium had been replaced with drug-free medium. **B** Clonogenicity assay of HeLa cells exposed to different concentrations of gemcitabine for 24 h (*bars* SD)

Table 2 Chemosensitivity of cervical cancer cell lines exposed to gemcitabine for 48 h. IC_{30}, IC_{50} and IC_{70} are the concentrations of drugs required to inhibit cell growth by 30%, 50% and 70%, respectively. Values are means ± SD

Cell line	IC_{30} (μ*M*)	IC_{50} (μ*M*)	IC_{70} (μ*M*)
SiHa	0.1 ± 0.05	203 ± 12.3	1132 ± 32.4
CaLo	0.2 ± 0.06	0.89 ± 0.10	11 ± 1.32
InBl	0.07 ± 0.01	0.63 ± 0.07	9.4 ± 1.68
HeLa	0.05 ± 0.01	0.32 ± 0.02	9.0 ± 1.64
C33A	0.04 ± 0.01	0.27 ± 0.08	0.70 ± 0.12
CasKi	0.02 ± 0.09	0.11 ± 0.04	0.72 ± 0.11

with cytosols and supernatants in conjunction with morphological and nuclear staining analysis (Cancino et al., manuscript in preparation; [14, 15] has demonstrated that the cell death measured is apoptotic.

As gemcitabine is a potent radiosensitizer in a variety of solid tumors [4, 16, 17], we investigated this phenomenon in HeLa cells. As shown in Fig. 3, gemcitabine proved to be an effective radiosensitizer in these cells,

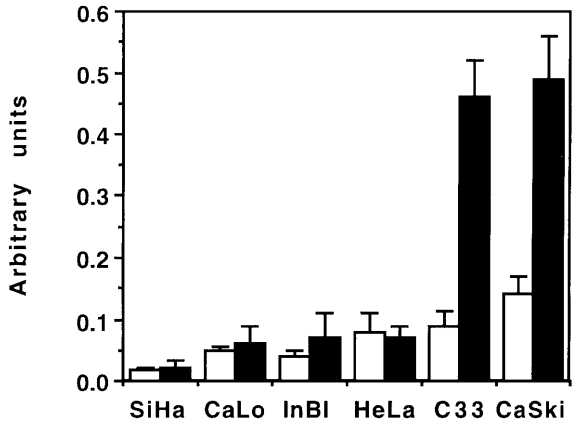

Fig. 2 DNA fragmentation analysis. Cells exposed to gemcitabine for 24 h analyzed for DNA fragmentation. The values indicated by the bars are means + SD (*empty bars* control cells, *solid bars* cells exposed to gemcitabine)

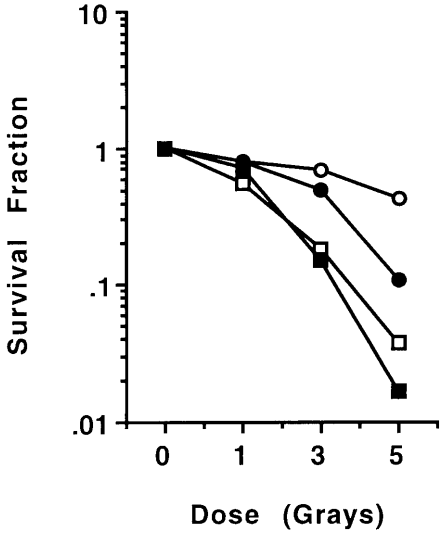

Fig. 3 Radiosensitizer effect of gemcitabine. HeLa cells were exposed to different gemcitabine concentrations for 24 h, irradiated at the indicated doses, and clonogenicity assays performed to estimate the survival fraction (*open circles* control, *black circles* 3 n*M*, *open squares* 10 n*M*, *black squares* 30 n*M*)

because it effectively synergized with radiation in a dose-dependent manner. The enhancement ratios obtained were 1.48 ± 0.05, 1.87 ± 0.02, and 2.03 ± 0.06 with 3, 10 and 30 n*M* of gemcitabine, respectively.

Because it has been reported that gemcitabine produces significant synergistic effects with other antineoplastic drugs in a variety of tumor cell lines [8, 18], we performed in vitro combination analysis in the cervical cell lines. Figure 4A, B shows a concentration response curve for cisplatin, demonstrating its activity against cervical cancer cell lines, as reported previously [9, 11]. Table 1 shows that the combination of cisplatin and gemcitabine produced consistent synergy throughout the effect levels tested ($P < 0.05$) as demonstrated by Wilcoxon's Signed Ranks test. Similar results were obtained

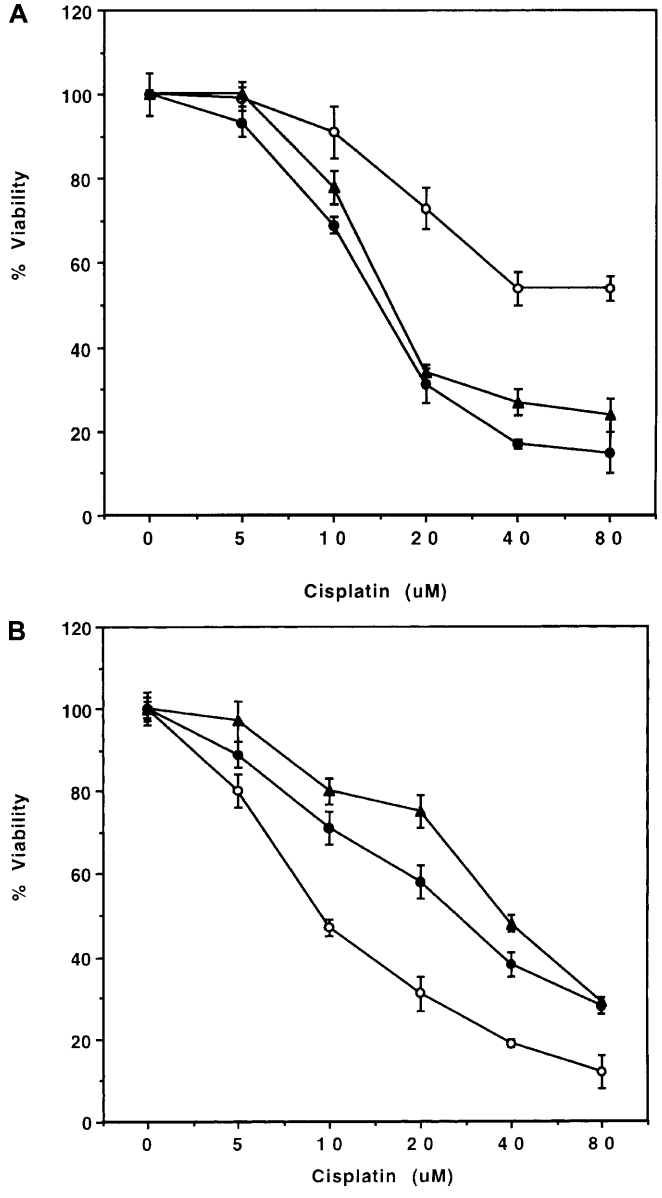

Fig. 4 A Cisplatin activity against cervical cancer cell lines. SiHa cells (*open circles*), InBl cells (*black circles*) and CaLo cells (*black triangles*) were exposed to cisplatin for 24 h. Viability, expressed as a percentage of control, was measured 24 h after exposure. **B** HeLa cells (*open circles*), CasKi cells (*black circles*) and C33A cells (*black triangles*) were exposed to cisplatin for 24 h. Viability, expressed as a percentage of control, was measured 24 h after exposure. Assays were performed in triplicate in at least four independent experiments as described in Materials and methods (*bars* SD)

using short-term analysis and clonogenicity assays (not shown). The synergy increased continuously with no significant differences among the mean effect levels ($P > 0.05$). It is noteworthy that in SiHa cells, which are resistant to gemcitabine at nanomolar concentrations, gemcitabine synergized with cisplatin at the 30% and 50% effect levels. As an example, a representative plot from which the data in Table 1 were derived is presented in Fig. 5.

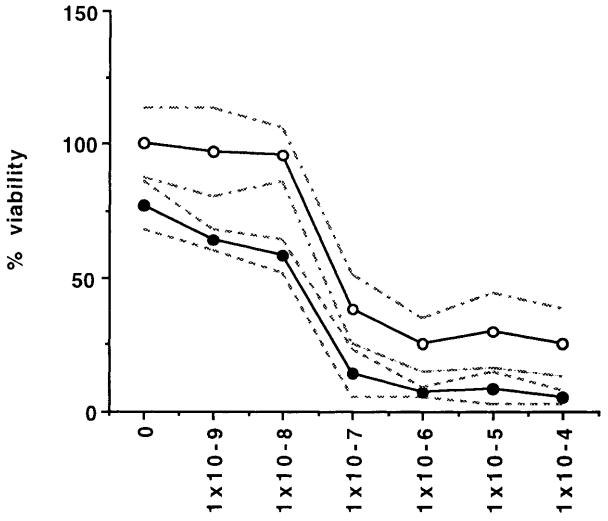

Fig. 5 Representative plot of gemcitabine and cisplatin combinational effects. InBl cells were exposed to gemcitabine for 24 h before treatment with cisplatin at 10 μM for an additional 24 h. The means and 95% confidence intervals for each dose-response curve are shown. Procedures were as described in Materials and methods

Discussion

Gemcitabine is a novel nucleoside analogue with activity in a variety of solid tumors in vitro and in vivo. We found that gemcitabine is cytotoxic in the most sensitive cervical cancer cell lines, although it also has cytostatic effects in the other cell lines (see Fig. 2). More importantly, gemcitabine has radiosensitizing effects in HeLa cells in accordance with the findings of Mose et al. [19]. Interestingly, the doses of gemcitabine needed to achieve radiosensitization of tumor cells are far below standard therapeutic doses as shown in pancreatic and head and neck cancer [20]. Thus far, there are no reported studies in which the radiosensitizing effects of gemcitabine in cervical cancer patients have been evaluated. Our results suggest that this drug is a promising agent to test in this setting.

The activity of gemcitabine as a single agent in cervical carcinoma is modest. In a phase II study [21], an 11% response rate was observed in previously treated patients with advanced or recurrent cervical cancer. In an early phase II cooperative study, Fukuoka et al, [22] observed partial responses to gemcitabine in only 8.7% of patients (2/23). In a multicenter trial conducted by the Gynecology Oncology Group [23], objective responses were achieved in only 8% of patients ($n = 26$); however, stable disease was observed in 21%.

Cisplatin and gemcitabine have activity in solid tumors, such as non-small-cell lung, ovarian, and head and neck cancers [24]. Because these agents have different mechanisms of cytotoxicity and their side effects are non-overlapping, they are suitable candidates for combination chemotherapy studies. In contrast to the low activity of gemcitabine as a single agent, in combination

with cisplatin in the same pretreated population of cervical cancer patients at standard doses it produces a much better response rate (41%, 7/17), which is even higher (57%) at non-irradiated sites [25]. A similar response rate (44%) has been observed in a recent study in which weekly cisplatin/gemcitabine was given [26]. These results suggest that the strong synergy of gemcitabine and cisplatin observed in our cervical cancer cell lines has clinical relevance.

It is noteworthy that in our in vitro studies only nanomolar concentrations of gemcitabine were required for the effect reported, suggesting that when used in combination with cisplatin, a lower dose of gemcitabine could be effective. In fact, the weekly cisplatin and low-dose gemcitabine regimen we used has demonstrated significant activity but negligible toxicity in advanced and recurrent cervical cancer patients [26]. However, this suggestion should be tested in larger comparative studies.

In conclusion, our results demonstrate that gemcitabine shows significant activity against a panel of cervical cancer cell lines, has potent radiosensitizing effects, and has effective cytotoxic synergy with cisplatin. Because cisplatin is considered the most active single agent for cervical cancer and the combination of the two agents shows remarkable activity in vitro and in advanced or recurrent patients, a clinical trial as first-line chemotherapy is clearly warranted. Moreover, the radiosensitizing properties of gemcitabine should be tested in cervical cancer patients either alone or in combination with cisplatin.

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