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Clinical report

Eradication of osteosarcoma lung metastasis using intranasal gemcitabine

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We sought to determine whether gemcitabine, a new pyrimidine antimetabolite, could inhibit the growth of human osteosarcoma cells (OS) *in vitro* and *in vivo*. Four human OS cell lines (MG-63, TE-85, SAOS-2 and SAOS-LM7) were used to assess the activity of the drug *in vitro*. Gemcitabine caused growth inhibition and cell death in all four cell lines as measured using the MTT and colony-forming assays (IC_{50} = 6.5 nM–9 μM and 7–14 nM, respectively). Using our newly developed human SAOS-LM7 OS lung metastasis mouse model, we assessed the *in vivo* activity of gemcitabine given i.p. and intranasally (i.n.). Mice were treated twice weekly for 3 weeks and then once weekly for 3 weeks using either i.p. or i.n. gemcitabine starting 4 weeks after tumor cell injection. The i.p. injection, at 120 mg/kg, resulted in a decrease in lung weights and the size of the nodules. However, no significant reduction in the number of metastatic nodules was seen (control median: >200 versus gemcitabine median: 150, $p = 0.084$). In contrast, the number of lung metastases was significantly decreased in mice that received i.n. gemcitabine at 15 (median: 1; range: 0–115, $p < 0.005$) and 12 mg/kg (median: 41; range: 7–163, $p = 0.005$) when compared with control mice (median: >200). Intranasal therapy is a non-invasive method of drug delivery and has the advantage of targeting the lung, resulting in a higher drug concentration in the tumor area. In our study, i.n. instillation of gemcitabine inhibited the growth of lung metastases at an 8- to 10-fold lower dose than that used i.p. and appeared to be more effective in eradicating OS lung nodules. Because the lung is the most common site of OS metastasis, our data suggest that i.n. gemcitabine may be a novel therapeutic approach in the treatment of OS lung metastases. [© 2002 Lippincott Williams & Wilkins.]

Key words: Gemcitabine, intranasal therapy, lung metastasis, osteosarcoma.

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Introduction

Osteosarcoma (OS), which is the most frequently diagnosed primary malignant bone tumor, continues to be a challenge in oncology. Most patients with OS die of pulmonary metastasis. The use of aggressive combination chemotherapy plus surgery for OS results in a 2-year metastasis-free survival rate of 60–65%.[1–6] However, this rate has not changed over the past 15 years despite alterations in the chemotherapeutic regimens used. Patients who have a relapse are particularly difficult to manage as their therapeutic options are limited.

Gemcitabine (2′,2′-difluorodeoxycytidine), an agent that is not routinely used in initial chemotherapeutic regimens for OS, is metabolized intracellularly by nucleoside kinases to the active diphosphate and triphosphate nucleosides. The cytotoxic effect of this agent is attributed to a combination of the two actions of these nucleosides which inhibit DNA synthesis and cell proliferation in S phase, resulting in cell accumulation in late G_1/S phase.[7] Gemcitabine has proven efficacy against human leukemia cell lines *in vitro*, and a number of solid murine and human tumors *in vivo*.[7,8] In addition, animal toxicology studies have demonstrated that myelosuppression is the major adverse reaction and dose-limiting toxic effect of gemcitabine.[9] Phase I clinical trials confirmed this dose-limiting toxic effect and found minimal non-hematologic toxicity.[10,11] Furthermore, prolonged infusions of low-dose gemcitabine have been well tolerated, and antitumor activity has been demonstrated in pretreated advanced soft tissue sarcoma and breast cancer patients.[12,13]

Gemcitabine is routinely administered via i.v. infusion. However, a major limitation of systemic administration of drugs is reduced drug

concentration in the tumor area. Because OS metastasizes almost exclusively to the lung, we hypothesized that direct delivery of gemcitabine intranasally may enhance the concentration of it in the tumor area, allowing the use of lower doses and yielding less systemic toxicity.

Materials and methods

Reagents and drugs

Eagle's minimal essential medium (EMEM), Hanks' balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+}, non-essential amino acids (NEAA), sodium pyruvate (SP), MEM vitamins, L-glutamine and 2.5%. trypsin were purchased from Biowhittaker (Walkersville, MD). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA). All reagents were free of endotoxins as determined using the Limulus amebocyte lysate assay (sensitivity limit, 0.025 ng/ml), which was purchased from Sigma (St Louis, MO). Gemcitabine, which was purchased from Eli Lilly (Indianapolis, IN), was dissolved in normal saline.

Cell lines

Three human OS cell lines (MG-63, SAOS-2 and TE-85) were purchased from ATCC (Rockville, MD). SAOS-LM7 cells were derived from lung metastases of nude mice that were injected with SAOS-LM6 cells.[14] All of these cells were maintained in EMEM supplemented with 1 mM NEAA, 1 mM SP, 2 mM L-glutamine, 2-fold MEM vitamins and 10% heat-inactivated FBS (56°C for 30 min). The monolayer cultures were maintained in 75-cm^2 tissue culture flasks (Costar, Cambridge, MA) at 37°C in a humidified 5% CO_2 incubator and tested periodically for mycoplasma contamination using the reverse transcriptase-polymerase chain reaction assay (primer purchased from Sigma-Genosys, The Woodlands, TX). SAOS-2 cells were verified to be free of pathogenic murine viruses (Microbiological Associates, Bethesda, MD).

Cytotoxicity assay

The level of cytotoxicity was determined using the MTT assay.[15] First, tumor cells were seeded into 96-well tissue culture plates. After an attachment period of 6 h at 37°C in a 5% CO_2 humidified incubator, the cells were treated with fresh medium alone or medium containing gemcitabine for 48 h. During the last 2 h of incubation, 40 μl MTT (2.5 mg/ml; Sigma) was added into each well (0.42 mg/ml). At the end of the incubation, the MTT was removed and the cells were lysed with dimethylsulfoxide. Metabolically viable cells were monitored for conversion of MTT to formazan using a Titertek Multiskan 96-well microtiter plate reader at 570 nm (Flow Laboratories, Helsinki, Finland). The level of cytotoxicity was calculated using the following formula: cytotoxicity (%) = $(A-B/A) \times 100$, in which A is the 570-nm absorbance of cells treated with medium alone and B is the 570-nm absorbance of cells treated with gemcitabine. Each experiment was repeated at least 2 times.

Colony-forming assay

Target cells (300 cells/well) were seeded into six-well plates (Costar, Cambridge, MA) and incubated overnight. The cultures were then exposed to medium alone or media containing different concentrations of gemcitabine for 24 h. At the end of treatment, the conditioned media were removed, and the cultures were washed twice with HBSS, re-fed with fresh medium and incubated for 12–14 days. Also, triplicate wells were used for each group. At the end of this incubation period, the cells were fixed with 10% formalin and stained with 0.04% crystal violet. A colony was defined as growth of more than 30 cells. Additionally, the plating efficiency and percentage of surviving cells were calculated using colony counts and formulas. Specifically, the plating efficiency (%) = (number of colonies present in the control well/number of cells seeded into well) $\times$ 100, while the survival fraction (%) = (number of colonies present in the drug-treated well/number of colonies present in the untreated control well) $\times$ 100. Each experiment was repeated at least 3 times.

Mouse model

Specific pathogen-free athymic male nude mice (4–5 weeks old) were purchased from Charles River (Wilmington, MA). The mice were maintained in an animal facility approved by the American Association of Laboratory Animal Care in accordance with the current regulations and standards of the US Department of Agriculture, Department of Health and Human Services, and National Institutes of Health. The mice were housed five to a cage and kept in a laminar flow cabinet under specific pathogen-free

conditions for 1–2 weeks before being used. Intravenous injection of SAOS-LM7 cells (1×10^6 cells/0.2 ml) harvested in subconfluence and in mid-log phase resulted in microscopic lung metastases by 3–5 weeks and visible lung nodules by 6 weeks (data not shown).

Intraperitoneal injection of gemcitabine

The optimal i.p. gemcitabine dose for antitumor activity has been shown to be 120 mg/kg given twice weekly.[8,16] In the present study, gemcitabine was dissolved in 0.9% NaCl to reach a final concentration of 15 mg/ml. Four weeks after the injection of SAOS-LM7 cells, the mice received an i.p. injection of 120 mg/kg gemcitabine in 0.2 ml of normal saline twice weekly for 3 weeks and then once weekly for 3 weeks; mice in the control group received an i.p. injection of with 0.2 ml of normal saline on the same schedule. The mice were sacrificed 24 h after completing therapy. Next, their lungs were removed, weighed and fixed in Bouin's solution. The effect of gemcitabine was determined by counting the lung metastases under a dissecting microscope and measuring the size of the metastatic nodules using calipers.

Intranasal therapy using gemcitabine

Four weeks after the tumor cell injection, mice were anesthetized using isoflurane (Burns Veterinary Supply, Farmers Branch, TX) with the Multistation Rodent Anesthetic Delivery and Scavenging System (MDS Matrix, Orchard Park, NY) and then held vertically upright by the scruff of the neck. Gemcitabine (12 or 15 mg/kg) in 25 μl of normal saline was administered i.n. drop by drop[17] twice weekly for 3 weeks and then once weekly for 3 weeks. The mice were sacrificed the day after completing therapy. Then their lungs were removed, weighed and fixed in Bouin's solution. Metastases were counted and measured as described above.

Cell cycle analysis

Target cells (0.8×10^6) were set into T75 flasks. After 6 h, the cells were synchronized by incubation with serum-free medium for 24 h and then treated with medium alone, medium containing 2 or 5 nM gemcitabine for 45 h. At the end of incubation, the cells were washed twice with PBS (4°C), harvested by trypsinization, washed again with PBS (4°C), resuspended as single cells with PBS (4°C), and then fixed with ethanol (final concentration was 70%) at 4°C overnight. The cells were washed with PBS (4°C), resuspended with PBTB (0.5 ml Tween 20, 0.5 g bovine serum albumin and PBS up to 100 ml), incubated with 0.01% final concentration of RNase (Sigma) at 37°C for 30 min (shaking the tube twice during incubation), and then washed again with PBTB. The cells (1×10^6) were resuspended in 1 ml PBTB (4°C) and stained with 20 μl of propidium iodide (solved in 95% ethanol; Sigma).[18] The samples were analyzed using an Epics Profile flow cytometer (Coulter, Hialeah, FL).

Results

Effect of gemcitabine on human OS cells *in vitro*

The level of cytotoxicity was determined using the MTT and colony-forming assays. As shown in Figures 1 and 2, all of the OS cell lines were sensitive to gemcitabine. The TE-85 cell line was the most sensitive, having an IC_{50}=6.5–7.0 nM.

Effect of i.n. gemcitabine on experimental OS lung metastases

We next determined whether OS lung metastases were sensitive to gemcitabine *in vivo* using our

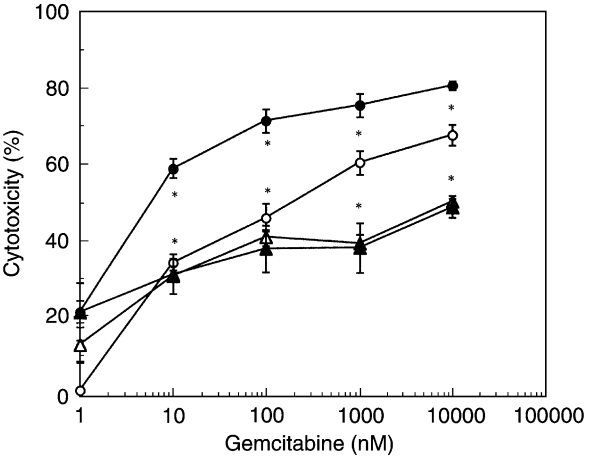

Figure 1. Cytotoxicity of gemcitabine against MG-63 (open circles), TE-85 (filled circles), SAOS-2 (open triangles) and SAOS-LM7 (filled triangles) cells. Target cells (5×10^3) were incubated with gemcitabine for 48 h. Cytotoxicity was then determined using the MTT assay. Triplicate wells were used for each group. Each point represents the mean ± SD. One of at least three independent experiments. *p=0.017–0.001.

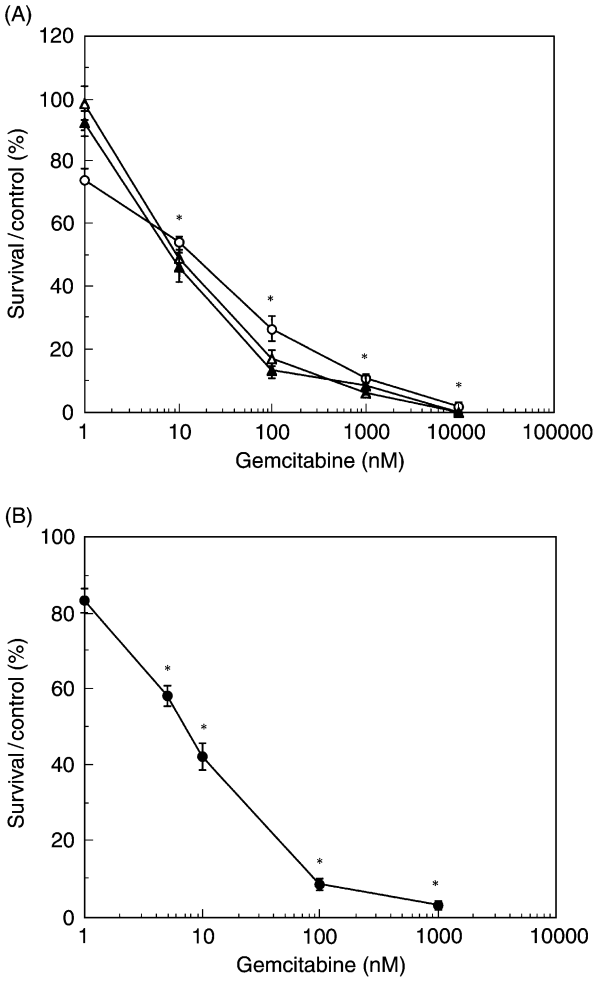

Figure 2. Effect of gemcitabine on MG-63 (open circles), TE-85 (filled circles), SAOS-2 (open triangles) and SAOS-LM7 (filled triangles) cells. Target cells (300 cells/well) were exposed to gemcitabine for 24 h. Survival fractions were determined using the colony-forming assay after 12–14 days. Triplicate wells were used for each group. Each point represents the mean ± SD. One of at least three independent experiments. (A) *p=0.0001–0.0003; (B) *p=0.0001–0.0054.

newly developed nude mouse model. Four weeks after the injection of SAOS-LM7 cells, mice received gemcitabine either via i.n. instillation or i.p. injection twice weekly for 3 weeks and then once weekly for 3 weeks.

Intranasal gemcitabine was effective in reducing the number of pulmonary metastases at both 15 and 12 mg/kg (Table 1 and Figure 3). The median lung weights and number of pulmonary lesions in the mice that received gemcitabine were both significantly reduced compared with those in the control animals. In addition, the metastatic nodules in the former group were significantly smaller as 97% of them were 0.5 mm or less. In contrast, the control mice had large tumors throughout their lungs, ranging in size from 3.5 to 7.4 mm. Gemcitabine given via i.p. injection also resulted in a significant reduction in lung weights. There appeared to be a reduction in the number of lung metastases as well, but it was not statistically significant (p=0.084). Tumor size was affected, however, with 99% of the tumor nodules measuring 0.5 mm or less. Taken together, we interpret the reduction in lung weights and tumor sizes to mean that i.p. gemcitabine was indeed efficacious. However, a dose 8–10 times greater than that of the i.n. one was used.

Effect of gemcitabine on cell cycle progression

As shown at Figure 4 and Table 2, the SAOS-2 and SAOS-LM7 cells were blocked at the G_1/S phase and cells accumulated in the S phase. The fraction of cells in the S phase was significantly increased from 20.3 to 76.1% and 16.4 to 76% in SAOS-2 and SAOS-LM7 cells, respectively. The fraction of cells in the G_1 and G_2 phase was decreased from 54 to 11% (G_1) and 26 to 13% (G_2) for the SAOS-2 cells; and 64 to 20% (G_1) and

Table 1. Effect of gemcitabine administration on OS lung metastases[a]

Treatment	Incidence[b]	Median number of nodules (range)	Median lung weight mg (range)
Control	5/5	>200 (>200–>200)	795 (365–1336)
i.p. (120 mg/kg)	5/5	150 (40–235)[c]	246 (231–274)[d]
i.n. (15 mg/kg)	3/5	1 (0–115)[e]	213 (191–321)[d]
i.n. (12 mg/kg)	5/5	41 (7–163)[e]	242 (234–280)[d]

[a]Mice were injected i.v. with 10^6 SAOS-LM7 cells, separated into groups of four on week 5, then treated with i.n. or i.p. gemcitabine twice weekly for 3 weeks and then once weekly for 3 weeks. Mice were sacrificed on week 11, 24 h after completing therapy. One of two representative experiments.
[b]Number of tumor-positive mice/total number of mice.
[c]p=0.084.
[d]$p < 0.025$.
[e]$p < 0.01$.

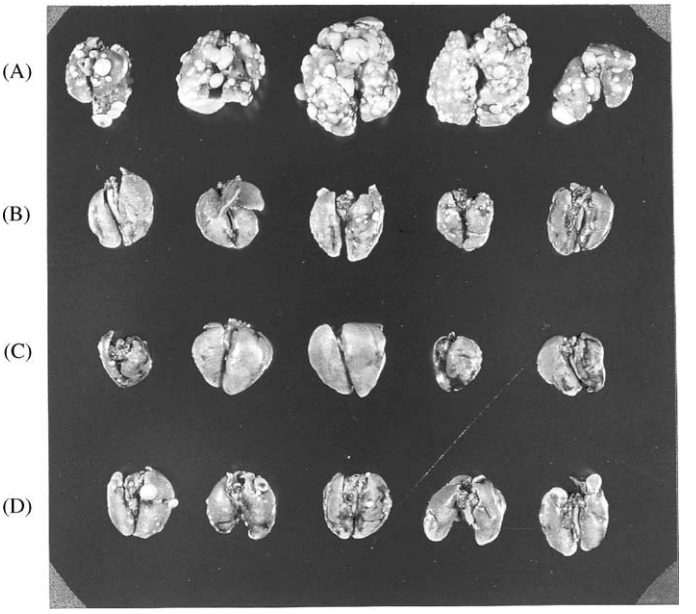

Figure 3. Effect of i.n. gemcitabine on OS lung metastases. Mice were injected i.v. with 10^6 SAOS-LM7 cells. Four weeks later, the mice were injected i.p. with (A) normal saline or (B) 120 mg/kg gemcitabine twice weekly for 3 weeks and then once weekly for 3 weeks. Intranasal gemcitabine was given at (C) 15 or (D) 12 mg/kg twice weekly for 3 weeks and then once weekly for 3 weeks. Mice were sacrificed 24 h after completing therapy, and their lungs were removed and fixed in Bouin's solution. One of two independent experiments.

20 to 4% (G_2) for the SAOS-LM7 cells. The increase in the S phase and decrease in the G_1/G_2 phase was dose dependent for gemcitabine (Figure 4 and Table 2). This change in cell cycle for the SAOS-2 and SAOS-LM7 cells was not evident in MG-63 or TE-85 cells.

Discussion

Gemcitabine is a pyrimidine antimetabolite that inhibits both RNA and DNA viruses in cell culture.[19,20] It had been shown to have broad-spectrum antitumor activity.[7,8,16] Also, preclinical studies in mice demonstrated activity against melanoma, lymphosarcoma, squamous cell carcinoma of the head and neck, human soft tissue sarcoma, and ovarian cancer.[7,8,16] The maximum tolerated dose in these preclinical studies was established to be 120 mg/kg.[8,16] However, the present study demonstrated that gemcitabine has significant activity against human OS cells both *in vitro* and *in vivo*.

The antitumor effect of gemcitabine appears to be schedule dependent, as 3-day-interval injections were more effective than daily injections in several preclinical models.[7,8,16] Therefore, in our *in vivo* studies, we adopted the twice-weekly schedule for 3 weeks followed by the once-weekly schedule for 3 additional weeks. Therapy was started 4 weeks after tumor cell injection, which is when microscopic tumors are present in the lungs (data not shown). Additionally, the animals were sacrificed at week 11, which is when tumors are expected to be visible as described previously (data not shown). Indeed, our control animals had multiple large tumor nodules and lung weights 3–4 times greater than that of normal animals on week 11 (expected normal lung weight: approximately 200 mg) (Table 1). Our studies also demonstrated that i.p. gemcitabine at 120 mg/kg was somewhat effective against OS lung metastases. In addition, animals that received i.p. gemcitabine had smaller tumors. We also observed a decrease in lung weights in these mice, although their median number of metastases was not significantly different from that in the control animals.

In the majority of published *in vivo* studies, gemcitabine was administered i.p.[8,16] Our study is the first to evaluate the i.n. route as a potential method of delivering this drug. The administration of 8- to 10-fold less of the drug i.n. resulted in superior efficacy (Table 1) in that the median number of pulmonary metastases was significantly reduced in addition to the size of these metastases and total lung weights. Following treatment using 15 mg/kg gemcitabine, two of five animals had no visible or microscopic tumors. We hypothesize that this was a result of achieving a higher drug concentration in the lung compared with that achieved using i.p. administration.

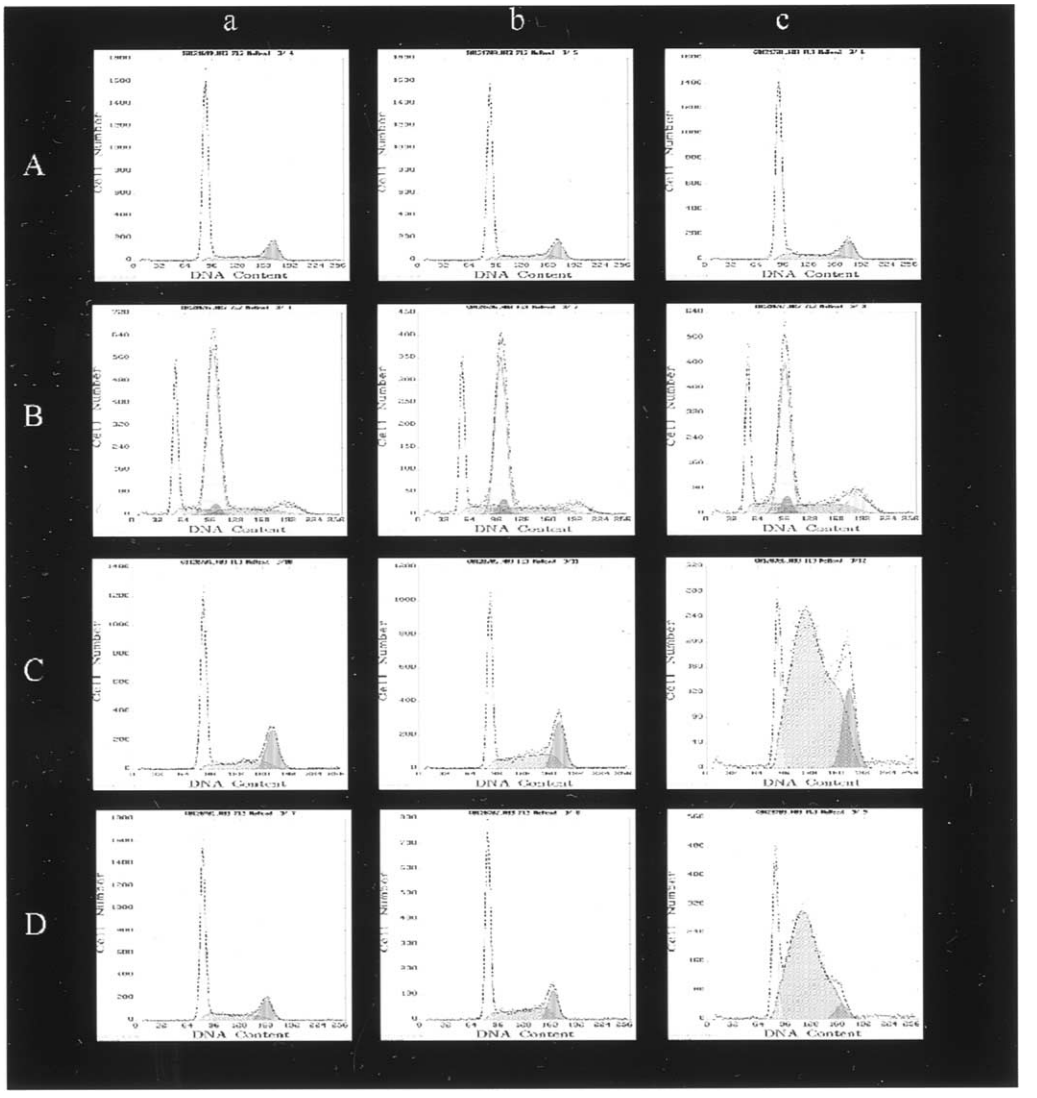

Figure 4. Effect of gemcitabine on cell cycle. Mg-63 (A), TE-85 (B), SAOS-2 (C) and SAOS-LM7 (D) cells were treated with medium alone (a), 2 nM (b) or 5 nM (c) of gemcitabine for 45 h. At the end of incubation, the cells were washed and fixed with ethanol overnight. Then the cells were incubated with RNase and stained with propidium iodide. The cell cycle was analyzed by flow cytometry. One of three independent experiments.

Table 2. Effect of gemcitabine on cell cycle progression (percent in each phase)

Gemcitabine (nM)	MG-63			TE-85			SAOS-2			SAOS-LM7[a]		
	G_1	S	G_2	G_1	S	G_2	G_1	S	G_2	G_1	S	G_2[b]
0	72	14	14	71	18	11	54	20	26	64	16	20
2	69	17	14	64	24	12	42	32	26	55	29	16
5	69	17	14	61	26	14	11	76	13	20	76	4

[a]Target cells were treated with 0, 2 and 5 nM of gemcitabine for 45 h.
[b]The DNA fractions of cell cycle phase were measured by propidium iodide staining and flow cytometry.
[c] One of three independent experiments.

Phase II trials with gemcitabine have shown activity against breast cancer,[13] advanced transitional cell carcinoma of the bladder,[21] pancreatic cancer,[22] soft tissue sarcoma,[12] leiomyosarcoma[23] and non-small cell lung cancer;[24] stabilization of chemoresistant pelvic OS was also demonstrated in one patient.[25] Our data indicate that gemcitabine should be considered in the treatment of OS pulmonary metastases. In addition, we suggest that i.n. or perhaps aerosol drug delivery may be superior to

other types of delivery for this disease due to the location of the tumor. Aerosol delivery has long been used in the treatment of asthma[26] and it is an efficient way to deliver a drug to a target organ. In particular, we have demonstrated that the efficacy of aerosol delivery of liposome-encapsulated 9-nitro-camptothecin (9-NC) using an identical mouse model system.[27] A phase I aerosol trial of 9-NC is now under way. Having demonstrated the principle and efficacy of aerosol therapy in preclinical models, we believe it is time to explore this approach in the treatment of OS in the lung.

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