Non-toxic and short treatment with gemcitabine inhibits *in vitro* migration of HT-1080 cells

Corinne Alcouffe[a], Jean Boutonnat[a], Jocelyne Clément-Lacroix[b], Mireille Mousseau[c] and Xavier Ronot[a]

Gemcitabine has demonstrated clinical activity in solid tumors. Several *in vitro* studies were carried out regarding its metabolism, toxicity and cell cycle effects, but none was done on the anti-metastasis potential of the drug. We sought to determine the effect of gemcitabine concentrations on migration velocity of HT-1080 cells at concentrations which do not alter cell cycle progression and proliferation. Cells were treated for 1 or 5 h at IC_{10-70} of gemcitabine in order to estimate its effects on viability, proliferation and migration capacity using flow cytometry and microscopy imaging, respectively. The gemcitabine treatment for 1 h had no effect on cell proliferation, viability, cycle or migration on HT-1080 cells. Even though the 5 h of exposure at IC_{10}, IC_{20} and IC_{50} concentrations did not affect cell viability, proliferation and cell cycle repartition, the mean velocity of HT-1080 dramatically decreased by 50 and 30%, respectively. Gemcitabine at IC_{70} concentrations for 5 h of exposure first induced a time course inhibition of proliferation, together with a decrease in viability and altered cell morphology, and then inhibited cell migration by 50%. These data suggest the possibility to couple the anti-migratory property of gemcitabine with the known anti-tumoral effect in the treatment of tumors with high metastatic potential. *Anti-Cancer Drugs* **15:803–807**
© 2004 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2004, **15**:803–807

Keywords: antimetabolite, cytometry, metastasis, proliferation

[a]Laboratoire de Dynamique Cellulaire, Ecole Pratique des Hautes Etudes, Grenoble, France, [b]Equipe Dynacell, UMR CNRS 5525, Grenoble, France and [c]Service d'Oncologie Médicale, Centre Hospitalier Universitaire de Grenoble, Grenoble, France.

Sponsorship: This research was supported by La Ligue Nationale Contre le Cancer (Comité de la Drôme et de l'Isère), GEFLUC Grenoble-Dauphiné-Savoie, Association ESPOIR Isère contre le Cancer and AGARO (Association Grenobloise d'Aide à la Recherche en Oncologie).

Correspondence to X. Ronot, Laboratoire de Dynamique Cellulaire, EPHE, UMR CNRS 5525, IFRT 130, Domaine de la Merci, 38706 La Tronche Cedex, France. Tel: +33 4 56 52 00 48; fax: +33 4 56 52 00 22; e-mail: xavier.ronot@ujf-grenoble.fr

Received 26 January 2004 **Revised form accepted** 4 June 2004

Introduction

Gemcitabine [2′,2′-difluorodeoxycytidine (dFdC)] is a cytotoxic deoxycytidine analog with proven clinical activity against non-small cell lung, ovarian, pancreas and breast carcinoma [1–3].

After intracellular diffusion, the drug is activated by phosphorylation in active gemcitabine diphosphate (dFdCDP) and gemcitabine triphosphate (dFdCTP) metabolites. The dFdCTP is incorporated into DNA and RNA [4,5]. It then allows the incorporation of a single subsequent nucleotide, blocking DNA polymerase. The proofreading enzymes are unable to remove gemcitabine from this position [5]. Consequently, cells are arrested in $G_{0/1}$ or S phase and apoptosis in solid tumor cells is triggered [6–8].

dFdCDP inhibits the deoxyribonucleotide reductase resulting in a depletion of the cellular dCTP pool that potentiates the effects of the drug. Indeed dCTP (i) competes with dFdCTP for DNA incorporation, (ii) inhibits deoxycytidine kinase, which phosphorylates dFdC, and (iii) activates dCMP deaminase, which deaminates dFdCMP into the inactive dFdUMP [9–12]. Consequently, the depletion of the dCTP pool enhances the incorporation of dFdCTP into DNA and also the retention of dFdC into cells.

In order to inhibit proliferation and induce apoptosis, effective chemotherapy requires exposure to active drug concentrations for a period of time, sufficient enough to target a maximum number of tumor cells which are in the DNA synthesis phase. However, some anti-neoplasics combine anti-proliferative and anti-metastasis activity. Concerning cell migration, no proven relationship was demonstrated between cell growth and invasion for gemcitabine; that is why this drug is usually combined with taxol or cisplatin in breast and lung cancers [13–17]. However, in pancreatic cancer, which gives highly hepatic and peritoneal metastases, as chemotherapy combination is still inefficient, gemcitabine is used in mono-chemotherapy [18,19]. Using a newly developed human SAOS-LM7 OS lung metastasis model, Jia *et al.* showed a decrease in the number of lung metastases in gemcitabine-treated mice, suggesting this drug as a novel therapeutic approach in the treatment of osteosarcoma lung [20].

0959-4973 © 2004 Lippincott Williams & Wilkins

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

This approach reveals whether or not gemcitabine, independent of its anti-proliferative activity, has an anti-migratory potential on HT-1080, a cell line known to display high migratory potential [21].

Material and methods

Chemical

Gemcitabine (Gemzar, Lilly France, Saint-Cloud, France) was dissolved in PBS and stored at –20°C until use.

Cell culture

The HT-1080 cell line (ATCC, CCL-121) was grown in antibiotic-free RPMI 1640 (Life Technologies, Cergy-Pontoise, France) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies) and 2 mM L-glutamine (Life Technologies). Cells were cultured at 37°C in a humidified 5% CO_2 incubator and tested to ensure freedom from mycoplasma contamination.

Determination of inhibitory concentrations (IC)

Cytotoxicity of gemcitabine was assessed by cell numeration. Cells were cultured, in triplicate, at 6×10^4 cells/well density in 24-well plates (Falcon, BD Biosciences, Le Pont de Claix, France), in 700 µl medium and grown overnight. Twenty-four hours later, cells were treated with 0, 0.1, 1, 10, 100 and 1000 nM gemcitabine, and the plates were incubated for 24 h with the drug. The cells were detached with 0.5 ml 0.1% trypsin/0.021% EDTA (Life Technologies) and counted to determine inhibitory concentrations IC_{10}, IC_{20}, IC_{50} and IC_{70}. Each value represents the mean of three independent experiments.

Cell proliferation and cell viability

Drug treatment was performed with gemcitabine at the different IC during 1 and 5 h in order to analyze cell proliferation and viability.

For proliferation experiments, cells were labeled with the PKH67 kit, containing a 1 mM PKH67 solution in ethanol and an iso-osmotic aqueous solution Diluent C (Sigma-Aldrich, St-Quentin Fallavier, France). PKH67 was described to be equally divided between each daughter cell upon cell division [22,23]. To summarize, cells were washed with 1 ml PBS in 15-ml centrifuge tubes (Falcon) and resuspended in Diluent C at 10^7 cells/ml. The cells were then rapidly mixed with a freshly prepared PKH67 solution at a concentration of 5 µM in Diluent C and incubated for 3 min to ensure homogeneous staining. The labeled step was stopped by addition of an equal volume of FBS for 1 min and followed by an equal volume of complete culture medium. After three rinses in PBS, the cells were resuspended in 5 ml culture medium.

After cell detachment by 0.1% trypsin/0.02% EDTA (Life Technologies), PKH67-labeled cells were incubated with 2.5 µg/ml propidium iodide (PI) (Sigma-Aldrich) and analyzed everyday for 3 days. PKH67 and PI associated fluorescences were measured by flow cytometry (FACS-Calibur; BD Biosciences). This combination of fluorescent probes allows us to measure the proliferation of living cells. The proliferation index of each cell suspension was determined by calculating the PKH mean fluorescence intensity at each point and dividing this value by the mean of PKH67 fluorescence intensity at time 0 (mean $\text{intensity}_{\text{day 0}}$/mean $\text{intensity}_{\text{day 1–3}}$) [23].

Cell cycle analysis

Drug treatment was performed with gemcitabine at the different IC during 1 and 5 h in order to analyze the cell cycle.

On the first and third day following treatment, cells were detached, enumerated and fixed in 70% ethanol for at least 30 min. Fixed cells were washed with PBS to remove ethanol and stained with 10 µg/ml PI and 0.5 mg/ml RNase (Sigma-Aldrich). Cells were kept at room temperature for 30 min before being analyzed by flow cytometry.

Flow cytometry

For flow cytometry, 10^4 events for each sample analyzed using a FACSCalibur (BD Biosciences) equipped with a 15 mW 488 nm argon ion laser. PKH67 green fluorescence (FL1) was collected through a 530/30 nm bandpass filter and PI red fluorescence (FL2) through a 585/42 nm bandpass filter. Data were elaborated using CellQuest II software (BD Biosciences). Electronic compensation FL2 – FL1 was around 40%.

Cell migration analysis

Drug treatment was performed with gemcitabine at the different IC during 1 and 5 h in order to analyze cell migration.

Real-time digital image processing was used to acquire images and calculate centroid position as a function of time. Phase contrast image sequences were acquired using an imaging workstation composed of an inverted microscope (Axiovert 100M; Carl-Zeiss, Le Pecq, France) equipped with a temperature and CO_2 controlled humidified chamber (37°C, 5% CO_2) (M Incubator; Carl-Zeiss), automated shutters (Uniblitz), a CCD camera (Micromax 1300-Y; Ropper Scientific, Evry, France) and a motorized stage (Marzhauser; Carl-Zeiss). The system is coupled with image processing software (Metamorph; Ropper Scientific), which identifies boundaries from phase-contrast images, and measures cell centroid position and cell speed between each field. Between 10 and 20 cells per field in eight different fields were scanned every 15 min for 8 h. Mean velocity was

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

determined for at least 200 cells per sample and calculated with Microsoft Excel software.

Statistics analysis

Samples were run in triplicate and each experiment was repeated 3 times. Student's *t*-test was used to evaluate differences between treated groups. The comparisons giving $p < 0.05$ were regarded as significantly different.

Results

Determination of inhibitory concentrations of gemcitabine

IC_{10}, IC_{20}, IC_{50} and IC_{70} were determined in treated HT-1080 cells after a 24-h exposure to graded concentrations of the drug. Proliferation rapidly decreased for low concentrations of gemcitabine: 1, 3 and 8.5 nM was respectively required for inhibiting cell growth by 10, 20 and 50%. Then proliferation decreased slowly and 70% inhibition of cell growth was obtained with a gemcitabine concentration of 95 nM.

Cell viability and morphology

We next assessed cell viability during 3 days of culture after 1 and 5 h of gemcitabine treatment, using the PI exclusion test (Fig. 1).

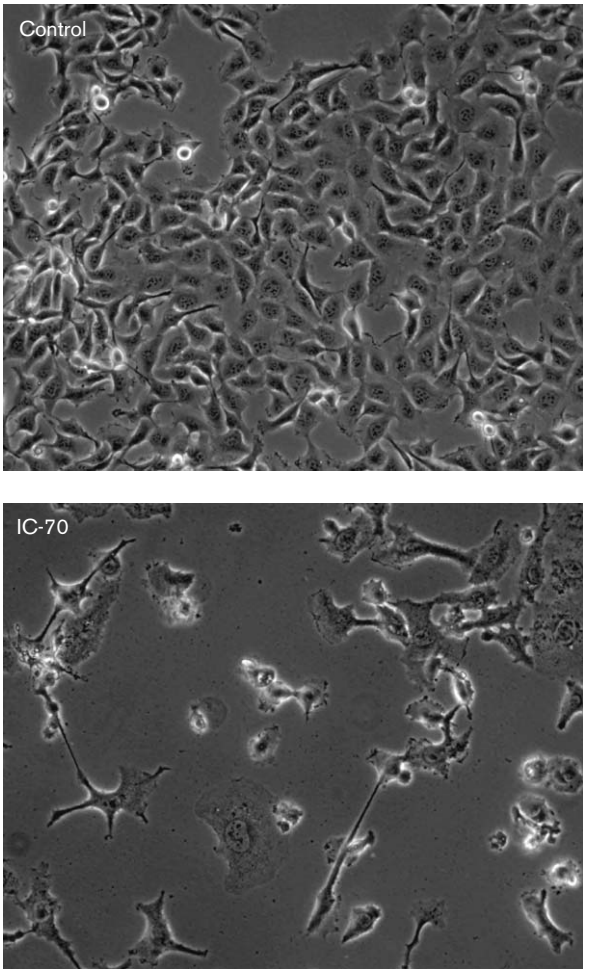
The mean fraction of viable cells in control samples was 95% on day 0 and 95–97% during the subsequent culture period (days 1–3). No significant effect of 1 h of gemcitabine treatment was observed ($0.05 < p < 0.93$). Only 5 h treatment by IC_{70} concentration slightly altered cell viability ($p = 0.003$). The fraction of viable cells decreased from 94% on day 1 to 82 and 73% on days 2 and 3, respectively. Although the viability decrease was low after 5 h of exposure, HT-1080 exhibited an altered morphology characterized by an increase in cell size, large cytoplasmic expansions and cell debris.

Cell proliferation

To explore the effect of gemcitabine on cell kinetics, proliferation was measured using the vital fluorescent probe PKH67 which displays the ability to be partitioned equally between each daughter cell upon cell division. Figure 2 shows the PKH67 fluorescence intensity distributions during 3 days of culture following the 1- and 5-h gemcitabine treatments. For both control, 1 and 5 h drug-treated samples with IC_{10}, IC_{20}, IC_{50} and IC_{70}, the fluorescence distribution profiles displayed an active proliferation from day 0 to 3, with a decrease of mean fluorescence intensity by half between each day of culture.

Proliferation indexes were 2, 5 and 11 for control samples on days 1–3, respectively. After 1 h of exposure, no significant effect on HT-1080 proliferation was observed on any of the concentration used ($0.49 < p < 0.98$). For a 5-h treatment, similar proliferative profiles were observed for IC_{10}, IC_{20} and IC_{50} ($0.06 < p < 0.94$). However, IC_{70} induced a proliferation arrest for 2 days followed by recovery on day 3 ($p = 0.03$). For this concentration and treatment duration, proliferation indexes were then 1, 1 and 4 on days 1–3.

Fig. 1



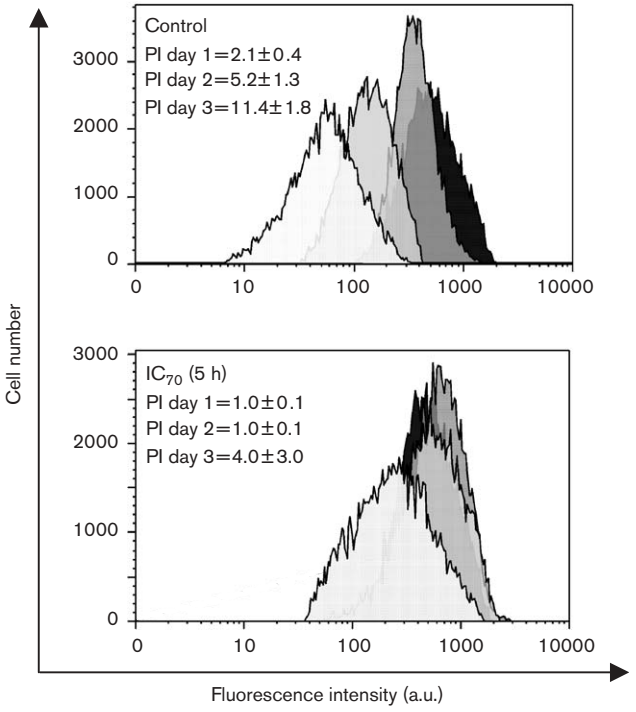
Effect of 5 h gemcitabine exposure at 0 and IC_{70} concentration on cell morphology, after 3 days of culture (magnification × 100).

Cell cycle distribution

We next evaluated cell cycle distribution at day 1 and at day 3 after 5 h of exposure to gemcitabine (Table 1). Gemcitabine did not induce significant variation in cell cycle distribution at IC_{10}, IC_{20} and IC_{50} ($0.2 < p < 0.84$, data not shown). Changes in cell cycle distribution were observed for IC_{70} drug exposure ($p = 0.01$). Cell cycle distributions were 8/77/15% and 29/63/8% for $G_{0/1}$, S and $G_2 + M$ phases, respectively, on days 1 and 3. Therefore, the majority of cells accumulate in the S phase. However, on day 3, the fraction of cells in S and $G_2 + M$ phases was

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

Fig. 2



Flow cytometric analysis of PKH67-associated fluorescence of HT-1080 cells during 3 days of culture after 1 (A) and 5 (B) h exposure at 0 and IC_{70} concentration of gemcitabine. Proliferation indexes (Pi) were determined by ratio of mean fluorescence intensities ($intensity_{day\ 0}/intensity_{day\ 1-3}$).

Table 1 Cell cycle distribution of HT-1080 control cells and treated at IC_{70} for 5 h (samples were analyzed by flow cytometry on days 1 and 3)

Phase	Control		IC_{70}	
	Day 1	Day 3	Day 1	Day 3
$G_{0/1}$	46 ± 0.5	56 ± 2.7	8 ± 0.5	29 ± 1.2
S	36 ± 1.5	26 ± 3.0	77 ± 0.6	63 ± 2.6
G_2 + M	18 ± 2.0	18 ± 0.5	15 ± 0.4	8 ± 4.0

slightly decreased and the $G_{0/1}$ fraction of cells increased in parallel.

Cell migration

Since proliferation kinetics and viability of HT-1080 cells were not modified after 1 and 5 h of exposure from IC_{10} to IC_{70} of gemcitabine, cell migration was performed by real-time digital image processing. No significant differences in mean velocity between the control and the 1-h treated cells were observed ($p = 0.89$) The control and IC_{10}- to IC_{70}-treated cells migrated with a mean velocity of 25 μm/h (25.5 ± 10, 26.3 ± 11, 25.7 ± 10, 25.2 ± 10 and 24.6 ± 9.7 μm/h, respectively, for control and IC_{10}, IC_{20}, IC_{50} and IC_{70}).

On the contrary, the mean velocity of cells treated for 5 h at IC_{10}, IC_{20}, IC_{50} and IC_{70}, respectively, decreased to 12 ± 6.5, 12.5 ± 7, 17.6 ± 9.7 and 13.9 ± 7.3 μm/h, whereas the migration speed was 25 ± 10 μm/h for control cells ($p = 0.0001$). Therefore, the mean velocity of cells treated for 5 h was decreased by about 50% at IC_{10}, IC_{20} and IC_{50}, and by 30% at IC_{70}.

Discussion

Gemcitabine is an antiproliferative agent with proven clinical activity in solid tumors. Accordingly, it is of interest to determine the effect of gemcitabine separately on cell behavior at preclinical level using a cellular model (fibrosarcoma cells) known to display a high metastasis potential [21].

Our study was undertaken to gauge the possible relevance of gemcitabine as an anti-metastasis agent independently of the anti-proliferative properties. Many authors have shown cell cycle effects induced by anticancer drugs, but only a few of these drugs, such as biphosphonates, inhibit both proliferation and cell migration *in vitro* [24]. According to clinical protocols, gemcitabine IC_{10-70} were tested through short treatment of 1 and 5 h in order to study the relationship between cell proliferation and migration. In the literature, the IC values determined for the HT-1080 cells were lower than those determined for lung cell lines like H460, H322 and LL [6], demonstrating the higher sensitivity of HT-1080 cells to gemcitabine.

One hour of gemcitabine treatments had no effect on cell proliferation, viability, cycle or migration on HT-1080 cells. On the other hand, cell behavior following a 5-h treatment was quite different. Even though IC_{10}, IC_{20} and IC_{50} concentrations did not affect cell viability, proliferation and cycle repartition, the mean velocity of HT-1080 cells dramatically decreased by 50% for IC_{10} and IC_{20}, and by 30% for IC_{50} concentrations. A slightly mean velocity decrease observed with IC_{50} concentration could be explained by a larger cell distribution in the different classes of velocity. Cells exposed at IC_{50} concentration migrated at a lower velocity than control cells but most of them were ranging from 10 to 30 μm/h while the majority of cells exposed at IC_{10} and IC_{20} concentrations had a velocity of 10–20 μm/h (data not shown).

Gemcitabine at IC_{70} concentration for 5 h of exposure induced a time course inhibition of proliferation together with a decrease in viability and altered cell morphology, and also inhibited cell migration by 50%. Proliferation inhibition was correlated with a cell cycle accumulation in the S phase but HT-1080 growth was partially recovered. Using a human ovarian cell line, Cappela *et al.* have described this proliferation recovery, which follows a cell

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

cycle freezing by gemcitabine [8]. In their study, they showed that low concentrations of gemcitabine (10 nM) induced temporary cell cycle arrest followed by a complete growth recovered within a few hours.

Since there are no reported studies on effects of gemcitabine on *in vitro* cell migration, the contribution from our study is that: (i) gemcitabine, when used in monotreatment, shows a specific effect on cell migration, which is independent of cell proliferation kinetics, and (ii) the cell velocity is not correlated with the S cell cycle phase perturbation.

In addition to the known anti-tumoral effect of gemcitabine, our data suggest an effect on the metastatic dissemination like those used in used in chemoradiotherapy based on gemcitabine in the treatment of advanced squamous cell carcinoma of the head and neck; gemcitabine is delivered at a dosage of 100 mg/m^2, i.e. 10-fold less than the concentration used in monotherapy [25].

Acknowledgments

The authors are particularly grateful to Magali Barbier, Brigitte Peyrusse, IAB-UJF and Shoma Chatterjee.

References

1 Hui YF, Reitz J. Gemcitabine: a cytidine analogue active against solid tumors. *Am J Health Syst Pharm* 1997; **54**:162–170.
2 Thomas A, Steward W P. Gemcitabine—a major advance? *Ann Oncol* 1998; **9**:1265–1267.
3 Boven E, Schipper H, Erkelens CA, Hatty SA, Pinedo HM. The influence of the schedule and the dose of gemcitabine on the anti-tumour efficacy in experimental human cancer. *Br J Cancer* 1993; **68**:52–56.
4 RuizvanHaperen VW, Veerman G, Boven E, Noordhuis P, Vermorken JB, Peters GJ. Schedule dependence of sensitivity to 2′,2′-difluorodeoxycytidine (Gemcitabine) in relation to accumulation and retention of its triphosphate in solid tumour cell lines and solid tumours. *Biochem Pharmacol* 1994; **48**:1327–1339.
5 Huang P, Chubb S, Hertel LW, Grindey GB, Plunkett W. Action of 2′,2′-difluorodeoxycytidine on DNA synthesis. *Cancer Res* 1991; **51**: 6110–6117.
6 Kroep JR, Giaccone G, Tolis C, Voorn DA, Loves WJ, Groeningen CJ, *et al.* Sequence dependent effect of paclitaxel on gemcitabine metabolism in relation to cell cycle and cytotoxicity in non-small-cell lung cancer cell lines. *Br J Cancer* 2000; **83**:1069–1076.
7 Tolis C, Peters GJ, Ferreira CG, Pinedo HM, Giaccone G. Cell cycle disturbances and apoptosis induced by topotecan and gemcitabine on human lung cancer cell lines. *Eur J Cancer* 1999; **35**: 796–807.
8 Cappella P, Tomasoni D, Faretta M, Lupi M, Montalenti F, Viale F, *et al.* Cell cycle effects of gemcitabine. *Int J Cancer* 2001; **93**: 401–408.
9 Heinemann V, Hertel LW, Grindey GB, Plunkett W. Comparison of the cellular pharmacokinetics and toxicity of 2′,2′-difluorodeoxycytidine and 1-beta-D-arabinofuranosylcytosine. *Cancer Res* 1988; **48**: 4024–4031.
10 Heinemann V, Xu YZ, Chubb S, Sen A, Hertel LW, Grindey GB, Plunkett W. Cellular elimination of 2′2′-difluorodeoxycytidine 5′-triphosphate:a mechanism of self-potentiation. *Cancer Res* 1992; **52**:533–539.
11 Heinemann V, Schulz L, Issels RD, Plunkett W. Gemcitabine: a modulator of intracellular nucleotide and deoxynucleotide metabolism. *Semin Oncol* 1995; **22**:11–18.
12 Kroep JR, van Moorsel CJ, Veerman G, Voorn DA, Schultz RM, Worzalla JF, *et al.* Role of deoxycytidine kinase (dCK), thymidine kinase 2 (TK2), and deoxycytidine deaminase (dCDA) in the antitumor activity of gemcitabine (dFdC). *Adv Exp Med Biol* 1998; **431**:657–660.
13 Kroep JR, Giaccone G, Voorn DA, Smit EF, Beijnen JH, Rosing H, *et al.* Gemcitabine and paclitaxel: pharmacokinetic and pharmacodynamic interactions in patients with non-small-cell lung cancer. *J Clin Oncol* 1999; **17**:2190–2197.
14 Murad AM, Guimaraes RC, Aragao BC, Scalabrini-Neto AO, Rodrigues VH, Garcia R. Phase II trial of the use of paclitaxel and gemcitabine as a salvage treatment in metastatic breast cancer. *Am J Clin Oncol* 2001; **24**:264–268.
15 Jassem J, Krzakowski M, Roszkowski K, Ramlau R, Slominski JM, Szczesna A, *et al.* A phase II study of gemcitabine plus cisplatin in patients with advanced non-small cell lung cancer: clinical outcomes and quality of life. *Lung Cancer* 2002; **35**:73–79.
16 Zoli W, Ricotti L, Barzanti F, Dal Susino M, Frassineti GL, Milandri C, *et al.* Schedule-dependent interaction of doxorubicin, paclitaxel and gemcitabine in human breast cancer cell lines. *Int J Cancer* 1999; **80**:413–416.
17 Ricotti L, Tesei A, De Paola F, Ulivi P, Frassineti GL, Milandri C, *et al.* *In vitro* schedule-dependent interaction between docetaxel and gemcitabine in human gastric cancer cell lines. *Clin Cancer Res* 2003; **9**:900–905.
18 Moreaux J, Catala M, Marzano L. Results of the surgical treatment of pancreatic cancer. Study of a series of 96 surgically treated patients. *Gastroenterol Clin Biol* 1984; **8**:11–16.
19 Kozuch P, Petryk M, Evans A, Bruckner HW. Treatment of metastatic pancreatic adenocarcinoma: a comprehensive review. *Surg Clin North Am* 2001; **81**:683–690.
20 Jia SF, Worth LL, Turan M, Duan XP, Kleinerman ES. Eradication of osteosarcoma lung metastasis using intranasal gemcitabine. *Anticancer Drugs* 2002; **13**:155–61.
21 Haga A, Nagase H, Kito H, Sato T. Invasive properties of cadmium-resistant human fibrosarcoma HT-1080 cells. *Cancer Biochem Biophys* 1997; **15**:275–284.
22 Boutonnat J, Barbier M, Muirhead K, Mousseau M, Ronot X, Seigneurin D. Optimized fluorescent probe combinations for evaluation of proliferation and necrosis in anthracycline-treated leukaemic cell lines. *Cell Prolif* 1999; **32**:203–213.
23 Rousselle C, Barbier M, Comte V, Alcouffe C, Clement-Lacroix J, Chancel G, *et al.* Innocuousness and intracellular distribution of PKH67: a fluorescent probe for cell proliferation assessment. *In Vitro Cell Dev Biol Anim* 2001; **37**:646–655.
24 Senaratne SG, Pirianov G, Mansi JL, Arnett TR, Colston KW. Bisphosphonates induce apoptosis in human breast cancer cell lines. *Br J Cancer*, 2000; **82**:1459–1468.
25 Aguilar-Ponce J, Granados-Garcia M, Villavicencio V, Poitevin-Chacon A, Green D, Duenas-Gonzales A, *et al.* Phase II trial of gemcitabine concurrent with radiation for locally advanced squamous cell carcinoma of the head and neck. *Ann Oncol* 2004; **15**:301–306

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.