Cancer Chemother Pharmacol (2008) 61:291–299
DOI 10.1007/s00280-007-0474-z

ORIGINAL ARTICLE

Pharmacodynamic characterization of gemcitabine cytotoxicity in an in vitro cell culture bioreactor system

Mark N. Kirstein · Richard C. Brundage · Megan M. Moore · Brent W. Williams · Lisa A. Hillman · Jason W. Dagit · James E. Fisher · Paul H. Marker · Robert A. Kratzke · Douglas Yee

Received: 30 October 2006 / Accepted: 14 March 2007 / Published online: 12 April 2007

Abstract
Purpose Gemcitabine, a pyrimidine nucleoside, is approved for the treatment of non-small cell lung cancer, pancreatic carcinoma, and breast cancer. Chemotherapy regimens are determined experimentally with static tissue culture systems, animal models, and in Phase I clinical trials. The aim of this study was to assess for gemcitabine-induced cell death following infusion of drug under clinically-relevant conditions of infusion rate and drug exposure in an in vitro bioreactor system.
Methods To estimate an appropriate harvest time for cells from the bioreactor after drug treatment, we estimated the temporal relationship between gemcitabine treatment for 1 h and cell death at a later time point with monolayer growth assays (i.e., static culture). Afterward, 5.3 mg gemcitabine was infused over 0.5 h in the bioreactor, followed by mono-exponential decay, simulating patient concentration–time profiles ($n = 4$). Controls were run with drug-free media ($n = 4$). Cells were harvested from the bioreactor at a later time point and assessed for cell death by flow cytometry.
Results According to monolayer growth assay results, cytotoxicity became more apparent with increasing time. The E_{Max} for cells 48 h after treatment was 50% and after 144 h, 93% ($P = 0.022$; t test), while flow cytometry showed complete DNA degradation by 120 h. Gemcitabine was infused in the bioreactor. The gemcitabine area under the concentration–time curve (AUC) was 56.4 μM h and the maximum concentration was 87.5 ± 2.65 μM. Flow cytometry results were as follows: the G1 fraction decreased from 65.1 ± 4.91 to 28.6 ± 12% ($P = 0.005$) and subG1 increased from 14.1 ± 5.28 to 42.6 ± 9.78% ($P = 0.004$) relative to control. An increase in apoptotic cells was observed by TUNEL assay.
Conclusions The in vitro bioreactor system will be expanded to test additional cell lines, and will serve as a useful model system for assessing the role of drug pharmacokinetics in delivery of optimized anticancer treatment.

Keywords Gemcitabine · Bioreactor · SubG1 · MDA-MB-231 cells · Pharmacokinetics

M. N. Kirstein (✉)
Department of Experimental and Clinical Pharmacology, College of Pharmacy and Comprehensive Cancer Center, University of Minnesota, 308 Harvard St SE, Minneapolis, MN 55455, USA
e-mail: kirst002@umn.edu

R. C. Brundage · M. M. Moore · B. W. Williams · L. A. Hillman · J. W. Dagit · J. E. Fisher
Department of Experimental and Clinical Pharmacology, College of Pharmacy, University of Minnesota, 308 Harvard St SE, Minneapolis, MN 55455, USA

J. W. Dagit · J. E. Fisher
Clinical Pharmacology Analytical Services, University of Minnesota, Minneapolis, USA

P. H. Marker
Department of Medicine and Stem Cell Institute, University of Minnesota, Minneapolis, USA

R. A. Kratzke · D. Yee
Department of Medicine and Cancer Center, University of Minnesota, Minneapolis, USA

Introduction

Gemcitabine, a pyrimidine nucleoside, is approved for the treatment of non-small cell lung cancer, pancreatic carcinoma, breast, and ovarian cancer. The agent undergoes metabolism by plasma and liver cytidine deaminase to

form 2′,2′-difluorodeoxyuridine (dFdU), a compound with little antitumor activity. Greater than 75% of administered gemcitabine is excreted either unchanged, or as the dFdU metabolite into the urine. Membrane transport of gemcitabine is mediated by equilibrative nucleoside transporters [1–3]. Once inside the cell, the parent molecule undergoes intracellular phosphorylation by deoxycytidine kinase at the tumor site to form difluoro-dCMP, and is phosphorylated further by other intracellular kinases to form difluoro-dCDP and difluoro-dCTP [4–6]. The diphosphate metabolite (dFdCDP) inhibits ribonucleotide reductase, an enzyme that catalyzes formation of deoxynucleosides required for DNA synthesis [7]. The triphosphate (dFdCTP) is incorporated into DNA, followed by one more deoxynucleotide molecule. This stops DNA polymerase, resulting in chain termination [8–10]. It is theorized that continuous infusions are superior to shorter infusions due to greater active metabolite accumulation.

Plasma gemcitabine clearance varies 4–30-fold between patients receiving the same dose, and dFdU production also varies 2–11-fold between patients [11, 12]. It is unclear how this variability affects intracellular metabolite production and antitumor activity. A strong relationship has been associated between the AUC for dFdU and cytidine deaminase expression levels and activity. Progression rate and survival are also significantly related to expression levels and activity of this enzyme for pancreatic tumors [13]. Though it has not been directly demonstrated, production of active phosphorylated metabolites in patients with high cytidine deaminase activity are likely decreased, leading to poorer response rates. However, measurement of phosphorylated metabolites at the tumor site is difficult, meaning that most investigation of phosphorylated metabolite levels are conducted with peripheral blood mononuclear cells as surrogate [11, 14].

We recently reported the development of an in vitro bioreactor system to evaluate chemotherapy drug regimens [15]. This system allows for growth of cells in a bioreactor cartridge, and the ability to infuse chemotherapy to simulate concentrations measured in biological samples (i.e., human plasma). The gemcitabine concentrations can be controlled through dose, infusion rate, or pump flow rate to simulate concentration–time curves that are clinically relevant. Later, the cells can be harvested and analyzed to determine relative cytotoxicity. In the case of agents that are activated at the tumor site (e.g., 5′-FU, gemcitabine) the ability to control these drug concentrations could optimize exposure and enhance antitumor effect, making this in vitro system useful for determining infusion rate–cytotoxicity relationships. We have previously shown that MDA-MB-231 breast cancer cells grown in this system and treated with gemcitabine infused over 30 min results in a significant increase of cells in S-phase and no change in subG1. For cytotoxic drugs, cell death is a more desirable endpoint than S-phase arrest. Agents such as hydroxyurea and cisplatin in addition to gemcitabine are known to cause cell cycle arrest during S-phase without necessarily leading to cell death, depending upon the concentrations used [16, 17]. Here, we have analyzed whether gemcitabine, infused to give clinically relevant AUCs can result in the death of tumor cells grown in the bioreactor system.

Materials and methods

Cell culture

All cell culture operations were carried out in a sterile class II biological safety cabinet (Sterilgard III Advance, Baker Company, Sanford, Maine, USA). The MDA-MB-231 cell line was cultured in Roswell Park Memorial Institute (RPMI) 1640 media (Invitrogen, Carlsbad, CA, USA) containing 5% fetal bovine serum (Biosource, Rockville, MD, USA), 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). The cells were grown in 25 and 75-cm^2 flasks (Corning, NY, USA) in a humidified incubator at 37°C with 5% CO_2 (Forma Scientific, Marietta, OH, USA). Cells were stained with trypan blue (Sigma-Aldrich, St. Louis, MO, USA) and then counted on a hemacytometer.

Monolayer growth assays

To assess sensitivity of the cells to chemotherapy, monolayer growth assays were carried out using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) [18]. Using 24-well plates (Corning, NY, USA), cells were seeded at a density of 15,000 cells per well. After adhering overnight, the cells were treated with gemcitabine or drug-free media as control. The following serial dilutions of gemcitabine were used in triplicate: 0.01, 0.05, 0.1, 0.25, 0.75, 1, 5, 10, and 100 μM. Treatment was for 1 h in a humidified incubator at 37°C in 5% CO_2. Afterward, cells were washed once with phosphate buffered saline (PBS), and fresh drug-free media was added. Three independent experiments testing 48, 120, and 144 h incubation periods were done. After the incubation periods, cells were treated with 0.17 mg/ml MTT in 1× PBS for 4 h in a humidified incubator at 37°C in 5% CO_2. Afterwards, the MTT solution was removed and the MTT Formazon (formed by the live cells) was resuspended in isopropyl alcohol (99 + %) (Acros, Morris Plains, NJ, USA). The absorbance was measured by a spectrophotometer set at 550 nm. The triplicate values were averaged and the background absorbance was subtracted. The fraction of the control was calculated for each

treated set and plotted versus the concentration of gemcitabine treatment. The Hill Equation (shown below) [19] was fit to the absorbance data using maximum-likelihood estimation as implemented in ADAPT II [20]. Model parameters that were estimated included the maximum effect (E_{MAX}), the 50% inhibitory concentration (IC_{50}), and the effect slope (γ):

$$\text{Absorbance} = 1 - \left(\frac{E_{\max} \cdot C^{\gamma}}{IC_{50}^{\gamma} + C^{\gamma}}\right).$$

Static culture

The MDA-MB-231 cells were seeded (500,000) into 100 mm^2 plates (BD Falcon, Bedford, MA, USA), and allowed to adhere overnight. Cells were treated with gemcitabine (0.25 μM) for 1 h, and matched with controls, treated with drug free media in a humidified incubator at 37°C with 5% CO_2. After treatment, the cells were washed once with PBS, and fresh drug-free media was added. Cells were either harvested 2 or 5 days after treatment (three independent experiments). After trypsinization, 300,000 cells were treated with 1% paraformaldehyde for 15 min at 4°C, followed by washing with PBS. Cells were suspended into a 70:30 (v:v) ethanol:PBS mixture and stored at –20°C. Cells were later prepared for cell cycle analysis as described below.

Bioreactor preparation

Bioreactor procedures were developed using the manufacturer's specifications. The Polysulfone Plus cartridges (FiberCell Systems, Frederick, MD, USA) were prepared as described previously [15]. Briefly, 5×10^6 cells were seeded into the sterile enclosed extracapillary space (ECS) surrounding the hollow fiber capillaries. The hollow fiber cartridge was connected by gas permeable tubing to a reservoir bottle containing growth media. The cartridge was mounted on a duet pump that infuses sterile media from the reservoir bottle through the gas permeable tubing where it is oxygenated and saturated with CO_2. After the media passes through the fibers it returns to the reservoir bottle and is reinfused into the cartridge. The flow rate was approximately 20 ml/min on the duet pump. The duet pump and bioreactor cartridges were maintained in a humidified incubator at 37°C with 5% CO_2 for 2 weeks and then again for 120 h after gemcitabine ($n = 4$) or drug-free media (control) treatment ($n = 4$).

Gemcitabine infusions

The gemcitabine infusions were carried out as described previously [15]. From previous work, MDA-MB-231 cells grown in the bioreactor arrest in S-phase when harvested 2 days after gemcitabine treatment. Similar conditions of drug dose, infusion rate, and pump flow rates were used for these experiments. Briefly, 5.3 mg gemcitabine was infused over 0.5 h into a central reservoir by a programmable syringe pump (Cole Parmer, Vernon Hills, IL, USA). Compared to our previously published methods, we now examined cells at 5 days after drug treatment. The media within the central reservoir was continuously stirred and was pumped out through the fiber lumens at a flow rate of 5 ml/min, delivering the drug to the cells. Media in the central reservoir was maintained at approximately 80 ml. The flow through was collected in the elimination reservoir. Control experiments were set up in a similar manner with drug free media. The flow rates from the diluent reservoir and central reservoir were controlled with a Masterflex L/S digital drive peristaltic pump (Cole Parmer). After the infusion, the hollow fiber cartridge was restored to the duet pump in the humidified incubator at 37°C in 5% CO_2 until the date of cell harvest. At 120 h after treatment, the cells were harvested and prepared for cell cycle analysis similar to cells grown in static cultures. Total cell recovery and viability was also determined with trypan blue.

Pharmacokinetic analysis

Pharmacokinetic analysis was performed as described previously with modifications [15]. Briefly, 0.25 ml samples were collected from the extracapillary space (ECS) and lumen at the following time points: 0.08, 0.25, and 0.47 h during the infusion and 0.08, 0.17, 0.33, 0.5, 0.83, 1, 1.25, and 1.5 h after the end of drug infusion. Samples were stored at –80°C until analysis. The internal standard, 2′-deoxycytidine, was added, the samples acidified with 20 μl of 70% perchloric acid, and 25 μl of the resultant supernatant was injected onto an isocratic high-performance liquid chromatographic instrument with uv detection (267 nm). This method was determined to be accurate and precise (total assay %CV 3.7) at gemcitabine concentrations ranging from 2 to 200 μM. A three-compartment model was fit to the gemcitabine lumen and ECS concentration versus time data using maximum-likelihood estimation as implemented in Adapt II (Fig. 1) [20]. Lumen concentrations were modeled simultaneously with ECS concentrations. Estimated three-compartment model parameters included volume of the lumen (V_2) and the intercompartmental rate constants between the lumen and ECS (K_{23} and K_{32}). The volume of the central reservoir (V_1) was fixed to 80 ml, the intercompartmental flow rate from compartment 1 to 2 and the elimination flow rate from the lumen to the waste reservoir were fixed to the media flow rate that was calculated by dividing the total volume

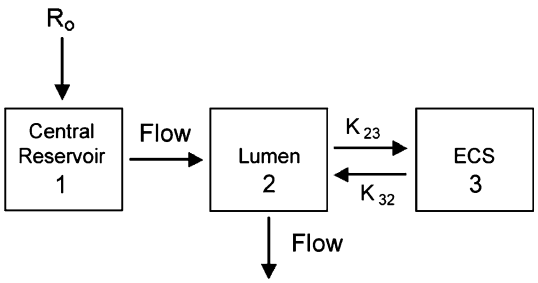

Fig. 1 Three-compartment model describing gemcitabine bioreactor disposition. Model parameters include the volume of the central reservoir that was fixed to 80 ml (V_1), volume of the lumen (V_2), volume of the ECS that was fixed to 2.5 ml (V_3), and the intercompartmental rate constants between the lumen and ECS (K_{23} and K_{32}). Flow from the central reservoir to the lumen, and from the lumen to the waste reservoir was fixed to the experimentally-determined flow rate

of media recovered in the waste bottle divided by the elapsed time from the start of infusion. The volume of the ECS (V_3) was fixed to 2.5 ml, which is the measured volume in the ECS as per manufacturer specifications. The model parameters for each experiment were used to simulate the concentration–time profiles for the lumen and ECS from which the area under the lumen and ECF concentration–time curves were calculated from zero to infinity ($AUC_{0\rightarrow\infty}$). The AUC from zero to infinity was also estimated with the linear trapezoidal method [21].

Cell cycle analysis

The MDA-MB-231 cells, which had been previously harvested and stored in 1× PBS/ethanol, were resuspended in 1 ml of 3.8 mM sodium citrate containing 50 μg/ml propidium iodide and 125 μg/ml RNAse A as described previously [15]. Briefly, the cells were analyzed on a Becton Dickinson FACSCalibur (Becton Dickinson, San Jose, CA, USA) flow cytometer gated on forward light scatter pulse height, and side scatter pulse height for analysis of cell cycle fractions, and ungated mode for detection of cells with subG1 DNA content. The histograms were then evaluated with Flow Jo Watson Pragmatic v. 6 software (Tree Star, Standford, CA, USA). Cell cycle fractions were compared between treatment and control groups with the unpaired *t*-test.

TUNEL analysis

Cells were also analyzed with the TUNEL assay. Cells stored in ethanol/PBS were prepared for TUNEL flow cytometry analysis using Apoptag® fluorescein DNA end-labeling (Chemicon, Temecula, CA, USA). Briefly, terminal deoxynucleotidyl transferase labeled the cell's DNA with fluorescein-conjugated nucleotides (FCN) with incubation at 37°C for 0.5 h. After labeling, cells were resuspended in 1 ml of 3.8 mM sodium citrate containing 50 μg/ml propidium iodide (PI) and 125 μg/ml RNAse A and incubated for 15 min at room temperature. To establish FACS Calibur settings, single-labeled positive controls were prepared from MDA-MB-231 cells grown in 100 mm plates. DNAse (Sigma, St. Louis, MO, USA) treated cells were used to generate a positive control. FCN and PI data was collected concurrently in FL-1 and FL-2 channels, respectively. Baseline PI for sub-G1 was set at 5% or less; FCN baseline values for TUNEL were established at 3% or less. The number of cells in each cell-cycle fraction was analyzed as previously described.

Results

Cytotoxicity assay

To establish a suitable time point to analyze cell death after gemcitabine exposure, we initially performed cytotoxicity assays for MDA-MB-231 cells after treatment in monolayer growth assays. The 1 h treatment time was chosen based upon the short half-life for gemcitabine, which is rapidly metabolized [11, 12, 22, 23]. After the 1-h exposure cells were then incubated in drug-free media for an additional 2 or 5 days, followed by cytotoxicity analysis. Shown in Fig. 2 are MTT cytotoxicity assay curves for MDA-231 cells treated with gemcitabine for 1 h, followed by incubation in drug-free media for 48, 120, or 144 h. Shown in Table 1 are the overall results of gemcitabine treatment as measured by the MTT assay. The E_{max} values between 48 and 120 h incubation times were significant (*t* test;

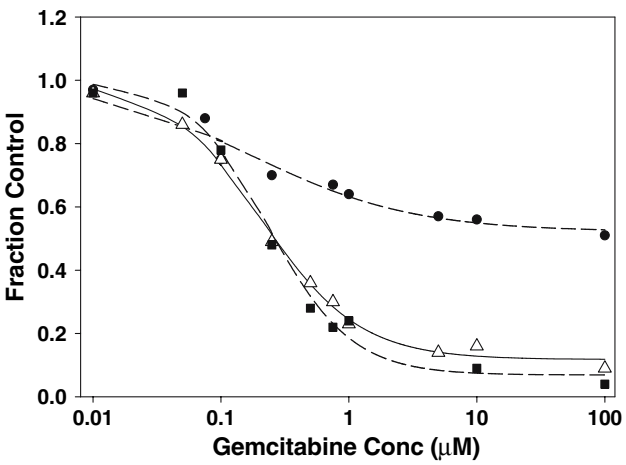

Fig. 2 Dose-response curves for MDA-MB-231 cells after treatment with gemcitabine for 1-h, followed by a wash step and incubation in drug-free media for either 48 h (*filled circle*), 120 h (*open triangle*), or 144 h (*filled square*). Point is mean of 3 independent experiments each in triplicate, coefficient of variation for all data points ranged from 1.23 to 31.8%

Table 1 Gemcitabine MTT cytotoxicity assay results (Mean ± SD) for MDA-MB-231 cells grown in static culture

No. hour after drug treatment	E_{max}	Slope (γ)	IC_{50} (μM)
48	0.50 ± 0.11	0.81 ± 0.29	1.18 ± 1.72
120	0.88 ± 0.02	1.19 ± 0.23	0.22 ± 0.11
144	0.93 ± 0.01	1.42 ± 0.19	0.28 ± 0.08

$P = 0.02$) as were the values between 120 and 144 h ($P = 0.009$), suggesting that cytotoxicity, particularly for high concentrations (i.e., greater than 1 μM), becomes more apparent with time. Since flow cytometry results (see next section) demonstrate complete degradation of DNA by day 5, we did not test incubation times longer than 144 h.

Static culture flow cytometry

To test the notion that E_{max} values, estimated from the MTT assay may be suitable for estimating harvest time from the bioreactor, we treated MDA-MB-231 cells with gemcitabine in static culture, and analyzed the cells by flow cytometry. Cells were treated with gemcitabine for 1 h using approximate IC_{50} concentrations (i.e., 0.25 μM). Following treatment, the cells were incubated in drug-free media for 48 or 120 h, and analyzed by flow cytometry (Fig. 3). Cell cycle analyses for control cells were as follows: 66.8 ± 1.05% G1, 18.9 ± 5.51% S, 7.81 ± 3.86% G2/M, and 2.10 ± 0.43% subG1. Cell cycle analysis for cells harvested 48 h after treatment were as follows: 20.0 ± 9.37% G1, 61.5 ± 8.60% S, 7.22 ± 0.53% G2/M, and 7.67 ± 2.29% subG1. By 120 h, we observed complete loss of the characteristic G1 and G2/M peaks, relative to controls, and cell cycle analysis was not possible.

Gemcitabine pharmacokinetics in the bioreactor system

As a quality control for verifying accurate drug concentrations, gemcitabine pharmacokinetics were evaluated. On day 14, 5.3 mg gemcitabine were infused over 30 min in 4 separate cartridges and media samples were collected from the lumen and ECS during and after the infusion. Drug-free media was infused for the control cartridges. The measured flow rate for all gemcitabine infusions was 4.57 ± 0.18 ml/min. A gemcitabine concentration–time plot from a single experiment is shown in Fig. 4. The gemcitabine area under the concentration–time curve (AUC) in the lumen was 56.4 μM h (Mean ± SD), and for the ECS, 58.8 ± 25.4 μM h. As calculated from the trapezoidal rule, the gemcitabine AUC in the lumen was 56.4 ± 4.14 μM h, and for the ECS, 54.3 ± 25.6 μM h. The gemcitabine C_{max} in the lumen was 87.5 ± 2.65 μM, and the ECS, 44.9 ± 18.2 μM. Based upon the dose infused, the predicted

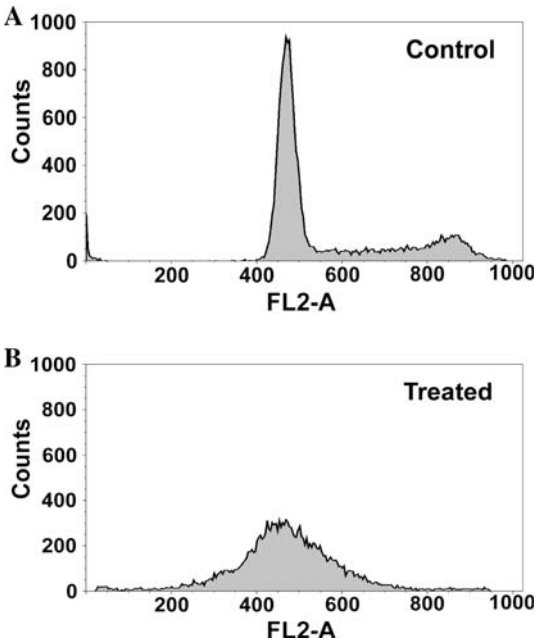

Fig. 3 Representative flow histograms of MDA-MB-231 cells recovered from separate 100-mm plates and treated with propidium iodide. **a** Histogram MDA-MB-231 cells treated with drug-free media as control, followed by incubation in drug free media for 48 h, **b** MDA-MB-231 cells after treatment with 0.25 μM (IC_{50}) gemcitabine for 1 h, followed by incubation in drug-free media for 48 h

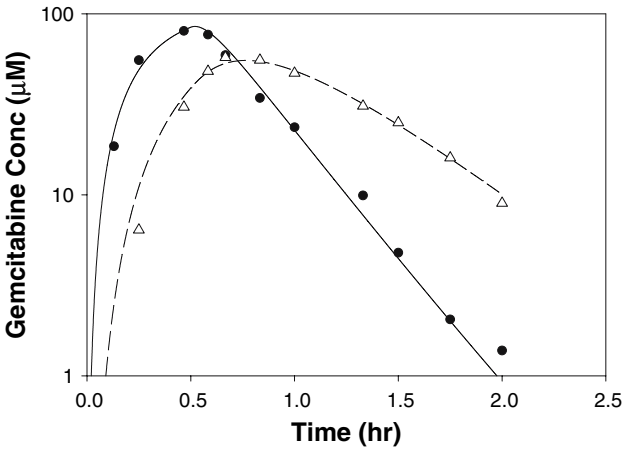

Fig. 4 Representative concentration–time plot for a single experiment of gemcitabine measured from the lumen (*filled circle*) and ECS (*open triangle*). Plot depicts gemcitabine infused as 5.3 mg over 0.5 h, followed by elimination from the bioreactor

value for C_{max} was 106 μM, while the AUC is a known quantity based upon the dose and fixed flow rate. Accuracy of the calculated versus predicted value was 82.5% for C_{max}.

Bioreactor flow cytometry

To determine viability and cell cycle analysis for the two cohorts of MDA-MB-231 cells grown in bioreactors, the

cells were collected 120 h after gemcitabine treatment. Shown in Fig. 5 are representative histograms for control and gemcitabine-treated cells grown in cartridges, and the data are summarized in Table 2. A significant increase in the fraction of cells in the subG1 fraction was observed after gemcitabine treatment (Fig. 5b), compared with untreated cells (Fig. 5a). Concomitantly, significant decreases in the fraction of cells present in the G1 and G2/M phases were observed for gemcitabine-treated versus untreated cells. As determined from trypan blue staining, $21.2 \pm 8.0 \times 10^6$ cells were recovered from bioreactors treated with gemcitabine (70.5% viable). This value was less than that for the control group ($29.4 \pm 9.0 \times 10^6$ cells), but these differences were not significant. Cells from the fourth cartridge in each group were also analyzed with the TUNEL assay (Fig. 5c). The assay showed a marked induction of apoptosis with 6.4% apoptotic index in the gemcitabine treated cells and 0.76% for control cells.

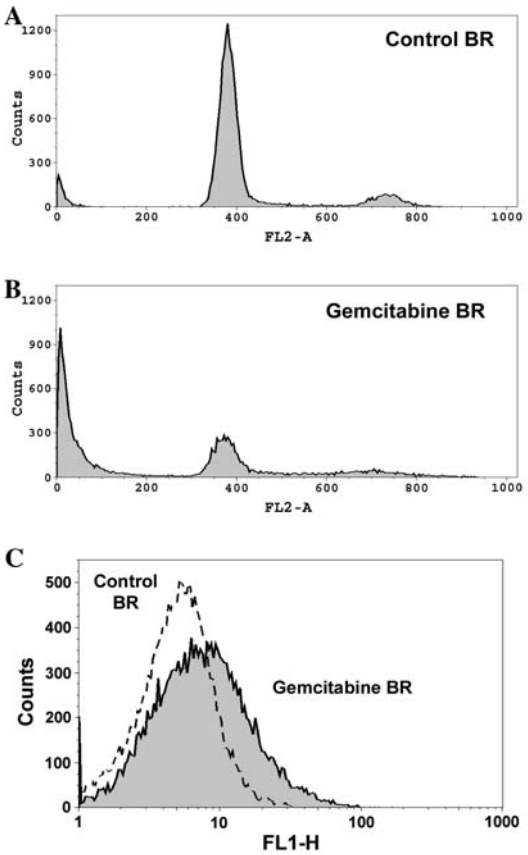

Fig. 5 Gemcitabine-induced cytotoxicity analysis of MDA-MB-231 cells recovered from separate bioreactors. **a** Histogram representative for MDA-MB-231 cells treated with drug-free media as control, **b** histogram for MDA-MB-231 cells after 5.3 mg gemcitabine infused over 0.5 h. After all drug was removed, cells were recovered 120 h later, and treated with propidium iodide. **c** TUNEL assay at 120 h after control and gemcitabine infusion. *BR* bioreactor

Discussion

An in vitro bioreactor system has been used to test the effect of infused gemcitabine on cell death for the anchorage-dependent MDA-MB-231 cell line. Since it is difficult to monitor cells directly, an indirect approach was used to estimate an appropriate time to harvest the cells after treatment. To determine a reasonable waiting time after drug treatment we tested gemcitabine treatment of MDA-MB-231 cells with traditional methods of cytotoxicity determination and flow cytometry. Our later time point E_{max} estimates (i.e., 120 and 144 h) from the MTT assay more closely approximate a true E_{max} value relative to the 48 h time point estimates, but we did not proceed beyond 144 h. The maximum effect results were already at 93%, thus testing longer time points would have only yielded small incremental increases for E_{max}. Since we observed complete DNA degradation by 120 h (flow cytometry), and since this assay is more suitable for bioreactor specimens, we did not continue with longer time points for the MTT assay. As shown by flow cytometry, harvesting cells 5 days after drug treatment appeared to be sufficient to allow for a significant increase in the subG1 fraction of cells, compared with control cartridges.

Since it is not possible to harvest anchorage-dependent cells at multiple time points from the same bioreactor, we chose the MTT cytotoxicity assay [24, 25] to assess gemcitabine's temporal effect on cell kill in the static system. These results would then enable us to estimate an acceptable time point to harvest treated cells from the bioreactor, and assess for drug induced cytotoxicity. The 1 h treatment time was chosen to approximate the plasma exposure time observed in patients treated with gemcitabine [11, 12, 22, 23]. Five days after treatment, cell death was nearly double

Table 2 Cell cycle analysis for MDA-231 cells grown in the ECS with and without gemcitabine treatment

		G1 (%)	S (%)	G2/M (%)	SubG1 (%)	Cell recovery (million)	Viability (%)
Controls ($n = 4$)	Mean ± SD	65.1 ± 4.91	11.9 ± 2.14	9.01 ± 0.77	14.1 ± 5.28	29.4 ± 8.97	82.8 ± 3.15
Gemcitabine ($n = 4$)	Mean ± SD	28.6 ± 12.0	22.5 ± 7.66	6.36 ± 1.40	42.6 ± 9.78	21.2 ± 8.00	70.5 ± 16.0
	P	0.005	0.08	0.02	0.004	0.22	0.23

that observed 2 days after treatment, approaching 90%. With flow cytometry, we observed that cells accumulated in S-phase on day 2, which was similar to results we published previously for cells harvested from the bioreactor 2 days after treatment [15]. Depending on treatment concentrations and length of treatment, accumulation of cells in S-phase has been demonstrated for various cell lines treated with gemcitabine concentrations ranging from 1 nM to 100 μM [26–30], whereas others have reported an accumulation of cells in subG1 and apoptotic fractions [31–33]. More recently, Vargas and colleagues [34] treated MDA-MB-231 cells with gemcitabine, and they demonstrated a temporal relationship between treatment and emergence of cell cycle arrest in S phase followed later by an increase in apoptosis. Cell doubling rates in static culture appear higher than that observed in our bioreactor, so this might render statically grown cells more sensitive to chemotherapy. Hence, our results would likely enable us to predict the earliest reasonable date to harvest treated cells from the bioreactor. Since we observed significant cell death at 5 days after treatment in the bioreactor system, we did not proceed to test later harvest dates.

The MDA-MB-231 cells grown in the control bioreactors show a higher subG1 fraction (~11–16%) than those grown in 100 mm plates (~2%), suggesting that overall viability in the bioreactor is less than for static culture. We reported previously that we can recover >99% of cells from the bioreactor, ruling out recovery variability [15]. Part of this cell death occurs during the 2 week incubation period in the bioreactor (data not shown), and may be related to the extent of fiber surface area that is covered with cells and macromolecules, and leading to possible membrane fouling. This could obstruct exchange of nutrients and waste products between the lumen and ECS, rendering the cells more susceptible to cell death. Furthermore, the infusion can be modified to fit part or all of the apparatus system into the 37° CO_2 incubator, since the cartridges contain approximately 7 feet of silicon tubing that is semi permeable to oxygen and CO_2. This underscores the necessity of running controls in order to account for any non-drug-related cell death. Furthermore, tumors contain variable amounts of dead tissue within [35], so non-drug-related cell death in our model system may actually enable us to study the role of dead cells on permeability of anti-tumor agents into solid tumors. Microscopy studies of the cell-laden fibers are warranted; to assess the nature of this mono-cellular or possible multiple layered cell growth.

To evaluate the treatment of MDA-MB-231 cells with clinically-relevant concentration-time exposure of gemcitabine, we compared four cartridges with MDA-231 cells that had been treated with 5.3 mg gemcitabine over 30 min, with cartridges, treated with drug-free media as control. This dose and infusion rate was the same as that used in the previous study in which we reported an increase in S-phase fraction after 2 days. Furthermore, the pharmacokinetics (C_{max}, half-life) measured for these experiments are similar to those we reported earlier [15] and are similar to measurements from plasma of patients receiving gemcitabine [11, 12, 22, 23]. The targeted AUC values were similar to values reported by Abbruzzese but less than those reported by Venook and colleagues. This depends on the dosage and individual clearance of drug for each subject enrolled in the studies. For the bioreactor, one can increase the AUC while keeping the C_{max} constant by decreasing the elimination rate constant (i.e., the pump flow rate). A small decrease for the gemcitabine dosage will keep the C_{max} values to similar values. For example, to increase the targeted AUC from 56 to 75 μM h while maintaining a C_{max} of 88 μM, the pump flow rate setting can be decreased from 5 ml/min down to approximately 3.5 ml/min, and the dosage decreased from 5.3 mg down to approximately 4.5 mg. HPLC analysis is recommended, to make further adjustments, if necessary for future experiments.

We did not observe dFdU production in this system. Hence, the elimination rate that we selected for these experiments is designed to simulate the total gemcitabine clearance (i.e., renal and metabolic). This elimination rate does not account for the additional clearance of gemcitabine that occurs intracellularly through the conversion to the phosphorylated metabolites. Saturation of the activation pathway is a significant limitation of treatment. Studies can be designed to assess the importance of dosage and infusion rate on this saturation, and the extent to which gemcitabine is metabolized to the active phosphorylated metabolites through direct measurement of dFdCDP and dFdCTP production. Once this production is estimated, it may be necessary to adjust the elimination rate in the system to more closely mimic only the clearance that occurs renally and through hepatic and plasma cytidine deaminase. The process is likely to be imperfect for a few reasons. Since we are able to harvest up to 40 million cells from the system, it is possible that more gemcitabine is phosphorylated in a system with a large number of tumor cells, relative to a small number. Second, dosing considerations in patients are also based upon toxicity, so even if we are able to optimize metabolite production in our system, further studies in animal models would be recommended. Third, the system in its current design has a limited volume of distribution relative to patients, so it may not allow for precise dosing until tissue distribution aspects are considered.

Five days after treatment, we observed a significant increase in subG1 fraction compared to controls, and S-phase fractions were also higher, though not as high as in cells recovered 2 days after treatment. Taken together, these results demonstrate that MDA-MB-231 cells grown in the

bioreactor system and treated with clinically-relevant gemcitabine exposures accumulate in S-phase first, followed by cell death after 5 days. These results are consistent with those we and others have observed with the static system, though cell death appears to occur more slowly in the bioreactor [36, 37].

In summary, this study has demonstrated the feasibility of cytotoxic treatment of anchorage-dependent MDA-231 cells with gemcitabine given over 30 min and concentration–time data are described by a three-compartment model. Whereas patient plasma gemcitabine kinetics are second order, the kinetics in the ECS are described as a first order compartment downstream from the lumen (also first order). It may be possible to simulate second order kinetics by redirecting the lumen flow back towards the central reservoir. The waste bottle would then be directly connected to the central reservoir. Even under those conditions, tissue distribution may not be evident, due to the limited tissue volume present in our system, relative to an organ system. Since desirable cell recovery time might vary, depending on the cell line and drug, static tissue culture experiments can be used to estimate the temporal relationship between drug treatment and cell death before beginning experiments in the bioreactor. Future directions for this in vitro bioreactor system include studies that compare the cytotoxicity of similar gemcitabine doses given over different infusion rates and evaluation of active intracellular metabolite accumulation. In order to understand the heterogeneity of the system, testing of additional cell lines (i.e., lung, breast, pancreatic, and ovarian) is also warranted. Once single agent studies are completed, further studies will evaluate the addition of adjunct agents to short and prolonged infusion gemcitabine.

Acknowledgments This work was supported in part by the Cancer Center Translational Breast Cancer Award to M.N.K.

References

1. Spratlin J, Sangha R, Glubrecht D, Dabbagh L, Young JD, Dumontet C, Cass C, Lai R, Mackey JR (2004) The absence of human equilibrative nucleoside transporter 1 is associated with reduced survival in patients with gemcitabine-treated pancreas adenocarcinoma. Clin Cancer Res 10(20):6956–6961
2. Mackey JR, Mani RS, Selner M, Mowles D, Young JD, Belt JA, Crawford CR, Cass CE (1998) Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines. Cancer Res 58(19):4349–4357
3. Garcia-Manteiga J, Molina-Arcas M, Casado FJ, Mazo A, Pastor-Anglada M (2003) Nucleoside transporter profiles in human pancreatic cancer cells: role of hCNT1 in 2′,2′-difluorodeoxycytidine-induced cytotoxicity. Clin Cancer Res 9(13):5000–5008
4. Krishnan P, Fu Q, Lam W, Liou JY, Dutschman G, Cheng YC (2002) Phosphorylation of pyrimidine deoxynucleoside analog diphosphates: selective phosphorylation of L-nucleoside analog diphosphates by 3-phosphoglycerate kinase. J Biol Chem 277(7):5453–5459
5. Bouffard DY, Laliberte J, Momparler RL (1993) Kinetic studies on 2′,2′-difluorodeoxycytidine (Gemcitabine) with purified human deoxycytidine kinase and cytidine deaminase. Biochem Pharmacol 45(9):1857–1861
6. Liou JY, Dutschman GE, Lam W, Jiang Z, Cheng YC (2002) Characterization of human UMP/CMP kinase and its phosphorylation of D- and L-form deoxycytidine analogue monophosphates. Cancer Res 62(6):1624–1631
7. Heinemann V, Xu YZ, Chubb S, Sen A, Hertel LW, Grindey GB, Plunkett W, (1990) Inhibition of ribonucleotide reduction in CCRF-CEM cells by 2′,2′-difluorodeoxycytidine. Mol Pharmacol 38(4):567–572
8. Ross DD, Cuddy DP (1994) Molecular effects of 2′,2′-difluorodeoxycytidine (Gemcitabine) on DNA replication in intact HL-60 cells. Biochem Pharmacol 48(8):1619–1630
9. Huang P, Chubb S, Hertel LW, Grindey GB, Plunkett W (1991) Action of 2′,2′-difluorodeoxycytidine on DNA synthesis. Cancer Res 51(22):6110–6117
10. Plunkett W, Huang P, Gandhi V (1995) Preclinical characteristics of gemcitabine. Anticancer Drugs 6(Suppl 6):7–13
11. Abbruzzese JL, Grunewald R, Weeks EA, Gravel D, Adams T, Nowak B, Mineishi S, Tarassoff P, Satterlee W, Raber MN et al (1991) A phase I clinical, plasma, and cellular pharmacology study of gemcitabine. J Clin Oncol 9(3):491–498
12. Venook AP, Egorin MJ, Rosner GL, Hollis D, Mani S, Hawkins M, Byrd J, Hohl R, Budman D, Meropol NJ, Ratain MJ (2000) Phase I and pharmacokinetic trial of gemcitabine in patients with hepatic or renal dysfunction: Cancer and Leukemia Group B 9565. J Clin Oncol 18(14):2780–2787
13. Bengala C, Guarneri V, Giovannetti E, Lencioni M, Fontana E, Mey V, Fontana A, Boggi U, Del Chiaro M, Danesi R, Ricci S, Mosca F, Del Tacca M, Conte PF (2005) Prolonged fixed dose rate infusion of gemcitabine with autologous haemopoietic support in advanced pancreatic adenocarcinoma. Br J Cancer 93(1):35–40
14. Tempero M, Plunkett W, Ruiz Van Haperen V, Hainsworth J, Hochster H, Lenzi R, Abbruzzese J (2003) Randomized phase II comparison of dose-intense gemcitabine: thirty-minute infusion and fixed dose rate infusion in patients with pancreatic adenocarcinoma. J Clin Oncol 21(18):3402–3408
15. Kirstein MN, Brundage RC, Elmquist WF, Remmel RP, Marker PH, Guire DE, Yee D (2006) Characterization of an in vitro cell culture bioreactor system to evaluate anti-neoplastic drug regimens. Breast Cancer Res Treat 96(3):217–225
16. Chen M, Hough AM, Lawrence TS (2000) The role of p53 in gemcitabine-mediated cytotoxicity and radiosensitization. Cancer Chemother Pharmacol 45(5):369–374
17. Matranga CB, Shapiro GI (2002) Selective sensitization of transformed cells to flavopiridol-induced apoptosis following recruitment to S-phase. Cancer Res 62(6):1707–1717
18. Twentyman PR, Luscombe M (1987) A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. Br J Cancer 56(3):279–285
19. Hill A (1913) Biochem J 7:471–480
20. D'Argenio DZaS A (1997) ADAPT II User's Guide: Pharmacokinetic/Pharmacodynamic Systems Analysis Software. In: Biomedical Simulations Resource, Los Angeles
21. Zhuang Y, Fraga CH, Hubbard KE, Hagedorn N, Panetta JC, Waters CM, Stewart CF (2006) Topotecan central nervous system penetration is altered by a tyrosine kinase inhibitor. Cancer Res 66(23):11305–11313
22. Dumez H, Louwerens M, Pawinsky A, Planting AS, de Jonge MJ, Van Oosterom AT, Highley M, Guetens G, Mantel M, de Boeck G, de Bruijn E, Verweij J (2002) The impact of drug adminis-

tration sequence and pharmacokinetic interaction in a phase I study of the combination of docetaxel and gemcitabine in patients with advanced solid tumors. Anticancer Drugs 13(6):583–593
23. Kroep JR, Giaccone G, Voorn DA, Smit EF, Beijnen JH, Rosing H, van Moorsel CJ, van Groeningen CJ, Postmus PE, Pinedo HM, Peters GJ (1999) Gemcitabine and paclitaxel: pharmacokinetic and pharmacodynamic interactions in patients with non-small-cell lung cancer. J Clin Oncol 17(7):2190–2197
24. Ferrari M, Fornasiero MC, Isetta AM (1990) MTT colorimetric assay for testing macrophage cytotoxic activity in vitro. J Immunol Methods 131(2):165–172
25. Gooch JL, Van Den Berg CL, Yee D (1999) Insulin-like growth factor (IGF)-I rescues breast cancer cells from chemotherapy-induced cell death-proliferative and anti-apoptotic effects. Breast Cancer Res Treat 56(1):1–10
26. Tolis C, Peters GJ, Ferreira CG, Pinedo HM, Giaccone G (1999) Cell cycle disturbances and apoptosis induced by topotecan and gemcitabine on human lung cancer cell lines. Eur J Cancer 35(5):796–807
27. Cappella P, Tomasoni D, Faretta M, Lupi M, Montalenti F, Viale F, Banzato F, D'Incalci M, Ubezio P (2001) Cell cycle effects of gemcitabine. Int J Cancer 93(3):401–408
28. Ng SSW, Tsao MS, Chow S, Hedley DW (2000) Inhibition of phosphatidylinositide 3-kinase enhances gemcitabine-induced apoptosis in human pancreatic cancer cells. Cancer Res 60(19):5451–5455
29. Pauwels B, Korst AE, Pattyn GG, Lambrechts HA, Van Bockstaele DR, Vermeulen K, Lenjou M, de Pooter CM, Vermorken JB, Lardon F (2003) Cell cycle effect of gemcitabine and its role in the radiosensitizing mechanism in vitro. Int J Radiat Oncol Biol Phys 57(4):1075–1083
30. Perabo FG, Lindner H, Schmidt D, Huebner D, Blatter J, Fimmers R, Muller SC, Albers P (2003) Preclinical evaluation of gemcitabine/paclitaxel-interactions in human bladder cancer lines. Anticancer Res 23(6C):4805–4814
31. Ali S, El-Rayes BF, Aranha O, Sarkar FH, Philip PA (2005) Sequence dependent potentiation of gemcitabine by flavopiridol in human breast cancer cells. Breast Cancer Res Treat 90(1):25–31
32. Chandler NM, Canete JJ, Callery MP (2004) Caspase-3 drives apoptosis in pancreatic cancer cells after treatment with gemcitabine. J Gastrointest Surg 8(8):1072–1078
33. Serrano MJ, Sanchez-Rovira P, Algarra I, Jaen A, Lozano A, Gaforio JJ (2002) Evaluation of a gemcitabine-doxorubicin-paclitaxel combination schedule through flow cytometry assessment of apoptosis extent induced in human breast cancer cell lines. Jpn J Cancer Res 93(5):559–566
34. Hernandez-Vargas H, Rodriguez-Pinilla SM, Julian-Tendero M, Sanchez-Rovira P, Cuevas C, Anton A, Rios MJ, Palacios J, Moreno-Bueno G (2006) Gene expression profiling of breast cancer cells in response to gemcitabine: NF-kappaB pathway activation as a potential mechanism of resistance. Breast Cancer Res Treat 102(2):157–172
35. Jain RK (2005) Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science 307(5706):58–62
36. Shi Z, Azuma A, Sampath D, Li YX, Huang P, Plunkett W (2001) S-Phase arrest by nucleoside analogues and abrogation of survival without cell cycle progression by 7-hydroxystaurosporine. Cancer Res 61(3):1065–1072
37. Huang P, Plunkett W (1995) Fludarabine- and gemcitabine-induced apoptosis: incorporation of analogs into DNA is a critical event. Cancer Chemother Pharmacol 36(3):181–188