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A spheroid-based 3-D culture model for pancreatic cancer drug testing, using the acid phosphatase assay

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Abstract

Current therapy for pancreatic cancer is multimodal, involving surgery and chemotherapy. However, development of pancreatic cancer therapies requires a thorough evaluation of drug efficacy *in vitro* before animal testing and subsequent clinical trials. Compared to two-dimensional culture of cell monolayer, three-dimensional (3-D) models more closely mimic native tissues, since the tumor microenvironment established in 3-D models often plays a significant role in cancer progression and cellular responses to the drugs. Accumulating evidence has highlighted the benefits of 3-D *in vitro* models of various cancers. In the present study, we have developed a spheroid-based, 3-D culture of pancreatic cancer cell lines MIAPaCa-2 and PANC-1 for pancreatic drug testing, using the acid phosphatase assay. Drug efficacy testing showed that spheroids had much higher drug resistance than monolayers. This model, which is characteristically reproducible and easy and offers rapid handling, is the preferred choice for filling the gap between monolayer cell cultures and *in vivo* models in the process of drug development and testing for pancreatic cancer.

Key words: Spheroid; Three-dimensional culture; Pancreatic cancer; Drug testing

Introduction

Pancreatic cancer has a poor prognosis. For about the last 10 years, gemcitabine has remained the first-line chemotherapeutic agent for advanced pancreatic cancer; however, the success of drug treatment is poor, and overall survival has not improved for decades. Drug resistance is thought to be a major reason for the limited benefit of most pancreatic cancer therapies. Increasing drug efficiency and decreasing drug resistance are current principal aims in pancreatic cancer research (1).

The most common drug model for *in vitro* study of cancer cells is the monolayer culture of cells. Although the two-dimensional (2-D) model has made significant contributions to cancer research, it has certain intrinsic limitations that have promoted the development of three-dimensional (3-D) culture models. Compared to 2-D culture models, 3-D culture models can provide a microenvironment that more closely mimics the microenvironment observed in tumor tissues. This feature is crucial for drug testing, since environmental cues can have profound effects on properties, behaviors, and functions of cancer cells, which may in turn affect cellular responses to drugs (2-4). Thus, 3-D culture models offer a more sophisticated means of mimicking *in vivo* environments, including the control of concentration gradients of signaling molecules and therapeutic agents, composition and structure of extracellular matrix surrounding the cancer cells, and the morphology and arrangement of individual cells.

Multicellular spheroids are probably the most widely accepted model for 3-D culture (5). Several studies have highlighted the potential of multicellular tumor spheroids (MCTS) in cancer research and treatment (6-12). Multicellular spheroid cultures can reflect the tumor microenvironment, volume growth kinetics, and cytoarchitecture, similar to those of avascular tumor nodules, micrometastasis, or the intervascular region of large, solid tumors. Previous research has studied MCTS-based drug screening in various types of tumors (13,14). MCTS in pancreatic cancer were first described by McLeod (15). However, MCTS remain poorly investigated in spheroid-based chemotherapy research.

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In the present study, a spheroid-based 3-D culture model of MIAPaCa-2 and PANC-1 MCTS, using the acid phosphatase assay, was established to investigate the chemotherapy characteristics of pancreatic cancer cells. As an important supplement to monolayer-based assays, well-controlled MCTS may provide new insights and a better estimation of antitumor efficacy for pancreatic cancer drug testing.

Material and Methods

Cell lines and spheroid culture

The MIAPaCa-2 and PANC-1 human pancreatic cancer cells were the kind gift of Professor Helmut Friess at the Department of Surgery, Klinikum Rechts der Isar, Technische Universität München, Germany. Cells were thawed from frozen stock and subcultured for $<$20 passages. Dulbecco's modified Eagle's medium (DMEM) containing 1 g/L glucose, 1% (w/v) sodium pyruvate, 1% (w/v) L-glutamine, and 3.7% (w/v) $NaHCO_3$, supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal calf serum (FCS), was used for culturing (HyClone, USA). All cultures were kept at 37°C in a humidified atmosphere with 5% CO_2. Cell transfer and preparation of single-cell suspensions were performed by mild enzymatic dissociation, using 0.05% trypsin and 0.02% EDTA in phosphate-buffered saline (HyClone). Spheroids were initiated in a liquid overlay by seeding 1.2×10^3 MIAPaCa-2 cells and 1.0×10^3 PANC-1 cells/well in 200 μL medium using agarose-coated, 96-well culture plates (50 μL 1.5% agarose/well). After an initiation interval of 4 days, 50% of supernatant was replaced with fresh medium, repeated every 48 h thereafter except for a 72 h drug treatment setup described in the drug treatment section.

Cell counting, flow cytometry, and microscopy

For the detection of viable cells, 15 to 30 spheroids were collected and dissociated using a spheroid dispersion solution (SCIVAX, Inc., USA), and the cell number was counted with a NucleoCounter (Chemometec, Denmark). Cell aliquots were stained with the DNA intercalating dye propidium iodide (PI, 2 μg/mL per 10^6 cells), and the cells were analyzed on an Accuri C6 Flow Cytometer using CFlow Plus software packages (Accuri Cytometers, Inc., USA).

Morphological analyses of spheroids and phase contrast imaging in 96-well microplates were carried out manually on a DM IL inverted phase-contrast microscope (Leica, Germany). The volumes of the spheroids were calculated as previously described (16,17). Generated data tables contained the following morphometric information: maximum diameter ($d1$), the diameter at right angles to maximum diameter ($d2$), mean spheroid diameter (D), and spheroid volume. Volumes were calculated with the AVD mass and volume calculator 7.2.1 (Avlan Design, Canada). Briefly, $d1$ and $d2$ were measured and recorded. D was calculated using the formula $D = \sqrt{d1 d2}$. Spheroid volume (mm^3) was calculated using the formula $V = \frac{4}{3}\pi\left(\frac{D}{2}\right)^3$.

Acid phosphatase assay

A modified acid phosphatase (APH) assay, which was based on quantification of cytosolic APH activity, was validated for determining viable cells in spheroids (18). Intracellular APH in viable cells hydrolyzed *p*-nitrophenyl phosphate to *p*-nitrophenol. Its absorbance at 405 nm was directly proportional to the cell number in the range of 10^3 to 10^5 cells/monolayer. MCTS cultures grown in liquid overlay were transferred with supernatant onto flat-bottom, 96-well microplates and centrifuged for 10 min at 1500 *g* to spin down spheroids, clusters, and single cells. The pellet was washed by carefully replacing 160 μL of supernatant with phosphate buffer solution (PBS). Centrifugation was repeated, and the supernatant was discarded to a final volume of 100 μL. Then, 100 μL of assay buffer was added per well and incubated for 90 min at 37°C. Following incubation, 10 μL NaOH was added to each well, and absorbance at 405 nm was measured within 10 min with a WellScan MK3 microplate reader (Labsystems Dragon, Finland).

Drug treatment

Treatment was performed with 5-fluorouracil (5-FU, Sigma-Aldrich, USA) and gemcitabine hydrochloride (Eli Lilly and Company, USA). Drugs (100 mM stock solutions in dimethyl sulfoxide) were applied in the following concentrations: 200, 100, 50, 25, 10, 1, 0.1, and 0.01 μM, in culture medium. Treatment with 10% Triton-X-100 (Sigma-Aldrich) in culture medium for 1.5 h at 37°C led to a 100% loss of cell membrane integrity in structurally intact spheroids, and served as positive control. For treatment, MIAPaCa-2 and PANC-1 monolayer cultures were grown in 96-well plates by plating 5×10^2 exponentially growing cells per well. Spheroids were grown in liquid overlay, as described above. Both monolayer and spheroid cultures were treated for 96 h after inoculation by replacing 50% (100 μL) of the culture supernatant with drug-supplemented fresh medium. The spheroid diameter at the onset of treatment was 370 to 410 μm. The treatment interval was 72 h. Untreated control cells and spheroids were always cultured in parallel, using 100 μL dimethyl sulfoxide-containing medium for the drug dilution.

Analysis of drug efficacy

Drug effects were documented after 72 h of treatment via the APH assay and spheroid volume analysis. All experiments were carried out in triplicate. APH data were corrected for background absorption at 405 nm. Drug effects were recorded relative to untreated controls, using the mean APH signal of untreated spheroids ($n = 9$) measured for each individual experiment.

Statistical analysis

Dose-response curves and IC_{50} values were calculated for each individual experiment by sigmoidal dose-response analysis, using the Hill fitting equation in the Prism 5 software (GraphPad Software, Inc., USA). The individual dose-response curves and IC_{50} values for each drug were averaged, and are reported as means ± SD.

Results

Characteristics of MIAPaCa-2 and PANC-1 pancreatic cancer spheroids

Single MCTS of about 400 μm in diameter were harvested after a 96-h initiation incubation of 1.2×10^3 MIAPaCa-2 cells and 1.0×10^3 PANC-1 cells per culture well. With the medium being refreshed every 48 h, spheroid volume increased according to the Gomertz equation, which classically and mathematically describes tumor and also spheroid growth kinetics (19). Maximum spheroid diameter of about 1 mm was reached after about 9 days for MIAPaCa-2 and 10 days for PANC-1 spheroids. Double spheroid volume was obtained after 29 h for MIAPaCa-2 and 28 h for PANC-1 spheroids (Figure 1A).

We compared the dead cells, calculated as the proportion of PI-stained cells in spheroids, as determined by flow cytometric analysis following dissociation (Figure 1B). The proportion of dead cells in MIAPaCa-2 spheroids of 400 and 600 μm in size was about 5 to 8.58% and 15.52 to 20.02%, respectively. On the other hand, the proportion of dead cells in PANC-1 spheroids with sizes of 400 and 600 μm was about 4.96 to 10% and 15.78 to 19.86%, respectively.

The number of viable cells per spheroid, was determined by automated counting following spheroid dissociation. For MIAPaCa-2 and PANC-1, a positive linear correlation between spheroid size and viable cells per spheroid was observed (Figure 1C). Meanwhile, the proportion of PI-positive cells (membrane-defective cells) monitored by flow cytometry increased during spheroid growth, reflecting the initiation of more cell death in the spheroid center at sizes >500 μm. On the other hand, spheroids of smaller size (e.g., 400 μm) contained only 5 to 10% membrane-defective cells.

Applicability and linearity of the APH assay in pancreatic cancer cell spheroid cultures

The linearity of the APH assay signal in MIAPaCa-2 and PANC-1 spheroids as a function of the viable cell count/spheroid is shown in Figure 2. The absorption signal was linear in MIAPaCa-2 spheroids, with cell number from 3.3×10^3 to 1.24×10^5 cells/spheroid, covering spheroid diameters of up to 1000 μm. In PANC-1 spheroids, the APH signal was linear up to a maximum cell number of 1.19×10^5 cells/spheroid and a diameter of about 1000 μm. These results showed applicability and well-fitting linearity of the APH assay in both MIAPaCa-2 and PANC-1 spheroid cultures.

Cytotoxicity in MCTS using the APH assay

In order to investigate the applicability of the APH method for determining drug-induced cytotoxicity in spheroids, MIAPaCa-2 and PANC-1 MCTS at day 4 with a size of 360 to 410 μm, that consisted of about 0.78 to 1.5×10^4 and 0.70 to 1.5×10^4 viable cells and <10% dead cells, were incubated with gemcitabine. Spheroids were analyzed after a 72-h treatment interval with 0.01 to 200 μM gemcitabine for the following parameters: spheroid size and volume, APH-assay signal, and cell count per spheroid. The effect of gemcitabine relative to untreated controls was calculated, and the results of the APH assay and cell count analysis were elevated (Figure 3). The results showed quite similar curves across the gemcitabine concentration range that was applied here. The results further indicated that the APH assay would be a reliable and valuable model to investigate drug efficacy in spheroids.

Drug efficacy in MCTS *vs* monolayer culture using the APH assay

Drug efficacy in 3-D cultures was revealed to be different from that observed in classical 2-D cell cultures. The APH assay is a novel tool used to investigate and quantify differences of drug sensitivity in spheroid and monolayer cultures. In this study, two drugs (gemcitabine and 5-FU) were compared in relation to application of the APH assay in spheroids. The drug dilutions used did not alter the background absorbance. Dose-response curves were documented in MIAPaCa-2 as well as PANC-1 monolayer cultures and in spheroids ranging in size from 360 to 410 μm at the initiation of treatment. Drug efficacy experiments were performed on a 96-well plate using 0.01 to 200 μM gemcitabine and 5-FU.

Drug effects in MIAPaCa-2 and PANC-1 cells were elevated after 72-h treatment intervals in three independent experiments per drug with n = 8 spheroids per condition in each experiment. After a 72-h drug treatment, the APH signal decreased in a dose-dependent manner relative to untreated controls. Treatment with 200 μM gemcitabine of MIAPaCa-2 cells resulted in >64% loss of cell viability, whereas PANC-1 monolayer cells generally showed a 45% survival rate, even at high gemcitabine concentrations. When treated with 200 μM 5-FU, MIAPaCa-2 and PANC-1 monolayer cells showed a 38.6% and 51.24% survival rate, respectively. The IC_{50} values in MIAPaCa-2 monolayer cultures were 47.6 ± 25.2 μM for gemcitabine and 61.9 ± 21.2 μM for 5-FU. PANC-1 monolayer cultures showed IC_{50} values of 64.9 ± 10.3 μM for gemcitabine. At the highest drug concentration of 200 μM, a reduction of cell viability >50% was not observed; thus the IC_{50} in PANC-1 monolayer culture could not be estimated for 5-FU (Figure 4).

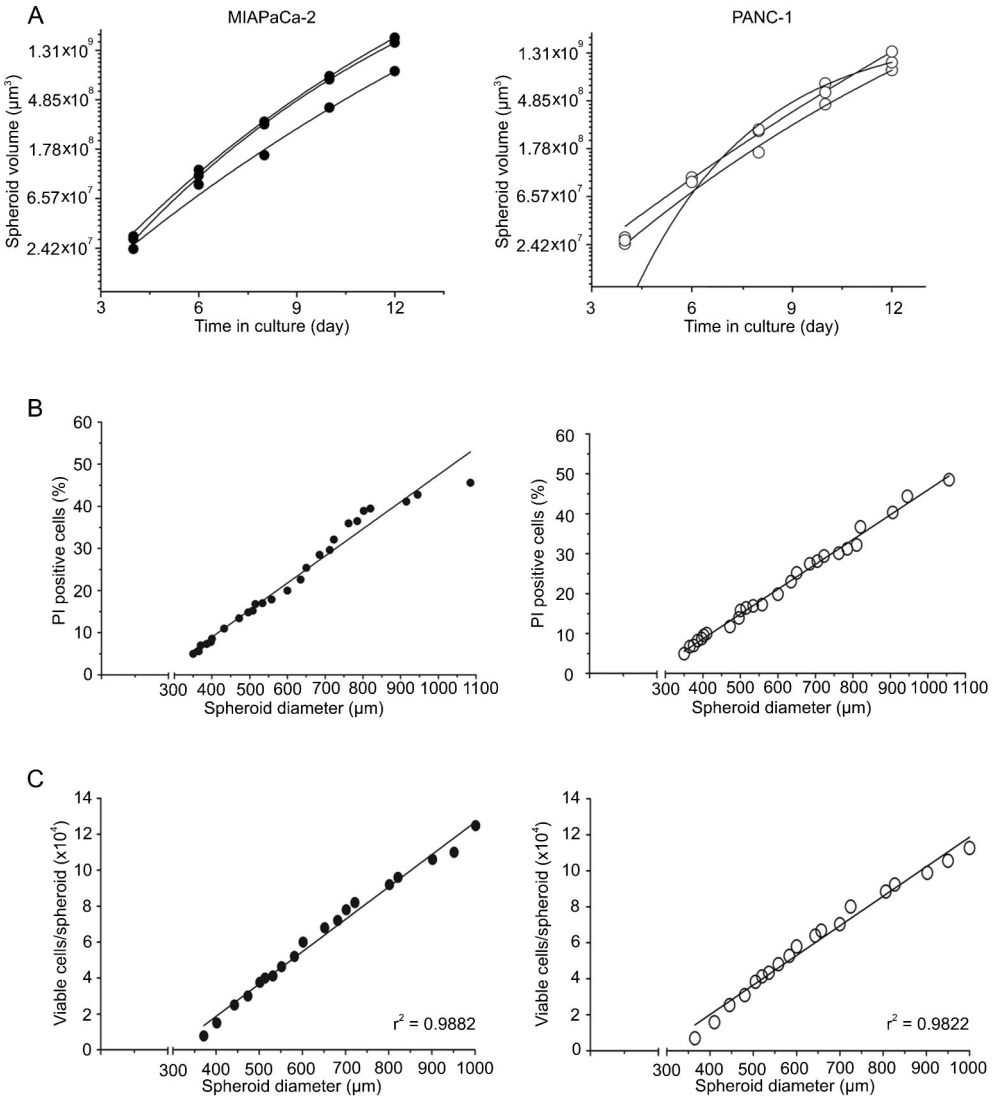
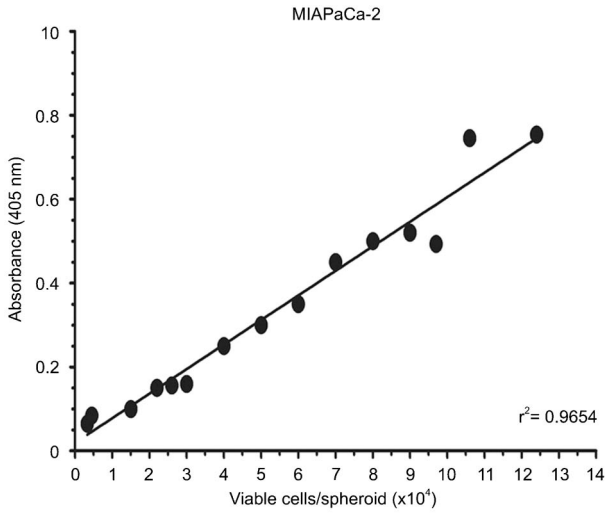


Figure 1. General characteristics of spheroid growth. *A*, Volume of MIAPaCa-2 and PANC-1 spheroids as a function of time in culture, with an initiation interval of 4 days and a seeding density of 1.2×10^3 MIAPaCa-2 cells and 1.0×10^3 PANC-1 cells per well. Data points are mean spheroid volumes for 8 to 16 spheroids. *B*, Proportion of propidium iodide (PI)-positive cells in MIAPaCa-2 and PANC-1 spheroids as a function of the average spheroid diameter determined by flow cytometry following dissociation of 15 to 30 spheroids. *C*, Viable cells in MIAPaCa-2 and PANC-1 spheroids as a function of the average spheroid diameter. Data are reported as average cell numbers determined from 3 aliquots of 15 to 30 spheroids.

MIAPaCa-2 and PANC-1 spheroid cultures demonstrated significantly reduced sensitivities to two different chemotherapeutic agents. Even at the highest drug concentration of 200 µM, a ⩾50% reduction of cell viability was not observed in either MIAPaCa-2 or PANC-1 spheroids (Figure 4). Thus, the IC_{50} in MIAPaCa-2 and PANC-1 spheroid cultures could not be estimated for gemcitabine and 5-FU. These results showed that the efficacy of the drugs tested in this study was generally lower in MIAPaCa-2 and PANC-1 spheroids than in monolayer culture.

Microscopic imaging was performed prior to the APH assay in order to test the hypothesis that the spheroid volume after treatment was not necessarily indicative of cell viability and the signal intensity of the APH assay. Phase contrast images of MIAPaCa-2 spheroids treated with gemcitabine and 5-FU are shown in Figure 5. Spheroid volume reduction and disruption reflected a cell loss. Evident spheroid shedding and disruption occurred in spheroids of MIAPaCa-2 cells treated with 100 µM gemcitabine, but viable cells could still be detected by the APH assay. Nevertheless, microscopic images might still



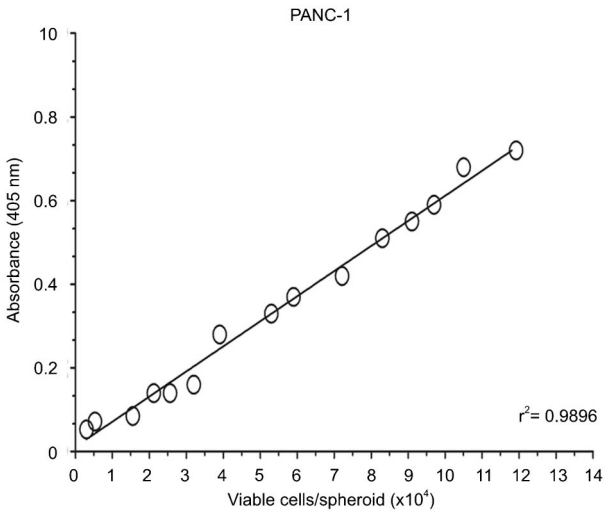
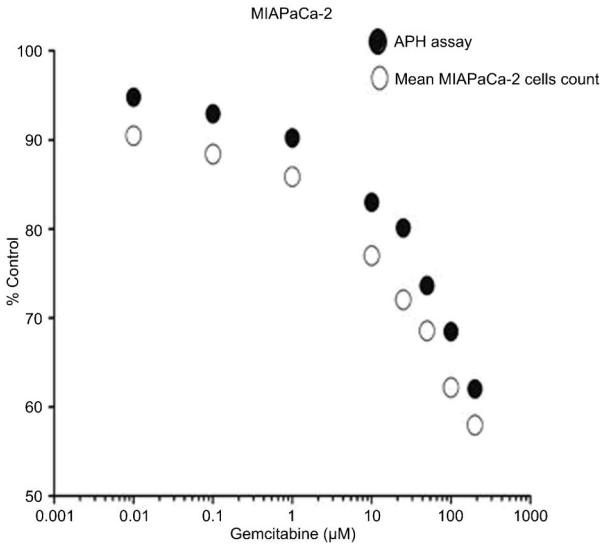


Figure 2. Linearity of the acid phosphatase (APH) assay in MIAPaCa-2 and PANC-1 spheroids. APH colorimetric measurement in MIAPaCa-2 and PANC-1 spheroids (mean, n⩾8 spheroids) as a function of the average number of viable cells per spheroid. Three aliquots of 20 spheroids were measured for each data point.



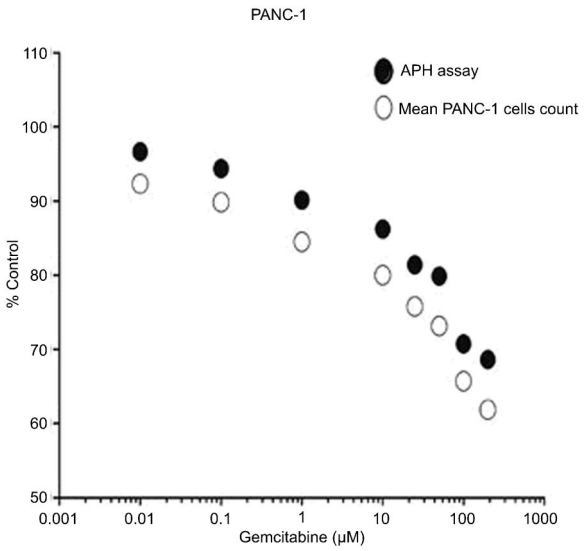


Figure 3. Acid phosphatase (APH) activity reflects cell viability in MIAPaCa-2 and PANC-1 spheroids after treatment. Comparison of APH activity and live cell counts following dissociation in MIAPaCa-2 and PANC-1 spheroids after treatment with different concentrations of gemcitabine for 72 h. Drug efficacy was documented relative to the respective untreated controls. Data are reported as means.

be supplementary parameters of 3-D cultures when they are used for drug-efficacy testing.

Discussion

The limitations of 2-D cell culture have motivated researchers to develop an *in vitro* model for the study of cancer and drug efficacy. Compared to animal models, *in vitro* models are conducive to systematic, repetitive, and quantitative investigation of cell or tissue physiology in drug discovery and development (20,21). These models can be more easily controlled and are usually less expensive and less time-consuming than animal models. *In vitro* models can be used to assess a large number of combinations of experimental parameters. Such high-throughput testing is usually not feasible with animal-based models. Here, we have established a spheroid-based 3-D culture model in MIAPaCa-2 and PANC-1 MCTS using the APH assay, and investigated the potential of the model as a spheroid-based drug assay for pancreatic cancer in 3-D cultures.

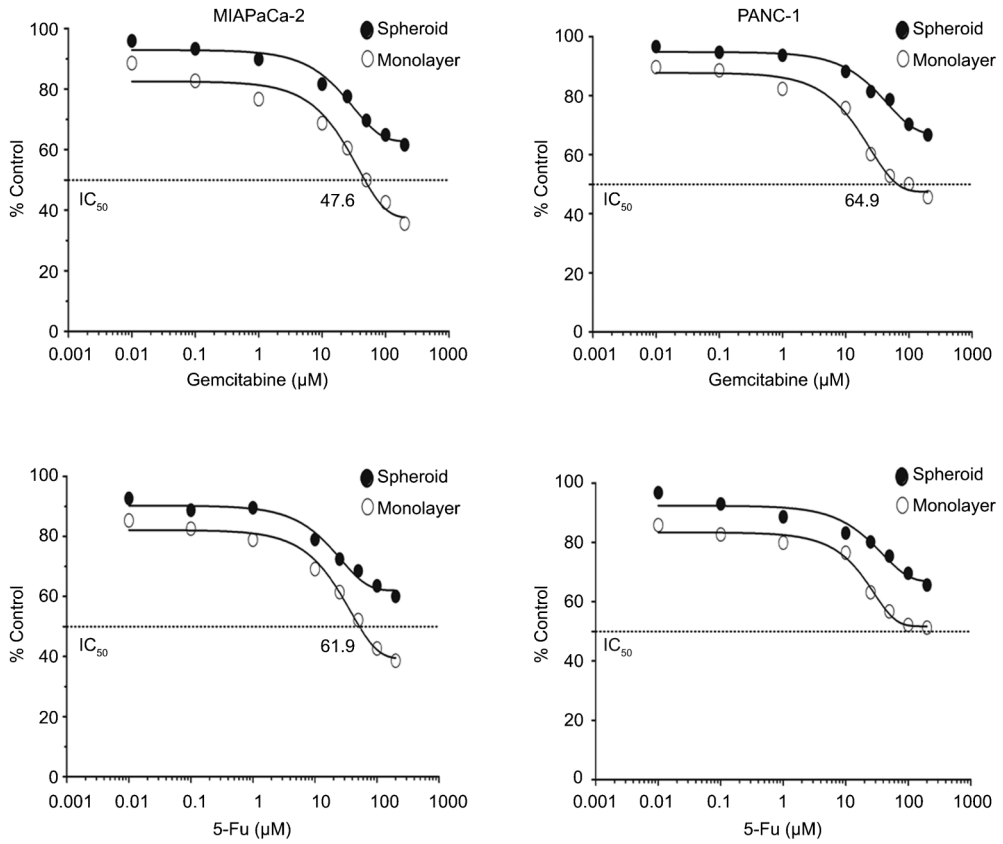


Figure 4. Application of the acid phosphatase (APH) assay to determine drug effects in MIAPaCa-2 and PANC-1 spheroid cultures. APH activity/cell viability in MIAPaCa-2 and PANC-1 monolayer and spheroid cultures after 72 h of treatment with gemcitabine and 5-fluorouracil (5-FU). Data are reported as means of ⩾3 individual experiments each with eight spheroids treated and measured per condition. IC_{50} values were calculated to investigate the difference of drug efficacy in 2-D *vs* 3-D culture and in these two pancreatic cancer cell lines.

The MCTS model was first described in the 1970s by Sutherland et al. (5) as a way to mimic the heterogeneity present in solid tumors and account for the effect of the tumor microenvironment on drug transport and efficacy. This model attempted to bridge the gap between standard monolayer cell culture and actual tumors. In fact, tumor cells grown as spheroids acquire some type of clinically relevant multicellular resistance to apoptosis-inducing drugs, which may mimic the chemoresistance found in solid tumors (22-24). Thus, experts in the field have proposed including MCTS as a mandatory model in major programs for drug screening and development. The underlying benefits of using MCTS in antitumor drug testing have frequently been alluded to (13,25,26). In our previous study (27), a 3-D culture method was found for assessing chemosensitivity. However, the spheroids used in that method were not homogeneous, and the complex method was not convenient for antitumor drug testing.

In this study, spheroids grown from MIAPaCa-2 and PANC-1 pancreatic cancer cell lines showed an exponential growth pattern in agarose-treated, 96-well plates. A maximum spheroid diameter of approximately 1 mm was reached after about 9 days of culture for MIAPaCa-2 and 10 days for PANC-1 spheroids. The physiological state of spheroids depends on the spheroid size, the individual and cell-type-specific behavior of pancreatic cancer cells, the cell density, and also the culture time. We intended to establish a spheroid-based screen from these two pancreatic cancer cell lines with clear pathophysiological gradients but without obvious cellular death at the initiation of drug treatment. According to the literature and our experience, a 4-day initiation interval for spheroid formation was found to establish spheroids of pancreatic cancer cells reproducibly under identical culture conditions (15,28). The spheroids would reach a standard size of 365 to 410 μm after 96 h of incubation at the initiation of drug treatment. Small spheroids with a size of up to 200 μm are often used for drug testing and may be sufficient to reflect 3-D cell-cell and cell-matrix interaction (29-31). It is clearly inappropriate, when attempting to create pathophysiological conditions, to have a hypoxic area in the spheroid center. Hypoxia is not

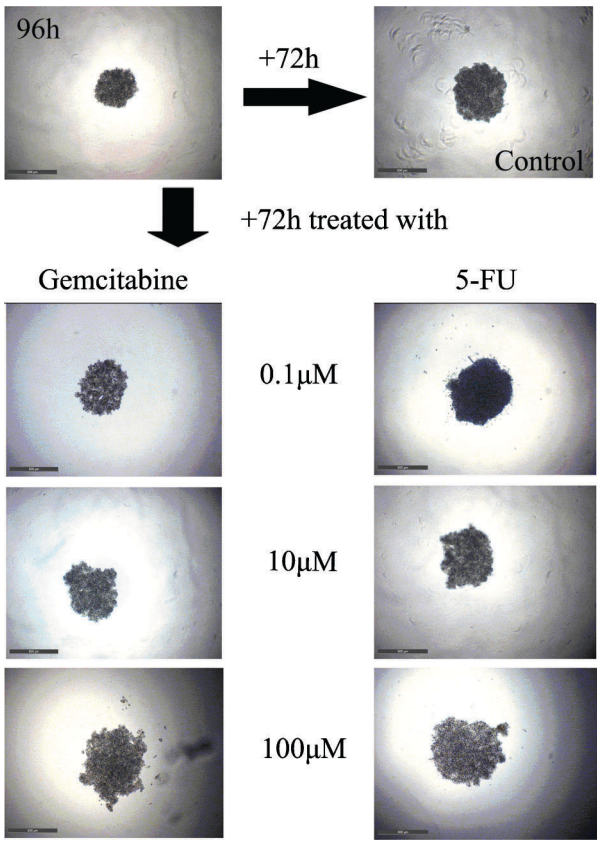


Figure 5. Spheroid integrity following treatment with gemcitabine and 5-fluorouracil (5-FU). Phase contrast images of MIAPaCa-2 spheroids at the initiation of drug treatment and after a 72-h treatment interval with 0.1, 10, and 100 μM (Bar: 500 μm).

only a well-established radio- and drug-resistance factor, but also leads to numerous indirect effects in tumor cells by modulating expression patterns (25,32). Larger spheroids with a diameter between 500 and 600 μm develop secondary central necroses, which make the pathophysiological conditions complex and difficult to control. Thus, we chose spheroids with a size of 365-410 μm for drug treatment.

Easy and rapid handling of both spheroid culture and analysis are crucial requirements of a model for drug testing of pancreatic cancer. Different techniques for spheroid culturing have been extensively described in studies, including advantages and disadvantages (25,26,33). We selected the easy and rapid agarose-overlay, 96-well plate approach to set up the spheroid-based model.

Several different cytotoxicity/viability assays for 2-D culture, based on absorption, luminescence, or fluorescence, were considered and tested in MCTS. Parameters included cellular uptake of membrane-impermeable dyes such as CCK-8, MTT, Alamar blue, and EtHD-1, release of substrates such as lactate dehydrogenase or glucose-6-phosphate dehydrogenase, activity of mitochondrial dehydrogenases or intracellular esterases, and cellular adenosine triphosphate level and the ratio of ATP/ADP. These commercially available systems have been successfully applied in monolayer cultures in various tumor cell lines, including pancreatic cancer cells (34-37). However, most of these tests could not be easily adapted to application in 3-D culture.

Recently, the APH assay was established as a reliable tool to determine cell viability in complex 3-D culture (18,33). The APH assay is simple, rapid, and high-throughput compatible, as it does not require spheroid dissociation. Our study also verified that the APH assay 1) is applicable for single MIAPaCa-2 and PANC-1 spheroids in 96-well plates, 2) does not require spheroid dissociation, and 3) is linear and highly sensitive for MIAPaCa-2 and PANC-1 spheroids up to 1.24×10^5 and 1.19×10^5 cells/spheroid, respectively (both up to a size of 1000 μm).

To establish a pancreatic cancer-specific, spheroid-based model, we used the APH assay by treating MIAPaCa-2 and PANC-1 spheroids with gemcitabine and 5-FU, two drugs that are commonly used for pancreatic cancer. The efficacy of gemcitabine and 5-FU in monolayer and spheroid cultures was compared. We observed a reduced sensitivity of spheroid cell cultures to gemcitabine and 5-FU compared to monolayer cultures, which should be reflected not only by spheroid integrity and size but also by APH signals and APH-dependent IC_{50} values. In the present study, monolayer controls of MIAPaCa-2 and PANC-1 showed dose-response curves and IC_{50} values consistent with the published literature, both for gemcitabine and 5-FU (35,38,39). Drug effects on MIAPaCa-2 spheroids were shown via the APH assay, but the incubation interval necessary to reach the IC_{50} demonstrated that spheroids of the MIAPaCa-2 pancreatic cancer cell line were less sensitive to gemcitabine and 5-FU than monolayer culture. Reduced drug efficacy was also seen in PANC-1 in the spheroids compared with monolayer culture. We conclude that our pancreatic cancer spheroid model more closely reflected the efficacy of antitumor drugs. With the APH assay, classic drugs for pancreatic cancer such as gemcitabine and 5-FU could be easily monitored in spheroids and compared with monolayer data. One of the potential applications of the spheroid-APH assay is the testing of single and combined therapeutic strategies with new target-, and/or pathway-specific treatment modalities.

In conclusion, we established a spheroid-based culture model in the MIAPaCa-2 and PANC-1 pancreatic cancer cell lines for drug testing with the APH assay. The model integrates reproducible, easy, and rapid handling for drug testing of pancreatic cancer. The model would help to fill the gap between monolayer cultures and *in vivo* models in the process of drug development and testing for pancreatic cancer.

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