RESEARCH ARTICLE

Development of an in vitro tumor spheroid culture model amenable to high-throughput testing of potential anticancer nanotherapeutics

Melani A. Solomon, Jenkins Lemera, and Gerard G. M. D'Souza

Department of Pharmaceutical Sciences, MCPHS University, Boston, MA, USA

Abstract

Context: Three-dimensional tumor spheroid cultures are a better representative of in vivo solid tumors than monolayer cultures and should be used for testing potential nanotherapeutics in vitro.

Objective: To develop techniques to test the disposition and efficacy of nanocarrier formulations in spheroids in a cost-effective manner amenable to high-throughput testing.

Methods: Spheroids were obtained using a modified liquid overlay technique in a 96-well plate. Several nanocarrier formulations were prepared and tested in the spheroid model. The disposition of the formulations in the spheroids was determined by confocal microscopy while the effect of the drug-loaded formulations was assessed in terms of the cell viability, loss of membrane integrity, induction of caspases and inhibition of growth of the spheroids.

Results: The surface charge of the formulations influenced the accumulation of the nanocarrier and drug in the spheroid, with the cationic formulation accumulating to the greatest extent. Also, the smallest particle size formulation, micelles, penetrated to the greatest extent in the spheroid. The iRGD tumor-penetrating peptide co-administered with unmodified liposomes exhibited both high accumulation and penetration. The effect studies revealed that the formulations that penetrated or accumulated to the highest extent in the spheroid exhibited better antitumor activity compared to the other formulations.

Conclusion: The 96-well plate format spheroid model developed in the study can be used toward the rational selection of nanocarrier therapeutics prior to their testing in in vivo models.

Keywords

Accumulation, apoptosis, cytotoxicity, liposomes, nanoparticles

Introduction

Monolayer cultures of cells are most commonly used as models to select optimal treatment regimens for solid tumors. However, although cancer cell lines exhibit similar mutations and gene expression profiles as in vivo tumors, they lack the three-dimensionality, heterogeneity, dense extracellular matrix and penetration barriers as in vivo solid tumors. As a result, effects seen in this model may be an overestimation or an underestimation of the actual effect that may be seen in vivo (Herrmann et al., 2008; Imamura et al., 2015; Sambale et al., 2015). Several three-dimensionally growing cultures of tumor cells have been generated and studied as suitable models for solid tumors. Some of these include the cylindroid model (Kim et al., 2010a), the toroid model utilized to study the self-assembly of microtissues (Youssef et al., 2011) and spheroids.

Spheroids are spontaneously aggregating three-dimensional cultures of tumor cells that have been extensively studied since the 1970s (Mueller-Klieser, 1987). Most of the studies in this regard had been restricted to the better understanding of cancer biology and growth patterns of tumors (Haji-Karim & Carlsson, 1978; Knuechel et al., 1990). Such studies established that the extracellular matrix of spheroids is chemically similar to that of solid tumors (Yamada & Cukierman, 2007). Further, the growth pattern of spheroids was found to be similar to the avascular stage of in vivo tumors. Spheroids have also been utilized as a model to study the intratumoral distribution characteristics of commonly used chemotherapeutic drugs (Erlanson et al., 1992; Nederman, 1984; Olive & Durand, 1994; Rofstad & Sutherland, 1989). The major limitation of spheroid-based systems is that currently they have not been adapted to high-throughput testing on a similar scale as monolayers have been (LaBarbera et al., 2012; Mehta et al., 2012). Some attempts have been made to study spheroids in a high-throughput manner. Friedrich et al. (2009) developed a semi-automated technique to produce spheroids and to study the effect of drugs on reducing the size of spheroids. Fayad et al. (2009) developed another high-throughput technique to generate spheroids and study the proapoptotic effect of drugs on them (Fayad et al., 2011) and more recently Yoshii et al. developed a technique to generate spheroids from patient cells in a high-throughput format and test the effect of drugs on them by...
positron emission tomography (Yoshii et al., 2015). However, screening of chemotherapeutic small molecules using the spheroid model is still limited.

The major reason for the lack of utility of the spheroid model in studying the effect of small molecules on solid tumors may be the lack of vascularization in the spheroid model which limits the predictability of specificity of the chemotherapeutic agent for the tumoral cells. However, unlike the small molecular drugs, nanocarriers have been shown to selectively extravasate to solid tumor sites by the enhanced permeability and retention (EPR) effect. Thus in this case, a spheroid perfectly represents the microenvironment that a nanocarrier would encounter on extravasation. A few studies have been performed to test the disposition of nanocarriers in the spheroid model (Goodman et al., 2008; Kostarelos et al., 2004), but in all these studies spheroids were only used as an extension of the monolayer cultures, i.e. to prove that an agent that was effective in monolayer cultures also showed effectiveness in a spheroid model (Ho et al., 2011; Kim et al., 2010b; Perche et al., 2012; Wang et al., 2011; Wolf et al., 2011; Yao et al., 2011; Ying et al., 2007). Some studies have also proposed that spheroids can be used to screen drug-loaded nanocarriers (Kang et al., 2015; Sambale et al., 2015). However, no high-throughput method to test the distribution of nanocarriers and the effect of drug-loaded nanocarriers on the spheroid model has yet been performed. Hence, the aim of this work was to implement in the spheroid model the evaluation of all possible nanocarrier treatment outcomes (cytotoxicity assays, apoptosis assays, cellular association, uptake and intracellular distribution) as are traditionally evaluated in monolayer cultures.

Materials and methods

Materials

Lewis lung carcinoma (LLC) cells were obtained from ATCC (Manassas, VA). All lipids unless specified were purchased from Avanti Polar Lipids (Alabaster, AL). Stearyl triphenyl phosphonium (STPP) was obtained as a synthesized product as described in Boddapati et al. (2005). Dioleoyl triphenyl phosphonium (DOTPP), dimyristoyl triphenyl phosphonium (DMTPP) and dipalmitoyl triphenyl phosphonium (DPTPP) were custom-synthesized in our laboratory. Triphenyl phosphonium bromide, staurosporine and gelatin 225 bloom were purchased from Sigma-Aldrich (St. Louis, MO). CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit and Caspase 3, 8, 9 luminescence assay kits were purchased from Promega (Madison, WI). The bicinchoninic acid (BCA) protein assay kit was obtained from Pierce (Rockford, IL). Tissue-tek® OCT matrix was from Sakura (Torrance, CA). The 96-well plate format analysis of accumulation and penetration in spheroids was obtained from MatTek Corporation (Ashland, MA).

Production of spheroids

LLC cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The cells were sub-cultured regularly and were utilized for experiments at a 70% confluence. LLC spheroids were produced using a liquid-overlay technique on agar surfaces. One percent of agar was coated in each well of a half-area 96-well plate and 1000 LLC cells were transferred to each well using a multichannel pipette. The plate was centrifuged using a Marathon 3000R centrifuge (Thermo Fisher Scientific, Rockville, MD) at 250 × g for 15 min and placed in 5% CO₂, 37 °C atmosphere to allow the spheroids to grow. The size of the spheroids was determined every 24 h by imaging the spheroids and determining the diameter of the best-fit ellipse region around the spheroid using a microscope (NIS Elements software®, Nikon, Tokyo, Japan).

Histology of spheroids

A 6-day old spheroid was removed from the agar-coated plate into a 1.5 ml conical tube. The media was carefully aspirated out and the spheroid was washed 2× with phosphate buffered saline (PBS). For fixation, 0.001% methylene blue dye in 4% paraformaldehyde was added to the tube and placed at 4 °C for 24 h. Following fixation, the spheroids were embedded in OCT matrix and 10 μm sections were obtained using a microtome (Reichert 855 HistoSTAT cryostat microtome). The sections were stained with hematoxylin and eosin and observed under a light microscope.

Determination of metabolic activity of spheroids

Spheroids were transferred from the agar-coated plate into wells of an uncoated 96-well plate. The medium surrounding the spheroids was aspirated out and the spheroid was washed 2× PBS. The spheroid was dissociated with treatment with 0.25% trypsin and gentle pipetting to obtain a suspension of cells in a final volume of 100 μl. Simultaneously, from a suspension of LLC cells, 860, 8600, 86 000 cells were also transferred to the plate in the same volume. Ten microliter of methyl tetrazolium salt (MTS) reagent was added to each well and the plate was incubated at 37 °C, 5% CO₂ for 1 h. The absorbance of the plate was read at 490 nm using a microplate reader (Synergy HTX Multi-mode microplate reader, BioTek, Winooski, VT).

Inter-plate transfer of spheroids

The agar-coated half-area plate containing the growing spheroids was inverted over a 96-well plate containing treatments and secured using bands. This set-up was centrifuged at 250 × g for 2 min.

The 96-well plate format analysis of accumulation and penetration in spheroids

Spheroids were treated with the respective rhodamine-labeled formulation or labeled drug-loaded formulation for 1 h. Following this, the spheroids were washed with PBS twice and treated with 100 μM Hoechst for 15 min. The spheroids were washed again with PBS, fixed in 4% paraformaldehyde and transferred to corresponding wells of an optical grade glass bottom plate. Thereafter, the spheroids were imaged using a spinning disk confocal microscope in an inverted configuration (Olympus IX81 equipped with a Yokogawa spinning disk) at 561, 488 and 405 nm to detect the
fluorescence of rhodamine, Oregon Green paclitaxel or Hoechst, respectively. The position of the spheroid in each well was specified using the multi-position capability in the instrument following which the Z-stacks were acquired in a high-throughput format at 100 × magnification. Each optical section was 5 μm. Using ImageJ, the integrated densities of the formulation or the drug in the Z-stack were normalized by the integrated densities of the Hoechst-stained nuclei in the same stack and plotted for comparison. For assessment of penetration, optical sections were obtained as described above. Thereafter, the optical section at 100 μm depth from the surface of the spheroid was chosen and intensity profiles along 6 diameters of the section were obtained using ImageJ. This was done for three spheroids in each treatment group. The intensities in each section were normalized by the maximal intensity in the respective section to give a term \( I / I_{\text{max}} \). The penetration distance at 0.3 \( I_{\text{max}} \) was used as an indicator of penetration depth.

Flow cytometry evaluation of association of nanocarriers with cells of the spheroid

Three spheroids were pooled together in a volume of 100 μl of medium in a conical tube. Thirty microliters of a rhodamine-labeled nanocarrier formulation was added to the medium and the spheroids were incubated for 1 h at 37°C, 5% CO₂. The spheroids were washed 2 × with PBS by gentle pipetting. Then, the spheroids were treated with 10 μM Hoechst for 15 min at 37°C and washed to remove any excess dye. Following this, spheroids were trypsinized with 0.25% trypsin at 37°C with gentle pipetting to disperse the spheroid. When the spheroids dispersed (15 min), fresh media was added to neutralize the trypsin and the spheroid cell pellet was obtained by centrifugation. The cells were fixed in 4% paraformaldehyde and were subjected to flow cytometry analysis (Becton Dickinson FACSCalibur, BD Biosciences, Franklin Lakes, NJ).

Histological assessment of penetration of nanocarriers in spheroids

Spheroids were treated with the respective rhodamine-labeled nanocarrier formulation for 30 min following which they were stained with Hoechst, 100 μM for 15 min. The spheroids were washed thoroughly with PBS and cryosectioned. (Note: The spheroids were sectioned in absence of direct fluorescent light.) The sections were imaged under a 4',6-diamidino-2-phenylindole (DAPI) filter and a rhodamine filter to visualize the Hoechst-stained nuclei and labeled nanocarriers, respectively, using an epifluorescence microscope (Nikon Eclipse E600). Unlabeled liposome-treated spheroid sections were used as controls to subtract the background for the liposome distribution. The widest-diameter section was chosen and the intensity profiles along 6 diameters of the section were obtained using ImageJ (National Institutes of Health). This was done for three spheroids in each treatment group. The average intensities in each section were normalized by the maximal intensity in the respective section to give a term \( I / I_{\text{max}} \). The penetration distance at 0.3 \( I_{\text{max}} \) was used as an indicator of penetration depth.

Determination of drug concentration in spheroids

High performance liquid chromatography (HPLC) conditions: Agilent 1200 system with UV detection; column: Ultrasphere C18, 5 μm, 250 × 4.6 mm; mobile phase composition: Acetonitrile: Methanol:Water = 30:30:40; flow rate: 1 ml/min; detection wavelength: 228 nm. The chromatograms were acquired in a high-throughput format at 100 × C213. The HPLC method was linear (r² = 0.99) in the concentration range of 1–500 μg/ml. Eight spheroids, 10-day old, were taken into a tube and treated for 24 h following which the spheroids were washed thoroughly with PBS to remove any loosely bound drug. Thereafter, the spheroids were disrupted using a cell lysis buffer (BioVision, Milpitas, CA) and pipetting harshly. After lysis of the cells, four volumes of tert-butylmethylether were added to the buffer and the tube was vortexed for 3 min. The samples were then centrifuged at 3000 × g for 10 min, the organic phase was transferred to a fresh tube and the solvent was evaporated off. Finally, the residue remaining after evaporation was reconstituted in the mobile phase and analyzed by the developed method. The data are represented as fold-accumulation of paclitaxel relative to the Plain liposomes-treated group.

Determination of subcellular distribution in spheroids

Spheroids were treated with the rhodamine-labeled formulations or Oregon Green paclitaxel-loaded formulations for 1 h. Following this, they were washed and mitochondria were stained with 200 nM Mitotracker® Green or 500 nM Mitotracker® Red for spheroids treated with the rhodamine-labeled formulations or Oregon Green paclitaxel-loaded formulations, respectively. Thereafter, the spheroids were washed with PBS twice and treated with 100 μM Hoechst for 15 min. The spheroids were washed again with PBS and transferred one each to each well of an optical grade glass bottom plate. Thereafter, the spheroids were imaged using spinning disk confocal microscopy at 561, 488 and 405 nm to detect the fluorescence of rhodamine or Mitotracker® Red CMXRoS, Oregon Green paclitaxel or Mitotracker® Green and Hoechst, respectively. The Z-stacks were acquired at 400 × magnification. Each optical section was 2 μm. Colocalization analysis was performed in ImageJ by determining the Pearson’s coefficient. Data are plotted as Pearson’s coefficient for comparison.

Determination of cell viability of spheroid cells

Cell viability was determined by the MTS assay with slight modifications to the manufacturer’s protocol. Spheroids were treated, in triplicate, with 50 μM paclitaxel-loaded formulations for 24 h following which the spheroids were washed thoroughly with PBS. Thereafter, 10 μl of the MTS reagent was added with 100 μl of media per well and the plate was incubated at 37°C, 5% CO₂ until a color change to deep red was seen in the control wells. The absorbance was measured at 490 nm using a multi-plate reader. The absorbance of 0.1 N HCl or lysis buffer-treated spheroids was used as an indicator of 0% viability while the absorbance of untreated spheroids indicated 100% viability. The effects due to the treatments were expressed as % cell viability of spheroids relative to the untreated control.
Determination of membrane integrity of spheroid cells

The LDH assay measures the LDH enzyme released by cells when they rupture. The spheroids were treated with the respective treatments for 24 h following which the plate was centrifuged at 73°C for 2 min. Thereafter, 50 μl of the cell-free supernatant was removed into a new plate and 50 μl of LDH substrate mix was added to it. The plate was incubated at 37°C for 10 min following which 50 μl of the stop solution was added and absorbance was read at 490 nm using a multi-plate reader. The absorbance of lysis buffer-treated cells served as the positive control for this assay, i.e. 100% loss of cell membrane integrity while the absorbance of untreated spheroids served as a measure of 0% loss of membrane integrity. The effects due to treatments were expressed as % loss of cell membrane integrity relative to the positive control.

Measurement of caspase activity in spheroid cells

Spheroids, 6-day old, were treated with the respective treatment for 24 h in a 96-well plate. Thereafter, the caspase-Glo 3/7 or caspase-Glo 8 or caspase-Glo 9 reagent (Promega) containing the lysis buffer and caspase 3/7 or caspase 8 or caspase 9 substrate, respectively, was added to the wells and gently pipetted to disperse the spheroid. The contents of each well were transferred to a corresponding white plate using a multichannel pipettor and the plates were incubated in the dark for 1 h. Thereafter, the luminescence was measured in a multi-plate format using a plate reader. The caspase activities were normalized by the protein contents. The results were expressed as fold-increase in caspase activity relative to an untreated control.

Determination of reduction in size of spheroids

Spheroids were transferred to the treatment plate and the initial size of the spheroids was obtained. Thereafter, the diameter was measured every 24 h until the end of the study (72 h post-treatment) by an observer blinded to the treatments. The results were expressed as the change in diameter of the spheroids from the initial diameter.

Preparation of nanocarrier formulations

Liposomes were prepared by the standard thin-film hydration method. Briefly, lipids were mixed in the desired ratio (Table 1) and evaporated to form a thin film using a rotary evaporator. The thin film was hydrated with PBS and probe sonicated to yield liposomes at a concentration of 5 mg/ml.

Micelles were formed by the thin-film hydration method. Briefly, the desired quantity of the PEG-PE-2000 lipid was evaporated to form a thin film using a rotary evaporator. The thin film was hydrated with PBS and sonicated for 5 min using a probe sonicator to yield micelles at a concentration of 5 mg/ml. For the preparation of drug-loaded liposomes and micelles, a solution of paclitaxel in chloroform was prepared and it was added to the lipid mixture at the desired concentration. Thereafter, the film was hydrated and liposomes were prepared. For fluorescence experiments, liposomes and micelles were surface modified with rhodamine (0.5%) or Oregon Green-labeled paclitaxel was loaded in the liposomes.

Gelatin nanoparticles were prepared by a desolvation method modified from Lu et al. (Lu et al., 2004). Briefly, 100 mg of gelatin (225 bloom) was dissolved in 10 ml of water containing 20% Tween-80. The solution was heated to 40°C with constant stirring. When a solution of gelatin was obtained,
8 ml of a 20% solution of sodium sulfate was added drop wise. Thereafter, 7 ml of distilled water was added until the solution turned clear. Following this, 250 µl of a 40% v/v solution of glyoxal was added to cross-link the gelatin. After 5 min, 5 ml of a 12% sodium metabisulfite solution was added to stop the cross-linking and the solution was stirred for 20 min. To prepare rhodamine-labeled gelatin nanoparticles, 400 µg of rhodamine-dextran dissolved in methanol was added to the gelatin solution during the addition of the sodium sulfate solution. Similarly, for the preparation of the paclitaxel-loaded gelatin nanoparticles, a solution of paclitaxel in methanol was added to the gelatin solution during the addition of sodium sulfate. The unencapsulated rhodamine or paclitaxel were separated from the preparation by dialysis against distilled water for 18 h with frequent changes in the external medium.

The nanocarriers were evaluated for their size and zeta potential using a Brookhaven particle size analyzer (Brookhaven Instruments Corporation, Holtsville, NY) while their encapsulation efficiency was determined by HPLC. The unencapsulated paclitaxel was precipitated by centrifugation at 3000 × g for 10 min. Thereafter, the liposomes and micelles were ruptured using 1% Triton X-100 while the gelatin nanoparticles were diluted with the HPLC mobile phase and the amount of paclitaxel incorporated was determined.

**Results**

**Spheroids were obtained reproducibly in a 96-well plate format**

Using the modified liquid overlay technique, spheroids of maximum size of 1 mm were obtained reproducibly in each well of the 96-well plate (Figure 1A). The growth pattern of the spheroids (Figure 1B and C) was found to be similar to the avascular phase of growth of in vivo solid tumors (Gimbrone et al., 1974). Further, histologically, the spheroid section was very similar to the section from an in vivo solid tumor (Figure 1D) (Fine et al., 2010). The metabolic assay...
revealed that a spheroid containing approximately 80 000 cells contained about 10 000 metabolically active cells (Figure 1E).

**Evaluation of nanocarrier disposition in the spheroid model revealed differences between the accumulation and penetration of nanocarrier treatments**

The size and zeta-potential of the various nanocarrier preparations used in the studies are shown in Table 1. The 96-well plate format testing of the nanocarriers in the spheroid model was conducted as per the scheme shown (Supplementary Figure 1).

Using a spinning disk confocal microscope in an inverted configuration, the spheroids in the glass bottom well plate could be imaged up to a maximum depth of 100 μm from the surface at 100 × magnification. Acquisition of images from all 96 wells took 6 h. A detailed analysis of the penetration of the formulations revealed that the micelles and iRGD peptide co-administered with the Plain liposomes (Plain + iRGD) penetrated to the greatest extent in spheroids (Figures 2 and 3A). In a separate experiment, spheroids treated with the respective nanocarrier formulation were sectioned and the penetration pattern was confirmed to be similar to the pattern we observed with the high-throughput imaging technique (Supplementary Figure 2).

Further, the accumulation of the nanocarrier was determined by measuring the integrated density of the formulation in each optical slice comprising the 100 μm distance. As can be seen in Figure 3(B), the cationic formulation (1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)-liposome) accumulated to a higher extent than the neutral (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)-liposome) or the anionic formulation (1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS)-liposome). The mitochondriotropic formulations, although cationic, did not accumulate to as high an extent as the DOTAP formulation. This was further confirmed by a study designed to assess the differential association of nanocarriers with the LLC spheroids by flow cytometry.
where the cationic liposomes (DOTAP-liposome) exhibited a higher association with cells of the LLC spheroids compared to the other formulations tested (Supplementary Figure 3). Although the micellar formulation penetrated into the spheroids more than the other formulations, the accumulation of the micelles was not as high. Co-administration of the iRGD peptide together with the Plain liposomes increased the accumulation of the Plain liposomes almost three-fold compared to the Plain liposomes alone.

Further, the 96-well plate format confocal imaging technique was also used to determine the accumulation and penetration of Oregon Green-labeled paclitaxel in spheroids (Figure 4A). The paclitaxel delivered by the iRGD peptide co-administered with the Plain liposomes (iRGD/Plain + Pxt-liposome) formulation both accumulated and penetrated to the maximum extent of all the formulations (Figure 4B), which correlated with the accumulation and penetration of the formulation. Surprisingly, however, the DOTAP formulation, which exhibited a high accumulation in the spheroid, did not deliver the drug to as high an extent. Similarly, although the micellar formulation penetrated to a depth of 120 µm in the spheroid, the drug loaded in the micelles only penetrated to a depth of about 70 µm (Figure 4C).

Finally, in order to verify the amount of drug accumulated in the spheroids, a liquid chromatographic technique was used to estimate the paclitaxel content extracted from spheroids at the end of a 24-h treatment with the respective paclitaxel containing formulation. The cationic liposome formulation, the mitochondriotropic formulations, the iRGD/Plain + Pxt-liposome and the micellar formulations delivered a higher amount of paclitaxel in spheroids compared to the other formulations (Figure 5).

**Spheroids can be utilized for studying the subcellular distribution of formulations**

At a high magnification (400×) in the spinning disk system, the LLC spheroids could only be imaged clearly till a maximum depth of 80 µm from the surface of the spheroid. Following a treatment for 1 h, only the STPP-liposome formulation exhibited a significantly higher level of colocalization with the mitochondria compared to the Plain liposomes (Figure 6A and C). Further, Oregon Green-labeled paclitaxel delivered intracellularly by STPP-liposomes showed significantly higher colocalization with mitochondria compared to when delivered by Plain liposomes (Figure 6B).
Evaluation of the effect of drug-loaded nanocarriers in spheroids revealed that highly accumulating nanocarriers exhibited higher antitumor activity.

In a study designed to determine the viability of cells in the spheroid following treatment with the nanocarriers, the final concentration of paclitaxel in the media was 50 μM. None of the drug-loaded nanocarriers exhibited a significant decrease in cell viability following the treatment relative to the untreated control (Figure 7A). In a separate study, the loss of membrane integrity of cells in the spheroid following treatment was assessed. Treatment with the cationic formulation (DOTAP + Pxt-liposomes) and the iRGD/Plain + Pxt-liposome caused a 70% cell membrane rupture, about three times more than the Plain + Pxt-liposome-treated group (Figure 7B) while the anionic formulation (DOPS + Pxt-liposomes), the micellar formulation...
Evaluation of the apoptotic potential of paclitaxel in each of the formulations that were used for the delivery of paclitaxel revealed that the cationic DOTAP + Pxt-liposomes, the iRGD/Plain + Pxt-liposome formulation and the Micelles + Pxt formulation induced effector caspases 3/7 at a statistically significant higher level (greater than four-fold increase in caspase induction) than the other formulations (Figure 8A). Although the PEGylated formulations and the mitochondriotropic formulations exhibited a significantly higher level of caspase-3 activity relative to the untreated control, the levels of caspase induced were not as high as those generally associated with apoptosis. Further, in order to determine if the intrinsic or extrinsic pathway of apoptosis was activated, activities of initiator caspases 9 or 8 were measured, respectively, following a similar protocol as a measurement of caspase 3 activity. As can be seen in Figure 8(B and C), it appears that initiator caspase 9 was eventually involved in the activation of caspase 3 implicating the role of the intrinsic pathway of apoptosis in the toxicity of paclitaxel in the iRGD/Plain + Pxt-liposome-treated group. The DOTAP + Pxt or the Micelles + Pxt formulations did not significantly induce caspase 9 at the end of the 24-h period (Figure 8C).

The eventual aim of any treatment strategy for solid tumors is the shrinking of the tumor mass. Hence, the influence of the paclitaxel-loaded nanocarriers in shrinking the spheroids was studied. Spheroids were utilized for this study while they were still actively growing. The decrease in diameter of the spheroids following the addition of various treatments was not significantly different in 4-day old versus 6-day old spheroids (Supplementary Figure 4). The preparations that led to a decreased growth of the spheroids compared to the empty nanocarrier were the DOTAP + Pxt-liposomes, the iRGD/Plain + Pxt-liposome formulation and the Micelles + Pxt formulation (Figure 9A). Among these formulations, only the iRGD/Plain + Pxt-liposome treatment resulted in shrinking of the spheroid, although at a much less level than the lysis buffer treatment (Figure 9B).
Discussion

Generally, studies of the antitumor effects of drug-loaded nanocarriers have been performed using the 2-D monolayer model (Ahmad & Allen, 1992; Ke et al., 2014; Ramadass et al., 2015; Zhou et al., 2013). Most often, these studies have shown an increase in the cytotoxicity of the drug-loaded formulations compared to an unloaded control. However, since cell monolayers lack the three-dimensionality seen in in vivo solid tumors, effect-based studies in this model may overestimate the true effect that may be seen in vivo. Hence, we attempted to develop an in vitro three-dimensional tumor spheroid model that would enable a high-throughput testing of potential nanocarrier therapies for treatment of solid tumors in vivo. Several 96-well plate format protocols were successfully developed that could test the disposition and effect of nanocarrier formulations in the spheroid model. The disposition of the nanocarriers in the spheroids was characterized both in terms of the ability of the nanocarrier to accumulate in the spheroid as well as to penetrate into the spheroid. The rank order of the formulations based on their ability to accumulate and penetrate is shown in Table 2. As can be seen, most of the formulations that accumulated to a high extent in the spheroid were not able to penetrate while the formulations that penetrated to a high extent did not accumulate. The surface charge of the formulation may be one of the factors responsible for this effect. The positive charge of the DOTAP conferred a high cell-binding ability to the DOTAP-liposomes, which may have resulted in higher accumulation in the spheroid. On the contrary, the anionic DOPS-liposome formulation did not bind as strongly to the cells but was able to penetrate more than the DOTAP-liposomes. Thus, it appears that the strongly bound formulations penetrate less while the weakly bound formulations penetrate more into spheroids. Another factor that influenced the penetration of the formulations in the spheroids was the particle size of the formulation. The micelles were significantly smaller in size than the liposomes and gelatin nanoparticles, which may be responsible for the high penetration ability of the micelles. On the other hand, the larger gelatin nanoparticles exhibited a low penetration and

Figure 7. Determination of cytotoxicity of nanocarrier treatments in LLC spheroids. (A) Cell viability of LLC spheroids on treatment with the respective nanocarrier preparation at the end of 24 h. Data represented as mean ± standard deviation (n = 3). No differences were observed between treatments by a one-way ANOVA followed by Tukey’s multiple comparison tests. (B) Loss of membrane integrity of LLC spheroids on treatment with the nanocarrier formulations at the paclitaxel concentration of 50 μM at the end of 24 h. Data represented as mean ± standard deviation (n = 4). *p < 0.05; **p < 0.0001 compared to the untreated group (Medium), one-way ANOVA, Tukey’s multiple comparison tests.

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accumulation into the spheroid. Since a 1-h treatment with the labeled nanocarrier preparations was optimum to detect differences between treatments by a high-throughput optical sectioning technique, all the estimations of accumulation and penetration were made following a 1-h treatment of the spheroids with the respective formulation. However, the amount of paclitaxel that accumulated in the spheroids was determined by a liquid chromatographic technique at the end of a 24-h treatment, the duration of treatment following which the effect-based assays were also conducted. The drug accumulation experiments revealed that all the paclitaxel-loaded cationic liposomes, the Micelles + Pxt and the iRGD/Plain + Pxt-liposome treatment accumulated a higher amount of paclitaxel than the other formulations.

The effect of the drug-loaded nanocarriers on the spheroids was studied using various assays. The formulations that accumulated to a higher extent in the spheroid were also associated with a significant loss in membrane integrity of the cells of the spheroid. The toxicity was also associated with an induction of effector caspases 3/7, which was a consequence of activation of the intrinsic mitochondrial pathway implicated by the induction of caspase 9. In previous studies conducted both in vitro and in vivo, paclitaxel has been shown to trigger the intrinsic pathway of apoptosis (Fulda & Debatin, 2006; Tan et al., 2005). Further, the effect of the paclitaxel-loaded nanocarriers on the size of the spheroids was also determined. The formulations that exhibited either the highest accumulation or the highest penetration were associated with a decreased growth of the spheroids relative to the untreated control or the empty nanocarrier. However, only the iRGD/Plain + Pxt-liposome treatment showed a decrease in the size of the spheroids 72 h following treatment. Of the parameters that we tested, it appeared that the accumulation of the nanocarrier in the spheroid played an important role in

Figure 8. Measurement of caspase activity in nanocarrier-treated spheroids. (A) Fold caspase 3 activity in LLC spheroids on treatment with 1 μM staurosporine (STS) or nanocarrier formulations at 50 μM paclitaxel concentration after 24 h relative to an untreated control. Data represented as mean ± standard deviation (n = 3). *p < 0.05, one-way ANOVA, Tukey’s multiple comparison tests. (B) Fold caspase 8 activity in LLC spheroids on treatment with 1 μM STS or nanocarrier formulations at 50 μM paclitaxel concentration after 24 h relative to an untreated control. Data represented as mean ± standard deviation (n = 3). No differences were observed between treatments by a one-way ANOVA followed by Tukey’s multiple comparison tests. (C) Fold caspase 9 activity in LLC spheroids on treatment with 1 μM STS or nanocarrier formulations at 50 μM paclitaxel concentration after 24 h relative to an untreated control. Data represented as mean ± standard deviation (n = 3). *p < 0.05, one-way ANOVA, Tukey’s multiple comparison tests.
influencing the effect of the drug loaded in the nanocarrier. It seems that the accumulation of the formulation, even if the accumulation is limited to the periphery, is associated with a greater cytotoxicity than the other formulations. On the other hand, if the formulation penetrates to a high extent in the spheroid, it can also exhibit cytotoxicity. The best case scenario exists when a formulation can accumulate and penetrate to a high extent in spheroids as was seen in case of co-administration of iRGD peptide with the paclitaxel loaded Plain liposomes. Although all the effect-based studies described exhibited differences between treatments, none of the formulations brought about any reduction in the cell viability of the spheroids relative to the untreated control. One plausible explanation for this phenomenon may be that on treatment with the formulations and loss of the proliferative layer, the quiescent layers of cells in the spheroid may start proliferating again. Since the MTS assay determines the metabolic activity of cells, the compensation of the spheroid cells on treatment may not permit the detection of any reduced metabolic activity. This observation also highlights the importance of performing multiple effect-based assays rather than relying on the results from one cytotoxicity assay toward establishing the activity of a therapeutic moiety. In all these studies, we did not use paclitaxel alone as a control due to the low aqueous solubility of paclitaxel. Instead Plain liposomes containing paclitaxel served as the control.

The 96-well plate format spheroid study selected the DOTAP-liposomes, micelles and the iRGD peptide co-administration with Plain liposomes for further testing in vivo. Cationic liposomes have been previously shown to accumulate at tumor vasculature and shrink tumors (Campbell et al., 2002; Kunstfeld et al., 2003; Schmitt-Sody et al., 2003; Strieth et al., 2004). Similarly, micellar formulations have also exhibited improved antitumor activity in vivo (Bae et al., 2005; Gong et al., 2012, 2013; Kim et al., 2010b). The iRGD peptide was used at a concentration similar to that reported in previous studies (Sugahara et al., 2010).
The co-administration of iRGD peptide with paclitaxel loaded Plain liposomes led to a three-fold increase in accumulation and penetration of the drug in the spheroids. This increased accumulation and penetration was associated with several cytotoxic effects and even led to a decrease in the size of the spheroids 72 h following treatment. These results are consistent with in vivo results reported in the literature. Thus, the spheroid model was able to select a therapeutic strategy that had shown to work in an in vivo environment. Surprisingly, the PEGylated formulation did not exhibit a significant effect in LLC spheroids. In view of some previously published in vivo studies which did not find any improvement in antitumor activity on surface modification with PEG (Hong et al., 1999; Parr et al., 1997), the spheroid model seemed to have been justified in not selecting the PEGylated liposomal formulation.

With subcellular targeting approaches gaining increased attention, we developed a high-throughput confocal-microscopy-based assay to determine the subcellular distribution of nanocarriers, specifically their colocalization with the mitochondria. Mitochondriotropic ligands such as STPP when incorporated into liposomes have been shown to increase the cytotoxicity of the encapsulated drug (Boddapati et al., 2005). A subcellular distribution study of the mitochondriotropic liposomes in the LLC spheroids revealed that only the STPP-liposomes formulation colocalized with the mitochondria to a significantly higher level compared to the other newly synthesized mitochondriotropic formulations. However, the paclitaxel-loaded STPP-liposomes did not exhibit any improvement in the activity of the encapsulated paclitaxel in LLC spheroids. In a separate study conducted on Ovcar-3 (paclitaxel-resistant cell line) spheroids, a significant reduction in the viability of Ovcar-3 spheroids was observed on treatment with the STPP + Pxt-liposomes compared to the Plain + Pxt-liposomes (Solomon et al., 2013). Similarly, in an in vivo study, paclitaxel-loaded mitochondriotropic liposomes displayed an antitumor effect in drug-resistant lung cancer (Wang et al., 2011). Thus, mitochondriotropic liposomes may have a cell-line specific effect with a higher chance of improving the sensitivity of resistant tumors to chemotherapeutic drugs.

The aim of this work was to adapt testing of spheroids to a high-throughput system. Although we could not validate the techniques we developed in a robotic system, all the terminal steps in the protocols used involved semi-automated instruments or motorized stages for the imaging measurements. Moreover, all of the intermediate steps were performed using multi-channel pipettes or high-throughput scalable operations in a 96-well plate format. We were able to perform all the studies with as much ease and speed as performing them on monolayers.

Table 2. Rank order of the formulations based on the different parameters.

| Parameters                          | Low          | Intermediate | High          |
|-------------------------------------|--------------|--------------|---------------|
| Accumulation of formulation         | DOPE         | DOTAP, Plain + iRGD | DOTAP, Plain + iRGD |
| Penetration of formulation          | DOTAP, DTPP, DOTPP, NPs, Plain, DOPE | DOTAP, Plain + iRGD, Micelles, NPs, DOTAP | DOTAP, Plain + iRGD, Micelles, DOTAP |
| Drug accumulation                   | DOPE, DTPP, DOTPP, NPs, Micelles, DOTPP | DOTAP, Plain + iRGD, Micelles, DOTAP | DOTAP, Plain + iRGD, Micelles, DOTAP |
| Loss of membrane integrity          | DOPE, DOPE, DOTAP, DTPP, NPs, Micelles, NPs | Plain, PEG, DOPE, DOTPP, NPs, Micelles, NPs, DOPS | Plain, PEG, DOTPP, NPs, Micelles, NPs, DOPS |
| Cell viability                      | Caspase induction | Size reduction |
|                                     | DOTAP, DOTPP, DTPP, NPs, Micelles, NPs | DOTAP, DOTPP, DTPP, NPs, Micelles, NPs | DOTAP, DOTPP, DTPP, NPs, Micelles, NPs |

Conclusion

Spheroids were generated reproducibly in a high-throughput amenable manner using relatively inexpensive materials. The nanocarriers that were selected by the 96-well plate format spheroid model were also those that have previously been shown to exhibit antitumor effects in in vivo systems. Thus, using the techniques developed, testing of nanocarrier-based treatment strategies can be done in a more relevant in vitro
system with as much ease and speed as performing them in monolayer cultures.

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Supplementary material available online
Supplementary Figures 1–4