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Oswald Biological Sciences First Place: Development of Three-Dimensional Lung Multicellular Spheroids in Air and Liquid Interface Culture for the Evaluation of Anti-Cancer Therapeutics

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1. Abstract

The implementation of *in vitro* results to *in vivo* applications has limitations due to conventional two-dimensional (2D) *in vitro* conditions lacking the ability to create a physiologically representative model. This study investigated a three-dimensional (3D) cell culture technique to model lung tumors *in vitro*. A 3D lung cancer model was created by applying collagen (a semi-non-adhesive material) to a transwell, which allowed for nutrient transfer through the collagen. Two lung cancer cells lines (H358, a bronchioalveolar carcinoma and A549, a lung adenocarcinoma) were seeded on top of the collagen. The non-adhesive collagen allowed the cells to preferentially attach to one another rather than to the surface, thus creating multicellular spheroids (MCS). To better mimic the environment for lung cancer specifically, an air-interface culture (AIC) as opposed to the commonly used liquid-covered culture (LCC) was created. For AIC conditions, the cell media on the apical side of the Transwell was removed and the basolateral side of the well was filled with cell media to allow for effective nutrient transport to the cells while also exposing the cells to air. A comparison of 2D and 3D cell behavior and viability was completed using paclitaxel and aerosol particles containing paclitaxel as a representative model for drug delivery.

As evidenced with brightfield and fluorescent microscopy imaging, the AIC model proved to yield viable MCS at sizes similar to MCS formed in LCC conditions (100 to 200μm in diameter). With the optimized 3D model, LCC cells were exposed to paclitaxel in media. In another drug delivery method, paclitaxel-loaded dry powder aerosol particles were delivered to AIC cells through direct application with an insufflator. This alternative delivery method using direct delivery of dry powder particles was be used as the drug application method on AIC since paclitaxel cannot be delivered through media as in LCC conditions. The nanoparticles containing paclitaxel are comprised of a PEGylated phospholipid excipient mixture which encapsulates the drug. Using viability analysis, it was shown that the applications of paclitaxel in LCC conditions show variance in efficacy when comparing 2D and 3D culture conditions (where the IC_{50} values for paclitaxel were higher for 3D compared to 2D). Transepithelial electrical resistance (TEER)

across Calu-3 (another lung adenocarcinoma cell line) monolayers was evaluated before and after particle delivery to illustrate that the particle application does not affect the permeability of the cells, which indicated that this form of drug therapy will not affect the permeability of lung tissue. Overall, a much more representative *in vitro* model has been developed that is expected to be an improved predictor of efficacy of alternative drug delivery methods such as direct pulmonary delivery for lung cancer, which could lead to more efficient drug therapies for lung cancer patients.

2. Introduction

2.1 The Importance of Lung Cancer Research

People in the United States die from lung cancer more than any other cancer diagnosis [1]. It is a prominent cause of death and is therefore a large focus in the research community today. There are many methods of treatment in use including chemotherapy, radiation, surgery, photodynamic therapy, or a combination of these [2]. *In vitro* models are a major factor in analyzing the effectiveness of treatment of drug chemotherapeutics. In lung cancer specifically, there are many characteristic elements of a tumor and tumor site that require an *in vitro* model to be altered and manipulated in order to make the model as physiologically representative as possible.

2.1 Purpose, Project Objectives, and Hypothesis

The purpose of this project was to develop an *in vitro* lung tumor model that could mimic *in vivo* tumor shape, behavior, and environment more closely and effectively and observe the effects of this model on chemotherapeutic drug studies. In order to do this, many aspects of the cell culturing methods to form a tumor model had to be altered. Specifically, a model that was three-dimensional, possessed a surrounding environment like an *in vivo* tumor, and could also be exposed to air (as cancer cells are in the lung) and continue to yield proliferating cells needed to be established. Therefore, the first objective of the project was to develop and optimize the

culturing conditions of three-dimensional lung multicellular spheroids in air interface and liquid covered culture conditions, ensuring proper spheroid development and viability. The next step was to evaluate the difference in cellular response of two-dimensional (2D) and three-dimensional (3D) cultured cells to the chemotherapeutic drug paclitaxel delivered under liquid covered culture conditions. Furthermore, in considering that lung cancer cells uniquely grow in the presence of air, the final objective was to evaluate the difference in cellular response of 2D and 3D cultured cells to paclitaxel-loaded dry powder aerosol particles under air interface culture conditions.

The two different culturing methods of air interface and liquid covered culture were expected to yield similarly sized multicellular spheroids with equally viable cells. Once each of these models was established, the delivery of paclitaxel to the 3D cultured cancer cells under liquid covered culture conditions was predicted to return results that show that there was less of a response, in the 3D model to the drug than in the 2D model. Similarly, in the delivery of paclitaxel-loaded dry powder aerosol particles to the 3D model under air interface culture conditions, it was conjectured that the results would show less of a response in the 3D model than in the 2D model as well.

There are many benefits of this study and gathering this information. Determining the difference between 3D and 2D would allow more predictable transitions from initial *in vitro* studies to *in vivo* studies. In gathering knowledge about cancer drug efficacy and potency with this specific tumor model that considers pulmonary conditions, which is more physiologically representative for lung tumors, it will reveal valuable information about the potential of direct pulmonary chemotherapeutic delivery. Ultimately, the possibility of a more effective and efficient application of drug therapy will be an option for lung cancer patients.

2.2 Multicellular Spheroids and Development

The typical way to form a 3-dimensional tumor model is the formation of multicellular spheroids. Multicellular spheroids (MCS) are a three-dimensional configuration of cells. They

are largely beneficial when utilized as a 3D tumor model because they recreate a tumor and its surrounding environment quite closely. MCS form as cells grow and attach to each other. When they aggregate the integrin of the cells begin to interact with each other forming cadherin-cadherin bonds. The more bonds formed, the more compact the cells become, forming a 3D spheroid shape. The integrins also react to form an extracellular matrix (ECM) consisting of fibrous proteins and proteoglycans that are made in cell and secreted by exocytosis. The ECM provides structure for the cell formation, regulates the cell to cell interactions, and assists in cell hydration. The innermost layer of the spheroid consists of a necrotic core, surrounded by a layer of quiescent cells, with an outermost layer of proliferating cells [3]. The specific advantages of these characteristics of MCS are discussed in the next section. In general, all of these characteristics create a model that closely mimics that of a tumor model, allowing a smoother transition from *in vitro* to *in vivo* drug treatment studies and an ability to better predict *in vivo* results.

There are many methods of creating multicellular spheroids. Some common methods include liquid overlay, hanging drop, micromolding, spinner flask, rotary culture, and centrifugation. In liquid overlay, well plates are coated with a non-adhesive, partially hydrophilic material to create a surface on which the cells, once seeded, are more often inclined to attach to each other than to the surface. For the hanging drop method, cells in solution are seeded in multiple drops on the lid of a Petri dish or well plate. When the lid is turned over on top of the plate, the droplet does not fall due to adhesion, while gravity gathers the cells at the bottom of the droplet. Micromolding cells involves seeding the cells in solution in a plate that contains a series of very small rounded “molds” so that cells can gather at the bottom of the mold. In spinner flask culture, cells in excess solution are placed in a flask and constantly spun at certain speeds in order to keep the cells suspended in solution. Having nothing else on which to attach, the cells begin to attach to each other. In a rotary culture, cells are seeded in media into a rounded wheel-like container that constantly turns so that the cells gathered at the bottom of the container never have enough time to attach to the surface of the container. Therefore, again, with

nothing else to attach to and gathered together, they begin to attach and interact with each other. Finally, in centrifugation, cells are placed in media in a centrifuge tube and spun so that the cells pack into the bottom of the tube and begin to interact. [3] The method used in this particular project was the primarily mentioned liquid overlay. This method tends to work contingent upon the response of the cell line to this form of cell culture [4]. This is also one of the only methods that allows the use of an air interface conditions as a variable characteristic, which is discussed later. Overall, the formation of an MCS is the ideal 3D model and its desired characteristics for this project.

2.3 Three-Dimensional and Two-Dimensional Models

Drug testing is most often performed on cancer cell lines that are cultured in the conventional two-dimensional (2D) method. This form of cell culturing can be compared to the more complex but at many times more beneficial three-dimensional (3D) method. The benefits of a 3D model are numerous when considering that the goal of the model is to recreate tumor-like behavior and environment. The many characteristics of MCS mimic real *in vivo* tumors. The MCS contain necrotic cores, allowing many of the cells to be fairly "protected" by the outermost layer of cells. Since dug therapeutics will often be designed to attack proliferating cancer cells, a frequent occurrence is that the drug will successfully cause apoptosis in the outermost layer of a tumor, however, the necrotic core and quiescent cells are left alone. Once the outermost layer of cells die and break away, the quiescent cells begin to receive nutrients and proliferate [5]. This is an important characteristic to recreate so that certain drugs are not incorrectly labeled with a high efficacy when they are only effective on an outer layer of proliferating cancer cells. This characteristic may also be recreated with a 3D MCS model again because of its ability to create compact MCS that are separated from media by each other, an ECM, as well as other surfactant proteins secreted by the cells [7]. Furthermore, the ECM present provides structure and assists in cell hydration throughout the MCS with lessened exposure to the nutrients in media [5]. Directly following, this avascular model allows the buildup of metabolism including carbonic and lactic

acid causing a lower pH in and around the MCS, which also mimics *in vivo* tumors [14]. These aspects that mimic *in vivo* tumors are important when the goal of the model is to closely predict cellular response to certain anti-cancer drugs.

These advantages become noticeably more meaningful when a comparison to a 2D model is observed. The 2D method simply involves seeding cells grown in a flask with cell media for nutrients into well plates with a rough plastic surface on the bottom, so that the cells are able to attach to and grow on this uneven surface. This method of cell culturing has advantages and disadvantages when utilizing the cells for testing cancer drug efficacy and potency. This model allows quick and easy tests that can be done in a 96-well plate. Since many more trials can be done at once, it is consequently less expensive to do these tests on a large scale. However, the cells seeded in a 2D configuration will usually grow in the form of a monolayer of cells. This implies that, since the cells attach to the rough plastic surface of the well, they do not attach to or react with each other. Without cell to cell interactions, the cells are not induced to produce proteins or any surfactant. This results in a lack of an extracellular matrix, in turn resulting in no structure or support or extra hydration that an ECM brings. The fact that the cells do not attach to one another and build up means a lack of protection from other cells; each cell is just as exposed to its surroundings (including drug exposure) as any other cell. For this reason, a 2D model often displays results with a more sensitive response to a drug than what ends up being the case *in vivo*. [8]

2.4 Air Interface Culture and Liquid Covered Culture

The method of using liquid overlay to create MCS can be varied in another way. Liquid covered culture (LCC) is the condition of liquid overlay in which the cells are seeded onto a non-adhesive surface by applying the cells in media on top of the surface. The media that brings nutrients remains on top of the surface and the cells to keep nutrients available to the cells. Air interface culture (AIC) differs in that once the cells are no longer suspended in the media and have attached to the surface and one another, the media is removed so that the cells may be

exposed to air as well. In order to retrieve nutrients under these conditions, the non-adhesive material and the cells are often seeded in transwells that sit in the wells of a well plate. Media may be applied to the well itself, and the cells are able to retrieve nutrients as the media passes through the permeable bottom of the transwell [9] and also through the non-adhesive material to the cells. An asset of this type of culture is that the surfactant that is produced by lung cancer cells can often be washed away in cell media when LCC conditions are utilized. In AIC conditions, this surfactant would be able to remain intact, creating yet another aspect of an *in vivo* lung tumor to be represented in the MCS model [10]. In addition, AIC conditions allow the obvious but unique aspect of lung tumors to be mimicked; these cancer cells are exposed to air in the lungs.

2.5 Cell Lines

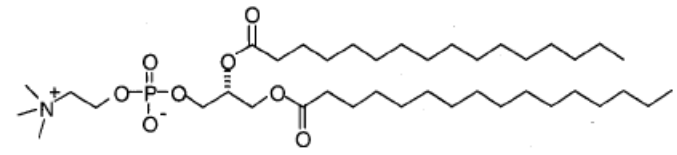
There were three cell lines used for this project. *H358* cells are Bronchioalveolar carcinoma cells, which is a type II alveolar non-small cell lung cancer. *A549* are Lung adenocarcinoma, which is also a type II alveolar cancer cell. [4] Both of these cell lines are known as type II alveolar cells, also known as pneumocytes, which are known to secrete a characteristic mucous layer when interacting with each other, as well as surfactant proteins such as A(SP-A), a pulmonary surfactant. A(SP-A) counteracts inhibitory proteins during lung injury. It also reduces surface tension among the cells, acting as a host site for particles and allowing for an attractive landing site for these particles. Harmful particles are bound by this protein in order to protect the lung [6]. These cell lines have proven to be good candidates for creating MCS through the use of the liquid overlay method [4]. The last cell line, Calu-3, are also a lung adenocarcinoma. This cell line forms very tightly-formed monolayers that allows for high levels of resistance, which was used ideally for TEER analysis studies [13].

2.6 Paclitaxel and DPPC:PTX Particles

Paclitaxel (PTX), also known as Taxol or Taxoprexin, is a largely popular, new upcoming anti-cancer drug (structure seen in Figure 1). It is one of the top five cancer drugs in research in the past few years. This drug was extracted and developed from the Pacific Yew Tree, a rare plant, which has led to its synthesis in the laboratory to be researched. It has proven to be a very effective drug, but it is nearly insoluble in water, and therefore must be dissolved in Dimethyl Sulfoxide (DMSO) in order to be applicable in solution. Paclitaxel functions as a mitotic inhibitor, stabilizing microtubules in cells to prevent cell division. Also, the apoptotic activity of this drug is able to influence penetrability of the outer layer of cells, allowing the drug to penetrate solid tumors depending on the tumor's cellularity and density. For these reasons, PTX is an ideal drug to use for this project. [11]

For a direct pulmonary delivery model, aerosol nanocomposite microparticles loaded with paclitaxel were made via spray drying. The components of the lipospheres formed are Dipalmitoyl phosphatidylcholine (DPPC) and the drug paclitaxel (PTX). These components molecular structures can be seen in Figure 1.

(1)



(2)

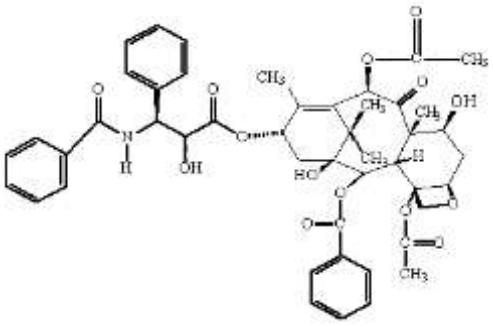


Figure 1. Molecular structures of: (1) DPPC, (2) Paclitaxel.

DPPC is a major constituent in lung surfactant (about 80% DPPC), so it is safe in the lungs upon degradation. This also assists in decreasing toxicity because no additional agents are needed for solubility since this component is already largely soluble. When spray dried, the DPPC forms a spherical shape called a liposphere. These particles can be loaded with many different therapeutic agents, and for this project PTX was used. These components combine to form particles approximately 400-1500nm in diameter and can penetrate into the deep lung regions. The location of particle deposition can also be tuned by varying the particle size slightly. These dry powder particles can be applied to cells cultured in AIC conditions *in vitro* via an insufflator (Figure 2), mimicking a direct pulmonary delivery through a dry powder inhaler. These particles would then make a path through the airway interstitial tissue to the epithelial lining, and finally really the tumor or local blood supply at which it would release the therapeutic drug. [12]

Figure 2. Schematic of insufflator.

2.7 TEER

Transepithelial Electrical Resistance (TEER) is a measurement of the electrical resistance across a cell monolayer. This measurement can be used to analyze the packing density and

permeability of the cells in a monolayer. It is often utilized to ensure that packing density does not decrease with the addition of any treatments to the cells being used in experimentation. The purpose of this analysis for this project is to ensure that the permeability of Calu-3 cells grown under AIC and LCC conditions are not significantly different, as well as to ensure that the delivery of any chemotherapeutic particles to the cells would not significantly increase the cells' monolayer permeability. If this were to occur, the cell tissue in the lung would become compromised and allow fluid to permeate the tissue. TEER across cells in AIC and LCC conditions has been established to have statistically equal resistance levels [13]. For this project, equal resistance levels are desired for cells grown under AIC conditions without any nanoparticle exposure, cells exposed to blank (DPPC only) aerosolized lipospheres, and cells exposed to drug-loaded (DPPC:PTX) lipospheres. This analysis involves measuring the resistance of the cells by growing a cell monolayer under both LCC and AIC conditions, exposing the AIC cells to nanoparticles through direct delivery, and placing the monolayer between two electrodes in order to gather the resistance measurement across the monolayer before and after exposure. Although this study involves a 3D model for drug delivery, in order to obtain a resistance measurement the cells must be confluent, otherwise the resistance would not be uniform between the electrodes. Also, the Calu-3 monolayer is meant to represent healthy lung tissue in this study because it has many characteristics of lung cells despite the fact that it is technically a lung cancer cell line. For these reasons, the cells were grown in a 2D model [13].

3. Materials and Methods

3.1 Cell Seeding

In order to have a steady supply of cells to seed and plate into wells for each experiment, each cell line was broken out into flasks and grown with phenol-red free cell media until they were ready to be seeded. This media used throughout the project was made my mixing 445mL Dulbecco's Modified Eagle Medium (DMEM), 50mL Fetal Bovine Serum (FBS), 292mg L-Glutamine, and five milliliters Sodium Pyruvate, and vacuum-filtering it for sterilization. This

media is ideal for this experiment in that it is a clear, pale yellow color and is transparent for the viewing/imaging of cells. The cells were allowed to grow in an incubator at 37 °C until about 60% confluence and were split. At this time, the cells were washed with 2mL of trypsin, a digestive enzyme that enables the cells to detach from the rough plastic surface of the flask. Another 3mL of trypsin was added to the flask and the flask was allowed to incubate for 15-20 minutes to allow full detachment of cells. When all cells were detached, 7mL of DMEM media was added to the flask for a total of a 10mL cell solution. Twenty microliters of this solution was placed into the Cell-O-Meter (cell counting instrumentation/software) in the culture lab. This software provided cell density information, and allowed a dilution to the preferred cell seeding density to be calculated (varying according to each experiment and cell line). The cell solution was then diluted with media to this preferred density and pipetted into the well plates for each experiment.

3.2 Two-Dimensional Study with Paclitaxel

Both H358 and A549 cell lines were diluted to 75,000 cells/mL and 30,000 cells/mL, respectively, and seeded in a 96-well plate at 200μL/well with half of the plate seeded with H358 and the other half A549. These seeding densities were chosen based on knowledge of each cell line's doubling growth rate. The cells were allowed to grow for 48 hours and then paclitaxel was added in eight different concentrations (varying from 5μM to .0001μM) across the plate. Paclitaxel, as previously mentioned, needed to be dissolved in DMSO. Therefore, a stock solution was made of 5000μM PTX in DMSO (or 4.27mg PTX/mL DMSO). The paclitaxel being fully dissolved, the solution was then diluted with DMEM media to a nontoxic amount of DMSO (.10% vol.). A DMSO/media control as well as a media only control was also used. The PTX was then applied to the cells in the 96-well plate with half the cells seeded with A549 and another half seeded with H358, each column containing a different concentration of PTX or a control. With this format, cell viability would be able to be calculated with the proper controls.

Amount of rat tail collagen to add:

$$\frac{(volume\ initial\ collagen\ soln)*(concentraion\ collagen\ needed)}{(initial\ collagen\ concentration)}$$

Amount of 1N NaOH to add: $(Amount\ of\ collagen\ to\ add) * (\frac{.023mL}{mL})$

Amount of dH_2O to add: Volume initial collagen solution – Amount of PBS to add – Amount of rat tail collagen to add – Amount of NaOH to add

All ingredients were then combined, the rat tail collagen was slowly added last, and the solution mixed well. The final collagen solution was then prepared, which consisted of the initial collagen solution at 70% of the volume of the volume of final collagen solution needed, 20% DMEM media, and 10% FBS. These components were again combined, slowly adding the collagen solution last, and mixed well. The collagen was added to the wells at 900µL/well and allowed to incubate at 37 °C for 20-30 minutes for solidification. This procedure was followed for the preparation of the non-adhesive surface on which the cells would be seeded. For 3D liquid covered culture conditions only, which were to be compared to the 2D study, collagen was plated

in two 24-well plates. H358 and A549 cells were seeded in two separate well plates with seeding densities of 40,000cells/mL and 15,000cells/mL, respectively, on top of the collagen after it was solidified. The schematic of the well can be seen in Figure 3.

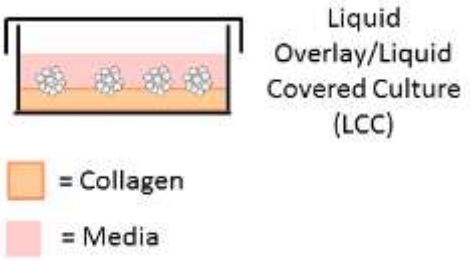


Figure 3. Schematic of LCC well for 3D culture.

MCS were allowed to grow for about 9 days, or until they reached an average diameter of about 50-100μm, and then paclitaxel was delivered to the cells in five different concentrations and left exposed to the drug for 72 hours. The paclitaxel was dissolved in DMSO in the same way as the 2D study and diluted the same way. Five different concentrations (from 1μM to .0001μM) were delivered to the cells (4 wells each concentration), and the last four wells acted as controls and were exposed to only a DMSO/media solution. Only one control containing DMSO and media was used because the differences in the 2D study between the DMSO/media control and the media only control were insignificant.

3.4 Air Interface and Liquid Covered Culturing

The ability to optimize an air interface cultured MCS was an important part of this project. In order to compare the success of this type of culture to the liquid covered culture, cells were seeded very similarly to the procedure described in section 3.2, with some slight alterations. In this part of the study, collagen was plated in transwells that fit into 12-well plates. Six transwells were seeded with the A549 cell line and six were seeded with the H358 cell line at a seeding density of 15,000cells/mL and 40,000cells/mL, respectively. 500μL of cell solution was

added to the apical side of the transwell, and 1,500µL of DMEM media was added to the basolateral side of the transwell. In order to create AIC conditions, after 24 hours, the cells were attached to the collagen and each other, and the media was removed from the apical side of three of the transwells containing A549 cells and three of the transwells containing H358 cells. This layout can be seen in Figure 4. The media continued to permeate through the bottom of the transwell as well as through the collagen in order for the cells to continue to receive nutrients. The growth of the MCS were compared between the wells that continued to receive nutrients from the media on the apical side of the well (LCC) and the wells that were exposed to air and received nutrients from the basolateral side of the well (AIC).

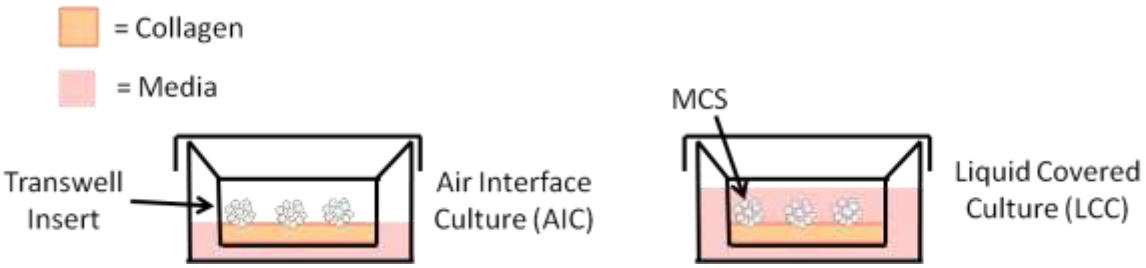


Figure 4. AIC and LCC conditions schematic.

In addition to seeding the A549 cell line in 3D on top of collagen, they were also seeded with the same procedure as described above, but without the addition of collagen. This allowed a monolayer of A549 cells to grow in both AIC and LCC conditions. This experiment was done in order to perform another type of analysis to ensure that the permeability of air interface cultured cells exposed to the drug-loaded nanoparticles is not affected, indicating that the lung tissue *in vivo* would not be compromised when the drug-loaded nanoparticles were delivered to a patient's lungs.

3.5 Live/Dead Assay Preparation

In analyzing the viability of cells after drug delivery, a live/dead fluorescent assay was prepared. This procedure started with the removal of all media from the wells. Then each well was washed with 500µL of sterile PBS. The assay was then prepared in a centrifuge tube of necessary size with 4,970µL PBS, 10µL Calcein AM (CAM), and 20µL Ethidium Homodimer-1 (Eth-D). These ingredients were added in multiples of these amounts if more solution was needed. This solution was added to each well plate and left to incubate at 37 °C for 20-30 minutes. CAM and Eth-D are fluorescent materials. This assay allows analysis of the amount of live and dead cells. CAM is able to permeate through a cell membrane and reacts with live cells' enzymes to become entrapped inside the cell where it fluoresces. Eth-D is only able enter a cell membrane and fluoresce if the cell is dead and has a compromised membrane. This assay could be analyzed with the BioTek® Gen 5 2.00 Plate Reader by reading the amount of fluorescence of each material (CAM and Eth-D) as well as imaged with fluorescent microscopy to view viable and dead MCS.

3.6 Resazurin Assay Preparation

Another fluorescent assay was used in analyzing the viability of cells after drug delivery called Resazurin. For this analysis, 50mL (or 10% volume) of Resazurin was simply added to the wells containing the MCS after 72 hours of exposure to PTX. The assay was allowed to incubate at 37 °C for 3 hours. Then 100µL from each well was transferred to a well in a 96-well plate so that the fluorescence of each well could be read by the plate reader. Resazurin becomes fluorescent when it reacts with the substances in the cell that contribute to mitochondrial activity. These substances are only produced when a cell is alive; therefore, the amount of fluorescence of Resazurin is directly proportional to the amount of live cells or the amount of cell viability.

3.7 TEER Analysis

During TEER analysis, the electrical resistance across the Calu-3 cell monolayer—this cell line used due to its inclination to form a tightly packed monolayer—is measured using an

electrode set on either side of the monolayer and measuring voltage resulting from a set current flow from one end to the other. The resistance is then calculated within the instrumentation. A description of the process of measuring the TEER across the cell monolayer using World Precision Instruments Epithelial Voltohmeter (Evom^2) can be seen in Figure 5.

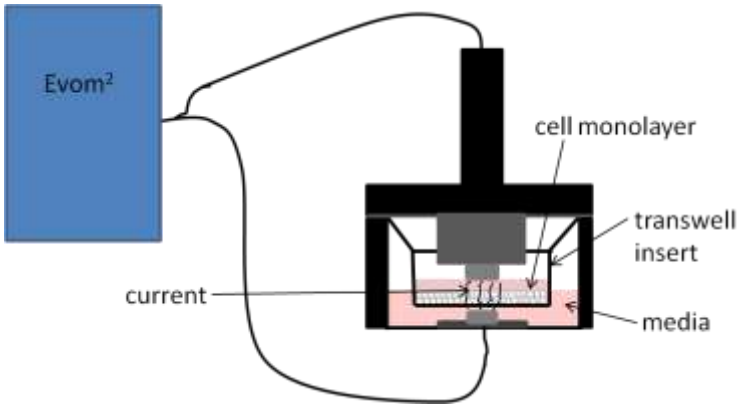


Figure 5. Schematic of TEER analysis. Each side of the electrode is placed on either side of the cell monolayer seeded in the transwell. This electrode is connected to the Epithelial Voltohmeter, which displays the resistance (Ω).

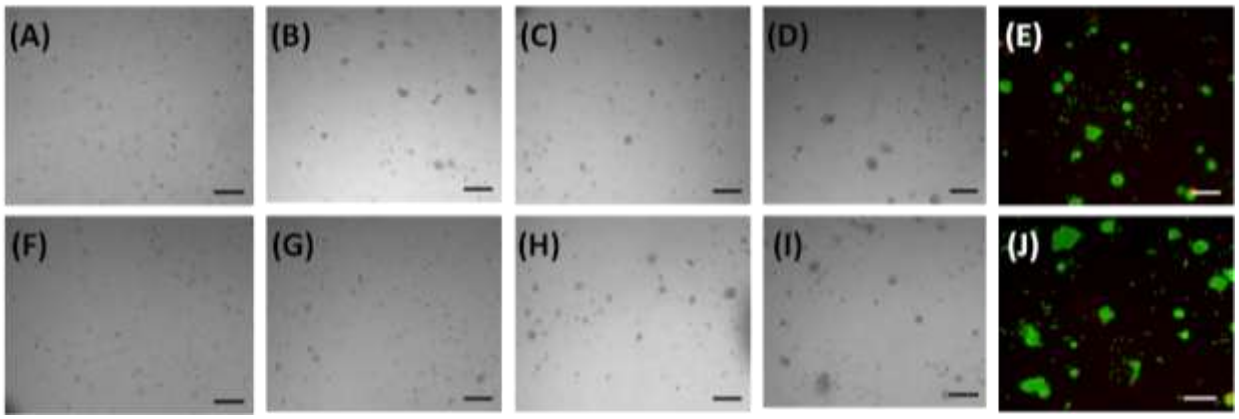
Once an equal resistance level is found for both LCC and AIC, blank lipospheres and PTX-loaded lipospheres were exposed to AIC wells, and TEER was measured again in each well after 24 hours.

4. Results and Discussion

4.1 MCS Development and Growth

In order to fulfill the first objective of the project, a 3D model cultured in both AIC and LCC was optimized. The growth of the two cell lines was monitored using brightfield microscopy. On the last day, a Live/Dead Assay was performed so that the amount of live cells could be viewed using fluorescent microscopy. A summary of these images can be seen in Figure 6.

(i)



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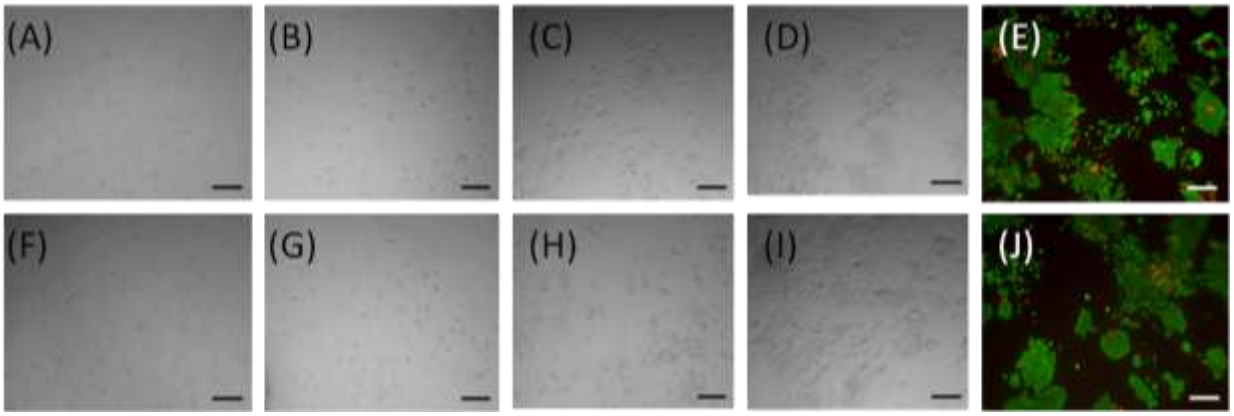

Figure 6. 3D spheroid formation brightfield images and final day Live/Dead images (with respect to time): (i) H358 and (ii) A549 where A through E are LCC conditions, F through J are AIC conditions. Images A and F, B and G, C and H, D and I, and E and J correspond to days 1, 4, 6, 8 and 9, respectively. Throughout the growth period the MCS grow to sizes varying from 50-250 µm. On the final day, Live/Dead imaging shows fully viable MCS. (Scale bar: 200µm)

The development of the MCS proved to be quite successful. The Live/Dead images on the final day displayed very few dead cells, which provided a good control when compared to MCS exposed to drug. Depending on the passage number of the cells when they were seeded, the cells sometimes grew and formed spheroids faster. The A549 cells did form fairly large MCS by day 9 at around 200-400µm, whereas the H358, which have a longer doubling time in general,

formed MCS at around 100-250μm in the same amount of time. The average diameters of the MCS were measured using Nikon imaging software. A graph showing the actual diameter growth is displayed in Figure 7. Both cell lines proved to form viable MCS of reasonable size.

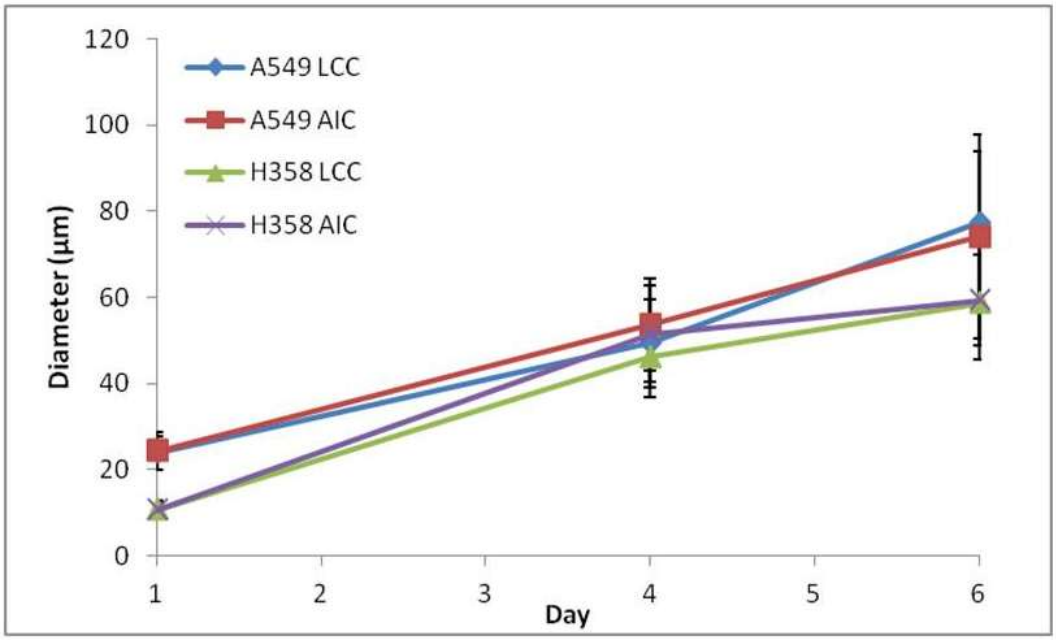

Figure 7. Spheroid diameter/growth curves. Both A549 and H358 cell lines show similar diameter growth patterns in both LCC and AIC conditions. (Ave +/- SE, n=25).

Although the A549 formed MCS faster, both cell lines showed a similar, significant, and steady increase in diameter over the elapsed time. This data confirmed that a successful 3D model had been reached in both AIC and LCC conditions.

4.2 Comparison of 2D and 3D models in LCC conditions with Exposure to PTX

The differences in response to drug delivery between a 2D model and the developed 3D model from this project were hypothesized to be significant. The responses were analyzed using viability curves of the cells when exposed PTX and IC50 values from these dose response

curves. IC50 values are defined as the concentration of an inhibitory drug at which the biological system and functions of the cells are inhibited by half. This value allows the effectiveness of a drug to be compared analytically in a quantitative way. The viability of the cells when exposed to multiple concentrations was measured using Live/Dead and Resazurin assays as described in the methods and materials section. A BioTek® Gen 5 2.00 plate reader examined and measured fluorescence given off by each well. In the Live/Dead assay, the level of Calcein AM fluorescence was an indication of viable the cells, while the level of Ethidium Homodimer-1 fluorescence indicated the presence of dead cells. The percent viability was calculated using the two fluorescent measurements gathered by the plate reader, and comparing them to the control well's fluorescence. The calculation for viability analysis used these calculations:

$$\%\, Live\ Cells = \frac{CAM\ fluorescence_{(sample)} - CAM\ fluorescence_{(blank)}}{CAM\ fluorescence_{(control)} - CAM\ fluorescence_{(blank)}}$$

$$\%\, Dead\ Cells = \frac{EthD\ fluorescence_{(sample)} - EthD\ fluorescence_{(blank)}}{EthD\ fluorescence_{(control)} - EthD\ fluorescence_{(blank)}}$$

$$\%\, Viability = \frac{\%Live\ Cells}{\%Live\ Cells + \%\, Dead\ Cells}$$

The average percent viability of the wells for each concentration was then found and used as the final data. Similarly, in a Resazurin assay, the more fluorescent the well was the more alive and viable the cells were. Percent viability was calculated using a very simple calculation:

$$\%\, Viability = \frac{Fluorescence\ of\ well\ plate\ for\ one\ concentration}{Average\ Fluorescence\ of\ Control\ wells} * 100\%$$

Again the average percent viability of the wells for each concentration was used as the final data. After calculating the viability at the multiple concentrations of PTX delivered, the data was

combined to form a viability curve that displays the cell viability as a response to dose concentrations. The responses of the cells in the 2D model compared to the responses of the 3D model can be compared in Figure 8.

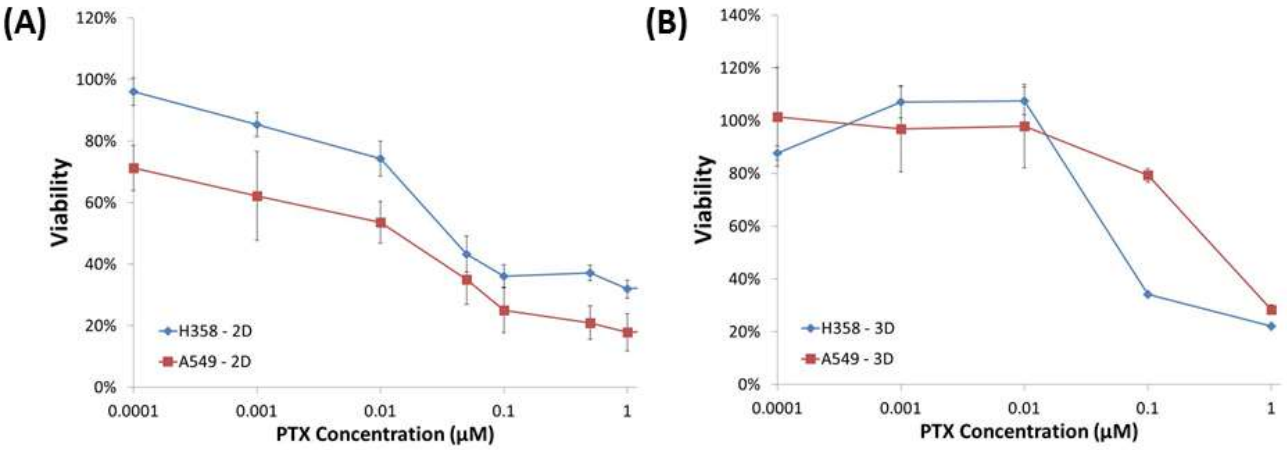

Figure 8. Comparison between 2D and 3D model viability curves of A549 and H358 cell lines for: (A) 2D model and (B) 3D model. (Ave +/- SE, 2D n=4, 3D n=3).

Figure 8 illustrates that a transition from a 2D model to a 3D model can cause varying amounts of differences in dose response in differing cell lines. Overall both cell lines show less response to the drug paclitaxel in the 3D model than in the 2D model. This is to be expected since the 3D model allows many more characteristics of an *in vivo* tumor including more protection for cells in an MCS, as well as more stability and structure. These differences in response can be seen more quantitatively in the IC50 values in Table 1.

Table 1. IC50 values for 2D and 3D model under LCC conditions.

Cells	Culture Condition	PTX (μM)
H358	2D	0.0152 ± 0.0039
A549	2D	0.0046 ± 0.0018
H358	3D	0.0836 ± 0.0159
A549	3D	0.3335 ± 0.1258

The IC50 values undeniably show that a larger concentration of PTX is needed to get the same cellular response to the drug in the 3D model.

Beyond the viability of the cells, the growth of the MCS was also observed to see the affects of the drug on the actual growth of the MCS model. Images of the MCS were taken using brightfield and final day Live/Dead fluorescent microscopy. The Live/Dead images can be seen for both cell lines in Figure 9.

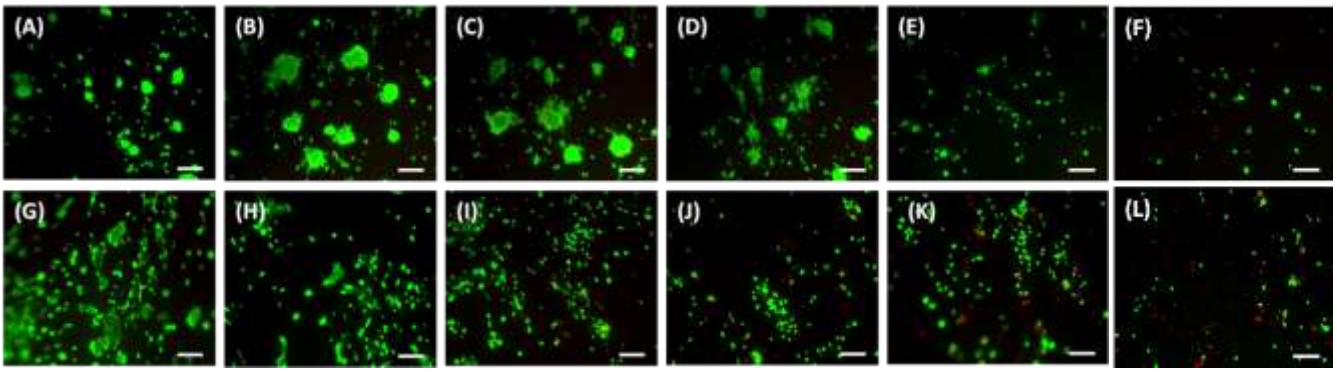

Figure 9. Final day Live/Dead images of 3D cells exposed to PTX under LCC conditions where A through F are H358 cells and G through L are A549 cells. A and G, B and H, C and I, D and J, E and K, and F and L correspond to 0, 0.0001, 0.001, 0.01, 0.1, and 1 μM PTX respectively. (Scale bar: 200μm)

The images show that the higher concentrations of paclitaxel clearly began to break up the MCS as they were inhibited. The termination of growth of the MCS and further breaking down of the

MCS is evidenced by measuring the average diameter of the MCS throughout the drug delivery process. Figure 10 illustrates the MCS growth of the two cell lines when exposed to paclitaxel.

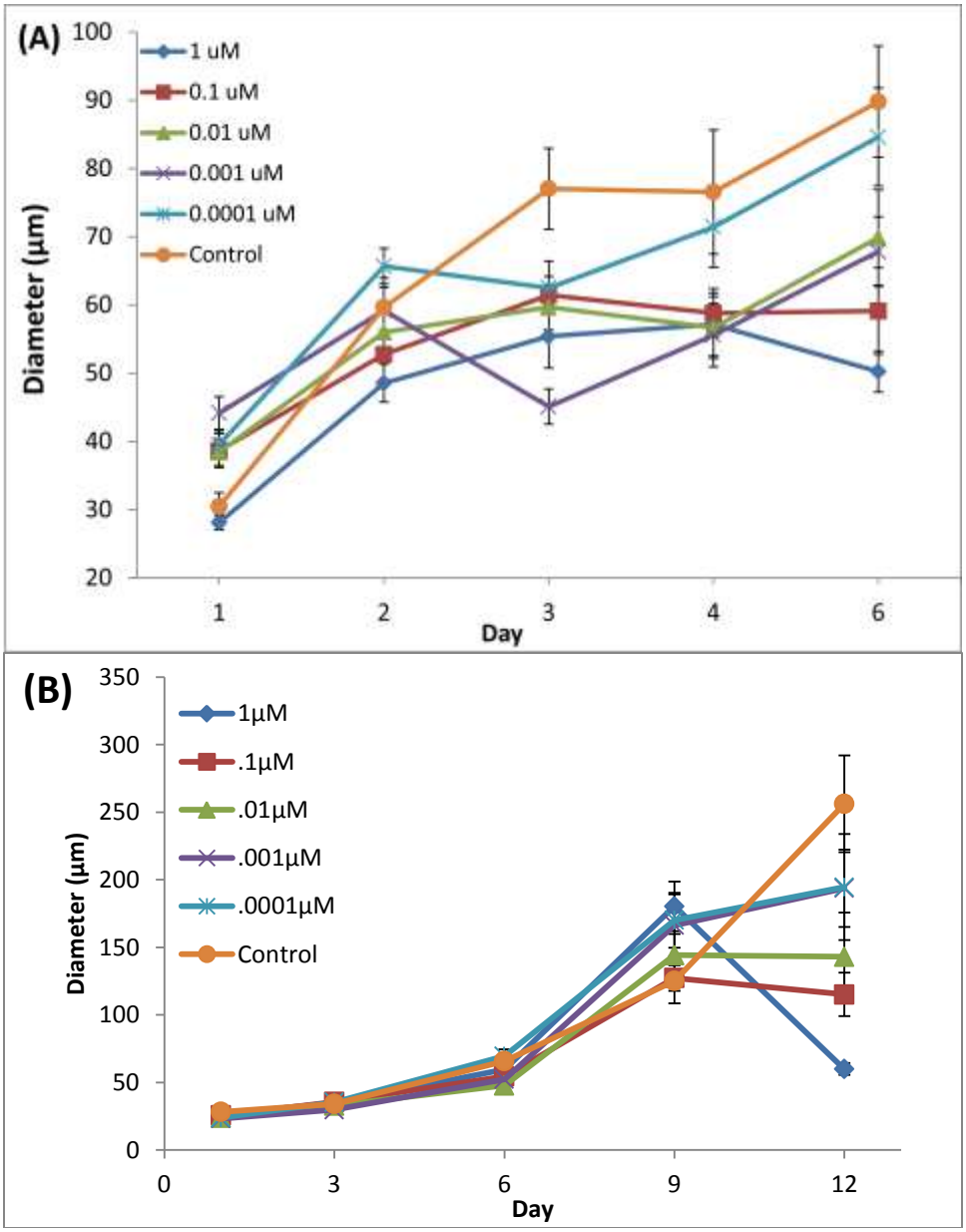

Figure 10. MCS growth curves when exposed to PTX with respect to time where: (A) H358 and (B) A549. (Ave +/- SE, n=25).

4.3 TEER Results

The TEER levels across a Calu-3 cell monolayer under LCC and AIC conditions were slightly different, but most of these results can be contributed to the fact that LCC conditions provide more nutrients to the monolayer after it is formed because the cells are exposed to media from the top and bottom of the monolayer. In AIC conditions, the cells on top of the monolayer cease to receive as much nutrient exposure. Then the comparison of TEER levels was made between control AIC monolayers and AIC monolayers exposed to blank DPPC lipospheres. The progression of TEER over time as the monolayers form and are exposed to lipospheres can be seen in Figure 11.

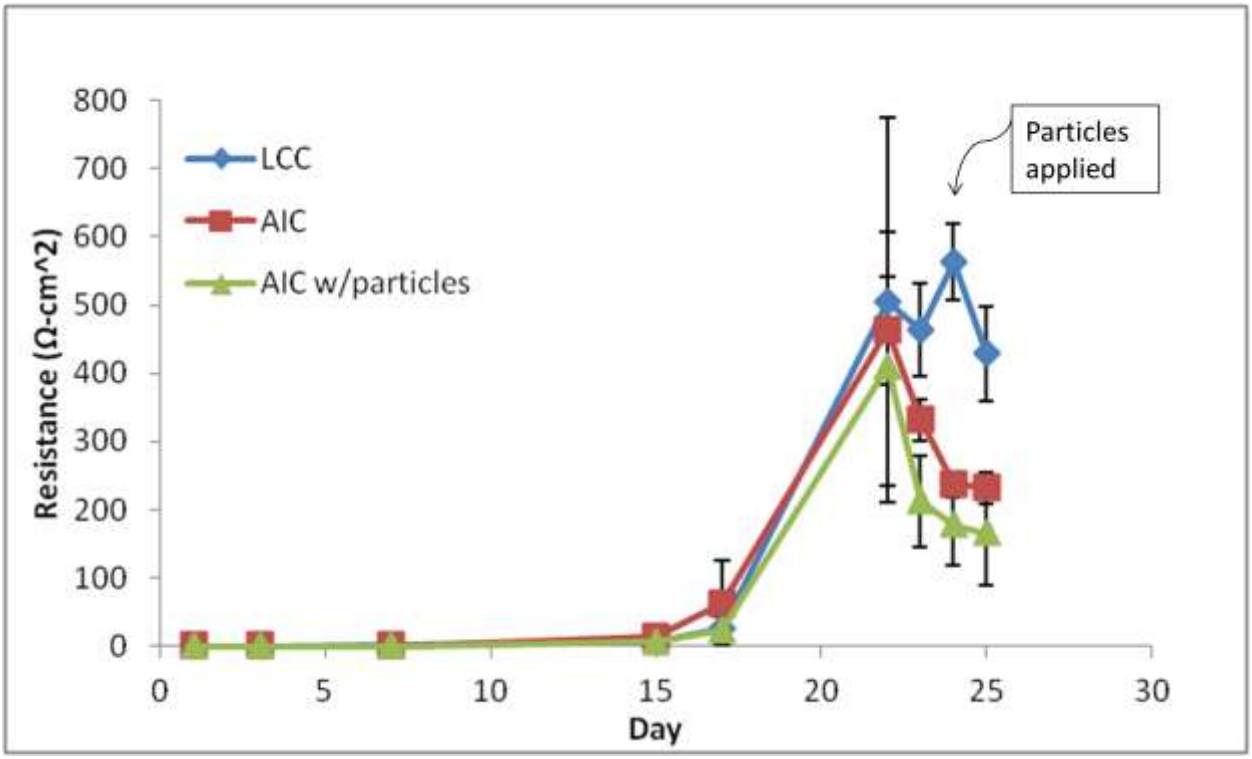

Figure 11. TEER analysis for Calu-3 cell line with exposure to DPPC lipospheres. The wells with and without exposure to the lipospheres have statistically insignificant differences in TEER readings. (Ave +/- SD, n=3).

As a monolayer formed, the resistance was insignificant until about day 22 when the monolayer fully covered the Transwell surface. The higher amount of error on that day was due to the fact that some wells

had formed a full monolayer while others were still in the process of forming one. After all wells formed the monolayer, the cells continue to grow on top of each other, and the topmost cells do not receive nutrients from media in the AIC, resulting in slight cell death. This occurrence provides an explanation for the more significant decrease in TEER for AIC monolayers. When the DPPC lipospheres were exposed on day 24, however, the TEER remains statistically consistent with the control AIC wells even after 24 hours. This provides evidence that the exposure to lipospheres does not decrease cell packing density or increase monolayer permeability.

4.4 Liposphere Application to 3D AIC

As a preliminary study, A549 and H358 spheroids were exposed to lipospheres of only two different amounts of PTX loaded onto the lipospheres. This study was to gauge where the concentration gradient will need to be for the dry powder lipospheres in order to get viability curves and IC50 values comparable to the 3D LCC study. The results of the preliminary study can be seen in Figure 12.

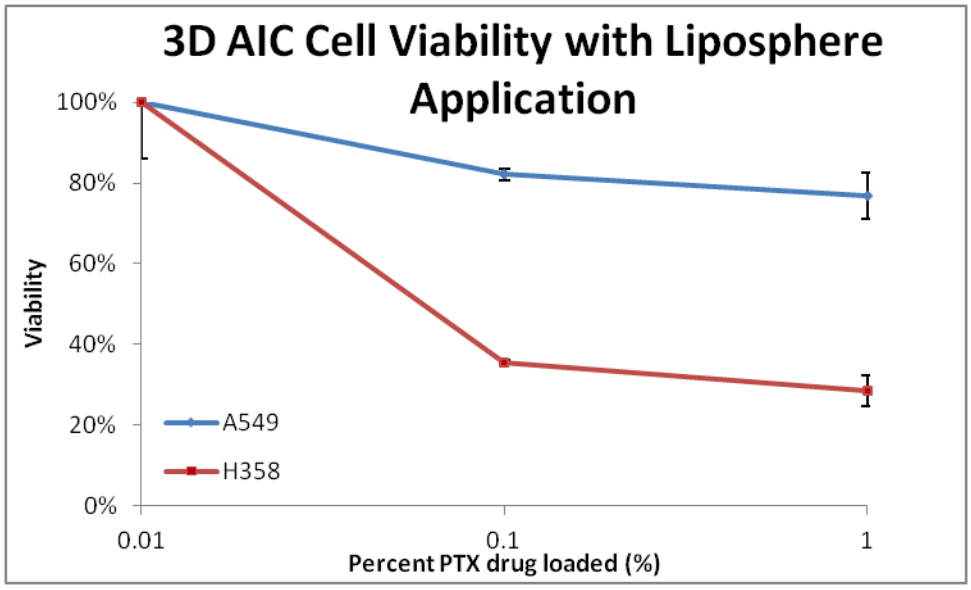

Figure 12. Viability analysis on A549 and H358 spheroids after exposure to 0.1% and 1.0% (of the total liposphere weight) PTX-loaded lipospheres.

The preliminary study already suggests that the A549 cell line is much more resistant to this form of drug delivery. This confirms the hypothesis that A549 cells have more protection in an AIC environment because they secrete significantly more protective surfactants that form a mucosal layer. This aspect of their characterization would not be represented in the 3D LCC model. This study serves as a starting point for more viability analysis in 3D AIC studies with liposphere application.

5. Conclusions

In recreating a closely physiologically representative model of an *in vivo* lung tumor, a plausible model is developed in seeding lung cancer cells on collagen in air interface culture conditions. This model yields viable multicellular spheroids at optimal sizes from about 100-400μm in diameter depending upon the length of time they are allowed to grow. This model being more similar to an *in vivo* tumor in many ways, it better predicts the affects of a drug on a tumor in an *in vivo* study. In addition, a 3D MCS model in general creates a tumor-like environment and offers protection to the spheroids, resulting in a greater resistance to inhibition by inhibitory anti-cancer drugs like paclitaxel than a more sensitive 2D model would suggest. Furthermore, when using a 2D model, one could assume that when transferring the results to an *in vivo* study the cells exposed to the drug will show a different response and most likely be less sensitive to the same dosage. If a 3D model were to be used, the *in vitro* results would transfer much more smoothly and exhibit much more similar results to *in vivo.* This may lead to less extensive research needed *in vivo*, and a quicker transition of a chemotherapeutic drug into clinical trials. The implications that this more useful 3D model may result in less *in vivo* studies also assists in ethical as well as monetary issues with *in vivo* experimentation, considering the moral issues and expenses of animal testing. The differences in drug response which this 3D model denotes require acknowledgment by any researcher developing a chemotherapeutic drug. The fact that paclitaxel began to break up the MCS before causing apoptosis in all the cells further suggests that the chemotherapeutic must work its way through the many layers of a tumor before it can inhibit the cancer cells. The differences in 2D and 3D model drug response in LCC

conditions leads to the assumption that a similar pattern will occur in AIC conditions. AIC culture also has other characteristics to consider. TEER significantly increases across a monolayer of the Calu-3 cell line. The resistance is due to a tightly packed structure that prevents leakage of fluids in the lung. This resistance remains the same with the application of direct PTX-loaded nanoparticle delivery to this cell monolayer, implying that the nanoparticles can be safely delivered to lung tissue without comprising the tissue and causing it to become leaky with fluid or permeable to other harmful particles. Lastly, the 3D AIC model proves to represent more characteristics similar to *in vivo* (such as protective musical layers) when analyzing cellular response to direct pulmonary delivery of PTX-loaded lipospheres.

6. Future Work

After the successful development of a dependable 3D model to specifically mimic a lung tumor that is exposed directly to air, the possibility of direct pulmonary delivery arises. Experimentation with this *in vitro* model using aerosolized lipospheres loaded with paclitaxel is likely to contribute valuable information concerning plausible direct pulmonary delivery. MCS grown in AIC conditions will be exposed to the DPPC:PTX particles in varying concentrations with an insufflator to model a direct pulmonary application of the anti-cancer drug. Cell viability curves and IC50 values will also be measured and analyzed similar to the analysis done on the MCS grown in LCC conditions. The differences in AIC and LCC responses will provide information on the effects of two different growth conditions as well as the effects which the two different methods of drug delivery have on the drug response. In addition, a distinction will be made between drug response in the 2D compared with the 3D model in this more unique culturing method that is specific to recreating a lung tumor and tumor microenvironment. Much is still to be learned from this new lung tumor model.

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