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Multicellular spheroids of A549 and A549-iRFP as an in vitro model of lung cancer

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MULTICELLULAR SPHEROIDS OF A549 AND A549-IRFP CELLS AS AN IN VITRO MODEL OF LUNG CANCER

By

Xinyu Pei

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Drug Delivery and Targeting

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2020
MULTICELLULAR SPHEROIDS OF A549 AND A549-IRFP CELLS AS AN IN VITRO MODEL OF LUNG CANCER

By

Xinyu Pei

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MULTICELLULAR SPHEROIDS OF A549 AND A549-IRFP CELLS AS AN IN VITRO MODEL OF LUNG CANCER

Abstract

By Xinyu Pei
University of the Pacific
2020

Lung cancer is the second most common cancer in both men and women around the world, and 85% of it is non-small cell lung cancer (NSCLC). It is estimated that in 2020, there will be 228,820 cases of lung cancer and 135,720 deaths from lung (American Cancer Society, 2020). The prognosis of lung cancer is poor (<10% 5-year survival rate for advanced NSCLC), which can be partly attributed to limitations in bio-models that screen for drug candidates against lung cancer.

Traditionally, the most commonly used in vitro method for screening therapeutic drugs is monolayer cell cultures, which are reproducible, convenient and of low cost. However, monolayer cell culture models are unable to reproduce many properties of in vivo solid tumors such as the morphological features and the microenvironment including cellular heterogeneity, cell-cell interactions, and gradients of oxygen, pH, and nutrients. Consequently, excessive ineffective drug candidates would proceed to animal studies, which would prolong the time for drug development and increase the overall cost of drug discovery.

In consideration of the foregoing, in vitro models of cancer based on three-dimensional multicellular spheroids (MCS) have been developed in our group to characterize drug candidates and drug delivery systems. Compared to monolayer cells, the multicellular spheroids can better simulate drug penetration and drug resistance in solid tumors. Therefore, the multicellular
spheroids represent a more clinically relevant in vitro model to evaluate the efficacy of anticancer drugs.

This project aims to characterize MCS of lung cancer cells as an improved platform to evaluate drug candidates against lung cancer. Cell viability assays on cisplatin, carboplatin, gemcitabine, and doxorubicin have been conducted to compare the anticancer activities between conventional monolayer cells and the corresponding MCS of human lung cancer cell lines, A549 and A549-iRFP (fluorescently labeled A549 cells). Higher concentrations of the tested anticancer drugs is consistently needed to inhibit 50% the cell viability in MCS than the corresponding monolayer cells of A549 and A549-iRFP.

Cycled dosing schedules based on guidelines for NSCLC from National Comprehensive Cancer Network have been designed and used to treat A549-iRFP MCS. The A549-iRFP MCS have been exposed to anticancer drugs either continuously, or in pulsed concentrations according to the drugs’ pharmacokinetics (PK). The continuous drug exposure has been found to inhibit more cell growth in MCS than the corresponding PK-mimetic drug exposure. Such phenomenon would bring significant positive bias to the activity of many anticancer drug candidates during their early discovery and development.

Taken together, MCS of A549 and A549 iRFP cells better represent the efficacy of anticancer drugs in clinic than the monolayer. MCS can also be used to evaluate anticancer drug candidates by pulsed drug exposure based on their pharmacokinetics, and by commonly used cycled dosing regiments to better predict their efficacy in clinical settings.
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CHAPTER 1: INTRODUCTION

1.1. Cancer and Anticancer Drug Development Process

Drug development is a long process where a potential new drug must be identified and evaluated in preclinical studies which involves in vitro cell culture and in vivo animal studies. Then the drug candidates must go through four phases of clinical trials before being approved by the FDA. In theory, the preclinical studies at the early stages of drug discovery process should improve the success rate of the clinical trials but in reality, the approval rate of anticancer drug is exceptionally low despite of the promising preclinical results. A recent report given by Biotechnology Innovation Organization (BIO) entitled “Clinical Development Success Rates 2006-2015” pointed out that oncology drug candidates had only 5.1% rate of success from phase I compared to the success rate of 11.9% for drug candidates of all other indications (Thomas et al., 2016). This low success rate of oncology drug candidates could be partially explained by the preclinical cancer models that pharmaceutical companies use for the first-pass screening of the drug candidates. The main strategy to select drug candidates in vitro is to evaluate their activities in cultures of two-dimensional (2D) monolayer cells. It is only in recent years that more sophisticated, three-dimensional (3D) cell cultures were introduced as an improved model to screen anticancer drugs in vitro.

1.2. Traditional in vitro Drug Screening in Monolayer Cell Culture

Monolayer cell culture is the most commonly used drug screening method in vitro. Advantages of monolayer cell cultures include fast assessment, simplicity, convenience, good reproducibility and low cost. However, this method does not reproduce many properties of solid tumor in vivo. The monolayer cells have poor cell-cell interactions, poor cell-extracellular interactions, negligible cancer cell heterogeneity, incorrect morphology and substantially
different microenvironment. Consequently, a lot of unviable drug candidates have been forwarded from such cell culture studies to further studies in vivo, which caused extensive waste of time, money and experimental animals.

1.3. Improved in vitro Drug Screening in Three-Dimensional Cell Culture

Because of the above-mentioned limitations of the monolayer cells, it is necessary to reproduce 3D architecture of solid tumor to better understand the biology of cancer, and to better identify anticancer drug candidates. 3D cell cultures can meet these needs and can be developed into better platforms of in vitro drug screening. In 3D cell cultures, cells can be aggregated to form clusters in a spheroid shape, which is called multicellular spheroid (MCS). In this way, multicellular spheroids of cancer cells can be fabricated to mimic many more features of solid tumors than monolayer cells in culture.

1.3.1. Morphological Features

Multicellular spheroids are cell aggregates with a three-dimensional structure that is more similar to solid tumor in morphology than monolayer cells. Well-formed spheroids normally show a spheroid shape under scanning electron microscope or a round shape under phase contrast microscope. The MCS morphology of different cell lines vary; some form tight spheroids while others form loose MCS (as shown in Figure 1).

Figure 1. Morphology of tight and loose MCS after 24h cultivation. (A, B). Highly compact MCS formed by MCF7 and T-47D cells; (C, D) Loose MCS formed by MDA-MB-231 and MDA-MB-361. Scale bar = 500 µm. (Andrea & Manfred, 2006).
1.3.2. **Hypoxia Microenvironment**

Hypoxia is one of the hallmarks in solid tumors, where the oxygen level gradually reduced from outer regions to inner areas. This occurs as a consequence of multiple factors, including rapid oxygen consumption, increased diffusion distance from vasculature to cells, irregular vascular geometry, and low oxygen capacity in blood (Hammond et al., 2014; Höckel & Vaupel, 2001). Such an oxygen gradient can also be seen in MCS, as confirmed in many studies using microelectrodes, ethidium-calcein staining, and/or measurements of hypoxia inducible factor (HIF) and its downstream target gene, VEGF. (Bredel-Geissler, Karbach, Walenta, Vollrath, & Mueller-Klieser, 1992; Riffle & Hegde, 2017; Zanoni et al., 2016). For example, a 3.3-fold increase of HIF-1α level in MCS was detected compared with monolayer of HeLa cells (Rodríguez-Enríquez et al., 2008). One possible reason for the oxygen gradient in MCS would be that cells at the peripheral regions consumes most oxygen as it diffuses to inner cells (Mueller-Klieser & Sutherland, 1982).

1.3.3. **Altered Cell Metabolism**

Cells normally obtain energy through oxidative phosphorylation under aerobic environment, whereas in solid tumor, even in the presence of oxygen, energy is preferably obtained from glycolysis, when lactate is generated as by-product (Vander Heiden, Cantley, & Thompson, 2009; Warburg, 1956). This alteration of metabolism in solid tumors is known as the Warburg. MCS also shows the Warburg effect by the increased mRNA expression of glucose transporter 1 (GLUT-1) and lactate dehydrogenase (LDHA). For example, GLUT-1 expressed on PANC-1 MCS was around 6.5 times higher than the corresponding monolayer cells and the LDHA expression was 2.8 times higher (Longati et al., 2013). Another example, GLUT-1
expression increased 2.6-3.4 times in HeLa MCS compared to the corresponding monolayer cells (Rodríguez-Enríquez et al., 2008).

1.3.4. Acidic Microenvironment

In solid tumor, cancer cells generate excess amount of lactate (pKa=3.9) and hydrogen ions (H\(^+\)) as a result of glycolysis, which leads to a fall in intracellular pH (pHi). In order to counter the acidic pH, protons are exported across the plasma membrane by various transporters such as H\(^+\)-ATPase, Na\(^+\)-H\(^+\) exchange, carbonic anhydrases and monocarboxylate transporter (MCT), which maintains a favorable pHi of 7.0-7.2 but generates an acidic extracellular microenvironment (Parks, Chiche, & Pouysségur, 2013; Swietach, Vaughan-Jones, & Harris, 2007). This acidic microenvironment in tumors has been verified in many studies. In one MCF-7 breast cancer tumor, extracellular pH in the peripheral region of the tumor about 10 μm away from capillary blood vessel wall is around 7.3, but drops to 6.7 in a region about 400 μm away from the blood vessel wall (G. Helmlinger, Yuan, Dellian, & Jain, 1997). Acidification also takes place inside MCS as a pH difference of 0.3–0.4 units is seen between the center (pH 6.9–7.05) and the periphery (pH 7.3–7.4) in MCS of many cancer cell lines including HT29, V79-379A, EMT6, U-138 MG, U-251 MG U-118 MG and HTh7 (Carlsson & Acker, 1988).

1.3.5. Cell-Cycle Arrest

Rapidly growing cancer cells experience hypoxia, nutrient deficiency and acidic microenvironment, which induces necrotic cell death especially in the inner region of solid tumors or spheroids. Many studies have demonstrated that MCS possesses a necrotic zone in the center, a proliferating zone at the periphery and some quiescent cells in between, which cannot be seen in monolayer cell cultures. MCS of various types of cancers, such as human breast cancer, colon cancer and ovarian cancer is found to overexpress p27, a quiescence marker, by
1.2~15 fold compared to the corresponding monolayer cells (Croix et al., 1996). In addition, DLD-1 human colon adenocarcinoma MCS is found to express the proliferation marker Ki-67 mostly at the periphery, and yet the quiescence marker p27 at the center (Mellor, Ferguson, & Callaghan, 2005).

1.3.6. Cellular Components

Besides carcinoma cells, solid tumors are heterogenic, and consist of many other types of cells including immune cells (e.g., T lymphocytes, B lymphocytes, macrophages, NK cells and lymphatic endothelial cell), tumor vascular endothelial cells, cancer-associated fibroblasts, pericytes and adipocytes (Balkwill, Capasso, & Hagemann, 2012). Even though these cells are non-malignant, they play important roles in tumor angiogenesis, progression, invasion, metastasis and resistance by interacting with the surrounding cancer cells and with one another (M. Wang et al., 2017). Cellular heterogeneity can also be achieved in MCS by co-culturing stromal cells with cancer cells. For example, the coculture of colorectal tumor spheroids with immune cells presented a relevant tool to study antitumor potential of immunomodulatory antibodies (Courau et al., 2019); a triple co-culture of pancreatic cancer cells with fibroblasts and endothelial cells closely mimicked the resistance to anticancer treatments (Lazzari et al., 2018).

1.3.7. Extracellular Matrix (ECM) Deposition

Extracellular matrix is a non-cellular matrix of biopolymers in all tissues. ECM is composed of fibrous proteins (e.g., collagens, elastins, fibronectins, and laminins) and proteoglycans (e.g., glycosaminoglycan). In normal tissues, ECM provides both structural and biochemical support in regulating cell proliferation, cell adhesion and cell signaling (Frantz, Stewart, & Weaver, 2010). In tumors, ECM protein dynamics become abnormal and the deposition is elevated, such as increased collagen found in breast cancer (Levental et al., 2009;
Provenzano et al., 2006). The ECM proteins are also found overexpressed in U-118 MG glioma and HTh-7 thyroid spheroids (Nederman, Norling, Glimelius, Carlsson, & Brunk, 1984). ECM proteins (e.g., proteoglycan and fibronectin) are expressed more in three dimensional spheroids than in the corresponding monolayers (Glimelius, Norling, Nederman, & Carlsson, 1988).

1.3.8. Cell-ECM and Cell-Cell Physical Interactions

In a solid tumor, cell-ECM interactions (indicated by β1-integrin level) and cell-cell interactions (indicated by E-cadherins level) are elevated compared to normal tissues. Such elevation is associated with cancer cell signaling, proliferation, migration and invasion (Casey et al., 2001; Walker, Mojares, & Del Río Hernández, 2018; Xiong & Xu, 2016). Cell-ECM and cell-cell physical interactions are found to be more pronounced in MCS than the corresponding monolayer cell cultures due to MCS’ spatial architecture. β1-integrins was significantly increased in 3D epithelial ovarian cancer cell lines (OV-MZ-6 and SKOV-3) compared to 2D (Loessner et al., 2010). E-cadherin was significantly higher in Huh7 spheroid compared to 2D (Jung et al., 2017).

1.3.9. Physical Barrier

Elevated ECM protein deposition, cell-ECM interactions and cell-cell physical interactions in solid tumors are also important concerns for anticancer drug development because they increase tissue density and yield a physical barrier that limits the penetration of anticancer drugs (Minchinton & Tannock, 2006).

1.4. Lung Cancer

1.4.1. Lung Cancer and its Epidemiology

According to American Cancer Society, lung cancer is the second most common cancer in both men and women (excluding skin cancer) and the leading cause of cancer death, making
up nearly 25% of all cancers. It is estimated that in 2020, there will be about 228,820 new cases of lung cancer and about 135,720 deaths from lung cancer (American Cancer Society, 2020).

### 1.4.2. Types of Lung Cancer

There are two types of lung cancers, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which need different treatment. About 15% of lung cancers are small cell lung cancer (SCLC) and about 85% are non-small cell lung cancer (NSCLC). NSCLC has three main subtypes: adenocarcinoma, squamous cell carcinoma and large cell carcinoma.

### 1.4.3. Staging

The progression of non-small cell lung cancer consists of 4 stages:

- **Stage I**: Cancer is localized only in one lung and has not spread to any lymph nodes.
- **Stage II**: Cancer is localized in lung and nearby lymph nodes.
- **Stage IIIA**: Cancer has spread to lymph nodes but only on the same side of the chest where cancer started growing.
- **Stage IIIB**: Cancer has spread to lymph nodes on the opposite side of the chest, or above the collar bone.
- **Stage IV**: Cancer has spread to both lungs, or to fluid in the proximity, or to distant organs.

### 1.4.4. Treatment

Surgery, radiation, chemotherapy (chemo), targeted treatment and immunotherapy are used to treat NSCLC, either alone or in combination. Surgery is mostly applied to stage I and stage II patients to remove tumor. Radiation therapy is used alone or in combination with chemo.

Chemotherapy can be given after surgery to kill remaining cancer cells, a strategy known as adjuvant chemotherapy. Moreover, chemotherapy can be given before surgery to shrink the
tumor size, a strategy known as neoadjuvant chemotherapy. In addition, chemotherapy is also used to treat unresectable cancers. The commonly used chemo drugs include cisplatin, carboplatin, vinorelbine, docetaxel, gemcitabine, etoposide and pemetrexed. Combination of two chemo drugs are usually used, which often includes cisplatin or carboplatin plus one other drug. Patients with certain gene mutations can be treated with targeted therapy drugs. For example, Osimertinib, Afatinib, Erlotinib or Gefitinib is used for patients who are positive of EGFR mutations, Alectinib or Brigatinib for ALK mutations, Crizotinib or Entrectinib for ROS1 mutations, and Dabrafenib plus Trametinib for BRAF mutations. Patients who have high level of PD-L1 protein expression can be treated with immunotherapy, such as pembrolizumab.

1.5. Hypothesis and Specific Aims

1.5.1. Statement of Purpose

Because of the afore-mentioned advantages of MCS to mimic many more features of solid tumors than monolayer cell cultures, the purpose of this project is to characterize MCS of lung cancer cells as an improved platform to evaluate drug candidates against lung cancer. Two human lung cancer cell lines will be used, A549, which is one of the most investigated cell lines of NSCLC, and A549-iRFP-Puro (A549-iRFP), which is derived from A549 and stably expresses near-infrared fluorescent protein for convenient detection.

1.5.2. Hypothesis

This project aims to test the hypothesis that the sensitivity of MCS A549 and A549-iRFP to anticancer drugs better reflect the drugs’ efficacy against lung cancer in clinic than the corresponding monolayer cells.

1.5.3. Specific Aims
This project has the following three specific aims. Aim 1, to construct A549 MCS and A549-iRFP MCS; Aim 2, to compare the sensitivity of A549 and A549-iRFP MCS to several well-established chemotherapy drugs with the sensitivity of the corresponding A549 and A549-iRFP monolayers; Aim 3, to evaluate the viability of the fluorescent A549-iRFP MCS after treatment with the anti-lung cancer drugs based on their clinical regimens.
CHAPTER 2: CONSTRUCTION OF MCS OF A549 AND A549-iRFP

2.1. Introduction

2.1.1. A549 and A549-iRFP Cells

A549 is adenocarcinoma human epithelial cells, developed from the lung of a 58-year old male Caucasian. It has been used as a model of NSCLC in many studies, from basic mechanism of lung cancer to novel drug development (Liebmann et al., 1993). A549-iRFP-Puro is a polyclonal population of the human lung carcinoma cell line A549 (ATCC® CCL-185™) that stably express near-infrared fluorescent protein (iRFP) and can be selected using puromycin. The fluorescence ex/em is 690/713 nm which makes in vivo and ex vivo imaging of implanted cells easier, since biological tissues have lower absorption and less autofluorescence at 600 to 1000 nm (Hilderbrand & Weissleder, 2010).

2.1.2. Methods for MCS Formation

In recent years, various methods have been developed for the formation of MCS. It is important to fabricate multicellular spheroids with controllable and uniform size, biological functions and 3D microenvironment that best mimics tumors in vivo. Conventional and commonly used methods can be divided into scaffold-based and scaffold-free methods.

In a scaffold-based method, as its name suggests, scaffold is applied to help form MCS that provides not only the 3D structure but also cell-cell and cell-matrix interactions. Hydrogels are widely used as scaffold, which includes natural hydrogel (e.g., collagen, Matrigel™ and hyaluronic acid-based hydrogels) and synthetic hydrogel (e.g., Poly(ethylene glycol) (PEG), Poly(N-isopropylacrylamide) (PNIPAM) and Poly(ε-carpoalacton) (PCL)) (Cui, Hartanto, & Zhang, 2017). There are two types of scaffold-based methods: matrix-based method and microfabrication method. A typical type of scaffold-based methods is matrix-based method, in
which MCS could be either formed on top of the matrix or inside the matrix. A more complex scaffold-based method is the microfabrication method, it involves hydrogel and needs to use specially made equipment such as micropatterned plates or microfluidic platforms.

In a scaffold-free method, spinner flasks, NASA rotating flasks and pellet culture method involves agitation, while other methods such as hanging drop, ultra-low cell attachment plate method, liquid overlay and magnetic levitation and 3D bioprinting are agitation-free. Descriptions and comparisons of the above-mentioned methods are listed in Table 1. Even though each method has significant limitations, they could be combined to complement one another and thus produce better MCS.
<table>
<thead>
<tr>
<th>Methods</th>
<th>Description</th>
<th>Advantage</th>
<th>Disadvantage</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scaffold-based</td>
<td>Matrix on-top and matrix-embedded</td>
<td>Provide 3D support that mimics <em>in vivo</em></td>
<td>Variable sizes;                Spheroids overlapping with each other;  difficult to retrieve cells</td>
<td>(Ingeson-Carlsson, Martinez-Monleon, &amp; Nilsson, 2015; Lee, Kenny, Lee, &amp; Bissell, 2007)</td>
</tr>
<tr>
<td>Microfabrication</td>
<td>Micropatterned plates</td>
<td>Controllable and uniform size; Suitable for HTS</td>
<td>Need to prepare micropatterned chamber</td>
<td>(Markovitz-Bishitz et al., 2010; Sunami, Yokota, &amp; Igarashi, 2014)</td>
</tr>
<tr>
<td></td>
<td>Microluidic platforms</td>
<td>Long term culture; Provide dynamic 3D microenvironment</td>
<td>Difficult to harvest; Specialized equipment required; Not suitable for HTS</td>
<td>(Lim &amp; Park, 2018; Wu et al., 2011)</td>
</tr>
<tr>
<td>A agitation-free</td>
<td>Spinneflasks</td>
<td>Simple to perform; Long term culture; Mass production</td>
<td>Variable sizes and morphologies due to shear stress; Not for large scale</td>
<td>(Lazar et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>NASA Rotating flasks</td>
<td>Simple to perform; Rapid formation</td>
<td>Cells may be damaged by shear stress</td>
<td>(Ingram et al., 1997)</td>
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<tr>
<td></td>
<td>Pellet culture</td>
<td>Simple to perform; Rapid formation</td>
<td>Limited culture period</td>
<td>(Li, He, &amp; Pei, 2011)</td>
</tr>
<tr>
<td>Scaffold-free</td>
<td>Hanging drop</td>
<td>Low cost; Uniform size and shape; Simple to perform; Rapid formation</td>
<td>Fragile; Hard to change media; Low throughput; Limited culture period</td>
<td>(Tung et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>Ultra-low cell attachment plates</td>
<td>Easy to image; Easy to change media; Easy to harvest; Long term culture; Suitable for HTS</td>
<td>Expensive</td>
<td>(Vinci et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>Liquid Overlay</td>
<td>Low cost; Simple to perform</td>
<td>Variable sizes size and morphologies; Hard to harvest; Low throughput</td>
<td>(Costa, de Melo-Diogo, Moreira, Carvalho, &amp; Correia, 2018)</td>
</tr>
<tr>
<td></td>
<td>Magnetic levitation</td>
<td>Rapid formation</td>
<td>Not for large scale; Cells need to be pretreated with magnetic nanoparticles</td>
<td>(Haisler et al., 2013)</td>
</tr>
</tbody>
</table>


(Table 1 Continued)

<table>
<thead>
<tr>
<th>Magnetic 3D bioprinting</th>
<th>Magnetically labeled cells (incubated with magnetic nanoparticles) are pulled down under magnetic forces where magnet is placed beneath the plate</th>
<th>(Tseng et al., 2015)</th>
</tr>
</thead>
</table>
2.1.3. Characterization and Evaluation of MCS

Many assays have been used to evaluate MCS growth and its response to growth factors and drug treatments (listed in Table 2). Methods usually used on monolayer can also be used on MCS. However, although MTT assay has been used on MCS, it is not highly recommended since it is harder to penetrate MCS. Instead, CellTiter-Glo® can be a substitute assay to quantify MCS viability (Riss, 2017).
2.2. Materials and Methods

Table 2
Comparison of Assay Methods Used in MCS

<table>
<thead>
<tr>
<th>Cellular activity</th>
<th>Assay</th>
<th>Assay example</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis assay/ cell death analysis</td>
<td>DNA fragmentation</td>
<td>TUNEL assay</td>
<td>(Gunther, Pawlak, Damasceno, Arnold, &amp; Terzis, 2003; Gabriell Helmlinger, Netti, Lichtenbeld, Melder, &amp; Jain, 1997)</td>
</tr>
<tr>
<td></td>
<td>Caspase activity</td>
<td>Caspase assays</td>
<td>(Kessel et al., 2017)</td>
</tr>
<tr>
<td></td>
<td>Phosphatidylserine exposure</td>
<td>Annexin V</td>
<td>(Andrea &amp; Manfred, 2006; Yamaguchi et al., 2010)</td>
</tr>
<tr>
<td>Cytotoxicity assay</td>
<td>Released intracellular protein</td>
<td>G6PD</td>
<td>(W. Wang et al., 2018)</td>
</tr>
<tr>
<td></td>
<td>Damage of cell membrane</td>
<td>LDH assay</td>
<td>(Xu, Ma, &amp; Purcell, 2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7-AAD</td>
<td>(Patra, Peng, Liao, Lee, &amp; Tung, 2016)</td>
</tr>
<tr>
<td>Cell proliferation and cell cycle assay</td>
<td>Vital dyes</td>
<td>Calcein-AM</td>
<td>(Patra et al., 2016)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trypan blue</td>
<td>(Ivanov et al., 2014)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Propidium iodide</td>
<td>(Andrea &amp; Manfred, 2006)</td>
</tr>
<tr>
<td></td>
<td>Combined dye</td>
<td>Live/dead cell viability assay</td>
<td>(Sirenko et al., 2015)</td>
</tr>
<tr>
<td>Cell viability assay</td>
<td>ATP</td>
<td>CellTiter-Glo®</td>
<td>(Kessel et al., 2017; Vinci et al., 2012)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ViaLight™ plus kit</td>
<td>(Nie, Garner, &amp; D'Souza, 2017)</td>
</tr>
<tr>
<td></td>
<td>Tetrazolium</td>
<td>MTT assay</td>
<td>(Ho, Yeap, Ho, Rahim, &amp; Alitheen, 2012)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MTS assay</td>
<td>(Yeon et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>Resazurin</td>
<td>Alamar blue assay</td>
<td>(Ivanov et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>Acid Phosphatase</td>
<td>APH assay</td>
<td>(Ivanov et al., 2014)</td>
</tr>
<tr>
<td>Morphological changes</td>
<td>Morphology, diameter and volume</td>
<td>Inverted microscope</td>
<td>(Vinci et al., 2012)</td>
</tr>
</tbody>
</table>
2.2.1. **Materials**

A549 cell line was purchased from ATCC (VA, US). A549-iRFP cell line was purchased from Imanis Life Sciences (MN, US). RPMI 1640, DMEM, Collagen, Trypsin-EDTA, 1% penicillin-streptomycin and Live/DEAD™ Cell Imaging Kit were purchased from Thermo-Fisher. Puromycin was purchased from Alfa Aesar (MA, US). Fetal bovine serum was purchased from Gemini Bio-Products (CA, US). Ultra Cruz® Black/Clear Flat bottom 96-well microplates were purchased from Santa Cruz (CA, US). White Flat Bottom 96-well microplates and 96-well Spheroid microplates were purchased from Corning Life Science (NY, US). CellTiter 96® AQueous One Solution Cell Proliferation Assay and CellTiter-Glo® 3D Cell Viability Assay kits were purchased from Promega Corporation (Madison, WI, US). Cisplatin, Doxorubicin Hydrochloride was purchased from BIOTANG Inc. (MA, US). Carboplatin was purchased from ChemScene llc (NJ, US). Paclitaxel and Gemcitabine Hydrochloride were purchased from LC Laboratory (MA, US).

2.2.2. **Cell Culture Maintenance**

A549 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Gemini, CA, US) and 1% Penicillin-Streptomycin (Corning Life Science, NY, US). A549-iRFP cells were maintained in DMEM supplemented with 10% fetal bovine serum (Gemini, CA, US), 1% Penicillin-Streptomycin (Corning Life Science, NY, US), and 1 µg/ml puromycin (Alfa Aesar, MA, US). Cells were grown in 5% CO₂ at 37 °C and passaged at 85% confluence (3~4 days).

2.2.3. **Formation of 3D MCS**

NSCLC cell lines A549 and A549-iRFP were seeded at 5000 cells in 100 µl growth media for each well with 0.3% collagen onto Corning 96-well spheroid microplates (Corning
Life Science, US), which were then centrifuged at 300 × g for 7 min by Eppendorf Centrifuge 5810R. Plates were incubated for 48 hours then supplemented with another 100 µl growth media. Media was changed every other day by replacing 100 µl media in microplates with 100 µl fresh media to ensure a total volume of 200 µl. The growth and morphology of MCS were observed by Keyence (US) BZ-X700 fluorescence microscope.

2.2.4. Characterization and Optimization of 3D MCS Viability Assay

A549-iRFP cells were seeded at 3000 cells/well in 96-well spheroid microplates to form A549-iRFP MCS as described above. Certain amount MCS were collected each time for experiment when diameter reaching around 350 µm, 550 µm, 750 µm, and 950 µm. MCS were divided into 6 groups, according to the volume ratio between MCS with media and CellTiter-Glo® 3D Cell Viability Assay varying from 1:1 to 1:6. The total working volume was 200µl for each well. Each group had 2 subgroups of a 5-minute shaking time as described in 3D cell viability assay’s protocol and another group of 10 minutes shaking time (see Table 1). Assay was used as protocol except 5 more additional ratios of sample volume to reagent volume were added from 1:2 up to 1:6, and another set of groups was vigorously shaking for additional 5 minutes other than the protocol. In general, MCS were collected and transferred to opaque-welled plates, assay was added to each well as calculated (see Table 1). Microplates were vigorously mixed as its designated time group and incubated at room temperature for additional 25 minutes. The luminescence was then recorded by a Synergy HTX microplate reader (BioTek, US). Samples were evaluated in at least replicates.
Table 3
3D Assay Validation Detail

<table>
<thead>
<tr>
<th>$V_{\text{sample}}$ (µl)</th>
<th>5 min</th>
<th>10 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{assay}}$ (µl)</td>
<td>$V_{\text{sample}}$(µl)</td>
<td>$V_{\text{assay}}$(µl)</td>
</tr>
<tr>
<td>1:1</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>1:2</td>
<td>66.7</td>
<td>133.3</td>
</tr>
<tr>
<td>1:3</td>
<td>50.0</td>
<td>150.0</td>
</tr>
<tr>
<td>1:4</td>
<td>40.0</td>
<td>160.0</td>
</tr>
<tr>
<td>1:5</td>
<td>33.3</td>
<td>166.7</td>
</tr>
<tr>
<td>1:6</td>
<td>28.6</td>
<td>171.4</td>
</tr>
</tbody>
</table>

2.2.5. Imaging of Live/Dead in A549 3D MCS

A549 cells were seeded at 5000 cells/well in 96-well spheroid microplates to form A549 3D MCS as described above. A549 MCS were transferred to a glass Petri dish when the diameter reached 500 µm and incubated with Live/DEAD™ Cell Imaging Kit at room temperature for 45 minutes before washed 3 times with PBS buffer. A549 3D MCS were imaged by a Leica DMIRE2 confocal microscope. Excitations at 491nm and 561nm were used for live and dead dyes, respectively; since the live dye can produce an intensive green fluorescence in live cells at ex/em ~494 nm/~517 nm, and dead dye gives a bright red fluorescence at ex/em ~528 nm/~617 nm. Images were acquired with MetaMorph and analyzed by using ImageJ software.

2.2.6. Data Analysis.

Data are presented as mean ± standard deviation (SD) for assay characterization study. The statistical analysis is performed by two-way analysis of variance (ANOVA) for assay characterization study using GraphPad Prism 8.0 Software (GraphPad Software, La Jolla, CA). Tukey’s multiple comparisons test analysis was performed to compare between groups. Results with $p < 0.05$ were accepted as statistically significant.
2.3. Results and Discussion

2.3.1. Formation of 3D A549 and A549-iRFP MCS

Among methods described above, matrix, centrifugation and ultra-low attachment plate methods are used together in our lab to form spheroids. Controllable and uniform size MCS were formed in both A549 and A549-iRFP with the use of 0.3% collagen and centrifugation at 300 × g. As is shown in Figure 2, MCS were tight and relatively round shape, and the size of MCS grew bigger over time in both cell lines. Growth curves were also validated in volume and cellular viability, both of which increased over days (Figure 3).

*Figure 2.* Morphology of MCS on day 1, 3, 5, 7, 9 and 11. Tight and relatively round shape 3D MCS were formed. Size of MCS grew bigger over days (represented by diameter). (A) 3D A549 MCS; (B) A549-iRFP MCS; cells were seeded 5000 cells/well.
2.3.2. Characterization and Optimization of 3D Cell Viability Assay Condition

According to the 3D cell viability assay’s protocol, a volume of reagent equal to cell culture media present in each well should be added, and then vigorously mix for 5 minutes to induce cell lysis. Plate is incubated for 25 minutes to stabilize the luminescent signal before reading. However, considering MCS will probably grow very big at the end of our studies, 1:1 ratio of MCS to reagent volume and 5 minutes mixing time may not be enough mixing. Insufficient mixing will result in inaccurate readouts, because less ATP will be released and result in underestimated luminescent signal level. Therefore, in order to produce effective ATP extraction from MCS so that optimum assay performance can be achieved, higher ratios of reagent volume were added and longer mixing time was also being tested. Among all the size ranges, there are no significant differences between 1:1 and each tested ratio (1:2, 1:3, …, 1:6), and no difference is seen between 5 minutes and 10 minutes mixing time (results shown in Figure 4), which means the amount of reagent used and time for mixing in protocol are enough
to lyse cells and can react thoroughly. Therefore, 3D viability assay can be carried out as specified in the protocol’s steps, no adjustment needed to be done.

Figure 4. 3D cell viability assay validated on different sizes of 3D A549-iRFP spheroids. There is no significant difference among different volume ratio between sample and assay in all sizes and shaking time being tested, and no significant difference under 5 minutes and 10 minutes shaking time in all sizes and ratio. (P>0.05 for two-way ANOVA). (A) 3D A549-iRFP spheroids at diameter ~350μm; (B) 3D A549-iRFP spheroids at diameter ~550μm; (C) 3D A549-iRFP spheroids at diameter ~750μm; (D) 3D A549-iRFP spheroids at diameter ~950μm.

2.3.3. Distribution of Live and Dead Cells in 3D A549 MCS

Figure 5A shows a cross section of a 3D MCS A549 that is 500 μm in diameter by confocal microscope, taken 200 μm from the bottom which is close to the core. The green fluorescence is from fluorescent calcein representing live cells, red fluorescent comes from EthD-1 which represents dead cells. From both confocal image (Figure 5A) and relative fluorescent intensity (Figure 5B), green signals can be seen more in peripheral area while red signals are more centered in the core. This means live cells are located at outside layer while
dead cells are more accumulated in core area, which confirms that necrotic core is formed in 3D A549-iRFP at the size of 500 µm.

This phenomenon can also be seen in 3D MCS A549-iRFP done by our lab previously, shown in Figure 6 (Yingbo, 2019). The diameter of chosen MCS was also around 500 µm, and the cross section was also taken 200 µm from the bottom, using same experimental conditions. The distribution of green and red fluorescence signal in MCS shown in Figure 6 confirms the existence of necrotic core in 3D MCS of A549-iRFP.

*Figure 5.* Confocal image of live/dead cells in A549 3D MCS. (A) Confocal image shows the distribution of live and dead cells in A549 3D MCS; (B) Relative fluorescent intensity profile indicates the distribution of live cells and dead cells vary in 3D A549 MCS; green fluorescent represents live cells; red fluoresce represents dead cells.
2.4. **Summary**

Round, tight and uniform MCS of both A549 and A549-iRFP are successfully constructed at selected seeding density on ultra-low attachment plates using collagen and centrifugation. Their sizes are controllable and grows bigger over days. Necrotic cores are located at the center of MCS on both cell lines when sizes reach 500 µm. CellTiter-Glo®, a 3D cell viability assay, can be used to evaluate MCS viability after drug treatment under specified protocol procedures even when diameter is larger than 1000 µm.

Of interest is that these MCS only consist of carcinoma cells, which does not have other type of cells that a solid tumor has, for example fibroblasts and endothelial cells. Those components are important in mimicking solid tumor heterogenic cellular constitution which further support cell-cell and cell-matrix interactions. However, because of the collagen we used, it provided the matrix for cells to grow and aggregate, leading to cells diversifying into proliferating cells, quiescent cells and non-proliferating cells. Even though there is only one type of cell, we still developed MCS that mimics solid tumor better than monolayer cells.

*Figure 6.* Confocal image of live/dead cells in A549-iRFP 3D MCS. (A) red fluorescent signal (B) green fluorescent signal (C) merged image of two signals (D) image of 3D A549-iRFP MCS taken in brightfield.
In this way, our lab combined three methods to form A549 and A549-iRFP MCS successfully which are ready to be used in drug screening. This method is quite novel and has high success rate of providing good spheroids in large amount, especially ideal for HTS.
3.1. Introduction

After the successful construction of A549 and A549-iRFP MCS, four anticancer drugs, namely cisplatin, carboplatin, gemcitabine and doxorubicin were chosen to be assessed on their ability to inhibit the growth of MCS. These four anticancer drugs were chosen because they are widely used in A549 related studies, cisplatin and carboplatin are drugs of choice in clinical settings (Edelman, Quam, & Mullins, 2001; Punia, Raina, Agarwal, & Singh, 2017; Shali et al., 2018; Teng et al., 2018; Varbanov, Kuttler, Banfi, Turcatti, & Dyson, 2019). Gemcitabine is more frequently used in squamous cell NSCLC while doxorubicin is indicated in small-cell lung cancer. These two drugs are used as negative controls. Four types of cell culture were used: A549 monolayer cells, A549 MCS, A549-iRFP monolayer cells, and A549-iRFP MCS due to the afore-mentioned advantages of MCS to mimic more features of solid tumors than monolayer cell cultures (Chapter I).

3.1.1. Cisplatin

Cisplatin is the first platinum-based anticancer agent. It was approved by FDA in 1978. It exhibits broad spectrum of antitumor activity in various cancer types including testicular, ovarian, lung, head and neck, and bladder cancers. It has several side effects, for example, serious emesis, neurotoxicity, and nephrotoxicity (which is often dose-limiting toxicity). The mechanism of action is due to its interaction with DNA. The chloride ligands of cisplatin are replaced by H₂O molecules once inside cells, then covalently bind to the N7 site of purine base of DNA to form intrastrand crosslinks. This cisplatin-DNA adducts distort the DNA duplex structure which block DNA replication and transcription (D. Wang & Lippard, 2005).
3.1.2. Carboplatin

Carboplatin is the second FDA-approved antitumor agent from the Platinum drug family. It has a similar antitumor spectrum to cisplatin, and has cross-resistance with cisplatin. Carboplatin is generally more tolerable than cisplatin, and its dose limiting toxicity is myelosuppression. Carboplatin’s mechanism of action is similar to cisplatin, however, cyclobutane-1,1-dicarboxylate in carboplatin makes it more resistant to aquation and therefore slower to form carboplatin-DNA adducts (Knox, Friedlos, Lydall, & Roberts, 1986).

3.1.3. Doxorubicin

Doxorubicin is an anthracycline anticancer drug. It was first extracted from *Streptomyces peucetius var. caesius* (Arcamone et al., 1969). It is used for the treatment of variety of cancers, such as breast cancer, small-cell lung cancer, ovarian cancer, acute lymphoblastic leukemia, Hodgkin lymphoma, etc. There are two main mechanisms of its cytotoxicity, intercalation into DNA and generation of free radicals. The first mechanism inhibits the DNA synthesis while the second leads to DNA and cell membrane damage (Thorn et al., 2011).

3.1.4. Gemcitabine

Gemcitabine is a nucleoside analog of deoxycytidine that exhibits anticancer activity in breast cancer, non-small cell lung cancer and bladder cancer. Gemcitabine is cell phase specific, which kills cells during DNA synthesis (S-phase). It is metabolized intracellularly into diphosphate (dFdCDP) and triphosphate (dFdCTP) nucleosides, both of which attribute to inhibition of DNA synthesis (Eli Lilly, 1996).

3.2. Materials and Methods

3.2.1. Materials
A549 cell line was purchased from ATCC (VA, US). A549-iRFP cell line was purchased from Imanis Life Sciences (MN, US). RPMI 1640, DMEM, Collagen, Trypsin-EDTA, and 1% penicillin-streptomycin were purchased from Thermo-Fisher. Puromycin was purchased from Alfa Aesar (MA, US). Fetal bovine serum was purchased from Gemini Bio-Products (CA, US). Ultra Cruz® Black/Clear Flat bottom 96-well microplates were purchased from Santa Cruz (CA, US). White Flat Bottom 96-well microplates and 96-well Spheroid microplates were purchased from Corning Life Science (NY, US). CellTiter 96® AQueous One Solution Cell Proliferation Assay and CellTiter-Glo® 3D Cell Viability Assay kits were purchased from Promega Corporation (Madison, WI, US). Cisplatin and Doxorubicin Hydrochloride were purchased from BIOTANG Inc. (MA, US). Carboplatin was purchased from ChemScene llc (NJ, US). Gemcitabine Hydrochloride was purchased from LC Laboratory (MA, US).

3.2.2. **Cell Culture Maintenance**

A549 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Gemini, CA, US) and 1% Penicillin-Streptomycin (Corning Life Science, NY, US). A549-iRFP cells were maintained in DMEM supplemented with 10% fetal bovine serum (Gemini, CA, US), 1% Penicillin-Streptomycin (Corning Life Science, NY, US), and 1 μg/ml puromycin (Alfa Aesar, MA, US). Cells were grown in 5% CO₂ at 37 °C and passaged at 85% confluence (3~4 days).

3.2.2.1. **Cytotoxicity Assay for Selected Anticancer Drugs on 2D Monolayer Cells.**

A549 and A549-iRFP cells were seeded onto Ultra Cruz® Clear and Black flat-bottom 96-well microplates (Santa Cruz, CA, US), respectively, at seeding density of 5000 cells/well. Cells were grown overnight to be prepared for the cytotoxicity assay. The growth media was then replaced by media containing the drug at decremental concentrations, for which each selected
drug was dissolved in water and diluted with media by 10 fold to prepare the highest concentration of the drug solution in media, followed by further dilution with media to decremental concentrations. Drug solutions in media were replaced by growth media after 72 hours and/or 48 hours incubation. Reagent solution (20 µl/well) of the CellTiter 96® AQueous One Solution Cell Proliferation Assay was then added and incubated for 3 hours, after which UV absorbance (λ=490 nm) was read by a Synergy HT microplate reader. One set of cells were treated with drug-free growth media for each experiment and assayed in parallel as control, whose viability was taken as 100%. Wells in microplates with no seeded cells were also assayed in parallel as the blank. Viability for each assay sample at various drug concentrations were calculated as the following equation (Equation 3.1):

\[
Viability(\%) = \frac{(A_1-A_{blank})}{(A_{control}-A_{blank})} \times 100\%
\]

Equation 3.1

Samples were evaluated in triplicates or more.

3.2.2.2. **Cytotoxicity Assay for Selected Anticancer Drugs in 3D MCS Cell Model.**

A549 and A549-iRFP cells were seeded 5000 cells/well onto Corning 96-well spheroid microplates (Corning Life Science, NY, US) to form MCS as described previously (Chapter section 2.2.3). Spheroids were grown to diameter ~ 500 µm in 5 to 6 days after seeding to be prepared for the assay. Media was then replaced by drug solutions in media at decremental concentrations, for which the drug (except cisplatin) was dissolved in water and diluted by 10 fold with media to prepare the highest concentration, followed by further dilution with media to decremental concentrations. Because of its poor solubility in water, cisplatin was directly dissolved in media and then further diluted with media to decremental concentrations. Drug solutions in media were replaced by 100 µl growth media after 48-72 hours incubation with cells. Reagent solution (100 µl) of CellTiter-Glo® 3D Cell Viability Assay was added to each
well, and the plates were shaken for 5 min and then incubated for 25 min, after which
luminescence was recorded by a Synergy HT microplate reader. One set of cells were treated
with drug-free growth media for each experiment and assayed in parallel as control, whose
viability was taken as 100%. Viability for each sample at various drug concentrations were
calculated by the following equation (Equation 3.2):
\[
\text{Viability(\%)} = \frac{A_x}{A_{\text{control}}} \times 100\% \quad \text{Equation 3.2}
\]
Samples were evaluated in triplicates or more.

3.2.3. Data Analysis

Data are presented as mean ± standard deviation (SD) for all cytotoxicity assay studies.
IC$_{50}$ values were estimated by nonlinear regression [log(inhibitor) vs. response, Variable slope,
four parameters] using GraphPad Prism 8.0 Software (GraphPad Software, La Jolla, CA). The
statistical analysis is performed by unpaired t test to compare statistical difference of IC$_{50}$
between monolayer and MCS using GraphPad Prism 8.0 Software (GraphPad Software, La Jolla,
CA). Results with P < 0.05 were accepted as statistically significant.

3.3. Results

3.3.1. Sensitivity of A549 Monolayer Cells and MCS to Anticancer Drugs

A549 cells, either in monolayer or in MCS, were treated with different anticancer drugs
at decremental concentrations and their viability is presented as dose-response curves in Figure
7. Concentration of different anticancer drugs that is required to inhibit fifty percent growth
(IC$_{50}$) of A549 monolayer cells and MCS were then estimated from data in Figure 7 using the
GraphPad Prism software and are shown in Table 4. The four selected chemotherapy drugs were
firstly tested in 2D monolayer cells. Three of the drugs, namely cisplatin, carboplatin and
doxorubicin inhibited most viability of the monolayer cells below 200 µM except for
gemcitabine. The four drugs were then evaluated in 3D MCS cell culture. Except for gemcitabine, which was not active at the highest dose tested, the drugs generated typical dose-response curves in 3D MCS. IC₅₀ values in MCS was estimated with high reproducibility. Therefore, the 3D MCS viability assay can be readily used to reliably evaluate drug activity. Moreover, table 4 compares IC₅₀ values between A549 monolayer cells and A549 MCS, which reveals higher IC₅₀ values in MCS (e.g., 10-fold higher IC₅₀ of doxorubicin for A549 MCS than A549 monolayer cells).

MCS have properties that are similar to that of solid tumor, which monolayer cells don’t have, such as hypoxic and acidic microenvironment, increased cell-cell and cell-matrix interactions and cellular heterogeneity. Those properties may lead to inability of compounds to penetrate through cells or to alter cell signaling, which in turn induces drug resistance in 3D MCS. However, the literature has also reported cases of increased drug sensitivity in 3D MCS over monolayer cells. Trastuzumab, a targeted anticancer drug, can reduce 48% of SKBR-3 3D MCS growth but only 16% of the corresponding monolayer cells at the same concentration. The authors of this investigation concluded it was because the 3D MCS was more representative of the HER2 signaling pathway in tumors in vivo (Pickl & Ries, 2009). Still, another example is that 2D A431.H9 cells maintained 72% viability after treatment with tirapazamine (TPZ), while the corresponding 3D MCS maintained only 40% viability. This higher sensitivity of 3D MCS to the drug was explained by the need of TPZ to be activated by hypoxia, which was prominent in 3D MCS but not in 2D monolayer cells (Tung et al., 2011). These two examples highlight the fact that MCS do not necessarily induce drug resistance, but the combination of the drug and the three dimensional cellular environment in MCS can substantially alter the drug effect and probably better reflect the drug’s effect in vivo.
Figure 7. Representative dose-dependant viability curves of A549 monolayer cells (blue) and MCS (red) after exposure to anticancer drugs. (A) cisplatin, (B) carboplatin, (C) doxorubicin, and (D) gemcitabine. Data presented as mean ± SD, N ≥3.

Table 4
IC50 Values of Different Anticancer Drug on A549 Monolayer Cells and MCS. Data Presented as Mean ± SD Of Three Separate Experiments. (P>0.05 for Unpaired T Test).

<table>
<thead>
<tr>
<th>Drug</th>
<th>2D (μM) ± SD</th>
<th>3D (μM) ± SD</th>
<th>IC50(3D)/IC50(2D) (fold)</th>
<th>P value between IC50 on 2D and 3D</th>
</tr>
</thead>
<tbody>
<tr>
<td>cisplatin</td>
<td>9.07 ± 3.27</td>
<td>20.71 ± 2.63</td>
<td>2.28</td>
<td>0.0040</td>
</tr>
<tr>
<td>carboplatin</td>
<td>131.80 ± 10.66</td>
<td>188.90 ± 18.68</td>
<td>1.43</td>
<td>0.0100</td>
</tr>
<tr>
<td>doxorubicin</td>
<td>0.63 ± 0.23</td>
<td>6.57 ± 2.88</td>
<td>10.37</td>
<td>0.0302</td>
</tr>
<tr>
<td>gemcitabine</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

3.3.2. Sensitivity of A549 Monolayer Cells and MCS to Anticancer Drugs

A549-iRFP cells, either in monolayer or in MCS, were treated with different anticancer drugs at decremental concentrations and their viability is presented as dose-response curves in Figure 8. IC50 of different anticancer drugs on A549-iRFP MCS and monolayer cells were estimated from data shown in Figure 8 using the GraphPad Prism software. The resultant IC50
values are shown in Table 5. The four anticancer drugs showed similar results in A549-iRFP cells compared to A549. Specifically, gemcitabine is still not active at the highest dose tested while dose-response curves are prominent for cisplatin, carboplatin, and doxorubicin in both 2D and 3D cultures. Similar to the data for A549 cells (Table 4), the IC_{50} value of cisplatin, carboplatin, and doxorubicin against A549-iRFP 3D MCS was substantially higher than that against A549-iRFP monolayer cells (Table 5). However, as is shown in Table 6, parts of the IC_{50} values of A549-iRFP for both the 2D monolayer cells and the 3D MCS are comparable to those in A549 (P > 0.05 for unpaired t test), but some are statistically different (P < 0.05 for unpaired t test). A549-iRFP is parental A549 cells transduced with LV-iRFP-P2A-Puro (LV032), so it does not necessarily exhibit exact same drug response as A549, there are still some limitations of using A549-iRFP to represent A549. But in general, A549-iRFP shows same trend as A549 that 3D MCS needs higher concentration to inhibit 50% of cell growth than the corresponding 2D monolayer cells.
Figure 8. Representative dose-dependant viability curves of A549-iRFP monolayer cells (blue) and MCS (red) after exposure to anticancer drugs. (A) cisplatin, (B) carboplatin, (C) doxorubicin, and (D) gemcitabine. Data presented as mean ± SD, N ≥3.
Table 5
IC50 Values of Different Anticancer Drugs on A549-iRFP Monolayer Cells and MCS. Data Presented as Mean ± SD of Three Separate Experiments. (P>0.05 for Unpaired T Test).

<table>
<thead>
<tr>
<th>Drug</th>
<th>2D (μM)</th>
<th>3D (μM)</th>
<th>$\frac{IC_{50(3D)}}{IC_{50(2D)}}$ (fold)</th>
<th>P value between IC50 on 2D and 3D</th>
</tr>
</thead>
<tbody>
<tr>
<td>cisplatin</td>
<td>12.25 ± 1.04</td>
<td>50.67 ± 15.29</td>
<td>4.14</td>
<td>0.0010</td>
</tr>
<tr>
<td>carboplatin</td>
<td>197.24 ± 33.61</td>
<td>265.63 ± 23.31</td>
<td>1.35</td>
<td>0.0221</td>
</tr>
<tr>
<td>doxorubicin</td>
<td>0.9 ± 0.45</td>
<td>4.18 ± 0.76</td>
<td>4.65</td>
<td>0.0008</td>
</tr>
<tr>
<td>gemcitabine</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 6
Comparison of IC50 Between A549 and A549-iRFP of Different Drugs on Their Corresponding Culture Type. (P>0.05 for Unpaired T Test).

<table>
<thead>
<tr>
<th>Drug</th>
<th>P value between A549 and A549-iRFP on 2D</th>
<th>$\frac{IC_{50(2D, A549)}}{IC_{50(3D, A549)}}$ (fold)</th>
<th>P value between A549 and A549-iRFP on 3D</th>
<th>$\frac{IC_{50(3D, A549-iRFP)}}{IC_{50(3D, A549)}}$ (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cisplatin</td>
<td>P&gt;0.05</td>
<td>1.35</td>
<td>0.0233</td>
<td>2.45</td>
</tr>
<tr>
<td>carboplatin</td>
<td>0.0297</td>
<td>1.50</td>
<td>0.0112</td>
<td>1.41</td>
</tr>
<tr>
<td>doxorubicin</td>
<td>P&gt;0.05</td>
<td>1.42</td>
<td>P&gt;0.05</td>
<td>0.64</td>
</tr>
</tbody>
</table>

3.4. Summary

Drug response curves of cisplatin, carboplatin and doxorubicin were successfully generated in MCS of both A549 and A549-iRFP cell lines. The 3D cytotoxicity protocol described in this chapter can be used to evaluate compounds as potential anti-lung cancer drugs because it is reproducible and easy to operate. Higher concentration of the tested anticancer drugs are needed to inhibit 50% the cell viability in MCS than monolayer A549 and A549-iRFP cells, which can be explained by the MCS properties that are absent in monolayer cells (Chapter I), such as hypoxic and acidic microenvironment, cellular heterogeneity and increased cell-cell and cell-matrix interactions.
CHAPTER 4: TREATING MCS OF LUNG CANCER CELLS WITH CHEMOTHERAPY AGENTS BASED ON CLINICAL REGIMENS

4.1. Introduction

4.1.1. Clinical Regimens

Cisplatin, carboplatin, vinorelbine, docetaxel, etoposide, gemcitabine, pemetrexed and paclitaxel are commonly used chemotherapy drugs against NSCLC. These drugs are usually used in combinations in clinical regimens to treat NSCLC. Table 7 lists preferred chemotherapy regimens for NSCLC neoadjuvant and adjuvant therapy based on National Comprehensive Cancer Network® (NCCN®) Guidelines on non-small cell lung cancer (Version 3.2020) (NCCN, 2020).

Table 7

| NCCN Guidelines® NSCLC Chemotherapy Regimens for Neoadjuvant and Adjuvant Therapy |
|----------------------------------|------------------|------------------|------------------|
| cisplatin 75 mg/m² day 1         | +     pemetrexed 500 mg/m² | day 1 every 21 days for 4 cycles |
| cisplatin 75 mg/m² day 1         | +     gemcitabine 1250 mg/m² | day 1 and 8, every 21 days for 4 cycles |
| cisplatin 75 mg/m² day 1         | +     docetaxel 75 mg/m² | day 1 every 21 days for 4 cycles |
| cisplatin 50 mg/m² days 1 and 8  | +     vinorelbine 25 mg/m² | days 1, 8, 15, and 22, every 28 day for 4 cycles |
| cisplatin 100 mg/m² day 1        | +     vinorelbine 30 mg/m² | days 1, 8, 15, and 22, every 28 day for 4 cycles |
| cisplatin 75-80 mg/m² day 1      | +     vinorelbine 25-30 mg/m² | days 1 and 8, every 21 days for 4 cycles |
| cisplatin 100 mg/m² day 1        | +     etoposide 100 mg/m² | days 1-3 every 28 day for 4 cycles |

Chemotherapy Regimens for Patients with Comorbidities or Patients Not Able to Tolerate Cisplatin

| carboplatin AUC 6 day 1           | +     paclitaxel 200 mg/m² | day 1 every 21 days for 4 cycles |
| carboplatin AUC 5 day 1           | +     gemcitabine 1000 mg/m² | days 1 and 8, every 21 days for 4 cycles |
| carboplatin AUC 5 day 1           | +     pemetrexed 500 mg/m² | day 1 for nonsquamous every 21 days for 4 cycles |

4.1.2. Incubation Time

In most in vitro cytotoxicity studies, cells are exposed to drug for 48- or 72-hour continuous exposure (Larsson et al., 2020; Nordin et al., 2019). In fact, many small molecule
drugs are metabolized or eliminated quickly, so that their half-life in plasma is much shorter than 48 hours. Therefore, it is irrational to use 48- or 72-hour continuous treatment for all drugs and important to consider the PK of a drug when designing experiments to treat MCS with the drug. Take cisplatin as an example, the peak plasma concentration is about 10 µM and its half-life is less than 1 hour in patients (Himmelstein et al., 1981). A rational experimental design with cisplatin would be to incubate cells with low concentrations for only a few hours instead of using 100 µM and with incubations of 24-72 h (C. Wang & Youle, 2012).

In this study, because it is exceedingly cumbersome for a cell culture system to precisely mimic the exponential decrease of the drug concentration as we see in the in vivo PK, an approximation of the PK was simulated by lowering the concentration to ¼ of the original after two half-lives, and then from ¼ of the original concentration to 0 after another 2 half-lives.

4.2. Materials and Methods

4.2.1. Materials

A549-iRFP cell line was purchased from Imanis Life Sciences (MN, US). DMEM, Collagen, Trypsin-EDTA, 1% penicillin-streptomycin were purchased from Thermo-Fisher. Puromycin was purchased from Alfa Aesar (MA, US). Fetal bovine serum was purchased from Gemini Bio-Products (CA, US). White Flat Bottom 96-well microplate and 96-well Spheroid microplate were purchased from Corning Life Science (NY, US). CellTiter-Glo® 3D Cell Viability Assay kits were purchased from Promega Corporation (Madison, WI, US). Carboplatin was purchased from ChemScene llc (NJ, US). Gemcitabine Hydrochloride was purchased from LC Laboratory (MA, US).

4.2.2. Cell Culture Maintenance
A549-iRFP was maintained in DMEM supplemented with 10% fetal bovine serum (Gemini, CA, US), 1% Penicillin-Streptomycin (Corning Life Science, NY, US) and 1 μg/ml puromycin (Alfa Aesar, MA, US). Cells were grown in 5% CO₂ at 37 °C and passaged at 85% confluence every 3~4 days.

4.2.3. 3D MCS

A549-iRFP cells were seeded at 5000 cells/well onto Corning 96-well spheroid microplates (Corning Life Science, NY, US) to form MCS as described previously (Chapter II 2.2.3). Spheroids were ready for experiment when the diameter reached 500 μm, typically in 5~6 days. At the beginning of the drug treatment, the growth media were replaced by drug solutions in media. Besides drug treatment, media was partially exchanged every other day by replacing 100 μl/well media in microplates with 100 μl/well fresh media to ensure a total media volume of 200 μl.

4.2.4. Scheduled Treatment of MCS with Anticancer Drugs

4.2.4.1. Targeted levels of drug exposure based on clinical regimen. Among all the regimens NCCN recommend, carboplatin AUC=5 on day 1, gemcitabine 1000 mg/m² on days 1 and 8, every 21 days for 4 cycles was chosen to be mimicked on MCS because both drugs are in the list of the anticancer drugs under this thesis’ studies.

4.2.4.2. Grouping. Experimental groups were designed following the criteria discussed below. Control groups were also prepared for each drug/drug combination per treatment duration. The grouping details are listed in Table 7.

4.2.4.3. Duration of drug exposure. Based on our previous lab experience, MCS has been cultured for 20 days (Yingbo, 2019). So, in this study, the total selected time frame of drug treatment on MCS was 28 days which is 7 days/cycle, 4 cycles in total, instead of 21 days x
4 cycles = 84 days for the clinical regimen. Each cycle for the MCS treatment is 7 days, where carboplatin was given on day 1 of each cycle and gemcitabine on day 1 and day 3 of each cycle.

In order to mimic drug concentration decrease due to metabolism and elimination, some groups of MCS were scheduled to have media change according to half-life of the drug. Specifically, the drug concentration was lowered to ¼ of the original after 2 half-lives, and then further lowered to 0 after another 2 half-lives. Half-lives of gemcitabine and carboplatin are considered 1 hour and 2 hours, respectively, according to prior PK studies (Eli Lilly, 1996; van der Vijgh, 1991). For comparison to the PK-based, cycled drug exposure, additional groups of MCS were also continuously exposed to drug(s) for 48 hours. The schematic diagram of concentration changes is shown in Figure 9.

4.2.4.4. **Drug Concentration.** The concentration of the drugs to treat MCS are determined based on two criteria. One criterion is to use clinically relevant concentrations. According to NCCN guideline, carboplatin is dosed to target AUC=5, which corresponds to 44.65 μM initial concentration for groups that are treated in cycles according to the drug half-life, or corresponds to 4.65 μM in case of 48-hour continuous drug exposure. Gemcitabine’s initial concentration is based on its peak plasma concentration (PPC) of about 30 μM when dosed at 1000 mg/m² (Ciccolini, Serdjebi, Peters, & Giovannetti, 2016). The other criterion is to use IC₅₀ values of different drugs on the monolayer cells which obtained from the *in vitro* studies of this thesis. Therefore, 150 μM was used on carboplatin.

To start the treatment of MCS with a drug, the media (200 µl/well) in microplates was replaced by 200 µl/well freshly made drug solutions in media. In order to decrease the concentration of a single drug to ¼ of the initial level at the double half-life time, 150 of 200 µl drug-containing media in each well was replaced by fresh, drug-free media. To mimic the PK for
a two-drug combination, assuming gemcitabine and carboplatin is evenly distributed in the media, so 150 µl of the initial 200 µl drug-containing media was replaced by freshly prepared, more diluted carboplatin solution in media at gemcitabine’s first and second half-life so that the concentration of each of the two drugs in the combination reaches the target concentration. At the end of drug exposure, all drug-containing media was replaced by fresh, drug-free media (see Table 8).
<table>
<thead>
<tr>
<th>group number</th>
<th>drug</th>
<th>treatment criteria</th>
<th>treatment action</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>concentration</td>
<td>duration</td>
</tr>
<tr>
<td>experimental groups</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>carboplatin</td>
<td>AUC=5</td>
<td>t½</td>
</tr>
<tr>
<td>2</td>
<td>carboplatin</td>
<td>continuous exposure</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>IC₅₀</td>
<td>t½</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>continuous exposure</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>gemcitabine</td>
<td>PPC</td>
<td>t½</td>
</tr>
<tr>
<td>6</td>
<td>gemcitabine</td>
<td>continuous exposure</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>gemcitabine</td>
<td>PPC</td>
<td>continuous exposure</td>
</tr>
<tr>
<td>8</td>
<td>carboplatin</td>
<td>PPC</td>
<td>t½</td>
</tr>
<tr>
<td>control groups</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>follow group 1 and 3 (t½ of carboplatin)</td>
<td>change media at 2nd carboplatin half-life (4 hours), then incubate for another 2 carboplatin half-life (4 hours)</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>follow group 2 and 4 (continuous exposure)</td>
<td>incubate for 48 hours</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>follow group 5 (t½ of gemcitabine)</td>
<td>change media at 2nd gemcitabine half-life (2 hours), then incubate for another 2 gemcitabine half-life (2 hours)</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>follow group 6 and 7 (continuous exposure)</td>
<td>incubate for 48 hours</td>
</tr>
</tbody>
</table>
(Table 8 Continued)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>follow group 8 (t$_1$/2 of gemcitabine and carboplatin)</th>
<th>change media at 2$^{nd}$ gemcitabine half-life (2 hours), incubate for another 2 gemcitabine half-life (2 hours) followed, then change media again after 2 carboplatin half-life (4 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.2.4.5. Viability. The fluorescent signal ($\lambda_{ex} = 685$ nm, $\lambda_{em} = 700$ nm) of the near-infrared fluorescent protein iRFP from A549-iRFP was recorded by an Odyssey® Infrared Imaging 205 System (LI-COR® Biosciences, Lincoln, NE, USA) at the 700 nm channel to measure the iRFP expression as a dynamic indicator of MCS viability. On the last day of viability monitoring, all the MCS were also assessed by the CellTiter-Glo® 3D Cell Viability Assay, which measures the cellular ATP level.

The iRFP level and the cellular ATP level of MCS on the last day of viability monitoring were used to calculate the iRFP inhibition rate and the cellular ATP inhibition rate using the following equations:
\[ iRFP \text{ inhibition rate} = \frac{iRFP \text{ level}_{(\text{drug-treatment group})}}{iRFP \text{ level}_{(\text{control group})}} \times 100\% \quad \text{Equation 4.1} \]

\[ \text{cellular ATP inhibition rate} = \frac{ATP \text{ level}_{(\text{drug-treatment group})}}{ATP \text{ level}_{(\text{control group})}} \times 100\% \quad \text{Equation 4.2} \]

4.2.5. Data Analysis

Data are presented as mean ± standard deviation (SD). When there were more than two groups, the statistical analysis was performed by Brown-Forsythe and Welch one-way analysis of variance (ANOVA) using GraphPad Prism 8.0 Software. Tamhane’s T2 multiple comparisons test analysis was performed to compare between groups, unpaired t-test was performed when there were only two groups to compare. Results with \( P < 0.05 \) were accepted as statistically significant.

4.3. Results and Discussion

4.3.1. A549-iRFP MCS’s Response to Carboplatin

Figure 10 shows the growth curves of carboplatin treated MCS as shown by the increase of the iRFP fluorescence. Viability of MCS on the last day was extracted from Figure 10 and plotted in Figure 11 (A). The inhibition of viability (Figure 11 B) was calculated from the iRFP level of the last day, where the iRFP level of a treatment group was divided by the iRFP level from its drug-free control group (Equation 4.1). 3D viability assay was also used to independently validate the iRFP-based MCS viability and plotted in Figure 11 (C). The data in
Figure 13 (C) was extracted and used to calculate the inhibition of viability according to the 3D viability assay of cellular ATP (Figure 11 D, Equation 4.2).

Figure 10. Growth curves of carboplatin treated MCS, according to the fluorescence of cellular iRFP.

There is no statistic difference (P>0.05 by Brown-Forsythe and Welch ANOVA) between two drug-free control groups (both treated with drug-free media, one incubated for 48 hours, one changed media according to half-life) in Figure 11 (A, C), indicating changing media at half-live doesn’t affect MCS viability. The 150 µM, 48-hour treatment group statistically inhibited more cell growth (P<0.05 by Brown-Forsythe and Welch ANOVA) than 150 µM, PK simulation.
group in Figure 11 (A-D). Even though these two groups share the same concentration, they have different treatment duration, which resulted in different inhibition effect. Continuous drug exposure for 48-hour or 72-hour is commonly used in many cell culture assays for drug-screening. However, most drugs metabolize quickly in body and thus does not maintain the initial concentration at the site of action for 48 hours to 72 hours. Therefore, changing media to mimic the decrease of drug concentration *in vivo* would improve the clinical relevance of drug screening in cell culture. The 4.65 µM, 48-hour treatment group inhibit a little bit more than 44.65 µM, PK simulation group, but the difference is not statistically significant (P>0.05 by Brown-Forsythe and Welch ANOVA, Figure 11 A-D). Although these two groups were treated with different concentrations and duration of drug exposure, they had the same AUC. Their similar viability inhibition rates indicate that neither longer duration of drug exposure nor higher drug concentration is superior. The similar viability inhibition also suggest that AUC is a better predictor of the efficacy of carboplatin. Results in Figure 11 A, B are consistent with Figure 11 C, D, indicating that, similar to cellular ATP level, the iRFP fluorescent signal is a reliable indicator of MCS growth and viability. It also very convenient and can be assessed repeatedly, whereas by measuring ATP level, MCS will have to be sacrificed. However, large standard deviations are seen using iRFP signal shown in Figure 10. This is probably because the media change sometimes moved MCS off the center in a well, which increased the absorption of the fluorescent signal by the black wall of the 96-well plate, which in turn artificially lowered the reading and increased the g variances. According to drug responses from MCS, it shows that MCS can be used in intensive and long-term cytotoxicity study.
4.3.2. A549-iRFP MCS’s Response to Gemcitabine

Figure 12 shows the growth curves of gemcitabine treated MCS, as indicated by the change of viability determined by the fluorescence of cellular iRFP. Moreover, viability of MCS on the last day of the experiment was re-plotted in Figure 13 A. Figure 13 A is then re-plotted in Figure 13 B, where the decrease of iRFP fluorescence was divided by the iRFP fluorescence of control MCS that were not exposed to the anticancer drug to indicate growth inhibition (Equation 4.1). For cross-validation, cellular ATP of MCS at the end of the experiment was also measured by the 3D viability assay (Figure 13 C) and re-plotted into growth inhibition in Figure 13 D by dividing the decrease of cellular ATP with that of control MCS (Equation 4.2).

![Figure 12](image)

*Figure 12. Growth curves of gemcitabine treated A549iRFP MCS, according to the fluorescence of cellular iRFP.*
Figure 13. Viability and inhibition rate of gemcitabine-treated A549-iRFP MCS. Data presented as mean ± SD, N ≥4. (A) Viability indicated as iRFP level; (B) Inhibition rate calculated from iRFP level; (C) Viability read by CellTiter-Glo® 3D Cell Viability Assay; (D) Inhibition rate calculated from 3D cell viability assay. **P< 0.002, ****P<0.0001 for Brown-Forsythe and Welch ANOVA. ++P< 0.002, ++++P<0.0001 for unpaired t test.

There is no statistic difference (P>0.05 by Brown-Forsythe and Welch ANOVA) between the two drug-free control groups (both treated with drug-free media, one incubated for 48 hours, one changed media according to half-life) in Figure 13 (A, C), indicating that changing media at the half-live time points did not affect the MCS viability. Gemcitabine that was treated at 30 µM for 48- inhibit most cell growth in this study, which is not consistent with IC50 viability curve done in Chapter III. In this case, it is almost complete inhibition whereas in previous scenario, none can inhibit more than 50%. This could probably relate to batch difference of gemcitabine. The 30 µM, 48-hour treatment group shows statistically lower viability (P<0.05 by Brown-Forsythe and Welch ANOVA) than the 30 µM, PK simulation group in Figure 13 (A, C) according to iRFP fluorescence, which is consistent with the significantly different cellular ATP levels between the two groups (Figure 13 B and D), both strongly indicating that continuous drug exposure over 48 hours inhibited MCS viability significantly more (P<0.05 by Brown-Forsythe and Welch ANOVA) than pulsed exposure according to the drug’s PK in vivo. This phenomenon is also consistent with what is seen in carboplatin treated MCS groups (Figure 11).
Similar to results on carboplatin-treated MCS groups (Figure 10), reproducible and easily assessed iRFP signal shows relatively large standard deviations in gemcitabine-treated MCS (Figure 12). Furthermore, MCS show sensitive drug responses, this indicates its ability to be used in intensive, long-term cytotoxicity study.

4.3.3. Response of A549-iRFP MCS to Drug Combinations

Figure 14 shows the growth curves of MCS that are treated with a combination of carboplatin and gemcitabine based on the fluorescence of cellular iRFP. Viability of such MCS on the last day was re-plotted in Figure 15 A, from which growth inhibition was derived (Figure 15 B) by dividing the decrease of iRFP fluorescence with iRFP fluorescence of control MCS that was not exposed to any drug (Equation 4.1). The 3D viability assay based on cellular ATP measurements was also used to cross-validate the MCS viability (Figure 15 C), from which growth inhibition was derived (Figure 15 D) by dividing the decrease of cellular ATP with that of control MCS that was not exposed to any drug (Equation 4.2).

![Figure 14. Growth curves of MCS treated with a combination of carboplatin and gemcitabine according to the fluorescence of cellular iRFP.](image)
Figure 15. Viability and inhibition rate of carboplatin and gemcitabine-treated A549-iRFP MCS. Data presented as mean ± SD, N ≥4. (A) Viability indicated as iRFP level; (B) Inhibition rate calculated from iRFP level; (C) Viability read by CellTiter-Glo® 3D Cell Viability Assay; (D) Inhibition rate calculated from 3D cell viability assay. **P<0.002, ****P<0.0001 for Brown-Forsythe and Welch ANOVA. ++P<0.002, ++++P<0.0001 for unpaired t test.

There is statistically significant difference (P<0.05 by Brown-Forsythe and Welch ANOVA) between drug-free control groups of 48-hour and the one that changes media according to gemcitabine and carboplatin’s half-life. The lower viability in the PK simulation group in Figure 15 (A, C) is probably related to the fact that PK simulation group had media changes three times as often as the continuous exposure control group. This result indicates that the more time the media is changed the more MCS viability is affected negatively. Statistic difference (P<0.05 by Brown-Forsythe and Welch ANOVA) is also seen between MCS under continuous exposure to 30 µM gemcitabine plus 4.65 µM carboplatin group and MCS under
half-life-based exposure to 30 µM gemcitabine plus 44.65 µM carboplatin group, as shown in Figure 15 (A, C). The continuous-exposure group has lower viability comparing to PK mimetic group, which is consistent with the finding in single carboplatin and single gemcitabine group (Figure 11 B, D and Figure 13 B, D). By changing media at half-lives, although this is not a perfect mimetic of PK changes, it still reduces positive bias from 48-hour or 72-hour continuous exposure and gives us a brief insight of how drugs will work in vivo. Results in Figure 15 A and B are consistent with Figure 15 C and D, indicating that the iRFP fluorescent is a reliable signal to monitor MCS growth. Large standard deviations can also be seen in Figure 16. Similar to results on carboplatin-treated MCS and gemcitabine-treated MCS, MCS co-treated with carboplatin and gemcitabine shows good viability response at the end of this study, and is able to under through intense dosing schedule, indicating its promising future in long-term cytotoxicity study.

4.4. Summary

MCS of the fluorescent lung cancer cell line A549 iRFP have been treated with chemotherapy agents not only by continuous exposure, but also by schedules that mimic the drug pharmacokinetics and the clinical chemotherapy regimen. A number of conclusions can be drawn from this study. Firstly, MCS can be cultured for more than 30 days while under complicated drug treatment. Secondly, all continuous drug exposure inhibited more cell growth than the corresponding PK-simulating drug exposure. Thirdly, changing media at half-lives can simulate the drug PK roughly. Even though it is not a perfect simulation, it better reflects the drug efficacy, especially when comparing drugs of different half-lives. If only continuous drug exposure is used to assess anticancer activity, it would bring bias for the potency of certain drugs over others. Fourthly, all cell viability/growth inhibition results based on the near-infrared signal
of the fluorescent cellular protein iRFP is consistent with the luminescent signal of cellular ATP in the 3D viability assay. The fluorescent signal of iRFP can be measured multiple times over the long course of the experiment without sacrificing the MCS and thus can provide the dynamic data of MCS growth. However, the fluorescent signals carry large standard deviations. To improve the data precision in future study, extra attention is needed to make sure that MCS is still placed in the center of the well after each media change.

Overall, dynamic treatment of MCS with anticancer drugs based on drug PK and clinical regimen can be used as platform in drug discovery.
REFERENCES


oncology (Royal College of Radiologists (Great Britain)). 26(5), 277-288.
doi:10.1016/j.clon.2014.02.002

the growth of multicellular tumor spheroids. Nature Biotechnology, 15(8), 778-783.
doi:10.1038/nbt0897-778

tumors in vivo: high-resolution measurements reveal a lack of correlation. Nature medicine, 3(2),
177-182. doi:10.1038/nm0297-177


doi:10.1038/clpt.1981.91

tumor spheroid (MCTS) culture from breast cancer cell and a high throughput screening method
using the MTT assay. PLoS One, 7(9), e44640. doi:10.1371/journal.pone.0044640

Höckel, M., & Vaupel, P. (2001). Tumor hypoxia: definitions and current clinical, biologic, and
molecular aspects. Journal of the National Cancer Institute, 93(4), 266-276.
doi:10.1093/jnci/93.4.266

pathway inhibitors on migration and invasiveness of BRAF(V600E) mutant thyroid cancer cells
in 2D and 3D culture. Exp Cell Res, 338(2), 127-135. doi:10.1016/j.yexcr.2015.08.003

Ingram, M., Techy, G. B., Saroufeem, R., Yazan, O., Narayan, K. S., Goodwin, T. J., & Spaulding, G. F.
(1997). Three-dimensional growth patterns of various human tumor cell lines in simulated
doi:10.1007/s11626-997-0064-8

Ivanov, D. P., Parker, T. L., Walker, D. A., Alexander, C., Ashford, M. B., Gellert, P. R., & Garnett, M.
C. (2014). Multiplexing spheroid volume, resazurin and acid phosphatase viability assays for
high-throughput screening of tumour spheroids and stem cell neurospheres. PLoS One, 9(8),
e103817. doi:10.1371/journal.pone.0103817

Spheroids with Enhanced Aggressiveness to Mimic Human Liver Cancer In Vitro and In Vivo.
Scientific Reports, 7(1), 10499. doi:10.1038/s41598-017-10828-7

Kessel, S., Cribbes, S., Bonasu, S., Rice, W., Qiu, J., & Chan, L. L. (2017). Real-time viability and
apoptosis kinetic detection method of 3D multicellular tumor spheroids using the Celigo Image

platinum drugs: evidence that cis-diamminedichloroplatinum(II) and cis-diammine-(1,1-
cyclobutane dicarboxylato)platinum(II) differ only in the kinetics of their interaction with DNA.

Parris, T. Z. (2020). Optimization of cell viability assays to improve replicability and
reproducibility of cancer drug sensitivity screens. Scientific Reports, 10(1), 5798.
doi:10.1038/s41598-020-62848-5

specific functions of porcine hepatocyte spheroids entrapped in collagen gel. In Vitro Cell Dev Biol

spheroid based on a triple co-culture: A novel 3D model to mimic pancreatic tumor complexity.


