Development of transferrin targeted NCL-240 micelles and their evaluation using in-vitro 3D cancer cell culture (spheroid) models

Master’s Thesis

By,
Mr. Srikar Goud Nagelli

Advisor: Vladimir P. Torchilin, Ph.D., D.Sc.
Co-Advisors: Bhushan S. Pattni
Bhawani Aryasomayajula

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Author: Srikar Goud Nagelli

Department: Department of Pharmaceutical Sciences

Approval for thesis requirements for the Master of Science Degree in Pharmaceutical Sciences with specialization in Pharmaceutics and Drug Delivery

Thesis Committee (Chairman): Dr. Vladimir P. Torchilin Date__________________

____________________

Other committee members:

Dr. Eugene A. Bernstein Date__________________

____________________

Dr. Anne van de Ven Date__________________

____________________

Dean of the Bouvé College Graduate School of Health Sciences:

____________________ Date__________________
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1. ABSTRACT

The main objective of this project was to develop targeted micellar delivery systems of a novel cytotoxic drug (NCL-240; a second generation DM-PIT-1 analog) and to evaluate their efficacy using optimized 3D cell culture spheroid models. Spheroids were optimized for several cancer cell lines using a range of techniques such as non-adhesive liquid overlay method, hanging drop method, and co-culturing. Transferrin (Tf)-conjugated NCL-240 micelles were prepared with varying Tf amounts and their cytotoxicities were evaluated using the optimized spheroid models. The uptake and penetration of the formulations were also studied using confocal microscopy. The results indicated that the concentration of NCL-240 micelles required to achieve the same cytotoxicity was relatively higher in spheroids compared to the monolayers. Also, in NCI-ADR-RES, Tf-targeted NCL-240 micelles were shown to have a significant increase in cytotoxicity compared to untargeted NCL-240 micelles. Even the penetration and uptake studies indicated that targeting improves the uptake and penetration of formulations. However, in U87-MG spheroids, there was a significant difference in cell viability among micelles compared to free drug but no significant benefit due to targeting was observed. The same formulations penetrated lesser in U87-MG spheroids compared to NCI-ADR-RES spheroids. This study thereby emphasizes the importance of drug screening in spheroid models as the penetration dynamics are varying from cell line to cell line because of the 3D structure.
2. SPECIFIC AIMS

2.1. Problem statement

Cancer is a devastating disease that is the second leading cause of death worldwide. There have been a lot of scientific developments in understanding the pathogenesis of the disease. Translation into better clinical modalities in the clinic has not been significant for several cancer types. Chemotherapy, which is the major treatment option for many patients, is hindered by several challenges such as multi-drug resistance and non-target side effects. Monolayer 2D cell culture has been widely used for screening novel drug candidates and delivery systems for their efficacy. However, monolayer cultures do not mimic the \textit{in vivo} tumor conditions exactly. Hence, 3D cancer cell culture spheroids have developed interest recently. However, spheroid cell culture has a lot of challenges in terms of consistency as well as techniques for evaluating drug cytotoxicity. Hence, there is a need to develop a suitable method for generation of consistent spheroids in larger quantities with low costs for high throughput screening of cytotoxic drugs.

2.2. Objective and specific aims

Having understood the need for generating consistent 3D cell culture spheroids for better evaluation of formulations, the major objective of this thesis is to optimize various cancer cell spheroids so that they are suitable for evaluating cytotoxicity of a novel anticancer agent (NCL-240). Since targeting is known to be associated with better internalization of payload, the other objective of this thesis is to study the effect of transferrin (Tf)-conjugated NCL-240 micelles in terms of cytotoxicity, internalization and penetration into the spheroids.
The specific aims of this thesis project are:

**Aim 1: Optimization of 3D cancer cell spheroids**

a. To develop cancer cell spheroids for a range of cell lines (NCI-ADR-RES, SKOV-3TR, U87-MG) using several techniques such as non-adhesive liquid overlay, hanging drop method.

b. To evaluate spheroid formation consistency by microscopy and cell number analysis.

c. To establish the suitable parameters (cell number, centrifugation speed and time, etc.) and the best technique for high throughput and consistent generation of spheroids.

**Aim 2: Preparation and characterization of Tf-targeted NCL-240 micelles**

a. To prepare NCL-240 micelles and Tf-conjugated NCL-240 micelles with different amounts of Tf-ligand (0.5 µg, 1 µg and 2 µg Tf).

b. To study the release of NCL-240 from the micelles in vitro.

c. Characterization of micelles for size, surface charge and encapsulation efficiency.

**Aim 3: Evaluation of in vitro cytotoxicity using the developed formulations**

a. To evaluate of cytotoxicity of the developed formulations in monolayer cell cultures and optimized spheroid cell cultures for various cell lines.

b. To study the effect of targeting on cytotoxicity of drug in micelles by modifying the micelles with a targeting ligand.

c. To compare the cytotoxicities between monolayer and spheroids.

**Aim 4: Study the cellular uptake and association of formulations**

a. To prepare rhodamine-labeled targeted and untargeted micelles.

b. To study the penetration and cellular uptake of formulations using confocal microscopy.
3. BACKGROUND AND SIGNIFICANCE

3.1. Introduction

Cancer can be defined as a progressive disease characterized by a very high genomic instability, leading to an imbalance between the cell proliferation and apoptosis pathways [1, 2]. According to WHO statistics, cancer is the second leading cause of death worldwide [3]. In the United States, it is estimated that one out of four deaths can be attributed to cancer. With significant advances in the understanding of the tumor biology, the diagnosis and treatment of cancers have undergone a great level of improvement in the past few decades [4]. However, some of the cancers still have really low 5-year survival rates (Pancreatic cancer 6%, Liver cancer 16%, Lung cancer 17%, Esophageal cancer 19%, stomach cancer 28% and Brain cancer 35%) [5].

Cancer treatment depends on the stage and severity of cancer at the time of diagnosis. Surgical resection of tumors is the most effective form of treatment but it is possible only for early stage cancers (Stage 0 and Stage I). The tumors in later stages (II, III) in which the tumors have grown in size and have spread to neighboring tissue are usually treated by a combination of surgery, radiation therapy and chemotherapy [6]. In advanced cancers (stage IIIB and IV), surgery and radiation are not always possible and chemotherapy is the mainstay of treatment. The major limitation of conventional chemotherapy is that the cytotoxic agents accumulate in all the tissues without distinguishing between normal cells and tumor cells. As a result of this, the effective dose reaching the tumor may be as little as 5-10% and is also associated with a lot of side effects [7, 8]. The treatment outcomes vary a lot from patient to patient as the tumors are associated with high degree of heterogeneity [6, 9].
The chain of events within the cell that lead to its growth and replication is termed as the cell cycle. It consists of five phases: G1, S, G2, M and a resting G0 phase. In normal cells there are multiple control points called cyclin dependent kinases (CDKs) that prevent the cells from harmful mutations while the cells progress from one phase to another [10]. However, sometimes few mutations get carried over unrecognized. When multiple genes within a cell are mutated, cancer develops. In cancer, some genes called oncogenes, which promote cell proliferation are constitutively activated while some genes that regulate cell proliferation (tumor suppressors) are turned off permanently [9, 11-13].

![Figure 1: Schematic representation of the cell cycle and the role of CDK control (Adapted from ref. [10])](image)

Recent advances suggest that tumor microenvironment (TME; environment around the tumors comprising of cells, signaling molecules and extracellular matrix) plays an important role in the progression of the tumors. Hence it becomes important to study the properties of the TME to understand the cancer biology better [13-15]. A typical cancer is characterized by ten major hallmarks as described by Hanahan et.al. (Figure 2) which are acquired during the tumor development process. Each cancer may comprise of more than one of these hallmarks and hence
to treat the cancers effectively, it is important to understand the underlying traits completely [14]. The important hallmark from this project point of view is “sustaining proliferative signaling” and “Evading growth suppressors”.

3.2. Multi-drug resistance

The major hurdle that affects the efficacy of cancer chemotherapy is multi-drug resistance (MDR). It can be present inherently within the tumor due to mutation of certain genes even prior to treatment or can be acquired on treatment with certain class of drugs [16-19]. It is one of the causes for occurrence of tumor metastasis and eventual recurrence. One surprising feature of the acquired resistance is that the cells become resistant not only to the class of drugs that were used in treatment but also to several other drugs [18]. The mechanisms that have a role in development of MDR can be classified into two main categories namely cellular factors and physiological factors [16, 20-22]. Decrease in drug influx into the cells, increase in drug efflux from the cells, enhanced DNA repair, lack of apoptosis and increase in anti-apoptotic factors
comprise the cellular factors [20-22] whereas conditions related to the TME such as hypoxia and high interstitial fluid pressure are the physiological factors that cause MDR [16, 20].

As discussed earlier, resistance is conferred to the cells even due to the microenvironment and the conditions in which tumor grows. The stromal components increase the interstitial pressure and make it difficult for drugs to penetrate into the tumors. Also, the abnormal vasculature causes limited blood supply to the interior parts of the tumors and therefore regions in the interior of the tumor are more hypoxic and acidic compared to the periphery as demonstrated in Figure 3. This is also another important reason for ineffectiveness of chemotherapy [16, 20, 23].

![Figure 3: Characteristics of a tumor: A) Representation of tumor and its microenvironment B) Gradient of oxygen concentration and pH in the tumors (Adapted from the ref. [16])](image)

All the above-mentioned factors indicate that the drugs that are screened for cytotoxicity based on monolayer cell culture are limited in predicting the exact response of these drugs in solid tumors in vivo and hence the technique of culturing cells in three dimensions known as spheroid cell culture has now gained interest [16, 20, 23].
3.3. Spheroid cell culture

3.3.1. Need for spheroid cell culture

The role of *in vitro* cell culture in cancer drug development is very crucial for the screening of lead molecules with cytotoxic effects, and studying their mechanisms of drug efficacy at cellular and molecular levels. But, in many instances the cytotoxicity observed through conventional monolayer culture (2D cell culture) doesn’t correlate well with the effects observed *in vivo* [24].

In tumors, failure of chemotherapy is not only due to lack of efficacy of drugs but also because the drugs have limited penetration due to the barriers such as high interstitial fluid pressure, gradient of blood flow and epithelial tight junctions (In tumors, plasma membranes of adjacent epithelial cells fuse to form tight junctions to prevent lateral diffusion unlike the vascular endothelial cells formed by angiogenesis which are convoluted and leaky).

Also, the cells on the periphery of the tumor are highly proliferative whereas the cells on the inside are quiescent or dead (in the region called necrotic core). Since, the monolayer cultures cannot represent this kind of resistant phenomenon and heterogeneity among cells whereas the spheroid cell cultures better mimic these conditions, they are considered to be more representative of the natural tumor [24-29]. One more important feature similar to *in vivo* tumors is that spheroids contain arrested cells in all the phases of cell cycle [10]. A gene expression analysis study conducted by Takagi *et al.* compared the genes up- and down-regulated among prostate cancer cells grown as monolayer, spheroids and *in vivo* tumors. It was observed that spheroids up-regulated more than 2-fold genes and down-regulated less than half fold genes compared to the monolayer cultures [30]. Also, in another study conducted by Weaver *et al.*, it was observed that inhibition of β1-integrin in breast cancer cells resulted in the reversal of the phenotype and morphology to normal breast epithelial cells only in 3D culture and not in
monolayers [31]. Since the cost of getting a new molecule into the market is very high, it becomes necessary to screen some of the ineffective compounds even before testing them in animals. The 3D cell culture models such as multicellular tumor spheroids (MCTS) can serve as an intermediate level of screening for testing cancer chemotherapeutics (as shown in Figure 4) [24, 32].

![Diagram](image)

**Figure 4:** Role of spheroid cell culture as an intermediate between monolayer and animal testing (Adapted from ref. [24])

While the spheroid cell culture doesn’t replace the role of animal testing, it can be an additional intermediate screening level. Animal models will still be required to study the pharmacokinetic parameters and accumulation of drug within the tumors.

### 3.3.2. Summary of the methods used to prepare spheroids

Sutherland and colleagues were the first to apply spheroid cell culture to cancer research in 1970s. Since then, a lot of methods have been developed to grow cancer cells as spheroids (Figure 5). Each of these methods has their own advantages and disadvantages [29, 32]. They are summarized in the **Table 1** listed on the adjoining page.
<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non adhesive liquid overlay [28, 29, 32-34]</td>
<td>• Simple</td>
<td>• Non uniform spheroid sizes</td>
</tr>
<tr>
<td></td>
<td>• High throughput</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Doesn’t require specialized equipment</td>
<td></td>
</tr>
<tr>
<td>Hanging drop method [28, 29, 32-34]</td>
<td>• Can generate uniform spheroid shapes and sizes</td>
<td>• Tedious and time consuming</td>
</tr>
<tr>
<td></td>
<td>• Doesn’t require specialized equipment</td>
<td>• Not high throughput</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Small culture volumes make it difficult to change the media</td>
</tr>
<tr>
<td>Spinner Flasks [29, 32-34]</td>
<td>• Can be used for mass production</td>
<td>• No control over cell number and size of the spheroids</td>
</tr>
<tr>
<td></td>
<td>• Can form large spheroids</td>
<td>• Requires specialized equipment and is costly</td>
</tr>
<tr>
<td></td>
<td>• Spheroids produced are easily accessible</td>
<td>• Cells subjected to high shear forces</td>
</tr>
<tr>
<td>Matrices/Scaffolds [28, 29, 32]</td>
<td>• Can be used to generate spheroids of cells which don’t form spheroids easily</td>
<td>• Expensive for large scale production</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Difficult to retrieve cells post formation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Issues with biocompatibility and biodegradability of scaffold material</td>
</tr>
<tr>
<td>Microfluidic platforms [28, 29, 32]</td>
<td>• Suitable for high throughput testing</td>
<td>• Expensive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Further analysis maybe difficult</td>
</tr>
</tbody>
</table>

Table 1: Summary of spheroid formation techniques along with their advantages and disadvantages

Figure 5: Methods involved in spheroid preparation A) Forced floating or non-adhesive liquid overlay method, B) Hanging drop method and C) Agitation based technique or Spinner flask technique. (Adapted from ref. [32])
3.3.3. Challenges and future scope of spheroid cell culture

Even though there have been several new techniques for rapid generation of spheroids, the spheroid culture is not widely adopted because it still faces some challenges. They are: a) difficult to form consistent spheroids, b) forming spheroids with all the cancer cell types, c) no available established protocols for drug testing and efficacy in spheroids and d) generating spheroids with less number of cells [29]. Currently a large focus is towards generation of homogenous spheroids (spheroids with only one cell type). In the future, the scope of spheroid cell culture would be towards generating co-culture spheroids with multiple cell types (mesenchymal stem cells, fibroblasts, signaling molecules etc.) which resemble cancer tissues along with TME in vivo. More and more research groups have started focusing on this area [27, 35].

3.4. Micellar delivery systems for cancer therapeutics

In the recent years, nanotechnology has been showing a lot of promise in overcoming the challenges faced by conventional chemotherapy for treating cancers. A lot of nanocarriers or delivery systems in which the cytotoxic drugs can be encapsulated have been developed. The most commonly investigated nanocarriers for cancers are liposomes, micelles, polymeric nanoparticles, and polymer drug conjugates [36-39]. For the purpose of this study, focus will be given largely to micellar drug delivery systems. The advantages of the nanocarriers compared to the free drug delivery are: a) enhanced stability by preventing the drug from degradation or interacting with the surrounding environment; b) improved solubility of a large number of potential drug candidates that are rejected due to solubility issues; c) improved pharmacokinetics and controlled distribution of the drug; d) increased absorption and intracellular penetration [40].
Polymeric micelles are formed by the self-assembly of amphiphilic block co-polymers in the presence of an aqueous environment. They contain hydrophobic core in which lipophilic drugs can be solubilized and a hydrophilic shell that make them suitable for intravenous administration [41, 42]. The typical size of the micelles is in the range of 10-100 nm, because of which they accumulate in pathological areas such as tumors and infarcts based on a phenomenon known as Enhanced Permeability and Retention (EPR) effect. Matsumara and Maeda were the first people to study the EPR effect in cancers. When tumors reach the size of 2mm³, angiogenesis (formation of new blood vessels from existing blood vessels) occurs to support the growing nutrient and oxygen requirements. The newly formed vasculature in the tumor is defective compared to the normal vessels, being leaky and disorganized with large openings. Also, the tumors are characterized by low venous return and poor lymphatic drainage. Therefore, more and more nanoparticles accumulate in the tumors selectively with every circulation, due to passive targeting caused by the EPR effect [40-44].

Surface modification of nanoparticles with PEG is known to increase the longevity of the drug in vivo (first observed in liposomes). When long chain polymers of PEG are used to modify nanocarriers, they prevent the mononuclear phagocyte system (MPS) from binding due to steric hindrance. This prevents opsonization and clearance from the body by the reticuloendothelial system (RES). As a result, the carriers circulate in the blood for a longer duration of time giving more scope for extravasation into the tumors. Another reason for long circulation of the nanocarriers is their size, which is larger than glomerular filtration cutoff size of 10 nm. Hence, PEG-PE copolymer based micelles have triggered lot of interest because of their long circulation and better safety profile [37, 45, 46].
3.5. PI3K pathway in cancer and its role in development of a novel therapeutic

Several researches have shown that the phosphatidylinositol 3-kinase (PI3K) pathway is one of the most frequently activated pathways in cancer. This pathway plays a crucial role in tumor growth and survival. Hence, better understanding of this pathway can be helpful in improving the treatment options for cancer [47, 48]. The PI3K pathway can be activated (as shown in Figure 6) by growth factor stimulation through receptor tyrosine kinases (RTKs) or through receptors coupled to G protein (GPCRs). When growth factors activate the PI3K pathways, the PI3K phosphorylates PI(3,4)P₂ to PI(3,4,5)P₃ [47-50].

The PIP₃ binds to pleckstrin homology domain (PH) and causes a cascade of signaling, involving Akt phosphorylation. Akt activation causes activation and inhibition of several proteins that have roles in cell survival, proliferation and growth. The tumor suppressor protein phosphatase and tensin homolog (PTEN) which causes dephosphorylation of PIP₃ to PIP₂ prevents the activation of PI3K pathway and is the negative regulator of PI3K pathway. In cancers, the PI3K pathways are constitutively activated even without the presence of growth factors due to gain of function mutations in the oncogenes such as PI3K, AKT or due to loss of function mutations in tumor suppressors such as PTEN [47-50].
Figure 6: Activation of the PI3K pathway causing several downstream signals (Adapted from ref. [48])

There has been a lot of thrust towards development of newer targets within the PI3K pathway [51]. One such approach is small molecule inhibition of the binding of PIP₃ to PH, which prevents the downstream signaling caused by Akt activation [52]. Miao et al. discovered the first of its class drug (termed DM-PIT-1) by high throughput screening and binding efficacy studies of 50,000 different molecules. The drug had very good anti-proliferative, anti-migratory and apoptotic potential but however, it was very unstable in the biological fluids. Therefore, second generation analogs of DM-PIT-1 (namely NCL-176, NCL-179, NCL-198 and NCL-240 shown in Figure 7) were developed which had better stability in the body. NCL-240 is the drug of main focus for my study. The second-generation analogs of DM-PIT-1 are poorly soluble in water making them suitable candidates for micellar encapsulation [53]. Studies showed that the druggability and potency of second-generation DM-PIT-1 analogs increased compared to DM-PIT-1 indicating that they hold promise in inhibiting the Akt levels in cancers [52, 53].
3.6. Targeted delivery of NCL-240 micelles by surface modification of PEG-PE micelles

The goal of targeted delivery systems is to actively deliver the payload to the intended site of therapy and not at any other site, thereby preventing adverse side effects [40, 44]. Also targeting is useful for improving the internalization of the nanocarriers, which is very important in delivering certain chemotherapeutic and ribonucleic acid drugs. Tumor targeting can be achieved by surface modification of the nanocarriers with ligands specific to the receptors over-expressed in cancers or antibodies specific to the tumor surface antigens [41, 45]. Tumors are known to over-express certain receptors such as folate, transferrin, EGFR and therefore surface modification of the PEG-PE micelles with the ligands of these receptors can facilitate the entry of the delivery vehicle through receptor mediated endocytosis [36, 44, 54, 55].

Transferrin binds to iron and transports it to the cell by binding to the transferrin receptor. In tumor cells, the transferrin receptors are over-expressed owing to the increase in demand for
nutrient and blood supply requirements [44, 55]. In some tumors, the transferrin receptors are at least 100 to 300 times more expressed than the normal cells [56]. Some of the cell lines that over-express transferrin receptors are SKOV-3 (human ovarian carcinoma), LNCaP (human prostate adenocarcinoma), U87-MG (human primary glioblastoma), and HeLa (human cervical adenocarcinoma) [57, 58]. Hence, conjugating transferrin ligand to the drug loaded PEG-PE micelles can be used as a strategy to actively target tumors [55]. The method developed by Sawant et. al. to prepare ligand conjugated micelles requires the conjugation of holo-transferrin to distal ends of PEG-PE monomer by activated p-nitrophenyl carbonyl groups (pNP) [59].

Figure 8: Schematic representation of passive and active targeting of nanocarriers for cancer therapy (Adapted from ref. [40])
4. MATERIALS AND METHODS

4.1. Optimization of cancer cell spheroids

4.1.1. Cell culture conditions

NCI-ADR-RES (doxorubicin resistant human ovarian adenocarcinoma) cell line was obtained from the National Cancer Institute (Frederick, MD). SKOV-3 TR (taxol resistant human ovarian adenocarcinoma) cell line was a kind gift from Dr. Duan Zhenfeng (MGH, Boston, MA). The human epithelial glioblastoma cell line U87-MG was purchased from American Type Culture Collection (ATCC, Manassas, VA). NCI-ADR-RES, and U87-MG cells were cultured in Dulbecco’s modification of Eagles Medium (DMEM; CellGro, Kansas City, MO) supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic solution (10,000 I.U./ml Penicillin, 10,000 µg/ml Streptomycin, and 25ug/ml Amphotericin B). Similarly, SKOV-3 TR cells were cultured in RPMI-1640 medium (Cellgro, Kansas City, MO) supplemented with 10% FBS and 1% antibiotic solution. All the cells were maintained in an incubator at 37°C with 5% CO₂. Cells were passaged by detaching the cells using 0.25% Trypsin EDTA 1X (Cellgro, Kansas City, MO).

4.1.2. Spheroid cell culture

For spheroid cell culture, the cells were grown as monolayers in T75 and T150 flasks until 80% confluence and then detached by adding 0.25% Trypsin EDTA. A cell pellet was obtained by centrifugation at 1,200 rpm for 5 minutes, which was then re-suspended in 5ml of fresh media and cells were counted using a hemocytometer. Cell suspension was diluted according to the required cell concentration for spheroid culture. For any cell line, spheroids were first grown adopting the non-adhesive liquid overlay method developed by Friedrich et.al. [60] and slightly modifying the conditions depending on the cell line. Other spheroid culture
techniques such as hanging drop method, non-adhesive liquid overlay method and hanging drop method with use of external matrices like reconstituted basement matrix (RBM; for example Matrigel™ (BD Biosciences, San Jose, CA)), and co-culture with fibroblasts were used if the non-adhesive liquid overlay method failed to generate consistent and compact spheroids. Several cell numbers ranging from 5,000 cells/well to 25,000 cells/well were grown and the lowest cell number and the most suitable method that gave good and consistent spheroids on repetitions was established for each of the cell lines mentioned above.

4.1.2.1. Preparation of NCI-ADR-RES spheroids

**Non-Adhesive Liquid Overlay Method:** The non-adhesive liquid overlay method that was optimized in our lab for generating NCI-ADR-RES spheroids as mentioned in [61] was used to determine if the results were reproducible and also to optimize the treatment day and cell number suitable for the formulations. Each well of the 96 well plates was coated with 50µl of sterile 1.5% agar (Fischer Scientific, New Haven, NJ) in serum free media (DMEM; depends on the media in which cells were grown). The plates were then cooled for 40 minutes to form a non-adhesive coating. 10,000 cells per 100µl were seeded into each well and then the plates were centrifuged at a speed of 1500g for 15 minutes. The media was changed every 2 days by removing 50µl of old media and replacing it with the same volume of fresh serum containing media, without disturbing the spheroid. Images were taken at regular intervals using a Nikon ECLIPSE E400 microscope and analyzed to understand the spheroid growth profile.
4.1.2.2. Preparation of SKOV-3 TR spheroids:

Non-adhesive liquid overlay method: The spheroids were formed using non-adhesive liquid overlay method as described above except that RPMI agar was used instead of DMEM agar. Several cell numbers were tried ranging from 5,000 to 15,000 cells/well. The speed of centrifugation and time of centrifugation were also varied. Consistent spheroids were obtained at 10,000 cells/well and centrifugation at 1500 g for 15 minutes. Media was changed as previously explained. Images were taken at regular intervals using a Nikon ECLIPSE E400 microscope to analyze the spheroid growth profile.

Hanging Drop Method The cell suspension needed for hanging drop method was more concentrated and it was adjusted to make 10,000 cells per every 20µl of media. The lid of a 96-well plate was inverted and using a multi-channel pipette small drops were placed on the lid in such a way that each drop perfectly aligns with the well of the 96-well plate. The drops were formed more accurately if the pipette was kept at 90° angle. The lids were then placed inside a large (500cm²) cell culture plate containing around 30 ml of media to prevent dehydration of the drops. These plates were then kept inside an incubator undisturbed for 3 days to allow aggregation of cells due to surface tension and gravity. For harvesting the spheroids, after 3 days the bottom portion of the 96-well plates were coated with agar as described for non-adhesive liquid overlay method and around 200-250µl of media was filled in each plate until the brim. The lids were taken out from the larger plate and placed on the 96-well plate and due to the perfect alignment, the hanging spheroid drops perfectly fell into the respective wells. These plates were further centrifuged at 1000g for 5 minutes to allow for complete compaction and formation of spheroids. Media change was difficult in this method during the 3 days when the spheroids were hanging, and hence media was changed as described previously after the spheroids were
harvested into 96 well plates after 3 days of hanging. It was observed that it is difficult to focus the spheroids while taking images in this method. There are commercially available plates (for example Perfecta 3D® Hanging Drop plates by 3DBiomatrix, Ann Arbor, MI) for growing spheroids by hanging drop method but the method developed in this project seems to be more economical and high throughput.

Non-adhesive liquid overlay method with Matrigel™ This method was same as previously explained except that the suspension of cells was made in media containing varying Matrigel™ (BD Biosciences, San Jose, CA) concentrations (0%, 1.25%, 2.5% and 5%). The cell suspension and plates were maintained at 4°C in ice to prevent solidifying of the matrigel at room temperature. The plates were centrifuged at 4°C. After 2 days media was changed with same concentration of Matrigel™ containing media. The lowest Matrigel™ concentration (1.25%) formed consistent spheroids and it was adopted to generate spheroids for analysis.

Hanging drop method with Matrigel™ The spheroids were formed as described above using hanging drop method. The only modification made was in the step of harvesting spheroids. To harvest spheroids, Media containing Matrigel™ was filled in the plates after 3 days of spheroid formation and then centrifuged at 4°C.

Development of co-culture spheroids: Co-culture spheroids of SKOV-3 TR were grown using a combination of SKOV-3 TR cells and lung fibroblast cells. For co-culture, one cell line was dyed with Vybrant® CM-DiI cell-labeling solution (Molecular Probes®, Invitrogen, USA), while the other cell line was not dyed with any label. The CM-DiI dye has shown to be reliable and reproducible in long-term labeling and tracking of cells both in vitro and in vivo [62]. The steps to dye the cells were as per manufacturer’s protocol. All the steps were carried out in dark conditions. Briefly, human lung fibroblast CCL210 cells (ATCC) were grown in T75 flasks till
80-90% confluence. They were trypsinized and counted to form a suspension of 1 million cells/ml in serum-free RPMI-1640 media. 2. 5 μl of CM-DiI was added per ml of cell suspension and gently pipetted to mix. This mixture was incubated for 20 minutes at 37°C. At 37°C, this mixture was centrifuged at 1,500 rpm for 5 minutes and the supernatant was removed. The cells were gently re-suspended with media and the centrifugation step was repeated to wash off excess dye. The above steps were carried out twice for efficient washing. After re-suspending in serum-containing media, the cells were seeded in a T150 flask and allowed to grow for a few days until confluent. On achieving optimum confluence of both, SKOV3-TR and labeled CCL210 cells, in their respective flasks, were used for forming co-culture spheroids. These cells were harvested and counted for next steps.

Co-culture cell suspensions of SKOV3-TR: CCL210 were prepared in ratios 10:1, 5:1, 2:1 and 50:1 to cover a range of cancer cell: fibroblast ratios. These co-culture cell suspensions were seeded at a total of 10,000 cells/well in agar-coated 96-well plates to perform non-adhesive liquid overlay spheroid culture as explained previously.

4.1.2.3. Preparation of U87-MG spheroids

Non-adhesive liquid overlay method: The spheroids were formed using non-adhesive liquid overlay method as described above. Several cell numbers were tried ranging from 5,000 to 15,000 cells/well. The speed of centrifugation and time of centrifugation were also varied. Consistent spheroids were obtained at 10,000 cells/well and centrifugation at 1,000 g for 10 minutes. Media was changed as previously explained. Images were taken at regular intervals using a Nikon ECLIPSE E400 microscope to analyze the spheroid growth profile.
4.1.3. Spheroid consistency evaluation

The spheroids grown with different techniques were analyzed regularly for consistency in shape, size and integrity. The consistency in shape was determined by visual observation of images taken using a NIKON ECLIPSE E400 microscope whereas for the size, the diameters of the spheroids were measured on different days (as shown in Figure 10 below). Compactness of the spheroids was determined on observation under a microscope by the formation of dense tissue like appearance and tight junctions on the periphery. The spheroids were tested for integrity on the day they attained maximum compactness. The formation of spheroids was confirmed if they were intact even after applying a slight mechanical force, by pipetting several times. The method was deemed optimized if the shape, size and integrity of the spheroids were reproducible and consistent with several repetitions.

![Image](image.png)

**Figure 9:** Measurement of diameter using SPOT 5.0 software of NIKON ECLIPSE E400 microscope (scale bar is 50 µm)

4.1.4. Spheroid cell number and linearity analysis

The cell number is another parameter that determines the consistency of the spheroids. If a spheroid has almost same number of cells on a particular day and if that number is maintained with several repetitions, then the technique used for the generation of spheroids could be termed consistent. The cell number analysis was performed by using acid phosphatase assay (APH)
initially and later by CellTiter-Glo® luminescence assay (Promega, Madison, WI). This cell number analysis helped in understanding how many active cells are actually present in the spheroid. Cell concentrations ranging from 2,500 cells/well to 80,000 cells/well were seeded in triplicates in 96-well plate and a standard curve for absorbance vs. cell number was prepared. Cell number of the spheroid was determined by extrapolating the standard curve obtained from the respective method.

**Acid Phosphatase Assay:** The phosphatase assay used spheroids were collected using large orifice pipette tips from the agar-coated plates into 96-well plates (one spheroid per plate). The media was removed by vacuum and the spheroids were washed twice with 1X PBS pH 7.4 carefully without disturbing the spheroids. 100 µl of the phosphatase assay buffer (containing 0.1 M sodium acetate, 0.1% Triton-X-100 along with p-nitrophenyl phosphate which is the substrate for the phosphatase enzyme) was added to each well. The plates were incubated for 90 minutes at 37°C and constant stirring. At the same time, buffer was also added to 96 well plates containing monolayer cells for standard curve. The reaction was stopped by addition of 1N NaOH and absorbance was measured at 405 nm on a Biotek synergy HT plate reader (Biotek, Winooski, VT). The acid phosphatase assay though is a widely accepted assay failed to provide consistent results and there was a lot of inter-replicate variability. Hence, the cell number study was performed by the use of CellTiter-Glo® Luminescence Assay.

**CellTiter-Glo® Luminescence Assay:** Five spheroids were collected into each eppendorf tube per replicate following the same procedure as described above (Total 15 spheroids for n=3). The spheroids were dissociated into individual cells by adding 200 µl of Accumax® Cell Dissociation solution (Innovative Cell Technologies, San Diego, CA) into each eppendorf tube. The tubes were incubated at 37°C with constant stirring for 10 minutes. The spheroids were further dissociated by slight pipetting.
Equal volume of FBS was added to stop the reaction. A cell pellet was formed which was resuspended in 200 µl of media: CellTiter-Glo® solution (added in 1:1 ratio). The cells were lysed by vortexing. 100 µl of the suspension from each replicate was added to a 96-well black plate with clear bottom and luminescence was measured on a Biotek Synergy HT plate reader (Biotek, Winooski, VT). For standard curves the same procedure was followed from the step in which 1:1 media: CellTiter-Glo® was added. 

**Linearity Analysis:** The linearity of the cell number method was analyzed to determine the cell number range between which the cell number linearly correlates with absorbance in case APH assay or luminescence in case of Cell-Titer Glo assay. Only if the method generated a linear correlation with cell number, it could be used for cell viability studies in spheroids.

### 4.2. Preparation and characterization of untargeted and targeted NCL-240 micelles

PEG_{2000}-PE which is synthetically called 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000], and 1,2- dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). pNP-PEG_{3400}-pNP was obtained from Laysan Bio (Arab, AL). The commercial lipids were used directly without any further purification. The DM-PIT-1 analog NCL-240 was synthesized at National Chemical Laboratory (Pune, India) and was provided by Dr. Alexei Degterev (Tufts University, Boston, MA). Human holo-transferrin used for surface modification of the micelles was obtained from Sigma Aldrich (St. Louis, MO).

#### 4.2.1. Preparation of drug-loaded micelles

The micelles were prepared using thin film hydration technique. Briefly, DSPE-PEG_{2000}-PE dissolved in anhydrous chloroform was added to a round bottom flask. 6% weight of NCL-240 dissolved in acetonitrile was added to DSPE-PEG_{2000}-PE. The organic solvents were evaporated using a rotary evaporator to form a thin layer of lipid containing drug. The films were
further dried using Freezone 4.5 Freeze Dry System (Labconco, Kansas City, MO) for at least 4 hours. The lipid films were then rehydrated using 1X Phosphate buffered saline (PBS) pH 7.4 such that a lipid concentration of 15 mg/ml was achieved followed by vortexing for a few minutes. The micelles were centrifuged at 1,200 rpm for 5 minutes to remove any non-encapsulated drug by precipitation. The preparation was then sterilized by passing it through a 0.2-µm filter (Nalgene, Rochester, NY).

4.2.2. Drug encapsulation efficiency for NCL-240 loaded PEG-PE micelles

HPLC was used both for analysis of samples obtained from release studies and also to analysis the encapsulation efficiency of the drug loaded micelles. Reverse phase Xbridge C$_{18}$ (2.1cm x 250cm) column (Waters Corporation, Milford, MA) was used on a Hitachi Elite LaChrom HPLC with autosampler (Pleasanton, CA) for my studies. The mobile phase used for 80:20 acetonitrile to water with an isocratic elution flow rate of 1 ml/min. The drug was detected at a wavelength of 300 nm (wavelength which gave maximum absorbance) using the UV detector of HPLC. The retention time of the drug was in between 5 to 5.5 minutes. The sample injection was kept constant at 50 µl and the sample runtime was 8 minutes. Concentration of the drug was obtained by measuring the area under the curve of the chromatogram. A standard curve was plotted with 4 standard concentrations ranging from 5µg/ml to 20 µg/ml.

For encapsulation efficiency, 5 µl of micellar preparation was dissolved in 495 µl of acetonitrile to solubilize the lipids, making the micelles release the free drug. The sample was run using the HPLC and the area under the chromatogram was calculated. The standard curve linear equation was used to calculate the drug encapsulation efficiency of NCL-240 in PEG-PE micelles for a series of batches.
4.2.3. Drug release from of NCL-240 micelles

The NCL-240 loaded micelles were evaluated for their in-vitro drug release profile at 37°C in 1X PBS pH 7.4 over a period of 24 hours. The micelles were prepared as described above. 500 µl of micelles were taken after centrifugation into a Spectra/Pro® regenerated cellulose dialysis membrane (Spectrum Laboratories Inc., Rancho Dominguez, CA) with a molecular weight cut-off (MWCO) of 3,500 Da and dialyzed in a beaker containing large excess of 1x PBS pH 7.4 containing 0.02% Tween 20. At regular intervals of time, the dialysis membrane was opened and 5 µl of sample was taken. The sample was added to 495 µl of acetonitrile and stored at 4°C until the time of analysis by HPLC. Throughout the dialysis process, the beakers were kept in a continuous stirring condition. The drug concentration at each time point was determined by HPLC and a drug release profile was plotted.

4.2.4. Synthesis of pNP-PEG_{3400}-PE

pNP-PEG_{3400}-PE was synthesized based on a standardized protocol previously developed in our lab [63]. Briefly, pNP-PEG_{3400}-pNP (Laysan Bio, Arab, AL) dissolved in anhydrous chloroform was added to a pear shaped flask. DOPE in chloroform and triethylamine (TEA) were added to the flask in such a way that pNP-PEG_{3400}-pNP is 10 fold molar excess of DOPE and TEA is 2 fold molar excess to pNP-PEG_{3400}-pNP. The reaction mixture was left overnight under nitrogen with constant stirring. The organic solvents were evaporated using a rotary evaporator and the film formed was further dried using a Freezone 4.5 Freeze Dry system (Labconco, Kansas city, MO) for atleast 4 hours. The film was then rehydrated using 0.001 M HCl and separated on a sepharose column. Several samples that were eluted were collected and analyzed using Thin Layer Chromatography (TLC) to determine the fractions in which the conjugation product pNP-PEG_{3400}-pE was present. The fractions were spotted on a silica gel
TLC plate (Sigma-Aldrich, St. Louis, MO) and was run in a mobile phase containing CHCl₃: MeOH (80:20). Molybdenum blue, ninhydrin and dragendorf’s reagent were sprayed to confirm the presence of phospholipid, peptide bond and PEG components respectively. The fractions that tested positive for all the reagents were pooled, freeze-dried, weighed and reconstituted in anhydrous chloroform to make the appropriate stock solution. The stock was stored in -80°C until future use.

4.2.5. Preparation of transferrin-conjugated micelles (Tf-conjugated micelles)

Tf-conjugated micelles were prepared by thin film rehydration technique. Briefly, PEG₂₀₀₀-DSPC dissolved in chloroform was added to the round bottom flask. 10% (by moles of PEG₂₀₀₀-DSPC) of pNP-PEG₃₄₀₀-PE stock was added and the organic solvents were evaporated using a rotary evaporator. The film formed was further dried on a Freezone 4.5 Freeze Dry system (Labconco, Kansas city, MO) for 4 hours. The films were hydrated so as to achieve a lipid concentration of 15 mg/ml. The film was rehydrated with 20:80 composition of citrate buffered saline (CBS) pH 5.5 and Tf stock made in 1x PBS pH 7.4. The amount of Tf used for the stock was decided based on the molar ratio of pNP-PEG-PE: PE of 2.5:1. After the rehydration, pH of the solution was checked and adjusted to 8.5 by adding NaOH if necessary. The reaction was allowed to proceed for 4 hours at room temperature to allow sufficient Tf conjugation and complete hydrolysis of unreacted pNP groups. The Tf-conjugated micelles were dialyzed overnight in 4°C using a 100,000 MWCO membrane in a beaker containing 1L of 1X PBS pH 7.4 under constant stirring, to remove any unconjugated Tf.
4.2.6. Conjugation efficiency of transferrin-micelles using BCA assay

The conjugation efficiency of the Tf in the micelles was measured using a micro BCA assay kit (Pierce, Rockford, IL). The protocol given by the manufacturer was used. Briefly, standard concentrations of bovine serum albumin (BSA) ranging from 200 µg/ml to 1.5 µg/ml were prepared in a 96 well plate by serial dilutions. Three serial dilutions of Tf-micelles with dilution factors 20, 40 and 80 were also added to the plate. Working reagent provided in the kit was added to each well and the plate was incubated at 37°C with constant shaking for 2 hours. After incubation, the absorbance of the plates was measured using a Biotek Synergy HT (Biotek, Winooski, VT) plate reader at 562 nm. Standard curve of absorbance vs. BSA concentrations was plotted and the concentration of the Tf in the micelles was calculated by extrapolation. The Tf-micelles were normalized to attain the same lipid concentration of 15 mg/ml by adding appropriate amounts of DSPE-PEG\textsubscript{2000} plain micelles.

4.2.7. Preparation of transferrin-conjugated NCL-240 micelles

To prepare transferrin conjugated NCL-240 micelles, appropriate amounts of NCL-240 micelles and Tf-micelles were mixed, vortexed slightly and the reaction was incubated at room temperature for 4 hours or overnight at 4°C. Since micelles are dynamic systems, they assemble and disassemble to form Tf conjugated micelles (as shown in Figure 10).
4.2.8. Size and zeta potential of the micelles

All the micelles prepared were analyzed for their size, using Coulter N4 plus (Beckman Coulter, Pasadena, CA) and zeta-potential using Zetaplus (Brookhaven Instruments Corporation, Holtsville, NY). The size and zeta potential were measured as per the respective manufacturer’s protocols. Briefly, 50 µl of micelles after centrifugation and sterile filtration were diluted to 1 ml total volume by distilled water and were added to the cuvettes and their size and surface charge distributions were measured using the respective software.

4.3. Evaluation of in vitro cytotoxicity of targeted micelles

As previously discussed, PI3K pathway is the most commonly activated pathway in cancers. Different cancer cell lines have different levels of PI3K mutations and hence the efficacy of formulations can be better evaluated if it is studied across several cell lines. Therefore, the goal of this study was to evaluate the efficacy of NCL-240, in micellar drug delivery system across several cell lines grown in 2D and 3D. Also, targeting ligand transferrin is
known to increase the internalization of micelles, and comparing the results at 4 hour and 24 hour treatments with varying Tf amounts can assess its effect. The cytotoxicity achieved from monolayers was compared to that of 3D spheroids to understand the effect of limited penetration.

4.3.1. Cell viability studies in 2D monolayer cell culture

The cells needed for cell viability studies were cultured as explained previously. CellTiter-Blue® (Promega, Madison, WI) viability assay was used to measure the viability of the cells. The procedure followed was as per the kit manufacturer’s protocol. Briefly, cells were trypsinized, pelleted and counted using a hemocytometer. 96-well plates were seeded with 3,000 cells per well. After 24 hours incubation at 37°C and 5% CO₂ conditions, cells were treated with the formulations and were maintained at appropriate conditions. For 4-hour treatment, the cells were treated initially with formulations dissolved in serum free media and after the 4-hour incubation period, the treatment media was replaced with complete media. For 24-hour treatment groups, the cells were treated with formulations dissolved in complete media. After the treatment duration, media was removed and replaced with 90µl of complete media and 10 µl of CellTiter-Blue®. The plates were incubated for 90 minutes to 2 hours at 37°C and 5% CO₂, following which fluorescence was measured (Ex: 550nm and Em: 590 nm) using a Biotek Synergy HT plate reader (Biotek, Vinooski, VT). The cell viability was calculated relative to the fluorescence of the untreated controls and expressed as % cell viability. Statistical analysis was performed using one-way ANOVA with Tukey’s multiple comparison tests.

4.3.1.1. Transferrin activity assay

The transferrin activity assay was conducted in NCI-ADR-RES cells to determine if the NCL-240 micelles had any additional activity due to the surface modification with Tf. The
cells needed for this study were cultured as explained previously. The cells were seeded in 96-well plates similar to cell viability studies. Two treatment groups were made. For the first group, cells were treated with high initial dose of free Tf to completely block the Tf receptors and after 1-hour incubation treated with Tf targeted and untargeted NCL-240 micelles. For the second group, the cells were treated with Tf targeted and untargeted NCL-240 micelles but without blocking the Tf receptors. The assay was conducted at both the time points (4-hour and 24-hour incubation) similar to the 2D cell viability studies. Cell viability was used as a measure of Tf-activity and hence, cell viability of both treatment groups was calculated as previously.

**4.3.2. Cell viability studies in optimized 3D cell culture spheroids**

The spheroids were grown as per the methods that were optimized as explained previously. CellTiter-Glo® Luminescence assay (Promega, Madison, WI) was performed to evaluate the viability of the cells within the spheroids. After the spheroids reached their optimum compactness, around 15 spheroids were treated with each treatment type (5 spheroids per replicate). For 4-hour treatment group, the spheroids were treated with formulations dissolved in serum free media and was replaced with complete media after 4-hour incubation period. For 24-hour treatment group, the spheroids were treated with formulations dissolved in complete media. After the appropriate treatment duration, the spheroids corresponding to each replicate were collected into separate eppendorf tubes. The treatment media was removed and the spheroids were washed with 1X PBS pH 7.4. 200 µl of Accumax® Cell Dissociation solution (Innovative Cell Technologies, San Diego, CA) was added to the eppendorf tubes and incubated at 37°C for 5 minutes. After the spheroids were dissociated to individual cells, an equal volume of FBS was added to stop the reaction. The tubes were then centrifuged to form a cell pellet. The supernatant was discarded and the pellet was re-suspended with 200 µl of composition containing
media: CellTiter-Glo® ratio of 1:1. The cells were lysed by vortexing for 10 minutes. From this suspension, 100 µl was added to a 96-well black plate with clear bottom and the luminescence was measured after 10 minutes. The cell viability was calculated relative to the luminescence of the untreated spheroids (controls) and expressed as % cell viability. Statistical analysis was performed using one-way ANOVA with Tukey’s multiple comparison tests.

4.4. Penetration and cellular uptake studies in spheroids

4.4.1. Preparation of rhodamine-labeled targeted and untargeted micelles

1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B Sulfonyl) (Rh-PE) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). For preparing rhodamine labeled Tf-targeted micelles, the same procedure described earlier for the preparation of Tf-micelles was used, except that 1 % (mol) of Rh-PE was added along with the PEG2000-PE and pNP-PEG3400-PE. For preparation of rhodamine labeled untargeted micelles, 1% (mol.) of Rh-PE was incorporated into the plain micelles.

4.4.2. Penetration studies in spheroids

The spheroids were grown as per the optimized methods described previously. The spheroids were treated on the day they attained maximum compactness with rhodamine-labeled targeted and untargeted micelles dissolved in serum free media (3 spheroids per treatment). After 2 hours of incubation, the spheroids were collected and washed by transferring them into a petri dish containing excess of 1X PBS pH 7.4. They were then transferred into 8 well optical chamber slides (Lab-Tek Chamber Slide system, Fisher Scientific) containing around 200 µL of 1X PBS. The spheroids were analyzed on a Zeiss LSM 700 confocal microscope by using 575 nm for excitation and 595 nm for emission of rhodamine B present in Rh-PE. Z-stack imaging
was performed by taking slices at regular intervals of 11µm from the periphery into the spheroid. A constant area was taken inside each slice and the fluorescence intensity due to rhodamine was quantified using ImageJ software.

4.4.3. Uptake studies in spheroids

For uptake studies the treatment method used was similar to the penetration studies described previously. After 2 hours incubation, the spheroids were washed using sterile 1X PBS and were stained with Hoechst dye (Hoechst 33342, Molecular probes, Inc. Eugene, OR) using the manufacturer’s protocol. Briefly, the spheroids were added to 12 well plates containing 10 µg equivalent of Hoechst dye prepared in sterile 1X PBS, from a stock solution of 10 mg/ml concentration. The spheroids were then incubated at 37°C for around 30 to 45 minutes. After the incubation period, the spheroids were washed with sterile 1X PBS pH 7.4 buffer and transferred into 8 well optical chamber slides (Lab-Tek Chamber Slide system, Fisher Scientific) containing around 200 µL of 1X PBS. The spheroids were analyzed on a Zeiss LSM 700 confocal microscope by using two filters (Ex/Em of 575/595 nm for rhodamine B and 350/461 nm for Hoechst). The Z-stack images of both the filters were taken, and merge analysis was performed using ImageJ software.
5. RESULTS AND DISCUSSIONS

5.1. Optimization of cancer cell spheroids

5.1.1. NCI –ADR-RES spheroids

The images of the NCI-ADR-RES spheroids at different days, grown using non-adhesive liquid overlay method with 10,000 cells/well and centrifugation at 1,500 g for 15 minutes are shown in the Figure 11 on the adjoining page. The spheroids became dense on day 4 and started shedding off cells from day 7.
Figure 11: Images of NCI-ADR-RES spheroids grown using non-adhesive liquid overlay method after 1, 3, 4, 7 and 10 days. (Scale bars indicate 50 μm)
5.1.2. SKOV-3 TR spheroids

*Non-adhesive liquid overlay method:* The SKOV-3 TR spheroids grown using non-adhesive liquid overlay method are shown in Figure 12 below. The spheroids became dense on day 7 as they were comparatively slow growing than NCI-ADR-RES cells.

![Figure 12: Images of SKOV-3 TR spheroids grown using non-adhesive liquid overlay method taken at 1, 2, 5 and 7 days (Scale bars indicate 50 µm)](image-url)
**Hanging drop method:**

The spheroids were hanged for 3 days and they were harvested into 96-well plates on day 3. The images of the hanging drop spheroids after day 5 are as shown below in Figure 13.

![Spheroid images](image)

**Figure 13:** The images of SKOV-3 TR spheroids grown using hanging drop method after day 5 (Scale bars indicate 50 µm)

**Non-adhesive liquid overlay method with Matrigel™:**

The spheroids formed using non-adhesive liquid overlay method with varying extracellular matrix concentrations were able to form dense spheroids on day 3 itself and the spheroids become more compact with lower concentration of Matrigel out of all the concentrations that were investigated (1.25% in our case).

![Spheroid images](image)

**Figure 14:** Images of SKOV-3 TR spheroids grown using non-adhesive liquid overlay method with varying Matrigel concentration at day 3 (Scale bars indicate 50 µm)
**Hanging drop method with Matrigel™:**

The spheroids formed using hanging drop method with extracellular matrix addition after harvesting the spheroids didn’t show any difference in the appearance even after day 7 (as shown in Figure 15.)

![Figure 15: Images of SKOV-3 TR spheroids grown using hanging drop method with Matrigel™ 1.25% taken after day 7 (Scale bars indicate 50 µm)](image)

**Co-culture spheroids of SKOV-3 TR with CCL-210 lung fibroblasts:**

![Figure 16: Images of SKOV-3 TR spheroids grown using co-culture of lung fibroblasts with varying co-culture ratios for 3 days (Scale bars indicate 50 µm)](image)
The spheroids formed using co-culture, especially of ratios 5:1 and 2:1 were compact and could even sustain mechanical stress by pipetting. The ratio of cancer cells to fibroblasts required to exactly mimic the tumor in vivo needs to be optimized and that itself can be another separate project by itself and this thesis serves as a platform for future development of completely heterogeneous spheroids.

5.1.3. U87-MG spheroids

The images of the NCI-ADR-RES spheroids at different days grown using non-adhesive liquid overlay method with 10,000 cells/well and centrifugation at 1,000 g for 10 minutes are shown in Figure 17 on the adjoining page. The spheroids became dense on day 4 and started shedding off cells from day 7. These spheroids appeared to be more compact than NCI-ADR-RES spheroids, and were also smaller in appearance.
5.1.4. Consistency of spheroids

For NCI-ADR-RES spheroids grown using the non-adhesive liquid overlay technique, the spheroid diameters varied from 940 µm on day 1 to 542.5 µm on day 4 on which maximum compactness was observed. After day 6, the cells started shedding off and the spheroid diameters...
increased gradually to around 568.83 μm on day 10 (Figure 16). The results were consistent with low standard deviation for several repetitions.

On pipetting, the spheroids remained intact and could withstand the mechanical force. Hence, it was concluded that the NCI-ADR-RES cell lines formed compact spheroids with consistent shape and size and that the method has been optimized and can be used for evaluating cytotoxicity.

![Mean diameter of the NCI-ADR-RES spheroids grown in non-adhesive liquid overlay method](image)

**Figure 18:** Mean diameters of NCI-ADR-RES spheroids grown using non-adhesive liquid overlay method.

For SKOV-3 TR spheroids grown using the non-adhesive liquid overlay methods, the mean diameters ranged from 1,119.33 μm on day 1 to 726.25 μm on day 7 (Figure 17). The diameter of the spheroids was larger than the typical NCI-ADR spheroids on any day.
The SKOV-3 TR spheroids grown using the hanging drop method were of relatively smaller diameter compared to the spheroids grown using non-adhesive liquid overlay method. However, on pipetting the liquid overlay and hanging drop spheroids completely dissociated. Hence, it was concluded that these were not spheroids but cell aggregates. On pipetting the Matrigel™ spheroids, the cells from the core of the spheroid seemed to disintegrate while the cells on the periphery containing Matrigel™ remained intact. So this concluded that these spheroids are not suitable for cytotoxicity studies, as they don’t mimic tumor like characteristics.

**Figure 20:** The mean diameters of SKOV-3 TR spheroids grown using various methods on day 5.
The mean diameters of the U87-MG spheroids ranged from 870 μm on day 1 to around 480-490 μm on day 4, on which they attained maximum compactness. The diameter increased to 550 μm on day 7. The spheroid sizes were consistent with low standard deviations on all days for several repetitions.

![Mean diameter of the U87-MG spheroids](image)

*Figure 21*: Mean diameters of the U87-MG spheroids grown using non-adhesive liquid overlay method.

### 5.1.5. Cell number and linearity analysis

A standard curve of luminescence vs. cell number was obtained for each of the cell lines as shown in Figure 22. There was a linear relation between luminescence and cell number for all the cell lines and hence it indicated that the Cell-Titer Glo method which measures ATP production of the live cells can be used as for quantifying the number of cells and thereby can also be used for cell viability studies.

Also, in cell number studies of NCI-ADR-RES and SKOV-3 TR spheroids, the luminescence was used to extrapolate and estimate the number of live cells in the spheroid.
Figure 22: Cell Titer Glo standard curve plotted as luminescence vs. cell number for all the three cell lines

![CellTiter-Glo standard curve](image)

- NCI-ADR-RES: $y = 6.7277x + 20027$, $R^2 = 0.98594$
- SKOV-3 TR: $y = 6.0092x + 11871$, $R^2 = 0.9613$
- U87-MG: $y = 2.7997x + 18855$, $R^2 = 0.9593$

Figure 23: Cell number analysis of NCI-ADR-RES spheroids (non-adhesive liquid overlay) and SKOV-3 TR spheroids (non-adhesive liquid overlay with Matrigel) (For n=3 replicates, 5 spheroids per replicate)

The cell number analysis revealed that as the number of days progressed, the cell number decreased for both the cell lines. This indicates that as the number of days progressed more and more cells became deprived of nutrients and undergo anaerobic respiration and decrease the ATP production (measure of luminescence). Hence it suggests the formation of gradients like in vivo tumor conditions. Also, the number of active cells was comparatively very low in SKOV-3 TR.
spheroids formed by non-adhesive liquid overlay method with Matrigel. This could be because the physical compaction produced by Matrigel is forming the dense spheroid rather than cell-cell contacts and hence could be hindering with the cell growth. Since the cell number was too low, this model is not suitable for cell viability assays, as it will be very difficult to perceive the changes in luminescence from the treated and untreated groups. Hence SKOV-3 TR cell viability studies were not pursued.

5.2. Characterization of Tf-targeted and untargeted NCL-240 micelles

5.2.1. Drug Encapsulation efficiency

The equation of the standard curve obtained using HPLC was $y = 168201x + 138614$ with a $R^2$ value of 0.995. The encapsulation was determined to be 84.86 ± 4.59% (n=3, Mean ± S.D.)
5.2.2. Drug release study

The drug release profile from NCL-240 micelles indicated that there was an initial burst release (shown in Figure 25) followed by a sustained release. Around 23% of the drug was released in the total period of 24 hours.

![Figure 25: Drug release profile for NCL-240 loaded micelles performed for a period of 24 hours (n=3, Mean ± S.D.)](image)

5.2.3. Tf-conjugation efficiency

The equation of the standard curve (Figure 26) obtained from BCA assay was \( y = 0.0077x + 0.0536 \), with a \( R^2 \) value of 0.99. The conjugation yield of Tf in Tf-micelles for a series of batches was obtained as 9.95%±2.2%.

![Figure 26: Standard curve for BSA concentrations which can be used to estimate the amount of Tf conjugated to the Tf-micelles](image)
5.2.4. Size and surface charge characterization

The Table 2 below summarizes the results of size and zeta potential of all the formulations.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Size (in nm)</th>
<th>Zeta potential (in mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain (DSPE-PEG&lt;sub&gt;2000&lt;/sub&gt;) micelles</td>
<td>17.2±0.4</td>
<td>-6.8±1.7</td>
</tr>
<tr>
<td>NCL-240 micelles</td>
<td>17.8±1.1</td>
<td>-3.9±1.4</td>
</tr>
<tr>
<td>0.5 µg Tf-conjugated micelles</td>
<td>36.8±3.6</td>
<td>-30.6±2.8</td>
</tr>
<tr>
<td>1 µg Tf-conjugated micelles</td>
<td>38.2±5.2</td>
<td>-31.4±0.9</td>
</tr>
<tr>
<td>2 µg Tf-conjugated micelles</td>
<td>45.8±5.6</td>
<td>-37.5±1.3</td>
</tr>
</tbody>
</table>

Table 2: Size and zeta potential characterization of all the formulations (n=3, in the form of Mean ± S.D.)

The size analysis confirmed the presence of micelles. Also since their size is large enough to avoid glomerular filtration and small enough to cause EPR effect, they are suitable for the study. Also, conjugation of Tf was successful and this can be explained by the increased size of the micelles because of its large size (Size of Tf is 80 KDa) and more negative zeta potential that can be attributed to the net negative charge of Tf protein.

5.3. Cell viability studies:

5.3.1. Tf-activity in NCI-ADR-RES cells

The Tf-activity study in NCI-ADR-RES monolayer cultures are shown in Figure 31 in the adjoining page. After 4-hour incubation, the cell viability of targeted micelles treated to cells supplemented with large excess of free Tf was similar to that of untargeted micelles, indicating that the Tf activity was not lost after conjugation with micelles.
5.3.2. Cell viability studies of NCI-ADR-RES cells in monolayer

The IC_{50} value of NCI-ADR-RES cells grown in monolayers was obtained as 12.5 µM and it was chosen as the base concentration for the studies. The cells were treated with varying concentrations of NCL-240 micelles and Tf-conjugated NCL-240 micelles at 4 hours and 24 hours incubations. The 4-hour treatment group was initially treated with formulations in serum free media and after 4 hours, the media was replaced with serum containing media. The results are shown in Figure 28 in the adjoining page.
Figure 28: Cell viability of NCI-ADR-RES cells in 2D at 4 hour and 24-hour treatments (n=3, Mean ± S.D; f=free; m=micelle; * denotes p≤0.05, ** denotes p≤0.01, *** denotes p≤0.001)

In NCI-ADR-RES monolayer cells, after 4 hours incubation, it was observed that micelle form of NCL-240 showed significant difference compared to the free NCL-240. After 24 hours incubation, it was observed that the targeted and untargeted NCL-240 showed better effect compared to the free NCL-240. Also, a dose dependent effect was observed in the targeted micelles. At both the treatment time points, the controls were not comparatively toxic.

5.3.3. Cell viability studies of NCI-ADR-RES cells grown as spheroids

Cell viability studies in NCI-ADR-RES spheroids were conducted by starting with the same concentrations used for the monolayer. However, no significant cytotoxicity was observed at those concentrations. On increasing the concentrations of NCL-240 to 50 µM, the cytotoxicity was improved. The results of the cell viability studies conducted with 50 µM drug are shown in Figure 29 on the adjoining page. It was observed that the PEG-PE micelles as well as empty Tf-targeted micelles (micelles without drug) didn’t have any cytotoxicity of their own but have
shown to promote the growth of cells in the spheroids at both time points. Also, there was no significant difference between free NCL-240 and NCL-240 micelles.

![NCI-ADR 3D cytotoxicity: 4h incubation](image)

**Figure 29:** Cell viability of NCI-ADR-RES cells in spheroids at 4 hour and 24-hour treatments (n=3, Mean ± S.D, f=free and m=micelles; * denotes p≤0.05, ** denotes p≤0.01)

While the 24 hour incubation indicated that there was significant improvement in toxicity between untargeted and 2µg/ml Tf-targeted micelles, the 4 hour incubation also showed significant improvements in toxicity between untargeted and 2µg/ml as well as 1µg/ml Tf-targeted micelles. This highlights the importance of targeting in enhanced uptake to the cells.

Using the Tf-targeted drug loaded micelles, significant improvement was observed in the cell death as compared to untargeted drug loaded micelles that allowed reducing the drug concentration by half from 50µM (of untargeted mNCL-240) to 25µM (targeted mNCL-240). This highlights the benefit of using targeted micelles in delivering drugs effectively and also helps reduce the total dose.
5.3.4. Cell viability studies of U87-MG cells in monolayer

The cell viability results of U87-MG cells in monolayers are shown in Figure 30 below. At 4 hours incubation, it was observed that micelle form of NCL-240 showed significant difference compared to the free NCL-240. But at 24-hour incubations, it was observed that targeted and untargeted NCL-240 showed better effect compared to the free NCL-240. However, a dose dependent effect was not observed in the targeted micelles as was observed in case of NCI-ADR-RES cells.

![Cell viability of U87-MG cells in 2D at 4 hour and 24-hour treatments](image)

*Figure 30:* Cell viability of U87-MG cells in 2D at 4 hour and 24-hour treatments (n=3, Mean ± S.D; f=free; m=micelle; * denotes p≤0.05, **** denotes p≤0.0001)

5.3.5. Cell viability studies of U87-MG cells in spheroids

The cell viability results of U87-MG cells grown in spheroids are shown in Figure 31 on the adjoining page. At 4 hours incubation, it was observed that micelle form of NCL-240 showed significant difference compared to the 2µg/ml and 1µg/ml Tf-targeted NCL-240 micelles. But at
24-hour incubations, it was observed that there was no difference between free and micellar form of the drug and also there was no significant difference between untargeted and targeted micelles.

![Figure 31: Cell viability of U87-MG cells in 3D at 4 hour and 24-hour treatments (n=3, Mean ± S.D; f=free; m=micelle; * denotes p≤0.05)](image)

**5.4. Penetration and uptake studies in spheroids**

**5.4.1. Penetration study in NCI-ADR-RES spheroids**

The Z-stack images of the spheroids taken at an interval of 11 µm (shown in Figure 32 on the adjoining page) were analyzed using ImageJ to calculate the rhodamine intensity of each slice and the plot of calculated rhodamine intensities is shown in Figure 33. (on Page 55). It was observed that targeted micelles were able to penetrate into deeper layers of the spheroid when compared to untargeted micelles.
<table>
<thead>
<tr>
<th>Distance from periphery (in μm)</th>
<th>Untargeted Rh-micelles</th>
<th>1 μg/ml Tf targeted Rh-micelles</th>
<th>2 μg/ml Tf targeted Rh-micelles</th>
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</thead>
<tbody>
<tr>
<td>0</td>
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<td><img src="image2.png" alt="Image" /></td>
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<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 32:** Penetration of targeted and untargeted micelles in NCI-ADR-RES spheroids across several slices taken from the periphery
5.4.2. Penetration study in U87-MG spheroids

The Z-stack images of U87-MG spheroids were taken at an interval of 11 µm from the periphery. The slices were compared across targeted and untargeted treatment group (as shown in Figure 34 on the adjoining page). The rhodamine intensity in each slice was quantified and plotted as shown in Figure 35 on Page 57.
Figure 34: Penetration of targeted and untargeted micelles in U87-MG spheroids across several slices taken from the periphery.
From the slices as well as the quantitative plot, it was observed that there was no additional benefit of targeting with Tf in case of U87-MG spheroids. This could also be the reason for not observing significant differences between targeted and untargeted micelles in cell viability studies. An important point to note is that the same formulation had different penetration profiles in different cell lines emphasizing the importance of screening therapeutics in spheroids.

**Figure 35:** Trends of penetration of targeted and untargeted micelles in U87-MG spheroids
5.4.3. Uptake studies in NCI-ADR-RES spheroids
Figure 36: Uptake studies in NCI-ADR spheroids A) Hoechst stain control B) Untargeted Rh-micelles group and C) Targeted Rh-micelles group
5.4.4. Uptake studies of U87-MG spheroids

A)

B)
From the uptake data, it was observed that the uptake was higher in targeted micelles compared to untargeted micelles in case of NCI-ADR-RES spheroids as observed by overlapping red and blue channels which gives a purple color, whereas in case of U87-MG there wasn’t any difference in the uptake in targeted compared to the untargeted micelles. From the combined evidence from penetration, uptake and cell viability studies, it can be speculated that the U87-MG spheroids are more tightly packed and hence could be one reason for low uptake and penetration into the spheroids.
6. CONCLUSIONS

In recent years, with increase in the understanding of tumor biology people are now able to appreciate the role of TME in the progression of cancer. It is very crucial to evaluate newer therapies by using models, which closely resemble tumor like properties in vivo. Spheroid models represent the in vivo phenomenon better, and hence it was the main emphasis of this thesis. Methods were optimized for high throughput generation of spheroids for cell lines such as NCI-ADR-RES, SKOV-3 TR and U87-MG. Several methods for spheroid generation were tried such as non-adhesive liquid overlay method, hanging drop method, co-culture and also external matrices. Micellar delivery system was developed for the drug of interest (NCL-240, a novel cytotoxic drug and a second generation analog of DM-PIT-1) because of its poor solubility in water. NCL-240 micelles and Tf-conjugated NCL-240 were prepared and characterized for evaluating the cytotoxicity in monolayer and spheroid cell culture. The Tf-targeted NCL-240 micelles penetrated more into the NCI-ADR spheroids, because of which there was a significant difference in cytotoxicity between targeted and untargeted micelles. However, in U87-MG, the same formulations were unable to penetrate and there was no difference in the cytotoxicity observed between targeted and untargeted micelles. Hence, because of the variable and dynamic penetration environment, spheroids make a very good model to study the penetration effects of formulations and also for screening novel cytotoxic drugs.

Future Directions: The spheroid models optimized as a part of this thesis could be employed for testing other therapeutics. The co-culture spheroids, which were developed but optimized completely, could be used as a platform to develop fully heterogeneous spheroids. NCL-240 drug has shown a lot of promise in inhibition of the ubiquitous PI3K pathway, and moreover the targeted formulations could be tested in in vivo models to explore the effect of targeting. Lastly,
since the spheroids have the similar gene expression profiles as tumors, they would play an important role in gene silencing studies. Hence, they can be developed as a reliable model for testing siRNA therapeutics.
7. BIBLIOGRAPHY


