Combination Anticancer Nanopreparations of Novel Proapoptotic Drug, TRAIL and siRNA

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Abstract

Development of drugs for the treatment of cancer is a challenging endeavor often hindered by the solubility and distribution of the drug in the body. Drug delivery systems have been used for many years to overcome these issues. Polyethylene glycol-phosphatidylethanolamine (PEG-PE) micelles in particular have shown utility as a nanosized drug delivery vehicle capable of incorporating poorly soluble drugs and preferentially delivering them to the tumor. Addition of PEG polymers to the surface prolongs the half-life of the particle in the blood by evading clearance by the reticuloendothelial system (RES) and increases tumor accumulation through the utilization of the enhanced permeability and retention (EPR) effect. Micelles have also been shown to successfully incorporate and protect modified siRNA, a notoriously challenging therapeutic to deliver. Additionally, co-delivery of multiple therapeutics in multifunctional micelles has emerged as an important area in combination therapy research. The main goal of this project was to develop a multifunctional PEG-PE micellar delivery system capable of delivering multiple therapeutics for increased anti-tumor activity.

Previous studies have indicated the utility of a DM-PIT-1, a member of a class of novel PIP3-PH inhibitors, and its potential in the treatment of cancer. The PIP3-kinase (PI3K) pathway has been shown to have serious implications in cancer. Inhibiting this pathway has been shown to sensitize the cell to apoptosis. A second generation of more potent and druggable compounds has been developed based on the structure of DM-PIT-1. However, it has been difficult to develop successful compounds inhibiting PIP3 signaling while maintaining the physicochemical properties necessary for an effective drug. Many of these compounds are limited by their poor solubility and rapid clearance in vivo. Incorporating these compounds into PEG-PE micelles allows for increased solubility, prolonged half-life and tumor accumulation.

The addition of TNFα-related apoptosis-inducing ligand (TRAIL) bound to the surface of the micelle creates a combination micelle with excellent cytotoxic effects. TRAIL has been shown to be an effective apoptosis inducing ligand in a variety of in vitro and in vivo studies. TRAIL receptors are preferentially expressed on many cancer cell types as compared to healthy cells making this ligand an intriguing potential
therapy. The combination of TRAIL and PIP₃-PH inhibitors in a micellar delivery system has the potential to create a powerful anti-cancer therapeutic.

Including modified siRNA to down regulate cancer defense mechanisms can further sensitize the cell to apoptosis. siRNA delivery has been shown to be a difficult task. Rapid metabolism and clearance in the blood hinders their ability to reach the tumor. Additionally, their large size and negative charge prevents them from crossing the cell membrane to reach their location of action. Reversibly conjugating a modified siRNA to a lipid thereby creating an siRNA-S-S-PE, allows for their incorporation into PEG-PE micelles. These mixed micelles have been shown to protect the siRNA and successfully transfect cells.

This study aimed to combine the aforementioned therapeutics into a multifunctional PEG-PE based micelle delivery system. Novel proapoptotic drugs targeting the PIP₃-PH binding domain have been successfully incorporated into the lipid core of the micelle. These drugs were able to effectively sensitize the cell to the effects of surface-bound TRAIL. Additionally, siRNA targeting the anti-apoptotic protein survivin was shown to be incorporated into the micelles and further sensitize the tumor to the effects of the above compounds. Lastly, conjugating transferrin (TF) to the surface of the micelle was shown increase the tumor cell targeting and cytotoxicity in vitro.

Critical evaluation of this system was performed along the following specific aims: (1) characterization of PIP₃-PH inhibition and cytotoxicity of proapoptotic drug DM-PIT-1 and its novel analogs in vitro with and without TRAIL; (2) preparation and characterization of TRAIL-modified micelles loaded with DM-PIT-1 or its analogs; (3) evaluation of in vitro cytotoxicity of combination formulations across a range of tumor cell types; (4) characterization of TF-modified micelles targeting potential and their effects on cytotoxicity in vitro; (5) formulation and characterization of siRNA-S-S-PE mixed micelles and evaluation of gene silencing in vitro and in vivo; (6) evaluation of combination micelles as a multifunctional delivery system utilizing in vivo mouse models of human cancer.
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1 Objective and Specific Aims

Many excellent chemotherapeutic drugs are limited by their solubility in biological fluids. Cosolvents to improve stability can be employed but they are limited by their toxicities. An alternative approach is to encapsulate the poorly soluble drug into nanosized micelles. Several classes of these particles are able to incorporate the drug into their hydrophobic core thereby increasing the amount of drug able to be delivered, while simultaneously protecting the drug from metabolism and excretion by the body’s natural protection mechanisms. A reduction in off target toxicities and an increased amount of drug at the tumor site are two of the most important properties in any tumor delivery system.

Polyethylene glycol-phosphatidylethanolamine (PEG-PE) micelles have shown utility as a nanoparticle drug delivery system capable of effectively solubilizing and delivering poorly soluble drugs to the tumor. Additionally, it is capable of incorporating and protecting siRNA from degradation in biological fluids. Further, surface modification of the micelles through well-established chemistries is yet another method to functionalize the micelles, increasing their anti-tumor activity and improving tumor localization.

The goal of this study was to create a targeted combination micellar preparation consisting of a novel proapoptotic drug, surface-modified TRAIL ligand and siRNA. To achieve this goal, the following specific aims were pursued:

Specific Aims

1) Characterization of PIP3-PH inhibition and cytotoxicity of proapoptotic drug DM-PIT-1 and novel analogs in vitro with and without TRAIL;

2) Preparation and characterization of TRAIL-modified micelles loaded with DM-PIT-1 or its analogs;

3) Evaluation of in vitro cytotoxicity of combination micelle formulations across a range of tumor cell types;

4) Characterization of TF-modified micelles targeting potential and their effects on cytotoxicity in vitro;
5) Formulation and characterization of siRNA-S-S-PE mixed micelles and evaluation of gene silencing \textit{in vitro} and \textit{in vivo};

6) Evaluation of combination micelles as a multifunctional delivery system utilizing \textit{in vivo} mouse models of human cancer.
2 Background and Significance

2.1 Micelles as a multifunctional drug delivery systems

Micelles are self-assembling nanosized colloidal particles consisting of component surfactant molecules. In aqueous media, hydrophobic tails of the surfactant are secluded into the core of the micelle and the hydrophilic head forms the shell. Micelles consisting of amphiphilic block co-polymers possessing a phospholipid tail and polyethylene glycol (PEG) conjugated head group have been extensively studied for their use in solubilizing and delivering poorly soluble drugs and diagnostic agents [1, 2]. In particular, PEG-PE micelles have been shown to be an effective delivery system possessing excellent in vitro and in vivo properties with good biocompatibility [3, 4].

PEG-PE micelles (Figure 1) demonstrate several important properties critical to any drug delivery system [1]: (1) incorporation of hydrophobic drugs into the lipid core resulting in increased solubility and bioavailability; (2) reduced toxicity and off-target effects; (3) reduced metabolism and inactivation by isolating the drug in the core of the micelle; (4) PEG shielding prevents uptake by the reticuloendothelial system (RES) and increases blood half-life; (5) small size (approx. 7-40 nm) allowing accumulation in areas with compromised vasculature; (6) functionalization of the surface of the micelle can impart targeting or signaling properties [5-8].
Micellar drug delivery has been shown to have significant utility in the treatment of cancer. Tumors possess poorly formed and tortuous blood vessels. This, coupled with poor lymphatic drainage, results in a phenomenon known as the enhanced permeability and retention (EPR) effect [10]. Particles with a size of less than ~250 nm are able to extravasate through the leaky vasculature and preferentially accumulate in tumor tissue. PEG-PE micelles are able to utilize the EPR effect due to their small size and increased circulation half-life that results from PEG shielding [11].

Functionalizing the surface of micelles has emerged as a method to further increase tumor accumulation and efficacy (Figure 2). A variety of targeting ligands such as antibodies, transferrin (TF), and folate have been conjugated to the surface of micelles resulting in increased targeting and accumulation in the tumor tissues [5, 8, 12-15]. A common method for conjugating targeting ligands to the surface of micelles utilizes an activated p-nitrophenylcarbonyl (pNP) group at the distal end of the PEG-PE monomer [16]. pNP reacts with amino-groups of various ligands yielding a stable urethane (carbamate) bond.

**Figure 1:** Diagram of the PEG-PE component and assembly of block co-polymer micelles incorporating hydrophobic drug [9].
Figure 2: Schematic of a multifunctional micelle and a pNP-PEG-PE micelle used for conjugation of targeting, reporting or functional moieties [2, 16].

Micelles have been shown to rapidly exchange their component monomers with neighboring micelles [17]. Thus, micelles modified with a targeting ligand can be mixed with drug-loaded micelles to form a homogenous population of multifunctional micelles possessing targeted ligand at the surface of a drug-loaded core.

Continued research into the survival and proliferation of cancer indicate a variety of simultaneously perturbed pathways and mechanisms [18]. Combination therapy aimed at separate targets of survival or proliferation has potential to overcome various barriers to apoptosis. Simultaneous delivery of separate drugs in one targeted micellar drug delivery system has potential to be an effective agent in the treatment of cancer.

2.2 Importance of PI3K pathway in cancer

Cancer is characterized by uncontrolled cell growth and division of abnormal cells. Cancer can be caused by a variety of factors and each tumor is unique, even those of the same tissue type. Despite tumor heterogeneity, there are several hallmarks of cancer that have been shown to be important drivers of malignancy (Figure 3) [18]. Each of these hallmarks results from a perturbation of normal cellular pathways. The phosphoinositide 3-kinase (PI3K) signaling pathway has been shown to be involved with several of these traits.
Repairing normal function of this pathway has been shown to result in cellular apoptosis and tumor inhibition [20].

**Figure 3:** The 10 hallmarks of cancer and the treatments associated with each [18].

The PI3K pathway regulates a variety of cellular functions including cell metabolism, survival and apoptosis [21, 22]. Upon activation, PI3K is recruited to the cell membrane and is free to phosphorylate phosphatidylinositols, including PIP$_2$ converting it into the second messenger PIP$_3$. Phosphatidylinositols are composed of long chain fatty acid groups attached to a phosphorylated inositol head group via a glycerol moiety. Fatty acids anchor the molecule in the membrane, while the inositol is exposed to the cytoplasm. Various kinases catalyze the addition or removal of phosphates to the head group at specific locations. PI3K phosphorylates PIP$_2$ at the 3’ position to form the second messenger PIP$_3$. The phosphatases PTEN and SHIP breakdown PIP$_3$ by removing phosphates from the 3’ and 5’ positions respectively (Figure 4).
Figure 4: Illustration of the chemical structure and formation of PIP₃ by PI3K. Inactivation of PIP₃ occurs by the phosphatases PTEN and SHIP2 [22].

Once formed, PIP₃ is capable of recruiting and activating various proteins including Akt and PDK1 through interaction with their pleckstrin-homology (PH) domains (Figure 5). Normal function of the PI3K pathway involves dephosphorylation and inactivation of PIP₃ by phosphatase and tensin homolog protein (PTEN).
One of the most common genetic alterations in human cancers affects PTEN [23]. Silencing or mutation of PTEN prevents inactivation PI3K signaling. PIP₃ is no longer inactivated and is free to constitutively activate Akt and PDK1 resulting in downstream malignant effects. Another common perturbation of the PI3K pathway is an increase in the expression and activity of Akt and PDK1 [19].

The PI3K pathway is an emerging target in the treatment of cancer [24, 25]. Most of the compounds in clinical development focus on inhibiting PI3K, thus preventing formation of PIP₃. An alternative approach is to prevent the binding of PIP₃ to the PH domains of downstream proteins. Miao et al. have demonstrated binding of a small molecule, DM-PIT-1 (N-[(2-hydroxy-5-nitrophenyl)amino]thioxomethyl]-3,5-dimethyl-benzamide), to the PH domain of both Akt and PDK1 resulting in decreased cell viability and inhibited tumor growth [20]. In addition, DM-PIT-1 has been shown to sensitize the cell and increase the activity of TNFα-related apoptosis inducing ligand (TRAIL) [6].

**Figure 5:** PI3K signaling pathway and downstream cellular effects [19].
DM-PIT-1 is a first generation molecule developed as a PIP₃-PH inhibitor. Though the molecule has good inhibitory properties, it is limited by its solubility and stability in biological tissues. Figure 6 outlines some of the functional groups on the molecule that have been identified as potential areas for optimization. A second generation of novel analogs of the compound was therefore developed to overcome these issues (Figure 7).

**Figure 6:** Directions for optimizing DM-PIT-1 stability and efficacy.
2.3 TRAIL and death receptors

TNFα-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor family and has been shown to selectively induce death in cancerous cells, but not normal cells in vitro [27, 28]. TRAIL initiates apoptosis by binding to one of several Death Receptors (DR) on the surface of the cell (Figure 8). Once activated, the DR oligomerizes and initiates the formation of the Death Inducing Signaling Complex (DISC). DISC formation is dependent on the presence of a death domain located on the cytoplasmic tail of the DR. Caspase-8 is recruited to the membrane and activated by DISC formation. Apoptosis then proceeds through further activation of various caspases ultimately resulting in cell death.
Figure 8: TRAIL signaling and the downstream cellular effectors [29].

TRAIL signaling and induction of apoptosis is complicated by the variety of DRs that are expressed by cell. There are 5 known receptors that have been shown to interact with TRAIL. Of these, only 2 (DR4 and DR5) contain a death domain and are capable of inducing apoptosis. The other DRs are decoy receptors with no known signaling processes related to TRAIL and are thought to mediate the apoptotic signaling of the ligand [30].

TRAIL has been studied extensively in vitro and in vivo because of its unique induction of apoptosis in cancerous cells but not normal cells. It is thought that the expression of decoy receptors on normal cells prevents TRAIL from binding and activating DR4 or DR5. Cancerous tissue has been shown to have lower mRNA of decoy receptors [31]. Other mechanisms mediating the apoptotic effects of TRAIL in normal tissue...
involve the increased expression of the intracellular protein c-Flip which may interact with the death domains and block caspase activity [32]. A variety of cancer cells have shown resistance to TRAIL treatment likely through expression of either decoy receptors or increased c-Flip activity [33]. To overcome TRAIL resistance and further sensitize the cell to apoptosis, combination therapy involving TRAIL and chemotherapeutics has been investigated [6, 34].

2.4 Survivin protein

Survivin is a member of the inhibitor of apoptosis protein (IAP) family of anti-apoptotic proteins. As a family, these proteins function by decreasing apoptotic signaling in the cell [35]. Specifically, survivin exerts its anti-apoptotic activity through inhibition of caspase activation (Figure 9). Normal expression of survivin is limited to fetal tissue and is completely absent in terminally differentiated cells [36]. However, survivin is highly expressed in most human cancers, making it an ideal target as both a diagnostic and therapeutic target in cancer treatment [37]. Additionally, survivin levels in biopsied tissues serves as a prognostic marker for cancer progression.
Figure 9: Extrinsic and intrinsic apoptosis pathways converge to activate effector caspases. Survivin and other IAPs inhibit these pathways by preventing processing of initiator caspase-9 from the apoptosome and inhibiting the activity of the effector caspases [38].

Survivin expression is highly dependent on the cell cycle and is only found in the G2-M phase [39]. The molecular mechanisms regulating the expression and activity of survivin are not completely understood though there is evidence linking p53, the Wnt pathway, beta-catenin and the PI3K pathway (Figure 10) [40, 41]. As indicated in Figure 9, survivin has several mechanisms of action. Tamm et al. concluded that expression of survivin is able to inhibit Bax and Fas induced apoptosis, likely through downstream inhibition of these pathways [42]. Binding experiments performed also indicated that survivin is able to directly bind and inhibit caspase-3 and caspase-7 inhibiting their ability to further propagate apoptotic signaling.
Investigator: Robert D. Riehl

**Figure 10:** Interaction of survivin with the PI3K pathway [40].

Despite the clear biologic rationale for targeting survivin as an anti-cancer therapy, there has been little clinical progress [43]. In contrast to other targets of chemotherapy, survivin exerts its anti-apoptotic effects intracellularly. Therefore, any therapy must cross the cell membrane. In light of this, there has been little advancement in small-molecule inhibitors of survivin. Approaches to target survivin have therefore focused on small-molecule inhibition of survivin transcription [44], antisense inhibition of survivin translation [41], and immunotherapy targeting survivin peptides [45].

Targeting survivin alone is often ineffective at inhibiting tumor growth. Therefore, combining survivin inhibition with other therapies has garnered significant interest and initial results look promising [46]. As indicated in Figure 8 and Figure 10, IAPs such as survivin, influence both TRAIL induced apoptosis and can be activated by PI3K signaling. It is therefore hypothesized that inhibition of survivin will sensitize the cell to the apoptotic actions of TRAIL signaling and PI3K inhibition.
2.5 siRNA mechanisms and delivery

RNA interference (RNAi) has emerged as an intriguing therapeutic for the treatment of a variety of diseases, including cancer [47-49]. Originally discovered as an endogenous method to silence genetic expression in *C. elegans* [50], RNAi now includes a variety of endogenous and exogenous methods to selectively and transiently silence specific genes implicated in pathogenesis. Focusing on exogenous methods of RNAi, delivery of short segments of double stranded RNA (siRNA) possesses sequence complementarity with an mRNA of interest has shown to have much promise. Double stranded siRNA is incorporated into RNA-induced Silencing Complex (RISC) and it is subsequently unwound (Figure 11). The antisense strand is then able to complex complementary sequences of mRNA. mRNA is then cleaved and RISC is able to seek out other strands of mRNA. Because of its highly specific gene knockdown and its transient effect (3-7 days), siRNA is an exciting potential therapeutic [47].

**Figure 11:** Schematic of siRNA incorporation into RISC and its subsequent interaction with complementary mRNA resulting in silencing of a gene of interest [47].
The considerable utility of RNAi and siRNA in particular is limited by the ability of the therapeutic to reach its target tissue and enter the subcellular space where it is capable of exerting its activity. To incorporate into RISC and silence genes, siRNA must reach the perinuclear space of the cytoplasm in cells of diseased tissues. Barriers to this delivery are numerous and have been extensively studied [47]: (1) endogenous nucleases in the blood rapidly degrade naked siRNA limiting the half-life; (2) siRNA is a large highly negatively charged molecule incapable of crossing the cellular membrane; (3) endocytosed siRNA must escape the lysosomal degradation pathway; (4) nucleic acids have been shown to have immunogenic activity through activation of Toll-like Receptors [51].

The significant delivery challenges associated with siRNA have been addressed through a variety of unique systems [48]. Polymers, liposomes and other nanoparticles have all been utilized to overcome the delivery barriers associated with siRNA to a varying degree of success. An effective delivery vehicle for siRNA must be biocompatible, biodegradable and non-immunogenic. The vehicle should also protect the siRNA from degradation, deliver it to the cytoplasm of target cells and release the payload to allow incorporation into RISC. Cationic polymers or liposomes are the most commonly used method of delivery, though they are limited due to their toxicity. In addition, efficacy of cationic-mediated delivery vehicles is limited by poor release of siRNA into the cytoplasm due to the strong ionic charge interaction between siRNA and the positively charged vehicle. An emerging approach is to reversibly conjugate the siRNA to mixed PEG-PE micelles via a disulfide bond (Figure 12) [52]. Upon delivery into the cytoplasm, the reducing environment allows for cleavage of the disulfide bond and release of siRNA.
2.6 Combination therapy in cancer

By the time a deformed cell has become cancerous it has undergone a number of mutations in a variety of proapoptotic and survival pathways ensuring its continued growth and proliferation [18]. It is therefore advantageous to target a number of these pathways with separate therapeutics. The development of effective therapeutic combinations is an important tool in treating cancer. A successful combination therapy should contain agents that: (1) have proven activity as a single agent; (2) have separate mechanisms of action; (3) have non-overlapping dose limiting toxicities; (4) have non-overlapping resistance mechanisms; (5) can be delivered with an appropriate dosing schedule as to maximize efficacy [53, 54]. There are numerous examples in the literature of effective usage of drug-drug combinations [55-57], siRNA-drug combinations [46, 58, 59] and peptide-drug combinations [6, 34].

The goal of the present study was to combine and evaluate a novel inhibitor of the PI3K pathway with TRAIL surface modified to the distal tip of pNP-PEG-PE, and reversibly conjugated siRNA targeting survivin into a multifunctional PEG-PE micelle. Tumor cell targeted micelles functionalized with transferrin (TF) were also investigated as a means to increase cell association and delivery of incorporated therapeutic. The
hypothesis of this project was that micelles are an effective anti-tumor delivery vehicle capable of more effectively inhibiting tumor growth when formulated to contain a combination of therapeutics.
3 CHARACTERIZATION OF PIP$_3$-PH INHIBITION AND CYTOTOXICITY OF PROPAPOPTOTIC DRUG DM-PIT-1 AND NOVEL ANALOGS IN VITRO WITH AND WITHOUT TRAIL

3.1 Introduction

Despite the excellent proapoptotic, anti-proliferative and migration suppressive properties of DM-PIT-1, several issues with the compound limit its therapeutic potential [20]. Several portions of the compound contribute to poor stability in biological samples, and efforts to optimize a second generation of compounds have been conducted (as described in Figure 6). Figure 7 illustrates several of the novel analogs developed, while Table 1 provides a summary of their physicochemical properties. It was hypothesized that these compounds have been optimized for their ability to inhibit PIP$_3$/PH binding and subsequent activation of Akt. Additionally, changes to several of the functional groups of the parent DM-PIT-1 compound were hypothesized to increase the stability of the compounds in the presence of biological samples. Direct examination of PIP$_3$/PH inhibition was conducted utilizing several in vitro assays, while inhibition of subsequent Akt activation was also analyzed utilizing functional assays. Additionally, the stability of the novel analogs in relation to the parent DM-PIT-1 was determined. Lastly, the ability of the novel analogs to inhibit cancer cell growth was characterized at various concentrations in the presence and absence of TRAIL. These results taken together determine the success of the development of the second generation analogs of DM-PIT-1.
3.2 Materials and Methods

3.2.1 DM-PIT-1 and novel analogs

DM-PIT-1 (N-[[2-hydroxy-5-nitrophenyl]amino]thioxomethyl]-3,5-dimethyl-benzamide) and the novel analogs NCL-176, NCL-179, NCL-198 and NCL-240 were synthesized at the National Chemical Laboratory (Pune, India) and provided by Dr. Alexei Degterev (Tufts University, Boston, MA). Recombinant His6-tagged human TRAIL ligand was produced in E. coli and purified using Ni\(^{2+}\) chromatography as previously described [31, 60] and provided by Dr. Alexei Degterev.

3.2.2 PIP-strip protein pull down assay

DM-PIT-1 and its analogs were analyzed for their ability to inhibit Akt-PH domain-PIP\(_3\) binding. Pre-spotted PIP-strips were purchased from Echelon Biosciences (Salt Lake City, UT). PIP-strips were blocked with 3% (wt/vol) BSA in TBST [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% (vol/vol) Tween 20] for 1 hr at room temperature, followed by incubation with 0.5 µg/mL His-Akt-PH domain with or without inhibitor (100 µM DM-PIT-1 or 100 µM NCL-240 in 3% (wt/vol) BSA in TBST for 1 hr at room temperature. PIP-strips were washed extensively in TBST, and subjected to western blot analysis using anti-His-HRP primary antibody (Cell Signaling Technology, Beverly, MA) to detect the bound fusion protein.

3.2.3 Akt phosphorylation western blot

To determine the effects of the novel analogs on Akt phosphorylation, A2780 cells were treated with varying concentrations of drug for 4 hrs in DMEM with 10% FBS. Cells treated with DMSO alone or with InSolution Akt Inhibitor VIII (Calbiochem, San Diego, CA) were used as negative and positive controls respectively. Cells were then harvested and lysed using 1x RIPA buffer (Cell Signaling Technology, Beverly MA) containing the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). Protein concentrations of all samples were determined using Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA) and equal amounts of
protein were subjected to SDS/PAGE electrophoresis and western blot analysis. Briefly, protein samples were transferred to polyvinylidene difluoride (PVDF) membranes, and then blocked in either 5% BSA (wt/vol) or 3% casein (wt/vol) in TBST for 30 min at room temperature. The membranes were then incubated for either 2 hr at room temperature or overnight at 4°C with various primary antibodies (Cell Signaling Technology, Beverly, MA) (1:1500 dilution). The membranes were then washed extensively in TBST and incubated with secondary HRP-conjugated antibody (1:5000) for 1 hr at room temperature, developed with Luminata™ chemiluminescence ECL reagent (Millipore, Billerica, MA) and exposed to autoradiography film (Denville Scientific Inc., Metuchen, NJ).

3.2.4 Wound assay

DM-PIT-1 and novel analogs were analyzed for their ability to inhibit cell migration, an effect of Akt activation. A common assay to determine cell migration is the wound healing assay [61]. Briefly, Ibidi microchambers (Ibidi Cells In Focus, Verona, WI) were adhered to 6-well plates. A2780 cells were seeded at a density of 8 x 10^5 cells per well in a volume of 70 µL, and allowed to grow to confluency. The Ibidi microchamber was then removed, leaving a 500 µm +/- 50 µm wound. The old media and dislodged cells were aspirated and replaced with fresh media containing either DMSO, DM-PIT-1 or novel analogs. Every 8-12 hr, images were taken and Spot Advanced 4.5 software was used to measure the wound widths, from which the rate of migration inhibition was determined.

3.2.5 Microsomal stability

The microsomal stability of the initial drug DM-PIT-1 was compared against the most recently developed analog NCL-240. Previous studies have shown poor biological stability of DM-PIT-1 [62]. Samples of DM-PIT-1 and NCL-240 were submitted to Apredica Contract Research for microsomal stability. The procedure has been given below.
Samples were incubated in triplicate with mouse microsomes at 37° C. The reaction mixture contained microsomal protein in 100 mM potassium phosphate, 2 mM NADPH, 3 mM MgCl₂ at pH 7.4. At the indicated time, aliquots were removed from each reaction mixture and a stop solution (0.3% acetic acid in acetonitrile containing haloperidol, diclofenac, or other internal standard) was added immediately. The samples were centrifuged to remove precipitated protein, and the supernatants are analyzed by LC/MS/MS to quantitate the remaining parent compound.

### 3.2.6 Cell viability assay

Viability of various cell lines was measured using CellTiter-Blue® (Promega, Madison, WI) viability assay according to manufacturer’s instructions. Briefly, cells were seeded in 96-well plates according to proper cell densities (3,000-5,000 cells). After 24 hr incubation in 5% CO₂ at 37° C, cells were then treated with formulation under the appropriate conditions. Following treatment, media was removed, the wells were washed with serum complete media and replaced with 50 µL media and 10 µL CellTiter-Blue®. Cell viability was evaluated after 2 hrs of incubation at 37° C at 5% CO₂ by measuring the fluorescence (ex. 550 nm, em. 590 nm) using Synergy HT multi-detection microplate reader (Biotek, Winooski, VT).

### 3.2.7 Statistical analysis

Wherever possible, data was generated in triplicates for proper statistical analysis. In vitro experiments are reported as mean +/- standard deviation. One-way ANOVA followed by Tukey's multiple comparison tests were performed with significance determined by a p-value < 0.05.
3.3 Results and Discussion

3.3.1 Characterization of DM-PIT-1 and its novel analogs

DM-PIT-1 was the first of several PIP3-PH inhibitors to be characterized. New analogs were developed to improve the potency and stability of the drug in biological fluids. NCL-176, NCL-179 NCL-198 and NCL-240 were identified as potential lead compounds to be tested in vitro and studied for their PIP3/PH inhibition activity. Figure 7 and Table 1 provide the structures and physicochemical properties of the novel analogs.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular weight (g/mol)</th>
<th>Calculated LogP</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM-PIT-1</td>
<td>351.76</td>
<td>3.22</td>
</tr>
<tr>
<td>NCL-176</td>
<td>299.71</td>
<td>3.34</td>
</tr>
<tr>
<td>NCL-179</td>
<td>495.32</td>
<td>4.97</td>
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<tr>
<td>NCL-198</td>
<td>631.31</td>
<td>6.82</td>
</tr>
<tr>
<td>NCL-240</td>
<td>435.71</td>
<td>5.18</td>
</tr>
</tbody>
</table>

Table 1: Summary of molecular weights and calculated LogP values of DM-PIT-1 and novel analogs. (LogP calculated using CambridgeSoft ChemDraw® software)

3.3.2 PIP-strip protein pull down assay

Figure 13: PIP-strip pull down assay. Akt-PH protein incubated on lipid strips both in the presence and absence of DM-PIT-1 or NCL-240.

Both DM-PIT-1 and NCL-240 inhibit the binding of the Akt-PH domain to the lipid PIP3 as indicated by a decrease in spot intensity in Figure 13. In addition, both compounds were specific to PIP3 and did not noticeably inhibit PIP2 binding.
3.3.3 Akt phosphorylation western blot assay

**Figure 14:** Western blot of A2780 ovarian carcinoma cells treated with NCL-240. Cells treated with drug or inhibitor for 4 hrs in serum complete media. αp308 and αp473 are antibodies against phosphorylated Akt capable of selectively binding to phosphorylated motifs on the protein.

Western blot analysis indicates that at a concentration of 50 µM, NCL-240 is able to inhibit Akt phosphorylation (Figure 14). This is likely due to the inability of the Akt-PH domain to bind to PIP₃ in the presence of NCL-240. It is thus incapable of being recruited to the membrane where it can be phosphorylated by one of the protein kinases (PDK1 or PDK2).
3.3.4 Migration wound assay

**Figure 15:** Wound (migration) assay performed on A2780 ovarian carcinoma cells. Cells were incubated with DM-PIT-1 or novel analog at a concentration of either 1.25 µM or 15 µM (n=3). Two-way ANOVA followed by Bonferroni post-tests were performed. (Values represent mean +/- SD; *p < 0.05, **p < 0.01, ***p < 0.001 as compared to DMSO)

Cell migration is one of the downstream effects of the Akt pathway. Akt phosphorylates proteins that regulate actin formation and subsequent cell motility [63]. The cell migration assay is therefore a useful indicator of Akt activity. In this assay, A2780 cells were treated with DM-PIT-1, NCL-176 or NCL-240 at either 1.25 µM or 15 µM concentrations. Figure 15 illustrates the percentage of wound remaining open was significantly higher at both concentrations in the NCL-240 group indicating inhibition of the Akt pathway. NCL-176 showed improvement over DM-PIT- at low concentrations (40 hrs p < 0.001, 50 hr p < 0.001), but was not significantly different at the higher concentrations.
3.3.5 Microsomal stability

![Mouse Microsome Stability of DM-PIT-1 and NCL-240](image)

**Figure 16:** Mouse microsomal stability of DM-PIT-1 and NCL-240 (n=3).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Clearance Rate (µL/min)</th>
<th>Half-life (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM-PIT-1</td>
<td>1262</td>
<td>1.8</td>
</tr>
<tr>
<td>NCL-240</td>
<td>19.4</td>
<td>119</td>
</tr>
</tbody>
</table>

**Table 2:** Summary of microsomal stability of DM-PIT-1 and NCL-240. (Assay performed by Aprelica Contract Research)

As previously discussed, one of the limitations of the first generation compound DM-PIT-1 is its instability in biological tissues. This study analyzed the stability of DM-PIT-1 and NCL-240 in mouse microsomes, an assay commonly performed to evaluate the ability of enzymes to break down drugs. Figure 16 and Table 2 illustrate the improved stability of NCL-240 against metabolism by microsomal enzymes. DM-PIT-1 is rapidly metabolized with a half-life of only 1.8 min while NCL-240 has an improved half-life of nearly 2 hr.
3.3.6 Cytotoxicity of novel analogs as compared to DM-PIT-1

**Figure 17:** Cell viability of A2780 ovarian carcinoma following 24 hr incubation with DM-PIT-1 or its novel analogs with or without free TRAIL. (n=3, mean +/- SD shown) All concentrations of NCL-179, NCL-198 and NCL-240 are significantly different from DM-PIT-1 both with and without free TRAIL (p < 0.001).

Cytotoxicity of the novel analogs was compared against DM-PIT-1 in the presence and absence of TRAIL. A2780 cells were exposed to treatment at various concentrations for 24 hr. Figure 17 shows an improvement in the new analogs NCL-179, NCL-198 and NCL-240 as compared to DM-PIT-1 over all concentrations (p < 0.001). NCL-176 has similar cytotoxic effects as DM-PIT-1 under most conditions. Additionally, inclusion of TRAIL in the treatments results in decreased cell viability, particularly at high doses of drug and in formulations containing NCL-240 (p < 0.001).
3.4 Conclusions

The *in vitro* characterization of the various second generation PIP<sub>3</sub>-PH inhibitors indicates that the modifications to DM-PIT-1 have yielded compounds with improved inhibition of Akt phosphorylation and its subsequent activity, while also improving the *in vitro* stability of the compound. *In vitro* cytotoxicity assays indicated that novel analogs more effectively inhibit cell growth as compared to DM-PIT-1 both alone and in the presence of TRAIL. NCL-240 has emerged as having the best *in vitro* properties and will play a large role in the following studies conducted.
4 PREPARATION AND CHARACTERIZATION OF TRAIL-MODIFIED MICELLES LOADED WITH DM-PIT-1 OR ITS ANALOGS

4.1 Introduction

The poor aqueous solubility of both DM-PIT-1 and its novel analogs necessitates the incorporation into a drug delivery system capable of increasing the solubilization and stability of the compound. Amphiphilic conjugates of a hydrophilic polyethylene glycol (PEG) chain and a hydrophobic diacyl lipid, typically phosphatidyl ethanolamine (PE), have been utilized previously to incorporate DM-PIT-1, thereby increasing its solubility [6]. These conjugates are able to form stable nanosized micellar colloids in aqueous environments with a high nM to low µM critical micelle concentration (CMC). The lipid core of the micelle is an ideal environment to efficiently solubilize hydrophobic drugs, thus increasing their solubility and provides aqueous stability to the formulation. Additionally, modifications to the surface of the micelle can be achieved utilizing reactive pNP-PEG-PE in the lipid mixture. Functionalization of the surface by TRAIL allows for increased potential cytotoxicity.

The ability of PEG-PE to incorporate novel analogs of DM-PIT-1 into nanosized colloidal particles was analyzed. Incorporation efficiency and overall concentration of drugs in micelles was determined. Physical properties of drug-loaded micelles were analyzed to ensure formation of stable nanosized particles. The release of drug from micelles was determined to assess the ability of the drug to be released from the micelle under proper conditions. TRAIL-modified micelles were prepared and analyzed for TRAIL reaction efficiency and overall concentration. Additionally, their physical properties were also analyzed to ensure formation of stable nanosized particles. Lastly, combination drug-loaded, TRAIL-modified micelles were prepared and characterized to ensure that PEG-PE micelles could effectively incorporate drugs and possess surface functionalization, creating a multifunctional delivery system.
4.2 Materials and Methods

4.2.1 Formulation and characterization of drug-loaded micelles

1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG$_{2000}$-PE), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B Sulfonyl) (Rh-PE) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL) and used without further purification. pNP-PEG$_{3400}$-pNP was purchased from Laysan Bio (Arab, AL).

4.2.1.1 Formulation of drug-loaded micelles

Drug-loaded micelles were prepared by the thin-film hydration method. Briefly, PEG$_{2000}$-DSPE dissolved in chloroform was added to round bottom flasks. Various weight % of drug dissolved in acetonitrile was added and the sample vortexed for complete mixing. Organic solvents were then evaporated under a rotary evaporator to form a thin film. Films were further dried by freeze-drying on a Freezone 4.5 Freeze Dry System (Labconco, Kansas City, MO) for at least 4 hrs. Films were rehydrated with 1x PBS pH 7.4 to a lipid concentration of 13.07 mg/mL and vortexed for at least 5 min. Drug-loaded micelles were then centrifuged at 14,000 rpm for 5 min to precipitate any unincorporated drug and further filtered using a 0.2 µm filter (Nalgene, Rochester, NY).

4.2.1.2 Characterization of drug-loaded micelles

Drug incorporation efficiency was measured by reverse phase HPLC using an Xbridge C$_{18}$ (2.1cm x 250cm) column (Waters Corporation, Milford, MA) on a Hitachi Elite LaChrom HPLC with autosampler (Pleasanton, CA). The mobile phase consisted of either 70:30 acetonitrile:water (DM-PIT-1) or 60:40 acetonitrile:water (NCL-176, NCL-179, NCL-198, NCL-240) with a flow rate of 1 mL/min. Detection of drug was performed at a wavelength corresponding to a peak on the absorption spectrum (DM-PIT-1: 320 nm, NCL-176: 260 nm, NCL-179: 295 nm, NCL-198: 295 nm, NCL-240: 300 nm). Sample injections were kept constant.
at 50 µL and the sample runtime was 10 min. Concentration of drug was determined by measuring the area under curve of the peak. Standard curves of stock drug in acetonitrile from concentrations of 10 µg/mL to 150 ng/mL were used to determine incorporation of drug in micelles. Five µL of dialyzed drug-loaded micelles were diluted in 495 µL acetonitrile to destroy micelles, freeing the incorporated drug, and then analyzed.

All micellar samples were measured for their size distribution and zeta-potential using the Zetaplus (Brookhaven Instruments Corporation, Holtsville, NY). Briefly, 50 µL of micelle sample was dissolved in 1 mL of distilled water and micelle size and zeta-potential were analyzed according to manufacturer’s protocol.

4.2.1.3 Micellar drug release

Drug-loaded micelles were analyzed for their in vitro drug release profile at 37° C in 1x PBS pH 7.4 over 48 hrs. Micelles were prepared as described previously. 500 µL of drug-loaded micelles was aliquoted into a Spectra/Pro® regenerated cellulose dialysis membrane (Spectrum Laboratories Inc., Rancho Dominguez, CA) with a molecular weight cut-off (MWCO) of 1,000 Da and dialyzed against a large excess of 1x PBS pH 7.4. At predetermined times, dialysis bags were opened and aliquots of sample were taken and diluted with 200 µL acetonitrile. Drug concentration was determined by HPLC using previously described methods.

4.2.2 Formulation and characterization of TRAIL-modified micelles

4.2.2.1 Synthesis of pNP-PEG₃₄₀₀-PE

pNP-PEG₃₄₀₀-PE was synthesized according to standardized in-lab procedures [16]. pNP-PEG₃₄₀₀-pNP (Laysan Bio, Arab, AL) was dissolved in dry chloroform and DOPE in chloroform was added followed by triethylamine (TEA). The reaction mixture was left overnight at room temperature under nitrogen with stirring. Solvents were then removed by rotary evaporation and films were further dried on a Freezone 4.5 Freeze Dry System (Labconco, Kansas City, MO) for at least 4 hrs to remove any residual solvents. The dried film was then rehydrated with 0.001 M HCl and separated on sepharose column. Fractions were collected and analyzed by thin-layer chromatography (TLC) to confirm pNP-PEG₃₄₀₀-PE product. Fractions were spotted on silica gel.
TLC plates (Sigma-Aldrich, St Louis, MO) and run in a mobile phase of CH$_3$:MeOH (80:20). Molybdenum blue was sprayed on the plates to stain for the presence of phospholipid, while Dragendorff’s reagent was sprayed to stain for PEG. Product containing fractions were frozen, lyophilized, weighed and reconstituted with chloroform to appropriate stock concentrations and stored at -80° C.

4.2.2.2 Formulation of TRAIL-modified micelles

TRAIL-modified micelles were prepared by the thin-film hydration method as described previously [6]. Briefly, PEG$_{2000}$-DSPE dissolved in chloroform was added to round bottom flasks. 5% (by mole of DSPE-PEG$_{2000}$) pNP-PEG$_{3400}$-PE dissolved in chloroform was added and the sample was vortexed for complete mixing. Chloroform was then evaporated under a rotary evaporator to form a thin film. Films were further dried by freeze-drying on a Freezone 4.5 Freeze Dry System (Labconco, Kansas City, MO) for at least 4 hrs. Films were rehydrated with stock TRAIL (0.9 - 1.36 mg/mL) at a molar ratio of pNP-PEG-PE:TRAIL 40:1. 1x PBS pH 8.5 was added to achieve a final lipid concentration of 13.07 mg/mL and samples were vortexed for 5 min. The pH of the solution was determined and adjusted with 1.0 N NaOH to 8.5 if necessary. Reaction then proceeded for 4 hrs at room temperature to allow sufficient TRAIL conjugation and complete hydrolysis of unreacted pNP groups. TRAIL-modified micelles were then dialyzed using a 100,000 MWCO membrane (Spectrum Laboratories Inc., Rancho Dominguez, CA) against 1 L 1x PBS pH 7.4 for 1 hr followed by another 4 hrs of dialysis in 1 L fresh 1x PBS pH 7.4.

4.2.2.3 Characterization of TRAIL-modified micelles

Conjugation efficiency of TRAIL was measured using a micro BCA kit (Pierce, Rockford, IL) according to the provider’s procedure. Protein content was determined by comparing TRAIL micelles to BCA standards. Signals from TRAIL samples were normalized with plain micelle samples at the same lipid concentration to account for any increased signal from the presence of PEG-PE.
All micelle samples were measured for their size distribution and zeta-potential using the Zetaplus (Brookhaven Instruments Corporation, Holtsville, NY). Briefly, 50 µL of micelle sample was dissolved in 1 mL of distilled water and micelle size and zeta-potential were analyzed according to manufacturer’s protocol.

4.2.3 **Formulation and characterization of combination drug-loaded/TRAIL-modified**

Multifunctional combination micelles were prepared by coincubating drug-loaded micelles with TRAIL-modified (Figure 18). Samples were vortexed and allowed to mix for at least 4 hr at room temperature. Samples were measured for size distribution and zeta-potential as previously described.

![Figure 18: Schematic of formulation of drug-loaded TRAIL-modified micelles [26].](image-url)
4.3 Results and Discussion

4.3.1 Size and zeta-potential of drug-loaded/TRAIl combination micelles

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Size (nm)</th>
<th>Zeta-potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSPE-PEG\textsubscript{2000} micelles</td>
<td>17.5 +/- 0.1</td>
<td>-6.1 +/- 1.4</td>
</tr>
<tr>
<td>DM-PIT-1 micelles</td>
<td>18.1 +/- 0.5</td>
<td>-1.4 +/- 1.1</td>
</tr>
<tr>
<td>NCL-176 micelles</td>
<td>16.2 +/- 0.7</td>
<td>-3.6 +/- 1.4</td>
</tr>
<tr>
<td>NCL-179 micelles</td>
<td>16.0 +/- 0.8</td>
<td>-1.8 +/- 0.6</td>
</tr>
<tr>
<td>NCL-198 micelles</td>
<td>16.4 +/- 0.8</td>
<td>-2.1 +/- 0.7</td>
</tr>
<tr>
<td>NCL-240 micelles</td>
<td>17.4 +/- 0.9</td>
<td>-3.4 +/- 1.2</td>
</tr>
<tr>
<td>TRAIL micelles</td>
<td>40.1 +/- 3.1</td>
<td>-20.3 +/- 1.0</td>
</tr>
<tr>
<td>Combination DM-PIT-1/TRAIl (0.5 µg/mL) micelles</td>
<td>27.2 +/- 1.2</td>
<td>-13.6 +/- 0.6</td>
</tr>
</tbody>
</table>

Table 3: Size and zeta-potential characterization of drug-loaded and TRAIL-modified micelles. (n=3, mean +/- SD shown)

Size and zeta-potential characterization of drug-loaded and TRAIL-modified formulations confirm the formation of nanosized micelles (Table 3). Addition of drug into the core of the micelle does not appreciably change the size or zeta-potential properties of the micelles. As expected, TRAIL modification yields a micelle with a larger size and lower zeta-potential. TRAIL is a 50 kDa protein with a negative surface charge and will therefore increase the size of the micelles while also lowering the zeta-potential.

Though a direct assay to confirm that our combination drug-loaded, TRAIL-modified micelles have sufficiently mixed to form a homogenous population of multifunctional micelles (as illustrated in Figure 18) has not been performed, the size and zeta-potential shifts indicate TRAIL-micelle mixing. Additionally, there is only one population of micelles seen during size analysis.
4.3.2 Drug loading efficiency

**Figure 19:** Standard curve of DM-PIT-1 concentration as determined by HPLC.

**Figure 20:** Standard curve of NCL-176 concentration as determined by HPLC.

**Figure 21:** Standard curve of NCL-179 concentration as determined by HPLC.
**Figure 22:** Standard curve of NCL-198 concentration as determined by HPLC.

**Figure 23:** Standard curve of NCL-240 concentration as determined by HPLC.

Figures 19-23 provide examples of the standard curves used to analyze the concentration of incorporated drug in micellar preparations. The HPLC methods described previously provide repeatable, accurate and sensitive detection of drug over a range of ~150 ng/mL to 10 µg/mL. All standard curves had $R^2$ values of at least 0.999.
Table 4: Drug loading of DM-PIT-1 and its novel analogs in PEG-PE micelles. PEG-PE concentration is constant at 13.07 mg/mL. (n=3 with SD shown)

Utilizing the standard curves shown previously, Table 4 provides a summary of the loading properties of the various compounds in DSPE-PEG<sub>2000</sub> micelles. Lipid films containing drug were hydrated to a lipid concentration of 13.07 mg/mL. Under these conditions the loading efficiency and final drug concentration are reported. The wt% used was determined in part by the loading efficiency of each drug, and in part by the desire to have drug-loaded micelles at similar concentrations for side-by-side in vitro analysis of the various analogs. NCL-179 and NCL-198 have close to 100% loading efficiency under these conditions, likely due to their low hydrophobicity as indicated by their LogP values (Table 1). These results demonstrate that PEG-PE micellar preparations are capable of efficiently incorporating sufficient drug for further in vitro studies.

4.3.3 TRAIL conjugation to pNP-PEG-PE micelles

![Standard Curve of TRAIL Content determined by BCA](image)

Figure 24: Standard curve of TRAIL concentration as determined by BCA assay.
TRAIL-modified micelles were analyzed for their protein concentration as determined by the BCA assay. Following extensive dialysis, TRAIL-modified micelles were incubated with BCA reagent. Subtracting plain DSPE-PEG2000 micelle absorbance from the TRAIL-modified micelle absorbance normalized any increased signal from the presence of micelles in the system. Figure 24 demonstrates that the BCA method is capable of accurately correlating absorbance to TRAIL concentration. Using the standard curve similar to the one above provides the ability to quantify the amount of TRAIL conjugated to the micelles.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Reaction yield (%)</th>
<th>TRAIL concentration on Micelles (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAIL-modified micelles</td>
<td>92.4% +/- 6.8%</td>
<td>271.0 +/- 23.8 µg/mL</td>
</tr>
</tbody>
</table>

**Table 5:** Average reaction yield and concentration of TRAIL modified to micelles. (n=3 with SD shown)

Table 5 shows the average reaction yield and total TRAIL concentration of the conjugation reaction. This high reaction yield is likely the result of a high pNP-PEG-PE to TRAIL ratio used in the reaction (40:1). The concentration of TRAIL on the micelles is sufficient for further *in vitro* analysis, though it is possible to increase TRAIL concentration by either decreasing the pNP-PEG-PE to TRAIL ratio or increasing the mol% of pNP-PEG-PE used in the formulation.
4.3.4 Micellar drug release study

Figure 25: Percentage of drug released from micelles at 37° C. Drug-loaded micelles were dialyzed in 1,000 MWCO dialysis membranes against excess of 1x PBS.

All of the drugs evaluated possess rapid drug release under the experimental conditions with both DM-PIT-1 and NCL-176 completely released from micelles at 10 hrs. NCL-179 and NCL-198 micelles release only 40% of their payload after 48 hrs. 60% of NCL-240 was released from the micelles following 48 hrs. DM-PIT-1 and NCL-176 both have lower LogP values (Table 1) and are therefore more hydrophilic than NCL-179, NCL-198 and NCL-240, helping to explain the more rapid and complete release of drug from micelles.
4.4 Conclusions

PEG-PE micelles were demonstrated to successfully incorporate DM-PIT-1 and its novel analogs into nanosized colloidal particles. The concentrations of drug capable of being incorporated were sufficiently high for future *in vitro* and *in vivo* studies. Additionally, TRAIL-modified micelles were successfully prepared with sufficient TRAIL concentration on the surface of the micelles for future studies. The combined drug-loaded/TRAIL modified micelles were also found to be of appropriate size and zeta-potential. Lastly, the micellar drug release assay indicated that NCL-179 and NCL-198 were unable to be fully released from micelles following 48 hr at 37° C indicating potential issues for future *in vitro* and *in vivo* studies. NCL-176 and NCL-240, however, demonstrated rapid and sufficient release from micelles.
5 EVALUATION OF IN VITRO CYTOTOXICITY OF COMBINATION MICELLE FORMULATIONS ACROSS A RANGE OF TUMOR CELL TYPES

5.1 Introduction

The ability of combination drug-loaded, TRAIL-modified micelles to inhibit tumor cell growth was characterized across a range of tumor cell types. It is important to characterize the cytotoxicity of both drug-loaded micelles alone, TRAIL-modified micelles alone and the combination of drug-loaded and TRAIL-modified micelles. Several cell lines were employed to adequately determine the effect of these nanoformulations across a range of tumor tissue types. PI3K pathway mutations and TRAIL-sensitivity varies depending on the cell line and probing the effect of these formulations against these cell types allows for better elucidation of effect. Initial studies utilizing DM-PIT-1 were performed to determine the effect of PI3K pathway inhibition in combination with TRAIL. The effect of formulating these compounds into a micelle-based nanoformulations was also determined to ensure no loss of activity. A comparison of DM-PIT-1 and its novel analogs in the presence and absence of TRAIL was performed to determine the effect of incorporation of the compounds into micelles and evaluate any increased cell growth inhibition of the novel analogs. Additionally, many cells develop resistance against chemotherapy, often resulting in increased PI3K pathway activity. Treating drug-resistant variants of cell lines was also performed to determine if reversal of drug resistance was possible utilizing these combination formulations.
5.2 Materials and Methods

5.2.1 Cell culture

Human ovarian carcinoma cell lines A2780 and SKOV-3, human breast carcinoma cell lines A549 and MCF-7 and human glioblastoma cell line U-87 MG were purchased from ATCC (Manassas, VA). SKOV-3 TR cells were a gift from Dr. Duan Zhenfeng (MGH, Boston, MA). DU145 human prostate cancer cells were provided by Dr. Alexei Degterev (Tufts University, Boston, MA) A2780, A549, MCF-7, U87-MG and DU145 were all grown in Dulbecco's modified Eagle's media (DMEM), with 10% fetal bovine serum (FBS) and penicillin-streptomycin solution (CellGro, Kansas City, MO). SKOV-3 sensitive and SKOV-3 TR were grown in RPMI supplemented with 10% FBS and penicillin-streptomycin solution. All cell culturing was performed at 37° C with 5% CO₂.

5.2.2 Cell viability assay

Viability of various cell lines was measured using CellTiter-Blue® (Promega, Madison, WI) viability assay. Briefly, cells were seeded in 96-well plates according to proper cell densities (3,000-5,000 cells). After 24 hr incubation in 5% CO₂ at 37° C, cells were then treated with formulation under the appropriate conditions. Following treatment, media was removed, the wells were washed with serum complete media and replaced with 50 µL media and 10 µL CellTiter-Blue®. Cell viability was evaluated after 2 hrs of incubation at 37° C at 5% CO₂ by measuring the fluorescence (ex. 550 nm, em. 590 nm) using Synergy HT multi-detection microplate reader (Biotek, Winooski, VT).

5.2.3 Statistical analysis

Wherever possible, data was generated in triplicates for proper statistical analysis. In vitro experiments are reported as mean +/- standard deviation. One-way ANOVA followed by Tukey's multiple comparison tests were performed with significance determined by a p-value < 0.05.
5.3 Results and Discussion

5.3.1 Cell viability

Previously described formulations of drug-loaded, TRAIL-modified and combination micelles were analyzed for their cytotoxic effects in a variety of human cancer cell lines. DM-PIT-1 was the primary drug studied to analyze the effective concentration and utility of combining TRAIL with a novel proapoptotic drug. Additional studies involving formulations containing the novel analogs were performed and compared to DM-PIT-1 containing formulations.

5.3.1.1 Drug-loaded/TRAIL modified combination micelles in A2780 cells

![Figure 26: Comparison of % cell viability of A2780 ovarian carcinoma cells 24 hr following administration of formulation. DM-PIT-1 and TRAIL given in either free form (f) or micellar form (m). (n=3, error bars indicate SD, **p < 0.01)](image)

Initial cytotoxicity studies (Figure 26) focused on analyzing the effects of delivery method of DM-PIT-1 and TRAIL while also analyzing proper dosing concentrations to achieve optimal combination therapy. TRAIL
formulations given alone were all non-toxic to cells regardless of concentration or whether they were conjugated to micelles. Incorporating DM-PIT-1 into micelles was shown to increase cytotoxicity. Most interesting is the high cytotoxicity seen with the combination treatments. mTRAIL combined with mDM-PIT-1 (shown in red) at all concentrations of TRAIL achieved cell viability of less than 10%. This effect was seen even at the low TRAIL concentration of 0.5 µg/mL. Comparing the individual treatments of TRAIL and DM-PIT-1 to the combination treatments show more than additive effects. This provides evidence for the potential utility of codelivering these agents. A clearer representation of these data is shown in Figure 27.

![Figure 27](image)

**Figure 27:** Comparison of % cell viability of A2780 ovarian carcinoma cells 24 hr following administration of formulation. (n=3, error bars indicate SD, **p < 0.001)**
5.3.1.2 Drug-loaded/TRAIL modified combination micelles in various cell lines

**Figure 28**: Comparison of % cell viability of U87-MG glioblastoma cells 24 hr following administration of formulation. (n=3 error bars indicate SD, *p < 0.05)

**Figure 29**: Comparison of % cell viability of DU145 prostate cancer cells 24 hr following administration of formulation. (n=3 error bars indicate SD, ***p < 0.001)
Figure 30: Comparison of % cell viability of MCF-7 breast cancer cells 24 hr following administration of formulation. (n=3 error bars indicate SD **p < 0.01)

Figures 28-30 show that the effects seen in A2780 ovarian cells can be seen in a variety of other cancer cells of different tissues, indicating the utility of this combination treatment across a broad spectrum of cancer types. In most cases there is more than an additive improvement by combining TRAIL and DM-PIT-1 into one multifunctional micellar formulation.
Figure 31: Comparison of % cell viability of SKOV-3 sensitive (white) and resistant (black) cells 24 hr following administration of formulation. (n=3 error bars indicate SD, **p < 0.01)

The cytotoxicity of combination DM-PIT-1 micelles was analyzed in the sensitive and resistant variants of SKOV-3. SKOV-3 resistant cells (SKOV-3 TR) were cultured with low doses of paclitaxel and resistance was confirmed by challenging cells with paclitaxel prior to the study (data not shown). Figure 31 illustrates an interesting observation that the resistant variant of SKOV-3 is more susceptible to both DM-PIT-1 and combination treatments than the sensitive cells. An exact explanation of this phenomenon has not been elucidated, though it is has been previously demonstrated that the resistant variant of SKOV-3 possesses increased Akt signaling which could indicate a dependence on the PI3K signaling pathway for survival [64].
5.3.1.3 Comparison of cytotoxicity between micellar DM-PIT-1 and its novel analogs in A2780 ovarian carcinoma

Figure 32: (A) Comparison of % cell viability of A2780 ovarian carcinoma cells 24 hr following administration of formulation containing DM-PIT-1 or novel analog. (B) Combination of DM-PIT-1 or novel analog with 0.5 µg/mL micellar loaded TRAIL. (n=3 error bars indicate SD, *p < 0.05, ***p < 0.001) [26]

This study was conducted to demonstrate the efficacy of micellar preparations of the novel analogs while comparing their cytotoxic activities to micellar DM-PIT-1. Focusing in Figure 32 (A) on the comparison between free drug and micellar formulations of compounds illustrates that the efficacy of DM-PIT-1, NCL-176 and NCL-240 is at least maintained when drug is incorporated in micelles. In the case of NCL-179 and NCL-198, the incorporation of drug into micelles almost completely abrogates the cytotoxicity of the compounds. Increasing the incubation time from 24 hrs to 48 or 72 hrs had little effect in increasing the toxicities of micelle loaded NCL-179 and NCL-198 (data not shown). These results combined with the micelle release study (Figure 25) indicate that these formulations are stable in cell culturing conditions preventing release of free drug.

Figure 32 (A) also indicates that NCL-176 has similar cytotoxic activity to DM-PIT-1 across most formulations. In the 50 µM drug-loaded TRAIL-modified micelle formulations, NCL-176 is shown to have significantly higher cytotoxicity than DM-PIT-1. However, NCL-240 demonstrates improved cytotoxicity across all formulations and the incorporation of NCL-240 into micelles has little effect on the cytotoxic activity.
The excellent cytotoxicity profile and modifications to the compound resulting in increased stability in tissues demonstrated both NCL-176 and NCL-240 as promising candidates for *in vivo* tumor reduction studies.

### 5.3.1.4 Cytotoxicity of NCL-240 loaded/TRAIL-modified micelles against various sensitive and resistant cell lines

**Figure 33:** Comparison of % cell viability of SKOV-3 sensitive (blue) and resistant (red) cells 24 hr following administration of formulation. (n=3 error bars indicate SD, **p < 0.01, ***p < 0.001)

Following upon the results of the administration of DM-PIT-1 loaded/TRAIL-modified micelles on SKOV-3 sensitive and resistant cells outline in Figure 31, NCL-240 loaded/TRAIL-modified micelles were tested for their ability to overcome drug resistance and inhibit SKOV-3 TR cell growth. Figure 33 illustrates the effect of NCL-240 loaded micelles and combination NCL-240/TRAIL-modified micelles. TRAIL alone is relatively non-toxic to sensitive cells, though there is some effect seen against resistant cells. Both NCL-240 micelles and combination micelles illustrate dose-dependent cytotoxicity against SKOV-3 sensitive and resistant variants with significant toxicities seen down to 12.5 μM concentrations of drug. Additionally, all
formulations were more effective against the resistant variant of SKOV-3 as compared to the sensitive form (p < 0.001).

**Figure 34:** Comparison of % cell viability of A549 sensitive (blue) and resistant (red) cells 24 hr following administration of formulation. (n=3 error bars indicate SD, **p < 0.01, ***p < 0.001)

The breast adenocarcinoma A549 and its paclitaxel resistant variant were also studied for their susceptibility to NCL-240 and NCL-240/TRAIL-modified micelles. Confirmation of effective growth inhibition in multiple drug resistant cell lines is important to establish proof of effect. As seen in Figure 34 both the sensitive and resistant variants were more susceptible to TRAIL than other cell lines tested. As a result, combination NCL-240/TRAIL-modified micelles were extremely effective at inhibiting cell growth. As seen with SKOV-3 TR cells, A549 TR cells were also more susceptible to NCL-240, likely due to their increased PI3K/Akt signaling activity [65]. Once again a clear dose-dependent toxicity is illustrated in both NCL-240 and combination NCL-240/TRAIL-modified formulations.
**Figure 35:** Comparison of % cell viability of MCF-7 sensitive (blue) and resistant (red) cells 24 hr following administration of formulation. (n=3 error bars indicate SD, ***p < 0.001)

MCF-7 breast adenocarcinoma sensitive and doxorubicin resistant cells were also employed to determine the effect of NCL-240 and combination NCL-240/TRAIL-modified micelles on cell viability. As expected, TRAIL had little effect on MCF-7 cells as they have been documented to possess significant TRAIL resistance [66, 67]. NCL-240, however, displays significant cytotoxic effects against the resistant variant of MCF-7 at doses down to 12.5 µM. Akt has been demonstrated to have high levels of activity in MCF-7 doxorubicin resistant cells and the inhibition of activity by NCL-240 is likely the reason for decreased cell viability [68].
5.4 Conclusions

These studies taken together indicate that drug-loaded, TRAIL-modified micelles are effective against a variety of tumor cell types treated in vitro. Initial studies with DM-PIT-1 indicate that the inclusion of drug into the micelle and addition of TRAIL onto the surface of micelles did not significantly alter the cytotoxic activity of the compounds. Additionally, plain PEG-PE micelles do not have large effects on cell viability in keeping with previously reported results [3]. Evaluation of the cytotoxic effects of second generation DM-PIT-1 analogs loaded into PEG-PE micelles yielded successful cytotoxic profiles for both NCL-176 and NCL-240 indicating their potential application in future in vivo applications. Micellar loaded NCL-179 and NCL-198 were not effective at inhibiting A2780 growth in vitro. It is hypothesized that this is likely an effect of poor micellar release as evidenced in Figure 25.

NCL-240 was additionally tested against several sensitive/resistant pairs. In all three cell lines examined, NCL-240 and NCL-240/TRAIL-modified micelles exhibited increased cytotoxicity against the resistant variant. This is likely due to the increased PI3K/Akt activity present in these resistant variants as has been reported in previously published studies [64, 65, 68]. Further elucidation of the precise effect of these combination formulations is warranted to confirm this hypothesis.
6 CHARACTERIZATION OF TF-MODIFIED MICELLES TARGETING POTENTIAL AND THEIR EFFECTS ON CYTOTOXICITY IN VITRO

6.1 Introduction

The addition of targeting ligands onto the surface of nanoparticles continues to be one of the most attractive features of their use in anticancer applications. It is well established that many cancer cells differentially express cell surface receptors and epitopes that are capable of being targeted [69, 70]. The targeting of drug-loaded nanoparticles allows for preferential accumulation at the tumor site and increased uptake of the drug. Increased accumulation allows for better therapeutic outcomes at similar doses. Alternatively, the ability to maintain chemotherapeutic activity with less drug administered is also possible. Both of these scenarios have the potential to significantly improve patient outcomes.

Many tumor cell types express increased amounts of the cell surface transferrin receptor (TfR). Increased metabolic activity of cancer cells necessitates increased iron uptake [71]. Transferrin protein is able to bind iron in the blood stream and allows for the recognition and binding to TfR. This receptor functions by binding free transferrin and initiating endocytosis resulting in the uptake of TF. The iron is then released in the cell and the TfR is recycled back to the surface [72]. Several examples of effective nanoparticle targeting of the TfR have been demonstrated [7, 15, 73]. The studies outlined below attempt to analyze the effect of TF-targeting on the cellular association and cytotoxicity of PEG-PE micelle formulations. Additionally, TF-modification effects on the targeting and toxicity of drug-loaded and TRAIL-modified micelles are examined.
6.2 Materials and Methods

6.2.1 Formulation of TF-modified micelles

TF-modified micelles were prepared by the thin-film hydration method as described previously [6]. Briefly, PEG_{2000}-DSPE dissolved in chloroform was added to round bottom flasks. 50% (by mole of DSPE-PEG_{2000}) pNP-PEG_{3400}-PE dissolved in chloroform was added and the sample vortexed for complete mixing. Chloroform was then evaporated under a rotary evaporator to form a thin film. Films were further dried by freeze-drying on a Freezone 4.5 Freeze Dry System (Labconco, Kansas City, MO) for at least 4 hr. Films were rehydrated with stock TF (Sigma, St. Louis, MO) in 200 µL 1x PBS pH 8.5 at a molar ratio of pNP-PEG-PE:TF 2.5:1. Samples were then vortexed for 5 min. The pH of the solution was determined and adjusted with 1.0 N NaOH to 8.5 if necessary. Reaction proceeded for 4 hrs at room temperature to allow sufficient TF conjugation and complete hydrolysis of unreacted pNP groups. TF-modified micelles were then dialyzed using a 100,000 MWCO membrane against 1 L 1x PBS pH 7.4 for 1 hr followed by another 4 hrs of dialysis in 1 L fresh 1x PBS pH 7.4.

6.2.2 Characterization of TF-modified micelles

Conjugation efficiency of TF was measured using a micro BCA kit (Pierce, Rockford, IL) according to the provider’s procedure. Protein content was determined by comparing TF-modified micelles to BCA standards. Signals from TF samples were normalized with plain micelle samples at the same lipid concentration.

All micelle samples were measured for their size distribution and zeta-potential using the Zetaplus (Brookhaven Instruments Corporation, Holtsville, NY). Briefly, 50 µL of micelle sample was dissolved in 1 mL of distilled water and micelle size and zeta-potential were analyzed according to manufacturer’s protocol.
6.2.3 Preparation of final formulations

Following conjugation of TF and TRAIL to the micelle and characterization of the concentration of ligands on the surface, micelles were combined to form final formulations. For the cell association studies, TF and TRAIL-modified micelles were coincubated with Rhodamine labeled micelles at appropriate amounts resulting in formulations possessing known concentrations of TF and/or TRAIL. Formulations for the cytotoxicity studies were prepared by coincubating TF and or TRAIL with NCL-240 loaded or plain micelles.

6.2.4 FACS analysis of micellar targeting by TRAIL-modified and TF-modified micelles

Cell association of TRAIL-modified, TF-modified or combination TRAIL/TF-modified micelles was compared to unmodified micelles using fluorescence associated cell sorting (FACS) (BD FACS Caliber flow cytometer, San Jose, CA). Briefly, cells were seeded in 6-well plates at a concentration of 200,000 cells/well and placed in an incubator overnight. 1% (mol) Rhodamine-PE was included in plain micelles, TRAIL-modified and TF-modified micelles. Following dialysis, samples were coincubated according to appropriate concentrations of targeting ligand (0.5, 1, 2 or 8 µg/mL). All samples were normalized based on their fluorescent signal prior to cell treatments resulting in each sample containing comparable fluorescent formulation addition. Micelles were added to serum complete media at a concentration of 0.3 mg/ml lipid. Cells were washed, trypsinized and prepared for analysis following either 4 hr or 24 hr incubation.

6.2.5 Cell viability assay

Viability of various cell lines was measured using CellTiter-Blue® (Promega, Madison, WI) viability assay. Briefly, cells were seeded in 96-well plates according to proper cell densities (3,000-5,000 cells). After 24 hr incubation in 5% CO₂ at 37° C, cells were then treated with formulation for 2 hrs. Following treatment, media was removed, the wells were washed with serum complete media and replaced fresh serum complete media. 24 hr following incubation with formulations, cells were washed again and incubated with 50 µL media and 10 µL CellTiter-Blue®. Cell viability was evaluated after 2 hrs of incubation at 37° C at 5% CO₂ by
measuring the fluorescence (ex. 550 nm, em. 590 nm) using Synergy HT multi-detection microplate reader (Biotek, Winooski, VT).

6.2.6 Statistical analysis

Wherever possible, data was generated in triplicates for proper statistical analysis. *In vitro* experiments are reported as mean +/- standard deviation. One-way ANOVA followed by Tukey's multiple comparison tests were performed with significance determined by a p-value < 0.05.
6.3 Results and Discussion

6.3.1 Size and zeta-potential of TRAIL-modified and TF-modified micelles

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Size (nm)</th>
<th>Zeta-potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 µg TRAIL-modified micelles</td>
<td>18.5 +/- 0.7</td>
<td>-17.4 +/- 0.8</td>
</tr>
<tr>
<td>2 µg TRAIL-modified micelles</td>
<td>21.7 +/- 0.7</td>
<td>-17.4 +/- 1.2</td>
</tr>
<tr>
<td>8 µg TRAIL-modified micelles</td>
<td>40.1 +/- 3.1</td>
<td>-20.3 +/- 1.0</td>
</tr>
<tr>
<td>Freshly prepared TF-conjugated micelles</td>
<td>84.2 +/- 2.1</td>
<td>-37.7 +/- 1.7</td>
</tr>
<tr>
<td>0.5 µg TF-modified micelles</td>
<td>37.4 +/- 4.0</td>
<td>-30.1 +/- 3.1</td>
</tr>
<tr>
<td>1 µg TF-modified micelles</td>
<td>37.5 +/- 5.2</td>
<td>-30.4 +/- 0.7</td>
</tr>
<tr>
<td>2 µg TF-modified micelles</td>
<td>46.5 +/- 6.1</td>
<td>-35.5 +/- 1.6</td>
</tr>
</tbody>
</table>

Table 6: Size and zeta-potential of TRAIL-modified and TF-modified micelles used in FACS cell association studies. (n=3 with SD shown)

The size and zeta-potential analysis of TF and TRAIL-modified targeted micelles confirm that they are able to form nanosized micelles with a size consistent with surface modified micelles. Freshly prepared TF-conjugated micelles had a size of 84.2 nm and an increasing concentration of TF or TRAIL included in the formulation results in a larger size and a lower zeta-potential.

6.3.2 TF conjugation to pNP-PEG-PE micelles

![Standard Curve of TF Content determined by BCA](image)

**Figure 36**: Standard curve of TF concentration as determined by BCA assay.
TF-modified micelles were analyzed for their protein concentration as determined by the BCA assay. Following extensive dialysis, TF-modified micelles were incubated with BCA reagent. Subtracting plain DSPE-PEG2000 micelle absorbance from the TF-modified micelle absorbance normalized any increased signal from the presence of micelles in the system. Figure 36 indicates that the BCA method is capable of accurately correlating absorbance to TF concentration. Using the standard curve similar to the one above provides the ability to quantify the amount of TF conjugated to the micelles.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Reaction yield (%)</th>
<th>TF concentration on Micelles (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TF-modified micelles</td>
<td>33.1% +/- 2.9%</td>
<td>12.01 +/- 1.0 mg/mL</td>
</tr>
</tbody>
</table>

Table 7: Average reaction yield and concentration of TF modified to micelles. (n=3 with SD shown)

Table 7 shows the average reaction yield and total TF concentration. Reaction yield is lower than that seen for TRAIL as a result of the reaction conditions. The ratio of pNP-PEG-PE to TF is 2.5:1 rather than the 40:1 ratio for TRAIL—modified micelle preparation. The concentration of TF on the micelles is sufficient for further \textit{in vitro} analysis of cell targeting by FACS.

6.3.3 FACS analysis of cell association of TRAIL-modified, TF-modified and combination micelles

![Figure 37: Cell association of unmodified and TRAIL-modified Rhd-labeled micelles treated on A2780 ovarian carcinoma in serum complete media over 4 and 24 hrs as measured by FACS. (n=3 error bars indicate SD, *p < 0.05 as compared to non-targeted micelles)](image)
TRAIL-modified Rhd-labeled micelles containing various concentrations of TRAIL were incubated for 4 or 24 hrs in serum complete media. The left panel of Figure 37 shows that after 4 hrs there is no significant difference in cell association between plain micelles and TRAIL-modified micelles. However 24 hrs of incubation leads to a significant increase in cell association of all TRAIL-modified formulations. It is also clear that 0.5 μg/mL of TRAIL is sufficient to achieve a higher cell association at 24 hrs.

It is reasonable to conclude that an increase in cytotoxic activity of combination drug-loaded/TRAIL-modified micelles could be the result of increased cellular accumulation of drug. This however does not explain the similar cytotoxicity results between micellar-loaded drug given with free TRAIL and combination micelles with TRAIL conjugated to the micelle (Figure 26). Any additional cellular accumulation of drug is an additional advantage of TRAIL conjugation.

**Figure 38:** Cell association of targeted and non-targeted Rhd-labeled micelles treated on A2780 ovarian carcinoma in serum complete media over 4 hrs as measured by FACS. (n=3 error bars indicate SD, *p < 0.05, ***p < 0.001)

A2780 cells were chosen as a model cell line to evaluate TF-targeting as this cell line has been identified to express high levels of TfR [74]. TF-modified Rhd-labeled micelles were formulated with and without 0.5 μg/mL TRAIL included in the formulation. This study aimed to increase the cell association seen at 4 hrs. As
shown in Figure 38, TF-modified micelles all showed significantly higher cell association than both non-targeted micelles and TRAIL-modified micelles. There was a small increase in targeting when TRAIL was combined with TF (only 1.0 µg TF was significantly different). It is clear from these results that including TF-conjugate as a targeting agent increases cell association at concentrations as low as 0.5 µg/mL (corresponds to 0.17 wt% TF/lipid). Additionally, the presence of TRAIL-conjugate does not interfere with TF-targeting; therefore illustrating TF-modified micelles could have utility in tumor cell targeting and drug delivery.

6.3.4 Cytotoxicity of TF-targeted drug-loaded and TRAIL-modified micelles

The ultimate goal of including a targeting ligand in a drug delivery formulation is to increase the ability of the nanoparticle to associate and enter the cells resulting in an increased cellular accumulation of drug. This study aimed to determine the effects of TF-modification on the cytotoxicity of TRAIL-modified and NCL-240 loaded micelles on A2780 and U87-MG cells. With the exception of experiments identifying the cytotoxic effect of TF-micelles alone on the cells (Figure 39 and Figure 42) all TF-modified micelles contain 0.5 µg/mL TF.

**Figure 39:** Comparison of % cell viability of A2780 cells 24 hr following 2 hr incubation of either TF-targeted formulation (red) or plain micelles (black). (n=3 error bars indicate SD)
Figure 40: Comparison of % cell viability of A2780 cells 24 hr following 2 hr incubation of either TF-targeted formulation (red) or non-targeted TRAIL formulation (black). (n=3 error bars indicate SD)

Figure 41: Comparison of % cell viability of A2780 cells 24 hr following 2 hr incubation of either TF-targeted formulation (red) or non-targeted NCL-240 micelles (black). (n=3 error bars indicate SD, *p < 0.05)
**Figure 42:** Comparison of % cell viability of U87-MG cells 24 hr following 2 hr incubation of either TF-targeted formulation (red) or plain micelles (black). (n=3 error bars indicate SD)

**Figure 43:** Comparison of % cell viability of U87-MG cells 24 hr following 2 hr incubation of either TF-targeted formulation (red) or non-targeted TRAIL formulation (black). (n=3 error bars indicate SD)
Figure 44: Comparison of % cell viability of U87-MG cells 24 hr following 2 hr incubation of either TF-targeted formulation (red) or non-targeted NCL-240 micelles (black). (n=3 error bars indicate SD, *p < 0.05, **p < 0.01)

Both U87-MG and A2780 cells showed no effect of addition of TF to plain micelles up to 8 µg/mL of TF. Additionally, TF-targeted formulations also containing surface bound TRAIL (TF-TRAIL micelles) did not show any difference as compared to non-targeted TRAIL micelles. TF-targeting occurs through receptor-mediated endocytosis, which likely inhibits the ability of TRAIL to bind to their target Death Receptor on the surface. Thus, there is limited effect of TRAIL in these formulations. Only the higher doses of NCL-240 loaded micelles showed an improvement in cytotoxicity following the addition of TF-targeting. This is likely because of the exposure time of the cells to the formulation is relatively short at 2 hrs.
6.4 Conclusions

The pNP-PEG-PE conjugate is able to effectively react with TF resulting in micelles that have the ability to target A2780 cells and increase cellular association. TRAIL alone does not have any increased cellular association over a 4 hr incubation though increases in association can be seen following 24 hr. Additionally, combination TRAIL/TF-modified micelles do not significantly differ in cellular association from TF-modified micelles indicating TRAIL does not interfere with TF-mediated cellular association. It does not, however, enhance the association in anyway. TF-modified micelles do not show any cytotoxicity against both A2780 and U87-MG cells at concentrations up to 8 µg/mL. There are also no increases in cytotoxicity when included onto TRAIL-modified micelles. Lastly, TF-modified/NCL-240 micelles possess significantly increased cytotoxicity against U87-MG and A2780 indicating the potential of these targeted micelles in future *in vitro* and *in vivo* studies.
7 FORMULATION AND CHARACTERIZATION OF SIRNA-S-S-PE MIXED MICELLES AND EVALUATION OF GENE SILENCING

7.1 Introduction

Mussachio et al. established the use of siRNA-S-S-PE mixed micelles as a novel siRNA delivery system in initial proof-of-concept studies [52]. These previous studies have indicated that this system can be used to successfully silence GFP production in a GFP-expressing cell line in vitro. There were limitations with the system however, and further optimization was hypothesized to lead to higher silencing of target genes at lower concentrations of lipid and siRNA. Utilizing various chain-lengths of PEG in the micelle forming PEG-PE lipid may have the ability to increase siRNA-S-S-PE incorporation into the micelle thus allowing for increased delivery of siRNA at comparable lipid concentrations.

Attempts at characterizing siRNA-S-S-PE into micelles have proven difficult using traditional reverse-phase HPLC. Size-exclusion chromatography, allowing separation based on the size of the analytes in the system, was thought to be an improved method to qualitatively and quantitatively evaluate the incorporation of siRNA-S-S-PE in mixed micelles. Development of a method to analyze siRNA-S-S-PE mixed micelles was critical for the characterization of this delivery system.

Additionally, silencing of functional genes in a tumor cell model had not yet been attempted. The survivin gene has been demonstrated to be effective target of siRNA silencing, possessing the ability to inhibit tumor growth, especially in combination with chemotherapy [46]. It is therefore hypothesized that combining siRNA against survivin formulated into siRNA-S-S-PE micelles, with the drug-loaded/TRAIL modified micelles evaluated previously, that an increase in cytotoxicity is possible at lower doses of therapy.

Utilizing siRNA-S-S-PE mixed micelles to silence a target gene of interest had yet to be evaluated in an in vivo system. This work aims to confirm the utility of this system by silencing a target protein in the liver of mice. The liver is an excellent organ to target as it is highly perfused allowing the exposure of the siRNA-containing micelles to the cells of interest [75-77]. The blood clotting agent Factor VII was chosen as the target
for *in vivo* evaluation of siRNA-induced silencing as it is expressed solely in hepatocytes, rather than the Kupffer cells of the reticuloendothelial system (RES) [78]. Additionally, Factor VII is secreted into the serum allowing for simple detection through blood collection.
7.2 Materials and Methods

All siRNA experiments were performed under nuclease free conditions. Nuclease free water (Qiagen, Germantown, MD) and 10x PBS (Invitrogen, Carlsbad, CA) were utilized in formulation preparation. RNaseZap (Invitrogen, Carlsbad, CA) was employed to clean all laboratory equipment and glassware.

7.2.1 Formulation and characterization of siRNA-S-S-PE mixed micelles

7.2.1.1 Synthesis of siRNA-S-S-PE

Synthesis of siRNA-S-S-PE was done according to previously published protocols (Figure 45) [52]. A solution of the SPDP-activated siRNA (20 nmol) (Invitrogen, Carlsbad, CA and ThermoFisher Biosciences, Lafayette, CO) in nuclease free water, pH 7.4 (120 µL), was added drop wise to a solution of 1,2-dipalmitoyl-sn-glycero-3-phosphothioethanol (DPPE-SH) (2 µmol) (Avanti Polar Lipids Inc., Alabaster, AL) in DMSO and CHCl₃ (total volume of organic solvents 350 µL). The reaction was carried out for 48 hr at room temperature with continuous shaking. The unreacted DPPE-SH was removed on a d-salt dextran desalting column (Pierce, Rockford, Il). The collected samples containing the siRNA-conjugate were centrifuged for 5 min at 14,000 rpm to further remove mixed solvents and/or unreacted DPPE-SH. The siRNA-S-S-PE conjugate was stored at -20° C. Concentration of siRNA-S-S-PE was determined using UV/Vis absorbance at 280 nm on a NanoDrop 2000c Spectrophotometer (Thermo Scientific, Wilmington, DE). Samples were analyzed against a standard curve of absorbance at known siRNA concentrations.

The following siRNA sequences were employed to target the genes of interest. Anti-GFP siRNA was utilized as a scrambled control siRNA for in vitro and in vivo controls.

Anti-Survivin siRNA [46]:

Sense: 5’-GCAUUCGUCCGCUUGCGCU(dTdT)-SPDP-3’

Antisense: 5’-AGCGCAACCGGACGAAUGC(dTdT)-3’
Anti-Factor VII siRNA [75]:

Sense: 5′-GGAUCAUCUCAAGUCUUAC(dTdT)-SPDP-3′

Antisense: 5′-GUAAGACUUGAGAUGAUCC(dTdT)-3′

Anti-GFP (scramble) siRNA:

Sense: 5′-AUGAACUUCAGGGUCAGCU(dTdT)-SPDP-3′

Antisense: 5′-AGCUGACCCUGAAGUCAU(dTdT)-3′

Figure 45: Schematic of siRNA-S-S-PE formation [52].

The siRNA-S-S-PE conjugate was analyzed for the cleavability of the disulfide bond under physiologically relevant conditions. Briefly, siRNA-S-S-PE conjugates were incubated in 10 mM glutathione at 37° C for 4 hrs. The reaction mixture was then spotted on silica gel TLC plates (Sigma-Aldrich, St Louis, MO). 1 µL was spotted 3 times with sufficient time allowed for drying between spotting. Uncleaved siRNA-S-S-PE at identical concentrations was spotted and DPPE-SH was also spotted as an additional control. The plate was run.
in a mobile phase of CHCl₃:MeOH (80:20) and stained with molybdenum blue for the presence of phospholipid.

7.2.1.2 Formulation of siRNA-S-S-PE mixed micelles

Lipid films were prepared by adding various chain-length (1000, 2000 or 3000 Da) PEG-PE in chloroform to round bottom flasks. Chloroform was evaporated using a rotary evaporator and further dried by freeze-drying on a Freezone 4.5 Freeze Dry System (Labconco, Kansas City, MO) for at least 4 hr. Lipid films were then rehydrated with siRNA at 1:250, 1:500 or 1:750 (siRNA-S-S-PE/DSPE-PEG) weight ratios in nuclease free water and vortexed for 5 min. Rehydrated lipid concentration was held constant at 10 mg/mL in all samples.

Combination micelle formulations were prepared by rehydrating appropriate aliquots of lyophilized siRNA-S-S-PE with NCL-240 loaded/TRAIL-modified micelles. Additional plain PEG-PE micelles were added to maintain appropriate siRNA-S-S-PE:PEG-PE weight ratios. Incorporation of drug and siRNA-S-S-PE was not affected in the presence of the new components in the system and incorporations amounts were similar to those analyzed previously.

7.2.1.3 Characterization of siRNA-S-S-PE mixed micelles

Encapsulation efficiency of siRNA-S-S-PE into PEG-PE micelles composed of various PEG-chain lengths was analyzed using size exclusion chromatography (SEC) with a Shodex protein KW-800 column (Showa Denko America, New York, NY). Samples in nuclease free water were injected into the column containing a mobile phase of 50 mM NaCl and 50 mM Tris-HCl (pH 8.0) with a flow-rate of 1.0 mL/min. siRNA was measured via UV detection at 280 nm.

All micelle samples were measured for their size distribution and zeta-potential using the Zetaplus (Brookhaven Instruments Corporation, Holtsville, NY). Briefly, 50 µL of micelle sample was dissolved in 1 mL of nuclease free water and micelle size and zeta-potential were analyzed according to manufacturer’s protocol.
7.2.2 In vitro survivin silencing

A2780 cell lines were seeded into 24-well plates. Cells were treated with survivin siRNA-S-S-PE/PEG-PE mixed micelles (1:750 wt ratio) at a final concentration of 200 nM in serum complete media for 6 hr. Cells were washed, serum complete media was added and the cells were incubated for a further 48 hr. Survivin expression was then determined via survivin elisa kit (R&D systems, Minneapolis, MN) according to manufacturer’s protocol. Briefly, cells were rinsed three times with 1x PBS and treated with 200 mL of lysis buffer for 30 min on ice. Cell lysates were collected, vortexed, and incubated on ice for another 15 min. Cell debris was removed by centrifugation at 2000 g for 5 min and protein concentrations were determined by BCA assay (Pierce, Rockford, IL) after 6-fold dilution in 1x PBS. Following blocking of the elisa plate, samples were added into captured antibody pre-coated 96-well plates and incubated for 2 hr at room temperature. The plate was then rinsed twice and incubated with detection antibody for a further 2 hr. Streptavidin-HRP detection was then performed and plate was read at 450 nm (Synergy HT multi-detection microplate reader, Biotek, Winooski, VT).

7.2.3 Cell viability

Viability of various cell lines was measured using CellTiter-Blue® (Promega, Madison, WI) viability assay. Briefly, cells were seeded in 96-well plates according to proper cell densities (3,000-5,000 cells). After 24 hr incubation in 5% CO₂ at 37°C, cells were then treated with formulation under appropriate conditions depending on the experiment. Following treatment, media was removed, the wells were washed with serum complete media and incubated with 50 µL media and 10 µL CellTiter-Blue®. Cell viability was evaluated after 2 hrs of incubation at 37°C at 5% CO₂ by measuring the fluorescence (ex. 550 nm, em. 590 nm) using Synergy HT multi-detection microplate reader (Biotek, Winooski, VT).
7.2.4 siRNA-S-S-PE mixed micelle in vivo silencing

Mixed micelles containing siRNA-S-S-PE loaded into PEG\textsubscript{2000}-PE micelles or controls were administered as 200 μL intravenous (i.v.) injections into the tail vein of C57BL-6 mice. The formulations given were either 1.6 mg/kg Factor VII siRNA, 0.8 mg/kg Factor VII siRNA, 1.6 mg/kg Scramble siRNA or 1x PBS. All siRNA formulations were prepared as 80 mg/mL micelles in 1x PBS with an siRNA:PEG-PE ratio of 1:300. This was due to the limited dose volumes capable of administering i.v. in mice and the desire to deliver sufficient siRNA to achieve silencing. The 0.8 mg/kg Factor VII siRNA formulation was prepared by diluting 1.6 mg/kg siRNA formulation to the appropriate siRNA concentration. Therefore the lipid concentration of this formulation was only 40 mg/mL upon injection.

Following 48 hr, mice were anesthetized with 100 μL ketamine-xylazine and blood was collected into serum separator tubes (Becton Dickinson, Franklin Lakes, NJ) via cardiac puncture. Tubes were centrifuged for 10 min at 5000 g and serum was transferred to separate eppendorf tubes. Serum levels of Factor VII protein were determined in samples using the Biophen FVII (Aniara Corporation, West Chester, OH) chromogenic assay according to manufacturers' protocols. Serum Factor VII levels were compared against 1x PBS treated control mice and silencing is reported as percentage of serum Factor VII as compared to 1x PBS treated mice.

7.2.5 Statistical analysis

Wherever possible, data was generated in triplicates for proper statistical analysis. In vitro and in vivo silencing experiments are reported as mean +/- standard deviation. One-way ANOVA followed by Tukey's multiple comparison tests were performed with significance determined by a p-value < 0.05.
7.3 Results and Discussion

7.3.1 Formulation and optimization of siRNA-S-S-PE/PEG-PE mixed micelles

![Standard Curve of siRNA Concentration](image)

**Figure 46:** Standard curve of siRNA concentration as measured by Nanodrop UV/Vis Spectrophotometer.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>% siRNA recovered</th>
</tr>
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<tbody>
<tr>
<td>siRNA-S-S-PE</td>
<td>92.2 % +/- 2.1%</td>
</tr>
</tbody>
</table>

**Table 8:** siRNA-SPDP/DPPE-SH reaction yield measured as percentage of siRNA-S-S-PE recovered following separation on dextran desalting column.

The standard curve indicated in Figure 46 indicates that the method used to measure siRNA concentration is effective across a range of concentrations. The siRNA-SPDP/DPPE-SH conjugation reaction is highly efficient with siRNA-S-S-PE recovery of approximately 92.2% (Table 8). These reaction products can be rehydrated to any concentration in nuclease free water and can further be lyophilized and rehydrated in the presence of PEG-PE micelles to form mixed siRNA-S-S-PE/PEG-PE micelles.
**Figure 47:** Confirmation of cleavage of disulfide bond between siRNA and PE by thin-layer chromatography. The plate is stained with molybdenum blue following incubation with 10 mM glutathione at 37°C for 4 hrs. Evidence of cleavage indicated by the presence of free lipid in the cleaved sample (C=siRNA conjugate, R=siRNA conjugate following cleavage, P=PE-SH).

Figure 47 indicates that the siRNA-S-S-PE conjugate can be cleaved into component siRNA and PE-SH under a physiologically relevant reducing environment. The presence of free phospholipid in the sample labeled R indicates that the disulfide bond was cleaved and the PE-SH is free to run on the plate. Intracellular glutathione levels are found to be at approximately 10 mM while blood levels are approximately 2 mM. This is further evidence for the use of this system for siRNA delivery.
Figure 48: Overlay of size exclusion chromatography (SEC) chromatograms of separate injections of Plain PEG-PE Micelles, siRNA-SPDP and the siRNA-S-S-PE Conjugate. Absorbance measured at 260 nm.

Figure 49: Size exclusion chromatography (SEC) chromatogram of siRNA-S-S-PE/PEG-PE mixed micelles at a weight ratio of (1:750). Absorbance measured at 260 nm.

Size exclusion chromatography was utilized to better characterize the siRNA-S-S-PE conjugate and the incorporation into PEG-PE micelles. Previous methods relying on reverse phase HPLC were insufficient to accurately determine the incorporation of siRNA-S-S-PE into the micelles. Using this method it was determined that both siRNA-S-S-PE and siRNA-SPDP eluted with a longer retention time than micelles (Figure 48)
indicating they are of a smaller size and the siRNA-S-S-PE conjugate likely does not form a micelle when alone in an aqueous system. Additionally, the peak separation is adequate to determine the amount of siRNA-S-S-PE incorporated into the micelle (Figure 49).

<table>
<thead>
<tr>
<th>siRNA-S-S-PE + PEG_{1000}-PE micelles (weight ratio)</th>
<th>% siRNA incorporated</th>
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</thead>
<tbody>
<tr>
<td>1:200</td>
<td>0 %</td>
</tr>
<tr>
<td>1:500</td>
<td>46 %</td>
</tr>
<tr>
<td>1:750</td>
<td>96 %</td>
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<table>
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<tr>
<th>siRNA-S-S-PE + PEG_{2000}-PE micelles (weight ratio)</th>
<th>% siRNA incorporated</th>
</tr>
</thead>
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<tr>
<td>1:200</td>
<td>26 %</td>
</tr>
<tr>
<td>1:500</td>
<td>31 %</td>
</tr>
<tr>
<td>1:750</td>
<td>52 %</td>
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</table>

<table>
<thead>
<tr>
<th>siRNA-S-S-PE + PEG_{3000}-PE micelles (weight ratio)</th>
<th>% siRNA incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:200</td>
<td>12 %</td>
</tr>
<tr>
<td>1:500</td>
<td>23 %</td>
</tr>
<tr>
<td>1:750</td>
<td>34 %</td>
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</table>

**Table 9:** siRNA-S-S-PE conjugate incorporation into micelles composed of various PEG-PE chain lengths and at various weight ratios of siRNA-S-S-PE/PEG-PE as determined by size exclusion chromatography.

The effect of the chain length of PEG used in the PEG-PE component of the micelle on the incorporation of siRNA-S-S-PE and the size of the particles was determined. The newly developed SEC method of separation was utilized to determine the siRNA-S-S-PE incorporation concentration and efficiency (Table 9). The sizes of the micelles were determined as well and are indicated in Table 10. It was hypothesized that the chain length of PEG would affect the properties of the micelle and subsequent loading of siRNA-S-S-PE. It was determined that PEG_{2000}-PE is the optimum micelle forming material, as there is good loading across various weight ratios and the size of the micelles are consistent.
<table>
<thead>
<tr>
<th>siRNA-S-S-PE + PEG&lt;sub&gt;1000&lt;/sub&gt;-PE micelles (weight ratio)</th>
<th>Diameter (nm)</th>
<th>Polydispersity Index</th>
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<tr>
<td>1:200</td>
<td>24.2</td>
<td>0.28</td>
</tr>
<tr>
<td>1:500</td>
<td>15.1</td>
<td>0.17</td>
</tr>
<tr>
<td>1:750</td>
<td>13.0</td>
<td>0.19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>siRNA-S-S-PE + PEG&lt;sub&gt;2000&lt;/sub&gt;-PE micelles (weight ratio)</th>
<th>Diameter (nm)</th>
<th>Polydispersity Index</th>
</tr>
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<tbody>
<tr>
<td>1:200</td>
<td>18.7</td>
<td>0.19</td>
</tr>
<tr>
<td>1:500</td>
<td>18.3</td>
<td>0.19</td>
</tr>
<tr>
<td>1:750</td>
<td>20.2</td>
<td>0.27</td>
</tr>
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</table>

<table>
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<tr>
<th>siRNA-S-S-PE + PEG&lt;sub&gt;3000&lt;/sub&gt;-PE micelles (weight ratio)</th>
<th>Diameter (nm)</th>
<th>Polydispersity Index</th>
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<td>1:200</td>
<td>21.9</td>
<td>0.16</td>
</tr>
<tr>
<td>1:500</td>
<td>20.4</td>
<td>0.17</td>
</tr>
<tr>
<td>1:750</td>
<td>18.2</td>
<td>0.18</td>
</tr>
</tbody>
</table>

**Table 10:** Size and polydispersity index (PDI) of siRNA-S-S-PE mixed micelles composed of various PEG-PE chain lengths and at various weight ratios of siRNA-S-S-PE/PEG-PE.
7.3.2 In vitro characterization of siRNA-S-S-PE mixed micelles

![Survivin protein levels in A2780 following treatment with various formulations](image)

**Figure 50:** A2780 survivin silencing following treatment with various siRNA-containing formulations. Formulations were incubated for 6 hr on cells with survivin concentrations measured 48 hr later. Samples normalized to total protein content. (n=3 error bars indicate SD, ***p < 0.001)

Anti-survivin siRNA formulated as siRNA-S-S-PE/PEG-PE (1:750 weight ratio) mixed micelles was able to effectively silence survivin expression in A2780 cells. The inclusion of siRNA-S-S-PE into micelles is required for activity, as free survivin-S-S-PE has no silencing effect. Additionally, scrambled siRNA-S-S-PE formulated into mixed micelles at identical weight ratios has no effect on survivin silencing indicating silencing is not a result of off-target effects. These results indicate that siRNA-S-S-PE/PEG-PE mixed micelles are able to silence a therapeutic target of interest in an *in vitro* setting.
Figure 51: Cytotoxicity of A2780 ovarian carcinoma cells treated with various formulations for 6 hr in serum-complete media. Following incubation, cells were washed and media replaced. Cytotoxicity was determined 48 hrs following initial treatment. mSurvivin composed of siRNA-S-S-PE incorporated in PEG\textsubscript{2000}-PE micelles at a ratio of 1:750. (n=3 error bars indicate SD, **p < 0.01, ***p < 0.001).

Based on the previous optimization work, siRNA-S-S-PE was formulated in PEG\textsubscript{2000}-PE micelles at a weight ratio of 1:750. siRNA targeting the survivin was used to treat A2780 cells \textit{in vitro}. This study indicates that this system is able to effectively inhibit growth of A2780 in a dose dependent manner. Administration of plain micelles at comparable concentrations was not highly toxic indicating the cytotoxicity is the result of targeted anti-survivin siRNA-S-S-PE. Additionally, siRNA-S-S-PE administered free (without incorporation into micelles) did not have any significant effect on cell growth indicating the importance of micellar incorporation and protection.
Following successful cytotoxicity studies utilizing anti-survivin siRNA-S-S-PE/PEG-PE mixed micelles, studies were performed preincubating low doses of anti-survivin siRNA on A2780 cells followed by administration of low doses of NCL-240 loaded/TRAIL-modified micelles. This assay tests the hypothesis that silencing survivin prior to administration of drug-loaded/TRAIL-modified micelles will result in an increase in cytotoxicity due to the absence of the survivin pro-survival signaling. Figure 52 indicates that the addition of either 12.5 µM m240 – 0.1 µg/mL mTRAIL or 25 µM m240 – 0.1 µg/mL mTRAIL significantly increases cytotoxicity in a dose dependent manner as compared to siRNA treated cells alone. Though it is not indicated in Figure 52, siRNA pretreatment at 50 nM followed by administration of 25 µM m240 – 0.1 µg/mL mTRAIL results in significantly increased cytotoxicity as compared to samples not pretreated with siRNA (p < 0.001).
Figure 53: % cell viability of A2780 cells following treatment with combination survivin siRNA-S-S-PE/PEG-PE mixed micelles (1:750 weight ratio), NCL-240 loaded/TRAIL-modified micelles for 48 hr. Formulations contained either siRNA-S-S-PE/PEG-PE mixed micelles alone, or in combination with drug-loaded, TRAIL-modified micelles. siRNA sample in w/o siRNA indicates empty PEG-PE micelles (n=3 error bars indicate SD, ***p < 0.001)

Another dosing method combining anti-survivin siRNA-S-S-PE, NCL-240 and TRAIL was investigated in Figure 53. This study determined the cytotoxicity resulting from combining all the therapeutics into a single combination micelle on A2780 cells. Therapies were combined into a single micellar formulation and incubated with A2780 cells continuously for 48 hr. The presence of NCL-240 and TRAIL in the formulations led to significantly increased cytotoxicity across all the formulations of siRNA. Additionally, the addition of at least 10 nM siRNA resulted in significantly increased cytotoxic activity in the NCL-240/TRAIL-modified groups (p < 0.001).

Taken together Figure 52 and Figure 53 indicate utilizing anti-survivin siRNA in combination with NCL-240 and TRAIL is an effective method to induce cell death in A2780. Future examination in various cell lines and elucidation of optimal of dosing schedules and concentrations is needed to maximize combination effects. Additionally, examination of this system in an in vivo model of cancer is warranted.
7.3.3 *In vivo* silencing of Factor VII protein in the liver

![Graph showing % of FVII present in serum 48 hours following IV injection as compared to control mice. Formulations containing 1x PBS (Control), scramble siRNA at 1.6 mg/kg, Factor VII siRNA at 0.8 mg/kg or Factor VII siRNA at 1.6 mg/kg were injected i.v. Blood was collected 48 hr later and Factor VII serum levels were detected. (n=5 error bars indicate SD, *p < 0.05, **p < 0.01, ***p < 0.001.]

**Figure 54:** % of Factor VII protein in serum samples as compared to control treated mice. Formulations containing 1x PBS (Control), scramble siRNA at 1.6 mg/kg, Factor VII siRNA at 0.8 mg/kg or Factor VII siRNA at 1.6 mg/kg were injected i.v. Blood was collected 48 hr later and Factor VII serum levels were detected. (n=5 error bars indicate SD, *p < 0.05, **p < 0.01, ***p < 0.001)

The utility of any siRNA delivery system is ultimately based on the ability of the system to silence targeted genes in their *in vivo* tissue of interest. siRNA-S-S-PE/PEG-PE mixed micelles had not yet been tested *in vivo* and as a proof-of-concept *in vivo* silencing study, Factor VII protein was targeted as gene to silence. As evidenced in Figure 54, formulations of anti-Factor VII siRNA-S-S-PE/PEG-PE (1:300) mixed micelles were able to significantly down-regulate the expression of Factor VII in an *in vivo* mouse model. The modest silencing seen with scramble siRNA formulations could be the result of cross-reactivity of the anti-GFP sequence used as the scramble control. The dose dependent silencing of Factor VII, however, provides evidence of on target specific silencing of the target gene of interest. Additionally, all formulations were well tolerated with no apparent toxicities or weight loss in any of the mice (data not shown).
7.4 Conclusions

Based on the results shown above, siRNA-S-S-PE conjugates incorporated into PEG-PE micelles have demonstrated to be an effective means of siRNA delivery. Initial attempts to increase the loading efficiency and decrease the siRNA-S-S-PE:PEG-PE weight ratio by altering the PEG-chain length indicated that PEG_{2000}-PE micelles were the most effective means of incorporating siRNA-S-S-PE. These micelles were able to effectively incorporate siRNA-S-S-PE conjugates and *in vitro* silencing studies indicated that a target protein of interest can be effectively down regulated.

Following extensive characterization, it was determined that siRNA-S-S-PE:PEG-PE mixed micelles at weight ratio of 1:750 were optimal. Anti-survivin siRNA containing micelles were then analyzed for their cytotoxicity against A2780 cells and it was determined that 6 hr incubation with cells followed by 48 hr of further incubation results in significant dose-dependent toxicity at doses ranging down to 17.3 nM. Additionally, siRNA must be formulated into micelles for effective protection and cytotoxic effects. Preincubating anti-survivin siRNA at low doses followed by treatment with NCL-240/TRAIL-modified micelles proved to be an effective method of inhibiting cell growth. Providing extra time for siRNA to silence survivin likely increased the effect of the treatment of the drug-loaded/TRAIL-modified formulations. Combination siRNA/Drug/TRAIL-modified micelles also indicated effect *in vitro* with significant toxicities at low doses of drug. This formulation may not ultimately be the most effective, as the dosing schedules of siRNA and the other cytotoxic compounds must be optimized.

siRNA-S-S-PE/PEG-PE mixed micelles were able to effectively silence a targeted gene of interest in a proof-of-concept *in vivo* silencing experiment. This is the first evidence of *in vivo* silencing utilizing the reversibly conjugated siRNA-S-S-PE/PEG-PE mixed micelle delivery system. Future studies optimizing the dosing amounts and schedule should be conducted with the ultimate goal of silencing targeted genes in a tumor-bearing mouse model.
8 EVALUATION OF COMBINATION MICELLES AS A MULTIFUNCTIONAL DELIVERY SYSTEM UTILIZING \textit{IN VIVO} MOUSE MODELS OF HUMAN CANCER

8.1 Introduction

The utility of any drug delivery system is based on the ability of the system to effectively deliver their contents to the tissue of interest allowing for the therapeutic to exert its activity. Based on the previously described studies, the drug-loaded/TRAIL-modified micelles prepared illustrate the ability to effectively inhibit growth of tumor cells in an \textit{in vitro} setting. To confirm the ability of this system to effectively inhibit tumor growth in an \textit{in vivo} setting, several experiments were performed.

Previously published results indicated that one of the limitations of DM-PIT-1 was poor stability in biological samples. The second generation compounds were developed with several modifications aiming to impart additional stability to the compound. \textit{In vitro} stability studies indicated that NCL-240 was in fact more stable in biological samples. Confirmation of this result in an \textit{in vivo} system however, is required.

In order to demonstrate the ability of drug-loaded/TRAIL-modified micelles to inhibit tumor growth, several \textit{in vivo} experiments were performed. Confirmation that the therapeutics were performing their mechanism of action \textit{in vivo} was determined in addition to the overall effectiveness of the formulations at inhibiting tumor growth. Tolerability and toxicity of the formulations was also analyzed.

8.2 Materials and Methods

8.2.1 Animal Studies

All animal studies were conducted in accordance with the IACUC approved animal protocol on file with the Northeastern University DLAM.
8.2.2 Pharmacokinetic/stability of NCL-240 study

Healthy female athymic nude (Nu\(^{-}/\)Nu\(^{-}\)) mice aged 6-8 weeks were purchased from Charles River Laboratories (Cambridge, MA). A2780 cells grown in culture were trypsinized and \(\sim 5 \times 10^6\) cells were suspended in 200 \(\mu\)L 1x PBS. Cells were injected subcutaneously (s.c.) in the right flank of mice. Tumors were allowed to grow for approximately 14 days and mice were then injected with 20 mg/kg NCL-240 formulated in micelles as described previously. All formulations were administered as intaperitoneal (i.p.) injections. Following predefined time points (15 min, 30 min, 1 hr, 4 hr, 12 hr and 24 hr), mice were anesthetized with 100 \(\mu\)L ketamine-xylazine and blood was collected into serum separator tubes (Becton Dickinson, Franklin Lakes, NJ) via cardiac puncture. Tubes were then centrifuged for 10 min at 5000 g and serum was transferred to separate eppendorf tubes.

NCL-240 was separated from the serum using methods described previously [62]. Briefly, 200 \(\mu\)L of serum was mixed with 1 mL methylene chloride and placed on a shaker at room temperature for 3 hr. Organic fractions were collected, evaporated under nitrogen and further dried via freeze-drying. Samples were then rehydrated with 200 \(\mu\)L acetonitrile and analyzed for concentration of NCL-240 by HPLC using previously described protocols. Standard curves of NCL-240 concentration in blood were prepared by spiking known concentrations of NCL-240 dissolved in acetonitrile into blood collected from control mice.

8.2.3 Tumor response studies

Healthy female athymic nude (Nu\(^{-}/\)Nu\(^{-}\)) mice aged 6-8 weeks were purchased from Charles River Laboratories (Cambridge, MA). A2780 cells grown in culture were trypsinized and \(\sim 5 \times 10^6\) cells were suspended in 200 \(\mu\)L 1x PBS. Cells were injected s.c. in the right flank of mice. Tumors were allowed to grow approximately 14 days and mice were randomized to treatment groups upon tumors reaching \(\sim 200 \text{ mm}^3\). All formulations were administered as daily i.p. injections. Mice were monitored for changes in body weight and activity as indicators of toxicity. The tumors were measured daily using a vernier caliper (two perpendicular axes of the tumor were measured). Tumor volume was calculated according to the formula; \(\text{mm}^3 = 1/2\times(\text{length} \times \text{width} \times \text{height})\).
x width²). Following completion of the study, mice were euthanized and tumors were extracted. Tumors were then weighed and stored in freezing media at -80° C for further analysis.

### 8.2.4 Tumor histology

Immunohistochemistry assays were performed according to manufacturer’s protocols. Briefly, frozen tumors were sectioned (8-µm), placed on slides, fixed with paraformaldehyde and air-dried. Subsequently, slides were incubated with blocking solution for 1 hr followed by extensive washing and stained with either phospho-Akt (Ser-473) or cleaved caspase-3 (Asp175) primary rabbit mAbs (Cell Signaling, Danvers, MA) overnight at room temperature. Slides were extensively washed and anti-rabbit Alexa Fluor® fluorescent secondary antibody (Cell Signaling, Danvers, MA) was then incubated for 1 hr followed by washing and mounting of coverslips.

TUNEL assay (EMD Millipore Corporation, Billerica, MA) was performed similar to the method above. However, following fixation of tissue, slides were permeabilized with proteinase K for 15 min at RT, and then subjected to TUNEL assay using the FragEl™ DNA fragmentation Detection Kit following the manufacturer’s protocol. All slides were imaged by fluorescent microscopy and random images were obtained from slides prepared from two different tumors per group.

### 8.2.5 Statistical analysis

Wherever possible, data was generated in triplicates for proper statistical analysis. Pharmacokinetic data was analyzed by calculating the mean value of three mice using non-compartmental analysis. One-way ANOVA followed by Tukey's multiple comparison tests were performed with significance determined by a p-value < 0.05.

Tumor volumes and weights are reported as mean +/- standard deviation. One-way ANOVA followed by Tukey's multiple comparison tests were performed with significance determined by a p-value < 0.05. Two-
way ANOVA followed by Bonferroni post-tests were also performed on the tumor volume results with significance determined by a p-value < 0.05.
8.3 Results and Discussion

8.3.1 PK Study

The primary goal of this study was to determine if the second generation NCL-240 possessed superior stability in vivo as compared to published results of DM-PIT-1. Results of the stability study are outlined in Table 11 below.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Elimination rate constant $K_e$ (hr$^{-1}$)</th>
<th>Half-life $T_{1/2}$ (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micellar DM-PIT-1</td>
<td>1.29</td>
<td>0.54</td>
</tr>
<tr>
<td>Micellar NCL-240</td>
<td>0.70</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Table 11: Elimination rate constant and half-life comparison after single i.p. injection of Micellar NCL-240 as compared to published values of DM-PIT-1 [62].

These results coupled with the in vitro mouse microsomal stability data (Figure 16) indicate the second generation NCL-240 possesses superior stability in biological samples as compared to the first generation DM-PIT-1. An expanded pharmacokinetic and biodistribution study utilizing more early time points is warranted to confirm the results indicated in this study.
8.3.2 NCL-176 tumor inhibition study

**Figure 55:** Micellar loaded NCL-176 tumor inhibition of A2780 ovarian carcinoma xenografts. (A) Daily administration of 5 mg/kg NCL-176 (m176) alone and in combination with 2.67 mg/kg TRAIL (Combination) is able to successfully inhibit growth of A2780 xenografts over the course of 14 days ($p < 0.001$). Values are expressed as mean tumor volume with error bars indicating SD (n=4). (B) TUNEL histology performed on harvested tumor sections confirms the increased presence of apoptotic cells in the m176 and Combination groups. (C) The increased presence of cleaved caspase in tumor sections from mTRAIL, and Combination groups indicate the activity of TRAIL in the tumor. (D) Decreased phospho-Akt (p473) signal in m176 and Combination tumor sections indicate NCL-176 is successful at inhibiting the phosphorylation and activation of Akt in tumor cells [26].
Figure 56: % Body weight change of mice over the course of treatment (n=4).

The tumor inhibition effect of micelle loaded NCL-176 (m176) was tested in female nude mice bearing subcutaneous A2780 tumors alone and in combination with TRAIL. Both the m176 and Combination formulations were able to significantly inhibit the growth of tumors with a 76.5% and 72.9% reduction in tumor volume as compared to controls. All formulations were well tolerated by the mice with no signs of weight loss or overt toxicity. The limited effect of TRAIL addition on xenograft growth could be explained by the suboptimal NCL-176/TRAIL ratio and/or the low overall dose (2.67 mg/kg) as TRAIL was typically given at doses of 5 to 15 mg/kg in other xenograft studies [79, 80].

Tumor histology of harvested tumors confirmed the activity of NCL-176 and TRAIL in vivo. TUNEL staining was clear in the m176 and most pronounced in the combination group indicating significant induction of apoptosis. Additionally, cleaved caspase-3 signal was most pronounced in the combination group, confirming activation of apoptotic signaling. Phospho-Akt (p473) staining is decreased in NCL-176 containing groups demonstrating the efficacy of the drug at preventing Akt phosphorylation and activation. These results taken together indicate micellar NCL-176 alone and in combination with TRAIL can effectively inhibit tumor growth in vivo.
8.3.3 NCL-240 tumor inhibition study I

![A2780 Tumor Growth Inhibition graph](image)

**Figure 57:** Micellar loaded NCL-240 tumor inhibition of A2780 ovarian carcinoma xenografts. TRAIL administered in either free form (fTRAIL, fCombo) or in micellar form (mTRAIL, mCombo). Daily administration of 5 mg/kg NCL-240 (m240) alone and in combination with 2.67 mg/kg TRAIL (Combination) is able to successfully inhibit growth of A2780 xenografts over the course of 14 days (p < 0.001). Values are expressed as mean tumor volume with error bars indicating SD (n=5).

![Excised Tumor Weights bar chart](image)

**Figure 58:** Tumors treated with the combination of both NCL-240 and TRAIL resulted in significantly lower (p < 0.05) mass upon sacrifice as compared to the PBS group. The masses of excised tumors are expressed as means with error bars indicating SD (n=5).
Investigator: Robert D. Riehl

Figure 59: % Body weight change of mice over the course of treatment (n=5).

NCL-240 was also investigated for its tumor inhibition effects based on the in vitro data presented previously and the fact that NCL-176 showed promising results. Because of the low efficacy seen previously with mTRAIL (Figure 55), TRAIL was administered both in free and micellar forms ensure that the efficacy of TRAIL is not diminished when delivered on micellar surfaces. Results from this study indicated that the method of TRAIL delivery does not significantly affect its activity in vivo as seen in both the fTRAIL/mTRAIL comparison and the fCombo/mCombo comparison. Additionally, the Combination formulations of micellar loaded NCL-240 and TRAIL proved to be the most effective treatments (Figure 57). The tumor weights of these groups were also significantly lower than the PBS control (Figure 58). All formulations were well tolerated, as there was no significant change in body weight between groups (Figure 59).
8.3.4 NCL-240 tumor inhibition study II

**Figure 60:** Micellar loaded NCL-240 tumor inhibition of A2780 ovarian carcinoma xenografts. (A) Daily administration of 20 mg/kg NCL-240 (m240) alone and in combination with 10 mg/kg TRAIL (Combination) is able to successfully inhibit growth of A2780 xenografts over the course of 14 days (p < 0.001). Micelle associated TRAIL (mTRAIL) at the dose of 10 mg/kg is also able to significantly inhibit tumor growth (p < 0.05). Values are expressed as mean tumor volume with error bars indicating SD (n=5). (B) Tumors treated with the combination of both NCL-240 and TRAIL resulted in significantly lower (p < 0.05) mass upon sacrifice as compared to the PBS group. The masses of excised tumors are expressed as means with error bars indicating SD (n=5).

**Figure 61:** % Body weight change of mice over the course of treatment (n=5).

Doses of up to 50 mg/kg of NCL-240 proved to be non-toxic in mice (data not shown), so the dose of NCL-240 in the second in vivo tumor inhibition study was increased to 20 mg/kg. Micellar NCL-240 (m240)
and combination micelles significantly (p < 0.001) inhibited A2780 tumor growth in this study (Figure 60). Tumor growth was reduced by 63% in the m240 group while combination micelles inhibited growth 73% as compared to controls. The mTRAIL group also significantly inhibited tumor growth as compared to PBS (p < 0.05). Additionally, the administration of the combination of TRAIL and NCL-240 resulted in significantly reduced tumor weight. These formulations were also well tolerated in mice, as there was no apparent toxicity or loss of body weight (Figure 61).
8.4 Conclusions

Combination drug-loaded/TRAIL-modified micellar formulations were particularly successful at inhibiting growth of a subcutaneous A2780 tumor mouse model indicating that combinations of micellar inhibitors of the PI3K pathway and surface-bound TRAIL can be utilized as a therapy against TRAIL-resistant cancers. Micellar formulations of NCL-176 alone and in combination with TRAIL were able to significantly inhibit tumor growth and do so with no apparent toxicity. Additionally, histology results confirm that both NCL-176 and TRAIL exert tumor inhibition through their known mechanisms of action.

Results from the first NCL-240 tumor inhibition study indicate that the activity of TRAIL is not diminished when conjugated to the surface of the micelle, as both the free TRAIL and micellar TRAIL groups were statistically no different. This study confirmed that NCL-240 was effective at inhibiting tumor growth with no apparent toxicity. Lastly, the second NCL-240 tumor study indicated that higher doses of NCL-240 were still tolerated by the mice and that there was significant inhibition of tumor growth as compared to controls. Excised tumor weights also confirmed the ability of these formulations at inhibiting A2780 tumor growth.
9 Concluding Remarks

Advances in chemotherapy, particularly targeted therapies against individual tumors, have yielded significant improvements in patient survival. Despite this, conventional chemotherapy is rarely curative for solid tumors. Cancer cells possess several mechanisms promoting cell proliferation and preventing programmed cell death. As a result, combination therapies targeting separate pathways involved in cancer progression and tumor cell survival have emerged as the most effective treatments. Delivery of combination therapies is extremely important as significant dose limiting toxicities of both the chemotherapeutics and the cosolvents required for delivery of the drug, severely impact clinical potential.

Current research in drug delivery focuses primarily on the use of nanoparticles capable of incorporating therapeutics and delivering them to the site of interest. Lipid based PEG-PE micelles have been demonstrated to possess the ability to effectively incorporate insoluble lipophilic drugs into the core of the micelle and deliver the drugs to the tumor by utilizing the EPR effect. Additionally, the surface of the micelle can be decorated with both targeting and therapeutic ligands capable of imparting additional therapeutic benefit. Novel siRNA-S-S-PE conjugates can also be incorporated into the micelle further increasing therapeutic potential.

This work aimed to combine novel analogs of a pro-apoptotic inhibitor of the PI3K pathway, an apoptosis inducing ligand and siRNA into a single micellar Nano preparation with the anticipated benefit of increased anti-tumor effect. To achieve this objective, the novel analogs were characterized for their ability to inhibit PIP3-PH interaction and their anti-proliferative effects in vitro. They were then incorporated into PEG-PE micelles and combined with TRAIL to determine their effect on a variety of human cancer cell lines and an in vivo model of human cancer. Targeting these micelles with transferrin was also investigated to analyze any increased cytotoxic effects. Simultaneously, siRNA-S-S-PE was formulated into PEG-PE micelles of various chain-lengths to determine the optimal preparation. These siRNA-S-S-PE mixed micelles were then evaluated for their ability to silence the anti-apoptotic protein survivin in vitro and their ability to inhibit cell growth was
also characterized. Lastly, combination drug-loaded/TRAIL-modified/siRNA-containing micelles were prepared and evaluated as an anticancer Nano preparation.

Initial results indicated that the novel analogs or DM-PIT-1 were successful at inhibiting PIP3-PH interaction and possessed increased stability in biological samples. Cytotoxicity studies indicated that these analogs maintained tumor cell growth inhibition with each analog possessing at cytotoxic activity at least comparable to DM-PIT-1. Additionally, combining these drugs with TRAIL increased the cytotoxic activity indicating potential utility as a combination treatment.

Incorporating the novel analogs into micelles resulted in nanosized particles with good drug incorporation efficiency. Including reactive pNP-PEG-PE into the micellar formulation facilitated the TRAIL-modification to the surface of the micelle. Several of the micellar incorporated analogs demonstrated excellent cytotoxic effects against various cancer cell lines including resistant variants. NCL-176 and NCL-240 proved to possess optimal loading, cytotoxic and stability properties.

Studies indicated transferrin could also be conjugated to the surface of the micelle utilizing pNP-PEG-PE. These targeted micelles resulted in increased cellular association. The presence of TRAIL did not inhibit the ability of transferrin to target the cell indicating these multifunctional micelles could be utilized to increase tumor accumulation while not interfering with the cytotoxic properties of the therapeutics. Cytotoxicity studies indicated that the addition of transferrin could increase the cytotoxic effects of drug-loaded micelles as compared to non-targeted formulations.

siRNA-S-S-PE had previously been demonstrated to be effectively incorporated into PEG-PE micelles and could exert its silencing effects in an in vitro model. The studies outlined above aimed to optimize the incorporation of siRNA-S-S-PE by altering the PEG chain-length and the ratio of siRNA-S-S-PE to lipid. Utilizing an improved size-exclusion chromatography method, PEG2000-PE at an siRNA-S-S-PE to lipid ratio of 1:750 proved to be the optimal formulation. Preparations of siRNA-S-S-PE targeting the anti-apoptotic protein survivin demonstrated in vitro protein silencing and possessed cell growth inhibition. Combining siRNA-S-S-
PE mixed micelles with drug-loaded/TRAIL-modified micelles also increased cell growth inhibition indicating potential utility as a multifunctional nanoparticle preparation. Lastly, a proof-of-concept *in vivo* silencing study demonstrated for the first time that siRNA-S-S-PE mixed micelles were able to effectively decrease the expression of a target protein.

The NCL-176 and NCL-240 micellar formulations were studied in an *in vivo* mouse model of human cancer. Results indicated that these formulations alone and in combination with TRAIL were effective at inhibiting tumor growth as compared to controls. Additionally, histology indicated that tumor growth inhibition was a result of both the drug and TRAIL exerting their effects through their mechanisms of action.

In summary, PEG-PE micellar nanoparticle formulations incorporating a novel proapoptotic inhibitor of the PI3K pathway combined with surface-bound TRAIL are capable of effectively inhibiting solid tumor growth. Combining these with siRNA-S-S-PE conjugates targeting the anti-apoptotic protein surviving has the potential to further increases their cytotoxic effects. These studies indicate that PEG-PE micelles are an effective delivery system capable of combining several therapeutic agents into a stable nanosized particle that could be utilized for the treatment of solid tumors.
10 Bibliography

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